DETERGENT-SOLUBILIZED PATCHED PURIFIED FROM SF9 CELLS FAILS TO INTERACT STRONGLY WITH COGNATE HEDGEHOG OR IHOG HOMOLOGS

by
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Abstract

The Hedgehog (Hh) signaling pathway mediates key tissue patterning events during animal development, and abnormal pathway activity is associated with several cancers. Hh proteins are secreted morphogens that specify cell fates in neighboring tissues in a concentration-dependent manner. The twelve-pass transmembrane protein Patched (Ptc) has been identified as a key Hh receptor in genetic and cell-based binding studies. In addition to Ptc, the CDO/Ihog family of co-receptors, and other accessory proteins, are necessary for proper Hh signaling. Structures of Hh proteins bound to members of the CDO/Ihog family are known, but the nature of the full Hh receptor complex is not well understood. We have expressed Ptc proteins from Drosophila and Mouse in Sf9 cells and find that purified, detergent-solubilized Ptc proteins do not interact strongly with cognate Hh and CDO/Ihog homologs. These results may reflect a nonnative conformation of purified Ptc or that an additional factor or factors is required for high-affinity Ptc binding to Hh.

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Chapter 1 – Introduction

Hedgehog Signaling

The Hedgehog (Hh) signaling pathway mediates key tissue patterning events during animal development, and abnormal pathway activity is associated with several cancers [1, 2]. Hh proteins are secreted morphogens that specify cell fates in neighboring tissues in a concentration-dependent manner [3, 4]. The twelve-pass transmembrane protein Patched (Ptc) has been identified as a key Hh receptor in genetic [5–8] and cell-based binding studies [9–11]. In the absence of Hh, Ptc constitutively inhibits the activity of Smoothened (Smo) [6, 12], a seven-pass transmembrane protein. The mechanism of this inhibition is unknown but does not appear to involve a direct interaction between Ptc and Smo [12]. In the presence of Hh, this inhibition is relieved and the pathway is activated. For recent reviews, see [13–16].

Many additional proteins modulate Hh pathway activity, but their presence and activity are not always conserved across phyla. For instance, Gas1 positively regulates Hh signaling in mammals [17], but no Gas1 homolog exists in the fruit fly. Hhip, a cell surface protein that acts as a negative regulator by binding and sequestering Hh proteins in vertebrates [18] also has no apparent ortholog in the fly. The mammalian proteins CDO and BOC are orthologous to the fly proteins Ihog and Boi and each binds its cognate Hh protein, but the manner and co-factor dependence of Hh binding by fly and mammalian orthologs is not conserved [19].

Patched

Despite the central importance of the Hh signaling pathway in animal development and the identification of many key pathway components, little is known about the molecular details connecting these components. Ptc is presumed to control Smo activity by transporting a small sterol-like molecule, a hypothesis derived from an array of circumstantial evidence: the homology of Ptc to proton antiporters in the RND superfamily; the presence in Ptc of a sterol-sensing domain, which in other eukaryotic homologs is related to cholesterol trafficking; the indirect inhibition of Smo by Ptc, which suggests an intermediate [12]; and recent structural and biochemical studies of Smo showing that sterols
bind Smo and modulate its activity [20–22]. Nevertheless, ligand transport by Ptc has not been conclusively demonstrated, nor has a physiological ligand for Smo been identified. More generally, how Hh proteins modulate the activity of Ptc is not known [12].

A direct interaction between Hh and Ptc is clearly the simplest and most likely interpretation for high-affinity Hh binding to Ptc-expressing cells and Hh modulation of Ptc activity [9, 11]. Assays measuring binding to the cell surface leave open the possibility that other factors could be involved, however. For example, most cell-based binding studies predated knowledge of the importance of accessory proteins Gas1, CDO/Ihog and BOC/Boi for mediating interactions between Hh and the cell surface. These proteins are essential for both fly [23] and mammalian Hh signaling [17, 24]. Members of the CDO family of proteins bind Hh proteins directly with low micromolar affinities [19, 25, 26], whereas the affinity of mammalian Sonic Hedgehog (Shh) for binding to the surface of Ptc-expressing cells is in the low nanomolar range [9, 11]. This difference in affinity, as well as the fact that high-affinity cell-surface binding is dependent on the presence of Ptc, would seem to indicate that Ptc itself directly binds Shh but that the affinity for the binary Shh-Ptc interaction may be weaker than the affinity of Hh for Ptc-expressing cells. The role of the CDO/Ihog family of proteins appears to be to function as co-receptors that enhance binding affinity. In this case, a ternary complex between Ptc, Hh and members of the CDO/Ihog family may represent the initial signaling event at the cell surface. Evidence for such ternary complexes has been found for Drosophila Ptc and Ihog [23], but the evidence in the mammalian pathway is contradictory. Although CDO, BOC or Gas1 appear to be required along with Ptc for normal signaling in mammals [17, 24], the addition of the soluble Hh-binding domain of CDO (CDO Fn3) actually competes for ShhN binding to Ptc on the cell surface [19]. This observation suggests that the binding surfaces for Ptc and CDO on ShhN may overlap. This binding competition has been rationalized with the positive role of both Ptc and CDO in Hh signaling by the observation that physiological Hh is found in multivalent particles [27], allowing simultaneous Ptc and CDO binding. Multivalency is not required for high-affinity binding of Shh to Ptc on cells, however, as monomeric ShhN expressed in E. coli binds Ptc-expressing cells with high affinity [9, 11].
A major barrier to understanding Ptc activity and the nature of its interactions with Hh pathway components has been the difficulty of isolating functional forms of Ptc or Ptc fragments. Most information on the reported molecular mechanisms and binding partners of Ptc has been obtained indirectly, using cell, tissue or whole animal-based studies. We therefore undertook to express and purify mouse and *Drosophila* Ptc proteins with intact transmembrane and extracellular regions for binding studies with Hh and other Hh pathway components. Surprisingly, when extracted and purified in the presence of detergents, we find that Ptc proteins do not bind soluble, cognate Hh or CDO/Ihog homologs with high affinity as either binary or ternary complexes.
Chapter 2 – Detergent-Solubilized Patched Purified from Sf9 Cells Fails to Interact Strongly with Cognate Hedgehog or Ihog Homologs

Introduction

This project was initiated following work by Jason McLellan and others, who determined the structures of the *Drosophila* and mouse Hh proteins bound to their cognate Ihog/CDO homologs. These structures demonstrated that, although the individual proteins are homologous, the Hh:Ihog and Shh:CDO complexes use different binding modes. This work also showed an apparent competition between binding of Shh and CDOFn3 to cells expressing Ptc, a puzzling observation given that both CDO and Ptc are required for proper signaling, and both have a positive regulatory role in Hh signaling. To clarify the role of Ihog homologs and other accessory proteins with respect to signaling through Ptc, we decided to perform *in vitro* binding experiments on the relevant extracellular domains of these proteins.

Ptc is predicted to contain twelve transmembrane alpha-helices, cytoplasmic N- and C-termini, and large extracellular loops between transmembrane helices 1-2 and 7-8. Attempts have been made in the Leahy lab to express isolated soluble extracellular domains of Ptc, separate from its transmembrane helices, both as individual loops and connected with an artificial linker. Unfortunately, these soluble domains do not seem to be functionally separable from the transmembrane region of Ptc. While certain isolated loop constructs can be expressed and secreted from mammalian cell lines, and are well-behaved enough to purify from conditioned medium, they were not found to bind Hedgehog proteins in preliminary experiments (unpublished data).

Therefore, we believe that to understand the molecular role of accessory proteins such as Ihog, the expression and purification of Ptc as an intact membrane protein for *in vitro* binding experiments will be required. Ultimately, structural studies of Ptc bound to its protein ligands should be pursued. The work presented in Chapters 1 and 2 has been submitted in modified form for publication.
Materials and Methods

**Materials**—All chemicals were purchased from Sigma unless otherwise specified. Detergents were purchased from Affymetrix and included n-dodecyl-β-D-maltopyranoside (DDM), n-dodecylphosphocholine (fosc-choline 12, FC-12), and 2,2-didecylpropane-1,3-bis-β-D-maltopyranoside (lauryl maltose neopentyl glycol, LMNG). Low molecular weight heparin (LMWH, average molecular weight 3000 Da, sodium salt, from porcine sources) was purchased from P212121.com. Heparin Decasaccharide (DH) was purchased from Neoparin Inc. Antibodies for western blotting were mouse α-Myc monoclonal (9E10), which we isolated from hybridoma growth medium; rabbit α-Ihog polyclonal and mouse α-Hh monoclonal antibodies, which were gifts from P. Beachy; and appropriate HRP-conjugated secondary antibodies.

**Buffers**—Ni²⁺ Binding Buffer consisted of 35 mM NaH₂PO₄, 300 mM NaCl, and 15 mM imidazole adjusted to pH 8.0 with NaOH at 25°C. Ni²⁺ Wash Buffer consisted of 10 mM Tris base, 10 mM Tris HCl, and 300 mM NaCl. Ni²⁺ Elution Buffer was Ni²⁺ Wash Buffer with 250 mM imidazole. Strep Wash Buffer was 20 mM MOPS, 10 mM NaOH, and 200 mM NaCl. Strep Elution Buffer was Strep Wash Buffer with 2.5 mM desthiobiotin. Pull-Down Buffer was Strep Wash Buffer containing 0.01% LMNG and 0.2 mM TCEP. CD Buffer was 10 mM NaH₂PO₄, 150 mM NaF, titrated to pH 7.2 with NaOH. CPM Thermal Stability Buffer was 20 mM HEPES pH 7.5, 200 mM NaCl, 0.02% LMNG.

**Proteins and expression vectors**—IhogFn1, IhogFn2, IhogFn12 and IhogFn12ΔH are the first and/or second Type III Fibronectin (FnIII) domains from Ihog, with ΔH referring to a surface mutant with reduced heparin binding [25]. BOCFn3, BOCFn23 and BOCFn13 are FnIII domains 3, 2–3, and 1–3 respectively from BOC. These BOC and Ihog fragments were expressed as His-Myc-TEV- (HMT-) fusion proteins using the vector pT7HMT [28]. HhN, ShhN and ShhN-SC (The "Surface C" mutant of ShhN [11], which is deficient in binding to Ptc-expressing cells) were subcloned into a modified version of pMAL-c2x as described [19]. ShhFL and HhFL are the full-length Mouse and *Drosophila* Hh proteins including native signal sequences and C-terminal self-splicing domains. IhogFn12TM
consisted of the native Ihog signal sequence followed by the first and second FnIII domains and the transmembrane region, but with the intracellular region truncated. Ski and Hhat are the entire native Hh acyltransferases from *Drosophila* and Mouse, respectively. MmHhip consisted of the β-propeller and following two EGF domains of Mouse Hhip. MmPtcT1 and DmPtcT1 are the tagged Mouse and *Drosophila* Ptc proteins with their C-termini truncated immediately after the final predicted transmembrane helix. See Supplementary Table 1 for detailed protein sequence specifications.

Ptc proteins were expressed with a concatenated series of N-terminal tags including the Streptavidin Binding Peptide (SBP) [29], an HRV3C protease site, a 6×His tag, a Myc tag, and a TEV protease site. Tags and Ptc proteins were cloned into the transfer vector pFastBac1 (Invitrogen). For co-expression of ShhFL with Hhat, HhFL with Ski, and DmPtcT1 with IhogFn12TM, transfer vectors were constructed from pFastBacDual (Invitrogen). Hhip was cloned into a modified pFastBac1 vector containing the Honeybee Mellitin (HBM) signal sequence to target for secretion, followed by 8×His, SBP, Myc and TEV sequences.

*Production of recombinant baculoviruses*—Recombinant bacmids and baculoviruses for insect cell expression were constructed using the Bac-to-Bac system (Invitrogen) following manufacturer’s instructions. After transfecting Sf9 cells with bacmid DNA, the secreted virus (designated P1) was amplified two more times (P2 and P3 viruses) following manufacturer’s instructions. P3 virus was used for protein production.

*Bacterial expression and purification of HhN, IhogFn1, IhogFn2, IhogFn12, IhogFn12ΔH, ShhN, ShhN-SC, BOCFn3, BOCFn23 and BOCFn13*—All expression plasmids were transformed into BL21 and plated on LB with appropriate antibiotics. Single colonies were picked, grown overnight, and used to inoculate TB in baffled flasks at 225 RPM and 37°C. Bacteria were grown to an optical density (600 nm) of around 0.8, at which point the incubator temperature was lowered to 16°C and the cultures allowed to shake for an additional hour. IPTG was then added to a final concentration of 0.5 mM, followed by expression for 24 hr. Bacteria were harvested by centrifugation and cell pellets stored at -20°C or -80°C.
Individual bacterial pellets were resuspended in ice-cold Ni\textsuperscript{2+} Binding Buffer containing 0.1 μL/mL Benzonase Nuclease and 1 mM PMSF. Bacteria were then lysed using a French press, and extracts cleared by centrifugation and syringe filtration. All proteins were initially purified using 5 mL HisTrap columns (GE Healthcare) charged with Ni\textsuperscript{2+}. Where appropriate, tags were then removed by addition of His-tagged TEV or HRV3C and overnight incubation at 4°C. HhN, IhogFn12, IhogFn12ΔH, IhogFn1, and ShhN were then further purified by cation exchange chromatography as described [19, 25]. All proteins were desalted if necessary, then separated from tags and proteases by another round of HisTrap purification. All proteins were purified by a final size exclusion chromatography (SEC) step in Pull-Down Buffer, except for BOCFn13 for which the concentration of NaCl was 500 mM.

Expression and Purification of IL-6—A DNA fragment coding for mouse IL-6 was cloned into the vector pT7HMT (Table 2.1). This expression vector was transformed into BL21 and expressed similarly to other bacterial proteins, except that induction was carried out at 37°C. Cells were lysed using a French press and inclusion bodies isolated by centrifugation. Inclusion bodies were resuspended and solubilized with 35 mM NaH\textsubscript{2}PO\textsubscript{4}, 6 M guanidine, pH 8.0, then purified using 5 mL HisTrap columns with standard Ni\textsuperscript{2+} Wash/Elution buffers in the presence of 6 M guanidine. The purified, unfolded protein was left uncovered and allowed to oxidize in air while stirring for 48 hr at 4°C. Samples were then diluted with 35 mM NaH\textsubscript{2}PO\textsubscript{4}, 300 mM NaCl, pH 8.0 such that the imidazole concentration was 15 mM and purified using a 5 mL HisTrap column without guanidine. Refolded protein was purified by gel filtration in PBS.

Expression and purification of AcrB—The DNA fragment coding for AcrB was amplified by PCR from E. coli genomic DNA and cloned into pET21a. A fragment coding for the SbpPHMT tags was then inserted at the C-terminus of the AcrB coding sequence (see Table 2.1 for sequence details). AcrB was overexpressed and purified as described [30], except that 2% DDM was used for extraction, as in [31]. Briefly, the AcrB expression plasmid was transformed into BL21, and inoculated into Terrific Broth in 1 L shake flasks at 37°C. Cultures were grown to an optical density (600 nm) of approximately
0.7, when the growth temperature was changed to 30°C. Cultures were then grown to an optical density of 1.2, induced with 1 mM IPTG, grown for an additional 4 hr, and harvested. Cells were washed with PBS + 10% glycerol and frozen.

For purification, 5 mL of packed cells were resuspended to 30 mL in PBS containing benzonase and 1 mM PMSF, and lysed using a French press. Membranes were then harvested by ultracentrifugation. Membranes were resuspended using a Dounce homogenizer with 10 mL of buffer (35 mM NaH2PO4, 300 mM NaCl, 15 mM Imidazole, 10% glycerol, pH 8.0) per gram of membrane pellet. Integral membrane proteins were solubilized by adding 10% DDM solution to a final concentration of 2% followed by incubation on ice for 1 hour. Insoluble material was cleared by ultracentrifugation, and AcrB purified using a 5 mL Nickel HiTrap column in 20 mM Tris pH 8.0, 300 mM NaCl, 10% glycerol and 0.05% DDM followed by step elution with the same buffer containing 250 mM Imidazole.

Insect expression and purification of Hhip, DmPtcT1 and MmPtcT1—Sf9 and High Five cells were adapted and grown in suspension using a modification of the serum-free medium ISFM [32] containing 10 g/L glucose instead of 2.5 g/L. Sf9 or High Five cells were grown or split to a concentration of $2.0 \times 10^6$ cells/mL in air-sparged 1 L spinner flasks before infection with 20 mL of P3 baculovirus (for simultaneous infection with multiple viruses, 20 mL of each virus were added).

Hhip was expressed in High Five cells. After infection, expression was allowed to proceed for 72 hr before removing the cells by centrifugation. The conditioned medium was then adjusted to pH 8.0 with NaOH, resulting in a heavy precipitate of calcium phosphate that was removed by centrifugation and filtration. Up to 6 L of pH-adjusted, clarified medium was flowed by gravity through 15 mL of “cOmplete His” resin (Roche) at room temperature overnight, which was then washed and eluted using manufacturer’s recommended conditions. Eluted protein was further purified using a 5 mL StrepTrap column (GE Healthcare), and tags were cleaved by addition of TEV protease overnight. Cut tags, uncleaved fusion protein and TEV protease were then removed using a 5 mL HisTrap column, and the remaining Hhip was further purified by SEC.
MmPtcT1 and DmPtcT1 were expressed in Sf9 cells. After infection, expression was allowed to proceed for 72 hr before harvesting cells by centrifugation. If not used immediately, cell pellets were stored by washing with PBS containing 10% glycerol, followed by snap freezing in liquid nitrogen and storage at -80°C. Cells were resuspended to a final volume of 30 mL per L of culture with Ni²⁺ Bind Buffer containing 0.5 mM TCEP, 0.1 μL/mL Benzonase and one Complete Protease Inhibitor tablet, EDTA-free (Roche). Cells were lysed by French press, and nuclei and large debris removed by centrifugation at 10,000 rpm for 10 min in an SA-600 rotor. The supernatant was then transferred to a thin-walled ultracentrifuge tube and additional buffer added up to the maximum tube volume, around 40 mL, and centrifuged in an sw-28 rotor at 28,000 rpm for 1 hr to pellet membranes. Isolated membranes were weighed, added to 10 mL of Ni²⁺ Bind buffer per gram of membrane pellet, and resuspended using a Dounce homogenizer. 1 mL of 10% FC-12 per gram of membrane pellet was then added to the resuspended membranes, which were allowed to solubilize for 30 minutes on ice. Finally, detergent-solubilized protein was clarified with an additional ultracentrifugation spin.

