

INTRAMEMBRANE HELIX-HELIX ASSOCIATION IN OLIGOMERIZATION AND TRANSMEMBRANE SIGNALING

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KEY WORDS: dimerization, protein interaction, receptor conformation, signal
transduction, receptor structure

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INTRODUCTION

Many functions of cell surface membranes are carried out by proteins that are integrated across the lipid bilayer. These transbilayer proteins serve a large and diverse array of important biological functions critical for cell survival. Although investigators using cDNA cloning have deduced the sequences of numerous transmembrane proteins in the past several years, precise information regarding these proteins' secondary, tertiary, and quaternary structure is very limited (1). Moreover, the mechanisms by which these transmembrane proteins function, transmitting solutes or information across the lipid bilayer, are even less well defined (79).

In this review, we describe how a two-stage model of protein folding and helix-helix association (73) can be applied to models of signal transduction for proteins that contain a single transmembrane domain. We provide some general discussion of transmembrane structures and theoretical models as to their role in signaling. The focus is not on the primary formation or membrane insertion of the helices but on their assembly with other helices to form tertiary and quaternary structures. We present the emerging evidence that many single helix proteins oligomerize to transduce information to the cytoplasmic domain and the functional attributes of oligomerization and relate this evidence to recent structure discussions (72).

FOLDING OF MULTIHILIX TRANSMEMBRANE PROTEINS

Multiple-helix proteins as a group include molecules such as bacteriorhodopsin, rhodopsin, the anion transporter channels, sugar transporters, ATPases, and receptors including those that bind substance K, lutropin, follicle stimulating hormone, and the muscarinic receptor family (31, 42, 76, 105). The amino acid sequences of multiple helix proteins predicted from cDNA sequences allow provisional models for their arrangement in the membrane (105). Hydrophathy profiles and related algorithms can predict the location of the putative transmembrane segments reasonably well. The secondary structure of the aqueous domains of the extracellular and intracellular loops connecting the transmembrane helices can also be predicted somewhat less successfully (26, 42, 104). However, we have no useful algorithm to predict the tertiary arrangement of helices. Although all possible arrangements may be considered as formal possibilities, certain specific arrangements appear to be likely, as described below for bacteriorhodopsin.

To be useful, a view of folding necessarily contains a set of assumptions,

several of which are made in this review. The first assumption is that transmembrane proteins are folded in a stable state regardless of the specific mechanism of insertion into the bilayer. Second, the transmembrane orientation of proteins is stable, and such proteins do not flip from one side of the bilayer to the other (70). And last, the intramembranous domains of the transmembrane proteins are assumed to be α -helices where they contain hydrophobic regions sufficient to span a bilayer. The energy considerations of the hydrogen-bonding properties of a hydrophobic α -helix in an apolar environment indicate such helices (26). Obvious exceptions to this assumption are the transmembrane domains of the porins (111), which are β -barrels defining aqueous channels across bacterial outer membranes, but porins do not have the hydrophobic sequences characteristic of transmembrane helices.

Energetics of Folding in Two Stages

The transmembrane domains of multiple-helix proteins are characterized by hydrophobic stretches of about 20 amino acids. The primary structures indicate that each of the transmembrane domains might be independently stable as a separate transbilayer structure interacting with only the non-polar region of the lipid bilayer. In folded proteins, such as bacteriorhodopsin or reaction centers, the conformation of these segments is altered little from that which would be predicted as the conformation and transbilayer locus of the separate entities. Therefore, the interaction energies producing the tertiary structure of the folded molecule may not be as strong as those stabilizing the helices. The separation of the folding of independent helices from their association in higher order structures divides the energy constraints into two stages. Based on this view, a two-stage model of helix formation followed by helix-helix association was recently proposed by Popot & Engelman (73). Although the present review does not repeat the rationale of this model in detail, the general principles and arguments must be reiterated as they are the basis for the proposal that oligomerization and transmembrane signaling of single-helix membrane proteins may involve helix-helix interactions inside the bilayer.

In simple terms, the two-stage model predicts that a protein such as bacteriorhodopsin, which has seven transmembrane domains (41), could first fold each α -helix to an independent, stable conformation across a bilayer and then assemble these transmembrane helices into a tertiary structure in which the helices are not much altered. The first stage can be accounted for by the hydrophobic effect and hydrogen bonding that lead to the stability and conformation of the helices. Association of the helices, the second stage, requires the participation of other factors including polar interactions, links between helices, links to extra-membrane domains or

other proteins such as cytoskeletal proteins, interactions with prosthetic groups, and packing effects. Much experimentation has focused upon bacteriorhodopsin in order to test the propositions of the helix-association model.

