The SCHOOL of nature

II. Protein order, disorder and oligomericity in transmembrane signaling

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Recent reports have revealed that many proteins that do not adopt globular structures under native conditions, thus termed intrinsically disordered proteins (IDPs), are involved in cell signaling. Intriguingly, physiologically relevant oligomerization of IDPs has been recently observed and shown to exhibit unique biophysical characteristics, including the lack of significant changes in chemical shift and peak intensity upon binding. In this work, I summarize several distinct features of protein disorder that are especially important as related to receptor-mediated transmembrane signal transduction. I also hypothesize that interactions of IDPs with their protein or lipid partners represent a general biphasic process with the “no disorder-to-order” fast interaction which, depending on the interacting partner, may or may not be accompanied by the slow formation of a secondary structure. Further, I suggest signaling-related functional connections between protein order, disorder, and oligomericity and hypothesize that receptor oligomerization induced or tuned upon ligand binding outside the cell is translated across the membrane into protein oligomerization inside the cell, thus providing a general platform, the Signaling Chain HOmoOLigomerization (SCHOOL) platform, for receptor-mediated signaling. This structures our current multidisciplinary knowledge and views of the mechanisms governing the coupling of recognition to signal transduction and cell response. Importantly, this approach not only reveals previously unrecognized striking similarities in the basic mechanistic principles of function of numerous functionally diverse and unrelated surface membrane receptors, but also suggests the similarity between therapeutic targets, thus opening new horizons for both fundamental and clinically relevant studies.

Introduction

Cell surface receptors are integral membrane proteins and, as such, consist of three basic domains: extracellular (EC) ligand-binding domains, transmembrane (TM) domains and cytoplasmic (CYTO) signaling (or effector) domains. Upon recognition and binding of a specific ligand, cell surface receptors transmit this information into the interior of the cell, activating intracellular signaling pathways and resulting in a cellular response such as proliferation, differentiation, apoptosis, degranulation, the secretion of preformed and newly formed mediators, phagocytosis of particles, endocytosis, cytotoxicity against target cells, etc. The importance of receptors in health and disease^{1,2} makes the molecular understanding of signal transduction critical in influencing and controlling this process, thus modulating the cell response.

Ligand-induced receptor oligomerization is frequently employed as a key factor in receptor triggering.²⁻⁴ For many receptors, oligomerization is mediated by homointeractions between folded and well-ordered domains, representing a signaling-related functional link between protein order and oligomericity. On the other hand, intrinsic disorder serves as the native and functional state for many signaling proteins⁵ with phosphorylation, one of the critical and obligatory events in cell signaling, occurring predominantly within intrinsically disordered regions (IDRs).⁶ In addition, long IDRs preferentially reside on the CYTO side of many human TM proteins.^{7,8} In this context, the recently reported surprising ability of intrinsically disordered CYTO domains of immune receptor signaling subunits to form specific dimers⁹⁻¹¹ represents a functional link between protein intrinsic disorder and oligomericity. This phenomenon resolves a long-standing puzzle of receptor-mediated signaling and has important fundamental and clinical applications.

Here, I summarize our knowledge on the recently reported distinct features of signaling-related intrinsically disordered proteins (IDPs), including the lack of folding upon binding to protein and lipid partners. I also hypothesize that receptor oligomerization induced or tuned upon ligand binding outside the cell is translated across the membrane into protein oligomerization

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inside the cell, thus providing a general platform, the signaling chain homo-oligomerization (SCHOOL) platform, for receptor-mediated signaling. I also demonstrate how our improved understanding of the recently suggested functional link between protein intrinsic disorder and oligomericity as a key and missing element of transmembrane signal transduction provides novel insight into the molecular mechanisms of cell signaling and has important applications in biology and medicine.

Intrinsically Disordered Proteins

By definition, IDPs are proteins that lack a well-defined ordered structure under physiological conditions *in vitro*.¹² To predict whether a given protein or protein region assumes a defined fold or is intrinsically disordered, several computational methods have been developed.^{12–18} Experimentally, protein disorder can be detected by far-UV circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy. CD spectroscopy allows the estimation of the secondary structure content of a protein in solution. However, while for an ordered protein the CD signal gives information about each molecule in the sample, because nearly all the molecules are in the same structural state, it is different for an IDP that consists of a broad ensemble of molecules each having a different conformation.¹⁹ In this context, NMR is unparalleled in its ability to provide detailed structural and dynamic information on IDPs and has emerged as a particularly important tool for studies of IDP folding and interactions.^{20,21} NMR chemical shifts and line widths are extremely sensitive to subtle changes in protein conformational ensembles and are indispensable for detecting protein disorder (poor proton chemical shift dispersion and broad lines are indicative of disorder) and determining propensities of secondary structure formation on a residue-by-residue basis in unfolded and partly folded proteins.

Despite the fact that the existence of IDPs and IDRs has been recognized for many years, their functional role in crucial areas such as transcriptional regulation, translation and cellular signal transduction has only recently been reported due to progress in biochemical methodology.^{22–28} IDPs and IDRs are involved in various signaling and regulatory pathways, via specific protein-protein, protein-nucleic acid and protein-ligand interactions.^{22,24–26,28–32} Many of those post-translational modifications that rely on the low affinity, high specificity binding interactions (for example, phosphorylation) are associated primarily with IDPs and IDRs.^{6,25,27}

The major functional benefits of protein intrinsic disorder include increased binding specificity at the expense of thermodynamic stability, increased speed of interaction and the ability to recognize and bind multiple distinct partners without sacrificing specificity.^{22,23,27,28,31,32}

Binding with folding. The generally accepted view is that upon binding to their interacting partners and targets, IDPs undergo transitions to more ordered states or fold into stable secondary or tertiary structures—that is, they undergo coupled binding and folding processes (Fig. 1A).^{23,33–36}

Protein partner. Currently, the most characterized examples of folding being driven by binding are protein complexes formed by

IDPs with their folded (ordered) protein partners (Fig. 1A). This subject has been addressed in detail in many recent reviews and other articles.^{19,21–23,28,30,32,34,37,38} A classic example is binding of the kinase-inducible transcriptional-activation domain (KID) of cyclic AMP response element-binding protein (CREB) to CREB-binding protein (CBP). Upon binding to CBP, the intrinsically disordered KID polypeptide^{39,40} folds with the formation a pair of orthogonal helices.⁴¹ Coupled binding and folding can involve just a few residues^{41–45} or an entire protein domain.⁴⁶

Specific complex formation between IDPs is quite unusual, but not unprecedented.⁴⁷ Homodimerization of IDPs was first reported in 2004 for a novel class of signaling-related IDPs⁹ and later confirmed for other IDPs^{48–51} extending the phenomenon to different classes of IDPs and suggesting physiological relevance. It should be noted that in most of these studies, dimerization is accompanied by a mutual or “synergistic”⁴⁷ folding of two IDP molecules at the interaction interface (Fig. 1A). Thus, interactions between the constituents of such homodimers represent specific interactions between folded regions involved in complex formation.

