

ITC VI – Protein Receptor Studies

Andersen O. M., Schwarz F. P., Eisenstein E., Jacobsen C., Moestrup S. K., Etzerodt M., and Thogersen H. C. (2001) Dominant thermodynamic role of the third independent receptor binding site in the receptor-associated protein RAP. *Biochemistry* **40**, 15408-15417.

Abstract: The 39 kDa receptor-associated protein (RAP) is a three-domain escort protein in the secretory pathway for several members of the low-density lipoprotein receptor (LDLR) family of endocytic receptors, including the LDLR-related protein (LRP). The minimal functional unit of LRP required for efficient binding to RAP is composed of complement-type repeat (CR)-domain pairs, located in clusters on the extracellular part of LRP. Here we investigate the binding of full-length RAP and isolated RAP domains 1-3 to an ubiquitin-fused CR-domain pair consisting of the fifth and sixth CR domains of LRP (U-CR56). As shown by isothermal titration calorimetric analysis of simple RAP domains as well as adjoined RAP domains, all three RAP domains bind to this CR-domain pair in a noncooperative way. The binding of U-CR56 to RAP domains 1 and 2 is (at room temperature) enthalpically driven with an entropy penalty ($K(D) = 2.77 \times 10^{-6}$ M and 1.85×10^{-5} M, respectively), whereas RAP domain 3 binds with a substantially lower enthalpy, but is favored due to a positive entropic contribution ($K(D) = 1.71 \times 10^{-7}$ M). The heat capacity change for complex formation between RAP domain 1 and the CR-domain pair is $-1.65 \text{ kJ K}^{-1} \text{ mol}^{-1}$. There is an indication of a conformational change in RAP domain 3 upon binding in the surface plasmon resonance analysis of the interaction. The different mechanisms of binding to RAP domains 1 and 3 are further substantiated by the different effects on binding of mutations of the Asp and Trp residues in the LRP CR5 or CR6 domains, which are important for the recognition of several ligands.

Armstrong K. M., Insaïdo F. K. and Baker B. M. (2008) Thermodynamics of T-cell receptor-peptide/MHC interactions: progress and opportunities. *J Mol Recognit* **21**, 275-287.

Abstract: alphabeta T-cell receptors (TCRs) recognize peptide antigens presented by class I or class II major histocompatibility complex molecules (pMHC). Here we review the use of thermodynamic measurements in the study of TCR-pMHC interactions, with attention to the diversity in binding thermodynamics and how this is related to the variation in TCR-pMHC interfaces. We show that there is no enthalpic or entropic signature for TCR binding; rather, enthalpy and entropy changes vary in a compensatory manner that reflects a narrow free energy window for the interactions that have been characterized. Binding enthalpy and entropy changes do not correlate with structural features such as buried surface area or the number of hydrogen bonds within TCR-pMHC interfaces, possibly reflecting the myriad of contributors to binding thermodynamics, but likely also reflecting a reliance on van't Hoff over calorimetric measurements and the unaccounted influence of equilibria linked to binding. TCR-pMHC binding heat capacity changes likewise vary considerably. In some cases, the heat capacity changes are consistent with conformational differences between bound and free receptors, but there is little data indicating these conformational differences represent the need to organize disordered CDR loops. In this regard, we discuss how thermodynamics may provide additional insight into conformational changes occurring upon TCR binding. Finally, we highlight opportunities for the further use of thermodynamic measurements in the study of TCR-pMHC interactions, not only for understanding TCR binding in general, but also for understanding specifics of individual interactions and the engineering of TCRs with desired molecular recognition properties

Armstrong K. M. and Baker B. M. (2007) A comprehensive calorimetric investigation of an entropically driven T cell receptor-peptide/major histocompatibility complex interaction. *Biophys J* **93**, 597-609.

Abstract: The alphabeta T cell receptor (TCR) is responsible for recognizing peptides bound and "presented" by major histocompatibility complex (MHC) molecules. We recently reported that at 25 degrees C the A6 TCR, which recognizes the Tax peptide presented by the class I MHC human leukocyte antigen-A*0201 (HLA-A2), binds with a weak ΔH degrees, a favorable ΔS degrees, and a moderately negative $\Delta C(p)$. These observations were of interest given the unfavorable binding entropies and large heat capacity changes measured for many other TCR-ligand interactions, suggested to result from TCR conformational changes occurring upon binding. Here, we further investigated the A6-Tax/HLA-A2 interaction using titration calorimetry. We found that binding results in a $pK(a)$ shift, complicating interpretation of measured binding thermodynamics. To better characterize the interaction, we measured binding as a function of pH, temperature, and buffer ionization enthalpy. A global analysis of the

resulting data allowed determination of both the intrinsic binding thermodynamics separated from the influence of protonation as well as the thermodynamics associated with the pK(a) shift. Our results indicate that intrinsically, A6 binds Tax/HLA-A2 with a very weak ΔH degrees, an even more favorable ΔS degrees than previously thought, and a relatively large negative $\Delta C(p)$. Comparison of these energetics with the makeup of the protein-protein interface suggests that conformational adjustments are required for binding, but these are more likely to be structural shifts, rather than disorder-to-order transitions. The thermodynamics of the pK(a) shift suggest protonation may be linked to an additional process such as ion binding.

Babon J. J., Yao S., DeSouza D. P., Harrison C. F., Fabri L. J., Liepinsh E., Scrofani S. D., Baca M., and Norton R. S. (2005) Secondary structure assignment of mouse SOCS3 by NMR defines the domain boundaries and identifies an unstructured insertion in the SH2 domain. *FEBS J* **272**, 6120-6130.

Abstract: SOCS3 is a negative regulator of cytokine signalling that inhibits Janus kinase-signal transduction and activator of transcription (JAK-STAT) mediated signal transduction by binding to phosphorylated tyrosine residues on intracellular subunits of various cytokine receptors, as well as possibly the JAK proteins. SOCS3 consists of a short N-terminal sequence followed by a kinase inhibitory region, an extended SH2 domain and a C-terminal suppressor of cytokine signalling (SOCS) box. SOCS3 and the related protein, cytokine-inducible SH2-containing protein, are unique among the SOCS family of proteins in containing a region of mostly low complexity sequence, between the SH2 domain and the C-terminal SOCS box. Using NMR, we assigned and determined the secondary structure of a murine SOCS3 construct. The SH2 domain, unusually, consists of 140 residues, including an unstructured insertion of 35 residues. This insertion fits the criteria for a PEST sequence and is not required for phosphotyrosine binding, as shown by isothermal titration calorimetry. Instead, we propose that the PEST sequence has a functional role unrelated to phosphotyrosine binding, possibly mediating efficient proteolytic degradation of the protein. The latter half of the kinase inhibitory region and the entire extended SH2 subdomain form a single alpha-helix. The mapping of the true SH2 domain, and the location of its C terminus more than 50 residues further downstream than predicted by sequence homology, explains a number of previously unexpected results that have shown the importance of residues close to the SOCS box for phosphotyrosine binding.

Baldwin E. T., Sarver R. W., Bryant G. L., Jr., Curry K. A., Fairbanks M. B., Finzel B. C., Garlick R. L., Henrikson R. L., Horton N. C., Kelley L. L., Mildner A. M., Moon J. B., Mott J. E., Mutchler V. T., Tomich C. S., Watenpugh K. D., and Wiley V. H. (1998) Cation binding to the integrin CD11b I domain and activation model assessment. *Structure* **6**, 923-935.

Abstract: BACKGROUND: The integrin family of cell-surface receptors mediate cell adhesion through interactions with the extracellular matrix or other cell-surface receptors. The alpha chain of some integrin heterodimers includes an inserted 'I domain' of about 200 amino acids which binds divalent metal ions and is essential for integrin function. Lee et al. proposed that the I domain of the integrin CD11b adopts a unique 'active' conformation when bound to its counter receptor. In addition, they proposed that the lack of adhesion in the presence of Ca^{2+} ion reflected the stabilization of an 'inactive' I-domain conformation. We set out to independently determine the structure of the CD11 b I domain and to evaluate the structural effects of divalent ion binding to this protein. RESULTS: We have determined the X-ray structure of a new crystal form of the CD11 b I domain in the absence of added metal ions by multiple isomorphous replacement (MIR). Metal ions were easily introduced into this crystal form allowing the straight-forward assessment of the structural effects of divalent cation binding at the metal ion dependent adhesion site (MIDAS). The equilibrium binding constants for these ions were determined by titration calorimetry. The overall protein conformation and metal-ion coordination of the I domain is the same as that observed for all previously reported CD11 a I-domain structures and a CD11 b I-domain complex with Mn^{2+} . These structures define a majority conformation. CONCLUSIONS: Addition of the cations Mg^{2+} , Mn^{2+} and Cd^{2+} to the metal-free I domain does not induce conformational changes in the crystalline environment. Moreover, we find that Ca^{2+} binds poorly to the I domain which serves to explain its failure to support adhesion. We show that the active conformation proposed by Lee et al, is likely to be a construct artifact and we propose that the currently available data do not support a dramatic structural transition for the I domain during counter-receptor binding.

Bazarsuren A., Grauschopf U., Wozny M., Reusch D., Hoffmann E., Schaefer W., Panzner S., and Rudolph R. (2002) In vitro folding, functional characterization, and disulfide pattern of the extracellular domain of human GLP-1 receptor. *Biophys Chem* **96**, 305-318.

Abstract: The N-terminal, extracellular domain of the receptor for glucagon-like peptide 1 (GLP-1 receptor) was expressed at a high level in *E. coli* and isolated as inclusion bodies. Renaturation with concomitant disulfide bond formation was achieved from guanidinium-solubilized material. A soluble and active fraction of the protein was isolated by ion exchange chromatography and gel filtration. Complex formation with GLP-1 was shown by cross-linking experiments, surface plasmon resonance measurements, and isothermal titration calorimetry. The existence of disulfide bridges in the N-terminal receptor fragment was proven after digestion of the protein with pepsin. Further analysis revealed a disulfide-binding pattern with links between cysteines 46 and 71, 62 and 104, and between 85 and 126.

Benfield A. P., Whiddon B. B., Clements J. H. and Martin S. F. (2007) Structural and energetic aspects of Grb2-SH2 domain-swapping. *Arch Biochem Biophys* **462**, 47-53.

Abstract: The SH2 domain of growth factor receptor-bound protein 2 (Grb2) has been the focus of numerous studies, primarily because of the important roles it plays in signal transduction. More recently, it has emerged as a useful protein to study the consequences of ligand preorganization upon energetics and structure in protein-ligand interactions. The Grb2-SH2 domain is known to form a domain-swapped dimer, and as part of our investigations toward correlating structure and energetics in biological systems, we examined the effects that domain-swapping dimerization of the Grb2-SH2 domain had upon ligand binding affinities. Isothermal titration calorimetry was performed using Grb2-SH2 in both its monomeric and domain-swapped dimeric forms and a phosphorylated tripeptide AcNH-pTyr-Val-Asn-NH₂ that is similar to the Shc sequence recognized by Grb2-SH2 in vivo. The two binding sites of domain-swapped dimer exhibited a 4- and a 13-fold reduction in ligand affinity compared to monomer. Crystal structures of peptide-bound and uncomplexed forms of Grb2-SH2 domain-swapped dimer were obtained and reveal that the orientation of residues V122, V123, and R142 may influence the conformation of W121, an amino acid that is believed to play an important role in Grb2-SH2 ligand sequence specificity. These findings suggest that domain-swapping of Grb2-SH2 not only results in a lower affinity for a Shc-derived ligand, but it may also affect ligand specificity.

Blankenship J. W., Varfolomeev E., Goncharov T., Fedorova A. V., Kirkpatrick D. S., Izrael-Tomasevic A., Phu L., Arnott D., Aghajan M., Zobel K., Bazan J. F., Fairbrother W. J., Deshayes K. and Vucic D. (2008) Ubiquitin binding modulates IAP antagonist stimulated proteasomal degradation of c IAP1 and c IAP2. *Biochem J.* (publication)

Abstract: A family of anti-apoptotic regulators known as inhibitor of apoptosis (IAP) proteins interact with multiple cellular partners and inhibit apoptosis induced by a variety of stimuli. c IAP1 and c IAP2 are recruited to tumor necrosis factor receptor 1 (TNFR1)-associated signaling complexes where they mediate receptor-induced NF kappaB activation. Additionally, through their ubiquitin E3 ligase activities, c-IAP1 and c-IAP2 promote proteasomal degradation of NF kappaB-inducing kinase, NIK, and regulate the non-canonical NF-kappaB pathway. Herein, we describe a novel ubiquitin-binding domain of IAPs. The UBA (ubiquitin-associated domain) of IAPs is located between the baculovirus IAP repeat (BIR) domains and the caspase activation and recruitment domain (CARD) or the RING domain of c-IAP1 and 2 or XIAP, respectively. The c IAP1 UBA domain binds monoubiquitin and Lys48- and Lys63-linked polyubiquitin chains with low micromolar affinities as determined by surface plasmon resonance or isothermal titration calorimetry. Mutations of critical amino acid residues in the highly conserved Met-Gly-Phe binding loop of the UBA domain completely abrogate ubiquitin binding. These mutations in the UBA domain do not overtly affect the ubiquitin ligase activity of c IAP1 or the participation of c IAP1 and 2 in the TNFR1 signaling complex. Treatment of cells with IAP antagonists leads to proteasomal degradation of c-IAP1 and 2. Deletion or mutation of the UBA domain reduces this degradation, most likely by decreasing the interaction of the c-IAP proteins with proteasome. These results suggest that ubiquitin binding may be an important mechanism for rapid turn-over of auto-ubiquitinated c-IAP1 and 2 proteins

Bullock A.N., Debreczeni J.E., Edwards A.M., Sundstrom M., and Knapp S. (2006) Crystal structure of the SOCS2-elongin C-elongin B complex defines a prototypical SOCS box ubiquitin ligase. *Proc Natl Acad Sci U S A.* **103**, 7637-42.

Abstract: Growth hormone (GH) signaling is tightly controlled by ubiquitination of GH receptors, phosphorylation levels, and accessibility of binding sites for downstream signaling partners. Members of the suppressors of cytokine signaling (SOCS) family function as key regulators at all levels of this pathway, and mouse knockout studies implicate SOCS2 as the primary suppressor. To elucidate the structural basis for SOCS2 function, we determined the 1.9-Å crystal structure of the ternary complex of SOCS2 with elongin C and elongin B. The structure defines a prototypical SOCS box ubiquitin ligase with a Src homology 2 (SH2) domain as a substrate recognition motif. Overall, the SOCS box and SH2 domain show a conserved spatial domain arrangement with the BC box and substrate recognition domain of the von Hippel-Lindau (VHL) tumor suppressor protein, suggesting a common mechanism of ubiquitination in these cullin-dependent E3 ligases. The SOCS box binds elongin BC in a similar fashion to the VHL BC box and shows extended structural conservation with the F box of the Skp2 ubiquitin ligase. A previously unrecognized feature of the SOCS box is revealed with the burial of the C terminus, which packs together with the N-terminal extended SH2 subdomain to create a stable interface between the SOCS box and SH2 domain. This domain organization is conserved in SOCS1-3 and CIS1, which share a strictly conserved length of their C termini, but not in SOCS4, 5, and 7, which have extended C termini defining two distinct classes of inter- and intramolecular SOCS box interactions.

Bullock A. N., Rodriguez M. C., Debreczeni J. E., Songyang Z. and Knapp S. (2007) Structure of the SOCS4-ElonginB/C complex reveals a distinct SOCS box interface and the molecular basis for SOCS-dependent EGFR degradation. *Structure* **15**, 1493-1504.

Abstract: Tyrosine kinase signaling is tightly controlled by negative feedback inhibitors including suppressors of cytokine signaling (SOCS). SOCS assemble as SH2 domain substrate recognition modules in ElonginB/C-cullin ubiquitin ligases. In accordance, SOCS4 reduces STAT3 signaling from EGFR through increased receptor degradation. Variable C-termini in SOCS4-SOCS7 exclude these family members from a SOCS2-type domain arrangement in which a strictly conserved C terminus determines domain packing. The structure of the SOCS4-ElonginC-ElonginB complex reveals a distinct SOCS structural class. The N-terminal ESS helix functionally replaces the CIS/SOCS1-SOCS3 family C terminus in a distinct SH2-SOCS box interface that facilitates further interdomain packing between the extended N- and C-terminal regions characteristic for this subfamily. Using peptide arrays and calorimetry the STAT3 site in EGFR (pY(1092)) was identified as a high affinity SOCS4 substrate ($K(D) = 0.5 \mu\text{M}$) revealing a mechanism for EGFR degradation. SOCS4 also bound JAK2 and KIT with low micromolar affinity, whereas SOCS2 was specific for GH-receptor.

Calamini B., Santarsiero B. D., Boutin J. A. and Mesecar A. D. (2008) Kinetic, thermodynamic and X-ray structural insights into the interaction of melatonin and analogues with quinone reductase 2. *Biochem J* **413**, 81-91.

Abstract: Melatonin exerts its biological effects through at least two transmembrane G-protein-coupled receptors, MT1 and MT2, and a lower-affinity cytosolic binding site, designated MT3. MT3 has recently been identified as QR2 (quinone reductase 2) (EC 1.10.99.2) which is of significance since it links the antioxidant effects of melatonin to a mechanism of action. Initially, QR2 was believed to function analogously to QR1 in protecting cells from highly reactive quinones. However, recent studies indicate that QR2 may actually transform certain quinone substrates into more highly reactive compounds capable of causing cellular damage. Therefore it is hypothesized that inhibition of QR2 in certain cases may lead to protection of cells against these highly reactive species. Since melatonin is known to inhibit QR2 activity, but its binding site and mode of inhibition are not known, we determined the mechanism of inhibition of QR2 by melatonin and a series of melatonin and 5-hydroxytryptamine (serotonin) analogues, and we determined the X-ray structures of melatonin and 2-iodomelatonin in complex with QR2 to between 1.5 and 1.8 Å (1 Å=0.1 nm) resolution. Finally, the thermodynamic binding constants for melatonin and 2-iodomelatonin were determined by ITC (isothermal titration calorimetry). The kinetic results indicate that melatonin is a competitive inhibitor against N-methylidihydrocotinamide ($K(i)=7.2 \mu\text{M}$) and uncompetitive against menadione ($K(i)=92 \mu\text{M}$), and the X-ray structures shows that melatonin binds in multiple orientations within the active sites of the QR2 dimer as opposed to an allosteric site. These results provide new insights into the binding mechanisms of melatonin and analogues to QR2

Celie P.H., Kasheverov I.E., Mordvintsev D.Y., Hogg R.C., van Nierop P., van Elk R., van Rossum-Fikkert S.E., Zhmak M.N., Bertrand D., Tsetlin V. Sixma T.K, and Smit A.B. (2005) Crystal structure of nicotinic

acetylcholine receptor homolog AChBP in complex with an alpha-conotoxin PnIA variant. *Nat Struct Mol Biol.* **12**, 582-8.

Abstract: Conotoxins (Ctx) form a large family of peptide toxins from cone snail venoms that act on a broad spectrum of ion channels and receptors. The subgroup alpha-Ctx specifically and selectively binds to subtypes of nicotinic acetylcholine receptors (nAChRs), which are targets for treatment of several neurological disorders. Here we present the structure at a resolution of 2.4 Å of alpha-Ctx PnIA (A10L D14K), a potent blocker of the alpha(7)-nAChR, bound with high affinity to acetylcholine binding protein (AChBP), the prototype for the ligand-binding domains of the nAChR superfamily. Alpha-Ctx is buried deep within the ligand-binding site and interacts with residues on both faces of adjacent subunits. The toxin itself does not change conformation, but displaces the C loop of AChBP and induces a rigid-body subunit movement. Knowledge of these contacts could facilitate the rational design of drug leads using the Ctx framework and may lead to compounds with increased receptor subtype selectivity.

Chaudhury C., Brooks C. L., Carter D. C., Robinson J. M., and Anderson C. L. (2006) Albumin binding to FcRn: distinct from the FcRn-IgG interaction. *Biochemistry* **45**, 4983-4990.

Abstract: The MHC-related Fc receptor for IgG (FcRn) protects albumin and IgG from degradation by binding both proteins with high affinity at low pH in the acid endosome and diverting both from a lysosomal pathway, returning them to the extracellular compartment. Immunoblotting and surface plasmon resonance studies show that both IgG and albumin bind noncooperatively to distinct sites on FcRn, that the affinity of FcRn for albumin decreases approximately 200-fold from acidic to neutral pH, and that the FcRn-albumin interaction shows rapid association and dissociation kinetics. Isothermal titration calorimetry shows that albumin binds FcRn with a 1:1 stoichiometry and the interaction has hydrophobic features as evidenced by a large positive change in entropy upon binding. Our results suggest that the FcRn-albumin interaction has unique features distinct from FcRn-IgG binding despite the overall similarity in the pH-dependent binding mechanism by which both ligands are protected from degradation.

Chan E., Amon M., Marano R. J., Wimmer N., Kearns P. S., Manolios N., Rakoczy P. E. and Toth I. (2007) Novel cationic lipophilic peptides for oligodeoxynucleotide delivery. *Bioorg. Med. Chem* **15**, 4091-4097.

Abstract: In search of new oligodeoxynucleotide (ODN) delivery agents, we evaluated novel peptides derived from core peptide H-GLRILLKLV-OH (CP). CP is a fragment designed from the T-cell antigen receptor (TCR) alpha-chain transmembrane sequence. CP was able to enter cells including T-cells and inhibited interleukin-2 (IL-2) production. To examine the effect of increased lipophilicity on cellular uptake and activity of CP, a lipoamino acid (2-aminododecanoic acid) was incorporated into peptide CP resulting in 2-aminododecanoyl-CP (LP). The toxicity of CP and LP was assessed by measuring the haemolytic activity. Neither compound caused any haemolysis of red blood cells. We have also compared the biological activities of the CP and LP. Using a T-cell antigen presentation assay, the more lipophilic LP caused greater inhibition of IL-2 production than the parent CP in the antigen stimulated T-cells. The LP also showed increased permeability than CP in the Caco-2 cell assay. We utilised the enhanced cell permeability property of LP in oligodeoxynucleotide ODN1 delivery. Isothermal titration calorimetry (ITC) suggested that CP and LP complex with ODN1 in a 12:1 (CP:ODN1) and 15:1 (LP:ODN1) ratio. These complexes were then transfected into human retinal pigment epithelial cells. The level of transfection was measured by the decreased production of the protein human vascular endothelial growth factor (hVEGF). The results revealed greater transfection efficiency for both CP and LP (47%, 55% more inhibition) compared to commercially available transfection agent cytofectin GSV. These results suggested that the CP and particularly its lipophilic analogue LP have the potential to be used as oligodeoxynucleotide delivery systems.

Chen W., Lam S. S., Srinath H., Schiffer C. A., Royer W. E., Jr. and Lin K. (2007) Competition between Ski and CREB-binding protein for binding to Smad proteins in transforming growth factor-beta signaling. *J Biol Chem* **282**, 11365-11376.

Abstract: The family of Smad proteins mediates transforming growth factor-beta (TGF-beta) signaling in cell growth and differentiation. Smads repress or activate TGF-beta signaling by interacting with corepressors (e.g. Ski) or coactivators (e.g. CREB-binding protein (CBP)), respectively. Specifically, Ski has been shown to interfere with the interaction between Smad3 and CBP. However, it is unclear whether Ski competes with CBP for binding to Smads and whether they can interact with Smad3 at the same binding surface on Smad3. We investigated the interactions among purified constructs of Smad, Ski, and

CBP in vitro by size-exclusion chromatography, isothermal titration calorimetry, and mutational studies. Here, we show that Ski-(16-192) interacted directly with a homotrimer of receptor-regulated Smad protein (R-Smad), e.g. Smad2 or Smad3, to form a hexamer; Ski-(16-192) interacted with an R-Smad.Sm4 heterotrimer to form a pentamer. CBP-(1941-1992) was also found to interact directly with an R-Smad homotrimer to form a hexamer and with an R-Smad.Sm4 heterotrimer to form a pentamer. Moreover, these domains of Ski and CBP competed with each other for binding to Smad3. Our mutational studies revealed that domains of Ski and CBP interacted with Smad3 at a portion of the binding surface of the Smad anchor for receptor activation. Our results suggest that Ski negatively regulates TGF-beta signaling by replacing CBP in R-Smad complexes. Our working model suggests that Smad protein activity is delicately balanced by Ski and CBP in the TGF-beta pathway.

Chen X., Liu H., Focia P. J., Shim A. H. and He X. (2008) Structure of macrophage colony stimulating factor bound to FMS: Diverse signaling assemblies of class III receptor tyrosine kinases. *Proc. Natl. Acad. Sci U. S. A* **105**, 18267-18272.

Abstract: Macrophage colony stimulating factor (M-CSF), through binding to its receptor FMS, a class III receptor tyrosine kinase (RTK), regulates the development and function of mononuclear phagocytes, and plays important roles in innate immunity, cancer and inflammation. We report a 2.4 Å crystal structure of M-CSF bound to the first 3 domains (D1-D3) of FMS. The ligand binding mode of FMS is surprisingly different from KIT, another class III RTK, in which the major ligand-binding domain of FMS, D2, uses the CD and EF loops, but not the beta-sheet on the opposite side of the Ig domain as in KIT, to bind ligand. Calorimetric data indicate that M-CSF cannot dimerize FMS without receptor-receptor interactions mediated by FMS domains D4 and D5. Consistently, the structure contains only 1 FMS-D1-D3 molecule bound to a M-CSF dimer, due to a weak, hydrophilic M-CSF:FMS interface, and probably a conformational change of the M-CSF dimer in which binding to the second site is rendered unfavorable by FMS binding at the first site. The partial, intermediate complex suggests that FMS may be activated in two steps, with the initial engagement step distinct from the subsequent dimerization/activation step. Hence, the formation of signaling class III RTK complexes can be diverse, engaging various modes of ligand recognition and various mechanistic steps for dimerizing and activating receptors

Chrencik J. E., Brooun A., Kraus M. L., Recht M. I., Kolatkar A. R., Han G. W., Seifert J. M., Widmer H., Auer M., and Kuhn P. (2006) Structural and biophysical characterization of the EphB4*ephrinB2 protein-protein interaction and receptor specificity. *J Biol Chem* **281**, 28185-28192.

Abstract: Increasing evidence implicates the interaction of the EphB4 receptor with its preferred ligand, ephrinB2, in pathological forms of angiogenesis and in tumorigenesis. To identify the molecular determinants of the unique specificity of EphB4 for ephrinB2, we determined the crystal structure of the ligand binding domain of EphB4 in complex with the extracellular domain of ephrinB2. This structural analysis suggested that one amino acid, Leu-95, plays a particularly important role in defining the structural features that confer the ligand selectivity of EphB4. Indeed, all other Eph receptors, which promiscuously bind many ephrins, have a conserved arginine at the position corresponding to Leu-95 of EphB4. We have also found that amino acid changes in the EphB4 ligand binding cavity, designed based on comparison with the crystal structure of the more promiscuous EphB2 receptor, yield EphB4 variants with altered binding affinity for ephrinB2 and an antagonistic peptide. Isothermal titration calorimetry experiments with an EphB4 Leu-95 to arginine mutant confirmed the importance of this amino acid in conferring high affinity binding to both ephrinB2 and the antagonistic peptide ligand. Isothermal titration calorimetry measurements also revealed an interesting thermodynamic discrepancy between ephrinB2 binding, which is an entropically driven process, and peptide binding, which is an enthalpically driven process. These results provide critical information on the EphB4*ephrinB2 protein interfaces and their mode of interaction, which will facilitate development of small molecule compounds inhibiting the EphB4*ephrinB2 interaction as novel cancer therapeutics.

Chrencik J. E., Brooun A., Recht M. I., Kraus M. L., Koolpe M., Kolatkar A. R., Bruce R. H., Martiny-Baron G., Widmer H., Pasquale E. B., and Kuhn P. (2006) Structure and thermodynamic characterization of the EphB4/Ephrin-B2 antagonist peptide complex reveals the determinants for receptor specificity. *Structure* **14**, 321-330.

Abstract: The Eph receptor tyrosine kinases and their ligands, the ephrins, regulate numerous biological processes in developing and adult tissues and have been implicated in cancer progression and in

pathological forms of angiogenesis. We report the crystal structure of the EphB4 receptor in complex with a highly specific antagonistic peptide at a resolution of 1.65 angstroms. The peptide is situated in a hydrophobic cleft of EphB4 corresponding to the cleft in EphB2 occupied by the ephrin-B2 G-H loop, consistent with its antagonistic properties. Structural analysis identifies several residues within the EphB4 binding cleft that likely determine the ligand specificity of this receptor, while isothermal titration calorimetry experiments with truncated forms of the peptide define the amino acid residues of the peptide that are critical for receptor binding. These studies reveal structural features that will aid drug discovery initiatives to develop EphB4 antagonists for therapeutic applications.

Clark C., Bast D., Sharp A. M., St Hilaire P. M., Agha R., Stein P. E., Toone E. J., Read R. J., and Brunton J. L. (1996) Phenylalanine 30 plays an important role in receptor binding of verotoxin-1. *Mol Microbiol* **19**, 891-899.

Abstract: The homopentameric B subunit of verotoxin 1 (VT1) binds to the glycosphingolipid receptor globotriaosylceramide (Gb3). We produced mutants with alanine substitutions for residues found near the cleft between adjacent subunits. Substitution of alanine for phenylalanine 30 (Phe-30) resulted in a fourfold reduction in B subunit binding affinity for Gb3 and a 10-fold reduction in receptor density in a solid-phase binding assay. The interaction of wild-type and mutant B subunits with Pk trisaccharide in solution was examined by titration microcalorimetry. The carbohydrate binding of the mutant was markedly impaired compared with that of the wild type and was too weak to allow calculation of a binding constant. These results demonstrate that the mutation significantly impaired the carbohydrate-binding function of the B subunit. To ensure that the mutation had not caused a significant change in structure, the mutant B subunit was crystallized and its structure was determined by X-ray diffraction. Difference Fourier analysis showed that its structure was identical to that of the wild type, except for the substitution of alanine for Phe-30. The mutation was also produced in the VT1 operon, and mutant holotoxin was purified to homogeneity. The cytotoxicity of the mutant holotoxin was reduced by a factor of 10^5 compared to that of the wild type in the Vero cell cytotoxicity assay. The results suggest that the aromatic ring of Phe-30 plays a major role in binding of the B subunit to the Gal α 1-4Gal β 1-4Glc trisaccharide portion of Gb3. Examination of the VT1 B crystal structure suggests two potential carbohydrate-binding sites which lie on either side of Phe-30.

Chrencik J. E., Brooun A., Recht M. I., Kraus M. L., Koolpe M., Kolatkar A. R., Bruce R. H., Martiny-Baron G., Widmer H., Pasquale E. B., and Kuhn P. (2006) Structure and thermodynamic characterization of the EphB4/Ephrin-B2 antagonist peptide complex reveals the determinants for receptor specificity. *Structure* **14**, 321-330.

Abstract: The Eph receptor tyrosine kinases and their ligands, the ephrins, regulate numerous biological processes in developing and adult tissues and have been implicated in cancer progression and in pathological forms of angiogenesis. We report the crystal structure of the EphB4 receptor in complex with a highly specific antagonistic peptide at a resolution of 1.65 angstroms. The peptide is situated in a hydrophobic cleft of EphB4 corresponding to the cleft in EphB2 occupied by the ephrin-B2 G-H loop, consistent with its antagonistic properties. Structural analysis identifies several residues within the EphB4 binding cleft that likely determine the ligand specificity of this receptor, while isothermal titration calorimetry experiments with truncated forms of the peptide define the amino acid residues of the peptide that are critical for receptor binding. These studies reveal structural features that will aid drug discovery initiatives to develop EphB4 antagonists for therapeutic applications.