Solubilized protein was applied to a 5 mL HisTrap column, washed with 50 mL of Nickel Wash Buffer supplemented with 0.2 mM TCEP and 0.02% LMNG, and eluted with Nickel Elution Buffer supplemented with 0.2 mM TCEP and 0.02% LMNG. At this point, purified Ptc could be incubated in batch with a Strep-Tactin bead slurry for use in pulldown assays. Alternatively, to obtain higher concentrations and purity for Circular Dichroism (CD), analytical SEC, or for pulldown by ShhN- or HhN-coupled beads, an additional round of purification was performed using a 5 mL StrepTrap HP column. Purified Ptc from the nickel column was applied to the StrepTrap column, washed with Strep Bind Buffer supplemented with 0.2 mM TCEP and 0.01% LMNG, and eluted with Strep Elution Buffer containing 0.2 mM TCEP and 0.01% LMNG. For Ptc proteins intended for CD, the Strep-Tactin purification buffers were substituted with CD Buffer containing the same amounts of TCEP, LMNG and desthiobiotin. For Ptc proteins intended for 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) thermal melts, Ptc was purified as described above with the following modifications: 2 mg/ml iodoacetamide was added at membrane solublization; all
subsequent buffers did not include TCEP; and protein from Nickel column elution was desalted using a 5mL HiTrap desalting column equilibrated in CPM Thermal Stability Buffer. Freshly purified protein was used the same day for CPM thermal melt assays.

Size exclusion chromatography of detergent-exchanged Ptc—For each detergent screened, a membrane pellet expressing MmPtcT1 from 125 ml Sf9 culture was used. Membrane pellets were solubilized in 1% fos-choline 12 as described in Methods. Solubilized membranes were loaded onto a 1 mL HiTrap Nickel column. The column was washed with 20 column volumes of buffer containing 20 mM Tris pH 8.0, 300 mM NaCl, 50 mM imidazole, 0.2 mM TCEP and 0.1% fos-choline 12. Protein was eluted in 10 column volumes of elution buffer containing 20 mM Tris pH 8.0, 300 mM NaCl, 250 mM imidazole, 0.2 mM TCEP and the greater of 2 × C.M.C. or 0.05% (w/v) of the detergent of interest. Nickel elutions in the detergent of interest remained at 4°C overnight and the next day were injected onto a Superose6 column. The Superose6 column was pre-equilibrated in 20 mM Tris pH 8.0, 300 mM NaCl, 0.2 mM TCEP and the detergent of interest at the concentration described as follows. Detergents screened included octyl maltoside (1.78%), dodecyl maltoside (0.05%), decyl maltoside (0.345%), Cymal 4, Cymal 5 (0.24%), Cymal 6 (0.06%), Cymal 7 (0.05%), octyl glucose neopentyl glycol (0.12%), decyl maltose neopentyl glycol (0.05%), and lauryl dimethylamine N-oxide (0.05%). Concentrations of detergent during exchange and size exclusion chromatography are included in parentheses.

Ptc characterization—The behavior of Ptc proteins was analyzed by CD, CPM fluorescence thermal stability assays, and by analytical SEC using a Superose6 column. For CD analysis, spectra were collected at 4°C between 185 nm and 280 nm, using Ptc proteins in a cuvette with 1 mm pathlength. Thermal unfolding analysis was also performed by following the CD signal at 208 nm while increasing the temperature from 4°C to 95°C. CPM fluorescence thermal stability assays were performed as in [33]. In brief, a 1:40 dilution of a 4 mg/ml solution of CPM dye (Invitrogen) in DMSO was incubated in CPM Thermal Melt Buffer for 5 min at room temperature, protected from light. Ptc protein (10 μg) was diluted in CPM Thermal Melt Buffer to a final volume of 290 μL. After 5 min at room temperature,
10 μL of dilute dye was added and the sample was mixed and transferred to a quartz fluorometer cuvette. The cuvette was transferred to a Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon) equipped with a Peltier sample cooler (F-3004) and heated at a rate of 2°C/min. Emission at 463 nm was monitored with an excitation wavelength of 387 nm from 20°C to 80°C. For SEC analysis, Ptc was injected onto a Superose6 column equilibrated in Pulldown Buffer. Its elution profile was followed by UV absorption at 280 nm, and apparent molecular weights were estimated by comparison to Gel Filtration Standards (Bio-Rad).

**Immunostaining and confocal microscopy of Patched-expressing Sf9 cells**—Sf9 cells were plated on gelatinized glass coverslips in 6-well plates and simultaneously infected with the indicated viruses (Fig. 2.8). Expression was allowed to proceed for 24-36 hours. All steps were performed at room temperature unless otherwise noted. Cells were fixed by adding to the growth medium an equal volume of PFA (4% paraformaldehyde in PBS) for 5 min, followed by aspiration and addition of undiluted PFA for another 5 min. Cells were then permeabilized with 0.5% Tween-20 in PBS for 15 min, and blocked with PBST (PBS with 0.1% Tween-20) containing 1% BSA for 30 min. Staining was performed with α-Myc (1:200 in blocking solution) at 4°C overnight. Staining solution was removed and cells washed 3 times with PBST, incubating for 5 minutes each wash. Finally, cells were incubated in the dark for 1 hour with fluorescent secondary antibody solution (Cy3 Goat α-Mouse, 1:1000 in blocking solution), washed 3 times with PBST, and coverslips mounted on glass slides in aqueous mounting medium for confocal microscopy.

**Pulldown Assays with Ptc-coupled beads**—Bead-based pulldown assays were used to screen for interactions between purified, detergent-solubilized Ptc and other proteins. All steps were carried out on ice or at 4°C unless otherwise noted. Immediately after performing the nickel purification, Ptc resin was prepared by coupling the SBP-tagged protein to Strep-Tactin resin (IBA). An excess of Ptc protein solution (typically the elution peak from an entire 1 L prep) was added to enough Strep-Tactin resin to give 10 μL of Ptc resin for each desired pulldown condition; typically, this resulted in 2–5 μg of coupled Ptc protein per condition. Ptc was allowed to couple to completion for 2 hr. Resin was then pelleted
and resuspended in Pulldown Buffer, divided into separate tubes, and pelleted, such that 10 μL of packed Ptc resin were obtained in each tube. Pulldowns were set up in a total volume of 50 μL using the reaction compositions specified in Fig. 2.2 and Fig. 2.3. Generally, pulldown conditions contained 10 μM of each soluble protein, and around 0.75 μM of Ptc. These binding reactions were incubated for 1 hr. Ptc beads were then washed 4 times with 1 mL of Pulldown Buffer, and Ptc was eluted by adding 20 μL of Pulldown Buffer supplemented with 2.5 mM biotin. The presence of proteins that bound and eluted with Ptc was then analyzed using Coomassie-stained SDS-PAGE gels and Western blots.

**Pulldown assays with Hh-, ShhN- and ShhN-SC-coupled beads**—Uncleaved MHTSHP-Hh proteins were coupled to beads through their MBP tags, and incubated with the specified combinations of soluble Ptc and other proteins (Fig. 2.9 and 2.10). Ptc used in these pulldown assays was subjected to an additional Strep-Tactin purification step, mainly to increase its concentration, although purity was also slightly improved. To obtain HhN resin, amylose resin (NEB) was incubated with an excess of purified MHTSHP-HhN, -ShhN or -ShhN-SC. Control resin was either unconjugated amylose resin, or amylose resin conjugated to MHTSHP tags only. Resin was then pelleted, resuspended in pulldown buffer, and divided into separate tubes to give 10 μL of packed resin upon pelleting. Binding reactions were then set up in a total volume of 50 μL using the compositions specified in Fig. 2.9 and 2.10. The resulting concentration of coupled HhN fusion protein was approximately 15 μM. The volume of Patched added to the reaction was calculated to give a final concentration of 1.5 μM, typically around 20 μL of Patched per condition. Binding reactions were incubated and washed as above, before elution by addition of 20 μL of Pulldown Buffer containing 1 M maltose. The presence of bound protein was analyzed by Coomassie-stained SDS-PAGE and Western blot.

**Co-purification of Ptc with other proteins**—Ptc proteins were co-expressed with potential partners by infecting Sf9 cells with the following viruses or combinations of viruses: MmPtcT1 virus with ShhFL/Hhat dual expression virus; DmPtcT1/IhogFn12TM dual expression virus; DmPtcT1 virus with HhFL/Ski dual expression virus; and DmPtcT1/IhogFn12TM dual expression virus with
HhFL/Ski dual expression virus. In each case, the Ptc protein was fused with N-terminal SbpPHMT tags, and its potential binding partners were untagged. Sequential nickel, Strep-Tactin, and Superose6 chromatography steps were performed as outlined above, and the presence of co-purifying complexes evaluated by Coomassie-stained SDS-PAGE and Western blot.

Results

Ptc Purification and Characterization—Both Drosophila and Mouse Ptc expressed at levels of 0.5–3 mg of protein per liter of Sf9 cell culture. Detergent solubilization trials indicated, however, that Ptc proteins were only solubilized well by the fos-choline class of detergents (Fig. 2.6), and FC-12 was selected for routine extraction. Solubilized Ptc proteins were purified by a combination of Nickel and Strep-Tactin affinity chromatography (Fig. 2.1). Yields of purified Ptc ranged from 20–50% of total expressed protein as judged by Coomassie-stained gels and comparison to BSA standards. Losses resulted from incomplete solubilization, incomplete capture, or incomplete elution from Nickel and were dependent on flow rates and the amount of protein present initially. Although most detergents do not solubilize Ptc proteins efficiently, we found that once Ptc had been solubilized, FC-12 could be exchanged for a variety of other detergents during the initial HisTrap purification. We therefore evaluated the behavior of Ptc proteins by SEC after exchange into different detergents (Fig. 2.7). SEC behavior was qualitatively similar for most detergents tested, with a species eluting as a small oligomer, potentially consistent with a physiological trimer [34] (Fig. 2.1, arrow 1); a higher-order oligomer (arrow 2); and a small proportion in the void volume. Although the SEC behavior of Ptc after purification is similar in many detergents, Ptc in LMNG was less prone to precipitation over several days of storage, and all binding studies were therefore carried out with Ptc exchanged into LMNG.

Characterization of purified Ptc proteins by CD spectroscopy revealed spectra typical of proteins with high alpha-helical content. The secondary structure content of DmPtcT1 calculated from its CD spectrum was 47% helix and 13% sheet, similar to the values of 44% and 6% predicted from the primary sequence by PSIPRED [35]. CD at 208 nm was used to follow unfolding of DmPtcT1, giving an unfolding midpoint of 58°C. We also followed Ptc unfolding using CPM dye, a measure that
is more closely related to tertiary rather than secondary structure. By this method, apparent unfolding midpoints were approximately 42°C for MmPtCT1 and DmPtCT1 (Fig. 2.1). Confocal imaging of infected Sf9 cells expressing Ptc proteins indicated that Ptc was present at the cell surface (Fig. 2.8). Deglycosylation of purified MmPtCT1 with PNGaseF also resulted in a small but visible band shift on Coomassie-stained SDS-PAGE (Fig. 2.8), consistent with the molecular weight of several glycans; MmPtCT1 contains 6 consensus N-linked sites, with total expected glycan mass of 6-7 kDa, assuming pauci-mannose glycosylation profiles typical of Sf9 cells [36]. These results are consistent with proper processing and membrane localization of expressed Ptc proteins.

*Pulldown of potential binding partners by MmPtCT1*—MmPtCT1-coupled resin was tested for its ability to pull down soluble ShhN, ShhN-SC, HMT-BOCFn3, HMT-BOCFn23 and HMT-BOCFn13 individually as well as in ternary combinations (Fig. 2.2). 1 mM Ca\(^{2+}\) was included in most conditions as Ca\(^{2+}\) is known to be required for ShhN binding to BOCFn3. 200 μM LMWH was included in one condition with BOCFn13 and ShhN, since both are known to be heparin-binding proteins. We also included conditions with 5 mM EGTA, a Ca\(^{2+}\) chelator, as a control to eliminate ShhN:BOCFn13 binding. Finally, we included Hhip in certain conditions to test its ability to block potential binding of ShhN to MmPtCT1. Strep-Tactin resin without conjugated MmPtCT1 (Fig. 2.2) or with the tagged, irrelevant membrane protein AcrB conjugated (Fig. 2.12) was used as a control for nonspecific binding. We used Coomassie-stained SDS-PAGE to detect bound proteins, with Western blots also performed in some cases to detect weakly-bound proteins and confirm the identities of Coomassie-stained protein bands.

ShhN did not bind to detectably to MmPtCT1 beads as judged by Coomassie-stained SDS-PAGE gels of the eluted samples. Trace levels of ShhN binding to MmPtCT1 beads were detectable only by Western blotting; this binding was eliminated by EGTA. ShhN and BOCFn13 visibly precipitate in the presence of LMWH and Ca\(^{2+}\), resulting in high nonspecific binding to beads; we could therefore not evaluate specific binding of this complex to MmPtCT1. Since BOCFn13 and ShhN bind each other with micromolar affinity and each independently binds heparin, this precipitation was
probably due to heparin-mediated protein concatenation. Interactions between ShhN and MmPtcT1 beads were not enhanced by the presence of any BOC fragment, whether in the presence or absence of LMWH or Ca\(^{2+}\). Binding of ShhN to MmPtcT1 beads was also unaffected by the presence of Hhip.

By Western blot analysis, ShhN did not adhere to MmPtcT1 resin to a greater degree than the negative control surface mutant ShhN-SC. However, ShhN-SC showed no dependence on EGTA and no precipitation in the presence of LMWH and HMT-BOCFn13, consistent with its inability to bind BOC (Fig. 2.9).

HMT-BOCFn13 and HMT-BOCFn23 were not specifically pulled down by MmPtcT1-coupled beads. Although MmPtcT1-coupled beads did pull down BOC proteins as compared to blank beads (Fig. 2.2), we were surprised to find that this binding was dependent on the presence of 1 mM Ca\(^{2+}\) and was eliminated by EGTA, despite the lack of known calcium binding to BOC proteins. We therefore performed additional control experiments comparing the binding and solution behaviors of tagged and untagged BOC proteins in the presence of calcium (Fig. 2.11). These experiments showed that (i) His-tagged proteins oligomerize in the presence of 1 mM Ca\(^{2+}\) and that (ii) the binding of HMT-BOCFn13 to MmPtcT1 was dependent on the presence of a His tag. An irrelevant His-tagged protein was pulled down equally well by MmPtcT1 beads, and BOC proteins could also be pulled down equally by beads coupled with the irrelevant His-tagged membrane protein AcrB (Fig. 2.12). The presence of ShhN or ShhN-SC did not enhance the binding of BOC proteins to MmPtcT1 beads, nor did the addition of LMWH (Fig. 2.2).

We also performed the pulldown experiments in reverse (Fig. 2.9) by coupling ShhN and ShhN-SC to beads and attempting to pull down detergent-solubilized MmPtcT1 alone or in the presence of HMT-BOCFn3 and HMT-BOCFn13. Hhip was added to test for its ability to block potential Ptc binding, and soluble ShhN and ShhN-SC were added as specific competitors for Ptc binding to ShhN- and ShhN-SC-coupled resins. Consistent with our previous results (Fig. 2.2), we observed no binding above background of MmPtcT1 to ShhN resin. Aside from MmPtcT1, the resins
pulled down the expected binding partners: ShhN resin pulled down BOCFn3 and HMT-BOCFn13 in a Ca\textsuperscript{2+} dependent manner, and pulldown of HMT-BOCFn3 by ShhN was blocked by Hhip.

*Pulldown of potential binding partners by DmPtcT1*—We next used similar pulldown assays to screen for DmPtcT1 binding to potential partners. DmPtcT1-coupled beads were used to pull down HhN, IhogFn12, IhogFn12ΔH, IhogFn1 and IhogFn2. These proteins and their combinations were not found to interact strongly or specifically with DmPtcT1-coupled beads. Although IhogFn1, IhogFn12 and IhogFn12ΔH were pulled down by DmPtcT1 beads in the absence of LMWH and could be detected in Coomassie-stained gels (Fig. 2.3), this binding was only slightly greater than binding to the irrelevant membrane protein AcrB (Fig. 2.13). In the presence of 200 μM LMWH, mixing IhogFn1 or IhogFn12 with HhN induced visible precipitation, resulting in high non-specific binding to Strep-Tactin resin. We therefore could not evaluate specific binding to DmPtcT1 resin in these conditions. However, substituting 200 μM decaheparin (DH) for LMWH prevented the precipitation of IhogFn1 and HhN and eliminated binding of IhogFn1 to DmPtcT1. HhN binding to DmPtcT1 beads was not detectable on Coomassie-stained gels except in the cases where precipitation was present. By Western blotting HhN did not bind DmPtcT1 beads specifically or to a greater degree than uncoupled Strep-Tactin beads. The presence of IhogFn1 and DH with HhN did not enhance the binding of HhN to DmPtcT1 beads.