Bacteriorhodopsin Folding

Strong evidence in favor of the two-stage folding model has come from studies of the refolding and functional activity of proteolytically cleaved bacteriorhodopsin. Bacteriorhodopsin can be functionally reconstituted from proteolytic fragments in a variety of environments (41, 46, 48, 58, 74). When two proteolytic fragments are separately incorporated into two populations of lipid vesicles, each fragment forms a set of transbilayer helices. When the two populations of vesicles are fused, bacteriorhodopsin reforms from the association of the fragments and is functionally active (74). The crystal structure of such renatured molecules is indistinguishable from that of the native molecule at low resolution (75).

The significance of these kinds of experiments is that although the separate domains of bacteriorhodopsin are refolded under conditions that greatly differ from those occurring *in vivo*, the structures of the separate pieces are close to those expected if each helix forms independently. Furthermore, side-to-side interaction of the helices can occur as demonstrated by the fact that subunits recognize each other and form a folded molecule. The two-stage hypothesis predicts these data. Each individual helix must appropriately form and span the membrane prior to the association with neighboring helices to yield the complex, biologically active structure. These data also call attention to the role of polar forces and packing effects in helix-helix interactions. In this regard, helices are known to engage in detailed close packing (1, 22, 77, 115), and this packing interaction must overcome the entropy that favors helix separation (73).

Two-Stage Folding and Single-Helix Proteins

At the level of the lipid bilayer, the energetics required for packing helical transmembrane regions of a multiple-helix protein into a subunit are not different from packing transmembrane domains of separate single-helix proteins into an oligomer because stable helices can be regarded as folding domains (72). This is illustrated by the bacteriorhodopsin experiments in which refolded bacteriorhodopsin fragments behave as the two subunits of a heterodimer (73). The initial formation of independently stable helices is therefore critical for both multiple- and single-helix proteins. As a rule, genomic intron segments never occur in the coding sequence for transmembrane regions (47), supporting the idea that the helices are important domains. Furthermore, proteins such as the major intrinsic channel

protein (MIP) contain tandem sequence repeats indicative of the evolution of multiple-helix proteins from ancestral single-helix homodimers (111). The oligomerization of single-helix proteins is also probably of significance in signaling, as we discuss below.

TOPOLOGY OF SINGLE-HELIX PROTEINS

A wide variety of transmembrane proteins have a single apparent helix, including the influenza virus hemagglutinins, the histocompatibility antigens, the tyrosine kinase growth factor receptor family, immunoglobulin, the T-cell receptor, and the integrin receptor families (19, 72). Receptor proteins can be segregated into three chemically distinct domains: extracellular, transmembrane, and cytoplasmic. Notable diversity is found in the primary and secondary structures of the extra- and intracellular domains of these transbilayer proteins. However, the transmembrane segments generally are similar in length and hydrophobicity.

The transmembrane segments of single-helix proteins are composed of approximately 20 hydrophobic and uncharged amino acids, and are probably helical. The simplest argument for helicity comes from consideration of the geometry necessary to cross the lipid bilayer with about 20–22 amino acids. Twenty amino acids packed into an α -helical secondary structure would fold into six helical loops and extend about 30 Å, the length of the nonpolar region of an average lipid bilayer. Studies designed to test the minimum number of apolar residues needed for a protein to be stably maintained in the membrane showed that a lower limit of 12–14 amino acids maintains membrane insertion. This finding leaves open issues as to whether the thickness of the membrane can be locally dictated by the length of the transmembrane segment, whether potentially charged residues can be stably integrated into the bilayer, or whether alternative secondary structures might maintain the integrity of the transmembrane segments (21, 37). The energy cost of burying a potentially charged residue in its uncharged form can be offset by the net energy of burying the nonpolar residues of a 20-amino acid helix (26). When single-helix proteins are studied using circular dichroism in the presence of detergents, the transmembrane segments give spectra characteristic of α -helices (19).

SIGNALING THROUGH THE BILAYER

Given that the transmembrane regions of single-helix proteins are similar in overall composition, secondary structure, and length, it remains a conceptual problem to explain how signals are transmitted through the transmembrane helices and cellular functions are activated. When a simple

receptor such as that for the epidermal growth factor (EGFR) is bound to its ligand, EGF, the calculated stoichiometry of ligand binding to receptor is 1 : 1 (81, 98, 106). However, a Scatchard analysis of this binding event reveals nonlinear plots indicative of two receptor populations of different affinities. About 5% of the total receptor population is considered high affinity, and the remainder are low affinity (52). Given these observations for numerous single-helix membrane receptors, what is the physical difference, if any, between a high affinity receptor and a low affinity receptor if the primary and secondary structure of these receptors are identical?