Lipid partner. Prevalence of IDRs in the CYTO domains of many human TM proteins in general,^{7,8} and in particular, in the CYTO domains of signaling-related proteins (Table 1),^{9,10,52} raises the question if these regions exert membrane-binding activity and if affirmed, whether this activity has a physiological role. Recent studies of the intrinsically disordered CYTO domains of the ζ and CD3 ϵ signaling subunits (ζ_{cyt} and CD3 ϵ_{cyt} , respectively) of the T cell receptor (TCR) have demonstrated that these proteins bind to acidic dimyristoylphosphatidylglycerol (DMPG) vesicles and undergo a helical folding transition upon binding.^{35,36} ζ_{cyt} and CD3 ϵ_{cyt} contain an immunoreceptor tyrosine-based activation motif (ITAM), tyrosines of which are phosphorylated upon receptor triggering, and the authors^{35,36} hypothesized that helical folding of ITAMs upon membrane binding represents a conformational switch to control TCR activation.

On the other hand, it has been shown that binding of ζ_{cyt} and CD3 ϵ_{cyt} as well as ITAM-containing CYTO domain of Fc ϵ RI receptor γ subunit, Fc ϵ R γ_{cyt} , to acidic phospholipid vesicles is mediated by clusters of positively charged amino acids but not the ITAM residues.^{10,35,53} In contrast, helical folding of ITAMs observed in the presence of DMPG vesicles^{35,36,54} can be also promoted by physiologically irrelevant helical inducers such as trifluoroethanol and detergents.^{35,36,54,55} This supports a hypothesis that the association of these IDPs with negatively charged membranes is a biphasic process with a fast rate for an electrostatic-driven protein-liposome interaction and a slow rate for the hydrophobic-driven formation of an amphipathic helix.^{10,53} Similar biphasic process has been reported recently for binding of the HIV-1 Nef protein to model membranes.⁵⁶

Lipid bilayers are self-assembled structures, the mechanical properties of which are derived from noncovalent forces such as the hydrophobic effect, steric forces and electrostatic interactions. In this context, the electrostatic force is of special interest because biological membranes are rich in anionic lipids⁵⁷ and are therefore charged in aqueous solution. Importantly, electrostatic interactions play the critical role in membrane stability.⁵⁸

Thus, considering that net charges of ζ_{cyt} , $\text{CD3}\epsilon_{\text{cyt}}$ and $\text{Fc}\epsilon\text{R}\gamma_{\text{cyt}}$ are +5, +11 and +3 (Table 1), respectively, binding of these proteins to acidic phospholipids can potentially destabilize and disrupt lipid bilayers.

In lipid binding studies of ζ_{cyt} and $\text{CD3}\epsilon_{\text{cyt}}$ ^{35,36} the authors used DMPG vesicles to mimic the cell membrane. However, the lipid bilayers of these vesicles are not sufficiently stable and fuse upon binding ζ_{cyt} , as recently confirmed by dynamic light scattering (DLS) and electron microscopy (EM) experiments,⁵³ suggesting that the observed hydrophobic-driven helical folding of the ITAMs of ζ_{cyt} , $\text{CD3}\epsilon_{\text{cyt}}$ and $\text{Fc}\epsilon\text{R}\gamma_{\text{cyt}}$ likely occurs in the membrane stalk intermediates of fusion (Fig. 1A) and is similar to that promoted by physiologically irrelevant helical inducers such as trifluoroethanol and detergents (Fig. 1A).^{35,36,53-55} This not only questions the utility of micelles, detergents and unstable lipid vesicles (i.e., DMPG vesicles) as an appropriate model of the cell membrane but also highlights the importance of ensuring the integrity of model membranes upon protein binding in protein-lipid experiments in general, and in particular, in studies of IDP-lipid interactions.

Binding without folding. IDPs are often referred to as “remaining predominantly disordered” or “largely unfolded” upon dimerization or interaction with other proteins or lipids,^{35,39-45,48-51} meaning that the protein regions flanking the interaction interface but not the interface itself remain disordered. Recently, it has been suggested to term this mode of interaction “the flanking fuzziness” in contrast to “the random fuzziness” when the IDP remains entirely disordered in the bound state.⁵⁹ In this context, “the flanking fuzziness” is a part of the “coupled binding and folding” paradigm. Recent studies of a novel class of IDPs demonstrated that binding of IDPs is not necessarily accompanied by a disorder-to-order transition even within the interaction interface,^{9-11,53,60} thus going beyond the classical paradigm. The latter proteins are directly

Figure 1. Binding of an intrinsically disordered protein (IDP) to its target. Upon binding, an IDP may (A) or may not (B) fold on its interacting partner. (A) Binding of an IDP to another IDP or to a folded and well-ordered protein is coupled to secondary structure induction (left upper). In this widely observed scenario, interactions at the binding interface that mediate specific complex formation represent the interactions between folded domains (induction of helices is shown for illustrative purposes). Helical structure induction upon binding of an IDP to micelles or unstable lipid bilayers (left bottom) has been also widely reported.^{35,36,53,54} (B) Binding of an IDP to another IDP or to a folded and well-ordered protein is not accompanied by a disorder-to-order transition (right upper). In the recently discovered surprising scenario of IDP homo-oligomerization,^{9,11} interactions at the binding interface that mediate a specific oligomer formation represent the interactions between disordered domains making these interactions unusual and intriguing. The lack of secondary structure induction upon binding of an IDP to a folded and well-ordered protein has been also reported.⁶⁰ Binding of an IDP to stable lipid bilayers without a disorder-to-order transition (right bottom) has been also demonstrated.⁵³ Images were created using PyMol (www.pymol.org) from PDB file 1AVV for the HIV-1 Nef core domain (shown as an example of a folded interacting partner) and using arbitrary idealized structural elements to represent the ensemble of unfolded conformations of an IDP. Polar head groups and hydrophobic tails of detergent and lipid molecules are denoted by gray filled circles and lines, respectively. Adapted with permission from Sigalov AB. Protein intrinsic disorder and oligomericity in cell signaling. *Mol Biosyst* 2010; 6:451–61 and Sigalov AB, Hendricks GM. Membrane binding mode of intrinsically disordered cytoplasmic domains of T cell receptor signaling subunits depends on lipid composition. *Biochem Biophys Res Commun* 2009; 389:388–93.