Finally, we coupled HhN to amylose resin, and attempted to pull down soluble DmPtcT1 (Fig. 2.10). We also tested for binding to HhN of combinations of DmPtcT1 with IhogFn1, IhogFn2, IhogFn12, and IhogFn12ΔH, and with soluble non-conjugated HhN (as a specific competitor for DmPtcT1 binding to HhN beads). No binding of DmPtcT1 was detectable in any condition, even by Western blotting. Aside from DmPtcT1, the HhN resin pulled down its known binding partners as expected: IhogFn1 and IhogFn12 in a heparin-dependent manner, and IhogFn12ΔH much more weakly than IhogFn12. As expected, the HhN resin did not pull down IhogFn2.

*Co-expression and purification of Ptc proteins with potential binding partners*—Since *Drosophila* Ptc is believed to exist as a co-receptor complex with Ihog [23], we investigated whether the co-expression
and purification of IhogFn12TM with Ptc resulted in improved binding or a better monodispersity of Ptc as judged by SEC. In addition, since the Hh proteins used in our pulldown assays were expressed in *E. coli*, they lacked lipid modifications. We therefore also investigated the co-expression and purification of Ptc proteins with dually lipidated Hh proteins, referred to as HhNP and ShhNP. These were expressed using dual-expression HhFL/Ski and ShhFL/Hhat baculoviruses, which simultaneously drive expression of the full-length Hh proteins together with their respective palmitoyltransferases.

Co-expression of proteins did not result in co-purified stoichiometric complexes with tight apparent binding. When Sf9 cells were infected with a dual DmPtcT1/IhogFn12TM virus, both proteins were well-expressed. DmPtcT1 was purified using the standard Ni²⁺/Strep procedure (Fig. 2.4A), and the resulting purified protein was separated by analytical SEC (Fig. 2.4B). As judged by Western, some IhogFn12TM bound and eluted along with DmPtcT1 during each purification step, with significant amounts also dissociating during each wash step. Comparison of the Ihog Western band intensities to standards (data not shown) indicated that the amount of IhogFn12TM remaining after DmPtcT1 purification was sub-stoichiometric with respect to DmPtcT1. The SEC behavior of DmPtcT1 co-expressed with IhogFn12TM was similar to that of DmPtcT1 expressed alone. Coomassie-stained SDS-PAGE and Western blotting of the SEC fractions indicated that DmPtcT1 and IhogFn12TM elution peaks overlapped, but with IhogFn12TM eluting at slightly higher apparent molecular weight.

When DmPtcT1 and HhNP were co-expressed, HhNP was observed to co-purify with DmPtcT1 (Fig. 2.4A), with some losses during each affinity step. SEC followed by SDS-PAGE and Western blotting showed that the elution profile of HhNP was very broad, and its peak did not track the elution of DmPtcT1. Co-expression of all three proteins (DmPtcT1/IhogFn12TM with HhNP) did not appreciably alter the co-purification or SEC behavior of any protein.

Similarly, MmPtcT1 was co-expressed with ShhNP and purified (Fig. 2.5A). Significant amounts of ShhNP remained in the flowthrough during each affinity step, and although some residual
protein co-purified with MmPtcT1 and was detectable by Western, it was not visible on Coomassie-stained gels. By Western, the concentration of ShhNP clearly decreased during each affinity step, while the concentration of MmPtcT1 increased. The behavior of MmPtcT1 on SEC was not significantly altered by co-expression with ShhNP, and the elution peak of ShhNP did not match the peak of MmPtcT1 (Fig. 2.5B).

Discussion

In this study, Ptc proteins from mouse and Drosophila have been expressed in Sf9 cells and purified. These purified, detergent-solubilized Ptc proteins were characterized by SEC and CD. Ptc proteins contained the expected secondary structure content by CD. However, detergent-extracted and purified Ptc proteins did not elute as a single monodispersed peak on SEC, but as two apparent species; one consistent with a potential physiological trimer or small oligomer, and one of higher apparent molecular weight. Deglycosylation and confocal microscopy studies suggested that Ptc proteins were glycosylated and transported to the cell surface.

Despite our observations that Ptc proteins behaved as expected by CD, cell surface expression, and deglycosylation analysis, and that some purified protein ran at sizes potentially consistent with physiological oligomers by SEC, high-affinity binding to cognate Hh proteins or Ihog/BOC co-receptors could not be detected. Recent studies indicate that Ptc proteins have an absolute requirement for co-receptors in order to signal (BOC/CDO/Gas1 in mammals, Ihog/BOI in Drosophila), and that Ptc forms physical complexes with these co-receptors [17, 23, 24]. In addition, high-affinity binding of Shh to cells is Ptc-dependent [9, 11]. We were therefore surprised not to detect high-affinity binding between purified Ptc proteins and any of these putative binding partners, either pairwise or in ternary combinations.

While some bound ShhN was detected in Western blots after saturating amounts were incubated with MmPtcT1 beads, the amount of ShhN detected was not convincingly above levels of nonspecific binding to blank beads (Fig. 2.2). Based on the clearly observed binding of BOC/Ihog proteins to their cognate Hh proteins (Fig. 2.9 and 2.10), which are interactions with $K_d$'s of $\sim 2.5-5$
μM, binding to Ptc with any $K_d$ below ~5 μM would have resulted in the clear observation of Hh on Coomassie-stained gels. Furthermore, the surface mutant ShhN-SC, which has impaired binding to Ptc-expressing cells [11], showed roughly the same trace levels of binding to MmPtcT1 beads as native ShhN. The addition of Hhip also did not compete with Ptc beads for ShhN binding, further suggesting a lack of specific binding of ShhN to MmPtcT1 beads. Thus, we were not able to reproduce the observation of binding between ShhN and purified mouse Ptc [37]. In addition, HMT-BOCFn13 and HMT-BOCFn23 did not bind specifically to MmPtcT1. Binding in initial studies in the presence of Ca$^{2+}$ proved to be mediated by His tags (all BOC and Ptc proteins, but not Ihog proteins, were His-tag fusions) in the presence of Ca$^{2+}$ ions (Fig. 2.11–2.13). The presence of Shh together with BOC fragments did not alter the apparent binding of any of these proteins to MmPtcT1, thus failing to demonstrate the presence of a ternary complex between BOC/Shh/Ptc.

Like ShhN, Drosophila HhN did not bind DmPtcT1 beads at levels appreciably above background, as judged by Western blotting. No HhN was detectable on Coomassie-stained gels. IhogFn1, IhogFn12, and IhogFn12ΔH were bound by DmPtcT1 beads in the absence of both heparin and Hh, as detected by Coomassie-stained gels; however, for IhogFn12, roughly comparable binding was also seen to beads coupled with the irrelevant membrane protein AcrB. The addition of HhN and heparin decasachcharide eliminated binding of IhogFn1 to DmPtcT1 beads. In contrast with previous observations [23], no binding of IhogFn2 to DmPtcT1 beads was observed. In all cases where some interaction was observed, stoichiometry of the bound protein to Ptc was low, as judged by comparing Coomassie band intensities. Given the low stoichiometry of all observed complexes, and the nonspecific binding seen between IhogFn12 and AcrB, we conclude that specific binding of Ihog fragments to Ptc was not observed.

As a final effort at purifying high-affinity, stoichiometric complexes of Ptc proteins with putative ligands, we attempted several co-expression experiments: MmPtcT1 with ShhN$_P$, DmPtcT1 with HhN$_P$, DmPtcT1 with IhogFn12TM, and DmPtcT1 with HhN$_P$ and IhogFn12TM. In all cases, as Ptc proteins were purified using His and SBP tags, their expression partners increasingly dissociated
with each chromatographic step. A final Superose6 chromatography step showed some proportion of binding partner co-migrating with Ptc in each case, but with most having been lost in preceding purification steps. Qualitatively, this suggests at most a weak interaction between purified Ptc and these binding partners.

The absence of high-affinity interactions between purified Ptc and several potential binding partners observed here could be explained in several ways. Firstly, Ptc may not adopt a native or binding-competent conformation in this expression system, or it may lose these properties during detergent solubilization. Some studies have suggested that fos-choline detergents can solubilize misfolded membrane proteins, with this being a particular concern for proteins which can only be efficiently extracted by fos-choline detergents [38–40]. On the other hand, proteins have been crystallized directly from fos-choline detergents, including alpha helical membrane proteins [41]. The transmembrane protein SCAP was also well-extracted only by a fos-choline detergent, and was shown to retain function in detergent-extracted form [42]; this protein is similar to Ptc in its possession of a sterol-sensing domain, and in its overall topology and number of transmembrane helices (32 for a SCAP tetramer vs. 36 for a potential Ptc trimer), and was also expressed in Sf9 cells with N-terminal tags. Finally, it is possible that the lack of strong binding between Ptc and other pathway proteins under our assay conditions is indeed physiological. For instance, additional cofactors or binding mediators not present in our assays may be required for binding.

Acknowledgements

We thank Xiaoyan Zheng and Phil Beachy for kindly providing rabbit polyclonal antiserum against Ihog and hybridoma supernatants containing mouse monoclonal antibodies against Drosophila Hh. We also thank Hongjin Zheng and Tamir Gonen for advice and assistance with detergent screening by SEC, and for the use of reagents and facilities at the Gonen lab at HHMI Janelia Farms (Ashburn, VA).
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| HMT              | pTTHMT | pET28a        | MGSSHIIIIHHSSGLVPRGSQHMGMEQKLISEEDLNGENL
| SbpPHMT          | pSbpPHMT| pFastBac1     | MDEKTTGWREGHVEGLAGELEQLRLEHPQGQREP
| HbmHSbpMT        | pHbmHSbpMT| pFastBac1    | MKFLVNVALFVYISYYAAPHHHHHHHMDKTTTGWRGGHVVEGLAGELEQLRLEHHPQGQREPMEQKLISEEDLNENLYFQ

(Protease cut site used in purification shown with “/” between amino acids)

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(Native sequences shown in **bold**, tag and vector-derived shown as plain text)

**Table 2.1 – Protein Constructs**
Figure 2.1 – Purification and characterization of Ptc proteins. (A) DmPtcT1 and MmPtcT1 were purified by successive Ni\(^{2+}\) and Strep-Tactin (ST) chromatography steps. Gel Lanes: (M) resuspended membranes; (S) detergent-solubilized membranes; (F) flow-through; (E) elution. (B) SEC of purified, concentrated MmPtcT1 (140 kDa calculated) compared to tagged AcrB (123 kDa), a protein standard with the same predicted topology. MmPtcT1 elutes in two peaks: a small oligomer consistent with a potential physiological trimer (arrow 1) and a larger oligomer (arrow 2). (C) CD spectra of DmPtcT1 and MmPtcT1 show substantial alpha-helical character. (D) Thermal unfolding of Ptc proteins as measured by CD at 208 nm (correlated with alpha helical content) and by CPM dye binding to exposed cysteines.
Figure 2.2 – Pulldown of potential binding partners by MmPtCT1. Proteins and other cofactors were incubated with MmPtCT1-coupled Strep-Tactin resin and blank (uncoupled) Strep-Tactin resin in the indicated combinations. Bound proteins were eluted with biotin and analyzed by Coomassie-stained SDS-PAGE.
**Figure 2.3 – Pulldown of potential binding partners by DmPtcT1.** Proteins and other cofactors were incubated with DmPtcT1-coupled Strep-Tactin resin and blank (uncoupled) Strep-Tactin resin in the indicated combinations. Bound proteins were eluted with biotin and analyzed by Coomassie-stained SDS-PAGE.
Figure 2.4 – Co-expression and purification of DmPtcT1 with potential binding partners. Sf9 cells were co-infected with baculoviruses driving expression of DmPtcT1 along with IhogFn12TM, HhFL/Ski, or both IhogFn12TM and HhFL/Ski together. Expected positions of protein bands are marked, although these bands are not detectable in some cases. (A) DmPtcT1 was then purified, using Coomassie-stained SDS-PAGE for detection, while the presence of Hh and Ihog were followed by Western. The same Western blot membrane was sequentially probed with Ihog and Hh antibodies and imaged, and the images were then overlaid and color-coded accordingly (see key). Three major Hh bands are observed: one corresponding to the molecular weight of the unprocessed HhFL protein, one corresponding to the molecular weight of C-terminally processed Hh retaining its signal sequence, and one corresponding to the expected molecular weight of HhNp. (B) The resulting co-purified proteins were subjected to Superose6 SEC. The fractions were analyzed by Coomassie-stained SDS-PAGE for DmPtcT1 detection, and by Western blot, probing sequentially for Hh and Ihog as in (A).
Figure 2.5 – Co-expression and purification of MmPtcT1 with Shh. Sf9 cells were co-infected with baculoviruses driving expression of MmPtcT1 along with ShhFL/Hhat. (A) MmPtcT1 was then purified, using Coomassie-stained SDS-PAGE for detection, while the presence of Shh was followed by Western. Two major Shh bands are observed: one corresponding to the molecular weight of the unprocessed ShhFL protein, and one corresponding to the expected molecular weight of ShhNp. (B) The resulting co-purified proteins were subjected to Superose6 SEC. The fractions were analyzed by Coomassie-stained SDS-PAGE for MmPtcT1 detection, and by Western blot for Shh.
Figure 2.6 – Detergent Solubilization of DmPtcT1. Membranes were incubated with 1% of the indicated detergents, cleared by centrifugation (S), and incubated with a small volume of Ni2+ resin (FT) followed by elution with imidazole (E). Trial solubilization and small-scale purification were followed by Western blot against the Myc tag. OM: n-octyl β-D-maltopyranoside; DDM: n-dodecyl β-D-maltopyranoside; OG: n-octyl β-D-glucopyranoside; Cymal-6: 6-Cyclohexyl-1-hexyl-β-D-maltoside; FC16: n-hexadecylphosphocholine; TDAO: n-tetradecyl-N,N-dimethylamine-N-oxide; TX-100: Triton X-100.
Figure 2.7 – Gel filtration screening of Ptc proteins after detergent exchange. MmPtcT1 was solubilized in fos-choline 12 and exchanged during purification into the indicated detergents before analyzing by superose6 gel filtration. Similar behavior after initial purification was observed in most detergents, and for DmPtcT1 (not shown), with a Ptc oligomer peak slightly larger than the 670 kDa marker, and in some cases another peak between the 158 and 670 kDa markers.
Figure 2.8 – Ptc Proteins are expressed in Sf9 cells and reach the cell surface. (A) Sf9 cells were infected with the indicated viruses, immunostained using anti-Myc (9E10) primary and Cy3 secondary antibodies (red), and imaged by confocal microscopy. Nuclei were stained with DAPI (blue). (B) Purified MmPtcT1 was incubated with PNGaseF, and gel shift detected by Coomassie-stained SDS-PAGE.
Figure 2.9 – Pulldown of MmPtcT1 by Shh. Tagged MHTSHP-ShhN was coupled to amylose beads through its MBP fusion tag, and used to pull down MmPtcT1 in combination with other potential binding partners. The surface mutant MHTSHP-ShhN-SC (deficient in binding to Ptc-expressing cells [11]), as well as cleaved MHTSHP tags alone, were coupled similarly as negative controls. Proteins were incubated with ShhN beads or controls, washed, and eluted with maltose. ShhN, but not ShhN-SC or tag-only beads, was able to bind BOC fragments and Hhip (marked). ShhN did not bind MmPtcT1 to a greater degree than negative control resins.
Figure 2.10 – Pulldown of DmPtcT1 by HhN. Tagged MHTSHP-HhN was coupled to amylase beads through its MBP fusion tag, and used to pull down DmPtcT1 in combination with other potential binding partners. Uncoupled amylase beads were used as a negative control. Proteins were incubated with HhN beads or controls, washed, and eluted with maltose. HhN was able to specifically bind the expected IhogFn1-containing fragments in a heparin-dependent manner. No specifically bound DmPtcT1 was detectable by Coomassie-stained SDS-PAGE (not shown) or by Western blot (top row).
Figure 2.11 – Dimerization of His-Tags in the Presence of Calcium. (A) The Ca\(^{2+}\)-dependent dimerization of His tags was evaluated using HMT-BOCFn3 as a model protein. Tagged and untagged BOCF\(n\)3 was analyzed by Superdex75 gel filtration in the presence and absence of 1 mM Ca\(^{2+}\). Dimerization was detected only in the condition where the His-tagged protein was in the presence of Ca\(^{2+}\). (B) HMT-BOCFn13 and HMT-BOCFn3, but not the untagged BOCFn13 and BOCF\(n\)3, bind to MmPtcT1, which also contains a His-tag, in a Ca\(^{2+}\)-dependent manner. The likely explanation is His-tag/Ca\(^{2+}\) mediated interaction.
Figure 2.12 – His-tagged membrane proteins pull down his-tagged proteins. Both MmPtcT1 and the irrelevant membrane protein AcrB, which contained the same fusion tags (including a His-tag), were able to pull down HMT-BOCFn13 and the irrelevant his-tagged soluble protein HMT-IL6. The interaction was enhanced by Ca\(^{2+}\).
Figure 2.13 – Pulldown of IhogFn12 by DmPtcT1 beads vs. AcrB and blank control beads.

Blank Strep-Tactin beads, and beads coupled to AcrB and DmPtcT1, were used to pull down IhogFn12 and a mutant (ΔH) with disrupted heparin-binding properties, in the presence and absence of low molecular weight heparin (LMWH).
Chapter 3 – The Crystal Structure of the Third and Fourth Fibronectin Domains of Human Neogenin

Introduction

Like the CDO/Ihog family of proteins, Neogenin (Ngn) is a type I transmembrane protein with an extracellular domain consisting of immunoglobulin-like and Fibronectin type III (FnIII) domains (Fig. 3.1). Ngn has been shown to regulate a wide array of developmental processes, such as axon guidance, myogenesis and digit patterning [43], and binds several ligands, including members of the Netrin and Repulsive Guidance Module (RGM) families [44]. CDO also has reported roles in regulating these processes, although little is known regarding its relationship, if any, with Ngn. There is some evidence for binding between the third FnIII domain of CDO (CDOFn3), and the fourth FnIII domain of Ngn (NgnFn4) [45]. In addition, Ngn interacts genetically with the Shh pathway during digit patterning [43], and is a transcriptional target of Shh/Gli [46]. These lines of evidence suggest the potential for Ngn involvement with Hh signaling and CDO/BOC, potentially through a physical interaction with CDO.