Cooper M. A. (2004) Advances in membrane receptor screening and analysis. *J Mol Recognit* **17**, 286-315.

Abstract: During the last decade there has been significant progress in the development of analytical techniques for the screening of ligand binding to membranes and membrane receptors. This review focuses on developments using label-free assays that facilitate ligand-membrane-receptor screening without the need for chemical-, biological- or radiological-labelled reagents. These assays include acoustic, optical surface plasmon resonance biosensing, sedimentation (analytical ultracentrifugation), chromatographic assays, isothermal titration calorimetry and differential scanning calorimetry. The merits and applications of cell-based screening systems and of different model membrane systems, including planar supported lipid layers, bead-supported membranes and lipid micro-arrays, are discussed. Recent advances involving more established techniques including intrinsic fluorescence, FRET spectroscopy, scintillation proximity assays and automated patch clamping are presented along with applications to peripheral membrane proteins, ion

channels and G protein-coupled receptors. Novel high-throughput assays for determination of drug- and protein-partitioning in membranes are also highlighted. To aid the experimenter, a brief synopsis of the techniques commonly employed to purify and reconstitute membranes and membrane receptors is included.

Corbett P. T., Tong L. H., Sanders J. K., and Otto S. (2005) Diastereoselective amplification of an induced-fit receptor from a dynamic combinatorial library. *J Am Chem Soc* **127**, 8902-8903.

Abstract: A high-affinity, induced-fit receptor for NMe(4)I was discovered using dynamic combinatorial chemistry. The addition of the guest to a dynamic combinatorial library made using a racemic mixture of chiral building blocks caused the strong and highly diastereoselective amplification of the receptor at the expense of other library components. The receptor and its mode of binding were characterized by NMR, ITC, and re-equilibration experiments, from which it was deduced that the receptor probably forms a folded four-stave barrel shape on binding of the guest.

Cunningham B. C., Ultsch M., de Vos A. M., Mulkerrin M. G., Clauser K. R., and Wells J. A. (1991) Dimerization of the extracellular domain of the human growth hormone receptor by a single hormone molecule. *Science* **254**, 821-825.

Abstract: Human growth hormone (hGH) forms a 1:2 complex with the extracellular domain of its receptor-binding protein (hGHbp) as studied by crystallization, size exclusion chromatography, calorimetry, and a previously undescribed fluorescence quenching assay. These and other experiments with protein engineered variants of hGH have led to the identification of the binding determinants for two distinct but adjacent sites on hGH for the hGHbp, and the data indicated that there are two overlapping binding sites on the hGHbp for hGH. Furthermore, the binding of hGH to the hGHbp occurred sequentially; a first hGHbp molecule bound to site 1 on hGH and then a second hGHbp bound to site 2. Hormone-induced receptor dimerization is proposed to be relevant to the signal transduction mechanism for the hGH receptor and other related cytokine receptors.

Davis-Harrison R.L., Armstrong K.M., and Baker B.M. (2005) Two different T cell receptors use different thermodynamic strategies to recognize the same peptide/MHC ligand. *J Mol Biol.* **346**, 533-50. (Erratum in: *J Mol Biol.* 2005 **349**,906.)

Abstract: A6 and B7 are two alphabeta T cell receptors (TCRs) that recognize the Tax peptide presented by the class I major histocompatibility molecule HLA-A2 (Tax/HLA-A2). Despite the fact that the two TCRs have different CDR loops and use different amino acid residues to contact their ligand, both receptors bind ligand with similar diagonal orientations. Here we show that they also bind with very similar binding affinities and kinetics (the $\Delta\Delta G$ degrees for binding is approximately 0.3kcal/mol at 25 degrees C). The two receptors respond similarly to alterations in the MHC molecule, yet differ dramatically in their responses to ionic strength and temperature. The different responses to temperature indicate markedly different binding thermodynamics, which are not predictable from the surface area buried in the interfaces. A6 and B7 thus represent two TCRs that are both compatible with Tax/HLA-A2, although compatibility has been achieved through the use of different thermodynamic strategies. Finally, neither A6 nor B7 are predicted to undergo large conformational adaptations upon binding, distinguishing them from a number of other TCRs whose structure, thermodynamics, and kinetics have been characterized.

Datta A. and Stone M. J. (2003) Soluble mimics of a chemokine receptor: chemokine binding by receptor elements juxtaposed on a soluble scaffold. *Protein Sci* **12**, 2482-2491.

Abstract: Despite the broad biological importance of G protein-coupled receptors (GPCRs), ligand recognition by GPCRs remains poorly understood. To explore the roles of GPCR extracellular elements in ligand binding and to provide a tractable system for structural analyses of GPCR/ligand interactions, we have developed a soluble protein that mimics ligand recognition by a GPCR. This receptor analog, dubbed CROSS5, consists of the N-terminal and third extracellular loop regions of CC chemokine receptor 3 (CCR3) displayed on the surface of a small soluble protein, the B1 domain of Streptococcal protein G. CROSS5 binds to the CCR3 ligand eotaxin with a dissociation equilibrium constant of $2.9 \pm 0.8 \mu\text{M}$ and competes with CCR3 for eotaxin binding. Control proteins indicate that juxtaposition of both CCR3 elements is required for optimal binding to eotaxin. Moreover, the affinities of CROSS5 for a series of eotaxin mutants are highly correlated with the apparent affinities of CCR3 for the same mutants, demonstrating that CROSS5 uses many of the same interactions as does the native receptor. The strategy used to develop CROSS5 could be applied to many other GPCRs, with a variety of potential applications.

Deka R. K., Brautigam C. A., Yang X. F., Blevins J. S., Machius M., Tomchick D. R., and Norgard M. V. (2006) The PnrA (Tp0319; TmpC) lipoprotein represents a new family of bacterial purine nucleoside receptor encoded within an ATP-binding cassette (ABC)-like operon in *Treponema pallidum*. *J Biol Chem* **281**, 8072-8081.

Abstract: *Treponema pallidum*, the bacterial agent of syphilis, cannot be cultivated in vitro. This constraint has severely impeded the study of the membrane biology of this complex human pathogen. A structure-to-function approach thus was adopted as a means of discerning the likely function of Tp0319, a 35-kDa cytoplasmic membrane-associated lipoprotein of *T. pallidum* formerly designated as TmpC. A 1.7-Å crystal structure showed that recombinant Tp0319 (rTp0319) consists of two alpha/beta domains, linked by three crossovers, with a deep cleft between them akin to ATP-binding cassette (ABC) receptors. In the cleft, a molecule of inosine was bound. Isothermal titration calorimetry demonstrated that rTp0319 specifically binds purine nucleosides (dissociation constant (K_d) approximately 10⁻⁷ M). This predilection for purine nucleosides by rTp0319 is consistent with its likely role as a receptor component of a cytoplasmic membrane-associated transporter system. Reverse transcription-PCR analysis of RNA isolated from rabbit tissue-extracted *T. pallidum* additionally showed that tp0319 is transcriptionally linked to four other downstream open reading frames, thereby supporting the existence of an ABC-like operon (tp0319-0323). We herein thus re-name tp0319 as purine nucleoside receptor A (pnrA), with its operonic partners tp0320-0323 designated as pnrB-E, respectively. Our study not only infers that PnrA transports purine nucleosides essential for the survival of *T. pallidum* within its obligate human host, but to our knowledge, this is the first description of an ABC-type nucleoside transport system in any bacterium. PnrA has been grouped with a functionally uncharacterized protein family (HBG016869), thereby implying that other members of the family may have similar nucleoside-binding function(s).

Demarest S. J., Hopp J., Chung J., Hathaway K., Mertsching E., Cao X., George J., Miatkowski K., LaBarre M. J., Shields M., and Kehry M. R. (2006) An intermediate pH unfolding transition abrogates the ability of IgE to interact with its high affinity receptor FcεRIα. *J Biol Chem* **281**, 30755-30767.

Abstract: The interaction between IgE-Fc (Fcε) and its high affinity receptor FcεRI on the surface of mast cells and basophils is a key event in allergen-induced allergic inflammation. Recently, several therapeutic strategies have been developed based on this interaction, and some include Fcε-containing moieties. Unlike well characterized IgG therapeutics, the stability and folding properties of IgE are not well understood. Here, we present comparative biophysical analyses of the pH stability and thermostability of Fcε and IgG1-Fc (Fcγ). Fcε was found to be significantly less stable than Fcγ under all pH and NaCl conditions tested. Additionally, the Cε3Cε4 domains of Fcε were shown to become intrinsically unfolded at pH values below 5.0. The interaction between Fcε and an Fcγ-FcεRIα fusion protein was studied between pH 4.5 and 7.4 using circular dichroism and a combination of differential scanning calorimetry and isothermal titration calorimetry. Under neutral pH conditions, the apparent affinity of Fcε for the dimeric fusion protein was extremely high compared with published values for the monomeric receptor (K_D < 10⁻¹² M). Titration to pH 6.0 did not significantly change the binding affinity, and titration to pH 5.5 only modestly attenuated affinity. At pH values below 5.0, the receptor binding domains of Fcε unfolded, and interaction of Fcε with the Fcγ-FcεRIα fusion protein was abrogated. The unusual pH sensitivity of Fcε may play a role in antigen-dependent regulation of receptor-bound, non-circulating IgE.

Destoumieux-Garzon D., Duquesne S., Peduzzi J., Goulard C., Desmadril M., Letellier L., Rebuffat S., and Boulanger P. (2005) The iron-siderophore transporter FhuA is the receptor for microcin J25. Role of the microcin Val11-Pro16 beta-hairpin region in the recognition mechanism. *Biochem J* **389**, 869-876.

Abstract: The role of the outer membrane iron-transporter FhuA as a potential receptor for microcin J25 (MccJ25) was studied through a series of in vivo and in vitro experiments. The requirement for both FhuA and the inner membrane TonB/ExbB/ExbD complex was evidenced by antibacterial assays using complementation of a fhuA - strain, and by using isogenic strains mutated in the complex encoding genes, respectively. In addition, MccJ25 was shown to block phage T5 infection of *Escherichia coli*, in vivo, by inhibiting phage adhesion, which suggested that MccJ25 prevents the interaction between the phage and its receptor FhuA. This in vivo activity was confirmed in vitro as MccJ25 inhibited phage T5 DNA ejection triggered by purified FhuA. Direct interaction of MccJ25 with FhuA was demonstrated for the first time by size-exclusion chromatography and isothermal titration calorimetry. MccJ25 bound to FhuA with a 2:1

stoichiometry and a K_d of 1.2 μM . Altogether, our results demonstrate that FhuA is the receptor for MccJ25 and that the ligand-receptor interaction may occur in the absence of other components of the bacterial membrane. Finally, both differential scanning calorimetry and antimicrobial assays showed that MccJ25 binding involves FhuA external loops. Contrary to native MccJ25, a thermolysin-cleaved MccJ25 variant was unable to bind to FhuA and failed to prevent phage T5 infection of *E. coli*. Therefore, the MccJ25 Val11-Pro16 beta-hairpin region, which is disrupted upon thermolysin cleavage, is required for microcin recognition.

Deng Y., Zheng Q., Ketas T. J., Moore J. P. and Lu M. (2007) Protein design of a bacterially expressed HIV-1 gp41 fusion inhibitor. *Biochemistry* **46**, 4360-4369.

Abstract: Peptides derived from the carboxyl-terminal heptad repeat of the gp41 envelope glycoprotein ectodomain (C-peptides) can inhibit HIV-1 membrane fusion by binding to the amino-terminal trimeric coiled coil of the same protein. The fusion inhibitory peptide T-20 contains an additional tryptophan-rich sequence motif whose binding site extends beyond the gp41 coiled-coil region yet provides the key determinant of inhibitory activity in T-20. Here we report the design of a recombinant peptide inhibitor (called C52L) that includes both the C-peptide and tryptophan-rich regions. By calorimetry, C52L binds to a peptide mimic of the amino-terminal coiled coil with a K_d of 80 nM, reflecting the large degree of helicity in C52L as measured by circular dichroism spectroscopy. The C52L peptide potently inhibits in vitro infection of human T cells by diverse primary HIV-1 isolates irrespective of coreceptor preference, with nanomolar IC_{50} values. Significantly, C52L is fully active against T-20-resistant variants in a single-cycle HIV-1 infectivity assay. Moreover, because it can be expressed in bacteria, the C52L peptide might be more economical to manufacture on a large scale than T-20-like peptides produced by chemical synthesis. Hence the C52L fusion inhibitor may find a practical application, for example as a vaginal or rectal microbicide to prevent HIV-1 infection in the developing world.

Dey B., Pancera M., Svehla K., Shu Y., Xiang S. H., Vainshtein J., Li Y., Sodroski J., Kwong P. D., Mascola J. R. and Wyatt R. (2007) Characterization of human immunodeficiency virus type 1 monomeric and trimeric gp120 glycoproteins stabilized in the CD4-bound state: antigenicity, biophysics, and immunogenicity. *J Virol.* **81**, 5579-5593.

Abstract: The human immunodeficiency virus type 1 exterior gp120 envelope glycoprotein is highly flexible, and this flexibility may contribute to the inability of monomeric gp120 immunogens to elicit broadly neutralizing antibodies. We previously showed that an S375W modification of a critical interfacial cavity central to the primary receptor binding site, the Phe43 cavity, stabilizes gp120 into the CD4-bound state. However, the immunological effects of this cavity-altering replacement were never tested. Subsequently, we screened other mutations that, along with the S375W alteration, might further stabilize the CD4-bound state. Here, we define a selected second cavity-altering replacement, T257S, and analyze the double mutations in several gp120 envelope glycoprotein contexts. The gp120 glycoproteins with the T257S-plus-S375W double mutation (T257S+S375W) have a superior antigenic profile compared to the originally identified single S375W replacement in terms of enhanced recognition by the broadly neutralizing CD4 binding-site antibody b12. Isothermal titration calorimetry measuring the entropy of the gp120 interaction with CD4 indicated that the double mutant was also stabilized into the CD4-bound state, with increasing relative fixation between core, full-length monomeric, and full-length trimeric versions of gp120. A significant increase in gp120 affinity for CD4 was also observed for the cavity-filling mutants relative to wild-type gp120. The most conformationally constrained T257S+S375W trimeric gp120 proteins were selected for immunogenicity analysis in rabbits and displayed a trend of improvement relative to their wild-type counterparts in terms of eliciting neutralizing antibodies. Together, the results suggest that conformational stabilization may improve the ability of gp120 to elicit neutralizing antibodies.

Ding Z., Wang H., Liang X., Morris E. R., Gallazzi F., Pandit S., Skolnick J., Walker J. C. and Van D., Sr. (2007) Phosphoprotein and phosphopeptide interactions with the FHA domain from Arabidopsis kinase-associated protein phosphatase. *Biochemistry* **46**, 2684-2696.

Abstract: FHA domains are phosphoThr recognition modules found in diverse signaling proteins, including kinase-associated protein phosphatase (KAPP) from *Arabidopsis thaliana*. The kinase-interacting FHA domain (KI-FHA) of KAPP targets it to function as a negative regulator of some receptor-like kinase (RLK) signaling pathways important in plant development and environmental responses. To aid in the identification of potential binding sites for the KI-FHA domain, we predicted (i) the structure of a

representative KAPP-binding RLK, CLAVATA1, and (ii) the functional surfaces of RLK kinase domains using evolutionary trace analysis. We selected phosphopeptides from KAPP-binding Arabidopsis RLKs for in vitro studies of association with KI-FHA from KAPP. Three phosphoThr peptide fragments from the kinase domain of CLV1 or BAK1 were found to bind KI-FHA with KD values of 8-20 microM, by NMR or titration calorimetry. Their affinity is driven by favorable enthalpy and solvation entropy gain. Mutagenesis of these three threonine sites suggests Thr546 in the C-lobe of the BAK1 kinase domain to be a principal but not sole site of KI-FHA binding in vitro. The brassinosteroid receptor BRI1 and KAPP are shown to associate in vivo and in vitro. Further genetic studies indicate that KAPP may be a negative regulator of the BRI1 signaling transduction pathway. 15N-Labeled KI-FHA was titrated with the GST-BRI1 kinase domain and monitored by NMR. BRI1 interacts with the same 3/4, 4/5, 6/7, 8/9, and 10/11 recognition loops of KI-FHA, with similar affinity as the phosphoThr peptides.

Doyle M. L., Tian S. S., Miller S. G., Kessler L., Baker A. E., Brigham-Burke M. R., Dillon S. B., Duffy K. J., Keenan R. M., Lehr R., Rosen J., Schneeweis L. A., Trill J., Young P. R., Luengo J. I., and Lamb P. (2003) Selective binding and oligomerization of the murine granulocyte colony-stimulating factor receptor by a low molecular weight, nonpeptidyl ligand. *J Biol Chem* **278**, 9426-9434.

Abstract: Granulocyte colony-stimulating factor regulates neutrophil production by binding to a specific receptor, the granulocyte colony-stimulating factor receptor, expressed on cells of the granulocytic lineage. Recombinant forms of granulocyte colony-stimulating factor are used clinically to treat neutropenias. As part of an effort to develop granulocyte colony-stimulating factor mimics with the potential for oral bioavailability, we previously identified a nonpeptidyl small molecule (SB-247464) that selectively activates murine granulocyte colony-stimulating factor signal transduction pathways and promotes neutrophil formation in vivo. To elucidate the mechanism of action of SB-247464, a series of cell-based and biochemical assays were performed. The activity of SB-247464 is strictly dependent on the presence of zinc ions. Titration microcalorimetry experiments using a soluble murine granulocyte colony-stimulating factor receptor construct show that SB-247464 binds to the extracellular domain of the receptor in a zinc ion-dependent manner. Analytical ultracentrifugation studies demonstrate that SB-247464 induces self-association of the N-terminal three-domain fragment in a manner that is consistent with dimerization. SB-247464 induces internalization of granulocyte colony-stimulating factor receptor on intact cells, consistent with a mechanism involving receptor oligomerization. These data show that small nonpeptidyl compounds are capable of selectively binding and inducing productive oligomerization of cytokine receptors.

Eldredge J., Berkowitz S., Corin A. F., Day E. S., Hayes D., Meier W., Strauch K., Zafari M., Tadi M., and Farrington G. K. (2006) Stoichiometry of LTbetaR binding to LIGHT. *Biochemistry* **45**, 10117-10128.

Abstract: LTbetaR is a member of the TNF receptor family of proteins. It binds to two different cell surface ligands, LIGHT, a homotypic trimer, and LTalpha1beta2, a heterotypic trimer. We have measured the affinities of the dimeric IgG fusion protein, LTbetaRIgG, and monomeric LTbetaR protein binding to both LIGHT and LTalpha1beta2 using surface plasmon resonance and found values of <0.1 and 38 nM for LIGHT and <0.1 and 48 nM for LTalpha1beta2, respectively. We also determined the stoichiometries of binding for both forms of the receptor LTbetaRIgG and LTbetaR binding to LIGHT. The data obtained from several biophysical methods are consistent with receptor polypeptide to trimeric ligand ratios of 2:1. The determined masses of the complexes using SEC-LS corresponded to a single LTbetaRIgG bound to a LIGHT trimer, or two LTbetaR bound per LIGHT. Sedimentation velocity of varied ratios of LTbetaR to a fixed concentration of LIGHT were analyzed by SEDANAL and were successfully fit with a model with two tight binding sites on LIGHT and one poor affinity site. Isothermal calorimetric titration of LIGHT with either LTbetaR or LTbetaRIgG also demonstrated stoichiometries of 1:2 and 1:1, respectively. The binding of LTbetaR to LIGHT was endothermic and, hence, entropy-driven. TNFR p55 (extracellular domain) complexed with the trimeric ligand, TNFbeta, exhibits a 3:1 receptor/ligand stoichiometry. This complex has been used as the prototypical model setting the receptor-ligand complexation paradigm for the entire TNF family. The LTbetaR/LIGHT binding stoichiometry of 2:1 demonstrated here does not fit the paradigm. This has numerous implications for cell biology including signaling requiring only dimerization of LTbetaR rather than trimerization as expected from the structural paradigm.

Ely L.K., Beddoe T., Clements C.S., Matthews J.M., Purcell A.W., Kjer-Nielsen L., McCluskey J., and Rossjohn J. (2006) Disparate thermodynamics governing T cell receptor-MHC-I interactions implicate extrinsic factors in guiding MHC restriction. *Proc Natl Acad Sci U S A*. **103**, 6641-6.

Abstract: The underlying basis of major histocompatibility complex (MHC) restriction is unclear. Nevertheless, current data suggest that a common thermodynamic signature dictates alphabeta T cell receptor (TcR) ligation. To evaluate whether this thermodynamic signature defines MHC restriction, we have examined the thermodynamic basis of a highly characterized immunodominant TcR interacting with its cognate peptide-MHC-I ligand. Surprisingly, we observed this interaction to be governed by favorable enthalpic and entropic forces, which is in contrast to the prevailing generality, namely, enthalpically driven interactions combined with markedly unfavorable entropic forces. We conclude that extrinsic molecular factors, such as coreceptor ligation, conformational adjustments involved in TcR signaling, or constraints dictated by higher-order arrangement of ligated TcRs, might play a greater role in guiding MHC restriction than appreciated previously.

Evans L. J., Labeit S., Cooper A., Bond L. H., and Lakey J. H. (1996) The central domain of colicin N possesses the receptor recognition site but not the binding affinity of the whole toxin. *Biochemistry* **35**, 15143-15148.

Abstract: Colicin N is a three-domain pore-forming colicin which kills enterobacterial cells following an initial binding to its receptor, the outer membrane porin OmpF. The receptor-binding domain of colicin N alone, and attached to the translocation domain, was overexpressed and purified using a hexahistidine tag. The receptor domain attached to the pore-forming domain was obtained by enzymatic digestion. Circular dichroism spectroscopy showed that the domains have structure in keeping with the known structure of colicin N. The receptor domain was stable, retaining both secondary and tertiary structure in 2 M guanidine hydrochloride and at low pH. It bound to both OmpF and PhoE porin-producing *Escherichia coli* with no toxicity and protected sensitive *E. coli* against intact colicin N toxicity at high domain/ colicin N ratios. Its *in vitro* affinity for OmpF, as determined by isothermal titration microcalorimetry, was found to be approximately 50-fold weaker than that of native colicin N. The receptor domain was readily out-competed by native colicin N in *in vivo* fluorescence assays which, coupled with its structural stability, suggests that its interaction with OmpF is one of weak, reversible binding. Since neither of the double domain constructs shows wild-type binding affinity either, it appears that the molecular recognition is a property of the receptor domain but that affinity is influenced by the entire molecule.

Evans L. J., Cooper A., and Lakey J. H. (1996) Direct measurement of the association of a protein with a family of membrane receptors. *J Mol Biol* **255**, 559-563.

Abstract: A specific receptor is a requirement for most protein toxins and OmpF, a trimeric porin, was previously considered to be the unique membrane-receptor for colicin N. We show by qualitative *in vivo* analysis that the related porins OmpC or PhoE act as much less effective receptors. To elucidate receptor function, the *in vitro* binding of the 42 kDa toxin to each of the 120 kDa porin trimers was determined quantitatively using isothermal titration calorimetry. Colicin N binds to OmpF with K_a approximately $5 \times 10^5 \text{ M}^{-1}$ and a stoichiometry consistent with about three per trimer but it also binds to PhoE and OmpC with surprisingly similar affinities and stoichiometry. However, thermodynamic analysis of these hitherto unmeasured interactions suggests an unexpected entropic difference between these protein import receptors.

Farooq A., Plotnikova O., Zeng L., and Zhou M. M. (1999) Phosphotyrosine binding domains of Shc and insulin receptor substrate 1 recognize the NPXpY motif in a thermodynamically distinct manner. *J Biol Chem* **274**, 6114-6121.

Abstract: Phosphotyrosine binding (PTB) domains of the adaptor protein Shc and insulin receptor substrate (IRS-1) interact with a distinct set of activated and tyrosine-phosphorylated cytokine and growth factor receptors and play important roles in mediating mitogenic signal transduction. By using the technique of isothermal titration calorimetry, we have studied the thermodynamics of binding of the Shc and IRS-1 PTB domains to tyrosine-phosphorylated NPXY-containing peptides derived from known receptor binding sites. The results showed that relative contributions of enthalpy and entropy to the free energy of binding are dependent on specific phosphopeptides. Binding of the Shc PTB domain to tyrosine-phosphorylated peptides from TrkA, epidermal growth factor, ErbB3, and insulin receptors is achieved via an overall entropy-driven reaction. On the other hand, recognition of the phosphopeptides of insulin and interleukin-4 receptors by the IRS-1 PTB domain is predominantly an enthalpy-driven process. Mutagenesis and amino acid substitution experiments showed that in addition to the tyrosine-phosphorylated NPXY motif, the PTB domains of Shc and IRS-1 prefer a large hydrophobic residue at pY-

5 and a small hydrophobic residue at pY-1, respectively (where pY is phosphotyrosine). These results agree with the calculated solvent accessibility of these two key peptide residues in the PTB domain/peptide structures and support the notion that the PTB domains of Shc and IRS-1 employ functionally distinct mechanisms to recognize tyrosine-phosphorylated receptors.

Feese M. D., Tamada T., Kato Y., Maeda Y., Hirose M., Matsukura Y., Shigematsu H., Muto T., Matsumoto A., Watarai H., Ogami K., Tahara T., Kato T., Miyazaki H., and Kuroki R. (2004) Structure of the receptor-binding domain of human thrombopoietin determined by complexation with a neutralizing antibody fragment. *Proc Natl Acad Sci U S A* **101**, 1816-1821.

Abstract: The cytokine thrombopoietin (TPO), the ligand for the hematopoietic receptor c-Mpl, acts as a primary regulator of megakaryocytopoiesis and platelet production. We have determined the crystal structure of the receptor-binding domain of human TPO (hTPO(163)) to a 2.5-Å resolution by complexation with a neutralizing Fab fragment. The backbone structure of hTPO(163) has an antiparallel four-helix bundle fold. The neutralizing Fab mainly recognizes the C-D crossover loop containing the species invariant residue Q111. Titration calorimetric experiments show that hTPO(163) interacts with soluble c-Mpl containing the extracellular cytokine receptor homology domains with 1:2 stoichiometry with the binding constants of $3.3 \times 10^9 \text{ M}^{-1}$ and $1.1 \times 10^6 \text{ M}^{-1}$. The presence of the neutralizing Fab did not inhibit binding of hTPO(163) to soluble c-Mpl fragments, but the lower-affinity binding disappeared. Together with prior genetic data, these define the structure-function relationships in TPO and the activation scheme of c-Mpl.

Fernando H., Chin C., Rosgen J., and Rajarathnam K. (2004) Dimer dissociation is essential for interleukin-8 (IL-8) binding to CXCR1 receptor. *J Biol Chem* **279**, 36175-36178.

Abstract: Chemokines play a fundamental role in trafficking of immune cells and in host defense against infection. The role of chemokines in the recruitment process is highly regulated spatially and temporally and involves interactions with G protein-coupled receptors and cell surface glycosaminoglycans. The dynamic equilibrium between chemokine monomers and dimers, both free in solution and in cell surface-bound forms, regulates different components of recruitment such as chemotaxis and receptor signaling. The binding and activity of the chemokine interleukin-8 (IL-8) for its receptors, previously studied using "trapped" non-associating monomers and non-dissociating dimers, show that the monomer has a native-like function but support conflicting roles for the dimer. We have measured the binding of native IL-8 to the CXCR1 N-domain, using isothermal titration calorimetry and sedimentation equilibrium techniques. The N-domain constitutes a critical binding site, and IL-8 binding affinity to the receptor N-domain is in the same concentration range as the IL-8 monomer-dimer equilibrium. We observed that only the IL-8 monomer, and not the dimer, is competent in binding the receptor N-domain. Based on our results, we propose that IL-8 dimerization functions as a negative regulator for the receptor function and as a positive regulator for binding to glycosaminoglycans and that both play a role in the neutrophil recruitment process.

Fernando H., Nagle G. T., and Rajarathnam K. (2007) Thermodynamic characterization of interleukin-8 monomer binding to CXCR1 receptor N-terminal domain. *FEBS J* **274**, 241-251.

Abstract: Chemokines elicit their function by binding receptors of the G-protein-coupled receptor class, and the N-terminal domain (N-domain) of the receptor is one of the two critical ligand-binding sites. In this study, the thermodynamic basis for binding of the chemokine interleukin-8 (IL-8) to the N-domain of its receptor CXCR1 was characterized using isothermal titration calorimetry. We have shown previously that only the monomer of IL-8, and not the dimer, functions as a high-affinity ligand, so in this study we used the IL-8(1-66) deletion mutant which exists as a monomer. Calorimetry data indicate that the binding is enthalpically favored and entropically disfavored, and a negative heat capacity change indicates burial of hydrophobic residues in the complex. A characteristic feature of chemokine receptor N-domains is the large number of acidic residues, and experiments using different buffers show no net proton transfer, indicating that the CXCR1 N-domain acidic residues are not protonated in the binding process. CXCR1 N-domain peptide is unstructured in the free form but adopts a more defined structure in the bound form, and so binding is coupled to induction of the structure of the N-domain. Measurements in the presence of the osmolyte, trimethylamine N-oxide, which induces the structure of unfolded proteins, show that formation of the coupled N-domain structure involves only small ΔH and ΔS changes. These results together indicate that the binding is driven by packing interactions in the complex that are enthalpically favored, and

are consistent with the observation that the N-domain binds in an extended form and interacts with multiple IL-8 N-loop residues over a large surface area.

Fisher C., Abdul-Aziz D., and Blacklow S. C. (2004) A two-module region of the low-density lipoprotein receptor sufficient for formation of complexes with apolipoprotein E ligands. *Biochemistry* **43**, 1037-1044.
Abstract: The low-density lipoprotein (LDL) receptor transports two different classes of cholesterol-carrying lipoprotein particles into cells: LDL particles, which contain a single copy of apolipoprotein B-100 (apoB-100), and beta-migrating very low-density lipoprotein (beta-VLDL) particles, which contain multiple copies of apolipoprotein E (apoE). The ligand-binding domain of the receptor lies at its amino-terminal end within seven adjacent LDL-A repeats (LA1-LA7). Although prior work clearly establishes that LA5 is required for high-affinity binding of particles containing apolipoprotein E (apoE), the number of ligand-binding repeats sufficient to bind apoE ligands has not yet been determined. Similarly, uncertainty exists as to whether a single lipid-activated apoE receptor-binding site within a particle is capable of binding to the LDLR with high affinity or whether more than one is required. Here, we establish that the LA4-5 two-repeat pair is sufficient to bind apoE-containing ligands, on the basis of binding studies performed with a series of LDLR-derived "minireceptors" containing up to four repeats. Using single chain multimers of the apoE receptor-binding domain (N-apoE), we also show that more than one receptor-binding site in its lipid-activated conformation is required to bind to the LDLR with high affinity. Thus, in addition to inducing a conformational change in the structure of N-apoE, lipid association enhances the affinity of apoE for the LDLR in part by creating a multivalent ligand.

Fokkens M., Schrader T., and Klarner F. G. (2005) A molecular tweezer for lysine and arginine. *J Am Chem Soc* **127**, 14415-14421.

Abstract: Lysine and arginine play a key role in numerous biological recognition processes controlling, inter alia, gene regulation, glycoprotein targeting and vesicle transport. They are also found in signaling peptide sequences responsible, e.g. for bacterial cell wall biosynthesis, Alzheimer peptide aggregation and skin regeneration. Almost none of all artificial receptor structures reported to date are selective and efficient for lysine residues in peptides or proteins. An artificial molecular tweezer is introduced which displays an exceptionally high affinity for lysine (K(a) approximately 5000 in neutral phosphate buffer). It features an electron-rich torus-shaped cavity adorned with two peripheral anionic phosphonate groups. Exquisite selectivity for arginine and lysine is achieved by threading the whole amino acid side chain through the cavity and subsequent locking by formation of a phosphonate-ammonium/guanidinium salt bridge. This pseudorotaxane-like geometry is also formed in small basic signaling peptides, which can be bound with unprecedented affinity in buffered aqueous solution. NMR titrations, NOESY and VT experiments as well as ITC measurements and Monte Carlo simulations unanimously point to an enthalpy-driven process utilizing a combination of van der Waals interactions and substantial electrostatic contributions for a conformational lock. Since DMSO and acetonitrile compete with the amino acid guest inside the cavity, a simple change in the cosolvent composition renders the whole complexation process reversible.