Monomolecular Models

Several models have been advanced to explain signal transduction and high versus low affinity receptors based on monomeric receptors. We argue that such mechanisms are unlikely given the fluidity and deformability of the lipid bilayer. Bilayer fluidity has been explored by several means, and was well established at an early date (85). The viscosity of a lipid bilayer is approximately 1 centipoise, the approximate viscosity of olive oil. Molecular diffusion both of proteins and of lipids when they are unconstrained by linkage to other macromolecules is rapid. Further, the bilayer is known to be deformable; a thickness change of 30% corresponds to only about 1 kT in energy (62). Measurements of bacteriorhodopsin aggregation suggest that this local deformability is used to accommodate rather large mismatches between the hydrophobic thickness of a transmembrane protein and the thickness of the lipid bilayer (61). Thus, any proposed mechanism must be framed in terms of the deformable, fluid environment in which membrane proteins reside.

One mechanism assumes that a single helix can independently transmit information via an allosteric change in the α -helix. This model of single-helix transmission would include models that suggest that the α -helix unfolds, breaking one or more of its hydrogen bonds. The chain of energetic causality is not closely reasoned in such models, since the energy for local bilayer deformation is small compared to the energy required to break a hydrogen bond (5–6 kcal per mole) (73). A related mechanism posits that prolines within the α -helix may undergo *cis-trans* isomerization upon ligand binding (10), redirecting the protein chain. This model also suggests that proline carbonyl groups can act as intramembranous cation binding sites, the binding of which might also activate second messenger systems. Again, the energetic details of such mechanisms are not spelled out. To be credible, such proposals must invoke either known mechanisms derived from studies of related structures in other proteins or have a complete rationale in terms of the resistive as well as active elements in

the causal chain. Such arguments have not yet been put forward, and it is unclear how the events would be carried out in a fluid environment.

A third mechanism is based on the idea that a rotation of the helix in the membrane would change the relative geometry of the cytoplasmic domain. This model seems particularly unlikely given the fluidity of the lipid bilayer, because the helix is already in a low viscosity environment that permits rapid rotational diffusion (85).

A fourth mechanism, which might be referred to as the push-pull model, suggests that ligand binding causes in the extracytoplasmic domain a conformational change that either pulls or pushes on the transmembrane helix, thereby altering the cytoplasmic domain and triggering signaling. Given the deformability of the lipid bilayer (62), the proposed mechanism is far more likely to result in a change in the local bilayer thickness than a conformational change in the protein. The low energy of such deformation events would not provide sufficient specificity for information transfer to occur without a very high noise background.

The single-molecule models are diagrammed schematically in Figure 1.

SIGNAL TRANSDUCTION: MONOMOLECULAR MODELS

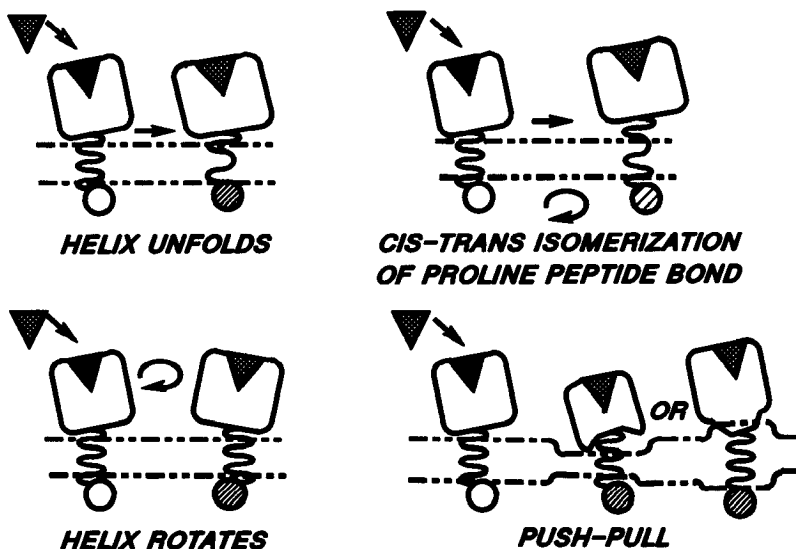


Figure 1 Signal transduction: single helix models. The schematic diagram shown suggests four mechanisms of transmitting information through the bilayer via a single-helical domain. Each diagram depicts the conformation of the receptor before and after interaction with ligand. The models, described in the text, suggest that the helix unfolds, proline peptide bonds isomerize, the helix rotates, and that the helix is pushed or pulled.

Given the known properties of lipid bilayers and the failure at this point to reason a causal chain in such an environment, none of these models seems a strong hypothesis for a mechanism of low noise information transfer across a membrane.