Table 1. Summary of disorder^a and secondary structure^b predictions for cytoplasmic domains of MIRR signaling subunits

Parameter	Protein								
	ζ	CD3 ϵ	CD3 δ	CD3 γ	Ig α	Ig β	Fc ϵ RI γ	DAP10	DAP12
net charge ^c	+5	+11	0	0	-9	-10	+3	+3	+3
$ \langle R \rangle ^d$	0.044	0.193	0.000	0.000	0.143	0.196	0.068	0.125	0.058
$\langle H \rangle_b^e$	0.429	0.483	0.413	0.413	0.465	0.484	0.438	0.458	0.434
$\langle H \rangle^f$	0.349	0.315	0.374	0.317	0.389	0.386	0.380	0.357	0.395
$\langle H \rangle_b - \langle H \rangle^g$	0.080	0.168	0.039	0.096	0.075	0.098	0.058	0.101	0.039
Results of secondary structure prediction ^{h,i}									
Alpha helix	40.87	14.04	19.57	2.17	22.22	0	25.00	0	19.23
Extended strand	5.22	0	0	0	0	21.57	18.18	20.83	3.85
Random coil	53.91	85.96	80.43	97.83	77.78	78.43	56.82	79.17	76.92

Adapted with permission from [Sigalov AB, Aivazian DA, Uversky VN, et al. Lipid-binding activity of intrinsically unstructured cytoplasmic domains of multichain immune recognition receptor signaling subunits. *Biochemistry* 2006; 45:15731–9]. ^aUsing the algorithm of Uversky et al.¹² ^bUsing the hierarchical neural network algorithm.¹⁸ ^cUsing the Swiss Institute of Bioinformatics (SIB) server ExPASy.¹⁴⁴ ^dThe mean net charge, defined by Uversky et al.¹² as the absolute value of the difference between the numbers of positively and negatively charged residues at pH 7.0, divided by the total residue number. ^eThe boundary $\langle H \rangle_b$ value, calculated using the Uversky equation $\langle H \rangle_b = (|\langle R \rangle| + 1.151)/2.785$. ^fThe mean hydrophobicity, defined using the Kyte/Doolittle scale,¹⁴⁵ as the sum of all residue hydrophobicities, divided by the total number of residues and rescaled to a range of 0–1. ^gThe positive difference between $\langle H \rangle_b$ and $\langle H \rangle$ indicates that a protein is unfolded. ^hThe values are indicated in %. ⁱNo significant fraction was predicted for 3_{10} helix, Pi helix, beta bridge, beta turn, bend region, ambiguous and other states.

involved in receptor-mediated signaling, which makes these findings particularly interesting and important.

Protein partner. Little structural information is available for complex formation of IDPs with disordered or ordered partners that is not accompanied by a disorder-to-order transition both outside and within the interaction interface (Fig. 1B). First example of this unusual phenomenon was reported in 2004,⁹ when using a variety of biophysical and biochemical techniques, the ITAM-containing CYTO domains of immune receptor signaling subunits namely, TCR ζ_{cyt} , CD3 ϵ_{cyt} , CD3 δ_{cyt} and CD3 γ_{cyt} , B cell antigen receptor Ig α_{cyt} and Ig β_{cyt} , and Fc ϵ RI γ_{cyt} , all were shown to form specific homodimers without a disorder-to-order transition upon dimerization, thus revealing for the first time the existence of specific interactions between disordered protein molecules. Interestingly, for ζ_{cyt} , the oligomerization behavior is best described by a two-step monomer-dimer-tetramer fast dynamic equilibrium with dissociation constants in the order of approximately 10 μ M (monomer-dimer) and approximately 1 mM (dimer-tetramer).⁹ In contrast to the other ITAM-containing proteins, Ig α_{cyt} forms stable dimers and tetramers even below 10 μ M.⁹ Phosphorylation of the ζ_{cyt} and Fc ϵ RI γ_{cyt} ITAM Tyr residues neither significantly alters their homo-oligomerization behavior nor is accompanied by folding.⁹ As shown by CD and NMR spectroscopy for random coil ζ_{cyt} , this IDP does not undergo a transition between disordered and ordered states upon dimerization and remains unfolded both outside and within the interaction interface(s) in the ζ_{cyt} dimer.^{9–11} Since its discovery in 2004,⁹ the unusual biophysical phenomenon of IDP homo-oligomerization has become of more and more interest to biophysicists and biochemists,^{32,61} and one can expect that further multidisciplinary studies will shed light on the possible structural basis of these interesting IDP features.

Later, NMR studies of a direct, physiologically relevant interaction between ζ_{cyt} and the well-structured core domain of the

simian immunodeficiency virus (SIV) Nef protein revealed that random coil ζ_{cyt} bound to Nef with micromolar affinity remains unfolded at the interaction interfaces in the 1:1 ζ_{cyt} -Nef complex,⁶⁰ thus extending the “binding without folding” mode observed in IDP-IDP complexes to interactions of IDPs with folded partner proteins (Fig. 1B).