This project was initiated following experiments communicated to us by Robert Krauss, as well as those reported in [45], demonstrating a potential physical interaction between CDOFn3 and NgnFn4. The interaction was identified by co-expressing the extracellular domains of CDO and Ngn in mammalian cells for secretion into tissue culture medium, followed by immunoprecipitation of one binding partner and western blotting for the other. The intact extracellular regions were shown to co-IP, and systematic deletion of individual domains from each protein identified CDOFn3 and NgnFn4 as those required for binding. This mapping of interaction domains by the Krauss lab, coupled with some evidence of a genetic interaction between Ngn and Hh signaling, prompted us to perform in vitro binding assays on purified Ngn and CDO fragments.

Results and Discussion

Interaction of NgnFn4 with CDOFn3—We expressed and purified Ngn fragments spanning the third and fourth FnIII domains (NgnFn34), and the third through fifth FnIII domains (NgnFn35).
Both fragments eluted at the expected apparent molecular weight of a monomer by SEC (Fig. 3.2). To screen for interactions with CDO, purified Ngn fragments were incubated in pulldown assays with CDOFn3-coupled CNBr resin (Fig. 3.3). Potential cofactors were included in some conditions, including low molecular weight heparin (LMWH), CaCl₂, and fetal bovine serum (FBS). ShhN was incubated with CDOFn3-CNBr resin in the presence and absence of Ca²⁺ to verify the proper binding activity of the resin. As expected, ShhN was pulled down by CDOFn3 resin only in the presence of 1 mM CaCl₂ or 10% FBS (which contains sufficient calcium for binding), demonstrating proper binding activity of the CDOFn3 resin. However, CDOFn3 resin failed to pull down NgnFn35 (Fig. 3.3A) and NgnFn34 (B) under all conditions tested. In addition to pulldown assays, we also analyzed complex formation in solution using SEC. Purified NgnFn34 and CDOFn23 were each chromatographed separately using SEC, then incubated together at a concentration of 50 μM and separated by SEC (Fig. 3.4). No peak shifts were observed, indicating a lack of detectable complex formation with a K_d tighter than approximately 25 μM.

As cell surface proteins, it remains possible that in their physiological context, confined to a membrane, CDO and Ngn may physically interact. However, despite the previously reported observation of binding using co-expressed and secreted protein fragments in mammalian tissue culture medium [45], binding could not be observed using purified soluble fragments containing the CDOFn3 and NgnFn4. It should be noted that the binding between CDOFn3 and ShhN is only moderately strong, with a reported K_d of 1.3 μM [19], but this interaction was readily observable as a positive control in pulldown assays. Interactions between CDOFn3 and NgnFn34, by contrast, could not be observed even at concentrations of up to 600 μM NgnFn34. Other possibilities are that an additional binding protein or co-factor not included in our assays could be required for complex formation between CDO and Ngn; or that additional CDO or Ngn domains are required outside of the ones included in our fragments (although this was not observed in [45], it could be that many weak binding contributions are made across several domains which are collectively required for tight binding).
Structure of NgnFn34—After purifying NgnFn4, NgnFn34 and NgnFn35 fragments, screening for crystallization conditions was performed. NgnFn4 and NgnFn35 failed to crystallize, but NgnFn34 crystallized in several conditions. The screen hits could be grouped into two sets: condition 1, around 10% PEG 6000, 2 M NaCl, 0.2 M KNO₃ or NaI, pH 8.0; and condition 2, around 20% PEG 3350, 0.2 M NaCl, 0.2 M KNO₃ or NaI, pH 8.0. Crystals from condition 1 and condition 2 appeared morphologically similar, and of similar sizes, with clusters of plates with dimensions around 250 Å × 125 Å × (thin). However, they proved to be different crystal forms, with condition 1 giving crystals in space group P1 and condition 2 giving crystals in P2₁. After optimizing condition 1 (Fig. 3.5), several crystals were cryoprotected and data were collected using an in-house Cu-Kα x-ray source. Data from two separate crystals were merged, and the structure was determined by molecular replacement using NMR models of the isolated NgnFn3 and NgnFn4 domains. Four molecules were present in the triclinic unit cell. The resolution cutoff was chosen at 2.55 Å using the “paired refinement” method as in [47], and the structure was refined to R/Rfree values of 0.243/0.267 (Fig. 3.6A and Table 3.1). The structure of NgnFn34 in the monoclinic crystal form was solved by molecular replacement using the individual FnIII domains from the triclinic structure; four molecules were present in the monoclinic asymmetric unit. The resolution of the monoclinic data was chosen at 2.65 Å, and the structure was refined to R/Rfree values of 0.212/0.253 (Fig. 3.6B).

The 8 independent NgnFn34 molecules (4 in each crystal form) were structurally similar to each other, with no significant differences between the corresponding individual FnIII domains. The Fn3 and Fn4 domains were arranged in a “bent” conformation relative to each other. Independent NgnFn34 molecules differed in the relative angle between Fn3 and Fn4 domains, indicating flexibility of the hinge between the Fn3 and Fn4 (Fig. 3.7).

Although NgnFn34 was monomeric in solution as determined by SEC, we examined the two independent crystal forms for any shared binding interfaces, since even weak binding can be physiologically relevant for proteins confined to a membrane. Contact interfaces in each crystal form were enumerated using PISA. To group equivalent surfaces, interfaces were compared within and
between crystal forms by tabulating the number of shared equivalent residues between pairs of interfaces. This analysis revealed that all interfaces involving more than approximately five residue pairs could be classified as one of about seven unique interface types. None of these interfaces were found to be shared between crystal forms, and none had a combination of buried surface area and surface complementarity that strongly suggested physiological relevance (Table 3.2). Interface A buried a relatively large surface area, but did not have high surface complementarity. Interface C was interesting as a potential heparin binding site; it brings together two highly basic surfaces, possibly allowed by the high salt concentration present in condition 1.

Materials and Methods

Protein expression and purification—NgnFn34 and NgnFn35 fragments were amplified by PCR and subcloned into the vector pMHTSHP. Expression plasmids were transformed into E. coli strain BL21 and grown at 37°C in Terrific Broth (TB) using baffled shake flasks to an optical density at 600 nm of approximately 0.6. The temperature was then changed to 16°C, and cultures were shaken for an additional hour before induction with 0.5 mM IPTG. Induction was allowed to proceed overnight, and cells were harvested and lysed using a French press. Lysis buffer was 35 mM NaH₂PO₄, 300 mM NaCl, 15 mM Imidazole, pH 8. Cell lysate was cleared by centrifugation and applied to a 5 mL. Nickel HiTrap (GE), followed by a wash of 5 column volumes with 20 mM Tris pH 8.0, 300 mM NaCl, 40 mM Imidazole, and elution with the same buffer containing 250 mM Imidazole. Elution fractions were pooled, and HRV-3C protease was added along with 5 mM EDTA and 20 mM β-mercaptoethanol, allowing tag cleavage to proceed overnight. Protein was then buffer exchanged into 20 mM Tris pH 8, 50 mM NaCl and applied to a 6 mL Resource Q column (GE), which retains the cleaved tags and allows the collection of flowthrough containing cleaved NgnFn34 or Fn35. NaCl was then added to 300 mM and imidazole to 15 mM, and protein was again passed over a 5 mL. Nickel HiTrap column to remove residual tagged protein, retaining the flowthrough containing purified NgnFn34 or Fn35. A final purification step was performed using preparative Superdex75 SEC in 20 mM Tris pH 8, 200 mM
NaCl before concentrating and flash-freezing protein stocks. ShhN, CDOFn3 and CDOFn23 were expressed and purified as described in Chapter 2.

**Pulldown of Ngn fragments by CDOFn3**—CDOFn3 was coupled to CNBr Sepharose 4 Fast Flow (GE) resin at a concentration of approximately 50 μM (concentration of protein in packed resin) following the manufacturer’s protocol. 10 μL of resin were incubated with Ngn or control proteins for 1 hour at 4°C under the specified conditions (Fig. 3.3) in a total volume of 50 μL using a buffer composition of 50 mM Tris pH 8, 200 mM NaCl. Resin was then rapidly washed 4 times with 1 mL of the same buffer, followed by elution of retained proteins from the 10 μL of packed resin by 20 μL of SDS-PAGE loading buffer. Eluted proteins were analyzed by Coomassie-stained SDS-PAGE.

**Analytical gel filtration**—NgnFn34 and CDOFn23 were added to 50 mM Tris pH 8, 200 mM NaCl at a final concentration of 50 μM each, and a final volume of 300 μL. Proteins were incubated for 1 hour at room temperature, followed by separation using analytical Superdex75 (GE) in the same buffer and analysis of the eluted fractions by Coomassie-stained SDS-PAGE. Proteins were also run separately under equivalent conditions, and elution volumes compared with the mixed proteins.

**Crystallization and structure solution**—two crystals from type 1 conditions (grown in 14% PEG 6000, 0.5 M NaCl, 0.4 M KNO3, 50 mM Tris pH 8.5; and 10% PEG 6000, 1 M NaCl, 0.1 M KNO3, 50 mM Tris pH 8.1 respectively) were cryoprotected by transferring them to drops with increased NaCl concentration (to 2 M and 3 M respectively) while retaining other solution parameters. One crystal from type 2 conditions (20% PEG 3350, 0.2 M KNO3, 0.2 M NaCl, 50 mM Tris pH 8.2) was cryoprotected by transferring it to drops containing mother liquor with added glucose (powder added to 30% w/v assuming no volume change after addition). Data were collected using a home Cu-Kα source, and images processed using XDS followed by scaling and merging with XSCALE. Data from the two type 1 conditions were merged and scaled together. Unmerged datasets were also produced for analysis of CC* by Phenix, which indicated an initial maximum resolution limit of 2.35 Å. This was further restricted to 2.55 Å and 2.65 Å for crystals of type 1 and 2 respectively after paired refinement in Phenix as described in [47].
The type 1 crystals were found to be triclinic, while type 2 crystals were in space group P21. Initial structure solution of the triclinic structure was by molecular replacement with Phaser, using isolated NMR structures of NgnFn3 and NgnFn4 with PDB codes 1x5h and 1x5i respectively, with their termini and flexible loops manually trimmed. Phaser successfully placed four Fn3 and four Fn4 domains, and after initial rounds of refinement with simulated annealing in Phenix, density was observable for the linkers between Fn3 and Fn4 domains. Linkers and missing residues were built in Coot, followed by additional rounds of refinement using optimization of geometry and b-factor weights, and using TLS groups identified automatically by Phenix. Rounds of refinement were alternated with manual structure correction and rebuilding in Coot.

Once refinement of the triclinic structure was nearly complete, individual core Fn3 and Fn4 domains were used as search models in Phaser to solve the monoclinic structure. Phaser again placed four Fn3 and four Fn4 domains in the asymmetric unit of the monoclinic cell. This solution was followed by additional rounds of refinement using weights optimization and automatically-identified TLS groups, alternated with manual structure correction and rebuilding in Coot.

Analysis of protein interfaces in each crystal form was performed using Qt-PISA, a program in the CCP4 package. Residues at interfaces were exported, and interfaces analyzed using a custom script, grouping any interfaces that were found to be equivalent. Buried ASA and surface complementarity values were calculated using the CCP4 program “sc”.

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### Crystal Parameters: Average (Last Shell)

- **Wavelength (Å)**: 1.542
- **Resolution range (Å)**: 40.96 - 2.55 (2.641 - 2.55)
- **Space group**: P 1
- **Unit cell**: 53.0, 63.5, 76.1, 83.9°, 84.6°, 75.6°

### Data Quality and Refinement:

- **Total reflections**: 144158 (7413)
- **Unique reflections**: 30424 (2727)
- **Multiplicity**: 4.7 (2.7)
- **Completeness (%)**: 98.01 (87.26)
- **Mean I/σ(I)**: 6.41 (0.64)
- **Wilson B-factor**: 47.05
- **R-merge**: 0.165 (1.17)
- **R-meas**: 0.1839
- **CC1/2**: 0.993 (0.672)
- **CC***: 0.998 (0.897)
- **R-work**: 0.243 (0.403)
- **R-free**: 0.267 (0.378)

### ASU Contents:

- **Non-hydrogen atoms**: 6297
- **macromolecules**: 6222
- **water**: 75
- **Protein residues**: 797

### Geometry:

- **RMS(bonds)**: 0.003
- **RMS(angles)**: 0.79
- **Ramachandran favored (%)**: 94
- **Ramachandran outliers (%)**: 0
- **Clashscore**: 2.26

### Average B-factor

- **macromolecules**: 60.3
- **solvent**: 37.8

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**Table 3.1 – Crystallographic data collection and refinement statistics.**
Figure 3.1 – The domain structure of CDO and Neogenin.

Figure 3.2 – Gel filtration of Ngn fragments. NgnFn34 and NgnFn35 were subjected to preparative SEC as a final purification step, and sequential fractions spanning the expected elution position of a monomer were analyzed by SDS-PAGE. The proteins were monodispersed, with no evidence for strong dimer formation.
Figure 3.3 – Pulldown of Ngn fragments by CDOFn3. CNBr-coupled CDOFn3 was used to pull down soluble NgnFn34 and NgnFn35, with pulldown of ShhN in the presence of 1 mM Ca\(^{2+}\) as a positive control for CDOFn3 resin activity. ShhN and NgnFn35 concentrations were 10 μM and 20 μM respectively. NgnFn34 concentrations were 300 μM (+) and 600 μM (++).
Figure 3.4 – Analysis of complex formation between Ngn and CDO by SEC. NgnFn34 and CDOFn23 were run separately by analytical SEC, then mixed together at a concentration of 50 μM each and analyzed for peak shift on SEC. No peak shift was observed, indicating a lack of detectable binding between the proteins.
**Figure 3.5 – Optimization of precipitant concentration for NgnFn34 crystallization.** NgnFn34 was crystallized by the hanging drop method to give the specified final concentrations in the equilibrated drop. All drops contained 0.25 M KNO$_3$, 50 mM Tris pH 8.1. Drops were then scored by eye according to crystal size and single growth, from red (no crystals) to green (large, single crystals). Crystals for data collection were chosen along the crystallization boundary of this phase diagram.

**Figure 3.6 – The structure of NgnFn34 in two crystal forms.** Individual NgnFn34 molecules are colored differently.
Figure 3.7 – Flexibility between the third and fourth FnIII domains of Ngn. Independent NgnFn34 molecules from the two crystal forms were aligned by their Fn3 domains. Flexibility in the linker between Fn3 and Fn4 domains is apparent.

Table 3.2 – Protein interfaces between NgnFn34 molecules in crystals. Buried solvent-accessible surface area (ASA) and surface complementarity (SC) are shown. No interfaces were duplicated across both crystal forms, and none had a combination of buried ASA and SC parameters that strongly suggested physiological relevance.
Chapter 4 – A Single Ligand is Sufficient to Activate EGFR Dimers

Introduction

Work on this project began following the publication by Mark Lemmon’s lab of the structure of *Drosophila* EGFR bound to its ligand, Spitz. This led us to perform cell-based assays using combinations of mutant human ErbB receptors, detailed below, to test the idea of a one-ligand, two-receptor activated dimer in the human system. Cell-based assays were performed by T. E. Cleveland IV, including mutagenesis, generation of stable cell lines, and ligand stimulation assays. Crystallization and structure determination of the Neuregulin-1β-bound soluble ErbB4 extracellular domain were performed by P. Liu; cell lines for the secretion of ErbB4 extracellular domain were generated by P. Liu, P. Longo and S. Bouyain; and P. Byrne performed stable cell line expression measurements (receptors per cell). The material presented in this chapter has been previously published and is reprinted with modifications from Liu, P., T. E. Cleveland IV, S. Bouyain, P. O. Byrne, P. A. Longo, and D. J. Leahy. 2012. A single ligand is sufficient to activate EGFR dimers. *PNAS* 109:10861–6, with permission from The National Academy of Sciences of the USA.

Human epidermal growth factor receptor (EGFR) and its homologs, known as ErbBs or HERs, are essential receptor tyrosine kinases that mediate cell proliferation and differentiation during animal development and are the targets of multiple cancer therapies [48]. EGFR is the archetype of single-pass membrane-spanning receptors thought to transmit signals by ligand-induced dimerization [49, 50], and structural studies show that ligand binding to human EGFR promotes rearrangement of its four extracellular domains from a tethered to an extended conformation in which a loop, termed the dimerization arm, becomes exposed and mediates formation of symmetric receptor dimers [51] (Fig. 4.1A). At odds with a ligand-induced dimerization model of EGFR signaling, however, are recent studies showing dimers of human EGFR in the absence of ligand [52–55] as well as negative cooperativity when EGF binds to EGFR [56]. Curiously, the single *Drosophila* EGFR homolog adopts an extended conformation in the absence of ligand and forms asymmetric receptor dimers with a single
high-affinity ligand bound [57, 58], suggesting different mechanisms may regulate EGFR activation in *Drosophila* and humans.

We report here evidence for active, singly-ligated homodimers of human EGFR and its homolog ErbB4. We also report the crystal structure of the ErbB4 extracellular region bound to its ligand Neuregulin-1β, which allows resolution of two types of human EGFR/ErbB dimers, one of which resembles the asymmetric *Drosophila* EGFR dimer and appears to reflect a singly-ligated ErbB dimer state. These results compel reappraisal of canonical views of ligand-induced dimerization and show that several previously anomalous properties of human EGFR and its homologs represent vertebrate innovations on a core signaling mechanism present in invertebrates.