Oligomerization Models

The second general concept of signaling through the transmembrane region requires more than a single-molecule receptor. One such model proposes that receptors are in an equilibrium between monomeric and oligomeric states. In the presence of a signal, the ligand molecule either physically bridges two receptor molecules or binds to a single receptor ectodomain, possibly changing the conformation of the ectodomain, to stabilize dimerization or oligomerization. In an oligomerization event, the sum of three energies is involved: the interaction of the ectodomains, the interaction of the transmembrane domains, and the interaction of the cytoplasmic domains. Thus, the alteration induced by receptor binding need only displace the sum of these energies to give a favorable free energy for interaction, which might be accomplished either by an additional favorable interaction of ectodomains or by a relaxation of an unfavorable interaction. Either mechanism of ligand action would result in the creation of a new oligomeric structure in the cytoplasm, which could trigger signaling. This hypothesis also predicts that, upon release of ligand, the oligomers would dissociate and return to their basal state of equilibrium. A difficulty with this model concerns the rapid diffusion kinetics of proteins in lipid, which would suggest that monomeric receptors might frequently collide and produce a signal background from transient oligomers. This would, in effect, compromise the controls or constraints on receptor activation that are overcome by ligand binding. However, the difficulty is avoided in the mechanism involving the removal of unfavorable interactions between the ectodomains as a consequence of ligation.

A corollary of the oligomerization model incorporates an oligomerization step with a subsequent allosteric global conformational change of the receptor complex upon interaction with ligand. This allosteric or conformational change could be a ligand-induced rotation of one subunit of the dimer in opposition to the other, creating a new relationship of cytoplasmic domains, and might require a close association or contact with all or some of the receptor domains. This model would also include receptors that exist as oligomeric, heterodimeric, or covalently linked dimers in their basal state, which would bind ligand and signal by an allosteric change. Figure 2 shows schematic diagrams of the multiple-helix models.

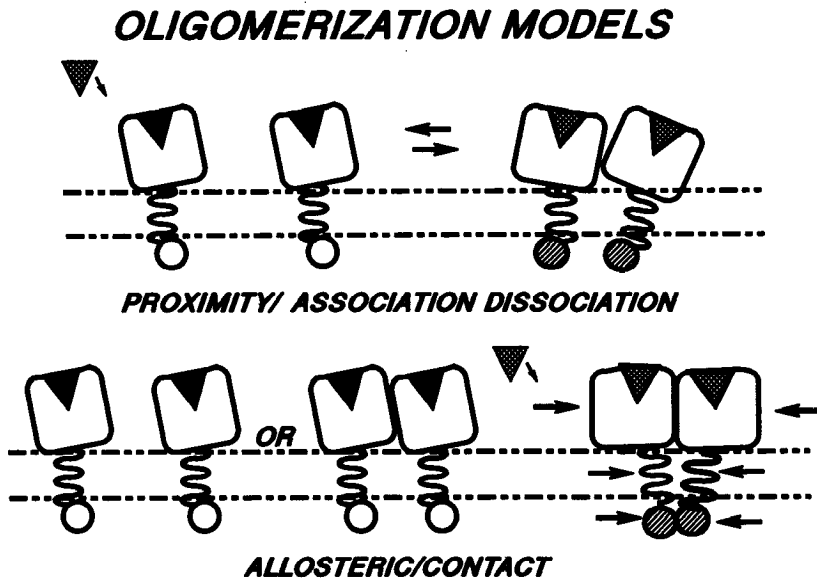


Figure 2 Oligomerization models. The upper schematic diagram suggests an association-dissociation equilibrium that favors oligomerization of proximal receptors in the presence of ligand (*top, right*). The lower diagram suggests that receptors may exist as monomers or oligomers and may oligomerize and/or alter their conformation by an allosteric mechanism in the presence of ligand. The conformational activation of the receptors would involve close association of all of the domains of the receptor protein as signified by the small arrows in the diagram.

EXAMPLES OF OLIGOMERIZATION AND SIGNAL TRANSDUCTION

Oligomerization and conformational change have been studied using a variety of methods in many biological systems. These methods include covalent cross-linking by insertion of unpaired cysteine residues, chemical cross-linking, separations by sucrose gradients, column chromatography or electrophoresis under nondenaturing conditions, and the construction and expression of truncated, mutated, and chimeric receptors. Studies of the statistical probability of protein-protein interaction in membranes involving the limited volume of the membrane and the true concentration of bilayer proteins present predict that the likelihood that membrane proteins would form oligomers is a million-fold greater than the probability that these proteins would exist in isolated states (35).

Examples of oligomeric signaling also include covalently linked dimers, like the insulin receptor, and heterodimeric proteins such as the integrin

family of molecules. The glycoporphin A protein is a stable dimer under a variety of conditions in vitro (10), and rotary shadowing techniques have also shown it to be a dimer (25). Complex receptors such as the T-cell receptor, lutropin receptor, and follicle stimulating hormone receptor are also thought to exist as dimers (76, 86).