Gokce I., Raggett E. M., Hong Q., Virden R., Cooper A., and Lakey J. H. (2000) The TolA-recognition site of colicin N. ITC, SPR and stopped-flow fluorescence define a crucial 27-residue segment. *J Mol Biol* **304**, 621-632.

Abstract: Colicins translocate across the *Escherichia coli* outer membrane and periplasm by interacting with several receptors. After first binding to the outer membrane surface receptors via their central region, they interact with TolA or TonB proteins via their N-terminal region. Colicin N residues critical to TolA binding have been discovered, but the full extent of any colicin TolA site is unknown. We present, for the first time, a fully mapped TolA binding site for a colicin. It was determined through the use of alanine-scanning mutants, glutathione S-transferase fusion peptides and Biacore/fluorescence binding studies. The minimal TolA binding region is 27 residues and of similar size to the TolA binding region of bacteriophage g3p-D1 protein. Stopped-flow kinetic studies show that the binding to TolA follows slow association kinetics. The role of other *E. coli* Tol proteins in colicin translocation was also investigated. Isothermal titration microcalorimetry (ITC) and in vivo studies conclusively show that colicin N translocation does not require the presence of TolB. ITC also demonstrated colicin A interaction with TolB, and that colicin A in its native state does not interact with TolAII-III. Colicin N does not bind TolR-II. The TolA protein is shown to be unsuitable for direct immobilisation in Biacore analysis.

Grauschopf U., Lilie H., Honold K., Wozny M., Reusch D., Esswein A., Schafer W., Rucknagel K. P., and Rudolph R. (2000) The N-terminal fragment of human parathyroid hormone receptor 1 constitutes a hormone binding domain and reveals a distinct disulfide pattern. *Biochemistry* **39**, 8878-8887.

Abstract: The N-terminal extracellular parts of human G-protein coupled receptor class B, for example, receptors for secretin, glucagon, or parathyroid hormone, are involved in ligand binding. To obtain structural and functional information on the N-terminal receptor fragment of human parathyroid hormone receptor 1 (PTHr1), the truncated receptor was expressed in the cytosol of *Escherichia coli* in the form of inclusion bodies. Oxidative refolding of inclusion body material resulted in stable, soluble, monomeric protein. Ligand binding was proved by surface plasmon resonance spectroscopy and isothermal titration calorimetry. Refolded receptor fragment was able to bind parathyroid hormone with an apparent dissociation constant of 3-5 μ M. Far-UV circular dichroism spectra showed that the refolded polypeptide contained approximately 25% alpha-helical and 23% beta-sheet secondary structures. Analysis of the disulfide bond pattern of the refolded receptor fragment revealed disulfide bonds between Cys170 and Cys131, Cys148 and Cys108, and Cys117 and Cys48. These results demonstrate that the extracellular N-terminal domain of the parathyroid hormone receptor (PTHr1) possesses a well-defined, stable conformation, which shows a significant ligand binding activity.

Hachet-Haas M., Balabanian K., Rohmer F., Pons F., Franchet C., Lecat S., Chow K. Y., Dagher R., Gizzi P., Didier B., Lagane B., Kellenberger E., Bonnet D., Baleux F., Haiech J., Parmentier M., Frossard N., renzana-Seisdedos F., Hibert M. and Galzi J. L. (2008) Small neutralizing molecules to inhibit actions of the chemokine CXCL12. *J Biol Chem* **283**, 23189-23199.

Abstract: The chemokine CXCL12 and the receptor CXCR4 play pivotal roles in normal vascular and neuronal development, in inflammatory responses, and in infectious diseases and cancer. For instance, CXCL12 has been shown to mediate human immunodeficiency virus-induced neurotoxicity, proliferative retinopathy and chronic inflammation, whereas its receptor CXCR4 is involved in human immunodeficiency virus infection, cancer metastasis and in the rare disease known as the warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis (WHIM) syndrome. As we screened chemical libraries to find inhibitors of the interaction between CXCL12 and the receptor CXCR4, we identified synthetic compounds from the family of chalcones that reduce binding of CXCL12 to CXCR4, inhibit calcium responses mediated by the receptor, and prevent CXCR4 internalization in response to CXCL12. We found that the chemical compounds display an original mechanism of action as they bind to the chemokine but not to CXCR4. The highest affinity molecule blocked chemotaxis of human peripheral blood lymphocytes *ex vivo*. It was also active *in vivo* in a mouse model of allergic eosinophilic airway inflammation in which we detected inhibition of the inflammatory infiltrate. The compound showed selectivity for CXCL12 and not for CCL5 and CXCL8 chemokines and blocked CXCL12 binding to its second receptor, CXCR7. By analogy to the effect of neutralizing antibodies, this molecule behaves as a small organic neutralizing compound that may prove to have valuable pharmacological and therapeutic potential

Handel, T. M., Johnson Z., Crown S.E., Lau E.K., Sweeney M., and Proudfoot A.E.(2005) REGULATION OF PROTEIN FUNCTION BY GLYCOSAMINOGLYCANS—AS EXEMPLIFIED BY CHEMOKINES. *Annual Review of Biochemistry* **74**, 385-410.

Abstract: Immune modulators such as cytokines and growth factors exert their biological activity through high-affinity interactions with cell-surface receptors, thereby activating specific signaling pathways. However, many of these molecules also participate in low-affinity interactions with another class of molecules, referred to as proteoglycans. Proteoglycans consist of a protein core to which glycosaminoglycan (GAG) chains are attached. The GAGs are long, linear, sulfated, and highly charged heterogeneous polysaccharides that are expressed throughout the body in different forms, depending on the developmental or pathological state of the organ/organism. They participate in many biological functions, including organogenesis and growth control, cell adhesion, signaling, inflammation, tumorigenesis, and interactions with pathogens. Recently, it was demonstrated that certain chemokines require interactions with GAGs for their *in vivo* function. The GAG interaction is thought to provide a mechanism for retaining chemokines on cell surfaces, facilitating the formation of chemokine gradients. These gradients serve as directional cues to guide the migration of the appropriate cells in the context of their inflammatory, developmental, and homeostatic functions. In this review, we discuss GAGs and their interaction with proteins, with a special emphasis on the chemokine system.

Harrington, A.E., Morris-Triggs, S.A., Ruotolo, B.T., Robinson, C.V., Ohnuma, S.I., and Hyvönen, M. (2006) Structural basis for the inhibition of activin signalling by follistatin. *EMBO J* **25**, 1035–1045.

Abstract: The secreted, multidomain protein follistatin binds activins with high affinity, inhibiting their receptor interaction. We have dissected follistatin's domain structure and shown that the minimal activin-inhibiting fragment of follistatin is comprised of the first and second Fs domains (Fs12). This protein can bind to activin dimer and form a stable complex containing two Fs12 molecules and one activin dimer. We have solved crystal structures of activin A alone and its complex with Fs12 fragment to 2 Å resolution. The complex structure shows how Fs12 molecules wrap around the back of the 'wings' of activin, blocking the type II receptor-binding site on activin A. Arginine 192 in Fs2 is a key residue in this interaction, inserting itself in between activin's fingers. Complex formation imposes a novel orientation for the EGF- and Kazal-like subdomains in the Fs2 domain and activin A shows further variation from the canonical TGF- β family fold. The structure provides a detailed description of the inhibitory mechanism and gives insights into interactions of follistatin with other TGF- β family proteins.

He X., Chow D., Martick M. M., and Garcia K. C. (2001) Allosteric activation of a spring-loaded natriuretic peptide receptor dimer by hormone. *Science* **293**, 1657-1662.

Abstract: Natriuretic peptides (NPs) are vasoactive cyclic-peptide hormones important in blood pressure regulation through interaction with natriuretic cell-surface receptors. We report the hormone-binding thermodynamics and crystal structures at 2.9 and 2.0 angstroms, respectively, of the extracellular domain of the unliganded human NP receptor (NPR-C) and its complex with CNP, a 22-amino acid NP. A single CNP molecule is bound in the interface of an NPR-C dimer, resulting in asymmetric interactions between the hormone and the symmetrically related receptors. Hormone binding induces a 20 angstrom closure between the membrane-proximal domains of the dimer. In each monomer, the opening of an interdomain cleft, which is tethered together by a linker peptide acting as a molecular spring, is likely a conserved allosteric trigger for intracellular signaling by the natriuretic receptor family.

He X. L. and Garcia K. C. (2004) Structure of nerve growth factor complexed with the shared neurotrophin receptor p75. *Science* **304**, 870-875.

Abstract: Neurotrophins are secreted growth factors critical for the development and maintenance of the vertebrate nervous system. Neurotrophins activate two types of cell surface receptors, the Trk receptor tyrosine kinases and the shared p75 neurotrophin receptor. We have determined the 2.4 Å crystal structure of the prototypic neurotrophin, nerve growth factor (NGF), complexed with the extracellular domain of p75. Surprisingly, the complex is composed of an NGF homodimer asymmetrically bound to a single p75. p75 binds along the homodimeric interface of NGF, which disables NGF's symmetry-related second p75 binding site through an allosteric conformational change. Thus, neurotrophin signaling through p75 may occur by disassembly of p75 dimers and assembly of asymmetric 2:1 neurotrophin/p75 complexes, which could potentially engage a Trk receptor to form a trimolecular signaling complex.

Heerklotz H. H., Binder H., and Epanand R. M. (1999) A "release" protocol for isothermal titration calorimetry. *Biophys J* **76**, 2606-2613.

Abstract: Isothermal titration calorimetry (ITC) has become a standard method for investigating the binding of ligands to receptor molecules or the partitioning of solutes between water and lipid vesicles. Accordingly, solutes are mixed with membranes (or ligands with receptors), and the subsequent heats of incorporation (or binding) are measured. In this paper we derive a general formula for modeling ITC titration heats in both binding and partitioning systems that allows for the modeling of the classic incorporation or binding protocols, as well as of new protocols assessing the release of solute from previously solute-loaded vesicles (or the dissociation of ligand/receptor complexes) upon dilution. One major advantage of a simultaneous application of the incorporation/binding and release protocols is that it allows for the determination of whether a ligand is able to access the vesicle interior within the time scale of the ITC experiment. This information cannot be obtained from a classical partitioning experiment, but it must be known to determine the partition coefficient (or binding constant and stoichiometry) and the transfer enthalpy. The approach is presented using the partitioning of the nonionic detergent C12EO7 to palmitoyloleoylphosphatidylcholine vesicles. The release protocol could also be advantageous in the case of receptors that are more stable in the ligand-saturated rather than the ligand-depleted state.

Hensley P., Doyle M. L., Myszka D. G., Woody R. W., Brigham-Burke M. R., Erickson-Miller C. L., Griffin C. A., Jones C. S., McNulty D. E., O'Brien S. P., Amegadzie B. Y., MacKenzie L., Ryan M. D., and Young P. R. (2000) Evaluating energetics of erythropoietin ligand binding to homodimerized receptor extracellular domains. *Methods Enzymol* **323**, 177-207.

Hippler-Mreyen S., Klare J. P., Wegener A. A., Seidel R., Herrmann C., Schmie G., Nagel G., Bamberg E., and Engelhard M. (2003) Probing the sensory rhodopsin II binding domain of its cognate transducer by calorimetry and electrophysiology. *J Mol Biol* **330**, 1203-1213.

Abstract: Sensory rhodopsin II, a repellent phototaxis receptor from *Natronobacterium pharaonis* (NpSR_{II}) forms a tight complex with its cognate transducer (NpHtr_{II}). Light excitation of the receptor triggers conformational changes in both proteins, thereby activating the cellular two-component signalling cascade. In membranes, the two proteins form a 2:2 complex, which dissociates to a 1:1 heterodimer in micelles. Complexed to the transducer sensory rhodopsin II is no longer capable of light-driven proton pumping. In order to elucidate the dimerisation and the size of the receptor-binding domain of the transducer, isothermal titration calorimetry and electrophysiological experiments have been carried out. It is shown, that an N-terminal sequence of 114 amino acid residues is sufficient for tight binding ($K_d=240\text{nM}$; $\Delta H=-17.6\text{kJmol}^{-1}$) and for inhibiting the proton transfer. These data and results obtained from selected site-directed mutants indicate a synergistic interplay of transducer transmembrane domain (1-82) and cytoplasmic peptide (83-114) leading to an optimal and specific interaction between receptor and transducer.

Hodel M. R., Corbett A. H., and Hodel A. E. (2001) Dissection of a nuclear localization signal. *J Biol Chem* **276**, 1317-1325.

Abstract: The regulated process of protein import into the nucleus of a eukaryotic cell is mediated by specific nuclear localization signals (NLSs) that are recognized by protein import receptors. This study seeks to decipher the energetic details of NLS recognition by the receptor importin alpha through quantitative analysis of variant NLSs. The relative importance of each residue in two monopartite NLS sequences was determined using an alanine scanning approach. These measurements yield an energetic definition of a monopartite NLS sequence where a required lysine residue is followed by two other basic residues in the sequence K(K/R)X(K/R). In addition, the energetic contributions of the second basic cluster in a bipartite NLS (approximately 3 kcal/mol) as well as the energy of inhibition of the importin alpha importin beta-binding domain (approximately 3 kcal/mol) were also measured. These data allow the generation of an energetic scale of nuclear localization sequences based on a peptide's affinity for the importin alpha-importin beta complex. On this scale, a functional NLS has a binding constant of approximately 10 nM, whereas a nonfunctional NLS has a 100-fold weaker affinity of 1 μM. Further correlation between the current in vitro data and in vivo function will provide the foundation for a comprehensive quantitative model of protein import.

Housden N. G., Loftus S. R., Moore G. R., James R., and Kleanthous C. (2005) Cell entry mechanism of enzymatic bacterial colicins: porin recruitment and the thermodynamics of receptor binding. *Proc Natl Acad Sci U S A* **102**, 13849-13854.

Abstract: Binding of enzymatic E colicins to the vitamin B12 receptor, BtuB, is the first stage in a cascade of events that culminate in the translocation of the cytotoxic nuclease into the *Escherichia coli* cytoplasm and release of its tightly bound immunity protein. A dogma of colicin biology is that the toxin coiled-coil connecting its functional domains must unfold or unfurl to span the periplasm, with recent reports claiming this reaction is initiated by receptor binding. We report isothermal titration calorimetry data of BtuB binding the endonuclease toxin Cole9 and a disulfide form (Cole9S-S) where unfolding of the coiled-coil is prevented and, as a consequence, the toxin is biologically inactive. Contrary to expectation, the thermodynamics of receptor binding, characterized by large negative values for ΔH , are identical for the two colicins, arguing against any form of BtuB-induced unfolding. We go on to delineate key features of the "colicin translocon" that assembles at the cell surface after BtuB binding by using a complex of histidine-tagged Im9 bound to Cole9S-S. First, we show that the porin OmpF is recruited directly to the BtuB.colicin complex to form the translocon. Second, recruitment is through the natively unfolded region of the colicin translocation domain, with this domain likely having two contact points for OmpF. Finally, the immunity protein is not released during its assembly. Our study demonstrates that although colicin unfolding is undoubtedly a prerequisite for *E. coli* cell death, it must occur after assembly of the translocon.

Houtman J.C., Barda-Saad M., and Samelson L.E. (2005) Examining multiprotein signaling complexes from all angles. *FEBS J.* **272**, 5426-35.

Abstract: Dynamic protein-protein interactions are involved in most physiological processes and, in particular, for the formation of multiprotein signaling complexes at transmembrane receptors, adapter proteins and effector molecules. Because the unregulated induction of signaling complexes has substantial clinical relevance, the investigation of these complexes is an active area of research. These studies strive to answer questions about the composition and function of multiprotein signaling complexes, along with the molecular mechanisms of their formation. In this review, the adapter protein, linker for activation of T cells (LAT), will be employed as a model to exemplify how signaling complexes are characterized using a range of techniques. The intensive investigation of LAT highlights how the systematic use of complementary techniques leads to an integrated understanding of the formation, composition and function of multiprotein signaling complexes that occur at receptors, adapter proteins and effector molecules.

Houtman, J.C.D., Yamaguchi, H., Barda-Saad, M., Braiman, A., Bowden, B., Appella, E., Schuck, P., and Samelson, L.E. (2006) Oligomerization of signaling complexes by the multipoint binding of GRB2 to both LAT and SOS1. *Nature Struct Molec Biol.* **13**, 798 – 805.

Abstract: Receptor oligomerization is vital for activating intracellular signaling, in part by initiating events that recruit effector and adaptor proteins to sites of active signaling. Whether these distal molecules themselves oligomerize is not well appreciated. In this study, we examined the molecular interactions of the adaptor protein GRB2. In T cells, the SH2 domain of GRB2 binds phosphorylated tyrosines on the adaptor protein LAT and the GRB2 SH3 domains associate with the proline-rich regions of SOS1 and CBL. Using biochemical and biophysical techniques in conjunction with confocal microscopy, we observed that the simultaneous association of GRB2, via its SH2 and SH3 domains, with multivalent ligands led to the oligomerization of these ligands, which affected signaling. These data suggest that multipoint binding of distal adaptor proteins mediates the formation of oligomeric signaling clusters vital for intracellular signaling.

Hu J. and Hubbard S. R. (2005) Structural characterization of a novel Cbl phosphotyrosine recognition motif in the APS family of adapter proteins. *J Biol Chem* **280**, 18943-18949.

Abstract: The Cbl adapter proteins typically function to down-regulate activated protein tyrosine kinases and other signaling proteins by coupling them to the ubiquitination machinery for degradation by the proteasome. Cbl proteins bind to specific tyrosine-phosphorylated sequences in target proteins via the tyrosine kinase-binding (TKB) domain, which comprises a four-helix bundle, an EF-hand calcium-binding domain, and a non-conventional Src homology-2 domain. The previously derived consensus sequence for phosphotyrosine recognition by the Cbl TKB domain is NXpY(S/T)XXP (X denotes lesser residue preference), wherein specificity is conferred primarily by residues C-terminal to the phosphotyrosine. Cbl is recruited to and phosphorylated by the insulin receptor in adipose cells through the adapter protein APS. APS is phosphorylated by the insulin receptor on a C-terminal tyrosine residue, which then serves as a binding site for the Cbl TKB domain. Using x-ray crystallography, site-directed mutagenesis, and calorimetric studies, we have characterized the interaction between the Cbl TKB domain and the Cbl recruitment site in APS, which contains a sequence motif, RA(V/I)XNqpY(S/T), that is conserved in the related adapter proteins SH2-B and Lnk. These studies reveal a novel mode of phosphopeptide interaction with the Cbl TKB domain, in which N-terminal residues distal to the phosphotyrosine directly contact residues of the four-helix bundle of the TKB domain.

Hu M., Gu L., Li M., Jeffrey P.D., Gu W., and Shi Y. (2006) Structural basis of competitive recognition of p53 and MDM2 by HAUSP/USP7: implications for the regulation of the p53-MDM2 pathway. *PLoS Biol.* **4**, e27.

Abstract: Herpesvirus-associated ubiquitin-specific protease (HAUSP, also known as USP7), a deubiquitylating enzyme of the ubiquitin-specific processing protease family, specifically deubiquitylates both p53 and MDM2, hence playing an important yet enigmatic role in the p53-MDM2 pathway. Here we demonstrate that both p53 and MDM2 specifically recognize the N-terminal tumor necrosis factor-receptor associated factor (TRAF)-like domain of HAUSP in a mutually exclusive manner. HAUSP preferentially forms a stable HAUSP-MDM2 complex even in the presence of excess p53. The HAUSP-binding elements were mapped to a peptide fragment in the carboxy-terminus of p53 and to a short-peptide region preceding

the acidic domain of MDM2. The crystal structures of the HAUSP TRAF-like domain in complex with p53 and MDM2 peptides, determined at 2.3-Å and 1.7-Å resolutions, respectively, reveal that the MDM2 peptide recognizes the same surface groove in HAUSP as that recognized by p53 but mediates more extensive interactions. Structural comparison led to the identification of a consensus peptide-recognition sequence by HAUSP. These results, together with the structure of a combined substrate-binding-and-deubiquitylation domain of HAUSP, provide important insights into regulation of the p53-MDM2 pathway by HAUSP.

Huber A. H., Kelley R. F., Gastinel L. N., and Bjorkman P. J. (1993) Crystallization and stoichiometry of binding of a complex between a rat intestinal Fc receptor and Fc. *J Mol Biol* **230**, 1077-1083.

Abstract: Fc receptors expressed in the gut of newborn rodents bind to maternal immunoglobulin in milk at pH 6.5, and transport it to the bloodstream of the neonate, where it dissociates at pH 7.4. The rat intestinal Fc receptor (FcRn) consists of a heavy chain, with significant sequence similarity to the heavy chain of class I MHC molecules, complexed to the class I light chain, beta 2-microglobulin. Although FcRn is predicted to contain a groove analogous to that which serves as the MHC peptide-binding site, the immunoglobulin ligand of FcRn is a macromolecule instead of a peptide. We have expressed and crystallized a secreted form of FcRn, and here report the crystallization of a complex between FcRn and its Fc ligand. Isolated FcRn-Fc complexes crystallize in space group I222 or I2(1)2(1)2(1) with unit cell dimensions $a = 125$ Å, $b = 152$ Å and $c = 216$ Å. The crystals diffract to 5.5 Å resolution with anisotropic diffraction to 3.5 Å. Data collection from cryopreserved crystals may allow the resolution limit to be extended, since the major reason for the poor resolution appears to be radiation decay. Even a low-resolution view of how FcRn binds Fc would be of interest to see if the binding site corresponds to the functional part of an MHC molecule. Since the structure of Fc is known, and a structure determination of FcRn is underway, it may be possible to locate the Fc binding site on FcRn at low resolution. As an initial characterization of the FcRn-Fc mode of interaction, and to facilitate the structure determination, we have determined the stoichiometry of binding of FcRn to Fc. We show that two FcRn molecules bind per Fc, as determined by analysis of gels of washed crystals, a column binding assay, and isothermal titration calorimetry.

Hung K. W., Kumar T. K., Kathir K. M., Xu P., Ni F., Ji H. H., Chen M. C., Yang C. C., Lin F. P., Chiu I. M., and Yu C. (2005) Solution Structure of the Ligand Binding Domain of the Fibroblast Growth Factor Receptor: Role of Heparin in the Activation of the Receptor(.). *Biochemistry* **44**, 15787-15798.

Abstract: The three-dimensional solution structure of the ligand binding D2 domain of the fibroblast growth factor receptor (FGFR) is determined using multidimensional NMR techniques. The atomic root-mean-square distribution for the backbone atoms in the structured region is 0.64 Å. Secondary structural elements in the D2 domain include 11 beta-strands arranged antiparallely into two layers of beta-sheets. The structure of the D2 domain is characterized by the presence of a short flexible helix that protrudes out of the layers of beta-sheets. Results of size exclusion chromatography and sedimentation velocity experiments show that the D2 domain exists in a monomeric state both in the presence and in the absence of bound sucrose octasulfate (SOS), a structural analogue of heparin. Comparison of the solution structure of the D2 domain with the crystal structure of the protein (D2 domain) in the FGF signaling complex reveals significant differences, suggesting that ligand (FGF) binding may induce significant conformational changes in the receptor. SOS binding sites in the D2 domain have been mapped on the basis of the (1)H-(15)N chemical shift perturbation data. SOS binds to the positively charged residues located in beta-strand III and the flexible helix. Isothermal titration calorimetry data indicate that the ligand (hFGF-1) binds strongly ($K(d)$ approximately $10^{(-)}(9)$ M) to the D2 domain even in the absence of SOS. Binding of SOS to either the D2 domain or hFGF-1 does not seem to be the driving force for the formation of the D2-hFGF-1 binary complex. The function of SOS binding appears to stabilize the preformed D2-FGF binary complex.

Hung K. W., Kumar T. K., Chi Y. H., Chiu I. M., and Yu C. (2004) Molecular cloning, overexpression, and characterization of the ligand-binding D2 domain of fibroblast growth factor receptor. *Biochem Biophys Res Commun* **317**, 253-258.

Abstract: Fibroblast growth factors (FGFs) regulate a wide range of important cellular processes. The biological activities of FGFs are mediated by cell surface receptors (FGFRs). In the present study for the first time we report the cloning, expression, and characterization of the ligand (FGF)-binding D2 domain of human FGFR2. D2 domain is expressed in *Escherichia coli* in high yields (10 mg/L) as inclusion bodies.

The protein is recovered by dissolving the inclusion bodies in 8 M urea and subsequently refolding on nickel affinity column. The protein is purified (to approximately 97% purity) to homogeneity using heparin-Sepharose affinity column. Far-UV circular dichroism data and chemical shift index plot based on ¹H-alpha, ¹³C-alpha, ¹³C-beta, and ¹³carbonyl group chemical shifts suggest that D2 domain is an all beta-sheet protein consisting of 9 beta-strands. Isothermal titration calorimetry and equilibrium urea unfolding experiments show that recombinant D2 domain is in a biologically active conformation and binds strongly to its ligand (FGF) and to the heparin analog, sucrose octasulfate (SOS). Using a variety of triple resonance NMR experiments, complete assignment of ¹H, ¹⁵N, and ¹³C resonances in D2 domain has been accomplished. The findings of the present study not only pave way for an in-depth investigation of the molecular mechanism(s) underlying the activation of FGF signaling but also provide avenues for the rational design of potent inhibitors against FGF-mediated pathogenesis.

Jansson M., Hallen D., Koho H., Andersson G., Berghard L., Heidrich J., Nyberg E., Uhlen M., Kordel J., and Nilsson B. (1997) Characterization of ligand binding of a soluble human insulin-like growth factor I receptor variant suggests a ligand-induced conformational change. *J Biol Chem* **272**, 8189-8197.

Abstract: Details of the signal transduction mechanisms of the tyrosine kinase family of growth factor receptors remain elusive. In this work, we describe an extensive study of kinetic and thermodynamic aspects of growth factor binding to a soluble extracellular human insulin-like growth factor-I receptor (sIGF-IR) variant. The extracellular receptor domains were produced fused to an IgG-binding protein domain (Z) in transfected human 293 cells as a correctly processed secreted alpha-beta'-Z dimer. The receptor was purified using IgG affinity chromatography, rendering a pure and homogenous protein in yields from 1 to 5 mg/liter of conditioned cell media. Biosensor technology (BIAcore) was applied to measure the insulin-like growth factor-I (IGF-I), des(1-3)IGF-I, insulin-like growth factor-II, and insulin ligand binding rate constants to the immobilized IGF-IR-Z. The association equilibrium constant, K_a , for the IGF-I interaction is determined to $2.8 \times 10^8 \text{ M}^{-1}$ (25 degrees C). Microcalorimetric titrations on IGF-I/IGF-IR-Z were performed at three different temperatures (15, 25, and 37 degrees C) and in two different buffer systems at 25 degrees C. From these measurements, equilibrium constants for the 1:1 (IGF-I:(alpha-beta'-Z)₂) receptor complex in solution are deduced to $0.96 \times 10^8 \text{ M}^{-1}$ (25 degrees C). The determined heat capacity change for the process is large and negative, $-0.51 \text{ kcal (K mol)}^{-1}$. Further, the entropy change (ΔS) at 25 degrees C is large and negative. Far- and near-UV circular dichroism measurements display significant changes over the entire wavelength range upon binding of IGF-I to IGF-IR-Z. These data are all consistent with a significant change in structure of the system upon IGF-I binding.

Janz J. M., Sakmar T. P. and Min K. C. (2007) A novel interaction between atrophin-interacting protein 4 and beta-p21-activated kinase-interactive exchange factor is mediated by an SH3 domain. *J Biol Chem* **282**, 28893-28903.

Abstract: Cross-talk between G protein-coupled receptors and receptor tyrosine kinase signaling pathways is crucial to the efficient relay and integration of cellular information. Here we identify and define the novel binding interaction of the E3 ubiquitin ligase atrophin-interacting protein 4 (AIP4) with the GTP exchange factor beta-p21-activated kinase-interactive exchange factor (beta PIX). We demonstrate that this interaction is mediated in part by the beta PIX-SH3 domain binding to a proline-rich stretch of AIP4. Analysis of the interaction by isothermal calorimetry is consistent with a heterotrimeric complex with one AIP4-derived peptide binding to two beta PIX-SH3 domains. We determined the crystal structure of the beta PIX-SH3.AIP4 complex to 2.0-A resolution. In contrast to the calorimetry results, the crystal structure shows a monomeric complex in which AIP4 peptide binds the beta PIX-SH3 domain as a canonical Class I ligand with an additional type II polyproline helix that makes extensive contacts with another face of beta PIX. Taken together, the novel interaction between AIP4 and beta PIX represents a new regulatory node for G protein-coupled receptor and receptor tyrosine kinase signal integration. Our structure of the beta PIX-SH3.AIP4 complex provides important insight into the mechanistic basis for beta PIX scaffolding of signaling components, especially those involved in cross-talk.

Johanson K., Appelbaum E., Doyle M., Hensley P., Zhao B., Abdel-Meguid S. S., Young P., Cook R., Carr S., Matico R., and . (1995) Binding interactions of human interleukin 5 with its receptor alpha subunit. Large scale production, structural, and functional studies of Drosophila-expressed recombinant proteins. *J Biol Chem* **270**, 9459-9471.

Abstract: Human interleukin 5 (hIL5) and soluble forms of its receptor alpha subunit were expressed in *Drosophila* cells and purified to homogeneity, allowing a detailed structural and functional analysis. B cell proliferation confirmed that the hIL5 was biologically active. Deglycosylated hIL5 remained active, while similarly deglycosylated receptor alpha subunit lost activity. The crystal structure of the deglycosylated hIL5 was determined to 2.6-Å resolution and found to be similar to that of the protein produced in *Escherichia coli*. Human IL5 was shown by analytical ultracentrifugation to form a 1:1 complex with the soluble domain of the hIL5 receptor alpha subunit (shIL5R alpha). Additionally, the relative abundance of ligand and receptor in the hIL5.shIL5R alpha complex was determined to be 1:1 by both titration calorimetry and SDS-polyacrylamide gel electrophoresis analysis of dissolved cocrystals of the complex. Titration microcalorimetry yielded equilibrium dissociation constants of 3.1 and 2.0 nM, respectively, for the binding of hIL5 to shIL5R alpha and to a chimeric form of the receptor containing shIL5R alpha fused to the immunoglobulin Fc domain (shIL5R alpha-Fc). Analysis of the binding thermodynamics of IL5 and its soluble receptor indicates that conformational changes are coupled to the binding reaction. Kinetic analysis using surface plasmon resonance yielded data consistent with the K_d values from calorimetry and also with the possibility of conformational isomerization in the interaction of hIL5 with the receptor alpha subunit. Using a radioligand binding assay, the affinity of hIL5 with full-length hIL5R alpha in *Drosophila* membranes was found to be 6 nM, in accord with the affinities measured for the soluble receptor forms. Hence, most of the binding energy of the alpha receptor is supplied by the soluble domain. Taken with other aspects of hIL5 structure and biological activity, the data obtained allow a prediction for how 1:1 stoichiometry and conformational change can lead to the formation of hIL5.receptor alpha beta complex and signal transduction.