**Results and Discussion**

We reasoned that if singly-ligated dimers of human EGFR exist as implied by negative cooperativity [56], an EGFR variant incapable of binding ligand may remain able to participate in signaling dimers. To test this idea we introduced debilitating amino-acid substitutions into the ligand-binding site of one EGFR variant and the kinase active site of another. These variants show negligible ligand-dependent phosphorylation when expressed individually in CHO cells, but co-expression restores phosphorylation in response to ligand as judged by both general and specific antiphosphotyrosine antibodies (Fig. 4.1B and 4.6). The simplest explanation for this observation is that ligand-binding deficient receptors are able to pair with kinase-deficient receptors to form active, singly-ligated EGFR dimers. Similar results were obtained for ErbB4/HER4 (Fig. 4.1C). Amino-acid substitutions in the ErbB4 dimerization arm in the context of either ligand-binding or kinase-activity deficient ErbB4 variants eliminates responsiveness when co-transfected (Fig. 4.7), implicating dimerization arms from both partners in formation of singly-ligated ErbB4 dimers. Participation of unliganded ErbBs in a signaling dimer despite burial of the dimerization arm in the tethered conformation likely reflects favorable energetics of the interreceptor dimer interface relative to the tethered state within a preformed dimer.
An essential feature of EGFR activation is an asymmetric dimer of EGFR kinase domains in which the C-terminal region of a ‘donor’ kinase contacts the N-terminal region of an ‘acceptor’ kinase and stimulates it [59] (Fig. 4.1A), and the question arises whether the extracellular asymmetry of singly-ligated EGFR dimers is coupled to this intracellular asymmetry. The ability of ligand-binding deficient EGFR to be activated by kinase-dead EGFR demonstrates that unliganded EGFRs can function as the acceptor kinase (Fig. 4.1B). To determine if ligand-binding deficient EGFR can function as a donor kinase, debilitating amino-acid substitutions were simultaneously introduced into the ligand-binding and kinase active sites of one EGFR and into the kinase donor site of another EGFR. Neither variant showed ligand-dependent phosphorylation when expressed on its own, but weak, ligand-dependent phosphorylation was observed when co-expressed (Fig. 4.1B). Similar results were obtained for ErbB4 (Fig. 4.1C). This observation suggests that unliganded EGFRs can serve as both a donor and an acceptor kinase and that extracellular asymmetry is not absolutely coupled to intracellular asymmetry, consistent with studies suggesting a loose linkage between ligand binding and kinase activation [60]. A recent report using a luciferase fragment complementation assay showed that normal activation of EGFR/ErbB2 heterodimers required the EGFR kinase to be active, suggesting that the liganded partner (EGFR) could initially only function as an acceptor kinase and that extra- and intra-cellular asymmetry are coupled [61]. In this case the intracellular kinases differ (vs. EGFR or ErbB4 homodimers), which may contribute to additional stabilization of the EGFR kinase in the acceptor role in the absence of phosphorylation. It will be interesting to determine if this is indeed the case or whether other factors underlie this apparent difference. The sites of all tested amino-acid substitutions are listed in Table 4.1. None of these sites impaired cell surface expression as judged by cell-surface biotinylation, and EGFR expression levels were estimated by Western blot (Fig. 4.8 and Table 4.2). Curiously, an original ligand-binding mutation introduced in EGFR, D355R, failed to express on the cell surface unless co-transfected with kinase-deficient EGFR. This observation suggests that EGFR molecules interact early in biogenesis and that this interaction can rescue otherwise nonviable forms of EGFR.
The presence of active, singly-ligated EGFR dimers on the cell surface raises the question of why crystal structures of the human EGFR extracellular region with ligand bound reveal symmetric, doubly-ligated EGFR dimers [62, 63]. To address this issue we sought additional crystal structures of liganded ErbB extracellular regions and report here the 3.0 Å crystal structure of the human ErbB4 extracellular region (sErbB4) complexed with Neuregulin-1β (Nrg) (Table 4.2). Three independent 2:2 sErbB4:Nrg dimers are present in the crystallographic asymmetric unit (Fig. 4.2). Domains I-III of the six sErbB4 subunits superimpose well among themselves (pairwise rmsds of 0.2-0.5 Å for Cαs) and with homologous regions of high-affinity ligand complexes of both human and Drosophila EGFR extracellular regions (sEGFR) (pairwise rmsds of 1.1-1.9 Å for Cαs) (Fig. 4.2 and 4.9, and Table 4.3) [58, 60, 62, 63]. The C-terminal juxtamembrane regions of domain IV are closely apposed when well ordered, suggesting interactions between transmembrane regions in active receptor dimers, but regions of domain IV homologous to those involved in direct dimer contacts in EGFR:EGF complexes [60] are mostly disordered and few specific intersubunit domain IV contacts are resolvable.

Although consisting of nearly symmetric 2:2 ligand:receptor complexes in each case, comparison of sErbB4:Nrg1β and human sEGFR:ligand dimers reveals two distinct dimer interfaces (Fig. 4.3). Dimers of sErbB4:Nrg1β and sEGFR:EGF [60, 63] are similar to one another but differ from TGFα-bound dimers of a truncated form of EGFR (tEGFR) comprising the N-terminal 3 extracellular domains [62] (Fig. 4.3). Superposition of a single receptor subunit of the tEGFR:TGFα dimer with a single subunit of either the sErbB4:Nrg1β or sEGFR:EGF dimers reveals the opposite ErbB subunits to differ by a 29° scissor-like rotation about the dimerization arms. This rotation disrupts dimer contacts made by the N-terminal regions of domain II in the tEGFR:TGFα complex, notably those mediated by two contiguous loops formed by residues 190-208 (187-205 in ErbB4). These loops are flush across the tEGFR:TGFα dimer interface but staggered in the sErbB4:Nrg1β and sEGFR:EGF dimers (Fig. 4.3 and 4.4).
The different ErbB dimers can be explained by truncation of domain IV in the tEGFR:TGFα complex [62]. The orientation of domain IV relative to domain III is conserved in EGFR structures whether liganded, unliganded, Drosophila, or human (Fig. 4.10), and modeling domain IV onto each subunit of the tEGFR:TGFα dimer shows that its presence would result in severe intersubunit clashes (Fig. 4.3). Accommodating domain IV in sErbB4:Nrg1β and sEGFR:EGF complexes while maintaining interreceptor dimerization arm contacts necessitates the scissor-like rotation of receptor subunits relative to their orientation in the tEGFR:TGFα dimer. A flush, tEGFR:TGFα-like arrangement of domain II loops is also observed in the asymmetric dimer of Drosophila sEGFR, in which only one receptor subunit has high affinity ligand bound (Fig. 4.4) [58]. In this case, the absence of a high-affinity ligand in one subunit effectively uncouples the relative orientation of the domain I/II and III/IV pairs and allows domain I and the N-terminal region of domain II of this subunit to shift and form the flush domain II interface without requiring domain IV clashes (Fig. 4.4). These results highlight the importance of the relative position of the distinct dimer contact regions in domains II and IV in forming optimal ErbB dimers (Fig. 4.1A and 4.2) as their relative position, and thus the nature of possible dimer contacts, changes when high-affinity ligand is bound.

Spontaneous formation of the flush dimer contact in human tEGFR dimers indicates that it is almost certainly more stable than the staggered contact observed in dimers of sErbB4 and sEGFR. The flush dimer would thus preferentially form in singly-ligated dimers of EGFR in which the relative positions of domains II and IV are uncoupled in the unliganded subunit. This observation, coupled with the results of our cell-based assays, strongly implies that asymmetric Drosophila sEGFR-like dimers are conserved in human ErbBs (Fig. 4.11). This conservation is satisfying from an evolutionary perspective and provides a structural rationale for negative cooperativity of ligand binding to EGFR [56]. Binding of ligand to the unliganded subunit of singly-ligated ErbB dimers requires conversion from a flush interface to the less stable staggered dimer interface, which necessarily reduces the apparent affinity of the second receptor for ligand relative to the first and results in negative cooperativity and a weaker receptor dimer [56]. The fact that intracellular regions are required for
negative cooperativity likely reflects the importance of these regions for stabilizing receptor dimers in the absence of ligand [64]. A theoretical study recently suggested interactions with the cell membrane may also induce a *Drosophila* EGFR-like dimer in human EGFR [65].

Our cell-based assays imply that singly-ligated EGFR dimers are signaling competent (Fig. 4.1). The fact that the Q194A mutation, which preferentially targets dimer contacts in the tEGFR:TGFα complex vs. the 2:2 sEGFR:EGF complex (Fig. 4.12), does not impair EGFR signaling [66] suggests that doubly-ligated EGFR dimers are also signaling competent. Additionally, the actual and modeled positions of domain IV are similar in 2:2 ErbB:ligand complexes and the asymmetric *Drosophila* sEGFR dimer, which we take to be an approximate model of singly-ligated human EGFR dimers [58, 60] (Fig. 4.13), suggesting equivalent arrangements of transmembrane and intracellular regions in both vertebrate dimer types.

Why then are physiological asymmetric *Drosophila* sEGFR-like dimers not observed in crystals of human sEGFR or sErbB4 complexed with ligand? The presence of asymmetric dimers in crystals of *Drosophila* sEGFR indicates that the extra stability of the asymmetric dimer interface more than compensates for the extra stability available from converting the low-affinity ligand interaction to a high-affinity interaction. That vertebrate ErbBs proceed to a weaker, symmetric dimer interface with two high-affinity ligand-receptor interactions implies that the energetic balance between high- and low-affinity dimer or ligand interactions has shifted during evolution, at least for the extracellular regions. One source for this shift is apparent. The tethered conformation of vertebrate ErbB extracellular regions, which buries the dimerization arm, necessarily reduces the ability of soluble, unliganded ErbB extracellular regions to dimerize with liganded partners. The presence of asymmetric, singly-ligated ErbB dimers in cells but not in crystals of isolated ErbB extracellular regions thus underscores the importance of human EGFR intracellular region-mediated preformed dimers, which are likely needed to stabilize asymmetric, singly-ligated dimers [64].

ErbB2/HER2 is an atypical ErbB that appears specialized to participate in asymmetric dimers. ErbB2/HER2 is the only EGFR homolog without known canonical ligands and serves as a universal
ErbB heterodimerization partner [67, 68]. Unlike all other unliganded human ErbBs, the ErbB2/HER2 extracellular region adopts a constitutively extended conformation in which its dimerization arm is exposed (Fig. 4.5). This conformation rationalized ErbB2’s role as a promiscuous heterodimerization partner but failed to explain the absence of ErbB2 homodimers in normal conditions [69, 70]. As noted by earlier authors [66], ligand binding to human ErbBs not only promotes conversion from the tethered to the extended conformation but also a change in conformation of domain II, which bridges ligand-binding domains I and III and bends to allow optimal contacts with ligand. By stabilizing formation of the asymmetric dimer, the change from a straight to a bent conformation in domain II in one dimer subunit appears to be the ligand-dependent on-off switch for Drosophila EGFR [58], which lacks the tethered conformation [57].

A direct contact between ErbB2 domains I and III that occludes canonical ligand binding surfaces and fixes the extended conformation also fixes a “straight” conformation of ErbB2 domain II. In this conformation the relative orientation of ErbB2 domain II and IV dimer contact regions is optimally aligned to pair with a liganded partner and serve as the unliganded subunit in asymmetric, singly-ligated ErbB heterodimers but precludes formation favorable ErbB2 homodimers (Fig. 4.4 and 4.5). Superposing ErbB2 extracellular regions on either domain II or domain IV contact regions of EGFR/ErbB dimers demonstrates that ErbB2 is not capable of simultaneously forming favorable domain II dimer contacts and bringing the juxtamembrane regions of domain IV into close proximity (Fig. 4.5), which appears to be a feature of ErbB signaling [51, 60, 71]. Unlike Drosophila EGFR, ligand binding is not required for ErbB2 to participate in active signaling complexes owing to the presence of ligand-binding homologs, which allowed ErbB2 to evolve a fixed straight domain II conformation and specialize as a heterodimerization partner. ErbB2 activation in cases of pathological overexpression thus seems likely to be mediated by intracellular regions [72, 73]. Curiously, a weak dimer of unliganded dmEGFR that looks similar to the leftmost dimer in Fig. 4.5B was observed in crystals and may serve as a model for weak, unliganded and presumably inactive ErbB extracellular region dimers [57].
Participation of unliganded ErbBs in active signaling complexes prompts reassessment of the role of the tethered conformation, which was first interpreted as keeping ErbBs “off” in the absence of ligand [51, 74, 75]. It is now apparent that the straight conformation of domain II is sufficient for this purpose, as evidenced by the absence of a tethered conformation in *Drosophila* EGFR and the failure of tether mutations in human EGFR to result in receptor activation [57, 76, 77]. In an organism with multiple EGFR homologs, however, the ability of an unliganded ErbB to participate in a signaling complex means that ligand binding to any ErbB could activate all co-expressed ErbBs. Such promiscuous activation is observed for ErbB2/HER2, for example, which is the only vertebrate ErbB not to adopt a tethered conformation. An additional inhibitory mechanism was thus needed to prevent indiscriminate ErbB responses to individual ligands in species with multiple ErbB homologs. By precluding unliganded EGFR, ErbB3, or ErbB4 from pairing with liganded forms of other ErbBs, the tethered conformation fulfills this role and likely facilitated diversification of ErbB function. The tethered conformation of human EGFR [75], ErbB3 [74], and ErbB4 [78] and the fixed, ligandless conformation of ErbB2 [69, 70] thus appear to have arisen following the appearance of multiple ErbB homologs as elaborations on the core signaling mechanism present in *Drosophila* EGFR. As tethered ErbBs appear able to convert to a signaling competent extended-straight conformation when dimerized with a liganded partner, which ErbB dimers form in the absence of ligand will govern the nature of ErbB responses and is an important avenue for future investigation.

ErbBs have evolved many mechanisms to safeguard and modulate their potent activity. The presence of inactive, singly-ligated, and doubly-ligated human ErbB dimers confers several advantages over a ligand-induced dimerization activation mechanism. Inactive dimers present a barrier to activation through random dimerization, and the presence of singly- and doubly-ligated dimers furnishes a mechanism to tune responses to different concentrations or affinities of ligands [58]. The results presented here show how specific intra- and intermolecular conformations combine to govern ErbB activity and lead to a unifying model of ErbB activation that rationalizes previously puzzling properties of EGFR and its homologs.
Materials and Methods

**Generation of ErbB-expressing Cell Lines**—Genes encoding ErbB mutants were generated by QuickChange mutagenesis (Stratagene) using PfuTurbo AD (Agilent) and sequenced. Variant ErbB genes were then subcloned with their native signal sequences into pSSX, a version of pSGHV0 modified to eliminate the growth hormone tag and add C-terminal Flag or HA tags [79]. CHO-S cells (Invitrogen) were maintained in adherent culture in DMEM:F12 supplemented with 5% FBS. Stably-transfected cell lines were created using FuGENE (Roche) according to manufacturer’s instructions. Cells were co-transfected with a total of 1 μg DNA per ml culture and 0.1 μg per ml of pCDNA 3.1 (Invitrogen), which contains the neomycin resistance gene. After 24 hours, fresh medium containing 1 mg/ml G418 was added, and the cells fed every three days until colonies appeared. Colonies were picked, expanded and screened for ErbB expression by Western using the appropriate tag antibody. Antibodies used for immunoprecipitation or Western detection were Flag-M2 (Sigma), 3F10 anti-HA (Roche), anti-EGFR (Santa Cruz sc-71033), anti-EGFR pY1068 (Abcam EP774Y), anti-ErbB4 (Santa Cruz sc-283), and 4G10 anti-phosphotyrosine (Millipore). The number of receptors per cell was estimated by comparison of anti-EGFR band intensities of cell lysates compared to intensities of known amounts of purified tEGFR [80]; cell lines with approximately equal expression of the ErbB variants were chosen for stimulation assays.

**ErbB Activity Assays**—ErbB-expressing cell lines were plated in 2 wells of a six-well plate at 0.2 \times 10^6 cells per well and grown 24 hr. On the day of the assay, cells were washed three times with 2 ml Ham’s F12 supplemented with 1 mg/ml BSA, then serum-starved for at least 3 hours at 37°C in the same medium. EGF or Nrg1β was added to one of the two wells at a final concentration of 100 ng ml\(^{-1}\), and the plates incubated at 37°C for 5 minutes. The wells were then washed once with ice-cold phosphate-buffered saline, and 250 μl of RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, and 0.1% SDS) supplemented with 1 mM activated Na\(_3\)VO\(_4\), 1 mM PMSF, and Benzonase nuclease (Sigma) was added directly to the wells. The cells were allowed to lyse for 30 minutes with gentle rocking, and the appropriate antibody was added to the lysate (using a 0.5
µg/ml for anti-Flag and 0.1 µg/ml for anti-HA). Lysates were next added directly to 20 µl Protein G Sepharose 4 Fast Flow (GE), and allowed to bind overnight at 4°C. Finally, beads were washed 3 times with 1 ml of RIPA buffer supplemented with 1 mM activated Na$_3$VO$_4$ and eluted by adding 20 µl of 5× SDS-PAGE loading buffer containing 10% fresh β-mercaptoethanol and incubating for 30 minutes at room temperature. Eluted proteins were separated on 4-20% Tris-Glycine SDS-PAGE gels (Invitrogen), transferred to PVDF membranes, and probed with the anti-phosphotyrosine antibody 4G10. A portion of crude lysate was reserved, run separately, and probed with antibodies against HA, Flag, EGFR, or ErbB4 to assess total receptor expression.