In a study designed to assess the mechanism of signal transduction in the bacterial aspartate receptor, unpaired cysteines were used to replace charged residues in various locations in the ecto-, transmembrane, and cytoplasmic domains (28). Upon stimulation with ligand, significant changes were observed in the rate of disulfide-bond formation at most of the locations that the cysteines were placed. This is direct evidence for a ligand-induced global conformational change affecting large structural portions of the receptor during transmembrane signaling.

The Tyrosine Kinase Receptor Family

The family of tyrosine kinase receptors is proposed to transduce signals by oligomerization and allosteric conformational changes after binding to ligand (99). This family includes the epidermal growth factor (EGFR), platelet-derived growth factor (PDGFR), colony stimulating factor (CSFR), and insulin receptors, and the HER2/*neu* and *trk* protooncogene-coded proteins (2, 40, 49, 97, 98, 100).

The EGFR and PDGFR have been postulated to exist at an equilibrium between monomers and dimers and to rapidly and reversibly form dimers upon stimulation with ligand (40, 113). The EGF and PDGF receptors have been chemically cross-linked in the presence of ligand on living cells, membrane preparations, and highly purified receptor preparations (6, 13, 16). The amount of covalently cross-linked EGF-induced dimer correlates with the concentration of ligand (29). The EGF-receptor has been studied using nondenaturing gel electrophoresis and sucrose gradient centrifugation and found to exist as monomers and noncovalent dimers with a significant increase in the amount of dimer in the presence of EGF (113). The dimeric fraction is of higher affinity for ligand and demonstrates higher levels of phosphorylation (8). Sedimentation analysis of the PDGFR has indicated that it exists exclusively as a dimer in the presence of ligand. The dimerizing effects of EGF and PDGF are both reversible and saturable (6, 113). These data support the hypothesis that oligomerization is necessary for signal transduction. The observation that several of the ligands for members of this family are bivalent, such as PDGF and CSF-1, suggests that these ligands are designed to mediate dimerization of neighboring receptors (38, 40, 83). Furthermore, if the bivalent ligand is a heterodimer, as found for PDGF, it may serve as a selective control by the cell that

mediates which second messenger pathway is activated depending on the composition of the ligand (99). The EGF ligand, however, is monomeric, which suggests that the ligand may not be simply bridging the receptor but instead may induce a conformational change (36).

A signaling mechanism using dimerization would also imply the possible existence of hybrid complexes between structurally similar receptors such as the α - and β -type PDGFR, EGFR, and HER2/*neu* or insulin and insulin-like growth factor receptor (IGF-1-R). Researchers have demonstrated hybrid complexes in several cases. PDGFR α forms heterodimers with PDGFR β , and insulin receptor forms a heterodimer with the IGF-1 receptor (87, 95). The EGFR-HER2/*neu* hybrid complex has also been detected by chemical cross-linking and immunoblotting (34, 103). It is not yet known if these hybrid receptors are functionally active (99).

Phosphorylation of the cytoplasmic domain on tyrosine appears to release an internal constraint by establishing a conformation of the receptor that is competent to interact with and phosphorylate many cellular substrates (99). The PDGFR, EGFR, and insulin receptor all reportedly phosphorylate between receptors (43, 99). For example, PDGF can stimulate a kinase-inactive β subunit when expressed with a normal α subunit (51, 95). The HER2/*neu* is also known to be a substrate of the EGFR (53, 90). Evidence indicates that synergistic interactions between HER2/*neu* and EGFR occur. When EGFR is coexpressed with non-transforming *neu* protein, the cells transform, while neither protein individually expressed has this effect (56). The EGFR when cotransfected with a kinase-negative point mutation becomes phosphorylated. These data are evidence for intermolecular phosphorylation, but cannot differentiate between intra- and interoligomeric mechanisms (44).

Some data suggest that the EGFR can signal via a single-helix mechanism. Purified EGFR preparations reportedly exist as inactive dimers that dissociate to active monomers upon binding ligand and ATP (7, 13). This report is supported by the observation that the EGFR isolated from human placenta, which is totally monomeric, is fully active (57, 71). Preparations of monomeric EGFR can also be activated under artificial conditions, for example in the presence of 0.25 M ammonium sulfate (57, 71). Furthermore, recent studies using resonance energy transfer in living cells, in contrast to membrane preparations, show no evidence of microaggregation (14). The lack of microaggregation in living cells suggests that the receptor may be constrained by neighboring proteins or cytoskeletal elements; such interactions could overcome the difficulties with monomolecular mechanisms mentioned above. Furthermore, a signal mechanism based on an inactive dimer and active monomer is plausible.

TOPOLOGY OF INTERACTIONS IN OLIGOMERS FOR SIGNALING

If oligomerization and allosteric conformational change of receptors is required upon ligand binding to trigger signaling, what portions of the receptor molecule are required to participate? Is the interaction of a part of a receptor with an intact receptor sufficient to form an oligomer and/or trigger signaling? These questions have been studied by constructing truncated and chimeric receptors that delete or replace specific domains.