Jones L. L., Colf L. A., Bankovich A. J., Stone J. D., Gao Y. G., Chan C. M., Huang R. H., Garcia K. C. and Kranz D. M. (2008) Different Thermodynamic Binding Mechanisms and Peptide Fine Specificities Associated with a Panel of Structurally Similar High-Affinity T Cell Receptors. *Biochemistry*. (publication)

Abstract: To understand the mechanisms that govern T cell receptor (TCR)-peptide MHC (pMHC) binding and the role that different regions of the TCR play in affinity and antigen specificity, we have studied the TCR from T cell clone 2C. High-affinity mutants of the 2C TCR that bind QL9-L (d) as a strong agonist were generated previously by site-directed mutagenesis of complementarity determining regions (CDRs) 1beta, 2alpha, 3alpha, or 3beta. We performed isothermal titration calorimetry to assess whether they use similar thermodynamic mechanisms to achieve high affinity for QL9-L (d). Four of the five TCRs examined bound to QL9-L (d) in an enthalpically driven, entropically unfavorable manner. In contrast, the high-affinity CDR1beta mutant resembled the wild-type 2C TCR interaction, with favorable entropy. To assess fine specificity, we measured the binding and kinetics of these mutants for both QL9-L (d) and a single amino acid peptide variant of QL9, called QL9-Y5-L (d). While 2C and most of the mutants had equal or higher affinity for the Y5 variant than for QL9, mutant CDR1beta exhibited 8-fold lower affinity for Y5 compared to QL9. To examine possible structural correlates of the thermodynamic and fine specificity signatures of the TCRs, the structure of unliganded QL9-L (d) was solved and compared to structures of the 2C TCR/QL9-L (d) complex and three high-affinity TCR/QL9-L (d) complexes. Our findings show that the QL9-L (d) complex does not undergo major conformational changes upon binding. Thus, subtle changes in individual CDRs account for the diverse thermodynamic and kinetic binding mechanisms and for the different peptide fine specificities

Kasper C., Pickering D. S., Mirza O., Olsen L., Kristensen A. S., Greenwood J. R., Liljefors T., Schousboe A., Watjen F., Gajhede M., Sigurskjold B. W., and Kastrop J. S. (2006) The structure of a mixed GluR2 ligand-binding core dimer in complex with (S)-glutamate and the antagonist (S)-NS1209. *J Mol Biol* **357**, 1184-1201.

Abstract: Ionotropic glutamate receptors (iGluRs) mediate fast synaptic transmission between cells of the central nervous system and are involved in various aspects of normal brain function. iGluRs are implicated in several brain disorders, e.g. in the high-frequency discharge of impulses during an epileptic seizure. (RS)-NS1209 functions as a competitive antagonist at 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionate receptors, and shows robust preclinical anticonvulsant and neuroprotective effects. This study explores 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionate receptor binding and selectivity of this novel class of antagonists. We present here the first X-ray structure of a mixed GluR2 ligand-binding core dimer, with the high-affinity antagonist (S)-8-methyl-5-(4-(N,N-

dimethylsulfamoyl)phenyl)-6,7,8,9,-tetrahydro-1H-pyrrolo[3,2-h]-isoquinoline-2,3-dione-3-O-(4-hydroxybutyrate-2-yl)oxime [(S)-NS1209] in one protomer and the endogenous ligand (S)-glutamate in the other. (S)-NS1209 stabilises an even more open conformation of the D1 and D2 domains of the ligand-binding core than that of the apo structure due to steric hindrance. This is the first time ligand-induced hyperextension of the binding domains has been observed. (S)-NS1209 adopts a novel binding mode, including hydrogen bonding to Tyr450 and Gly451 of D1. Parts of (S)-NS1209 occupy new areas of the GluR2 ligand-binding cleft, and bind near residues that are not conserved among receptor subtypes. The affinities of (RS)-NS1209 at the GluR2 ligand-binding core as well as at GluR1-6 and mutated GluR1 and GluR3 receptors have been measured. Two distinct binding affinities were observed at the GluR3 and GluR4 receptors. In a functional in vitro assay, no difference in potency was observed between GluR2(Q)(o) and GluR3(o) receptors. The thermodynamics of binding of the antagonists (S)-NS1209, DNQX and (S)-ATPO to the GluR2 ligand-binding core have been determined by displacement isothermal titration calorimetry. The displacement of (S)-glutamate by all antagonists was shown to be driven by enthalpy.

Kaufmann B., Baxa U., Chipman P. R., Rossmann M. G., Modrow S., and Seckler R. (2005) Parvovirus B19 does not bind to membrane-associated globoside in vitro. *Virology* **332**, 189-198.

Abstract: The glycosphingolipid globoside (globotetraosylceramide, Gb4Cer) has been proposed to be the cellular receptor of human parvovirus B19. Quantitative measurements of the binding of parvovirus B19 to Gb4Cer were performed to explore the molecular basis of the virus tropism. Solid-phase assays with fluorescence-labeled liposomes or 125iodine-labeled empty capsids were used to characterize the specificity of binding. In addition, surface plasmon resonance on lipid layers, as well as isothermal titration microcalorimetry, was utilized for real-time analysis of the virus-receptor interaction. These studies did not confirm binding of Gb4Cer to recombinant B19 VP2 capsids, suggesting that Gb4Cer does not function on its own as the cellular receptor of human parvovirus B19, but might be involved in a more complex recognition event. The biochemical results were further confirmed by cryo-electron microscopy image reconstructions at 10 Å resolution, in which the structures of empty capsids were compared with empty capsids incubated with Gb4Cer.

Kim M., Sun Z. Y., Byron O., Campbell G., Wagner G., Wang J., and Reinherz E. L. (2001) Molecular dissection of the CD2-CD58 counter-receptor interface identifies CD2 Tyr86 and CD58 Lys34 residues as the functional "hot spot". *J Mol Biol* **312**, 711-720.

Abstract: The heterophilic CD2-CD58 adhesion interface contains interdigitating residues that impart high specificity and rapid binding kinetics. To define the hot spot of this counter-receptor interaction, we characterized CD2 adhesion domain variants harboring a single mutation of the central Tyr86 or of each amino acid residue forming a salt link/hydrogen bond. Alanine mutations at D31, D32 and K34 on the C strand and K43 and R48 on the C' strand reduce affinity for CD58 by 47-127-fold as measured by isothermal titration calorimetry. The Y86A mutant reduces affinity by approximately 1000-fold, whereas Y86F is virtually without effect, underscoring the importance of the phenyl ring rather than the hydroxyl moiety. The CD2-CD58 crystal structure offers a detailed view of this key functional epitope: CD2 D31 and D32 orient the side-chain of CD58 K34 such that CD2 Y86 makes hydrophobic contact with the extended aliphatic component of CD58 K34 between CD2 Y86 and CD58 F46. The elucidation of this hot spot provides a new target for rational design of immunosuppressive compounds and suggests a general approach for other receptors.

Kouadio J. L., Horn J. R., Pal G., and Kossiakoff A. A. (2005) Shot-gun alanine-scanning shows that growth hormone can bind productively to its receptor through a drastically minimized interface. *J Biol Chem*.

Abstract: The high affinity binding site (Site1) of the human growth hormone (hGH) binds to its cognate receptor (hGHR) via a concave surface patch containing about 35 residues. Using 167 sequences from a shot-gun alanine scanning analysis of the Site1, we have determined that over half of these residues can be simultaneously changed to an alanine or a non-isosteric amino acid while still retaining a high affinity interaction. Among these hGH variants the distribution of the mutation is highly variable throughout the interface, although helix 4 is more conserved than the other binding elements. Kinetic and thermodynamic analyses were performed on 11 representative hGH Site1 variants that contained 14-20 mutations. Generally, the tightest binding variants showed similar associated rate constants (k_{on}) as the wild-type

(wt) hormone, indicating that their binding proceeds through a similar transition state intermediate. However, calorimetric analyses indicate very different thermodynamic partitioning: wt-hGH binding exhibits favorable enthalpy and entropy contributions, while the variants display highly favorable enthalpy and highly unfavorable entropy contributions. The heat capacities (DCp) on binding measured for wt-hGH and its variants are significantly larger than normally seen for typical protein-protein interactions, suggesting large conformational or solvation effects. The multiple Site1 mutations are shown to indirectly affect binding of the second receptor at Site2 through an allosteric mechanism. We show that the stability of the ternary hormone-receptor complex reflects the affinity of the Site2 binding, and is surprisingly exempt from changes in Site1 affinity, directly demonstrating that dissociation of the active signaling complex is a stepwise process.

Krell T., Renauld-Mongenie G., Nicolai M. C., Frayssé S., Chevalier M., Berard Y., Oakhill J., Evans R. W., Gorringer A., and Lissolo L. (2003) Insight into the structure and function of the transferrin receptor from *Neisseria meningitidis* using microcalorimetric techniques. *J Biol Chem* **278**, 14712-14722.

Abstract: The transferrin receptor of *Neisseria meningitidis* is composed of the transmembrane protein TbpA and the outer membrane protein TbpB. Both receptor proteins have the capacity to independently bind their ligand human transferrin (htf). To elucidate the specific role of these proteins in receptor function, isothermal titration calorimetry was used to study the interaction between purified TbpA, TbpB or the entire receptor (TbpA + TbpB) with holo- and apo-htf. The entire receptor was shown to contain a single high affinity htf-binding site on TbpA and approximately two lower affinity binding sites on TbpB. The binding sites appear to be independent. Purified TbpA was shown to have strong ligand preference for apo-htf, whereas TbpA in the receptor complex with TbpB preferentially binds the holo form of htf. The orientation of the ligand specificity of TbpA toward holo-htf is proposed to be the physiological function of TbpB. Furthermore, the thermodynamic mode of htf binding by TbpB of isotypes I and II was shown to be different. A protocol for the generation of active, histidine-tagged TbpB as well as its individual N- and C-terminal domains is presented. Both domains are shown to strongly interact with each other, and isothermal titration calorimetry and circular dichroism experiments provide clear evidence for this interaction causing conformational changes. The N-terminal domain of TbpB was shown to be the site of htf binding, whereas the C-terminal domain is not involved in binding. Furthermore, the interactions between TbpA and the different domains of TbpB have been demonstrated.

Kroe, R.R., Baker, M.A., Brown, M.P., Farrow, N.A., Elda Gautschi, E., Hopkins, J.L., LaFrance, R.R., Kronkaitis, A., Freeman, D., Thomson, D., Nabozny, G., Grygon, C.A., and Labadia, M.E. (2007) Agonist versus antagonist induce distinct thermodynamic modes of co-factor binding to the glucocorticoid receptor. *Biophys. Chem.* **128**, 156-164.

Abstract: The glucocorticoid receptor (GR) is involved in the transcriptional regulation of genes associated with inflammation, glucose homeostasis, and bone turnover through the association with ligands, such as corticosteroids. GR-mediated gene transcription is regulated or fine-tuned via the recruitment of co-factors including coactivators and corepressors. Current therapeutic approaches to targeting GR aim to retain the beneficial anti-inflammatory activity of the corticosteroids while eliminating negative side effects. Towards achieving this goal the experiments discussed here reveal a mechanism of co-factor binding in the presence of either bound agonist or antagonist. The GR ligand binding domain (GR-LBD(F602S)), in the presence of agonist or antagonist, utilizes different modes of binding for coactivator versus corepressor. Coactivator binding to the co-effector binding pocket of GR-LBD(F602S) is driven both by favorable enthalpic and entropic interactions whereas corepressor binding to the same pocket is entropically driven. These data support the hypothesis that ligand-induced conformational changes dictate co-factor binding and subsequent trans-activation or trans-repression.

Kwong P. D., Doyle M. L., Casper D. J., Cicala C., Leavitt S. A., Majeed S., Steenbeke T. D., Venturi M., Chaiken I., Fung M., Katinger H., Parren P. W., Robinson J., Van Ryk D., Wang L., Burton D. R., Freire E., Wyatt R., Sodroski J., Hendrickson W. A., and Arthos J. (2002) HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. *Nature* **420**, 678-682.

Abstract: The ability of human immunodeficiency virus (HIV-1) to persist and cause AIDS is dependent on its avoidance of antibody-mediated neutralization. The virus elicits abundant, envelope-directed antibodies that have little neutralization capacity. This lack of neutralization is paradoxical, given the

functional conservation and exposure of receptor-binding sites on the gp120 envelope glycoprotein, which are larger than the typical antibody footprint and should therefore be accessible for antibody binding. Because gp120-receptor interactions involve conformational reorganization, we measured the entropies of binding for 20 gp120-reactive antibodies. Here we show that recognition by receptor-binding-site antibodies induces conformational change. Correlation with neutralization potency and analysis of receptor-antibody thermodynamic cycles suggested a receptor-binding-site 'conformational masking' mechanism of neutralization escape. To understand how such an escape mechanism would be compatible with virus-receptor interactions, we tested a soluble dodecameric receptor molecule and found that it neutralized primary HIV-1 isolates with great potency, showing that simultaneous binding of viral envelope glycoproteins by multiple receptors creates sufficient avidity to compensate for such masking. Because this solution is available for cell-surface receptors but not for most antibodies, conformational masking enables HIV-1 to maintain receptor binding and simultaneously to resist neutralization.

Laakkonen L. J., Guarnieri F., Perlman J. H., Gershengorn M. C., and Osman R. (1996) A refined model of the thyrotropin-releasing hormone (TRH) receptor binding pocket. Novel mixed mode Monte Carlo/stochastic dynamics simulations of the complex between TRH and TRH receptor. *Biochemistry* **35**, 7651-7663.

Abstract: Previous mutational and computational studies of the thyrotropin-releasing hormone (TRH) receptor identified several residues in its binding pocket [see accompanying paper, Perlman et al. (1996) *Biochemistry* 35, 7643-7650]. On the basis of the initial model constructed with standard energy minimization techniques, we have conducted 15 mixed mode Monte Carlo/stochastic dynamics (MC-SD) simulations to allow for extended sampling of the conformational states of the ligand and the receptor in the complex. A simulated annealing protocol was adopted in which the complex was cooled from 600 to 310 K in segments of 30 ps of the MC-SD simulations for each change of 100 K. Analysis of the simulation results demonstrated that the mixed mode MC-SD protocol maintained the desired temperature in the constant temperature simulation segments. The elevated temperature and the repeating simulations allowed for adequate sampling of the torsional space of the complex with successful conservation of the general structure and good helicity of the receptor. For the analysis of the interaction between TRH and the binding pocket, TRH was divided into four groups consisting of pyroGlu, His, ProNH₂, and the backbone. The pairwise interaction energies of the four separate portions of TRH with the corresponding residues in the receptor provide a physicochemical basis for the understanding of ligand-receptor complexes. The interaction of pyroGlu with Tyr106 shows a bimodal distribution that represents two populations: one with a H-bond and another without it. Asp195 was shown to compete with pyroGlu for the H-bond to Tyr106. Simulations in which Asp195 was interacting with Arg283, thus removing it from the vicinity of Tyr106, resulted in a stable H-bond to pyroGlu. In all simulations His showed a van der Waals attraction to Tyr282 and a weak electrostatic repulsion from Arg 306. The ProNH₂ had a strong and frequent H-bonding interaction with Arg306. The backbone carbonyls show a frequent H-bonding interaction with the OH group of Tyr282 and strong, often multiple, interactions with Arg306. Three structures, which maintained these interactions simultaneously, were selected as candidates for ligand-receptor complexes. These show persistent interactions of TRH with Ile 109 and Ile 116 in HX 3 and with Tyr310 and Ser313 in HX 7, which will be tested to refine the structure of the ligand-receptor complex. The superposition of the three structures shows the extent of structural flexibility of the receptor and the ligand in the complex. The backbone of TRH inside the receptor is in an alpha-helical conformation, suggesting that the receptor, through its interaction with the ligand, provides the energy required for the conformational change in the ligand from an extended to the folded form.

Ladbury J. E. (2007) Measurement of the formation of complexes in tyrosine kinase-mediated signal transduction. *Acta Crystallogr D Biol Crystallogr* **63**, 26-31.

Abstract: Isothermal titration calorimetry (ITC) provides highly complementary data to high-resolution structural detail. An overview of the methodology of the technique is provided. Ultimately, the correlation of the thermodynamic parameters determined by ITC with structural perturbation observed on going from the free to the bound state should be possible at an atomic level. Currently, thermodynamic data provide some insight as to potential changes occurring on complex formation. Here, this is demonstrated in the context of in vitro quantification of intracellular tyrosine kinase-mediated signal transduction and the issue of specificity of the important interactions. The apparent lack of specificity in the interactions of domains

of proteins involved in early signalling from membrane-bound receptors is demonstrated using data from ITC.

Lambert L. J., Bobkov A. A., Smith J. W. and Marassi F. M. (2008) Competitive interactions of collagen and a jararhagin-derived disintegrin peptide with the integrin alpha2-I domain. *J Biol Chem* **283**, 16665-16672.

Abstract: Integrin alpha2beta1 is a major receptor required for activation and adhesion of platelets, through the specific recognition of collagen by the alpha2-I domain (alpha2-I), which binds fibrillar collagen via Mg(2+)-bridged interactions. The crystal structure of a truncated form of the alpha2-I domain, bound to a triple helical collagen peptide, revealed conformational changes suggestive of a mechanism where the ligand-bound I domain can initiate and propagate conformational change to the full integrin complex. Collagen binding by alpha2-I and fibrinogen-dependent platelet activity can be inhibited by snake venom polypeptides. Here we describe the inhibitory effect of a short cyclic peptide derived from the snake toxin metalloprotease jararhagin, with specific amino acid sequence RKKH, on the ability of alpha2-I to bind triple helical collagen. Isothermal titration calorimetry measurements showed that the interactions of alpha2-I with collagen or RKKH peptide have similar affinities, and NMR chemical shift mapping experiments with (15)N-labeled alpha2-I, and unlabeled RKKH peptide, indicate that the peptide competes for the collagen-binding site of alpha2-I but does not induce a large scale conformational rearrangement of the I domain

Landar A., Curry B., Parker M. H., DiGiacomo R., Indelicato S. R., Nagabhushan T. L., Rizzi G., and Walter M. R. (2000) Design, characterization, and structure of a biologically active single-chain mutant of human IFN-gamma. *J Mol Biol* **299**, 169-179.

Abstract: A mutant form of human interferon-gamma (IFN-gamma SC1) that binds one IFN-gamma receptor alpha chain (IFN-gamma R alpha) has been designed and characterized. IFN-gamma SC1 was derived by linking the two peptide chains of the IFN-gamma dimer by a seven-residue linker and changing His111 in the first chain to an aspartic acid residue. Isothermal titration calorimetry shows that IFN-gamma SC1 forms a 1:1 complex with its high-affinity receptor (IFN-gamma R alpha) with an affinity of 27(+/- 9) nM. The crystal structure of IFN-gamma SC1 has been determined at 2.9 Å resolution from crystals grown in 1.4 M citrate solutions at pH 7.6. Comparison of the wild-type receptor-binding domain and the Asp111-containing domain of IFN-gamma SC1 show that they are structurally equivalent but have very different electrostatic surface potentials. As a result, surface charge rather than structural changes is likely responsible for the inability of the His111-->Asp domain of to bind IFN-gamma R alpha. The AB loops of IFN-gamma SC1 adopt conformations similar to the ordered loops of IFN-gamma observed in the crystal structure of the IFN-gamma/IFN-gamma R alpha complex. Thus, IFN-gamma R alpha binding does not result in a large conformational change in the AB loop as previously suggested. The structure also reveals the final six C-terminal amino acid residues of IFN-gamma SC1 (residues 253-258) that have not been observed in any other reported IFN-gamma structures. Despite binding to only one IFN-gamma R alpha, IFN-gamma SC1 is biologically active in cell proliferation, MHC class I induction, and anti-viral assays. This suggests that one domain of IFN-gamma is sufficient to recruit IFN-gamma R alpha and IFN-gamma R beta into a complex competent for eliciting biological activity. The current data are consistent with the main role of the IFN-gamma dimer being to decrease the dissociation constant of IFN-gamma for its cellular receptors.

Laurine E., Lafitte D., Gregoire C., Seree E., Loret E., Douillard S., Michel B., Briand C., and Verdier J. M. (2003) Specific binding of dehydroepiandrosterone to the N terminus of the microtubule-associated protein MAP2. *J Biol Chem* **278**, 29979-29986.

Abstract: The effect of neurosteroids is mediated through their membrane or nuclear receptors. However, no dehydroepiandrosterone (DHEA)-specific receptors have been evidenced so far in the brain. In this paper, we showed by isothermal titration calorimetry that the DHEA specifically binds to the dendritic brain microtubule-associated protein MAP2C with an association constant of $2.7 \times 10^7 \text{ m}^{-1}$ and at a molar ratio of 1:1. By partial tryptic digestions and mass spectrometry analysis, we found that the binding involved the N-terminal region of MAP2C. Interestingly, MAP2C displays homologies with 17 beta-hydroxysteroid dehydrogenase 1, an enzyme required for estrogen synthesis. Based on these sequence homologies and on the x-ray structure of the DHEA-binding pocket of 17 beta-hydroxysteroid dehydrogenase 1, we modeled the complex of DHEA with MAP2C. The binding of DHEA to MAP2C

involved specific hydrogen bonds that orient the steroid into the pocket. This work suggests that DHEA can directly influence brain plasticity via MAP2C binding. It opens interesting ways for understanding the role of DHEA in the brain.

Lazic A., Dolmer K., Strickland D. K., and Gettins P. G. (2006) Dissection of RAP-LRP interactions: binding of RAP and RAP fragments to complement-like repeats 7 and 8 from ligand binding cluster II of LRP. *Arch Biochem Biophys* **450**, 167-175.

Abstract: The receptor associated protein (RAP) is a three domain 38kDa ER-resident chaperone that helps folding of LRP and other LDL receptor family members and prevents premature binding of protein ligands. It competes strongly with all known LRP ligands. To further understanding of the specificity of RAP-LRP interactions, the binding of RAP and RAP fragments to two domains (CR7-CR8) from one of the main ligand-binding regions of LRP has been examined by 2D HSQC NMR spectroscopy and isothermal titration calorimetry. We found that RAP contains two binding sites for CR7-CR8, with the higher affinity site (K(d) approximately 1microM) located in the C-terminal two-thirds and the weaker site (K(d) approximately 5microM) in the N-terminal third of RAP. Residues from both CR7 and CR8 are involved in binding at each RAP site. The presence of more than one binding site on RAP for CR domains from LRP, together with the previous demonstration by others that RAP can bind to CR5-CR6 with comparably low affinities suggest an explanation for the dual roles of RAP as a folding chaperone and a tight competitive inhibitor of ligand binding.

Lefevre J., Delepelaire P., Delepierre M. and Izadi-Pruneyre N. (2008) Modulation by substrates of the interaction between the HasR outer membrane receptor and its specific TonB-like protein, HasB. *J Mol Biol* **378**, 840-851.

Abstract: TonB is a cytoplasmic membrane protein required for active transport of various essential substrates such as heme and iron siderophores through the outer membrane receptors of Gram-negative bacteria. This protein spans the periplasm, contacts outer membrane transporters by its C-terminal domain, and transduces energy from the protonmotive force to the transporters. The TonB box, a relatively conserved sequence localized on the periplasmic side of the transporters, has been shown to directly contact TonB. While *Serratia marcescens* TonB functions with various transporters, HasB, a TonB-like protein, is dedicated to the HasR transporter. HasR acquires heme either freely or via an extracellular heme carrier, the hemophore HasA, that binds to HasR and delivers heme to the transporter. Here, we study the interaction of HasR with a HasB C-terminal domain and compare it with that obtained with a TonB C-terminal fragment. Analysis of the thermodynamic parameters reveals that the interaction mode of HasR with HasB differs from that with TonB, the difference explaining the functional specificity of HasB for HasR. We also demonstrate that the presence of the substrate on the extracellular face of the transporter modifies, via enthalpy-entropy compensation, the interaction with HasB on the periplasmic face. The transmitted signal depends on the nature of the substrate. While the presence of heme on the transporter modifies only slightly the nature of interactions involved between HasR and HasB, hemophore binding on the transporter dramatically changes the interactions and seems to locally stabilize some structural motifs. In both cases, the HasR TonB box is the target for those modifications

Lemmon M. A., Bu Z., Ladbury J. E., Zhou M., Pinchasi D., Lax I., Engelman D. M., and Schlessinger J. (1997) Two EGF molecules contribute additively to stabilization of the EGFR dimer. *EMBO J* **16**, 281-294.

Abstract: Receptor dimerization is generally considered to be the primary signaling event upon binding of a growth factor to its receptor at the cell surface. Little, however, is known about the precise molecular details of ligand-induced receptor dimerization, except for studies of the human growth hormone (hGH) receptor. We have analyzed the binding of epidermal growth factor (EGF) to the extracellular domain of its receptor (sEGFR) using titration calorimetry, and the resulting dimerization of sEGFR using small-angle X-ray scattering. EGF induces the quantitative formation of sEGFR dimers that contain two EGF molecules. The data obtained from the two approaches suggest a model in which one EGF monomer binds to one sEGFR monomer, and that receptor dimerization involves subsequent association of two monomeric (1:1) EGF-sEGFR complexes. Dimerization may result from bivalent binding of both EGF molecules in the dimer and/or receptor-receptor interactions. The requirement for two (possibly bivalent) EGF monomers distinguishes EGF-induced sEGFR dimerization from the hGH and interferon-gamma receptors, where multivalent binding of a single ligand species (either monomeric or dimeric) drives receptor

oligomerization. The proposed model of EGF-induced sEGFR dimerization suggests possible mechanisms for both ligand-induced homo- and heterodimerization of the EGFR (or erbB) family of receptors.

Leone M., Cellitti J. and Pellicchia M. (2008) NMR Studies of a Heterotypic Sam-Sam Domain Association: The Interaction between the Lipid Phosphatase Ship2 and the EphA2 Receptor(.). *Biochemistry*. (epublication)

Abstract: Sterile alpha motif (Sam) domains are protein interaction modules that are implicated in many biological processes mainly via homo- and heterodimerization. It has been recently reported that the lipid phosphatase Ship2 regulates endocytosis of the EphA2 receptor, a process that has been investigated as a possible route to reduce tumor malignancy. A heterotypic Sam-Sam domain interaction is mediating this process. Here, we report NMR and ITC (isothermal titration calorimetry) studies on the Sam domain of Ship2 revealing its three-dimensional structure and its possible mode of interaction with the Sam domain from the EphA2 receptor. These studies have also resulted in the identification of a minimal peptide region of Ship2 that retains binding affinity for the Sam domain of the EphA2 receptor. Hence, this peptide and the detection of key structural elements important for EphA2 receptor endocytosis provide possible ways for the development of novel small molecule antagonists with potential anticancer activity

Lewitzky M., Harkiolaki M., Domart M. C., Jones E. Y., and Feller S. M. (2004) Mona/Gads SH3C binding to hematopoietic progenitor kinase 1 (HPK1) combines an atypical SH3 binding motif, R/KXXXK, with a classical PXXP motif embedded in a polyproline type II (PPII) helix. *J Biol Chem* **279**, 28724-28732.

Abstract: Hematopoietic progenitor kinase 1 (HPK1) is implicated in signaling downstream of the T cell receptor. Its non-catalytic, C-terminal half contains several prolinerich motifs, which have been shown to interact with different SH3 domain-containing adaptor proteins in vitro. One of these, Mona/Gads, was also shown to bind HPK1 in mouse T cells in vivo. The region of HPK1 that binds to the Mona/Gads C-terminal SH3 domain has been mapped and shows only very limited similarity to a recently identified high affinity binding motif in SLP-76, another T-cell adaptor. Using isothermal titration calorimetry and x-ray crystallography, the binding of the HPK1 motif to Mona/Gads SH3C has now been characterized in molecular detail. The results indicate that although charge interactions through an RXXX motif are essential for complex formation, a PXXP motif in HPK1 strongly complements binding. This unexpected binding mode therefore differs considerably from the previously described interaction of Mona/Gads SH3C with SLP-76. The crystal structure of the complex highlights the great versatility of SH3 domains, which allows interactions with very different proteins. This currently limits our ability to categorize SH3 binding properties by simple rules.

Li C., Ni C. Z., Havert M. L., Cabezas E., He J., Kaiser D., Reed J. C., Satterthwait A. C., Cheng G., and Ely K. R. (2002) Downstream regulator TANK binds to the CD40 recognition site on TRAF3. *Structure (Camb)* **10**, 403-411.

Abstract: TRAFs (tumor necrosis factor receptor [TNFR]-associated factors) bind to the cytoplasmic portion of liganded TNFRs and stimulate activation of NF-kappaB or JNK pathways. A modulator of TRAF signaling, TANK, serves as either an enhancer or an inhibitor of TRAF-mediated signaling pathways. The crystal structure of a region of TANK bound to TRAF3 has been determined and compared to a similar CD40/TRAF3 complex. TANK and CD40 bind to the same crevice on TRAF3. The recognition motif PxQxT is presented in a boomerang-like structure in TANK that is markedly different from the hairpin loop that forms in CD40 upon binding to TRAF3. Critical TANK contact residues were confirmed by mutagenesis to be required for binding to TRAF3 or TRAF2. Binding affinity, measured by isothermal titration calorimetry and competition assays, demonstrated that TANK competes with CD40 for the TRAF binding site.

Li C., Chan J., Haeseleer F., Mikoshiba K., Palczewski K., Ikura M. and Ames J. B. (2008) Structural insights into Ca²⁺-dependent regulation of Inositol 1,4,5,-trisphosphate receptors by CaBP1. *J Biol Chem*. (epublication)

Abstract: Calcium-binding protein 1 (CaBP1), a neuron-specific member of the calmodulin (CaM) superfamily, modulates Ca²⁺-dependent activity of inositol 1,4,5-trisphosphate receptors (InsP3Rs). Here we present NMR structures of CaBP1 in both Mg²⁺-bound and Ca²⁺-bound states and their structural interaction with InsP3Rs. CaBP1 contains four EF-hands in two separate domains. The N-domain consists

of EF1 and EF2 in a closed conformation with Mg²⁺ bound at EF1. The C-domain binds Ca²⁺ at EF3 and EF4, and exhibits a Ca²⁺-induced closed-to-open transition like that of CaM. The Ca²⁺-bound C-domain contains exposed hydrophobic residues (L132, H134, I141, I144, V148) that may account for selective binding to InsP3Rs. Isothermal titration calorimetry analysis reveals a Ca²⁺-induced binding of the CaBP1 C-domain to the N-terminal region of InsP3R (residues 1-587), whereas CaM and the CaBP1 N-domain did not show appreciable binding. CaBP1 binding to InsP3Rs requires both the suppressor and ligand-binding core domains, but has no effect on InsP3 binding to the receptor. We propose that CaBP1 may regulate Ca²⁺-dependent activity of InsP3Rs by promoting structural contacts between the suppressor and core domains

Li J., Cook R., and Chaiken I. (1996) Interleukin 5 interactions with soluble and cell surface forms of its receptor. *J Mol Recognit* **9**, 347-355.

Li J., Cook R., Doyle M. L., Hensley P., McNulty D. E., and Chaiken I. (1997) Monomeric isomers of human interleukin 5 show that 1:1 receptor recruitment is sufficient for function. *Proc Natl Acad Sci U S A* **94**, 6694-6699.

Abstract: The normally dimeric human interleukin 5 (IL-5) was re-engineered into two monomeric isomer forms to investigate mechanistic features of receptor recognition. One form, denoted GM1-IL-5, is a CD-loop expanded form, in which an 8-residue linker designed for flexibility was inserted between residues 85 and 86. The second, denoted DABC-IL-5, is a circularly permuted form of human IL-5 in which a chain discontinuity was introduced in the CD loop and the two consequent chain fragments were joined at the normal N and C termini by a di-glycyl linker. Both IL-5 isomers folded into stable monomers in solution as shown by sedimentation equilibrium and CD and formed an intrachain disulfide bond predicted from the structure of wild type IL-5. From titration microcalorimetry and optical biosensor analyses, both monomers were shown to interact with the IL-5 receptor alpha chain with 1:1 stoichiometry and affinities 30- to 40-fold weaker than for the dimeric wild type protein. And both monomers stimulated cell proliferation of human IL-5 receptor positive cells with a concentration dependence close to that of wild type. The data show that both monomeric and dimeric forms of IL-5 function through similar 1:1 receptor alpha chain recruitment processes and that it is the helical packing of the monomeric four-helix bundle unit in IL-5, rather than the helical connectivity itself, that appears to play the major role in presenting structural epitopes to trigger functional receptor activation.