Expression of sErbB4 and Nrg-1β—A cDNA encoding residues 1-615 (numbering from the mature N-terminus) of the JM-a isoform of human ErbB4 (sErbB4) was subcloned into the pSGHV0 expression vector [79], transfected into Lec1 Chinese Hamster Ovary cells [81], and a cell line expressing ~1 mg/liter of sErbB4 selected. pSGHV0 directs expression of target proteins as fusion proteins with human Growth Hormone at the N-terminus followed by an octahistidine tag and a tobacco etch virus (TEV) protease recognition site. Following concentration and dialysis of sErbB4-conditioned medium, the sErbB4 fusion protein was purified using Ni-NTA chromatography, cleaved by TEV protease, deglycosylated using endoglycosidases H and F, and further purified using anion-exchange and size-exclusion chromatographies. ~0.7-0.8 mg of purified sErbB4 was obtained per liter of starting medium. A gene encoding the 55 amino-acid EGF repeat from human Neuregulin-1β (GTSHLVKCAEKFTFCVNGGECFMVKDLSNPSRYLCKCPNEFTGDRCQNYVMASF) was synthesized, subcloned into the pET32 expression vector, and expressed as a fusion protein with Thioredoxin, a histidine tag, and a TEV protease recognition site at its N-terminus in Origami cells (Novagen). The fusion protein was purified from lysates using Ni-NTA chromatography, cleaved using TEV protease, and further purified using cation exchange and size-exclusion chromatographies. A complex of sErbB4 and Nrg-1β was prepared by mixing a molar excess of Nrg-1β with sErbB4 and purifying the complex using size-exclusion chromatography.
The purified sErbB4:Nrg-1β complex was dialyzed into 2.5 mM Tris pH 8.0, 25 mM NaCl and concentrated to 4 mg/ml. Crystals were grown at 20°C by the hanging drop vapor diffusion method. 2 μl protein solution was mixed with 1 μl of a solution of 8% PEG 6000, 0.1 M Mg(OAc)2, and 0.1 M MES pH 6.0, and 0.5 μl Hampton Silver Bullet Bio condition F1 (0.25% w/v β-Nicotinamide adenine dinucleotide phosphate tetrasodium salt, 0.25% w/v Adenosine 5'-triphosphate disodium salt hydrate, 0.25% w/v N-Acetyl-D-galactosamine, 0.25% w/v Gentamicin sulfate salt hydrate, 0.02 M HEPES sodium pH 6.8) and 0.5 μl Hampton Silver Bullet Bio condition F7 (0.20% w/v Thymine, 0.20% w/v Sodium pyrophosphate tetrabasic decahydrate, 0.20% w/v D-Glyceric acid calcium salt dihydrate, 0.20% w/v β-Cyclodextrin, 0.20% w/v myo-inositol, 0.02 M HEPES sodium pH 6.8). Crystals grew as needles or blocks with a maximum size of 50 x 50 x 70 μm3. Crystals were soaked in 20% (v/v) PEG 200 prior to flash freezing in liquid nitrogen. X-ray diffraction data were collected from a single crystal at 23-ID-D-GM/CA of the advanced photon source at Argonne National Laboratory.

X-ray diffraction data were integrated, scaled, and merged using HKL2000 [82], and the structure determined by molecular replacement using the program MOLREP [83] with a single receptor:ligand subunit of the EGFR:TGFα complex used as a search model [62], which easily identified all six receptor subunits in the crystallographic asymmetric unit. Individual domains of the unliganded sErbB4 structure [78] were then superposed on the EGFR domains and fragments of domain IV placed in electron density as they became apparent during refinement. Refinement was carried out using the programs REFMAC [84], PHENIX [85], and autoBUSTER [86] alternated with model building using COOT [87]. Final X-ray data collection and refinement statistics are presented in Table 4.3.

Acknowledgments

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GM/CA CAT has been funded in whole or in part with Federal funds from the National Cancer Institute (Y1-CO-1020) and the National Institute of General Medical Sciences (Y1-GM-1104). Use of the APS was supported by the U.S. Department of Energy, Basic Energy Sciences, Office of Science, under contract No. DE-AC02-06CH11357. This work supported by R01GM099321 from the National Institutes of Health (DJL).
Figure 4.1 – Evidence for singly-ligated ErbB signaling dimers. (A) Schematic diagram showing tethered, extended, and dimeric conformations of EGFR with sites of function-targeting mutations indicated. (B) Anti-phosphotyrosine and anti-EGFR Western blots of tagged full-length EGFR immunoprecipitated from stably-transfected CHO cells. Wild-type (WT) EGFR was tagged with either hemagglutinin (HA) or Flag peptides, EGFR bearing an inactivating mutation in its kinase active site (Kin-) was tagged with HA, and EGFR bearing a mutation in its ligand-binding site (Lig-) was tagged with Flag. Mutation in the Kinase donor site (Do-) and combination of the Kinase- and Ligand-targeting mutations on a single EGFR (Kin::Lig) were also tested. Serum-starved cells were either untreated (-) or treated (+) with EGF for 5 minutes. Each WT or mutant EGFR was transfected singly; the Kin- and Lig- variant EGFRs were co-transfected as were the (Kin::Lig) and (Do-) variants. When co-transfected, the tag used for immunoprecipitation prior to Western blotting is indicated in red. (C) Similar experiments using ErbB4 and its ligand Neuregulin 1β (Nrg) are shown. The lanes shown were run on the same gel but rearranged electronically to match the order of experiments in (B). Bar graphs represent quantitation of bands from at least 3 independent experiments.
Figure 4.2 – The sErbB4:Nrg1β structure. Orthogonal views of a worm diagram of the three independent sErbB4:Nrg1β dimers in the crystallographic asymmetric unit following superposition of domains I, II, and III of sErbB4. Domains I, II, III, and IV are colored blue, green, yellow, and red, respectively, and Nrg1β is colored magenta. Lighter hues are used for the rightmost sErbB4 subunit.
Figure 4.3 – Two types of vertebrate ErbB dimer interaction. (Top) Orthogonal views of worm diagrams of sErbB4:Nrg1β and sEGFR:EGF [60] dimers following superposition of domains I, II, and III (see Fig. 4.1A for domain nomenclature). One receptor subunit is colored yellow, the other blue, and Nrg1β is colored magenta. (Bottom) Orthogonal views of worm diagrams of the tEGFR:TGFα complex [62] colored as in the top panel. The position of domain IV has been modeled on each subunit based on the domain III/IV relationship in the sEGFR:EGF complex and apo-sEGFR structures (Fig. 4.10). The modeled domain IV of one subunit is colored black and the other gray. The modeled regions are enclosed in a dashed red box with clashing regions indicated. Red asterisks mark the dimer interaction site mediated by the N-terminal regions of domain II, which differs in the two dimer types. Yellow and blue lines approximate the long axes of the receptor subunits to illustrate the relative scissoring of the subunits in the two dimer types.
Figure 4.4 – The tEGFR:TGFα domain II dimer interface is similar to the interface in Drosophila sEGFR:Spitz complexes and modeled ErbB2-containing heterodimers.

(Top row) In the leftmost panel a “side” view of an sErB4:Nrg1β dimer (equivalent to the top right image of Fig. 4.2) with one sErB4 subunit colored blue, another yellow, and Nrg1β magenta is shown. Moving rightwards one subunit of the EGFR:EGF [60], tEGFR:TGFα [62], or Drosophila EGFR:Spitz [58] complexes has been superposed on domains I, II, and III of the yellow sErB4 subunit. In the far right panel domain III of sErB2 [69] has been superimposed on domain III of the blue sErB4 subunit. Domains I and II of the non-ErbB4 receptors are colored red and domains III and IV light green. Colored arrows indicate shifts in unsuperposed subunit domains relative to sErB4 subunit domains, which in the case of tEGFR, Drosophila EGFR, and ErbB2 align the domain I/II interface regions directly opposite the corresponding regions of the opposite receptor subunit. (Bottom row) “Top” views of the superpositions shown in the top row following a 90° rotation about a horizontal axis in the plane of the page. The superposed receptor subunits are colored yellow, the unsuperposed sErB4 subunit is colored blue, and domains I and II of the unsuperposed EGFR or ErbB2 subunits are colored red. Red and green asterisks mark the two loops encompassed by ErbB4 residues 187-205 that are staggered in ErbB4 dimers but directly opposed in tEGFR, Drosophila EGFR, and modeled ErbB2-containing dimers.
Figure 4.5 – The fixed extended conformation of ErbB2 precludes formation of canonical homodimers. (A) Orthogonal views of worm diagrams of the six sErbB4:Nrg1β subunits (sErbB4 is colored blue and Nrg1β yellow) following superposition of domain III of sErbB4 with domain III of three independent sErbB2 structures (pink) [69, 70]. (B) Worm diagrams of a superposition of sErbB2 (light and dark pink) on domain III of an sErbB4:Nrg1β dimer (one sErbB4 subunit colored yellow and the other blue) (left panel); only domain III of the sErbB4:Nrg1β complex is shown for clarity, and red arrows indicate the shift in position of domains I and II of the ErbB2 subunits relative to their position in the sErbB4:Nrg1β complex. The sErbB4:Nrg1β complex is shown in the middle panel. In the rightmost panel domain I and the N-terminal part of domain II of ErbB2 is superposed on the corresponding regions of each subunit of the sErbB4:Nrg1β complex; only the superposed regions of the sErbB4:Nrg1β complex are shown for clarity. Red arrows indicate the shift in position of domains III and IV of the ErbB2 subunits relative to the position of the corresponding domains in the sErbB4:Nrg1β complex. The human sErbB2 structure was used for the superposition [69].
Figure 4.6 – Anti-EGFR pY1068 Western blots of EGFR immunoprecipitated from EGFR-transfected cell lines. Cell lines and labeling as described for Fig. 4.1B.
Figure 4.7 – The effect of dimerization arm mutations on ligand-dependent stimulation.

Antiphosphotyrosine and anti-ErbB Western blots of immunoprecipitated full-length EGFR (top) and ErbB4 (bottom) tagged and bearing the same mutations as in Fig. 4.1 except that a dimerization-arm (Di) mutation has been added as a double mutation with the kinase-deficient (Di:Kin) and ligand-targeting (Di:Lig) mutations. Bar graphs represent quantitation of bands from at least 3 independent experiments. The dimerization arm mutation of EGFR failed to eliminate ligand-driven activation completely, and the residual activation in the Di:Lig plus Kin transfected cells cannot be interpreted as independent of dimerization arm-mediated interactions in this case.
Figure 4.8 – Determination of receptor expression levels in cell lines. Anti-EGFR Western blots of lysates from known numbers of the EGFR-transfected cells used in cell-based assays as well as A431 cells. Cell lines are labeled as in Fig. 4.1. Known amounts of purified, truncated EGFR were loaded as standards (left 5 lanes).
Figure 4.9 – Ligand-bound ErbB ectodomain structures. Orthogonal views of worm diagrams of all structures of EGFR/ErbB ectodomains with high-affinity ligand bound following superposition of domains I, II, and III are shown. Superposed ErbBs include the six independent sErbB4:Nrg1β subunits reported here, the re-refined EGFR:EGF subunits [60], the tEGFR:TGFα subunits [62], and the high-affinity Drosophila EGFR:Spitz subunit [58].
Figure 4.10 – Alignment of Domain III/IV regions of EGFR. Orthogonal view of worm diagrams of domains III and IV of EGFR from both subunits of the EGFR:EGF complex [60], a low-pH tethered form of EGFR [75], and EGFR from a complex of EGFR and the Cetuximab Fab [88]. Only domain III (yellow) was used in the superposition, but the positions of domain IV (red) are very similar.
Figure 4.11 – Schematic diagram of ErbB dimer states. An inactive “preformed” dimer of EGFR, whose conformation is not known and may or may not adopt a tethered state, is able to bind a single ligand to form an active, asymmetric singly-ligated receptor dimer (middle panel). This asymmetric dimer has a liganded subunit with a bent domain II conformation and an unliganded subunit with a straight conformation shown in lighter hues. ErbB2/HER2 is ideally configured to serve as the unliganded partner in this dimer. The right panel shows a symmetric, doubly-ligated receptor dimer.
Figure 4.12 – Glutamine 194 participates differently in tEGFR:TGFα vs. sErbB4:Nrg1β and EGFR:EGF dimers. Worm diagrams of the “top” view of EGFR:ligand dimers, similar to the view in the bottom panels of Fig. 4.4, in which one EGFR subunit is colored yellow and the other blue. The tEGFR:TGFα dimer [62] is on the left and the EGFR:EGF dimer is on the right [60, 63]. The side chain of Q194 from each subunit is shown in red spheres.
Figure 4.13 – The position of domain IV region in the asymmetric *Drosophila* EGFR:Spitz dimer. The positions of the C-terminal regions of domain IV were modeled on each subunit of the asymmetric *Drosophila* EGFR:Spitz dimer by superimposing domain IV from the EGFR:EGF dimer [60] on the homologous regions of domain IV present in the *Drosophila* EGFR:Spitz dimer [58]. The *Drosophila* EGFR subunit with a high-affinity Spitz bound is colored yellow, the subunit with a low-affinity Spitz bound is blue, Spitz is magenta, and the modeled domain IV positions are light blue. The distance between the C-termini of the modeled domain IV regions is 12.8 Å, which compares to 8.0 Å in the EGFR:EGF dimer [60].
Table 4.1 – Cell lines and mutations.

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<td>Flag–D818N</td>
<td></td>
</tr>
<tr>
<td>HA-Dim</td>
<td>HA–F248A+R281S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA–Dim+Lig</td>
<td>HA–F248A+R281S+D351R</td>
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</tr>
<tr>
<td>Flag–Dim+Kin</td>
<td>Flag–F248A+R281S+D818N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA–Dim+Lig /Flag–Kin</td>
<td>HA–F248A+R281S+D351R</td>
<td>Flag–D818N</td>
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</tr>
<tr>
<td>Flag–Do</td>
<td>Flag–V929R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA–Lig+Kin/Flag–Do</td>
<td>HA–D351R+D818N</td>
<td>Flag–V929R</td>
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</tr>
<tr>
<td>Cell Line</td>
<td>EGF Receptors per cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT HA</td>
<td>130,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT Flag</td>
<td>128,000</td>
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</tr>
<tr>
<td>Lig-</td>
<td>60,000</td>
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</tr>
<tr>
<td>Kin-</td>
<td>53,000</td>
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<td></td>
</tr>
<tr>
<td>Kin- + Lig-</td>
<td>87,000</td>
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<td></td>
</tr>
<tr>
<td>Do-</td>
<td>179,000</td>
<td></td>
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</tr>
<tr>
<td>Do- + K:-L-</td>
<td>72,000</td>
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<tr>
<td>A431</td>
<td>1,900,000</td>
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Table 4.2 – Receptor expression levels for EGFR cell lines.
<table>
<thead>
<tr>
<th>ErbB4-Nrg1β</th>
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</thead>
<tbody>
<tr>
<td><strong>Data collection</strong></td>
</tr>
<tr>
<td>Wavelength</td>
</tr>
<tr>
<td>Space group</td>
</tr>
<tr>
<td><strong>Unit cell dimensions</strong></td>
</tr>
<tr>
<td>a (Å)</td>
</tr>
<tr>
<td>b (Å)</td>
</tr>
<tr>
<td>c (Å)</td>
</tr>
<tr>
<td>β (°)</td>
</tr>
<tr>
<td><strong>No. of unique reflections</strong></td>
</tr>
<tr>
<td>Completeness (%)</td>
</tr>
<tr>
<td>&lt;I/σ&gt;</td>
</tr>
<tr>
<td>R&lt;sub&gt;merge&lt;/sub&gt; (%)</td>
</tr>
<tr>
<td>Redundancy</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
</tr>
<tr>
<td>Resolution (Å)</td>
</tr>
<tr>
<td>R&lt;sub&gt;work&lt;/sub&gt; (%)</td>
</tr>
<tr>
<td>R&lt;sub&gt;free&lt;/sub&gt; (%)</td>
</tr>
<tr>
<td><strong>rmsd</strong></td>
</tr>
<tr>
<td>Bond length (Å)</td>
</tr>
<tr>
<td>Bond angle (°)</td>
</tr>
</tbody>
</table>

Values in parentheses are for the highest-resolution shell

Table 4.3 – Crystallographic data quality and refinement statistics.
### Table 4.4 – Comparison of ErbB ectodomain structures.