Intriguingly, NMR studies of ζ_{cyt} dimer^{9,11} and ζ_{cyt} -Nef complex⁶⁰ revealed a new, previously unknown NMR phenomenon—the lack of significant changes in chemical shift and peak intensity upon a specific protein complex formation.^{9,11,60} No chemical shift changes and significant changes in peak intensities are observed in the ^1H - ^{15}N heteronuclear single quantum coherence (HSQC) spectra of ^{15}N -labeled ζ_{cyt} upon dimerization (Fig. 2A)¹¹ or binding to the Nef protein (Fig. 2B).⁶⁰ Importantly, while the ζ_{cyt} dimerization interface is not yet known, the amino acid residues of ζ_{cyt} involved in the ζ_{cyt} -Nef interaction are well-established,⁶² but also do not exhibit chemical shift changes upon binding (Fig. 2B; cross-peak positions of SNID residues are marked).⁶⁰ ^1H - ^{15}N HSQC spectra represent a fingerprint of the protein backbone and are widely used as a quick, informative probe of changes in backbone conformation, particularly as it relates to structural studies of IDPs and their complexes.^{20,21} Thus, the unique and unprecedented NMR phenomenon observed^{9–11,60} likely highlights an unusual nature of specific interactions of IDPs upon binding without folding on their targets and opens a new line of research in the field of IDPs.

Lipid partner. In 2000,³⁶ it was shown that α -helical folding transition of random coil ζ_{cyt} upon binding to acidic phospholipids prevents ITAM phosphorylation. The authors concluded that this folding transition can represent a conformational switch to regulate TCR triggering.³⁶ Later, the other group of investigators extended these findings to intrinsically disordered CD3 ϵ_{cyt} and showed that binding of this protein to acidic phospholipids is accompanied by folding of ITAM, leading to inaccessibility of

the ITAM tyrosines for phosphorylation *in vitro*.³⁵ This led the authors^{35,63} to the conclusion that the conformational model of TCR activation previously suggested for ζ_{cyt} ³⁶ can be extended to CD3 ϵ_{cyt} . On the contrary, in other studies, it has been shown that binding of ζ_{cyt} , CD3 ϵ_{cyt} and Fc ϵ R γ_{cyt} to acidic phospholipids is not accompanied by a disorder-to-order structural transition.¹⁰ In contrast to micelles and DMPG vesicles,^{35,36,54} intrinsically disordered ζ_{cyt} , CD3 ϵ_{cyt} and γ_{cyt} have been shown to bind to acidic 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) vesicles without folding.¹⁰

A molecular explanation for this paradox was reported in 2009,⁵³ when two different membrane binding modes for ζ_{cyt} , CD3 ϵ_{cyt} and Fc ϵ R γ_{cyt} were shown, depending on the bilayer stability: (mode I) coupled binding and folding (Fig. 1A), and (mode II) binding without folding (Fig. 1B). It has been suggested⁵³ that in both modes, initially, clusters of basic amino acids in the regions outside ITAMs to bind to polar heads of acidic phospholipids while the ITAM residues do not contribute to binding at this stage. Then, in micelles (mode I), hydrophobic interactions between ITAMs and detergent tails promote folding of ITAMs, thus making ITAM tyrosines inaccessible for kinases as it has been shown for ζ_{cyt} /lysomyristoylphosphatidylglycerol (LMPG) micelles system.^{36,54} In vesicles, depending on the bilayer stability, initial protein binding to the membrane may (mode I) or may not (mode II) induce vesicle fusion and rupture and promote formation of ITAM helices stabilized by hydrophobic interactions with lipid tails in ruptured bilayers. As shown by DLS and EM, in the POPG vesicles used, protein binding does not disturb the lipid bilayer structure and does not cause vesicle fusion, thus explaining the lack of a disorder-to-order transition.^{10,53} Interestingly, phosphorylation of two and six ITAM tyrosines in Fc ϵ R γ_{cyt} and ζ_{cyt} , respectively, reduces the corresponding net charges from +3 to -0.5 for Fc ϵ R γ_{cyt} and from +5 to -5.5 for ζ_{cyt} but does not abrogate binding to POPG vesicles,¹⁰ further confirming a hypothesis

Binding of intrinsically disordered TCR ζ_{cyt}

No significant changes in chemical shift and peak intensity upon binding

A TCR ζ_{cyt} dimerization

Disordered partner

B TCR ζ_{cyt} binding to SIV Nef

Ordered partner

Figure 2. Intrinsically disordered cytoplasmic domain of T cell receptor ζ chain (ζ_{cyt}) does not fold upon binding to its unfolded (A) or folded (B) protein target. The ^1H - ^{15}N heteronuclear single quantum correlation (HSQC) spectra of ^{15}N -labeled ζ_{cyt} at 298 K (A) or 283 K (B) in the absence (blue) or presence (red) of its interacting partner, another ζ_{cyt} molecule (A) or the well-folded and ordered SIV Nef protein (B). In both scenarios, a new, previously unobserved NMR phenomenon, the lack of significant changes in chemical shift and intensity upon a specific protein complex formation, has been reported.^{9,11,60} Cross-peak positions of the SIV Nef interaction domains (SNIDs) residues are marked to highlight the lack of chemical shift changes for these residues upon binding to Nef. Adapted with permission from Sigalov AB, Zhuravleva AV, Orekhov VY. Binding of intrinsically disordered proteins is not necessarily accompanied by a structural transition to a folded form. *Biochimie* 2007; 89:419–21 and Sigalov AB, Kim WM, Salane M, et al. The intrinsically disordered cytoplasmic domain of the T cell receptor zeta chain binds to the Nef protein of simian immunodeficiency virus without a disorder-to-order transition. *Biochemistry* 2008; 47:12942–4.

that not only does the overall net charge but also the presence of clustered basic amino acid residues prove to be important for lipid-binding activity of these IDPs.¹⁰

Thus, these findings^{10,53} clearly demonstrate that binding to IDPs can induce membrane fusion and rupture in the lipid bilayer vesicles unstable under the experimental conditions used. Importantly, as shown,⁵³ the destructive effect of protein binding on bilayer lipid membrane is not dependent on vesicle size (small versus large unilamellar vesicles; SUV or LUV, respectively) or technique of SUV preparation (sonication vs. extruding). The membrane rupture is known to result in monolayer fusion of the membranes, i.e., in the formation of a bridge connecting the monolayers, which is usually named the stalk or hemifusion intermediate.⁶⁴ Interestingly, tight coupling between the loop-to-helix structural transition and stalk formation as a result of deformation of the target and viral membranes has been reported for influenza hemagglutinin.^{65,66} Thus, protein binding-induced membrane perturbation and disruption can represent a molecular basis for the observed formation of the ITAM helices in the presence of DMPG vesicles.^{35,36} It should be also noted that in mode II, ITAMs do not participate in binding to lipid bilayers⁵³ and the ITAM tyrosines are therefore likely to be easily accessible for phosphorylation (Fig. 1B).