Lim J. H., Kim M. S., Kim H. E., Yano T., Oshima Y., Aggarwal K., Goldman W. E., Silverman N., Kurata S., and Oh B. H. (2006) Structural basis for preferential recognition of diaminoimelic acid-type peptidoglycan by a subset of peptidoglycan recognition proteins. *J Biol Chem* **281**, 8286-8295.

Abstract: Drosophila peptidoglycan recognition protein (PGRP)-LCx and -LCa are receptors that preferentially recognize meso-diaminoimelic acid (DAP)-type peptidoglycan (PGN) present in Gram-negative bacteria over lysine-type PGN of gram-positive bacteria and initiate the IMD signaling pathway, whereas PGRP-LE plays a synergistic role in this process of innate immune defense. How these receptors can distinguish the two types of PGN remains unclear. Here the structure of the PGRP domain of Drosophila PGRP-LE in complex with tracheal cytotoxin (TCT), the monomeric DAP-type PGN, reveals a buried ionic interaction between the unique carboxyl group of DAP and a previously unrecognized arginine residue. This arginine is conserved in the known DAP-type PGN-interacting PGRPs and contributes significantly to the affinity of the protein for the ligand. Unexpectedly, TCT induces infinite head-to-tail dimerization of PGRP-LE, in which the disaccharide moiety, but not the peptide stem, of TCT is positioned at the dimer interface. A sequence comparison suggests that TCT induces heterodimerization of the ectodomains of PGRP-LCx and -LCa in a closely analogous manner to prime the IMD signaling pathway, except that the heterodimer formation is nonperpetuating.

Lin L. N., Li J., Brandts J. F., and Weis R. M. (1994) The serine receptor of bacterial chemotaxis exhibits half-site saturation for serine binding. *Biochemistry* **33**, 6564-6570.

Abstract: Ligand binding to the serine receptor of Escherichia coli has been studied using isothermal titration calorimetry. Bacterial inner membranes enriched in the serine receptor (Tsr) were titrated as sonicated membrane samples and after solubilization in octyl beta-D-glucopyranoside (OG) to determine the number of moles of ligand bound per mole of receptor (n), the binding constant (K_a), and the enthalpy of binding (ΔH) of serine to the receptor. The n value for serine binding to OG-solubilized Tsr protein (n =

0.5) was consistent with one molecule of serine binding to a receptor dimer, but in sonicated inner membrane samples, the n value was smaller (n approximately equal to 0.25), indicating that not all of the binding sites were accessible to added serine. At 7 and 27 degrees C, the values for K_a and ΔH were equivalent for the membrane and OG-solubilized samples and were found to be $4.7 \times 10^4 \text{ M}^{-1}$ and -15 kcal/mol, and $3.6 \times 10^4 \text{ M}^{-1}$ and -18 kcal/mol, respectively. The influence of covalent modification at the sites of methylation on the affinity of the receptor for serine was also investigated, and found to have only a modest effect. The property of half-site saturation is suggestive of models for transmembrane signaling where the receptor subunit interactions are modulated by ligand binding.

Lin S. H. and Lee J. C. (2002) Communications between the high-affinity cyclic nucleotide binding sites in *E. coli* cyclic AMP receptor protein: effect of single site mutations. *Biochemistry* **41**, 11857-11867.

Abstract: The binding of adenosine 3',5'-cyclic monophosphate (cAMP) and its nonfunctional analogue, guanosine 3',5'-cyclic monophosphate (cGMP), to the adenosine 3',5'-cyclic monophosphate receptor protein (CRP) from *Escherichia coli* was investigated by means of fluorescence and isothermal titration calorimetry (ITC) at pH 7.8 and 25 degrees C. A biphasic fluorescence titration curve was observed, confirming the previous observation reported by this laboratory (Heyduk and Lee (1989) *Biochemistry* **28**, 6914-6924). However, the triphasic titration curve obtained from the ITC study suggests that the cAMP binding to CRP is more complicated than the previous conclusion that CRP binds sequentially two molecules of cAMP with negative cooperativity. The binding data can best be represented by a model for two identical interactive high-affinity sites and one low-affinity binding site. Unlike cAMP, the binding of cGMP to CRP exhibits no cooperativity between the high-affinity sites. The effects of mutations on the bindings of cAMP and cGMP to CRP were also investigated. The eight CRP mutants studied were K52N, D53H, S62F, T127L, G141Q, L148R, H159L, and K52N/H159L. These sites are located neither in the ligand binding site nor at the subunit interface. The binding was monitored by fluorescence. Although these mutations are at a variety of locations in CRP, the basic mechanism of CRP binding to cyclic nucleotides has not been affected. Two cyclic nucleotide molecules bind to the high-affinity sites of CRP. The cooperativity of cAMP binding is affected by mutation. It ranges from negative to positive cooperativity. The binding of cGMP shows none. With the exception of the T127L mutant, the free energy change for DNA-CRP complex formation increases linearly with increasing free energy change associated with the cooperativity of cAMP binding. This linear relationship implies that the protein molecule modulates the signal in the binding of cAMP, even though the mutation is not directly involved in cAMP or DNA binding. In addition, the significant differences in the amplitude of fluorescent signal indicate that the mutations also affect the surface characteristics of CRP. These results imply that these mutations are not perturbing specific pathways of signal transmission. Instead, these results are more consistent with the concept that CRP exists as an ensemble of native states, the distribution of which can be altered by these mutations.

Lu J., Chen M., Dekoster G. T., Cistola D. P. and Li E. (2008) The RXRalpha C-terminus T462 is a NMR sensor for coactivator peptide binding. *Biochem Biophys Res Commun* **366**, 932-937.

Abstract: The C-terminal activation function-2 (AF-2) helix plays a crucial role in retinoid X receptor alpha (RXRalpha)-mediated gene expression. Here, we report a nuclear magnetic resonance (NMR) study of the RXRalpha ligand-binding domain complexed with 9-cis-retinoic acid and a glucocorticoid receptor-interacting protein 1 peptide. The AF-2 helix and most of the C-terminal residues were undetectable due to a severe line-broadening effect. Due to its outstanding signal-to-noise ratio, the C-terminus residue, threonine 462 (T462) exhibited two distinct crosspeaks during peptide titration, suggesting that peptide binding was in a slow exchange regime on the chemical shift timescale. Consistently, the $K(d)$ derived from T462 intensity decay agreed with that derived from isothermal titration calorimetry. Furthermore, the exchange contribution to the $(15)\text{N}$ transverse relaxation rate was measurable in either T462 or the bound peptide. These results suggest that T462 is a sensor for coactivator binding and is a potential probe for AF-2 helix mobility

Lubman O. Y., Ilagan M. X., Kopan R., and Barrick D. (2007) Quantitative Dissection of the Notch:CSL Interaction: Insights into the Notch-mediated Transcriptional Switch. *J Mol Biol* **365**, 577-589.

Abstract: Complex formation between the intracellular domain of the Notch receptor (NICD) and the transcription factor CSL is indispensable for transcriptional activation. To understand how NICD displaces

CSL-associated co-repressors, we have quantified the binding of different Notch1 ICD regions to a key interaction domain (the beta trefoil domain, or BTD) of human CSL. Electrophoresis, scattering, and titration calorimetry indicate that NICD and BTD combine to form a 1:1 heterodimer. Neither the Notch1 ankyrin domain (ANK) nor C-terminal region contributes binding energy towards BTD. In contrast, binding energy is attributed largely to a short segment including the conserved WFP sequence motif within the RAM region (the approximately 140 residue polypeptide segment N-terminal to the ANK domain); substitution of this motif substantially reduces affinity. Short (≤ 25 residues) WFP-containing peptides encoded by the four mammalian Notch genes have similar affinities to BTD; thus, activity differences between paralogues either result from other regions of NICD and CSL or from differences in interaction with downstream components. The importance of RAM was demonstrated by the ability of a short RAM peptides to dissociate NICD:CSL interaction in cellular lysates. These results support an emerging molecular mechanism for the displacement of co-repressors from DNA-bound CSL by NICD.

Lubman O. Y. and Waksman G. (2003) Structural and thermodynamic basis for the interaction of the Src SH2 domain with the activated form of the PDGF beta-receptor. *J Mol Biol* **328**, 655-668.

Abstract: Recruitment of the Src kinase to the activated form of the platelet-derived growth factor (PDGF) receptor involves recognition of a unique sequence motif in the juxtamembrane region of the receptor by the Src homology 2 (SH2) domain of the enzyme. This motif contains two phosphotyrosine residues separated by one residue (sequence pYIpYV where pY indicates a phosphotyrosine). Here, we provide the thermodynamic and structural basis for the binding of this motif by the Src SH2 domain. We show that the second phosphorylation event increases the free energy window for specific interaction and that the physiological target is exquisitely designed for the task of recruiting specifically an SH2 domain which otherwise demonstrates very little intrinsic ability to discriminate sequences C-terminal to the first phosphorylation event. Surprisingly, we show that water plays a role in the recognition process.

Lundback T. and Hard T. (1996) Sequence-specific DNA-binding dominated by dehydration. *Proc Natl Acad Sci U S A* **93**, 4754-4759.

Abstract: Fluorescence spectroscopy and isothermal titration calorimetry were used to study the thermodynamics of binding of the glucocorticoid receptor DNA-binding domain to four different, but similar, DNA-binding sites. The binding sites are two naturally occurring sites that differ in the composition of one base pair, i.e., an A-T to G-C mutation, and two sites containing chemical intermediates of these base pairs. The calorimetrically determined heat capacity change ($\Delta C_{p,obs}$) for glucocorticoid receptor DNA-binding domain binding agrees with that calculated for dehydration of solvent-accessible surface areas. A dominating effect of dehydration or solvent reorganization on the thermodynamics is also consistent with an observed linear relationship between observed enthalpy change (ΔH_{obs}) and observed entropy change (ΔS_{obs}) with a slope close to the experimental temperature. Comparisons with structural data allow us to rationalize individual differences between ΔH_{obs} (and ΔS_{obs}) for the four complexes. For instance, we find that the removal of a methyl group at the DNA-protein interface is enthalpically favorable but entropically unfavorable, which is consistent with a replacement by an ordered water molecule.

Lundback T., van Den B. S., and Hard T. (2000) Sequence-specific DNA binding by the glucocorticoid receptor DNA-binding domain is linked to a salt-dependent histidine protonation. *Biochemistry* **39**, 8909-8916.

Abstract: We used isothermal titration calorimetry in the temperature range 21-25 degrees C to investigate the effect of pH on the calorimetric enthalpy ($\Delta H(cal)$) for sequence specific DNA-binding of the glucocorticoid receptor DNA-binding domain (GR DBD). Titrations were carried out in solutions containing 100 mM NaCl, 1 mM dithiothreitol, 5% glycerol by volume, and 20 mM Tris, Hepes, Mops, or sodium phosphate buffers at pH 7.5. A strong dependence of $\Delta H(cal)$ on the buffer ionization enthalpy is observed, demonstrating that the DNA binding of the GR DBD is linked to proton uptake at these conditions. The apparent increase in the pK(a) for an amino acid side chain upon DNA binding is supported by the results of complementary titrations, where $\Delta H(cal)$ shows a characteristic dependence on the solution pH. $\Delta H(cal)$ is also a function of the NaCl concentration, with opposite dependencies in Tris and Hepes buffers, respectively, such that a similar $\Delta H(cal)$ value is approached at 300 mM NaCl. This behavior shows that the DNA-binding induced protonation is inhibited by increased concentrations of NaCl. A comparison with structural data suggests that the protonation involves a histidine (His451) in the

GR DBD, because in the complex this residue is located close to a DNA phosphate at an orientation that is consistent with a charged-charged hydrogen bond in the protonated state. NMR spectra show that His451 is not protonated in the unbound protein at pH 7.5. The pH dependence in $\Delta H(\text{cal})$ can be quantitatively described by a shift of the $pK(a)$ of His451 from approximately 6 in the unbound state to close to 8 when bound to DNA at low salt concentration conditions. A simple model involving a binding competition between a proton and a Na^+ counterion to the GR DBD-DNA complex reproduces the qualitative features of the salt dependence.

Machius M., Brautigam C. A., Tomchick D. R., Ward P., Otwinowski Z., Blevins J. S., Deka R. K. and Norgard M. V. (2007) Structural and biochemical basis for polyamine binding to the Tp0655 lipoprotein of *Treponema pallidum*: putative role for Tp0655 (TpPotD) as a polyamine receptor. *J Mol Biol* **373**, 681-694.

Abstract: Tp0655 of *Treponema pallidum*, the causative agent of syphilis, is predicted to be a 40 kDa membrane lipoprotein. Previous sequence analysis of Tp0655 noted its homology to polyamine-binding proteins of the bacterial PotD family, which serve as periplasmic ligand-binding proteins of ATP-binding-cassette (ABC) transport systems. Here, the 1.8 Å crystal structure of Tp0655 demonstrated structural homology to *Escherichia coli* PotD and PotF. The latter two proteins preferentially bind spermidine and putrescine, respectively. All of these proteins contain two domains that sandwich the ligand between them. The ligand-binding site of Tp0655 can be occupied by 2-(N-morpholino)ethanesulfanoic acid, a component of the crystallization medium. To discern the polyamine binding preferences of Tp0655, the protein was subjected to isothermal titration calorimetric experiments. The titrations established that Tp0655 binds polyamines avidly, with a marked preference for putrescine ($K_d=10$ nM) over spermidine ($K_d=430$ nM), but the related compounds cadaverine and spermine did not bind. Structural comparisons and structure-based sequence analyses provide insights into how polyamine-binding proteins recognize their ligands. In particular, these comparisons allow the derivation of rules that may be used to predict the function of other members of the PotD family. The sequential, structural, and functional homology of Tp0655 to PotD and PotF prompt the conclusion that the former likely is the polyamine-binding component of an ABC-type polyamine transport system in *T. pallidum*. We thus rename Tp0655 as TpPotD. The ramifications of TpPotD as a polyamine-binding protein to the parasitic strategy of *T. pallidum* are discussed.

Madrid K.P., and Jardim A. (2005) Peroxin 5-peroxin 14 association in the protozoan *Leishmania donovani* involves a novel protein-protein interaction motif. *Biochem J.* **391**, 105-14.

Abstract: Import of proteins with a PTS1 (peroxisomal targeting signal 1) into the *Leishmania* glycosomal organelle involves docking of a PTS1-laden LdPEX5 [*Leishmania donovani* PEX5 (peroxin 5)] receptor to LdPEX14 on the surface of the glycosomal membrane. In higher eukaryotes, the PEX5-PEX14 interaction is mediated by a conserved diaromatic WXXXY/F motif. Site-directed and deletion mutageneses of the three WXXXY/F repeats in LdPEX5 did not abolish the LdPEX5-LdPEX14 association. Analysis of the equilibrium dissociation constant ($K(d)$) revealed that *ldpex5*-W53A (Trp53-->Ala), *ldpex5*-W293A, *ldpex5*-W176,293A and *ldpex5*-W53,176,293A mutant receptors were capable of binding LdPEX14 with affinities comparable with wild-type LdPEX5. That the diaromatic motifs were not required for the LdPEX5-LdPEX14 interaction was further verified by deletion analysis that showed that *ldpex5* deletion mutants or *ldpex5* fragments lacking the WXXXY/F motifs retained LdPEX14 binding activity. Mapping studies of LdPEX5 indicated that the necessary elements required for LdPEX14 association were localized to a region between residues 290 and 323. Finally, mutational analysis of LdPEX14 confirmed that residues 23-63, which encompass the conserved signature sequence AX2FLX7SPX6FLKGKGL/V present in all PEX14 proteins, are essential for LdPEX5 binding.

Mason A. B., Woodworth R. C., Oliver R. W., Green B. N., Lin L. N., Brandts J. F., Savage K. J., Tam B. M., and MacGillivray R. T. (1996) Association of the two lobes of ovotransferrin is a prerequisite for receptor recognition. Studies with recombinant ovotransferrins. *Biochem J* **319 (Pt 2)**, 361-368.

Abstract: Different recombinant N-lobes of chicken ovotransferrin (oTF/2N) have been isolated from the tissue-culture medium of baby hamster kidney cells transfected with the plasmid pNUT containing the relevant DNA coding sequence. Levels of up to 40, 55 and 30 mg/l oTF/2N were obtained for constructs defining residues 1-319, 1-332 and 1-337-(Ala)₃ respectively. In addition, a full-length non-glycosylated oTF was expressed at a maximum of 80 mg/l and a foreshortened oTF consisting of residues 1-682 was expressed at a level of 95 mg/l. These preparations were then used to produce, proteolytically, two different C-lobes (oTF/2C) comprising residues 342-686 and 342-682. The purified recombinant N-lobes (oTF/2N)

are similar to the proteolytically derived half-molecule with regard to immunoreactivity and spectral properties; they show some interesting differences in thermal stability. A sequence analysis of the cDNA revealed six changes at the nucleotide level that led to six differences in the amino acid sequence compared with that reported by Jeltsch and Chambon [(1982) *Eur. J. Biochem.* 122, 291-295]. Electrospray mass spectrometry gives results consistent with these six changes. Interaction between the various N- and C-lobes was measured by titration calorimetry. Studies show that only those lobes that associate in solution are able to bind to the receptors on chick embryo red blood cells. These findings do not support a previous report by Oratore et al.

McMahon S.A., Miller J.L., Lawton J.A., Kerkow D.E., Hodes A., Marti-Renom M.A., Doulatov S., Narayanan E., Sali A., Miller J.F., Ghosh P. (2005) The C-type lectin fold as an evolutionary solution for massive sequence variation. *Nat Struct Mol Biol.* 12, 886-92.

Abstract: Only few instances are known of protein folds that tolerate massive sequence variation for the sake of binding diversity. The most extensively characterized is the immunoglobulin fold. We now add to this the C-type lectin (CLec) fold, as found in the major tropism determinant (Mtd), a retroelement-encoded receptor-binding protein of Bordetella bacteriophage. Variation in Mtd, with its approximately 10(13) possible sequences, enables phage adaptation to Bordetella spp. Mtd is an intertwined, pyramid-shaped trimer, with variable residues organized by its CLec fold into discrete receptor-binding sites. The CLec fold provides a highly static scaffold for combinatorial display of variable residues, probably reflecting a different evolutionary solution for balancing diversity against stability from that in the immunoglobulin fold. Mtd variants are biased toward the receptor pertactin, and there is evidence that the CLec fold is used broadly for sequence variation by related retroelements.

McNemar C., Snow M. E., Windsor W. T., Prongay A., Mui P., Zhang R., Durkin J., Le H. V., and Weber P. C. (1997) Thermodynamic and structural analysis of phosphotyrosine polypeptide binding to Grb2-SH2. *Biochemistry* 36, 10006-10014.

Abstract: A thermodynamic analysis using isothermal titration calorimetry (ITC) has been performed to examine the binding interaction between the SH2 (Src homology 2) domain of growth factor receptor binding protein 2 (Grb2-SH2) and one of its phosphotyrosine (pY) polypeptide ligands. Interaction of the Shc-derived phosphotyrosine hexapeptide Ac-SpYVNVQ-NH₂ with Grb2-SH2 was both enthalpically and entropically favorable ($\Delta H = -7.55 \text{ kcal mol}^{-1}$, $-\Delta S = -1.46 \text{ kcal mol}^{-1}$, $\Delta G = -9.01 \text{ kcal mol}^{-1}$, $T = 20$ degrees C). ITC experiments using five alanine-substituted peptides were performed to examine the role of each side chain in binding. The results were consistent with homology models of the Grb2-SH2-Shc hexapeptide complex which identified several possible hydrogen bonds between Grb2-SH2 and the phosphotyrosine and conserved asparagine(+2) side chains of the Shc hexapeptide. These studies also demonstrated that the hydrophobic valine(+1) side chain contributes significantly to the favorable entropic component of binding. The thermodynamic and structural data are consistent with a Grb2-SH2 recognition motif of pY-hydrophobic-N-X (where X is any amino acid residue). The measured heat capacity of binding ($\Delta C_p = -146 \text{ cal mol}^{-1} \text{ K}^{-1}$) was very similar to computed values using semiempirical estimates ($\Delta C_p = -106$ to $-193 \text{ cal mol}^{-1} \text{ K}^{-1}$) derived from apolar and polar accessible surface area values calculated from several homology models of the Grb2-SH2-Shc hexapeptide complex. The homology model which most closely reproduced the measured ΔC_p value is also the model which had the lowest RMS deviation from the subsequently determined crystal structure. Calculations based on the thermodynamic data and these semiempirical estimates indicated that the binding event involves burial of nearly comparable apolar (677 A²) and polar (609 A²) surface areas.

Miller P. J., Pazy Y., Conti B., Riddle D., Appella E. and Collins E. J. (2007) Single MHC mutation eliminates enthalpy associated with T cell receptor binding. *J Mol Biol* 373, 315-327.

Abstract: The keystone of the adaptive immune response is T cell receptor (TCR) recognition of peptide presented by major histocompatibility complex (pMHC) molecules. The crystal structure of AHIII TCR bound to MHC, HLA-A2, showed a large interface with an atypical binding orientation. MHC mutations in the interface of the proteins were tested for changes in TCR recognition. From the range of responses observed, three representative HLA-A2 mutants, T163A, W167A, and K66A, were selected for further study. Binding constants and co-crystal structures of the AHIII TCR and the three mutants were determined. K66 in HLA-A2 makes contacts with both peptide and TCR, and has been identified as a

critical residue for recognition by numerous TCR. The K66A mutation resulted in the lowest AHIII T cell response and the lowest binding affinity, which suggests that the T cell response may correlate with affinity. Importantly, the K66A mutation does not affect the conformation of the peptide. The change in affinity appears to be due to a loss in hydrogen bonds in the interface as a result of a conformational change in the TCR complementarity-determining region 3 (CDR3) loop. Isothermal titration calorimetry confirmed the loss of hydrogen bonding by a large loss in enthalpy. Our findings are inconsistent with the notion that the CDR1 and CDR2 loops of the TCR are responsible for MHC restriction, while the CDR3 loops interact solely with the peptide. Instead, we present here an MHC mutation that does not change the conformation of the peptide, yet results in an altered conformation of a CDR3.

Mimura Y., Sondermann P., Ghirlando R., Lund J., Young S. P., Goodall M., and Jefferis R. (2001) Role of oligosaccharide residues of IgG1-Fc in Fc gamma RIIb binding. *J Biol Chem* **276**, 45539-45547.

Abstract: Engagement of Fc gamma receptors (Fc gamma Rs) with the Fc region of IgG elicits immune responses by leukocytes. The recent crystal structure of Fc gamma RIII in complex with IgG-Fc has provided details of molecular interactions between these components (Sondermann, P., Huber, R., Oosthuizen, V., and Jacob, U. (2000) *Nature* 406, 267-273). One of the most intriguing issues is that glycosylation of IgG-Fc is essential for the recognition by Fc gamma Rs although the carbohydrate moieties are on the periphery of the Fc gamma RIII-Fc interface. To better understand the role of Fc glycosylation in Fc gamma R binding we prepared homogeneous glycoforms of IgG-Fc (Cri) and investigated the interactions with a soluble form of Fc gamma RIIb (sFc gamma RIIb). A 1:1 complex stoichiometry was observed in solution at 30 degrees C ($K(d)$, 0.94 μ m; ΔG , -8.4 kcal mol⁻¹; ΔH , -6.5 kcal mol⁻¹; $T \Delta S$, 1.9 kcal mol⁻¹; ΔC_p , -160 cal mol⁻¹ K⁻¹). Removal of terminal galactose residues did not alter the thermodynamic parameters significantly. Outer-arm GlcNAc residues contributed significantly to thermal stability of the C(H)2 domains but only slightly to sFc gamma RIIb binding. Truncation of 1,3- and 1,6-arm mannose residues generates a linear trisaccharide core structure and resulted in a significantly decreased affinity, a less exothermic ΔH , and a more negative ΔC_p for sFc gamma RIIb binding, which may result from a conformational change coupled to complex formation. Deglycosylation of the C(H)2 domains abrogated sFc gamma RIIb binding and resulted in the lowest thermal stability accompanied with noncooperative unfolding. These results suggest that truncation of the oligosaccharides of IgG-Fc causes disorder and a closed disposition of the two C(H)2 domains, impairing sFc gamma RIIb binding.

Mine S., Koshiba T., Honjo E., Okamoto T., Tamada T., Maeda Y., Matsukura Y., Horie A., Ishibashi M., Sato M., Azuma M., Tokunaga M., Nitta K., and Kuroki R. (2004) Thermodynamic analysis of the activation mechanism of the GCSF receptor induced by ligand binding. *Biochemistry* **43**, 2458-2464.

Abstract: The granulocyte colony-stimulating factor receptor (GCSFR), containing the Ig-like domain (Ig) and cytokine receptor homologous region (CRH), was prepared as a preformed dimer (Ig-CRH-Fc)(2) after fusion to the mouse Fc region via an eight-residue linker (approximately 55 A). Monomer Ig-CRH was also prepared after the Fc region was removed from (Ig-CRH-Fc)(2). GCSF binding to Ig-CRH and (Ig-CRH-Fc)(2) was investigated using light scattering and isothermal titration calorimetry. The average molecular mass determined by light scattering showed that both Ig-CRH and (Ig-CRH-Fc)(2) formed a 2:2 dimer with GCSF. Moreover, isothermal titration calorimetry showed that the thermodynamic parameters upon binding of GCSF to Ig-CRH and (Ig-CRH-Fc)(2) were comparable, suggesting a similar binding stoichiometry and interface [including similar buried surface area (5700-6000 A(2))] despite the presence of the eight-residue linker. The buried surface area is much larger than that calculated from our previous report of the crystal structure of the GCSF-CRH complex [Aritomi, M., et al. (1999) *Nature* 401, 713-717], suggesting a substantial contribution of the Ig domain to GCSF binding. The data also indicate that the distance (55 A) between two CRH domains in the 2:2 complex is much shorter than in our previous model (approximately 90 A) predicted from the same crystal structure of the GCSF-CRH complex.

Monaghan P., Woznica I., Moza B., Sundberg E. J. and Rosenblatt M. (2007) Recombinant expression and purification of the N-terminal extracellular domain of the parathyroid hormone receptor. *Protein Expr Purif* **54**, 87-93.

Abstract: Our goal is to elucidate the nature of the bimolecular interaction of parathyroid hormone (PTH) with its receptor, the parathyroid hormone receptor type-1 (PTH1R). In order to study this interaction, we are aiming to obtain a three-dimensional structure of the PTH-PTH1R bimolecular complex. Due to the

very low expression levels of endogenous PTHR1, a recombinant form is required for structural analysis. However, the extreme hydrophobicity of the transmembrane regions of PTHR1 makes heterologous expression of PTHR1 difficult. Therefore, we sought to express the N-terminal extracellular domain (N-ECD) of PTHR1, a region that plays a pivotal role in ligand interaction. We expressed the N-ECD in both bacterial (*Escherichia coli*) and insect (Sf9) cells. The form produced in *E. coli*, a fusion-protein with thioredoxin, is soluble. However, removal of the fusion partner from a partially purified preparation results in dramatic loss of yield of the N-ECD. Expression in Sf9 cells, however, facilitates purification of a soluble form of the N-ECD. Isothermal calorimetry demonstrates that this N-ECD binds PTH-(1-34), albeit with lower affinity than the full-length receptor. This report describes the expression and purification of milligram quantities of the isolated N-ECD of PTHR1. The receptor fragment retains the ability to bind its cognate peptide ligand, an important pre-requisite for subsequent structural studies.

Myszka D. G., Sweet R. W., Hensley P., Brigham-Burke M., Kwong P. D., Hendrickson W. A., Wyatt R., Sodroski J., and Doyle M. L. (2000) Energetics of the HIV gp120-CD4 binding reaction. *Proc Natl Acad Sci U S A* **97**, 9026-9031.

Abstract: HIV infection is initiated by the selective interaction between the cellular receptor CD4 and gp120, the external envelope glycoprotein of the virus. We used analytical ultracentrifugation, titration calorimetry, and surface plasmon resonance biosensor analysis to characterize the assembly state, thermodynamics, and kinetics of the CD4-gp120 interaction. The binding thermodynamics were of unexpected magnitude; changes in enthalpy, entropy, and heat capacity greatly exceeded those described for typical protein-protein interactions. These unusual thermodynamic properties were observed with both intact gp120 and a deglycosylated and truncated form of gp120 protein that lacked hypervariable loops V1, V2, and V3 and segments of its N and C termini. Together with previous crystallographic studies, the large changes in heat capacity and entropy reveal that extensive structural rearrangements occur within the core of gp120 upon CD4 binding. CD spectral studies and slow kinetics of binding support this conclusion. These results indicate considerable conformational flexibility within gp120, which may relate to viral mechanisms for triggering infection and disguising conserved receptor-binding sites from the immune system.

Naur P., Hansen K. B., Kristensen A. S., Dravid S. M., Pickering D. S., Olsen L., Vestergaard B., Egebjerg J., Gajhede M., Traynelis S. F. and Kastrup J. S. (2007) Ionotropic glutamate-like receptor delta2 binds D-serine and glycine. *Proc. Natl. Acad. Sci U. S. A* **104**, 14116-14121.

Abstract: The orphan glutamate-like receptor GluRdelta2 is predominantly expressed in Purkinje cells of the central nervous system. The classification of GluRdelta2 to the ionotropic glutamate receptor family is based on sequence similarities, because GluRdelta2 does not form functional homomeric glutamate-gated ion channels in transfected cells. Studies in GluRdelta2(-/-) knockout mice as well as in mice with naturally occurring mutations in the GluRdelta2 gene have demonstrated an essential role of GluRdelta2 in cerebellar long-term depression, motor learning, motor coordination, and synaptogenesis. However, the lack of a known agonist has hampered investigations on the function of GluRdelta2. In this study, the ligand-binding core of GluRdelta2 (GluRdelta2-S1S2) was found to bind neutral amino acids such as D-serine and glycine, as demonstrated by isothermal titration calorimetry. Direct evidence for binding of D-serine and structural rearrangements in the binding cleft of GluRdelta2-S1S2 is provided by x-ray structures of GluRdelta2-S1S2 in its apo form and in complex with D-serine. Functionally, D-serine and glycine were shown to inactivate spontaneous ion-channel conductance in GluRdelta2 containing the lurcher mutation (EC(50) values, 182 and 507 microM, respectively). These data demonstrate that the GluRdelta2 ligand-binding core is capable of binding ligands and that cleft closure of the ligand-binding core can induce conformational changes that alter ion permeation.

Ng C., Jackson R. A., Buschdorf J. P., Sun Q., Guy G. R. and Sivaraman J. (2008) Structural basis for a novel intrapeptidyl H-bond and reverse binding of c-Cbl-TKB domain substrates. *EMBO J* **27**, 804-816.