Ectodomains

We have several examples of an association site residing in the ectodomain of transmembrane proteins. Hemagglutinin lacking a transmembrane and cytoplasmic domain can form a trimer (23, 25). However, this oligomer derives significant stability from transmembrane-domain interactions, suggesting that the domain also has a complementary site (23). Histocompatibility-locus antigen heavy chains lacking transmembrane and cytoplasmic domains can form heterodimeric complexes with β 2-microglobulin (59). Soluble ectodomains derived from the cell adhesion molecule CD11b/CD18, a heterodimer, can form a soluble heterodimer when the ectodomains of each subunit are secreted from cotransfected cells (20). Peptides derived from the ectodomain of histocompatibility complex class I antigens are able to associate with the ectodomain of the insulin receptor and inhibit the activation of the receptor without inhibiting the binding of insulin (39).

Chemical cross-linking has shown that soluble ectodomains of the insulin, epidermal growth factor, and platelet-derived growth factor receptors associated with their respective native ectodomains when the soluble forms were coexpressed or incubated with the intact receptors in the presence of ligand (4, 96, 110). The soluble ectodomains inhibited the stimulation of the intact receptors by a mechanism other than competition for ligand. These observed associations of the ectodomains were specific. For example, the EGFR ectodomain did not inhibit the function of the PDGF, insulin, or HER2/*neu* receptors (4).

Constructs of the PDGF-receptor that contain the transmembrane and ectodomains have been made to look at the effect of a membrane-bound ectodomain. In the presence of ligand, this construct could form a heterodimer with full-length PDGFR as well as a truncated homodimer (96, 110). Neither the truncated homodimer nor the heterodimer with the wild-type receptor was functionally activated by ligand, as assayed by tyrosine phosphorylation and calcium mobilization. The data presented clearly show that the ligand-dependent association of the ectodomain segments of

the tyrosine kinase receptor family is insufficient to induce the appropriate conformational change in the cytoplasmic segment and trigger the tyrosine kinase activity. Thus, an activating conformational change in the receptor may require the association of the transmembrane and cytoplasmic domains in coordination with the ectodomains.

Transmembrane and Cytoplasmic Domains

Other examples show that the transmembrane domain has a significant role in mediating oligomerization. The best example is the demonstration that a synthetic peptide corresponding to the transmembrane domain of glycoporphin A can compete for the full-length molecule in order to form a heterodimer comprised of a subunit of full length and a subunit of peptide (9). This reaction was also determined to be sequence specific because peptides corresponding to homologous but nonidentical transmembrane domains did not inhibit the dimerization (9). The deletion of the transmembrane domain of gp160, the envelope glycoprotein of HIV-1, causes the protein to lose its propensity to dimerize (94). Manolios et al (65) recently demonstrated that the transmembrane sequence of the α -chain of the T-cell receptor is critical for the α -chain to associate with the CD3 δ -chain subunit of the T-cell receptor. A subdomain in this transmembrane segment, which contained two charged residues, was requisite for the T-cell receptor assembly, and the charge motif could be placed in an unrelated receptor transmembrane domain and restore assembly of the T-cell receptor subunits (18).

Replacement of the transmembrane domain of the PDGF receptor with that of the EGF receptor abolished ligand-mediated signaling of the PDGF receptor, indicating that the transmembrane domain plays a significant role in signaling (57). Replacement of the transmembrane domain of the neural growth factor receptor with the transmembrane domain of the EGFR inhibited the neural outgrowth of transfected cells, indicating that critical information was contained in the transmembrane domain sequence of the nerve growth factor (NGF) receptor (112). Proteolytic removal of the ectodomains of chicken asialoglycoprotein receptors have shown that the transmembrane domain mediates homodimerization of this receptor (63). Truncation of the ectodomain of the *c-ros* protooncogene product in viruses, the EGFR, and the nontransforming *neu* protein, constructs similar to the *v-erb-B* avian oncoprotein (24), activates tyrosine kinase activities. This observation suggests that the ectodomains may act to sterically hinder the transmembrane and cytoplasmic domains from close contact, which would be overcome by binding to ligand (3, 5, 32, 69). The transmembrane domains are required for this amplified activity because the

cytoplasmic domains expressed alone in baculovirus systems have extremely low activity (45, 107).

A recent study that specifically addressed the signaling mechanism by the EGFR transmembrane domain found that truncation of the transmembrane segment by six amino acids, the insertion of a charged residue similar to the *neu* protein mutation, and the placement of three proline residues in the transmembrane region had no effect on signaling (12). These mutations suggest that the monomolecular push-pull and rotational models are not relevant for the EGFR.