Thus, the use of not only micelles but also lipid vesicles can result in opposite conclusions regarding membrane-binding activity of IDPs and its physiological relevance. This highlights the importance of the choice of an appropriate membrane model in studies of protein-lipid interactions, particularly as it relates to IDPs. This also challenges the field of NMR studies where many lipid bilayer models cannot be used because of the particle size.

Summary. As widely discussed in the literature,^{23,28,33-36,59,61,67} IDPs often function through molecular recognition and binding to their folded or unfolded protein partners which is accompanied by induced folding of the IDP interaction (recognition) interface, the coupled folding and binding (or disorder-to-order transition) scenario (Fig. 1A). Another scenario, the “conformational selection” model, in which IDPs bind and fully fold through conformational selection following a two-state model, has been also proposed.⁶⁸⁻⁷⁰ In the consensus synergistic model,⁶⁸ conformational selection has been proposed to play the most important role in the specific encounter, while coupled folding and binding has been suggested to be essential for the formation of the fully-bound complexes.

Recently discovered functionally relevant binding of IDPs to unfolded and folded proteins without folding on the interacting partner^{9-11,60} revealed a novel insight into IDP interactions, thus demonstrating the existence of two different modes of IDP binding to their protein partners: with and without folding (the “disorder-to-order” and “no disorder-to-order” scenarios). In the context of signal transduction, the “no disorder-to-order” IDP interactions have been suggested as a novel therapeutic target for a variety of diseases,^{2,52,71-75} thus making studies of the basis of the interactions not only of fundamental scientific importance but also of great clinical value. These interactions of low affinity in the micromolar range^{9,60} could be of an electrostatic nature, which has been proposed to be exploited in IDPs by generating a “polyelectrostatic effect.”^{61,76}

The existence of two different modes of IDP binding to lipid bilayer membranes⁵³ raises an important question: which mode

of action is of physiological relevance? Considering that α -helical folding of ITAMs of signaling-related IDPs is not observed in the presence of those vesicles that are stable upon protein binding, this folding transition is unlikely to play a significant role in transmembrane signaling and cell activation. However, it does not necessarily mean that mode II (binding without folding) is also physiologically irrelevant. For example, within the SCHOOL model,^{71,74} homointeractions between CYTO domains of the ITAM-containing receptor signaling subunits are suggested to be necessary and sufficient to trigger the receptor. Thus, membrane binding of ζ_{cyt} , CD3 ϵ_{cyt} and Fc ϵ R γ_{cyt} might prevent homo-oligomerization of these CYTO domains⁹ in receptor clusters on the surface of resting cells and during random encounters of receptors diffusing in the cell membrane. Considering the reported prevalence of IDRs in the CYTO domains of many other human TM proteins,^{7,8} one can expect that the distinctive features of the ITAM-containing IDRs observed in membrane binding studies^{10,53} can be found for other CYTO IDRs, as well. Further studies will have to test this hypothesis of potential physiological significance.

Summarizing, I suggest a novel mechanistic hypothesis that describes interactions of IDPs with their protein or lipid partners as a general biphasic process with a fast rate for the electrostatic-driven “no disorder-to-order” Phase I interaction and a slow rate for the hydrophobic-driven (“disorder-to-order”) Phase II formation of a secondary structure (e.g., an amphipathic helix). Within this hypothesis, a Phase II may (folding upon binding) or may not (binding without folding) follow a Phase I depending on the interacting partner.

Receptor Signaling

Structural classification of receptors. Based on location of binding and signaling (effector) domains, functionally diverse and unrelated cell surface receptors can be structurally classified into two main families: those in which binding and signaling domains are located on the same protein chain, the so-called single-chain receptors (SRs, Fig. 3), and those in which binding and signaling domains are intriguingly located on separate subunits, the so-called multichain receptors (Fig. 4).^{2,52,71,74,77,78} Because many multichain activating receptors are immune receptors, they are all commonly referred to as multichain immune recognition receptors (MIRRs).^{2,72,77}

Assuming that the similar structural architecture of the receptors dictates similar mechanisms of receptor triggering and subsequent transmembrane signaling, one can suggest that the targets revealed by these mechanisms are similar in seemingly unrelated diseases. This builds the structural basis for the development of novel pharmacological approaches as well as the transfer of clinical knowledge, experience and therapeutic strategies between various disorders.

Protein intrinsic disorder and receptors. Signaling subunits of MIRRs contain in their cytoplasmic domains the ITAM or the YxxM motif, found in the DAP10 subunit (Fig. 4). Ligand binding results in phosphorylation of the ITAM/YxxM tyrosines, triggering the intracellular signaling cascade. Extracellular

Figure 3. Single-chain receptors (SRs). The extracellular portion of the receptors is on top and the cytoplasmic portion is on bottom. The lengths of the receptors as shown are only approximately to scale. The inset shows SR domain organization. Abbreviations: EpoR, erythropoietin receptor; G-CSF-R, granulocyte colony-stimulating factor receptor; TGF β , transforming growth factor-beta; TNF, tumor necrosis factor; JAK, Janus kinase; EGFR, epidermal growth factor receptor; InsR, insulin receptor; IGF1R, insulin-like growth factor I receptor; IRR, insulin receptor-related receptor; PDGFR, platelet-derived growth factor receptor; CSF1R, colony-stimulating-factor 1 receptor; FGFR, fibroblast growth factor receptor; MuSK, muscle-specific receptor tyrosine kinase; Eph, ephrin; DDR, discoidin domain receptor; Flt1, KDR and Flt4, vascular endothelial growth factor (VEGF) receptors. Adapted with permission from Sigalov AB. The SCHOOL of nature I. Transmembrane signaling. Self/Nonself 2010; 1.