Abstract: The c-Cbl tyrosine kinase binding domain (Cbl-TKB), essentially an 'embedded' SH2 domain, has a critical role in targeting proteins for ubiquitination. To address how this domain can bind to disparate recognition motifs and to determine whether this results in variations in substrate-binding affinity, we compared crystal structures of the Cbl-TKB domain complexed with phosphorylated peptides of Sprouty2, Sprouty4, epidermal growth factor receptor, Syk, and c-Met receptors and validated the binding with point-mutational analyses using full-length proteins. An obligatory, intrapeptidyl H-bond between the

phosphotyrosine and the conserved asparagine or adjacent arginine is essential for binding and orients the peptide into a positively charged pocket on c-Cbl. Surprisingly, c-Met bound to Cbl in the reverse direction, which is unprecedented for SH2 domain binding. The necessity of this intrapeptidyl H-bond was confirmed with isothermal titration calorimetry experiments that also showed Sprouty2 to have the highest binding affinity to c-Cbl; this may enable the selective sequestration of c-Cbl from other target proteins

Nisius L., Rogowski M., Vangelista L. and Grzesiek S. (2008) Large-scale expression and purification of the major HIV-1 coreceptor CCR5 and characterization of its interaction with RANTES. *Protein Expr Purif* **61**, 155-162.

Abstract: The G protein-coupled receptor CCR5 is a human chemokine receptor involved in the activation and migration of leukocytes. CCR5 is also the major HIV-1 coreceptor that, together with human CD4 and the viral glycoprotein gp120, promotes virus entry into host cells. Thus inhibition of the CCR5-gp120 interaction presents a promising route to prevent HIV infections. Atomic structural details of the interaction between CCR5 and its cognate chemokines or gp120 are presently unknown due to the general difficulties of membrane protein structure determination. Here, we report the high-yield expression of human CCR5 in baculovirus-infected Sf9 insect cells. Highly purified (>90%) CCR5 is obtained in detergent-solubilized form at yields of about 1 mg/l cell culture. The conformational integrity of recombinant CCR5 after purification is shown by immunoprecipitation with the conformation-dependent monoclonal antibody 2D7, CD and NMR spectroscopy. The detergent micelles contain CCR5 in monomeric and dimeric forms, which can be separated by size exclusion chromatography and characterized individually. Further functional characterization by isothermal titration calorimetry indicates that the recombinant receptor interacts with its cognate chemokine RANTES. This interaction is strongly suppressed when sulfation of CCR5 is inhibited in the insect cells

Ojida A., Mito-oka Y., Sada K., and Hamachi I. (2004) Molecular recognition and fluorescence sensing of monophosphorylated peptides in aqueous solution by bis(zinc(II)-dipicolylamine)-based artificial receptors. *J Am Chem Soc* **126**, 2454-2463.

Abstract: The phosphorylation of proteins represents a ubiquitous mechanism for the cellular signal control of many different processes, and thus selective recognition and sensing of phosphorylated peptides and proteins in aqueous solution should be regarded as important targets in the research field of molecular recognition. We now describe the design of fluorescent chemosensors bearing two zinc ions coordinated to distinct dipicolylamine (Dpa) sites. Fluorescence titration experiments show the selective and strong binding toward phosphate derivatives in aqueous solution. On the basis of (1)H NMR and (31)P NMR studies, and the single-crystal X-ray structural analysis, it is clear that two Zn(Dpa) units of the binuclear receptors cooperatively act to bind a phosphate site of these derivatives. Good agreement of the binding affinity estimated by isothermal titration calorimetry with fluorescence titration measurements revealed that these two receptors can fluorometrically sense several phosphorylated peptides that have consensus sequences modified with natural kinases. These chemosensors display the following significant features: (i) clear distinction between phosphorylated and nonphosphorylated peptides, (ii) sequence-dependent recognition, and (iii) strong binding to a negatively charged phosphorylated peptide, all of which can be mainly ascribed to coordination chemistry and electrostatic interactions between the receptors and the corresponding peptides. Detailed titration experiments clarified that the phosphate anion-assisted coordination of the second Zn(II) to the binuclear receptors is crucial for the fluorescence intensification upon binding to the phosphorylated derivatives. In addition, it is demonstrated that the binuclear receptors can be useful for the convenient fluorescent detection of a natural phosphatase (PTP1B) catalyzed dephosphorylation.

Pantoliano M. W., Horlick R. A., Springer B. A., Van Dyk D. E., Tobery T., Wetmore D. R., Lear J. D., Nahapetian A. T., Bradley J. D., and Sisk W. P. (1994) Multivalent ligand-receptor binding interactions in the fibroblast growth factor system produce a cooperative growth factor and heparin mechanism for receptor dimerization. *Biochemistry* **33**, 10229-10248.

Abstract: The binding interactions for the three primary reactants of the fibroblast growth factor (FGF) system, basic FGF (bFGF), an FGF receptor, FGFR1, and the cofactor heparin/heparan sulfate (HS), were explored by isothermal titrating calorimetry, ultracentrifugation, and molecular modeling. The binding reactions were first dissected into three binary reactions: (1) $FGFR1 + bFGF \rightleftharpoons FGFR1/bFGF$, $K_1 = 41$ (+/- 12) nM; (2) $FGFR1 + HS \rightleftharpoons FGFR1/HS$, $K_2 = 104$ (+/- 17) μ M; and (3) $bFGF + HS \rightleftharpoons bFGF/HS$,

$K_3 = 470$ (+/- 20) nM, where HS = low MW heparin, approximately 3 kDa. The first, binding of bFGF to FGFR1 in the absence of HS, was found to be a simple binary binding reaction that is enthalpy dominated and characterized by a single equilibrium constant, K_1 . The conditional reactions of bFGF and FGFR1 in the presence of heparin were then examined under conditions that saturate only the bFGF heparin site (1.5 equiv of HS/bFGF) or saturate the HS binding sites of both bFGF and FGFR1 (1.0 mM HS). Both 3- and 5-kDa low MW heparins increased the affinity for FGFR1 binding to bFGF by approximately 10-fold ($K_d = 4.9 \pm 2.0$ nM), relative to the reaction with no HS. In addition, HS, at a minimum of 1.5 equiv/bFGF, induced a second FGFR1 molecule to bind to another lower affinity secondary site on bFGF ($K_4 = 1.9 \pm 0.7 \mu\text{M}$) in an entropy-dominated reaction to yield a quaternary complex containing two FGFR1, one bFGF, and at least one HS. Molecular weight estimates by analytical ultracentrifugation of such fully bound complexes were consistent with this proposed composition. To understand these binding reactions in terms of structural components of FGFR1, a three-dimensional model of FGFR1 was constructed using segment match modeling. Electrostatic potential calculations confirmed that an elongated cluster, approximately $15 \times 35 \text{ \AA}$, of nine cationic residues focused positive potential (+2kBT) to the solvent-exposed beta-sheet A, B, E, C' surface of the D(II) domain model, strongly implicating this locus as the HS binding region of FGFR1. Structural models for HS binding to FGFR1, and HS binding to bFGF, were built individually and then assembled to juxtapose adjacent binding sites for receptor and HS on bFGF, against matching proposed growth factor and HS binding sites on FGFR1. The calorimetric binding results and the molecular modeling exercises suggest that bFGF and HS participate in a concerted bridge mechanism for the dimerization of FGFR1 in vitro and presumably for mitogenic signal transduction in vivo. (ABSTRACT TRUNCATED AT 400 WORDS).

Park S.Y., Borbat P.P., Gonzalez-Bonet G., Bhatnagar J., Pollard A.M., Freed J.H., Bilwes A.M., and Crane B.R. (2006) Reconstruction of the chemotaxis receptor-kinase assembly. *Nat Struct Mol Biol.* **13**, 400-7.

Abstract: In bacterial chemotaxis, an assembly of transmembrane receptors, the CheA histidine kinase and the adaptor protein CheW processes environmental stimuli to regulate motility. The structure of a *Thermotoga maritima* receptor cytoplasmic domain defines CheA interaction regions and metal ion-coordinating charge centers that undergo chemical modification to tune receptor response. Dimeric CheA-CheW, defined by crystallography and pulsed ESR, positions two CheWs to form a cleft that is lined with residues important for receptor interactions and sized to clamp one receptor dimer. CheW residues involved in kinase activation map to interfaces that orient the CheW clamps. CheA regulatory domains associate in crystals through conserved hydrophobic surfaces. Such CheA self-contacts align the CheW receptor clamps for binding receptor tips. Linking layers of ternary complexes with close-packed receptors generates a lattice with reasonable component ratios, cooperative interactions among receptors and accessible sites for modification enzymes.

Pearce K. H., Jr., Ultsch M. H., Kelley R. F., de Vos A. M., and Wells J. A. (1996) Structural and mutational analysis of affinity-inert contact residues at the growth hormone-receptor interface. *Biochemistry* **35**, 10300-10307.

Abstract: Mutational studies have shown that over two-thirds of the contact side chains at the human growth hormone (hGH)-receptor interface have little or no impact on binding affinity when converted to alanine [Cunningham, B. C., & Wells, J. A. (1993) *J. Mol. Biol.* 234, 554-563; Clackson, T., & Wells, J. A. (1995) *Science* 267, 383-386]. Herein, three of the most buried, yet functionally inert, residues on hGH (F25, Y42, and Q46) have been simultaneously mutated to alanine. Binding kinetics of the triple-alanine mutant shows that neither association nor dissociation rates are significantly affected and only slight, local disorder is seen in the crystal structure. However, large and compensating changes were observed in the enthalpy and entropy of binding as determined by isothermal titration calorimetry. The triple-alanine mutant bound with a more favorable enthalpy ($\Delta H = -12.2 \pm 0.7$ kcal/mol) and corresponding less favorable entropy [$\Delta S = -2.3 \pm 2.4$ cal/(mol.K)] compared to the wild-type interaction [$\Delta H = -9.4 \pm 0.3$ kcal/mol; $\Delta S = 7.7 \pm 1.2$ cal/(mol.K)]. Dissection of the triple-alanine mutant into the single F25A and double Y42A/Q46A mutant showed that the more favorable enthalpy was derived from the removal of the F25 side chain on helix-1 of the hormone. The ΔC_p values for both the triple-alanine mutant [-927 ± 10 cal/(mol.K)] and the individual mutants were significantly more negative than the ΔC_p for the wild-type interaction [-767 ± 34 cal/(mol.K)]. Such negative ΔC_p values are consistent with the proposal that the

hydrophobic effect is the primary contributor to the free energy of binding at this protein-protein interface. These results show that multiple-alanine mutations at contact residues may not affect binding kinetics, affinity, or global structure; however, they can produce local structural changes and can cause large compensating effects on the heat and entropy of binding. These studies emphasize that one cannot infer binding free energy from the existence of contacts alone and further support the notion that only a small set of contacts are crucial for the human growth hormone-receptor interaction.

Pickens J. C., Merritt E. A., Ahn M., Verlinde C. L., Hol W. G., and Fan E. (2002) Anchor-based design of improved cholera toxin and E. coli heat-labile enterotoxin receptor binding antagonists that display multiple binding modes. *Chem Biol* **9**, 215-224.

Abstract: The action of cholera toxin and E. coli heat-labile enterotoxin can be inhibited by blocking their binding to the cell-surface receptor GM1. We have used anchor-based design to create 15 receptor binding inhibitors that contain the previously characterized inhibitor MNPG as a substructure. In ELISA assays, all 15 compounds exhibited increased potency relative to MNPG. Binding affinities for two compounds, each containing a morpholine ring linked to MNPG via a hydrophobic tail, were characterized by pulsed ultrafiltration (PUF) and isothermal titration calorimetry (ITC). Crystal structures for these compounds bound to toxin B pentamer revealed a conserved binding mode for the MNPG moiety, with multiple binding modes adopted by the attached morpholine derivatives. The observed binding interactions can be exploited in the design of improved toxin binding inhibitors.

Qin H., Shi J., Noberini R., Pasquale E. B. and Song J. (2008) Crystal structure and NMR binding reveal that two small molecule antagonists target the high affinity ephrin-binding channel of the EphA4 receptor. *J Biol Chem* **283**, 29473-29484.

Abstract: The Eph receptor tyrosine kinases regulate a variety of physiological and pathological processes not only during development but also in adult organs, and therefore they represent a promising class of drug targets. The EphA4 receptor plays important roles in the inhibition of the regeneration of injured axons, synaptic plasticity, platelet aggregation, and likely in certain types of cancer. Here we report the first crystal structure of the EphA4 ligand-binding domain, which adopts the same jellyroll beta-sandwich architecture as shown previously for EphB2 and EphB4. The similarity with EphB receptors is high in the core beta-stranded regions, whereas large variations exist in the loops, particularly the D-E and J-K loops, which form the high affinity ephrin binding channel. We also used isothermal titration calorimetry, NMR spectroscopy, and computational docking to characterize the binding to EphA4 of two small molecules, 4- and 5-(2,5 dimethyl-pyrrol-1-yl)-2-hydroxybenzoic acid which antagonize ephrin-induced effects in EphA4-expressing cells. We show that the two molecules bind to the EphA4 ligand-binding domain with $K(d)$ values of 20.4 and 26.4 microm, respectively. NMR heteronuclear single quantum coherence titrations revealed that upon binding, both molecules significantly perturb EphA4 residues Ile(31)-Met(32) in the D-E loop, Gln(43) in the E beta-strand, and Ile(131)-Gly(132) in the J-K loop. Molecular docking shows that they can occupy a cavity in the high affinity ephrin binding channel of EphA4 in a similar manner, by interacting mainly with the EphA4 residues in the E strand and D-E and J-K loops. However, many of the interactions observed in Eph receptor-ephrin complexes are absent, which is consistent with the small size of the two molecules and may account for their relatively weak binding affinity. Thus, our studies provide the first published structure of the ligand-binding domain of an EphA receptor of the A subclass. Furthermore, the results demonstrate that the high affinity ephrin binding channel of the Eph receptors is amenable to targeting with small molecule antagonists and suggest avenues for further optimization

Ran X., and Song J. (2005) Structural insight into the binding diversity between the Tyr-phosphorylated human ephrinBs and Nck2 SH2 domain. *J Biol Chem.* **280**, 19205-12.

Abstract: The binding interaction between the Nck2 SH2 domain and the phosphorylated ephrinB initiates a critical pathway for the reverse signaling network mediated by Eph receptor-ephrinB. Previously, the NMR structure and Tyr phosphorylations of the human ephrinB cytoplasmic domain have been studied. To obtain a complete story, it would be of significant interest to determine the structure of the Nck2 SH2 domain that shows a low sequence identity to other SH2 domains with known structures. Here, we report the determination of the solution structure of the human Nck2 SH2 domain and investigate its interactions with three phosphorylated ephrinB fragments by NMR spectroscopy. The results indicate that: 1) although the human Nck2 SH2 domain adopts a core tertiary fold common to all SH2 domains, it owns some unique properties such as a shorter C-terminal helix and unusual electrostatic potential surface. However, the most

striking finding is that the C-terminal tail of the human Nck2 SH2 domain adopts a short antiparallel beta-sheet that, to the best of our knowledge, has never been identified in other SH2 domains. The truncation study suggests that one function of the C-terminal tail is to control the folding/solubility of the SH2 domain. 2) In addition to [Tyr(P)304]ephrinB2(301-322) and [Tyr(P)316]ephrinB2(301-322), here we identified [Tyr(P)330]ephrinB2(324-333) also capable of binding to the SH2 domain. The detailed NMR study indicated that the binding mechanisms for the three ephrinB fragments might be different. The binding with [Tyr(P)304]-ephrinB2(301-322) and [Tyr(P)316]ephrinB2(301-322) might be mostly involved in the residues over the N-half of the SH2 domain and provoked a significant increase in the backbone and side chain dynamics of the SH2 domain on the microsecond-millisecond time scale. In contrast, binding with [Tyr(P)330]ephrinB2(324-333) might have most residues over both halves engaged but induced less profound conformational dynamics on the mus-ms time scale.

Porter C. J., Matthews J. M., Mackay J. P., Pursglove S. E., Schmidberger J. W., Leedman P. J., Pero S. C., Krag D. N., Wilce M. C. and Wilce J. A. (2007) Grb7 SH2 domain structure and interactions with a cyclic peptide inhibitor of cancer cell migration and proliferation. *BMC. Struct. Biol* **7**, 58.

Abstract: BACKGROUND: Human growth factor receptor bound protein 7 (Grb7) is an adapter protein that mediates the coupling of tyrosine kinases with their downstream signaling pathways. Grb7 is frequently overexpressed in invasive and metastatic human cancers and is implicated in cancer progression via its interaction with the ErbB2 receptor and focal adhesion kinase (FAK) that play critical roles in cell proliferation and migration. It is thus a prime target for the development of novel anti-cancer therapies. Recently, an inhibitory peptide (G7-18NATE) has been developed which binds specifically to the Grb7 SH2 domain and is able to attenuate cancer cell proliferation and migration in various cancer cell lines. RESULTS: As a first step towards understanding how Grb7 may be inhibited by G7-18NATE, we solved the crystal structure of the Grb7 SH2 domain to 2.1 Å resolution. We describe the details of the peptide binding site underlying target specificity, as well as the dimer interface of Grb 7 SH2. Dimer formation of Grb7 was determined to be in the μM range using analytical ultracentrifugation for both full-length Grb7 and the SH2 domain alone, suggesting the SH2 domain forms the basis of a physiological dimer. ITC measurements of the interaction of the G7-18NATE peptide with the Grb7 SH2 domain revealed that it binds with a binding affinity of $K_d =$ approximately 35.7 μM and NMR spectroscopy titration experiments revealed that peptide binding causes perturbations to both the ligand binding surface of the Grb7 SH2 domain as well as to the dimer interface, suggesting that dimerisation of Grb7 is impacted on by peptide binding. CONCLUSION: Together the data allow us to propose a model of the Grb7 SH2 domain/G7-18NATE interaction and to rationalize the basis for the observed binding specificity and affinity. We propose that the current study will assist with the development of second generation Grb7 SH2 domain inhibitors, potentially leading to novel inhibitors of cancer cell migration and invasion.

Rana S., Kundu B. and Durani S. (2007) A mixed-alpha,beta miniprotein stereochemically reprogrammed to high-binding affinity for acetylcholine. *Biopolymers* **87**, 231-243.

Abstract: The protein-structure space is limited to L configuration in the asymmetric alpha-amino acid structures; the function space, on other hand, seems limitless because of the chemical diversity in the amino acid side chain structures. The chemical diversity in side chain structure may be multiplied beneficially with the stereochemical diversity in main chain structure; thus, de novo protein design may be explored for customizing molecular structures stereochemically and molecular functions chemically. Illustrating de novo design in the structure space of L and D alphabet, canonical all-beta folds of poly-L structure were reprogrammed as bracelet, boat, and canoe-shaped molecules-the "boat" as a receptor-like pocket and the "canoe" as a metal-ion receptor-simply by mutating specific L-amino acid residues to the corresponding D stereochemical structure. Demonstrating customization of molecular shape stereochemically and function chemically, a 15-residue mixed-alpha, beta miniprotein of canonical poly-L structure is now reprogrammed stereochemically as a cup-shaped receptor for acetylcholine via cation-pi interaction with a triad of aromatic side chains placed in mimicry of the acetylcholine-receptor sites both natural and artificial. Evidence from CD, fluorescence, NMR, DSC, ITC, MD, and molecular-docking studies is presented to show that a rationally designed 15-residue mixed-L, D peptide is a cooperatively ordered molecular fold in the stereochemically specified molecular morphology, submicromolar in affinity of acetylcholine and thus an acetylcholine receptor exceptionally small and simple.

Rickert M., Boulanger M. J., Goriatcheva N., and Garcia K. C. (2004) Compensatory energetic mechanisms mediating the assembly of signaling complexes between interleukin-2 and its alpha, beta, and gamma(c) receptors. *J Mol Biol* **339**, 1115-1128.

Abstract: Interleukin-2 is a key immuno-regulatory cytokine whose actions are mediated by three different cell surface receptors: the alpha, beta and the "common gamma" (gamma(c)) chains. We have undertaken a complete thermodynamic characterization of the stepwise assembly cycle for multiple possible combinations of the receptor-ligand, and receptor-receptor interactions that are necessary for formation of the high-affinity IL-2/alphabeta gamma(c) signaling complex. We find an entropically favorable high affinity interaction between IL-2 and its alpha receptor, a moderately entropically favorable low affinity interaction between IL-2 and its beta receptor, and no interaction between IL-2 and the shared receptor, gamma(c). Formation of the stable intermediate trimolecular complexes of IL-2 with alpha and beta receptors, as well as IL-2 with beta and gamma(c) receptors proceeds through enthalpy-entropy compensation mechanisms. Surprisingly, we see a moderate affinity interaction between the unliganded receptor alpha and beta chains, suggesting that a preformed alphabeta complex may serve as the initial interaction complex for IL-2. Reconstitution of the IL-2/Ralphabeta gamma(c) high-affinity quaternary signaling complex shows it to be assembled through cooperative energetics to form a 1:1:1:1 assembly. Collectively, the favorable entropy of the bimolecular interactions appears to be offset by the loss in rigid body entropy of the receptor components in the higher-order complexes, but overcome by the formation of increasingly enthalpically favorable composite interfaces. This enthalpic mechanism utilized by gamma(c) contrasts with the favorable entropic mechanism utilized by gp130 for degenerate cytokine interaction. In conclusion, we find that several energetically redundant pathways exist for formation of IL-2 receptor signaling complexes, suggesting a more complex equilibrium on the cell surface than has been previously appreciated.

Ross N. T., Mace C. R. and Miller B. L. (2007) Biophysical analysis of the EPEC translocated intimin receptor-binding domain. *Biochem Biophys Res Commun* **362**, 1073-1078.

Abstract: Enteropathogenic Escherichia coli (EPEC) are Gram (-) bacteria responsible for widespread illness in the form of diarrhea. EPEC cells attach to the intestinal epithelium using a Type III secretion system common to many Gram (-) bacteria. The translocated intimin receptor (TIR) is the first protein secreted through the EPEC secretion complex, and is absolutely required for pathogenesis. It inserts into the intestinal epithelium, serving as an anchor responsible for the attachment of EPEC to the host epithelial cell. Intimin is a transmembrane protein displayed on the EPEC cell surface with an extracellular domain that binds TIR. Observation of a TIR-TIR dimer in the X-ray co-crystal structure of the extracellular domains of intimin and TIR raised the question of how these protein domains interact and function in solution. Herein we report that the extracellular domain of TIR exists in a folded and active monomeric state in solution, as confirmed by analytical ultracentrifugation, analytical size-exclusion HPLC, isothermal titration calorimetry, and surface plasmon resonance.

Ross N. T. and Miller B. L. (2007) Characterization of the binding surface of the translocated intimin receptor, an essential protein for EPEC and EHEC cell adhesion. *Protein Sci* **16**, 2677-2683.

Abstract: The translocated intimin receptor (TIR) of enteropathogenic and enterohemorrhagic Escherichia coli (EPEC and EHEC) is required for EPEC and EHEC infections, which cause widespread illness across the globe. TIR is translocated via a type-III secretion system into the intestinal epithelial cell membrane, where it serves as an anchor for E. coli attachment via its binding partner intimin. While many aspects of EPEC and EHEC infection are now well understood, the importance of the intermolecular contacts made between intimin and TIR have not been thoroughly investigated. Herein we report site-directed mutagenesis studies on the intimin-binding domain of EPEC TIR, and how these mutations affect TIR-intimin association, as analyzed by isothermal titration calorimetry and circular dichroism. These results show how two factors govern TIR's binding to intimin: A three-residue TIR hot spot is identified that largely mediates the interaction, and mutants that alter the beta-hairpin structure of TIR severely diminish binding affinity. In addition, peptides incorporating key TIR residues identified by mutagenesis are incapable of binding intimin. These results indicate that hot spot residues and structural orientation/preorganization are required for EPEC, and likely EHEC, TIR-intimin binding.

Rudolph M. G., Luz J. G., and Wilson I. A. (2002) Structural and thermodynamic correlates of T cell signaling. *Annu Rev Biophys Biomol Struct* **31**, 121-149.

Abstract: The first crystal structures of intact T cell receptors (TCRs) bound to class I peptide-MHC (pMHCs) antigens were determined in 1996. Since then, further structures of class I TCR/pMHC complexes have explored the degree of structural variability in the TCR-pMHC system and the structural basis for positive and negative selection. The recent determination of class II and allogeneic class I TCR/pMHC structures, as well as those of accessory molecules (e.g., CD3), has pushed our knowledge of TCR/pMHC interactions into new realms, shedding light on clinical pathologies, such as graft rejection and graft-versus-host disease. Furthermore, the determination of coreceptor structures lays the foundation for a more comprehensive structural description of the supramolecular TCR signaling events and those assemblies that arise in the immunological synapse. While these telling photodocumentaries of the TCR/pMHC interaction are composed mainly from static crystal structures, a full description of the biological snapshots in T cell signaling requires additional analytical methods that record the dynamics of the process. To this end, surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), and ultracentrifugation (UC) have furnished both affinities and kinetics of the TCR/pMHC association. In the past year, structural, biochemical, and molecular biological data describing TCR/pMHC interactions have sublimely coalesced into a burgeoning well of understanding that promises to deliver further insights into T cell recognition. The coming years will, through a more intimate union of structural and kinetic data, allow many pressing questions to be addressed, such as how TCR/pMHC ligation is affected by coreceptor binding and what is the mechanism of TCR signaling in both early and late stages of T cell engagement with antigen-presenting cells.

Runge S., Schimmer S., Oschmann J., Schiodt C. B., Knudsen S. M., Jeppesen C. B., Madsen K., Lau J., Thogersen H. and Rudolph R. (2007) Differential structural properties of GLP-1 and exendin-4 determine their relative affinity for the GLP-1 receptor N-terminal extracellular domain. *Biochemistry* **46**, 5830-5840. **Abstract:** Glucagon-like peptide-1 (GLP-1) and exendin-4 (Ex4) are homologous peptides with established potential for treatment of type 2 diabetes. They bind and activate the pancreatic GLP-1 receptor (GLP-1R) with similar affinity and potency and thereby promote insulin secretion in a glucose-dependent manner. GLP-1R belongs to family B of the seven transmembrane G-protein coupled receptors. The N-terminal extracellular domain (nGLP-1R) is a ligand binding domain with differential affinity for Ex4 and GLP-1: low affinity for GLP-1 and high affinity for exendin-4. The superior affinity of nGLP-1R for Ex4 was previously explained by an additional interaction between nGLP-1R and the C-terminal Trp-cage of Ex4. In this study we have combined biophysical and pharmacological approaches thus relating structural properties of the ligands in solution to their relative binding affinity for nGLP-1R. We used both a tracer competition assay and ligand-induced thermal stabilization of nGLP-1R to measure the relative affinity of full length, truncated, and chimeric ligands for soluble refolded nGLP-1R. The ligands in solution and the conformational consequences of ligand binding to nGLP-1R were characterized by circular dichroism and fluorescence spectroscopy. We found a correlation between the helical content of the free ligands and their relative binding affinity for nGLP-1R, supporting the hypothesis that the ligands are helical at least in the segment that binds to nGLP-1R. The Trp-cage of Ex4 was not necessary to maintain a superior helicity of Ex4 compared to GLP-1. The results suggest that the differential affinity of nGLP-1R is explained almost entirely by divergent residues in the central part of the ligands: Leu10-Gly30 of Ex4 and Val16-Arg36 of GLP-1. In view of our results it appears that the Trp-cage plays only a minor role for the interaction between Ex4 and nGLP-1R and for the differential affinity of nGLP-1R for GLP-1 and Ex4.

Schiffmann D. A., White J. H., Cooper A., Nutley M. A., Harding S. E., Jumel K., Solari R., Ray K. P., and Gay N. J. (1999) Formation and biochemical characterization of tube/pelle death domain complexes: critical regulators of postreceptor signaling by the Drosophila toll receptor. *Biochemistry* **38**, 11722-11733. **Abstract:** In Drosophila, the Toll receptor signaling pathway is required for embryonic dorso-ventral patterning and at later developmental stages for innate immune responses. It is thought that dimerization of the receptor by binding of the ligand spatzle causes the formation of a postreceptor activation complex at the cytoplasmic surface of the membrane. Two components of this complex are the adaptor tube and protein kinase pelle. These proteins both have "death domains", protein interaction motifs found in a number of signaling pathways, particularly those involved in apoptotic cell death. It is thought that pelle is bound by tube during formation of the activation complexes, and that this interaction is mediated by the death domains. In this paper, we show using the yeast two-hybrid system that the wild-type tube and pelle death domains bind together. Mutant tube proteins which do not support signaling in the embryo are also unable to bind pelle in the 2-hybrid assay. We have purified proteins corresponding to the death domains of

tube and pelle and show that these form corresponding heterodimeric complexes in vitro. Partial proteolysis reveals a smaller core consisting of the minimal death domain sequences. We have studied the tube/pelle interaction with the techniques of surface plasmon resonance, analytical ultracentrifugation and isothermal titration calorimetry. These measurements produce a value of K_d for the complex of about $0.5 \mu\text{M}$.

Schon A. and Freire E. (1989) Thermodynamics of intersubunit interactions in cholera toxin upon binding to the oligosaccharide portion of its cell surface receptor, ganglioside GM1. *Biochemistry* **28**, 5019-5024.
Abstract: The binding and the energetics of the interaction of cholera toxin with the oligosaccharide portion of ganglioside GM1 (oligo-GM1), the toxin cell surface receptor, have been studied by high-sensitivity isothermal titration calorimetry and differential scanning calorimetry. Previously, we have shown that the association of cholera toxin to ganglioside GM1 enhances the cooperative interactions between subunits in the B-subunit pentamer [Goins, B., & Freire, E. (1988) *Biochemistry* 27, 2046-2052]. New experiments presented in this paper reveal that the oligosaccharide portion of the receptor is by itself able to enhance the intersubunit cooperative interactions within the B pentamer. This effect is seen in the protein unfolding transition as a shift from independent unfolding of the B promoters toward a cooperative unfolding. To identify the origin of this effect, the binding of cholera toxin to oligo-GM1 has been measured calorimetrically under isothermal conditions. The binding curve at 37 degrees C is sigmoidal, indicating cooperative binding. The binding data can be described in terms of a nearest-neighbor cooperative interaction binding model. In terms of this model, the association of a oligo-GM1 molecule to a B promoter affects the association to adjacent B promoters within the pentameric ring. The measured intrinsic binding enthalpy per promoter is -22 kcal/mol and the cooperative interaction enthalpy -11 kcal/mol . The intrinsic binding constant determined calorimetrically is $1.05 \times 10^6 \text{ M}^{-1}$ at 37 degrees C and the cooperative Gibbs free energy equal to -850 cal/mol .(ABSTRACT TRUNCATED AT 250 WORDS).

Schrader N., Kim E. Y., Winking J., Paulukat J., Schindelin H., and Schwarz G. (2004) Biochemical characterization of the high affinity binding between the glycine receptor and gephyrin. *J Biol Chem* **279**, 18733-18741.

Abstract: Gephyrin is an essential and instructive molecule for the formation of inhibitory synapses. Gephyrin binds directly to the large cytoplasmic loop located between transmembrane helices three and four of the beta-subunit of the glycine receptor and to microtubules, thus promoting glycine receptor (GlyR) anchoring to the cytoskeleton and clustering in the postsynaptic membrane. Besides its structural role, gephyrin is involved in the biosynthesis of the molybdenum cofactor that is essential for all molybdenum-dependent enzymes in mammals. Gephyrin can be divided into an N-terminal trimeric G domain and a C-terminal E domain, which are connected by a central linker region. Here we have studied the in vitro interaction of gephyrin and its domains with the large cytoplasmic loop of the GlyR beta-subunit (GlyRbeta-loop). Binding of gephyrin to the GlyR is exclusively mediated by the E domain, and the binding site was mapped to one of its sub-domains (residues 496-654). By using isothermal titration calorimetry, a high affinity ($K_d = 0.2-0.4 \mu\text{M}$) and low affinity ($K_d = 11-30 \mu\text{M}$) binding site for the GlyRbeta-loop was found on holo-gephyrin and the E domain, respectively, with a binding stoichiometry of two GlyRbeta-loops per E domain in both cases. Binding of the GlyRbeta-loop does not change the oligomeric state of either full-length gephyrin or the isolated E domain.