<table>
<thead>
<tr>
<th>Protein</th>
<th>HER4 A</th>
<th>HER4 B</th>
<th>HER4 C</th>
<th>HER4 D</th>
<th>HER4 E</th>
<th>HER4 F</th>
<th>Ogiso A</th>
<th>Ogiso B</th>
<th>Garr A</th>
<th>Garr B</th>
<th>DER C</th>
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</thead>
<tbody>
<tr>
<td>HER4 A</td>
<td>*</td>
<td>0.283</td>
<td>0.273</td>
<td>0.321</td>
<td>0.421</td>
<td>0.240</td>
<td>1.15</td>
<td>1.13</td>
<td>1.90</td>
<td>1.37</td>
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<tr>
<td>HER4 B</td>
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<td>*</td>
<td>0.371</td>
<td>0.220</td>
<td>0.472</td>
<td>0.231</td>
<td>1.24</td>
<td>1.15</td>
<td>1.89</td>
<td>1.34</td>
<td>1.40</td>
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<tr>
<td>HER4 C</td>
<td>494</td>
<td>494</td>
<td>*</td>
<td>0.348</td>
<td>0.438</td>
<td>0.367</td>
<td>1.21</td>
<td>1.18</td>
<td>1.89</td>
<td>1.32</td>
<td>1.41</td>
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<tr>
<td>HER4 D</td>
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<td>494</td>
<td>494</td>
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<td>0.203</td>
<td>1.20</td>
<td>1.17</td>
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<tr>
<td>HER4 E</td>
<td>494</td>
<td>493</td>
<td>494</td>
<td>493</td>
<td>*</td>
<td>0.496</td>
<td>1.18</td>
<td>1.26</td>
<td>1.74</td>
<td>1.29</td>
<td>1.55</td>
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<tr>
<td>HER4 F</td>
<td>494</td>
<td>494</td>
<td>494</td>
<td>494</td>
<td>*</td>
<td>1.16</td>
<td>1.13</td>
<td>1.90</td>
<td>1.37</td>
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<tr>
<td>Ogiso A</td>
<td>485</td>
<td>483</td>
<td>483</td>
<td>480</td>
<td>478</td>
<td>481</td>
<td>*</td>
<td>0.497</td>
<td>1.61</td>
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<tr>
<td>Ogiso B</td>
<td>483</td>
<td>481</td>
<td>483</td>
<td>480</td>
<td>483</td>
<td>481</td>
<td>497</td>
<td>*</td>
<td>1.707</td>
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<tr>
<td>Garr A</td>
<td>465</td>
<td>463</td>
<td>461</td>
<td>462</td>
<td>471</td>
<td>462</td>
<td>484</td>
<td>485</td>
<td>*</td>
<td>1.36</td>
<td>2.35</td>
</tr>
<tr>
<td>Garr B</td>
<td>487</td>
<td>484</td>
<td>486</td>
<td>485</td>
<td>485</td>
<td>486</td>
<td>496</td>
<td>495</td>
<td>488</td>
<td>*</td>
<td>1.70</td>
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<tr>
<td>DER C</td>
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<td>459</td>
<td>459</td>
<td>459</td>
<td>459</td>
<td>452</td>
<td>453</td>
<td>431</td>
<td>444</td>
<td>*</td>
</tr>
</tbody>
</table>

Number of matched residues
Chapter 5 – Discussion

The work presented in this thesis has advanced our methods for understanding the role of Ptc in Hh signaling in several practical ways. While the role of Ihog homologs as Hh binding proteins is now well established [19, 26], we continued to lack a molecular understanding of how Ihog homologs integrate Hh binding and signaling with the canonical Hh receptor, Ptc. In addition to binding Ihog homologs, our current understanding of Hh signaling indicates that Hh proteins also bind Ptc, either directly [9, 11] as in the mammalian pathway, or with the help of Ihog homologs [23, 89], as in the Drosophila pathway. A simple explanation is that signaling could proceed through a ternary complex such as Hh:Ihog:Ptc; but this may differ between phyla. Shh:CDO uses a different binding mode than Hh:Ihog, and soluble CDOFn3 apparently competes with Ptc on the surface of cells for binding to ShhN [19]. An additional complication is the potential multivalency of these proteins in vivo. The physiological Hh molecule is lipidated and may form multivalent signaling particles. Ihog homologs form heparin-mediated dimers [25]. Ptc itself is likely to form physiological oligomers, as seen in [34] and in our own gel filtration experiments with purified Ptc proteins. This combination of multivalent binding partners could lead to behaviors on the cell surface that are difficult to recapitulate in solution.

Clarifying the role of accessory proteins in signaling through Ptc will nevertheless probably require direct studies using purified, functional Ptc. In this work, we have successfully expressed and purified Ptc proteins from Drosophila and mouse, characterized them biophysically, and shown that Ptc proteins purified in this way do not bind to cognate Hh or Ihog homologs. It is unknown whether this result is physiologically “true,” reflecting the fact that Ptc requires some other factor or conditions to bind its partners, or if this result stems from our expression, solubilization, and/or purification methods. In the course of this project, we attempted a range of different purification and binding conditions, and this information will be necessary for future work attempting to demonstrate binding activity using purified Ptc. We have recently also performed additional studies on a different, C-terminally tagged Drosophila Ptc construct, with a different C-terminal truncation position suggested by Phil Beachy as being more stable and active when binding Hh/Ihog on the cell surface. While this C-
terminally tagged Ptc protein was more efficiently extracted by mild detergents such as DDM, and more monodispersed as judged by SEC, preliminary studies indicated that it still did not bind Hh/Ihog (preliminary data, not shown). Future studies will probably require further explorations of different Ptc constructs and expression systems other than the Sf9/baculovirus system; these studies should start with establishing positive binding to cells or to isolated Ptc-containing membranes, followed by the determination of which solubilization and purification methods, if any, are capable of preserving Ptc binding.

Work presented in this thesis also clarified the role of a proposed binding interaction between the third FnIII domain of the Hh pathway protein CDO, and the fourth FnIII domain of Ngn, a protein that has been tentatively linked to Hh signaling in a small number of experiments. We showed that these proteins do not bind as purified soluble domains. We also determined the structure of a fragment of Ngn spanning the third and fourth FnIII domains. This structure could be of use to other workers attempting to understand the role of Ngn in Hh signaling, or in other developmental pathways. We showed that the linker between the third and fourth FnIII domains is flexible, and that the third FnIII domain of Ngn contains a strongly basic patch that could potentially bind heparin or other negatively-charged molecules.

Finally, we have advanced the understanding of ErbB signaling in mammals by functionally demonstrating the presence of active one-ligand, two-receptor EGFR and ErbB4 dimers. While crystal structures of ligand-bound ErbB extracellular domains are symmetric, this result is consistent with curved Scatchard plots of ligand binding to ErbB-expressing cells, which have long suggested the possibility of negative cooperativity upon binding a second ligand in a dimer.
Appendix 1 – Production of monoclonal antibodies against mouse Patched

Results and Discussion

We wished to generate α-Ptc antibodies for potential future use as a crystallization aid. After initially obtaining Strep-Tactin-purified mouse Ptc exchanged into 0.02% DDM, we provided protein to the Monoclonal Antibody Core Facility (MACF) operated by the Department of Neuroscience for immunization of mice. Because this was a mouse protein, we anticipated that the extracellular region of Ptc would likely not be antigenic, since it is exposed to the mouse immune system during development. However, we thought it possible that antibodies might be obtained against the intracellular regions of Ptc. Such antibodies would, in fact, be particularly useful for the generation of crystal contacts on the intracellular face of Ptc, which lacks large ordered domains. The Ptc extracellular region is already thought to consist of a large, ordered domain with good potential for crystal contacts.

The MACF immunized mice, harvested splenocytes, performed the initial hybridoma fusion, and diluted the resulting fusions into 96-well plates seeded with thymocyte feeder cells for colony formation and initial cloning. These plates containing hybridoma clones (some wells with multiple colonies) were then transferred to our lab for all further screening and subcloning experiments.

I screened these plates for secreted antibodies by applying their supernatants to formalin-fixed and permeabilized MmPtc-expressing Sf9 cells, followed by washing and incubation with an appropriate Cy3-labeled secondary antibody. The screening plates were then washed and scanned using a Typhoon imager with appropriate filters for Cy3 detection (Fig. A1.1). I initially selected 96 positive hybridoma wells (which contained between 1–3 colonies each) and expanded them in 24-well plates. Colonies that survived expansion were frozen and stored in liquid nitrogen. These stored colonies were then subjected to multiple rounds of single-cell cloning by limiting dilution in 96-well plates (Fig.).

For screening antibodies produced by cloned hybridoma cell lines, we used ELISA plates coated with SbpPHMT-MmPtcT1. Because mice were immunized with tagged SbpPHMT-MmPtcT1, we also used plates coated with an irrelevant protein containing the same SbpPHMT tags to rule out antibodies against tags. Surprisingly, we found that the majority of hybridoma clones produced
antibodies only against tags, and not against Ptc. If this procedure is used by the lab for future antibody targets, it will be important to use de-tagged protein for initial mouse immunization if possible. Nevertheless, some hybridomas were identified that secreted antibodies against Ptc.

Work on this project is ongoing. We will continue to screen the original frozen hybridoma colonies for α-Ptc antibody production (current progress is shown in Fig. A1.3). Any α-Ptc antibodies identified will be tested for activity by ELISA, IP and Western. Antibodies will also be tested for use in immunofluorescence experiments on tissue sections in cooperation with the Beachy lab. It is our hope that any α-Ptc antibodies identified may be used to aid future crystallization experiments with Ptc.

Materials and Methods

Tissue culture—For subcloning and expansion of cell lines, we used Hybridoma-SFM (Life Technologies) supplemented with 10% FBS and 1 ng/mL of recombinant IL-6 (expressed and purified as described in Chapter 2). Ordinarily, the MACF uses feeder cells when single cell cloning hybridomas, but supplementation with IL-6 eliminates this requirement. The initial primary hybridoma cell lines from the first round of screening were expanded in medium containing hypoxanthine and thymidine, but this is no longer needed after the first few splits. Initial primary cell lines were slow-frozen in 90% FBS, 10% DMSO; but after additional passages and rounds of subcloning, cell lines were routinely frozen in Hybridoma SFM containing 20% FBS and 10% DMSO.

Antibody screening—We used immunofluorescence on formalin-fixed, Ptc-expressing Sf9 cells to screen the supernatants from the initial primary hybridoma colonies. Sf9 cells were suspended at a concentration of 1.53×10⁶ cells/mL, and 1:50 (v/v) MmPtcT1 baculovirus stock was added to the cell suspension. 100 μL of cells were plated per well in 96-well plates and cultured for 24–48 hr to allow for Ptc expression. To fix cells, 100 μL of 4% paraformaldehyde (PFA) in PBS was added directly to the culture medium and incubated for 5 minutes. Wells were then aspirated, and 100 μL of 4% PFA solution were added directly to the cells for another 5 minutes. Wells were aspirated and permeabilized for 15 minutes with PBS containing 0.5% Tween-20, followed by blocking for 30 minutes at room
temperature with PBS containing 0.1% Tween-20 and 1% BSA. Hybridoma supernatants were then
applied to the cells and incubated at 4°C overnight. Cells were washed 3 × 5 min. with PBS containing
0.1% Tween-20 (PBST), and secondary antibody solution containing 1:200 of an appropriate Cy3-
labeled antibody was applied to the cells for 1 hr at room temperature in the dark. Cells were again
washed 3 × 5 min. with PBST, followed by scanning using a Typhoon imager with appropriate settings
for Cy3 fluorescence detection.

After the initial round of screening, we used standard ELISA methods to screen antibody
supernatants. Purified SbpPHMT-MmpTcT1 was coated on medium-binding polystyrene ELISA plates
at a concentration of 2 μg/mL, with SbpPHMT-tagged Drosophila EGFR kinase coated on another set
of plates as a negative control to rule out tag-specific antibodies. Coated ELISA plates were then
incubated with hybridoma supernatants, followed by incubation with HRP-conjugated α-mouse
secondary antibody solution and detection using a colorimetric HRP substrate.
Figure A1.1 – Screening of primary α-Ptc hybridoma colonies. Hybridoma supernatants were used to perform immunofluorescence on formalin-fixed, mouse Ptc-expressing Sf9 cells. Hybridoma colonies found to be secreting α-Ptc antibodies were expanded and frozen.
Figure A1.2 – Subcloning of hybridoma cell lines. Hybridoma cell lines were single-cell cloned by serial dilution, and supernatants from wells with single colonies were screened by ELISA (color coded here from yellow = nonreactive, to red = reactive). ELISA plates were coated with BSA (negative control), SbpPHMT-DmEGFRK (tagged irrelevant protein) or SbpPHMT-MmPtcT1 and probed in parallel with hybridoma supernatants. Additional rounds of single-cell cloning were performed until uniformly secreting colonies resulted. Colonies from the parent cell line 8F11 are seen to uniformly secrete α-Ptc antibodies in rounds 2 and 3, indicating that this line is fully monoclonal and stable. Cell line 3A12 is unstable and has lost expression by round 3. Green boxes: α-Myc (positive control) antibody was used for ELISA detection. Red boxes: an irrelevant negative-control mouse antibody was used for detection.
Figure A1.3 – Summary of screening progress for all hybridoma cell lines. All original cloning plates are diagrammed. Green shaded boxes show the most reactive clones. Black-bordered boxes show primary cell lines that were placed into storage for later subcloning. Blue-bordered boxes show cell lines that have tested positive for specific α-Ptc antibodies, as opposed to α-tag antibodies.
Appendix 2 – General Materials, Methods and Protocols

Preparation of Insect Serum-Free Medium (ISFM)

Adapted by Thomas Cleveland from [32]. These are our working instructions.

Stock solutions

1. CaCl2 – 5% solution – dissolve 66.2g/liter (NOT 50g/liter, unless using anhydrous). We use dehydrate, F.W. 147, paper recommends a 5% anhydrous solution F.W. 111.

2. NaCl – 15% solution – 150 g/L

3. Ultrafiltered Yeastolate – 10% solution – we buy it already ultrafiltered; no need to ultrafilter
   a. Dissolve 100 g yeastolate ultrafiltrate into ~800mL ddH2O
   b. Stir and heat, using a thermometer, until it reaches 60°C. Immediately turn off heat.
   c. Continue stirring for about 10 minutes. Solution should be amber, but not cloudy.
   d. Bring volume up to 1L, immediately filter sterilize, and store at 4°C

4. Pluronic F-68 – 10% solution
   a. dissolve 100g in 800mL ddH2O overnight at 4°C on a magnetic stir plate
   b. Increase volume to 1L, filter sterilize and store at 4°C.

5. Lipid-Ethanol solution (1000x) – makes 100mL

<table>
<thead>
<tr>
<th>Component</th>
<th>Add</th>
<th>Preheat Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>i Cholesterol</td>
<td>450mg</td>
<td>---</td>
</tr>
<tr>
<td>ii Alpha tocopherol acetate (Vitamin E)</td>
<td>200mg</td>
<td>60°C</td>
</tr>
<tr>
<td>iii Cod liver oil fatty acid methyl esters</td>
<td>1g</td>
<td>37°C</td>
</tr>
<tr>
<td>iv Tween 80</td>
<td>2.5g</td>
<td>---</td>
</tr>
</tbody>
</table>

   a. Pre-heat the indicated components in a water bath to the indicated temperature. Do not heat too long.
   b. Weigh all components into a small glass beaker one at a time. Tare the beaker between additions. Components ii-iii are viscous. Transfer using a small syringe with long, large-bore needle
   c. Dissolve add 80-90mL 100% ethanol, stirring on a warm hot plate until cholesterol just dissolves.
   d. Bring volume to 100mL, aliquot 10.5mL into small glass vials, and store at -20°C
Making 10L ISFM:

Steps must be followed in this order and are performed on a magnetic stir plate

1. Autoclave the 10L carboy and add 9L ddH₂O. *(Total volume of other components is 850mL + 220g solids)*

2. On a magnetic stir plate, add 216.8g IPL-41 media. Allow >3 hours to dissolve completely.

3. Pre-warm both a 100 mL Pluronic aliquot and 10.5 mL lipid aliquot at 37°C at least 30 min.

4. Add the following components to the media in this order:
   a. 3.5g NaHCO₃. Wait until dissolved.
   b. 180mL 1N NaOH. Medium pH should be about 6.2.
   c. 100mL 5% CaCl₂ solution. If pH is wrong, calcium will precipitate
   d. 220mL 15% NaCl. Final medium osmolarity should be 360mOsm/kg.
   e. 50mL 10% Pluronic F-68
   f. 75g Glucose

5. Emulsify the lipid-ethanol solution with the Pluronic F-68 solution (this is the only step requiring technique!)
   a. Add 10.5mL aliquot of 1000x lipid-ethanol solution to 250 mL conical bottle.
   b. While vortexing at high speed (around setting 5), add the remaining 50mL of the Pluronic F-68 solution slowly (a constant stream of drops is OK). If using a variable-speed pipet-aid, set dispense to slow. It should take 2–3 minutes. Too slow, and the solutions may overcool and will not emulsify properly.
   c. The mixture will first be milky-white, but should become clear as the addition of Pluronic is complete. To check, pour into clear tube and let sit ~5 min to allow air bubbles from vortexing to settle.
   d. The final emulsion should be clear. If the emulsion is cloudy-white, the solutions were not adequately prewarmed, or the 10% Pluronic was added too slowly or quickly, or without sufficient agitation. In severe cases the emulsion may need to be remade.
   e. Pour the solution immediately into the media while stirring at high speed.

6. Add 400mL 10% Yeastolate Solution. Let stir for 10-15min

7. Sterile filter into 1L bottles using Stericup 0.2μm filters. One filter typically is enough for 5 L.

8. Rinse the carboy thoroughly. Autoclave if time permits.
CNBr Coupling Protocol

All steps should be done sequentially without breaks, as the resin is unstable as soon as it is hydrated. It is more unstable the higher the pH.

1. Swell CNBr resin in ice cold 1 mM HCl for around 30 min (resin is relatively stable at acidic pH, but will begin to lose reactive groups at neutral pH and higher).

2. Wash in gravity column (EconoColumn or similar) with 20 volumes or so of additional cold 1 mM HCl to remove cryoprotectants. Or, wash in batch with at least 5 washes totaling about 20 column volumes, allowing a couple of minutes of incubation with each wash before spinning out resin and performing the next wash.

3. Using cold 1 mM HCl, resuspend resin and transfer to the tube in which you intend to do your coupling reaction. Remove excess 1 mM HCl so that you have only packed resin in the tube.

---This part is different from the manufacturer’s manual---

4. Add protein DIRECTLY to the packed resin. I prefer *not* to wash the resin first with high-pH coupling buffer, because as soon as you do so, the resin starts to lose reactive groups. Naturally, your protein MUST be buffered well enough to tolerate addition to 1 mM HCl without significant pH shifts (but it doesn't really take that much buffer to be OK). I normally have my protein in 20 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$ or 20 mM MOPS/NaOH, pH ~ 7.5. Buffers and additives with free amino groups (such as Tris, ethanolamine, etc) cannot be in your sample or else they will compete for coupling. Mineral buffers (phosphate, carbonate) are best, and I have also personally used MOPS without problems. Your pH should be in the range of 7-9 for coupling to occur.