structure of signaling subunits varies from the short sequences found in ζ , γ , DAP10 and DAP12 to the Ig-like folds present in CD3 ϵ , CD3 δ , CD3 γ , Ig α and Ig β (Fig. 4). In contrast, as revealed by computational methods, CD and NMR spectroscopy, most of their cytoplasmic domains, namely, ζ_{cyt} , γ_{cyt} , CD3 ϵ_{cyt} , CD3 δ_{cyt} , CD3 γ_{cyt} , Ig α_{cyt} and Ig β_{cyt} , represent a novel class of IDPs (Table 1).⁹⁻¹¹ Interestingly, for DAP10_{cyt} and DAP12_{cyt}, secondary structure prediction using the hierarchical neural network algorithm¹⁸ exhibits high (about 80%) percentage of random

coil conformation (Table 1).⁵² Disorder prediction using the algorithm of Uversky et al.¹² reveals the boundary <H> values of 0.101 and 0.039 for DAP10_{cyt} and DAP12_{cyt}, respectively.⁵² These values are characteristic for IDPs and close to those calculated for other ITAM-containing sequences (Table 1).¹⁰ Thus, the cytoplasmic domains of signaling subunits of many different receptors expressed on various cells are surprisingly all intrinsically disordered (Fig. 4). This fits with recent findings that IDRs are prevalent in the cytoplasmic domains of human transmembrane

Figure 4. Multichain immune recognition receptors. Schematic presentation of the MIRRs expressed on many different immune cells including T and B cells, natural killer cells, mast cells, macrophages, basophils, neutrophils, eosinophils, dendritic cells and platelets. The inset shows MIRR assembly. Cytoplasmic domains of the MIRR signaling subunits represent a novel class of intrinsically disordered proteins and are shown to be dimeric. Curved line depicts protein disorder. Abbreviations: ITAM, immunoreceptor tyrosine-based activation motif; BCR, B-cell receptor; DAP-10 and DAP-12, DNAX adapter proteins of 10 and 12 kD, respectively; DCAR, dendritic cell immunoactivating receptor; GPVI, glycoprotein VI; ILT, Ig-like transcript; KIR, killer cell Ig-like receptor; LIR, leukocyte Ig-like receptor; MAIR-II, myeloid-associated Ig-like receptor; MDL-1, myeloid DAP12-associating lectin 1; NITR, novel immune-type receptor; NK, natural killer cells; SIRP, signal regulatory protein; TCR, T-cell receptor; TREM receptors, triggering receptors expressed on myeloid cells. Adapted with permission from Sigalov AB. The SCHOOL of nature I. Transmembrane signaling. Self/Nonself 2010; 1.

proteins⁷ and that protein phosphorylation predominantly occurs within IDRs,⁶ further suggesting an important role of intrinsic disorder in receptor-mediated signaling.

Receptor and protein oligomericity. Binding of multivalent but not monovalent ligand and subsequent receptor clustering are required for induction of the signaling cascade.^{2,3,71,79,80} This raises the question: What is the molecular mechanism by which clustering of the extracellular binding domains leads to the generation of the activation signal by intracellular signaling domains? For many receptors, ligand-induced clustering is known to result in oligomerization of receptor transmembrane and cytoplasmic domains.^{73,74,81-83} The subsequent formation of competent signaling oligomers in cytoplasmic milieu

provides the necessary and sufficient event to trigger the receptor. However, for receptors that signal through ITAM/YxxM modules (Fig. 4), this mechanism has been a long-standing open issue until recently, when formation of ITAM-containing cytoplasmic signaling oligomers was suggested to play a crucial role in transmembrane signaling mediated by these receptors.^{52,71,72,74,75} Interestingly, the homo-oligomerization of these IDPs is best described by a two-step monomer-dimer-tetramer fast dynamic equilibrium with monomer-dimer dissociation constants in the micromolar affinity range.^{9,11} These findings are in line with the known dependence of the overall binding affinity between proteins on the function of the protein complex. For example, obligate homodimers are strongly associated

Figure 5. SCHOOL principles of receptor signaling. Single- (A) or multichain (B) receptor oligomerization (clustering) induced upon ligand binding outside the cell is translated across the membrane into protein oligomerization inside the cell with cytoplasmic homointeractions representing the major driving force of receptor triggering. For SRs (A), small solid black and gray arrows indicate specific inter-unit homointeractions between transmembrane and cytoplasmic domains, respectively. For MIRRs (B), small solid black and gray arrows indicate specific inter-unit hetero- and homointeractions between transmembrane and cytoplasmic domains, respectively. Circular arrow indicates ligand-induced receptor reorientation. Curved line depicts disorder of the cytoplasmic domains of MIRR signaling subunits (B). Phosphate groups are shown as dark circles. Abbreviation: SCHOOL, signaling chain homo-oligomerization. Adapted with permission from Sigalov AB. The SCHOOL of nature I. Transmembrane signaling. Self/Nonsell 2010; 1.

with nano- or picomolar binding affinity while, in contrast, proteins that associate and dissociate in response to changes in their environment, such as the majority of signal transduction mediators, tend to bind more weakly.

As mentioned, the first evidence of an IDP's propensity for specific homodimerization distinct from non-specific aggregation behavior seen in many systems⁸⁴ has been recently reported⁹ and suggested to play an important role in transmembrane signaling.^{71,72,74} Later, other IDPs have also been found to form specific homodimers⁴⁸⁻⁵¹ and shown to function through dimer

formation,^{48,51} further demonstrating a direct functional link between protein intrinsic disorder and oligomericity. In the context of receptor-mediated signal transduction, this link represents a key and missing element in our understanding of transmembrane signal transduction. One can suggest that for the vast majority of receptors, receptor oligomericity (clustering upon binding of multivalent ligand) is translated across the membrane into protein oligomericity (formation of competent cytoplasmic signaling oligomers), thus providing a general platform for receptor-mediated signaling (Fig. 5).^{52,75}

Single-chain receptor signaling: functional link between protein order and oligomericity. Single-chain receptors (SRs) are receptors with binding and signaling domains located on the same protein chain (Fig. 3). Importantly, EC, TM and CYTO regions of these receptors represent folded and well-ordered domains.