In the case of the *neu* protein, a single point mutation in the transmembrane domain of the 185-kilodalton (kDa) protooncogene protein, valine to glutamic acid, induces transforming activity (2). Modeling and chemical cross-linking data suggest that this point mutation induces dimerization and subsequent transformation (91, 108, 109). The glutamic acid can be replaced by a glutamine and weakly by an aspartic acid but no other residues. Also, the glutamic acid residue cannot be moved from the location where it occurs without complete loss of transforming activity (3). The entire ectodomain and large portions of the cytoplasmic domain can be removed using molecular techniques with no diminution of transforming activity (3). The recent report of a valine-to-isoleucine point mutation in the transmembrane domain of amyloid precursor protein in patients with familial Alzheimer's disease has led to the speculation that a dimerizing event similar to that seen with the *neu* protein may be involved (33, 93).

Finally, a close association of the cytoplasmic domain, perhaps mediated by cytoskeletal proteins, may also play a part in signal transduction (78). Studies on the low density lipoprotein (LDL) receptor showed that the 30 terminal residues in the cytoplasmic domain were essential for the formation and stability of LDL receptor dimers (101). In the tyrosine kinase family, a chimeric molecule that contained the ectodomain of the EGFR with the PDGFR transmembrane and cytoplasmic domain lost activity from deletions in the cytoplasmic domain that were not the tyrosine kinase domain (27). This result was interpreted as indicating that regions of the cytoplasmic domain had important conformational effects on the tyrosine kinase domain.

Cotransfection of wild-type PDGF receptor with a truncated mutant that lacks a cytoplasmic domain led to dimerization and heterodimerization with full length PDGF receptor, but the heterodimer was not active. These data indicate that a close association of the ectodomain and transmembrane domain was not sufficient to activate the tyrosine kinase, suggesting a role for the close association of the cytoplasmic domain.

Constructs that placed the ectodomain of the EGFR onto the *v-erb-B*

oncprotein were not transforming except for deletions in the cytoplasmic domain that constitutively activated the receptor (66). And finally, a recent report indicated that tyrphostin molecules, which inhibit the tyrosine kinase activity of the EGFR, did not affect dimerization of the receptors (88). The data presented show that the propensity of specific receptors for oligomerizing and transducing signals may reside in a necessary allosteric change or in a close association in each of the three domains of the receptor.

MODELS OF TRANSMEMBRANE DOMAIN INTERACTION In surveys of proteins known to oligomerize, such as the trimeric structure of influenza virus hemagglutinin or the heterodimeric structure of the class II MHC antigens, the transmembrane regions are fairly well conserved across species. This indicates an evolutionary constraint on the sequence of the transmembrane segments. For example, in a survey of 20 hemagglutinins from several species, only one or two possible amino acids are allowed in some positions in the transmembrane domain, and these positions are spaced every 4 amino acids. The same motif is also found in Rous sarcoma virus gp37 and the poly-Ig receptor (60). As another example, there is remarkable identity between the different heavy-chain transmembrane regions of the MHC class II antigens as well as between the light chain hydrophobic regions (50). The juxtamembrane segments are not conserved, which suggests that a transmembrane association of the heavy and light chains produces a distinct selective pressure on the allowable composition of the transmembrane segments. The same observations have also been made for membrane IgG1, IgG2a, IgM, and the glycophorins (50).

Researchers have generally concluded that the tyrosine kinase receptor family has unremarkable transmembrane sequences (114). However, the transmembrane domain of the PDGF-receptor, for example, is highly conserved across species (110). Various models have been proposed to explain the propensity of the transforming *neu* protein to dimerize. Studies using conformational energy analyses based on empirical conformational energies for polypeptides programs (80) predicted that the non-transforming *neu* protein had a sharp bend at the valine position 664. In the transforming protein, this position is mutated to a glutamic acid and this study predicted that the glutamic acid allowed the transmembrane domain to form an α -helix (11). Gullick & Sternberg (57) have proposed that the side chain of the glutamic acid is protonated because of the hydrophobic environment and could form hydrogen bonds in the bilayer. This model also predicts a close packing of the α -helices to allow the formation of an interreceptor hydrogen bond (15).