Schuler J., Frank J., Trier U., Schafer-Korting M., and Saenger W. (1999) Interaction kinetics of tetramethylrhodamine transferrin with human transferrin receptor studied by fluorescence correlation spectroscopy. *Biochemistry* **38**, 8402-8408.

Abstract: We applied fluorescence correlation spectroscopy (FCS) to characterize the interaction dynamics of fluorescence-labeled transferrin with transferrin receptor (hTfR) associates isolated from human placenta. The dissociation constant for the equilibrium binding of TMR-labeled ferri-transferrin to hTfR in detergent free solution was determined to be $7 \pm 3 \text{ nM}$. Binding curves were compatible with equal and independent binding sites present on the hTfR associates. Under pseudo-first-order conditions, with respect to transferrin, complex formation is monophasic. From these curves, association and dissociation rate constants for a reversible bimolecular binding reaction were determined, with $(1.1 \pm 0.1) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for the former and $(6 \pm 4) \times 10^{-4} \text{ s}^{-1}$ for the latter. In dissociation exchange experiments, biphasic curves and concentration-independent reciprocal relaxation times were determined. From isothermal titration

calorimetry experiments, we obtained an enthalpy change of -44.4 kJ/mol associated with the reaction. We thus conclude that the reaction is mainly enthalpy driven.

Seelig A., Alt T., Lotz S., and Holzemann G. (1996) Binding of substance P agonists to lipid membranes and to the neurokinin-1 receptor. *Biochemistry* **35**, 4365-4374.

Abstract: Three new analogues of the neuropeptide substance P (SP) were synthesized. The C-terminal message segment was made more hydrophilic in (Arg9)SP or more hydrophobic in (Nle9)SP. In (AcPro2, Arg9)SP the charge at the N-terminal address segment was reduced, while that of the message segment was increased. The rationale underlying these substitutions was to correlate the physical-chemical properties of the SP-analogues, in particular their lipid-induced conformation and membrane-binding affinity, with receptor binding and functional activity. In solution, all three analogues exhibited random coil conformations as evidenced by circular dichroism spectroscopy. Addition of SDS micelles induced partially alpha-helical structures. The same structure was also produced by negatively charged lipid vesicles for (AcPro2, Arg9)SP and (Arg9)SP whereas both alpha-helix-like structures and beta-sheet structures were observed for SP and (Nle9)SP. The measurement of the Gibbs adsorption isotherms and monolayer expansion studies provided quantitative data on the surface area requirement and on the membrane penetration area of the SP analogues. The thermodynamic parameters for lipid binding were determined with monolayer expansion for measurements and high-sensitivity titration calorimetry. The apparent binding constants, K_{app} , for membranes containing 100% POPG were of the order of 10^3 - 10^5 M⁻¹. The binding was due to electrostatic attraction of the cationic peptides to the negatively charged membrane surface. The intrinsic (hydrophobic) binding constants, obtained after correcting for electrostatic effects, were much smaller with $K_p=10\pm 1$ M⁻¹ for (Arg9)SP, 9 ± 1 M⁻¹ for (AcPro2, Arg9)SP, and 39 ± 3 M⁻¹ for (Nle9)SP. The measurement of the binding affinities to the NK-1 receptor and of the in vitro activities showed that all three peptides behaved as agonists. Their binding affinity to the neurokinin-1 receptor decreased with the size of the side chains at position 9 of the amino acid sequence but was independent of the cationic charge of the peptides. The fact that even the highly charged (Arg9)SP has agonistic activity provides evidence that the binding epitope at the receptor is in a rather hydrophilic environment. This finding is in agreement with the low hydrophobic binding constants and the weak penetration of the three peptides into negatively charged membranes. It argues against a membrane mediated receptor mechanism and suggests that the agonist approaches the receptor binding, site from the aqueous phase.

Seet B. T., Berry D. M., Maltzman J. S., Shabason J., Raina M., Koretzky G. A., McGlade C. J. and Pawson T. (2007) Efficient T-cell receptor signaling requires a high-affinity interaction between the Gads C-SH3 domain and the SLP-76 RxxK motif. *EMBO J* **26**, 678-689.

Abstract: The relationship between the binding affinity and specificity of modular interaction domains is potentially important in determining biological signaling responses. In signaling from the T-cell receptor (TCR), the Gads C-terminal SH3 domain binds a core RxxK sequence motif in the SLP-76 scaffold. We show that residues surrounding this motif are largely optimized for binding the Gads C-SH3 domain resulting in a high-affinity interaction ($K(D)=8$ -20 nM) that is essential for efficient TCR signaling in Jurkat T cells, since Gads-mediated signaling declines with decreasing affinity. Furthermore, the SLP-76 RxxK motif has evolved a very high specificity for the Gads C-SH3 domain. However, TCR signaling in Jurkat cells is tolerant of potential SLP-76 crossreactivity, provided that very high-affinity binding to the Gads C-SH3 domain is maintained. These data provide a quantitative argument that the affinity of the Gads C-SH3 domain for SLP-76 is physiologically important and suggest that the integrity of TCR signaling in vivo is sustained both by strong selection of SLP-76 for the Gads C-SH3 domain and by a capacity to buffer intrinsic crossreactivity.

Sharma S. C., Rupasinghe C. N., Parisien R. B. and Spaller M. R. (2007) Design, synthesis, and evaluation of linear and cyclic peptide ligands for PDZ10 of the multi-PDZ domain protein MUPP1. *Biochemistry* **46**, 12709-12720.

Abstract: PDZ10 is the 10th of 13 PDZ domains found within MUPP1, a cytoplasmic scaffolding protein first identified as an endogenous binding partner of serotonin receptor type 2c (5HT2c). This association, as with those of several other interacting proteins that have subsequently been identified, is mediated through the C-terminal tail of the PDZ domain partner. Using isothermal titration calorimetry (ITC), we measured

the thermodynamic binding parameters [changes in Gibbs free energy (ΔG), enthalpy (ΔH) and entropy ($T\Delta S$)] of the isolated PDZ10 domain for variable-length N-acetylated peptides from the 5HT2c serotonin receptor C-terminal sequence, as well as for octapeptides of eight other putative partner proteins of PDZ10 (5HT2a, hc-kit, hTapp1, mTapp2, TARP, NG2, claudin-1, and HPV-18 E6). In length dependence studies of the 5HT2c sequence, the maximal affinity of the peptides leveled off rapidly and further elongation did not significantly improve the dissociation constant (K_d) of 11 μM observed with the pentapeptide. Among the native partners of PDZ10, octapeptides derived from the hc-kit and 5HT2c proteins were the strongest binders, with K_d values of 5.2 and 8.5 μM , respectively. The heat capacity change (ΔC_p) for the 5HT2c octapeptide was determined to be -94 cal/mol, and a calculated estimate indicates burial of polar and apolar surface areas in equal measure upon ligand binding. Peptides with phosphoserine at either the P-1 or P-2 position experienced decreased affinity, which is in accord with the hypothesis that reversible phosphorylation is a possible mechanism for regulating PDZ domain-mediated interactions. Additionally, two conformationally constrained side chain-bridged cyclic peptide ligands were also designed, prepared, evaluated by ITC, and shown to bind PDZ10 primarily through a favorable change in entropy.

Sharma V.A., Kan E., Sun Y., Lian Y., Cisto J., Frasca V., Hilt S., Stamatatos L., Donnelly J.J., Ulmer J.B., Barnett S.W., and Srivastava I.K. (2006) Structural characteristics correlate with immune responses induced by HIV envelope glycoprotein vaccines. *Virology* **352**, 131-144.

Abstract: HIV envelope glycoprotein (Env) is the target for inducing neutralizing antibodies. Env is present on the virus surface as a trimer, and, upon binding to CD4, a cascade of events leads to structural rearrangement exposing the co-receptor binding site and entry into the CD4⁺ host target cells. We have designed monomeric and trimeric Env constructs with and without deletion of the variable loop 2 ($\Delta V2$) from SF162, a subtype B primary isolate, and performed biophysical, biochemical and immunological studies to establish a potential structure-functional relationship. We expressed these Envs in CHO cells, purified the proteins to homogeneity and performed biophysical studies to define the binding properties to CD4, structural characteristics and exposure of epitopes recognized by b12 and CD4i mAb (17B) on both full-length and mutant HIV Env proteins. Parameters evaluated include oligomerization state, number and affinity of CD4 binding sites, enthalpy and entropy of the Env-CD4 interaction and affinity for b12 and 17b mAbs. We observed one CD4 binding site per monomer and three active CD4 binding sites per trimer. A 40-fold difference in affinity of the gp120 monomer vs. the o-gp140 trimer towards CD4 was observed ($K_d = 58$ nM and 1.5 nM, respectively), whereas only a 2-fold difference was observed for the V2 deleted Envs (K_d of gp120 $\Delta V2 = 19$ nM, K_d of o-gp140 $\Delta V2 = 9.3$ nM). Monomers had 3-fold higher affinity to the mAb 17b and at least 3-fold weaker affinity to b12 compared to trimers, with gp120 $\Delta V2$ having the weakest affinity for b12 ($K_d = 446$ nM). Affinity of CD4 binding correlated with proportion of the antibodies induced against the conformational epitopes by the corresponding Envs, and changes in mAb binding correlated with the induction of antibodies directed against linear epitopes. Furthermore, biophysical analysis reveals that the V2 deletion has broad structural implications in the monomer not shared by the trimer, and these changes are reflected in the quality of the immune responses induced in rabbits. These data suggest that biophysical characteristics of HIV Env, such as affinity for CD4, and exposure of important neutralizing epitopes, such as those recognized by b12 mAb, may be important predictors of its in vivo efficacy and may serve as important surrogate markers for screening Env structures as potential vaccine candidates.

Sharrow S. D., Vaughn J. L., Zidek L., Novotny M. V., and Stone M. J. (2002) Pheromone binding by polymorphic mouse major urinary proteins. *Protein Sci* **11**, 2247-2256.

Abstract: Mouse major urinary proteins (MUPs) have been proposed to play a role in regulating the release and capture of pheromones. Here, we report affinity measurements of five recombinant urinary MUP isoforms (MUPs-I, II, VII, VIII, and IX) and one recombinant nasal isoform (MUP-IV) for each of three pheromonal ligands, (+/-)-2-sec-butyl-4,5-dihydrothiazole (SBT), 6-hydroxy-6-methyl-3-heptanone (HMH), and (+/-)-dehydro-exo-brevicommin (DHB). Dissociation constants for all MUP-pheromone pairs were determined by isothermal titration calorimetry, and data for SBT were corroborated by measurements of intrinsic protein fluorescence. We also report the isolation of MUP-IV protein from mouse nasal extracts, in which MUP-IV mRNA has been observed previously. The affinity of each MUP isoform for SBT ($K(d)$ approximately 0.04 to 0.9 μM) is higher than that for DHB ($K(d)$ approximately 26 to 58 μM),

which in turn is higher than that for HMH (Kd approximately 50 to 200 μ M). Isoforms I, II, VIII, and IX show very similar affinities for each of the ligands. MUP-VII has approximately twofold higher affinity for SBT but approximately twofold lower affinity for the other pheromones, whereas MUP-IV has approximately 23-fold higher affinity for SBT and approximately fourfold lower affinity for the other pheromones. The variations in ligand affinities of the MUP isoforms are consistent with structural differences in the binding cavities of the isoforms. The data indicate that the concentrations of available pheromones in urine may be influenced by changes in the expression levels of urinary MUPs or the excretion levels of other MUP ligands. The variation in pheromone affinities of the urinary MUP isoforms provides only limited support for the proposal that MUP heterogeneity plays a role in regulating profiles of available pheromones. However, the binding data support the proposed role of nasal MUPs in sequestering pheromones and possibly transporting them to their receptors.

Sharrow S. D., Novotny M. V., and Stone M. J. (2003) Thermodynamic analysis of binding between mouse major urinary protein-I and the pheromone 2-sec-butyl-4,5-dihydrothiazole. *Biochemistry* **42**, 6302-6309. **Abstract:** The mouse pheromone 2-sec-butyl-4,5-dihydrothiazole (SBT) binds to an occluded, nonpolar cavity in the mouse major urinary protein-I (MUP-I). The thermodynamics of this interaction have been characterized using isothermal titration calorimetry (ITC). MUP-I-SBT binding is accompanied by a large favorable enthalpy change ($\Delta H = -11.2$ kcal/mol at 25 degrees C), an unfavorable entropy change ($-\Delta S = 2.8$ kcal/mol at 25 degrees C), and a negative heat capacity change [$\Delta C(p) = -165$ cal/(mol K)]. Thermodynamic analysis of binding between MUP-I and several 2-alkyl-4,5-dihydrothiazole ligands indicated that the alkyl chain contributes more favorably to the enthalpy and less favorably to the entropy of binding than would be expected on the basis of the hydrophobic desolvation of short-chain alcohols. However, solvent transfer experiments indicated that desolvation of SBT is accompanied by a net unfavorable change in enthalpy ($\Delta H = +1.0$ kcal/mol) and favorable change in entropy ($-\Delta S = -1.8$ kcal/mol). These results are discussed in terms of the possible physical origins of the binding thermodynamics, including (1) hydrophobic desolvation of both the protein and the ligand, (2) formation of a buried water-mediated hydrogen bond network between the protein and ligand, (3) formation of strong van der Waals interactions, and (4) changes in the structure, dynamics, and/or hydration of the protein upon binding.

Shi Y., Wang S., Krueger S., and Schwarz F. P. (1999) Effect of mutations at the monomer-monomer interface of cAMP receptor protein on specific DNA binding. *J Biol Chem* **274**, 6946-6956. **Abstract:** To determine the thermodynamic role of binding of an operon to cAMP receptor protein (CRP) in the activation of transcription, isothermal titration calorimetry measurements were performed on the binding of three 40-base pair DNA sequences to the cyclic nucleoside complexes of CRP and its mutants at 296 K. The three 40-base pair sequences consisted of a consensus DNA (conDNA) duplex derived from the CRP-binding site sequences of the operons activated by CRP and two DNA sequences based on the CRP-binding site sequences of the lac operon (lacDNA) and of the gal operon (galDNA). The mutants of CRP consisted of a T127L mutant, a S128A mutant, and a mutant containing both mutations (CRP*) which not only alter the transcriptional activity of the CRP complexes but also are involved in the monomer-monomer interfacial interactions of the CRP dimer. The binding reactions of the DNA duplexes to the fully cNMP-ligated CRP-mutant complexes were endothermic with binding constants as high as $6.6 \pm 1.1 \times 10^6 \text{ M}^{-1}$ (conDNA.CRP(cAMP)₂). ConDNA binding to the unligated T127L and CRP* mutants was observed as well as conDNA and lacDNA binding to CRP with cAMP bound to only one monomer. The reduction of the binding constants with increase in KCl concentration indicated the formation of two ion pairs for the cAMP-ligated CRP and S128A complexes and four ion pairs for the cAMP-ligated T127L and CRP* complexes. Reduction of the DNA binding constants upon substitution of D₂O for H₂O in the buffer, the large heat capacity changes, and the enthalpy-entropy compensation exhibited by the binding reactions indicate the importance of dehydration in the binding reaction. Small angle neutron scattering measurements on the lacDNA.CRP(cAMP)₂ complex in D₂O/H₂O mixtures show that the DNA is bent around the cAMP-ligated protein in solution.

Shiroishi M., Kuroki K., Tsumoto K., Yokota A., Sasaki T., Amano K., Shimojima T., Shirakihara Y., Rasubala L., van der Merwe P.A., Kumagai I., Kohda D., and Maenaka K. (2006) Entropically driven MHC class I recognition by human inhibitory receptor leukocyte Ig-like receptor B1 (LILRB1/ILT2/CD85j). *J Mol Biol.* **355**, 237-48.

Abstract: The human inhibitory receptor, leukocyte immunoglobulin (Ig)-like receptor B1 (also called Ig-like transcript (ILT) 2, CD85j), is broadly expressed on leukocytes. LILRB1 binds to a wide range of major histocompatibility complex class I molecules (MHCIs) and transduces negative signals that can, for example, prevent killing of MHC1-expressing cells. Here we report the kinetic, thermodynamic, NMR and crystallographic analyses of MHC1 recognition by LILRB1. Kinetic studies demonstrated that LILRB1 binds to MHCIs with fast association and dissociation rates, typical of cell-cell recognition receptors. Thermodynamic analyses showed that LILRB1-MHC1 interactions are entropically driven ($-T\Delta S = -9.4$ approximately -6.6 kcal mol⁻¹) with low heat capacity changes ($\Delta C(p) = -0.22$ approximately -0.10 kcal mol⁻¹ K⁻¹). The crystal structures of LILRB1 in the different crystal forms exhibited variation in the elbow angle between the two N-terminal Ig-like domains, indicating interdomain flexibility. Consistently, NMR analysis provided the direct evidence of the conformational changes of LILRB1 upon the MHC1 binding. These findings suggest that LILRB1-MHC1 interactions, while involving some conformational adjustment, are not accompanied by a very large reduction in conformational flexibility at the binding interface. This mode of binding is distinct from "induced-fit" binding, which is associated with large reductions in conformational flexibility, and would be suitable for rapid engagement of MHCIs to enable fast monitoring of the expression level of MHCIs on target cells.

Sleigh S. H., Tame J. R., Dodson E. J., and Wilkinson A. J. (1997) Peptide binding in OppA, the crystal structures of the periplasmic oligopeptide binding protein in the unliganded form and in complex with lysyllysine. *Biochemistry* **36**, 9747-9758.

Abstract: The periplasmic oligopeptide binding protein, OppA, acts as the initial receptor for the uptake of peptides by the oligopeptide permease (Opp) in Gram-negative bacteria. Opp will handle peptides between two and five amino acid residues regardless of their sequence. The crystal structures of a series of OppA-peptide complexes have revealed an enclosed but versatile peptide binding pocket and have illustrated how tri- and tetrapeptide ligands are accommodated. Here, the crystal structures of (i) OppA complexed with a dipeptide (lysyllysine) and (ii) unliganded OppA have been solved using X-ray data extending to 1.8 and 2.4 Å spacing, respectively. In the dipeptide complex, the alpha-amino group of the ligand is anchored through an ion pair interaction with Asp419, as observed in complexes with longer peptides. However, its alpha-carboxylate group forms water-mediated interactions with the guanidinium groups of Arg404 and Arg413 rather than the direct salt bridges to Arg413 and His371 observed in the tripeptide and tetrapeptide complexes, respectively. Isothermal titration calorimetric measurements of the binding of lysine-containing peptides of different lengths to OppA show that the dipeptide, KK, is bound with approximately 60-fold lower affinity than related tri- and tetrapeptides (KKK and KKKA, respectively). These data are discussed with reference to the calculated enthalpic and entropic contributions to ligand binding and the structures of the OppA peptide complexes. In the unliganded molecule, domain III has rotated as a rigid body through 26 degrees away from domains I and II, exposing the ligand binding site. The water structure in the binding cleft shows similarities to that in the various OppA-peptide complexes.

Spivak-Kroizman T., Lemmon M. A., Dikic I., Ladbury J. E., Pinchasi D., Huang J., Jaye M., Crumley G., Schlessinger J., and Lax I. (1994) Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation. *Cell* **79**, 1015-1024.

Abstract: Heparin is required for fibroblast growth factor (FGF) stimulation of biological responses. Using isothermal titration calorimetry, we show that acidic FGF (aFGF) forms a 1:1 complex with the soluble extracellular domain of FGF receptor (FGFR). Heparin exerts its effect by binding to many molecules of aFGF. The resulting aFGF-heparin complex can bind to several receptor molecules, leading to FGFR dimerization. In two cell lines lacking endogenous heparan sulfate, exogenous heparin is required for FGFR dimerization, tyrosine kinase activation, c-fos mRNA transcription, and cell proliferation. Moreover, a synthetic heparin analog that binds monovalently to aFGF blocks FGFR dimerization, activation, and signaling via FGFR. We propose that heparin causes oligomerization of aFGF such that its binding to FGFR results in dimerization and activation. This represents a novel mechanism for transmembrane signaling and may account for the action of many heparin-bound growth factors.

Spuches A. M., Argiros H. J., Lee K. H., Haas L. L., Pero S. C., Krag D. N., Roller P. P., Wilcox D. E. and Lyons B. A. (2007) Calorimetric investigation of phosphorylated and non-phosphorylated peptide ligand binding to the human Grb7-SH2 domain. *J Mol Recognit* **20**, 245-252.

Abstract: Grb7 is a member of the Grb7 family of proteins, which also includes Grb10 and Grb14. All three proteins have been found to be overexpressed in certain cancers and cancer cell lines. In particular, Grb7 (along with the receptor tyrosine kinase erbB2) is overexpressed in 20-30% of breast cancers. In general, growth factor receptor bound (Grb) proteins bind to activated membrane-bound receptor tyrosine kinases (RTKs; e.g., the epidermal growth factor receptor, EGFR) through their Src homology 2 (SH2) domains. In particular, Grb7 binds to erbB2 (a.k.a. EGFR2) and may be involved in cell signaling pathways that promote the formation of metastases and inflammatory responses. In previous studies, we reported the solution structure and the backbone relaxation behavior of the Grb7-SH2/erbB2 peptide complex. In this study, isothermal titration calorimetry studies have been completed by measuring the thermodynamic binding parameters of several phosphorylated and non-phosphorylated peptides representative of natural Grb7 receptor ligands as well as ligands developed through combinatorial peptide screening methods. The entirety of these calorimetric studies is interpreted in an effort to describe the specific ligand binding characteristics of the Grb7 protein.

Stanley W. A., Pursiainen N. V., Garman E. F., Juffer A. H., Wilmanns M. and Kursula P. (2007) A previously unobserved conformation for the human Pex5p receptor suggests roles for intrinsic flexibility and rigid domain motions in ligand binding. *BMC. Struct. Biol* **7**, 24.

Abstract: BACKGROUND: The C-terminal tetratricopeptide (TPR) repeat domain of Pex5p recognises proteins carrying a peroxisomal targeting signal type 1 (PTS1) tripeptide in their C-terminus. Previously, structural data have been obtained from the TPR domain of Pex5p in both the liganded and unliganded states, indicating a conformational change taking place upon cargo protein binding. Such a conformational change would be expected to play a major role both during PTS1 protein recognition as well as in cargo release into the peroxisomal lumen. However, little information is available on the factors that may regulate such structural changes. RESULTS: We have used a range of biophysical and computational methods to further analyse the conformational flexibility and ligand binding of Pex5p. A new crystal form for the human Pex5p C-terminal domain (Pex5p(C)) was obtained in the presence of Sr²⁺ ions, and the structure presents a novel conformation, distinct from all previous liganded and apo crystal structures for Pex5p(C). The difference relates to a near-rigid body movement of two halves of the molecule, and this movement is different from that required to reach a ring-like conformation upon PTS1 ligand binding. The bound Sr²⁺ ion changes the dynamic properties of Pex5p(C) affecting its conformation, possibly by making the Sr²⁺-binding loop - located near the hinge region for the observed domain motions - more rigid.

CONCLUSION: The current data indicate that Pex5p(C) is able to sample a range of conformational states in the absence of bound PTS1 ligand. The domain movements between various apo conformations are distinct from those involved in ligand binding, although the differences between all observed conformations so far can be characterised by the movement of the two halves of Pex5p(C) as near-rigid bodies with respect to each other.

Stauber D.J., Debler E.W., Horton P.A., Smith K.A., and Wilson I.A. (2006) Crystal structure of the IL-2 signaling complex: paradigm for a heterotrimeric cytokine receptor. *Proc Natl Acad Sci U S A*. **103**, 2788-93.

Abstract: IL-2 is a cytokine that functions as a growth factor and central regulator in the immune system and mediates its effects through ligand-induced hetero-trimerization of the receptor subunits IL-2R alpha, IL-2R beta, and gamma(c). Here, we describe the crystal structure of the trimeric assembly of the human IL-2 receptor ectodomains in complex with IL-2 at 3.0 Å resolution. The quaternary structure is consistent with a stepwise assembly from IL-2/IL-2R alpha to IL-2/IL-2R alpha/IL-2R beta to IL-2/IL-2R alpha/IL-2R beta/gamma(c). The IL-2R alpha subunit forms the largest of the three IL-2/IL-2R interfaces, which, together with the high abundance of charge-charge interactions, correlates well with the rapid association rate and high-affinity interaction of IL-2R alpha with IL-2 at the cell surface. Surprisingly, IL-2R alpha makes no contacts with IL-2R beta or gamma(c), and only minor changes are observed in the IL-2 structure in response to receptor binding. These findings support the principal role of IL-2R alpha to deliver IL-2 to the signaling complex and act as regulator of signal transduction. Cooperativity in assembly of the final quaternary complex is easily explained by the extraordinarily extensive set of interfaces found within the fully assembled IL-2 signaling complex, which nearly span the entire length of the IL-2R beta and gamma(c) subunits. Helix A of IL-2 wedges tightly between IL-2R beta and gamma(c) to form a three-way junction that coalesces into a composite binding site for the final gamma(c) recruitment. The IL-

2/gamma(c) interface itself exhibits the smallest buried surface and the fewest hydrogen bonds in the complex, which is consistent with its promiscuous use in other cytokine receptor complexes.

Stolt P. C., Chen Y., Liu P., Bock H. H., Blacklow S. C., and Herz J. (2005) Phosphoinositide Binding by the Disabled-1 PTB Domain Is Necessary for Membrane Localization and Reelin Signal Transduction. *J. Biol. Chem* **280**, 9671-9677.

Abstract: Disabled-1 (Dab1) is an essential adaptor protein that functions in the Reelin signaling pathway and is required for the regulation of neuronal migration during embryonic development. Dab1 interacts with NPXY motifs in the cytoplasmic tails of the lipoprotein receptors ApoER2 and very low density lipoprotein receptor through an amino-terminal phosphotyrosine binding (PTB) domain. Binding of Reelin to these receptors leads to tyrosine phosphorylation of Dab1 and the initiation of a signaling cascade that results in remodeling of the cytoskeleton. Structural and biochemical studies of the Dab1 PTB domain have demonstrated that this domain binds to both the NPXY peptide motif in the lipoprotein receptor tails as well as to the head group of phosphoinositide 4,5-P₂ through energetically independent mechanisms. Here we have investigated how phosphoinositide binding by the Dab1 PTB domain influences Reelin signal transduction. Our findings in cultured primary neurons that have been transduced with lentiviral constructs expressing mutant *Dab1* forms reveal that phosphoinositide binding by the Dab1 PTB domain is necessary for proper membrane localization of Dab1 and for effective transduction of a Reelin signal.

Sudandiradoss C., Priya Doss C. G., Rajasekaran R., Ramanathan K., Purohit R. and Sethumadhavan R. (2008) Investigations on the interactions of scorpion neurotoxins with the predicted structure of D1 dopamine receptor by protein-protein docking method. A bioinformatics approach. *C. R. Biol* **331**, 489-499.

Abstract: Dopamine receptors play a critical role in the cell signalling process responsible for information transfer in neurons functioning in the nervous system. Development of improved therapeutics for disorders like Parkinson's disease and schizophrenia would be significantly enhanced with the availability of the 3D structure for the dopamine receptors. Scorpion neurotoxins are unique source of structural templates from which new therapeutic agents might be developed. We report here the 3D structure of the human D1 dopamine receptor, predicted from primary sequence using computational techniques. The predicted structure of the human D1 dopamine receptor is used to understand the mechanism of interactions between scorpion neurotoxins through the protein-protein docking method. CHARMM force field was used for the energy minimization step before applying the docking method

Tamada T., Honjo E., Maeda Y., Okamoto T., Ishibashi M., Tokunaga M., and Kuroki R. (2006) Homodimeric cross-over structure of the human granulocyte colony-stimulating factor (GCSF) receptor signaling complex. *Proc Natl Acad Sci U S A*. **103**, 3135-40.

Abstract: A crystal structure of the signaling complex between human granulocyte colony-stimulating factor (GCSF) and a ligand binding region of GCSF receptor (GCSF-R), has been determined to 2.8 Å resolution. The GCSF:GCSF-R complex formed a 2:2 stoichiometry by means of a cross-over interaction between the Ig-like domains of GCSF-R and GCSF. The conformation of the complex is quite different from that between human GCSF and the cytokine receptor homologous domain of mouse GCSF-R, but similar to that of the IL-6/gp130 signaling complex. The Ig-like domain cross-over structure necessary for GCSF-R activation is consistent with previously reported thermodynamic and mutational analyses.

Touze T., Hayward R. D., Eswaran J., Leong J. M., and Koronakis V. (2004) Self-association of EPEC intimin mediated by the beta-barrel-containing anchor domain: a role in clustering of the Tir receptor. *Mol Microbiol* **51**, 73-87.

Abstract: Outer membrane intimin directs attachment of enteropathogenic *Escherichia coli* (EPEC) via its Tir receptor in mammalian target cell membranes. Phosphorylation of Tir triggers local actin polymerization and the formation of 'pedestal-like' pseudopods. We demonstrate that the intimin protein contains three domains, a flexible N-terminus (residues 40-188), a central membrane-integrated beta-barrel (189-549), and a tightly folded Tir-binding domain (550-939). Intimin was shown by electron microscopy to form ring-like structures with a approximately 7 nm external diameter and an electron dense core, and to form channels of 50picoSiemens conductance in planar lipid bilayers. Gel filtration, multiangle light scattering and cross-linking showed that this central beta-barrel membrane-anchoring domain directs intimin dimerization. Isothermal titration calorimetry revealed a high affinity, single-binding site

interaction of 2 : 1 stoichiometry between dimeric intimin and Tir, and modelling suggests that this interaction determines a reticular array-like superstructure underlying receptor clustering. In support of this model, actin rearrangement induced in Tir-primed cultured cells by intimin-containing proteoliposomes was dependent on the concentration of both intimin and Tir, and co-localized with clustered phosphorylated Tir.

Truneh A., Sharma S., Silverman C., Khandekar S., Reddy M. P., Deen K. C., McLaughlin M. M., Srinivasula S. M., Livi G. P., Marshall L. A., Alnemri E. S., Williams W. V., and Doyle M. L. (2000) Temperature-sensitive differential affinity of TRAIL for its receptors. DR5 is the highest affinity receptor. *J Biol Chem* **275**, 23319-23325.

Abstract: TRAIL is a member of the tumor necrosis factor (TNF) family of cytokines which induces apoptotic cell death in a variety of tumor cell lines. It mediates its apoptotic effects through one of two receptors, DR4 and DR5, which are members of the TNF receptor family, and whose cytoplasmic regions contain death domains. In addition, TRAIL also binds to 3 "decoy" receptors, DcR2, a receptor with a truncated death domain, DcR1, a glycosylphosphatidylinositol-anchored receptor, and OPG a secreted protein which is also known to bind to another member of the TNF family, RANKL. However, although apoptosis depends on the expression of one or both of the death domain containing receptors DR4 and/or DR5, resistance to TRAIL-induced apoptosis does not correlate with the expression of the "decoy" receptors. Previously, TRAIL has been described to bind to all its receptors with equivalent high affinities. In the present work, we show, by isothermal titration calorimetry and competitive enzyme-linked immunosorbent assay, that the rank order of affinities of TRAIL for the recombinant soluble forms of its receptors is strongly temperature dependent. Although DR4, DR5, DcR1, and OPG show similar affinities for TRAIL at 4 degrees C, their rank-ordered affinities are substantially different at 37 degrees C, with DR5 having the highest affinity ($K(D) \leq 2$ nm) and OPG having the weakest ($K(D) = 400$ nm). Preferentially enhanced binding of TRAIL to DR5 was also observed at the cell surface. These results reveal that the rank ordering of affinities for protein-protein interactions in general can be a strong function of temperature, and indicate that sizeable, but hitherto unobserved, TRAIL affinity differences exist at physiological temperature, and should be taken into account in order to understand the complex physiological and/or pathological roles of TRAIL.