Examples of SRs include receptor tyrosine kinases (RTKs) that are TM glycoproteins consisting of a variable EC N-terminal domain, a single membrane spanning domain, and a large CYTO portion composed of a juxtamembrane domain, the highly conserved tyrosine kinase domain and a C-terminal regulatory region (Fig. 3).⁸⁵ RTKs activate numerous intracellular signaling pathways, leading to a variety of cell responses. These receptors are triggered by the binding of their cognate ligands and transduce the recognition signal to the cytoplasm by phosphorylating CYTO tyrosine residues on the receptors themselves (autophosphorylation) and on downstream signaling proteins. The proteins of the tumor necrosis factor (TNF) receptor superfamily⁸⁶ are a group of SRs critically involved in the maintenance of homeostasis of the immune system (Fig. 3). Triggered by their corresponding ligands, these receptors either induce cell death or promote cell survival of immune cells. Transforming growth factor- β (TGF β) is a potent regulatory cytokine which inhibits the development of immunopathology to self or non-harmful antigens without compromising immune responses to pathogens.⁸⁷ The TGF β superfamily functions via binding to type I and II TM serine/threonine kinase receptors that belong to the SR family (Fig. 3).

According to the SCHOOL platform, signaling chain homooligomerization and formation of competent signaling oligomers in CYTO milieu provides the necessary and sufficient event to trigger receptors and induce cell activation (Fig. 5A).^{52,73-75} Within the consensus model of SR signaling, multivalent ligand binding results in receptor re-orientation and dimerization (oligomerization) and subsequent formation of competent signaling oligomers in the cytoplasm and trans-autophosphorylation at defined cytoplasmic tyrosines.^{3,52,73,75,79,83,88-91} Some SRs, such as members of the tumor necrosis factor (TNF) receptor superfamily,⁹⁰ exist as pre-assembled oligomers on the cell surface. In this scenario, multivalent ligand binding results in re-orientation of individual receptors in the pre-assembled oligomers to adopt an inter-unit geometry permissive for promotion of homointeractions between receptor CYTO domains and further receptor activation.^{52,73-75,91}

Thus, in terms of SR signaling, there exists the principal functional link between protein order and oligomericity in CYTO milieu.

Multichain Receptor Signaling: Functional Link Between Protein Disorder and Oligomericity

Functionally diverse members of the MIRR family are expressed on many different immune cells, including T and B cells, natural killer (NK) cells, mast cells, macrophages, basophils, neutrophils, eosinophils, dendritic cells (DCs) and platelets.^{2,72,77,92} Figure 4 shows typical examples of MIRRs including the T-cell receptor (TCR) complex, the B-cell receptor (BCR) complex, Fc receptors (e.g., Fc ϵ RI, Fc α RI, Fc γ RI and Fc γ RIII), NK receptors

(e.g., NKG2D, CD94/NKG2C, KIR2DS, NKp30, NKp44 and NKp46), immunoglobulin (Ig)-like transcripts and leukocyte Ig-like receptors (ILTs and LIRs, respectively), signal regulatory proteins (SRPs), dendritic cell immunoactivating receptor (DCAR), myeloid DNAX adapter protein of 12 kD (DAP12)-associating lectin 1 (MDL-1), blood DC antigen 2 protein (BDCA2), novel immune-type receptor (NITR), myeloid-associated Ig-like receptor (MAIR-II), triggering receptors expressed on myeloid cells (TREMs) and the platelet collagen receptor, glycoprotein VI (GPVI). For more information on the structure and function of these and other MIRRs, I refer the reader to recent reviews.^{2,93-112}

The MIRR ligand-binding subunits are integral membrane proteins with small CYTO domains that are themselves inert with regard to signaling. Signaling is achieved through the association of the ligand-binding chains with signal-transducing subunits that contain in their CYTO domains one or more copies of the ITAM regions with two appropriately spaced tyrosines (YxxL/I_xYxxL/I; where x denotes non-conserved residues)¹¹³ or the YxxM motif,^{114,115} found in the DAP10 CYTO domain¹¹⁵ (Fig. 4). The association of the MIRR subunits in resting cells is driven mostly by the noncovalent TM interactions between recognition and signaling components (Fig. 4) and plays a key role in receptor assembly, integrity and surface expression.^{72,99-101,103,108,111,116-127} Despite extensive studies in the field, the molecular mechanism linking extracellular clustering of MIRR binding subunits to intracellular phosphorylation of ITAM/YxxM tyrosines has been a long-standing mystery.

The intriguing ability of the intrinsically disordered ITAM-containing CYTO domains of MIRR signaling subunits to homooligomerize⁹ led to the development of a novel model of MIRR signaling, the Signaling Chain Homo-oligomerization (SCHOOL) model.^{2,52,71-75,78} The model suggests that formation of competent signaling subunit oligomers mediated by homotypic interactions in the cytoplasm, rather than receptor clustering/oligomerization per se, is necessary and sufficient to trigger the receptors and induce the downstream signaling sequence (Fig. 5B). Similar to SRs, some MIRRs such as TCR and major platelet collagen receptor glycoprotein VI (GPVI), can exist as pre-assembled oligomers on the cell surface.^{128,129} In these oligomers, multivalent ligand binding results in re-orientation of receptors to adopt an inter-unit geometry permissive for promotion of homointeractions between MIRR signaling subunit CYTO domains and further receptor activation (Fig. 5B).

Thus, in terms of MIRR signaling, there exists the principal functional link between protein disorder and oligomericity in CYTO milieu.

SCHOOL Platform of Receptor Signaling

According to the SCHOOL platform, signaling chain homooligomerization and formation of competent signaling oligomers in CYTO milieu provides the necessary and sufficient event to trigger receptors of both structural families (SRs and MIRRs) and induce cell activation (Fig. 5). Within the platform, receptor oligomerization induced or tuned upon ligand binding outside

the cell is translated across the membrane into protein oligomerization in CYTO milieu, thus providing a general platform for receptor-mediated signaling (Fig. 5).

The necessity and sufficiency of formation of competent signaling oligomers mediated by homointeractions between well-structured (SRs) or intrinsically disordered (MIRRs) cytoplasmic signaling domains to trigger receptor function dictates several important mechanistic principles of receptor signaling:

- sufficient interreceptor proximity in receptor dimers/oligomers,
- correct (permissive) relative orientation of the receptors in receptor dimers/oligomers,
- long enough duration of the receptor-ligand interaction that generally correlates with the strength (affinity/avidity) of the ligand,
- sufficient lifetime of an individual receptor in receptor dimers/oligomers.

These general principles are common for SRs and MIRRs and thus link mechanistically numerous structurally and functionally diverse receptors.