In a survey of the transmembrane domain of members of the tyrosine

Table 1 Survey of transmembrane domains with small aliphatic side-chain cluster motif^a

O	1	2	3	4																
A	L	I	V	G	T	L	S	G	T	I	F	F	I	L	L	I	I	F	L	C3B/C4B RECEPTOR
G	I	L	G	L	L	L	V	V	V	A	I	A	G	G	V	L	L	W		Fc RECEPTOR, p51
G	L	V	L	A	A	G	A	M	A	V	A	I	A	R						POLY Ig RECEPTOR
G	S	S	I	G	L	L	L	L	A	L	I	T	A	V	L	Y	K			ALPHA p150/95
P	I	V	A	G	V	V	A	G	I	V	L	I	G	L	A	L	L	L	I	BETA-3 INTEGRINS
G	V	M	A	G	V	I	G	T	I	L	L	I	S	Y	G	I	R			GLYCOPHORIN A
G	L	T	V	G	L	V	G	I	I	I	G	T	I	F	I	L	K			HLA-DR ALPHA
F	F	V	L	G	L	L	F	L	G	A	G	L	F	I	Y	F	R			HLA-DR BETA
S	A	V	V	G	M	S	L	L	A	L	I	S	I	F	A	S	C	Y	M	SEMLIKI FOREST VIRUS E2
G	G	V	A	G	L	L	L	F	I	G	L	G	I	F	F	C	V	R		T4 T-CELL SURFACE PROTEIN
A	A	I	V	G	T	V	A	G	I	V	L	I	G	I	L	L	V			BETA SUBUNIT LFA-1, MAC-1

^a A random survey of the transmembrane helices using the small aliphatic side-chain motif suggested by Sternberg & Gullick (92) was performed. The sequences shown are representative of large families of receptors that contain this motif. The residues that include the sequence in the motif are shown in bold type. C3B/C4B receptor (55); the Fc receptor, p51 (84); Poly Ig receptor (67); Alpha subunit of p150/95 (17); β -3 integrin subunit (30); glycophorin A (68); HLA-DR antigens α and β subunits (50); Semliki Forest virus protein E2 (102); T-cell T4 (CD4) protein (64); β subunits of LFA-1 and MAC-1 (54); and human cellular adhesion molecule-1 (89).

kinase receptor family, Sternberg et al (92) observed that clusters of small aliphatic side-chain residues were conserved in a motif. As glycines are infrequent in transmembrane regions, this motif appears to be significant (82). A survey of random transmembrane proteins, shown in Table 1, demonstrates that this motif is not particular to the tyrosine kinase receptor family but can also be found in the integrin and immunoglobulin families. The motif is found, for the most part, in receptor proteins known to form homo- or heterodimers. The exceptions to this rule are the observation of this motif in the CD4 and ICAM-1 molecules, neither of which is, at present, known to dimerize.

SUMMARY AND CONCLUSIONS

In spite of our greatly expanded knowledge of the primary structures of transbilayer receptor proteins, our knowledge of the tertiary and quaternary structures that define the biological activity of these receptors is scant. If we assume that the transmembrane regions of receptor proteins form stable α -helices regardless of the mechanism of insertion, a two-stage model of protein folding can be applied. For a multiple-helix protein, the two-stage model would predict that stable helical formation would be followed by an association of the helices to form the appropriate tertiary/quaternary structure. The two-stage model of protein folding is supported by various experiments with bacteriorhodopsin demonstrating that

separate proteolytic fragments of bacteriorhodopsin can be refolded separately and can specifically recognize each other in order to associate and form a biologically active molecule.

At the level of the bilayer, we propose that the energetics required for the association and packing of the helical transmembrane regions of a multiple-helix protein should not be significantly different from the association of separate single-helix proteins into an oligomer. Given the homogeneity in primary and secondary structure of the transmembrane regions of single-helix proteins, the association of multiple monomers may physically define high vs low affinity states and be a plausible mechanism of signal transduction.

Increasing data suggest that oligomerization of receptor proteins may be involved in signal transduction. The transmembrane domains of receptor proteins appear to contain information critical to signaling and may be involved in a close contact site between receptors. This observation allows the two-stage model of protein folding for multiple-helix proteins to be directly applied to the oligomerization of single-helix receptor proteins. In addition, significant data suggest that the ectodomains and cytoplasmic domains are also involved in signaling and oligomerization of receptor molecules. In effect, the present data suggest that the most plausible model, both mechanistically and energetically, is one that includes both oligomerization and a global allosteric conformational change involving all of the defined domains of the receptor molecule.

An oligomerization/conformational change model would predict that new sites of close contact would occur between the domains of the receptor molecule, some of which may be between the transmembrane helices. Therefore, experimenters should be able to generate peptides or small molecules that can specifically interfere with either the oligomerization or generation of new close-contact sites involved in the conformational change of the receptor that leads to signaling. In this way, specific receptors might be targeted for inhibition or possibly activation using binding events inside the bilayer.

ACKNOWLEDGMENTS

B. J. Bormann wishes to gratefully acknowledge the efforts of Drs. P. Jayaraj, J. Woska, P. Reilly, T. Kishimoto, and S. Marlin in the preparation of various portions of the manuscript. D. M. Engelman is grateful for support by the NIH, NSF, Boehringer Ingelheim, the National Foundation for Cancer Research, and to J.-L. Popot for discussions.

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