Remaining steps are according to the manual: quench, wash, and store for further use as directed. I use 1 M ethanolamine pH 8-8.3 for quenching.
Silver Stain

This protocol was adapted from [90]. Water should be of the highest purity available. Solutions R1, S3, S4 and S5 should be prepared fresh before use. All containers must be thoroughly cleaned. It is especially critical to clean any silver deposits from containers previously used for silver stain, because the deposition of metallic silver is highly autocatalytic; any traces of silver metal will cause large amounts of deposition. This can cause mirroring of the gel surface and will deplete your solution of silver ions, causing incomplete band development. The stripping solution described below can be used for thorough removal of silver metal deposits. Alternatively, use disposable containers such as pipette tip lids and discard after development.

For ultra-high sensitivity, silver staining can be repeated. After step 10, if there is no mirroring (silver deposition) on the gel surface, wash 3 × 10 min with water and go back to step 7. Any mirroring must first be removed using a quick wash of the silver destain solution below followed by transfer to destain stop solution. The gel can then be washed 3 × 10 min with water before returning to step 7.

Solutions

R1. 10% (w/v) sodium thiosulfate pentahydrate (prepare about 1 mL for small batch of gels)

S1. 30% (v/v) ethanol, 10% (v/v) acetic acid in H2O.

S2. 20% (v/v) ethanol

S3. Sensitizer: 8 mM NaS2O3 (100 μL of R1 per 50 mL water)

S4. Silver ion solution: 12 mM AgNO3 – about 0.2% (w/v)

S5. Basic Developer – 3% (w/v) K2CO3, 250 μL formalin, and 125 μL of R1 per liter.

S6. Stop Solution – 4% (w/v) Tris and 2% (v/v) acetic acid

Procedure

1. Fix gel(s) in S1 (same as our usual Coomassie destain) for at least 30 min. Three 30 min washes or similar may optionally be used for the best possible silver staining. Overnight or even longer works fine as a stopping point, if you run a gel but aren’t ready to do a complete silver stain yet.

2. Incubate gel 2 × S2 for 10 min

3. 2 × water for 10 min


5. 1 min in S3

6. 2 × water for 1 min

7. S4 with rocking for 20 min to 2 hr (no notable difference in quality for this time range)

9. Rinse in water for 10 s

10. Transfer to S5 and shake until any precipitate dissolves. Rock until bands are fully developed (5 to 45 min). Photograph quickly if desired. 3:00

11. Transfer to S6 for at least 30 min. 3:30

12. Transfer to water.

**Silver Stripping Solution:**

This is a very fast-acting solution for removing deposits of metallic silver. It is useful for removing silver mirroring from the surface of silver-stained gels to allow photographing, HOWEVER it will invariably remove many of the bands as well, within a few minutes. To use this solution, it is critical to have the gel imager turned on, focused, and with desired settings in place and ready to immediately take a picture. **Faint bands will start to fade within a minute or so,** so you must take your gel picture faster than that.

1. Dissolve 1.85 g of sodium chloride and 1.85 g of anhydrous cupric sulfate anhydrous (or equivalent amount of hydrate) in 42.5 ml of deionized water.

2. Add concentrated ammonium hydroxide until the precipitate that forms is completely dissolved to give a deep blue solution.

3. Adjust to 50 mL with deionized water. This solution may be prepared in advance and stored.

4. Freshly prepare a second solution containing 21.8 g sodium thiosulfate pentahydrate in 50 mL (final) deionized water.

5. Combine equal parts of the two solutions immediately prior to use.

6. Dip the silver-stained gel in stripping solution and shake for just long enough to remove mirroring (a few seconds at most), then immediately shake in a tray of water for a few seconds and take a picture.

7. Stop the destaining process by shaking or rocking with 10% acetic acid for at least 15 min, then transfer to water. It is difficult to fully stop the destaining process; bands may continue to fade slowly over the next day or so. Intense bands may remain intact, but nevertheless, you should not assume that the gel will be stable in the long term after using this solution. Immediate photographic documentation is required.
Protocols for the Expression and Purification of BocFn3, CdoFn3 and Hhip

Vectors:  
<table>
<thead>
<tr>
<th>Parent</th>
<th>Antibiotic</th>
<th>Sequencing Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT7HMT-BocFn3</td>
<td>pET28* Kan</td>
<td>1 &amp; 2</td>
</tr>
<tr>
<td>pT7HMT-CdoFn3</td>
<td>pET28* Kan</td>
<td>1 &amp; 2</td>
</tr>
<tr>
<td>pFastBac-HbmHSbpMT-MmHhip</td>
<td>pFastBac1 Amp</td>
<td>3 &amp; 4</td>
</tr>
</tbody>
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Primer:  
<table>
<thead>
<tr>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>T7 Universal F</td>
</tr>
<tr>
<td>T7 Terminator R</td>
</tr>
<tr>
<td>Polyhedrin F</td>
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<tr>
<td>EBV R</td>
</tr>
</tbody>
</table>

Buffers:  
Nickel Bind: 35 mM NaH$_2$PO$_4$, 300 mM NaCl, 15 mM Imidazole, pH 8.0
Nickel Wash: 20 mM Tris, 300 mM NaCl, 40 mM Imidazole, pH 8.0
Nickel Elution: Nickel Wash with 250 mM Imidazole
Strep-Tactin Bind: 20 mM MOPS acid, 10 mM NaOH, 200 mM NaCl. Final pH will be around 7.2 without additional adjustment. Many other buffers will work; consult the manufacturer’s information sheets if desired (companies that make Strep-Tactin resins are IBA and GE).
Strep-Tactin Elution: Strep-Tactin Bind with 2.5 mM desthiobiotin

BocFn3 and CdoFn3 Expression

2. Pick a colony and add to 2 mL LB-Kan in culture tube for starter culture. Shake overnight at 37°C.
3. The following morning, add starter culture to shake flask of LB-Kan. I normally use 0.5 L of media in a 2 L baffled flask shaking at 225 rpm at 37°C. You can scale up or down as desired. These proteins are well-expressed; you can expect several tens of mg of protein per L of culture.
4. Grow culture until the optical density at 600 nm is approximately 0.7.
5. Switch incubator to 16°C and shake for an additional hour to completely chill the culture. Alternatively, if you are in a hurry, you can chill the culture on wet ice. If you do not have
access to a chilled shaking incubator, I *believe* these proteins can be expressed at higher temperatures, but I have not done so personally, so you will have to try it yourself.

6. As soon as the culture is chilled to 16°C, induce with 0.5 mM of IPTG.

7. Allow induction to proceed for 18–24 hr.

8. Harvest the cells by centrifugation. Cell pellets can be lysed immediately for protein purification, or they may be frozen and stored for later. Pellets may be frozen directly in centrifuge bottles and stored at either -20°C or -80°C. They may also be resuspended in a small amount of PBS + 10% glycerol and transferred to falcon tubes before re-pelleting and freezing.

**BocFn3 and CdoFn3 Crude Purification**

1. As much as possible, carry out all steps on ice or at 4°C.

2. Resuspend cell pellet in Nickel Bind Buffer (25 mL buffer per 5 g of packed cells).

3. Add PMSF to 1 mM (from stock solution of 100 mM in methanol). Commercial protease inhibitor tablets can also be used.

4. Add benzonase (1 μL per 10 mL of cell suspension) or DNase.

5. Ideally, lyse cells using a French Press. A microfluidizer is a good substitute, but usually results in some dilution of your lysate, depending on your machine. Other lysis methods of your choice may be tried, but you may have to modify resuspension volumes and possibly other purification parameters.

6. Pellet debris by high speed centrifugation, and filter the supernatant through low-binding syringe filters (pore size 0.45 μm or smaller).

7. Apply cleared lysate to 5 mL of nickel resin (you may need more if you are using large culture volumes). Standard loose nickel resins may be used, such as Nickel-NTA agarose (Qiagen), or you may use pre-packed columns such as nickel-charged Chelating HiTraps or HisTraps (GE). Purifications are not sensitive to flow rates, small fluctuations in temperature, etc., and may be run by gravity, manual syringe, or FPLC.

8. Wash with 3 column volumes of Nickel Wash Buffer.

9. Elute with 3 column volumes of Nickel Elution Buffer.

Alternative to (9): For minimal volume elution (pre-packed columns only, such as HiTrap): After washing, elute with 0.8 column volumes and discard, then elute with 1.4 column volumes and retain. With a 5 mL HiTrap column, this will result in a 7 mL elution fraction containing most of the protein, very concentrated, with little loss. If desired, this can be applied directly to a preparative gel filtration column, if you don’t mind your protein having tags.

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If you do intend to proceed directly to gel filtration, I recommend adding 5 mM EDTA to the protein after elution, to prevent Nickel-mediated multimerization of the His-tagged protein (some amount of nickel is normally stripped from the column during elution).

At this point, the protein is pure enough for many applications. For additional purity, I often use the following sequence:

**Additional Purification**

1. Remove tags overnight at 4°C with His-tagged TEV protease in the presence of 5 mM EDTA and 5 mM DTT or TCEP.

2. Dialyze or desalt the de-tagged protein into Nickel bind buffer. Alternatively, if you intend to perform an anion-exchange step, dialyze or desalt into a low-salt buffer suitable for anion exchange, such as 20 mM Tris pH 8, 50 mM NaCl. Check the predicted pI of the protein and make sure your buffer pH is at least 1 pH unit higher.

3. (Optional) Apply protein to anion exchange column. Elute using a gradient from 50 mM to 1.0 M NaCl. Pool the fractions which contain your protein. If the protein eluted at an average salt concentration of less than 300 mM NaCl, you will need to add NaCl to a concentration of about 300 mM before binding to the nickel column in Step (4).

4. Using a system capable of gradient elution, apply the protein to a nickel column, and keep the flowthrough. This is a “reverse nickel” column, since you are isolating the NON-tagged protein.

5. Apply a linear imidazole gradient from 0 to 250 mM over 20 column volumes. This will separate de-tagged proteins that are weakly bound to the column (will generally elute before 60 mM Imidazole) from tagged proteins (uncut fusion protein and tagged TEV protease).

6. Analyze flowthrough and elution fractions by SDS-PAGE and pool those containing the de-tagged protein. It is possible that ALL de-tagged protein may bind the column and come off at low imidazole concentration, in which case you can simply discard the flowthrough. Alternatively, some of it may flow through, and some may come off at low imidazole. I do not advise skipping the SDS-PAGE step.

7. Concentrate to an appropriate volume for gel filtration (this depends on your column size but should be less than 5%, and ideally less than 3%, of your gel filtration column volume).

8. Purify by gel filtration (Superdex75 prep grade would be an appropriate choice) in a buffer of your choice. PBS and MOPS-based buffers with 150–300 mM NaCl, pH 7.5–8.3, are compatible with cyanogen bromide coupling, if you are trying to make BocFn3 or CdoFn3 CNBR resins.
**Hhip Expression**

I use ISFM in aerated spinner flasks to grow insect cells. For other media, you may have to alter and re-optimize cell concentrations and viral inoculation amounts. Prepare P3 virus according to instructions and calculations in the Bac-to-Bac system manual (Invitrogen). I normally do not titer viruses, because it is faster and simpler to just determine optimal infection volumes empirically.

1. Grow High Five cells in suspension to 1–2×10^6 cells/mL. We use 1–6 L aerated stir flasks for our growths.

2. Infect cells with ~20 mL of P3 virus per L of cells, or whatever amount you have determined is optimal.

3. Allow expression to proceed for 72 hr.

4. Remove cells by centrifugation and retain Hhip-conditioned medium.

**Hhip Crude Purification**

Hhip is secreted into the growth medium as a fusion with His and SBP (“Streptavidin Binding Peptide”) tags. Hhip is expressed at moderate levels, in the low single digit mg/L range, so you may have to process large amounts of media.

Unfortunately, insect media have components that interfere with purification. Chelators present in most insect media will strip typical nickel resins (Qiagen or generic NTA-agarose, HiTrap Chelating HP, HisTrap, etc. will all be stripped), and biotin present in the media will ruin streptavidin or Strep-Tactin columns. Dialysis may be used, but this is awkward and time-consuming when dealing with large volumes of media (my typical preps involve 1–6 L of media, which would all need to be dialized).

I have therefore worked out purification methods using special chelator-resistant nickel resins. For Hhip purifications using this method, you MUST use the exact nickel resin specified below—you CANNOT substitute other nickel resins.

1. Partially clear the medium (of intact cells and large particles) using a quick centrifugation (e.g. 5 min at 5,000 rpm in 1 L bottles).

2. Adjust pH of partially cleared medium to 8.0 by adding concentrated NaOH, while stirring vigorously and monitoring with a pH meter.

3. Many insect media are phosphate-buffered, and also contain Ca^{2+} ion. Thus, as you raise the pH, large amounts of calcium phosphate will precipitate (this will definitely occur with ISFM). Do not be alarmed when you see this precipitation.

4. Centrifuge the media at the maximum speed your bottles and rotor can handle for 30 min. We often use 30 min at 10,000 rpm in ~400 mL bottles; around 17,000 g for our rotor. This extensive centrifugation is critical for removing fine cell fragments that will otherwise clog filters. It will save many, many tedious hours of waiting and having to repeatedly change clogged filters.
5. Filter the media. We use 500 mL bottle-top filters, 0.22 μm PES. You should be able to quickly and easily filter multiple liters through a single bottle-top filter if you have centrifuged your media correctly.

6. Apply filtered media to 15 mL packed bed of Roche “cOmplete His-Tag Purification Resin.” I normally use a gravity-fed Econo-Column (such as Bio-Rad #737-2522, 2.5 × 20 cm). For multi-liter quantities, I often do the application at room temperature overnight. Set your column up to siphon from a large bucket of filtered media, and leave a loop of tubing that drops down to below the level of the column bed; this will ensure that the column doesn’t run dry at the end while unattended.

7. After all media is applied to the resin, wash with 5 column volumes of 20 mM Tris pH 8, 300 mM NaCl. Do NOT include any imidazole in the wash buffer, as this nickel resin is more sensitive than others to elution by imidazole.

8. Elute with 3 column volumes of Nickel Elution Buffer.

Hhip is usually still somewhat impure at this point, especially if you have included any serum in your growth media. Some additional purification is typically required. I usually use the following sequence:

**Additional Purification of Hhip**

1. Apply protein to a 5 mL StrepTrap column by syringe or using an FPLC system.

2. Wash with 3 column volumes of Strep-Tactin Bind Buffer.

3. Elute with Strep-Tactin Elution Buffer. Up to 5 column volumes may be needed for complete elution. You can collect fractions if desired and check by SDS-PAGE to decide which ones to pool.

4. Regenerate the column immediately by applying 5 column volumes of 0.5 M NaOH and 5 column volumes of H2O. Apply 20% ethanol for long-term storage. StrepTrap columns are relatively expensive, but can be re-used a large number of times if cleaned and maintained consistently in this way.

5. Pool protein and add His-tagged TEV protease overnight to remove tags. For Hhip, I have found that it is not always necessary to add EDTA and reducing agents for complete tag removal. It will save you a dialysis step if you don’t add them.

6. Adjust NaCl to 300 mM and pH to 8.0 and run a reverse nickel column, similar to instructions above for BocFn3.

7. Perform final gel filtration step in a buffer of your choice. PBS and MOPS-based buffers with 150–300 mM NaCl, pH 7.5–8.3, are compatible with cyanogen bromide coupling, if you are trying to make CNBR-Hhip resin.
Bibliography


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Curriculum Vitae

Thomas Cleveland

Birth

January 23, 1985 Covington, LA

Education:

2007-Present Ph.D. candidate, Johns Hopkins University School of Medicine, Baltimore, MD. Program in Molecular Biophysics. Anticipated completion in the Spring of 2014.

2003-2007 B.S., Tulane University, New Orleans, LA. Majors in Cell and Molecular Biology, Physics, minor in Mathematics, summa cum laude with departmental honors in physics.

Research

2007-Present Graduate Student, Johns Hopkins University School of Medicine, Baltimore, MD, under Prof. Daniel J. Leahy, Department of Biophysics and Biophysical Chemistry. Structural and biochemical studies of Hedgehog and EGFR signaling.


2004 – 2005 Research Assistant, Tulane University, New Orleans, LA under Prof. Alexander Kurganov, Dept. of Mathematics. Used FORTRAN to implement numerical schemes for solving certain classes of partial differential equations, which were applied to a reaction-convection-diffusion model of microbial growth and chemotaxis.

2004 – 2005 NIST SURF program, National Institute of Standards and Technology, Gaithersburg, MD under Dr. Muhammad Arif, Neutron Physics Group. Constructed an apparatus for the collection of images for neutron tomography. As proof of concept for the analysis of small biological samples, applied this technique to study grain compromised by fungal attack (Cleveland et al., 2008).

Peer-Reviewed Publications:


Presentations:

Poster. Detergent-Solubilized Patched Purified from Sf9 Cells Fails to Interact Strongly with Cognate Hedgehog or Ihog Homologs. 44th Mid-Atlantic Macromolecular Crystallography Meeting, Rockville, MD 2014.


Courses and Workshops:

2012    Mid-Atlantic Macromolecular Crystallography Meeting, Charlottesville, VA. Interactive workshop on lipidic cubic phase crystallization. Instructor: Dr. Vadim Cherezov, Scripps Research Institute


Teaching experience:

2009    Teacher’s Assistant to Prof. Juliette Lecomte, Johns Hopkins University Department of Biophysics, for the course "Spectroscopy and its application in biophysical reactions."

2007    Teacher's Assistant in Physics at the ADVANCE Program for Young Scholars, Northwestern State University, Natchitoches, Louisiana. ADVANCE is a three week residential summer program for students in grades 8-12.