Further, because of the ubiquitous nature of protein-protein interactions and the knowledge that inappropriate protein-protein binding can lead to disease, the specific and controlled inhibition and/or modulation of these interactions provides a promising novel approach for rational drug design. A number of recent reviews have addressed this topic.¹³⁰⁻¹³² Suggesting important role of TM interactions that mediate ligand-induced SR dimerization (oligomerization) and homo-interactions between CYTO domains that result in formation of competent signaling oligomers (Fig. 5A), the SCHOOL model of SR signaling reveals these interactions as important points for intervention to modulate SR signaling.^{52,73-75,133,134} Similarly, considering MIRR triggering as the result of the ligand-induced interplay between (1) intrareceptor TM interactions that stabilize and maintain receptor integrity, and (2) interreceptor homointeractions between the CYTO domains of MIRR signaling subunits that lead to formation of competent signaling oligomers (Fig. 5B), the SCHOOL models reveals these interactions as important points for intervention to modulate MIRR signaling.^{52,72-75,133,134} Importantly, these are common targets for all members of the MIRR family, which means that a general pharmaceutical strategy may be used to treat seemingly disparate disorders such, for example, as T-cell-mediated skin diseases and platelet disorders.^{52,72-75,133-135}

Applications in Biology and Medicine

By revealing specific protein-protein interactions critically involved in receptor-mediated signaling, current SCHOOL models that are based on functional connections between protein order (SRs), disorder (MIRRs) and oligomericity provide molecular explanations for many biological phenomena and processes, represent powerful tools for fundamental and applied research, and suggest novel avenues for drug discovery.^{2,52,71-75,133-136}

T-cell receptor signaling. Despite TCR being one of the most studied MIRRs, many of the models of TCR signaling suggested

to date are descriptive and often fail in trying to explain most of the known immunological data.

Structurally, TCR is a member of the MIRR family (Fig. 4) with its α and β antigen-binding subunits bound by TM interactions with three signaling homo- and heterodimers: $\zeta\zeta$, CD3 $\epsilon\delta$ and CD3 $\epsilon\gamma$.¹¹⁷ Within the SCHOOL model, distinct TCR signaling is achieved through the ζ and CD3 signaling oligomers,^{52,71,72,74,75} and interreceptor TM interactions represent not only a promising therapeutic target but also an important point of viral attack.^{72,73,133,137}

The TCR core peptide (CP), a synthetic peptide corresponding to the sequence of the TCR α transmembrane domain, is capable of inhibiting antigen-mediated T-cell activation, whereas T-cell activation via anti-CD3 antibodies is not affected by CP.¹³⁸ However, despite extensive studies, the mode of action of this clinically relevant peptide had not been elucidated until 2004 when the SCHOOL model was first introduced.⁷¹

Recently, inhibition of antigen- but not anti-CD3-stimulated T-cell activation has been reported for the fusion peptide (FP) found in the N terminus of the HIV envelope glycoprotein 41 (gp41).¹³⁹ However, the mode of action of this peptide had remained unknown until 2006 when the SCHOOL model was first applied to this area.⁷² Within the model, the molecular mechanisms of action for TCR CP and HIV gp41 FP are similar. Briefly, CP and FP compete with TCR α for binding to CD3 $\delta\epsilon$ and $\zeta\zeta$, resulting in functional disconnection of these subunits.^{72,133,137,140}

In summary, our current understanding of TCR signaling, together with the lessons learned from the viral pathogenesis,^{2,73,75,133,134,137} can be used not only for further fundamental research but also for rational drug design.

Glycoprotein VI signaling. Activation of circulating platelets by exposed vessel wall collagen is a primary step in the pathogenesis of thrombotic diseases. Despite intensive research efforts in antithrombotic drug discovery, uncontrolled hemorrhage still remains the most common side effect. Intriguingly, the selective inhibition of the GPVI collagen receptor may inhibit thrombosis without affecting hemostasis.² However, the mechanism of GPVI signaling has remained unknown until recently,^{135,141} therefore hindering the further development of this promising antithrombotic strategy. GPVI belongs to the MIRR family (Fig. 4) and signals through the associated ITAM-containing γ subunit. The application of the SCHOOL model^{73,74,133} resulted in the development of novel mechanistic concept of platelet inhibition and the invention of new platelet inhibitors.^{73,135,136,141}

NKG2D signaling. Despite advances in immune disorder research, there is still a great need for additional targets and agents for effectively reducing inflammatory bowel diseases (IBDs), namely ulcerative colitis (UC) and Crohns disease (CD) and affect millions of people worldwide. In 2007, a unique subset of CD4⁺ NKG2D⁺ T cells was identified in IBD patients.¹⁴² Later, inhibition of NKG2D, a member of the MIRR family (Fig. 4) that triggers through the associated YxxM motif-containing DAP10, has been proven to be of key importance in successful treatment of UC and CD.¹⁴³ Uncovering the molecular mechanisms of NKG2D signaling, the SCHOOL model suggests

the NKG2D-DAP10 transmembrane interactions as a promising point of intervention in IBD treatment.^{72-74,133,134} Further studies will have to test this concept.

Conclusions and Perspectives

The crucial role of receptor-mediated signaling in health and disease assumes that our understanding of the underlying molecular mechanisms and methods to modulate the cell response through control of TM signal transduction can contribute significantly towards the improvement of existing therapies and the design of new therapeutic strategies for a diverse set of disorders. For structurally related members of the MIRR family, the functional link between protein intrinsic disorder and oligomerization represents a missing piece of the long-standing puzzle of signaling and reveals striking similarities in the basic mechanistic principles of function of most SRs and MIRRs. In this context, the SCHOOL

model of MIRR signaling is similar to the consensus model of SR signaling in regards to both models suggesting that formation of competent signaling oligomers mediated by homointeractions between well-structured (SRs) or intrinsically disordered (MIRRs) CYTO signaling (effector) domains is a necessary and sufficient to trigger receptor function. This raises an interesting question: Why for MIRRs, where the recognition and signaling domains are located on separate protein chains, nature selected to use a functional link between protein disorder and oligomerization? One can expect that further multidisciplinary studies will clarify this question of great interest and practical utility.

In conclusion, recent fundamental advances uncovering the molecular mechanisms of receptor-mediated signaling have been accompanied by our improved understanding of unexplained biological phenomena. This opens new horizons in further fundamental and clinical research, research-based education and innovative drug design and discovery.

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