Uchida H., Banba S., Wada M., Matsumoto K., Ikeda M., Naito N., Tanaka E., and Honjo M. (1999) Analysis of binding properties between 20 kDa human growth hormone (hGH) and hGH receptor (hGHR): the binding affinity for hGHR extracellular domain and mode of receptor dimerization. *J Mol Endocrinol* **23**, 347-353.

Abstract: It has recently been shown that 20 kDa human growth hormone (hGH) forms the 1:2 hGH:hGHR receptor (hGHR) complex and expresses full agonistic activity, although it hardly forms the 1:1 hGH:hGHR complex as compared with 22 kDa hGH. To clarify this mechanism, we analyzed the mode of receptor dimerization of 20 kDa hGH using the intact form and mutants. Complex formation analysis between hGHR extracellular domain (hGHBP) and either site1 mutant (K157A) or site2 mutant (G105R) by gel-filtration showed that the site1 mutant apparently formed no 1:1 complex and that the site2 mutant formed only the 1:1 complex. Cell proliferation analysis revealed that the activity curve (vs ligand concentration) of 20 kDa hGH showed a bell-shaped pattern. This indicates that the receptor dimerization of 20 kDa hGH proceeds in a sequential manner. Based on this sequential binding we have produced a mathematical model for receptor dimerization as a function of [hGH], [hGHBP], $K(d)$ values for the first hGHBP binding ($K(d1)$) and the second hGHBP binding ($K(d2)$). The result of 20 kDa hGH binding to (S201C) hGHBP immobilized on biosensor tip showed that the $K(d1)$ value was 1.6×10^{-8} M. Adopting this value as a constant in the function described above, we have obtained calculative hGHR dimerization curves vs hGH concentration. Since the $K(d2)$ value could not be experimentally determined, the curves were simulatively obtained with varied $K(d2)$ values. The simulated curve pattern coincided with the experimental result of the cell proliferation in Ba/F3-hGHR when the value 2.5×10^{-10} M was adopted as $K(d2)$. In conclusion, although the affinity of 20 kDa hGH for the first hGHR binding is reduced to one-tenth, that for the second binding is increased ten-fold in comparison with those of 22 kDa hGH, indicating that 20 kDa hGH can be an effective hGH isoform in the presence of hGHBP.

Vogele L., Trivedi V. D., Sineshchekov O. A., Spudich E. N., Spudich J. L. and Luecke H. (2007) Crystal structure of the Anabaena sensory rhodopsin transducer. *J Mol Biol* **367**, 741-751.

Abstract: We present crystal structures of the Anabaena sensory rhodopsin transducer (ASRT), a soluble cytoplasmic protein that interacts with the first structurally characterized eubacterial retinylidene photoreceptor Anabaena sensory rhodopsin (ASR). Four crystal structures of ASRT from three different spacegroups were obtained, in all of which ASRT is present as a planar (C₄) tetramer, consistent with our characterization of ASRT as a tetramer in solution. The ASRT tetramer is tightly packed, with large interfaces where the well-structured beta-sandwich portion of the monomers provides the bulk of the tetramer-forming interactions, and forms a flat, stable surface on one side of the tetramer (the beta-face). Only one of our four different ASRT crystals reveals a C-terminal alpha-helix in the otherwise all-beta protein, together with a large loop from each monomer on the opposite face of the tetramer (the alpha-face), which is flexible and largely disordered in the other three crystal forms. Gel-filtration chromatography demonstrated that ASRT forms stable tetramers in solution and isothermal microcalorimetry showed that the ASRT tetramer binds to ASR with a stoichiometry of one ASRT tetramer per one ASR photoreceptor with a K(d) of 8 microM in the highest affinity measurements. Possible mechanisms for the interaction of this transducer tetramer with the ASR photoreceptor via its flexible alpha-face to mediate transduction of the light signal are discussed.

Weber A. N., Moncrieffe M. C., Gangloff M., Imler J. L., and Gay N. J. (2005) Ligand-receptor and receptor-receptor interactions act in concert to activate signaling in the Drosophila toll pathway. *J Biol Chem* **280**, 22793-22799.

Abstract: In Drosophila, the signaling pathway mediated by the Toll receptor is critical for the establishment of embryonic dorso-ventral pattern and for innate immune responses to bacterial and fungal pathogens. Toll is activated by high affinity binding of the cytokine Spatzle, a dimeric ligand of the cystine knot family. In vertebrates, a related family of Toll-like receptors play a critical role in innate immune responses. Despite the importance of this family of receptors, little is known about the biochemical events that lead to receptor activation and signaling. Here, we show that Spatzle binds to the N-terminal region of Toll and, using biophysical methods, that the binding is complex. The two binding events that cause formation of the cross-linked complex are non-equivalent: the first Toll ectodomain binds Spatzle with an affinity 3-fold higher than the second molecule suggesting that pathway activation involves negative cooperativity. We further show that the Toll ectodomains are able to form low affinity dimers in solution and that juxtamembrane sequences of Toll are critical for the activation or derepression of the pathway. These results, taken together, suggest a mechanism of signal transduction that requires both ligand-receptor and receptor-receptor interactions.

Wecksler W. R. and Norman A. W. (1980) A kinetic and equilibrium binding study of 1 alpha,25-dihydroxyvitamin D₃ with its cytosol receptor from chick intestinal mucosa. *J Biol Chem* **255**, 3571-3574.
Abstract: A kinetic and equilibrium binding study of the interaction of 1 alpha,25-dihydroxyvitamin D₃ (1 alpha,25-(OH)₂D₃) with its receptor system from chick intestinal mucosa has been carried out. The equilibrium dissociation constant (K_d) for the binding of 1 alpha,25-(OH)₂D₃ to the crude cytoplasmic receptor varies from 2.3 x 10⁻¹⁰ M at 4 degrees C to 5.0 x 10⁻¹⁰ M at 24 degrees C, yielding values for ΔG⁰, ΔH⁰ and ΔS⁰ for the dissociation process of 12.2 +/- 0.1 kcal/mol, 6.7 +/- 1.2 kcal/mol, and -19.9 +/- 3.2 cal/degree mol. This suggests that the binding of 1 alpha,25-(OH)₂D₃ to the receptor may be an entropy-driven process. Kinetic studies on the association and dissociation of the 1 alpha,25-(OH)₂D₃ receptor complex revealed that k_{assoc} varied from 0.6 x 10⁷ M⁻¹ min⁻¹ at 0 degrees C to 14.5 x 10⁷ M⁻¹ min⁻¹ at 30 degrees C and that k_{diss} varied from 4 x 10⁻⁵ min⁻¹ at 4 degrees C to 4 x 10⁻² min⁻¹ at 30 degrees C. The activation energy for the association process was found to be 17 kcal/mol, while that for the dissociation reaction was 45 kcal/mol.

Wegener K. L., Basran J., Bagshaw C. R., Campbell I. D., Roberts G. C., Critchley D. R. and Barsukov I. L. (2008) Structural basis for the interaction between the cytoplasmic domain of the hyaluronate receptor layilin and the talin F3 subdomain. *J Mol Biol* **382**, 112-126.

Abstract: Talin is a large cytoskeletal protein that is involved in coupling the integrin family of cell adhesion molecules to the actin cytoskeleton, colocalising with the integrins in focal adhesions (FAs). However, at the leading edge of motile cells, talin colocalises with the hyaluronan receptor layilin in what are thought to be transient adhesions, some of which subsequently mature into more stable FAs. During this maturation process, layilin is replaced with integrins, which are highly clustered in FAs, where localised

production of PI(4,5)P(2) by type 1 phosphatidyl inositol phosphate kinase type 1 gamma (PIP1gamma) is thought to play a role in FA assembly. The talin FERM F3 subdomain binds both the integrin beta-subunit cytoplasmic domain and PIP1gamma, and these interactions are understood in detail at the atomic level. The talin F3 domain also binds to short sequences in the layilin cytoplasmic domain, and here we report the structure of the talin/layilin complex, which shows that talin binds integrins, PIP1gamma and layilin in similar although subtly different ways. Based on structure comparisons, we designed a set of talin F3 mutations that selectively affected the affinity of talin for its targets, as determined by stopped-flow fluorescence measurements. Such mutations will help to assess the importance of the interactions between talin and its various ligands in cell adhesion and migration

Willcox B. E., Gao G. F., Wyer J. R., O'Callaghan C. A., Boulter J. M., Jones E. Y., van der Merwe P. A., Bell J. I., and Jakobsen B. K. (1999) Production of soluble alphabeta T-cell receptor heterodimers suitable for biophysical analysis of ligand binding. *Protein Sci* **8**, 2418-2423.

Abstract: A method to produce alphabeta T-cell receptors (TCRs) in a soluble form suitable for biophysical analysis was devised involving in vitro refolding of a TCR fusion protein. Polypeptides corresponding to the variable and constant domains of each chain of a human and a murine receptor, fused to a coiled coil heterodimerization motif from either c-Jun (alpha) or v-Fos (beta), were overexpressed separately in *Escherichia coli*. Following recovery from inclusion bodies, the two chains of each receptor were denatured, and then refolded together in the presence of denaturants. For the human receptor, which is specific for the immunodominant influenza A HLA-A2-restricted matrix epitope (M58-66), a heterodimeric protein was purified in milligram yields and found to be homogeneous, monomeric, antibody-reactive, and stable at concentrations lower than 1 μ M. Using similar procedures, analogous results were obtained with a murine receptor specific for an influenza nucleoprotein epitope (366-374) restricted by H2-Db. Production of these receptors has facilitated a detailed analysis of viral peptide-Major Histocompatibility Complex (peptide-MHC) engagement by the TCR using both surface plasmon resonance (SPR) and, in the case of the human TCR, isothermal titration calorimetry (ITC) (Willcox et al., 1999). The recombinant methods described should enable a wide range of TCR-peptide-MHC interactions to be studied and may also have implications for the production of other heterodimeric receptor molecules.

Wilson D., Perlson L., and Breslow R. (2003) Helical templating of oligopeptides by cyclodextrin dimers. *Bioorg Med Chem* **11**, 2649-2653.

Abstract: beta-cyclodextrin-based receptors were synthesized and tested for their ability to induce a helical fold in peptides bearing hydrophobic amino acid residues in the i, i+11- or i, i+14-positions. Circular dichroism experiments revealed that a dimeric beta-cyclodextrin receptor synthesized from a [1,1'-biphenyl]-4,4'-dithiol core demonstrated an ability to fold a designed peptide bearing the artificial amino acid L-p-t-butylphenylalanine in the i, i+11-positions, while other dimeric and monomeric receptors failed to do so. Titration studies were performed using both circular dichroism and calorimetry, the analysis of which yielded an apparent K_a on the order of 10^4 - 10^5 M^{-1} . However, no evidence could be obtained for helical folding with a peptide carrying tryptophan residues in place of the p-t-butylphenylalanine units. Our studies suggest that receptors of this type may be useful in molecular recognition of hydrophobic, already alpha-helical peptides in aqueous solution.

Wohlwend D., Strasser A., Dickmanns A., Doenecke D. and Ficner R. (2007) Thermodynamic analysis of H1 nuclear import: receptor tuning of importinbeta/importin7. *J Biol Chem* **282**, 10707-10719.

Abstract: The nuclear import of H1 linker histones is mediated by a heterodimer of transport receptors, known as importinbeta and importin7. Interestingly, both importins separately interact with H1, but only as a dimer they facilitate the translocation through the nuclear pore. We identified the H1 binding site of importin7, comprising two extended acidic loops near the C terminus of importin7. The analysis of the H1 import complex assembly by means of isothermal titration calorimetry revealed that the formation of a receptor heterodimer in vitro is an enthalpy-driven process, whereas subsequent binding of H1 to the heterodimer is entropy-driven. Furthermore, we show that the importinbeta binding domain of importin7 plays a key role in the activation of importin7 by importinbeta. This process is allosterically regulated by importinbeta and accounts for a specific tuning of the activity of the importinbeta.importin7 heterodimer. The results presented here provide new insights into cellular strategies to even energy balances in nuclear import and point toward a general regulation of importinbeta-related nuclear import processes.

Wollert T., Heinz D. W. and Schubert W. D. (2007) Thermodynamically reengineering the listerial invasion complex InlA/E-cadherin. *Proc. Natl. Acad. Sci U. S. A* **104**, 13960-13965.

Abstract: Biological processes essentially all depend on the specific recognition between macromolecules and their interaction partners. Although many such interactions have been characterized both structurally and biophysically, the thermodynamic effects of small atomic changes remain poorly understood. Based on the crystal structure of the bacterial invasion protein internalin (InlA) of *Listeria monocytogenes* in complex with its human receptor E-cadherin (hEC1), we analyzed the interface to identify single amino acid substitutions in InlA that would potentially improve the overall quality of interaction and hence increase the weak binding affinity of the complex. Dissociation constants of InlA-variant/hEC1 complexes, as well as enthalpy and entropy of binding, were quantified by isothermal titration calorimetry. All single substitutions indeed significantly increase binding affinity. Structural changes were verified crystallographically at ≤ 2.0 -Å resolution, allowing thermodynamic characteristics of single substitutions to be rationalized structurally and providing unique insights into atomic contributions to binding enthalpy and entropy. Structural and thermodynamic data of all combinations of individual substitutions result in a thermodynamic network, allowing the source of cooperativity between distant recognition sites to be identified. One such pair of single substitutions improves affinity 5,000-fold. We thus demonstrate that rational reengineering of protein complexes is possible by making use of physically distant hot spots of recognition.

Wright A. J., Higginbottom A., Philippe D., Upadhyay A., Bagby S., Read R. C., Monk P. N. and Partridge L. J. (2007) Characterisation of receptor binding by the chemotaxis inhibitory protein of *Staphylococcus aureus* and the effects of the host immune response. *Mol Immunol.* **44**, 2507-2517.

Abstract: The chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS) is reported to bind to the receptors for C5a and formylated peptides and has been proposed as a promising lead for the development of new anti-inflammatory compounds. Here we have examined the receptor specificity and mode of action of recombinant CHIPS(28-149) and also the immune response to CHIPS(28-149) in patients with *S. aureus* infections and in uninfected controls. Recombinant CHIPS(28-149) bound with high affinity to the human C5a receptor (C5aR), but had low affinity for the second C5a receptor, C5L2, and the formyl peptide receptor, FPR. Although ligand binding to C5aR was potently inhibited, CHIPS(28-149) had much weaker effects on ligand binding to C5L2 and FPR. Similarly, CHIPS(28-149) potently inhibited the ligand-induced activation of C5aR but was less potent at inhibition via FPR. NMR studies showed that CHIPS(28-149) bound directly to the N-terminus of C5aR but not C5L2, and CHIPS(28-149) residues involved in the interaction were identified by chemical shift analysis. All human sera examined contained high titres of IgG and IgA reactivity against CHIPS(28-149), and no correlation was observed between infection status at the time of serum collection and antibody titre. Individual serum samples promoted or inhibited the binding of CHIPS(28-149) to C5aR, or had no effect. IgG depletion of serum samples abrogated the effects on CHIPS binding, demonstrating that these were antibody mediated. Sera from infected individuals were more likely to inhibit CHIPS(28-149) binding than sera from healthy controls. However, high antibody titres correlated well with both inhibition and enhancement of CHIPS(28-149) binding to C5aR; this suggests that the inhibitory effect relates to epitope specificity rather than greater antibody binding. We conclude that CHIPS is likely to be too immunogenic to be used as an anti-inflammatory treatment but that some antibodies against CHIPS may be useful in the treatment of *S. aureus* infections.

Wright E., Vincent J. and Fernandez E. J. (2007) Thermodynamic characterization of the interaction between CAR-RXR and SRC-1 peptide by isothermal titration calorimetry. *Biochemistry* **46**, 862-870.

Abstract: The constitutive androstane receptor (CAR) enhances transcription of specific target genes that regulate several metabolic pathways. CAR functions as an obligate heterodimer (CAR-RXR) with the retinoid X receptor (RXR). Also part of the active receptor complex is the steroid receptor coactivator-1 (SRC-1) which interacts with the receptor complex via specific receptor interaction domains (RIDs). A peptide derived from SRC-1 RID2 is used to study the thermodynamic properties of the interaction with the CAR-RXR ligand binding domain (LBD) complex. In the absence of ligands for both CAR and RXR, binding of coactivator peptide to the CAR-RXR heterodimer is characterized by a favorable enthalpy change and an unfavorable entropy change. The addition of the CAR agonist, TCPOBOP, increases the affinity for coactivator by decreasing the unfavorable entropy and increasing the favorable intrinsic enthalpy of the interaction. The RXR ligand, 9-cis-RA, generates a second SRC-1 site and increases the affinity by improving the entropic component of binding. There is an additional increase in affinity for one

of the two sites in the presence of both ligands. The change in heat capacity (ΔC_p) is also investigated. A 2-fold difference in ΔC_p is observed between liganded and unliganded CAR-RXR. The observed thermodynamic parameters for binding of SRC-1 peptide to liganded and apo CAR-RXR as well as the difference in the ΔC_p data provide evidence that the apo CAR-RXR heterodimer is conformationally mobile. The more favorable enthalpic contribution for TCPOBOP-bound CAR-RXR indicates that preformation of the binding site improves the complementarity of the coactivator-receptor interaction.

Wu J., Li J., Li G., Long D. G., and Weis R. M. (1996) The receptor binding site for the methyltransferase of bacterial chemotaxis is distinct from the sites of methylation. *Biochemistry* **35**, 4984-4993.

Abstract: The principal locus for binding interactions between the aspartate and serine receptors of *Escherichia coli* and the methyltransferase was found to be in the last five amino acids of the receptor. The thermodynamic parameters of transferase-receptor interactions were determined by isothermal titration calorimetry. The serine receptor and three C-terminal fragments (C-fragments) of the aspartate receptor consisting of either the last 297, 88, or 38 amino acids gave comparable values for binding ($n=1$, ΔH approximately 13 kcal/mol, and K_a approximately $4 \times 10^5 \text{ M}^{-1}$). Truncating either 16 or 36 amino acids from the C-terminus eliminated observable interactions. Finally the pentapeptide Asn-Trp-Glu-Thr-Phe, which corresponds to the last five amino acids of the receptor and is strictly conserved among *E. coli* serine and aspartate receptors and the *Salmonella typhimurium* aspartate receptor, was found to have all the binding activity of the full-length receptor and the C-fragments. An *in vitro* methylation assay was used to obtain evidence for the physiological significance of this interaction in which excess peptide was able to completely block receptor methylation. The location of the binding site far from the methylation sites in the primary structure of the receptor suggests that the principle role of this interaction may be to hold the transferase in close proximity to all the methylation sites. Intersubunit methylation implication is proposed as plausible consequence of this "controlled proximity" mechanism since the ribose-galactose and dipeptide receptors lack the transferase binding sequence, and appear unable to bind transferase. Intersubunit methylation implies that transferase bound to either the serine or aspartate receptor subunit may catalyze methylation of receptor subunits in a neighboring dimer, including those that have ligand specificity.

Yan K. S., Kuti M., Yan S., Mujtaba S., Farooq A., Goldfarb M. P., and Zhou M. M. (2002) FRS2 PTB domain conformation regulates interactions with divergent neurotrophic receptors. *J Biol Chem* **277**, 17088-17094.

Abstract: Membrane-anchored adaptor proteins FRS2 α /beta (also known as SNT-1/2) mediate signaling of fibroblast growth factor receptors (FGFRs) and neurotrophin receptors (TRKs) through their N-terminal phosphotyrosine binding (PTB) domains. The FRS2 PTB domain recognizes tyrosine-phosphorylated TRKs at an NPXpY (where pY is phosphotyrosine) motif, whereas its constitutive association with FGFR involves a receptor juxtamembrane region lacking Tyr and Asn residues. Here we show by isothermal titration calorimetry that the FRS2 α PTB domain binding to peptides derived from TRKs or FGFR is thermodynamically different. TRK binding is largely enthalpy-driven, whereas the FGFR interaction is governed by a favorable entropic contribution to the free energy of binding. Furthermore, our NMR spectral analysis suggests that disruption of an unstructured region C-terminal to the PTB domain alters local conformation and dynamics of the residues at the ligand-binding site, and that structural disruption of the beta8-strand directly weakens the PTB domain association with the FGFR ligand. Together, our new findings support a molecular mechanism by which conformational dynamics of the FRS2 α PTB domain dictates its association with either fibroblast growth factor or neurotrophin receptors in neuronal development.

Yang Q., Alemany R., Casas J., Kitajka K., Lanier S.M., and Escriba P.V. (2005) Influence of the membrane lipid structure on signal processing via G protein-coupled receptors. *Mol Pharmacol.* **68**, 210-7.

Abstract: We have recently reported that lipid structure regulates the interaction with membranes, recruitment to membranes, and distribution to membrane domains of heterotrimeric G α betagamma proteins, G α subunits, and Gbetagamma dimers (*J Biol Chem* 279:36540-36545, 2004). Here, we demonstrate that modulation of the membrane structure not only determines G protein localization but also regulates the function of G proteins and related signaling proteins. In this context, the antitumor drug daunorubicin (daunomycin) and oleic acid changed the membrane structure and inhibited G protein activity in biological membranes. They also induced marked changes in the activity of the $\alpha(2A/D)$ -adrenergic receptor and adenylyl cyclase. In contrast, elaidic and stearic acid did not change the activity of the above-

mentioned proteins. These fatty acids are chemical but not structural analogs of oleic acid, supporting the structural basis of the modulation of membrane lipid organization and subsequent regulation of G protein-coupled receptor signaling. In addition, oleic acid (and also daunorubicin) did not alter G protein activity in a membrane-free system, further demonstrating the involvement of membrane structure in this signal modulation. The present work also unravels in part the molecular bases involved in the antihypertensive (Hypertension 43:249-254, 2004) and anticancer (Mol Pharmacol 67:531-540, 2005) activities of synthetic oleic acid derivatives (e.g., 2-hydroxyoleic acid) as well as the molecular bases of the effects of diet fats on human health.

Ye H. and Wu H. (2000) Thermodynamic characterization of the interaction between TRAF2 and tumor necrosis factor receptor peptides by isothermal titration calorimetry. *Proc Natl Acad Sci U S A* **97**, 8961-8966.

Abstract: The tumor necrosis factor receptor (TNFR) superfamily can induce diverse biological effects, including cell survival, proliferation, differentiation, and apoptosis. The major signal transducers for TNFRs are the family of TNF receptor associated factors (TRAFs). The direct interaction between TRAFs and the intracellular tails of TNFRs is the first step of this signal relay process. Structural studies have revealed a trimeric nature of TRAF2 and a symmetrical mode of receptor binding, suggesting the involvement of trivalent TNFR2-receptor interaction in the signal transduction. In this study, using isothermal titration calorimetry (ITC), we report thermodynamic characterization of the interaction between TRAF2 and monomeric peptide sequences from TNFR members, including TNFR2, CD40, CD30, Ox40, and 4-1BB, and the Epstein-Barr virus (EBV)-transforming protein, latent infection membrane protein-1 (LMP1). The dissociation constants of the interaction were shown to range between 40 μ M and 1.9 mM, which are substantially weaker than most protein-peptide interactions. The interaction is entirely driven by exothermic enthalpy, consistent with the abundance of polar contacts. The enthalpy of the interaction has a significant temperature dependence ($\Delta C_p = -245$ cal/mol small middle dotK). The unfavorable entropy in the interaction and the comparison with structural energetics calculations suggest the involvement of conformational rearrangement in the interaction. The low affinity of TRAF2 to monomeric receptor peptides further supports the importance of avidity contribution in TRAF2 recruitment by these receptors upon ligand-induced trimerization or higher order oligomerization.

Yi X. and Weis R. M. (2002) The receptor docking segment and S-adenosyl-L-homocysteine bind independently to the methyltransferase of bacterial chemotaxis. *Biochim Biophys Acta* **1596**, 28-35.

Abstract: To mediate adaptation to stimuli, the methyltransferase (CheR) catalyzes methyl group transfer from S-adenosyl-L-methionine (SAM) to glutamyl residues in the transmembrane receptors of the bacterial chemosensory signaling pathway. The interaction between receptors and CheR occurs at two sites: a methylation site-active site interaction, and a 'docking' site interaction that is separated both from the methylation sites and the CheR active site. It is not certain if the docking site interaction functions merely to localize the transferase in close proximity to the methylation sites, or if it also increases CheR catalytic activity. Isothermal titration calorimetry experiments are conducted to test for allosteric interactions between the docking and active sites on CheR, which are expected to be present if docking activates CheR. The binding parameters (ΔG , ΔH , ΔS) of a substrate analog of SAM, S-adenosyl-L-homocysteine (SAH), are measured both in the absence and presence of saturating concentrations of a pentapeptide (NWETF) that defines the docking receptor docking segment. SAH binding is unaffected by the presence of saturating NWETF, providing evidence that an allosteric activation of CheR does not take place upon docking, and thus supports the idea that the CheR-NWETF interaction merely functions to localize CheR near the sites of methylation.

Yip K. W., Godoi P. H., Zhai D., Garcia X., Cellitti J. F., Cuddy M., Gerlic M., Chen Y., Satterthwait A., Vasile S., Sergienko E. and Reed J. C. (2008) A TR3/Nur77 peptide-based high-throughput fluorescence polarization screen for small molecule Bcl-B inhibitors. *J Biomol. Screen.* **13**, 665-673.

Abstract: Nuclear receptor TR3/Nur77/NR4A1 binds several antiapoptotic Bcl-2-family proteins (Bcl-B, Bcl-2, Bfl-1) in a non-BH3-dependent manner. A 9-amino-acid peptide derived from full-length TR3 with polyarginine tail (TR3-r8) recapitulates TR3's binding specificity, displaying high affinity for Bcl-B. TR3-r8 peptide was used to screen for small molecule Bcl-B inhibitors. A fluorescence polarization assay (FPA) employing fluorescein isothiocyanate (FITC)-labeled TR3-r8 peptide (FITC-TR3-r8) and Bcl-B protein was optimized, with nonfluorescent TR3-r8 serving to demonstrate reversible, competitive binding.

Approximately 50,000 compounds were screened at 3.75 mg/L, yielding 145 reproducible hits with $\geq 50\%$ FITC-TR3-r8 displacement (a confirmed hit rate of 0.29%). After dose-response analyses and counterscreening with an unrelated FITC-based FPA, 6 candidate compounds remained. Nuclear magnetic resonance (NMR) showed that 2 of these compounds bound Bcl-B, but not glutathione S-transferase (GST) control protein. One Bcl-B-binding compound was unable to displace FITClabeled BH3 peptides from Bcl-B, confirming a unique binding mechanism compared with traditional antagonists of antiapoptotic Bcl-2-family proteins. This compound bound Bcl-B with K_d 1.94 \pm 0.38 μ M, as determined by isothermal titration calorimetry. Experiments using Bcl-B overexpressing HeLa cells demonstrated that this compound induced Bcl-B-dependent cell death. The current FPA represents a screen that can identify noncanonical inhibitors of Bcl-2-family proteins

Zabeau L., Defeau D., Iserentant H., Vandekerckhove J., Peelman F., and Tavernier J. (2005) Leptin receptor activation depends on critical cysteine residues in its fibronectin type III subdomains. *J Biol Chem.* **280**, 22632-40.

Abstract: The leptin receptor (LR) complex is composed of a single subunit belonging to the class I cytokine receptor family and exists as a preformed complex. The extracellular portion contains two cytokine receptor homology (CRH) domains, separated by an Ig-like domain and followed by two membrane-proximal fibronectin type III (FNIII) domains. The mechanisms underlying ligand-induced receptor activation are still poorly understood. LRs can exist as disulfide-linked dimers at the cell surface, even in the absence of leptin. We evaluated the role of the two unpaired cysteine residues (Cys-672 and Cys-751) in the FNIII domains in receptor clustering, leptin binding, and biological activity. Although mutation of cysteine on position 751 to serine has hardly any effect on ligand binding and receptor activation, the C672S mutant exhibits a marked reduction in STAT3-dependent signaling. The double mutant was completely devoid of biological activity, although leptin binding remained unaffected. Mutation of both residues resulted in complete loss of disulfide bridge formation of FNIII domains in solution. In contrast, no difference was observed in ligand-independent oligomerization of the membrane-bound receptor, suggesting a role for cysteines in the CRH2 domain in formation of the preformed LR complex. We propose a model wherein leptin-induced clustering of two preformed dimers forms the activated LR complex. Disulfide bridge formation involving Cys-672 and Cys-751 may be necessary for JAK activation and hence signaling.

Zaccheo O. J., Prince S. N., Miller D. M., Williams C., Kemp C. F., Brown J., Jones E. Y., Catto L. E., Crump M. P., and Hassan A. B. (2006) Kinetics of insulin-like growth factor II (IGF-II) interaction with domain 11 of the human IGF-II/mannose 6-phosphate receptor: function of CD and AB loop solvent-exposed residues. *J Mol Biol* **359**, 403-421.

Abstract: Ligands of the IGF-II/mannose 6-phosphate receptor (IGF2R) include IGF-II and mannose 6-phosphate modified proteins. Disruption of the negative regulatory effects of IGF2R on IGF-II-induced growth can lead to embryonic lethality and cancer promotion. Of the 15 IGF2R extracellular domains, domains 1-3 and 11 are known to have a conserved beta-barrel structure similar to that of avidin and the cation-dependent mannose 6-phosphate receptor, yet only domain 11 binds IGF-II with high specificity and affinity. In order to define the functional basis of this critical biological interaction, we performed alanine mutagenesis of structurally determined solvent-exposed loop residues of the IGF-II-binding site of human domain 11, expressed these mutant forms in *Pichia pastoris*, and determined binding kinetics with human IGF-II using isothermal calorimetry and surface plasmon resonance with transition state thermodynamics. Two hydrophobic residues in the CD loop (F1567 and I1572) were essential for binding, with a further non-hydrophobic residue (T1570) that slows the dissociation rate. Aside from alanine mutations of AB loop residues that decrease affinity by modifying dissociation rates (e.g. Y1542), a novel mutation (E1544A) of the AB loop enhanced affinity by threefold compared to wild-type. Conversion from an acidic to a basic residue at this site (E1544K) results in a sixfold enhancement of affinity via modification principally of the association rate, with enhanced salt-dependence, decreased entropic barrier and retained specificity. These data suggest that a functional hydrophobic binding site core is formed by I1572 and F1567 located in the CD loop, which initially anchors IGF-II. Within the AB loop, residues normally act to either stabilise or function as negative regulators of the interaction. These findings have implications for the molecular architecture and evolution of the domain 11 IGF-II-binding site, and the potential interactions with other domains of IGF2R.

Zhou X., Sun Q., Kini R. M. and Sivaraman J. (2008) A universal method for fishing target proteins from mixtures of biomolecules using isothermal titration calorimetry. *Protein Sci* **17**, 1798-1804.

Abstract: The most challenging tasks in biology include the identification of (1) the orphan receptor for a ligand, (2) the ligand for an orphan receptor protein, and (3) the target protein(s) for a given drug or a lead compound that are critical for the pharmacological or side effects. At present, several approaches are available, including cell- or animal-based assays, affinity labeling, solid-phase binding assays, surface plasmon resonance, and nuclear magnetic resonance. Most of these techniques are not easy to apply when the target protein is unknown and the compound is not amenable to labeling, chemical modification, or immobilization. Here we demonstrate a new universal method for fishing orphan target proteins from a complex mixture of biomolecules using isothermal titration calorimetry (ITC) as a tracking tool. We took snake venom, a crude mixture of several hundred proteins/peptides, as a model to demonstrate our proposed ITC method in tracking the isolation and purification of two distinct target proteins, a major component and a minor component. Identities of fished out target proteins were confirmed by amino acid sequencing and inhibition assays. This method has the potential to make a significant advancement in the area of identifying orphan target proteins and inhibitor screening in drug discovery and characterization