How proteins adapt to a membrane–water interface

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Membrane proteins present a hydrophobic surface to the surrounding lipid, whereas portions protruding into the aqueous milieu expose a polar surface. But how have proteins evolved to deal with the complex environment at the membrane–water interface? Some insights have been provided by high-resolution structures of membrane proteins, and recent studies of the role of individual amino acids in mediating protein–lipid contacts have shed further light on this issue. It now appears clear that the polar-aromatic residues Trp and Tyr have a specific affinity for a region near the lipid carbonyls, whereas positively charged residues extend into the lipid phosphate region.

THE MEMBRANE–WATER INTERFACIAL region comprises a relatively large part of the total bilayer thickness. In contrast to the hydrophobic core of the membrane, it presents a chemically complex environment, which offers many possibilities for noncovalent interactions with protein side chains. Therefore, the interface can play an important role in membrane association of proteins and peptides. Across the interfacial region, there is a steep polarity gradient; from highly apolar near the hydrocarbon region of the membrane to highly polar near the aqueous phase. The ester carbonyls of the lipids, the phospholipid head groups and water molecules around the lipid head groups present opportunities for dipole–dipole interactions and allow H-bonding with appropriate amino acid side chains. In addition, electrostatic interactions might occur between, for example, positively charged amino acid side chains and negatively charged lipid phosphate groups. Thus, the interactions of a protein with the membrane–water interface will strongly depend on specific properties of its amino acid side chains, including charge, hydrophobicity, polarity and potential for H-bonding.

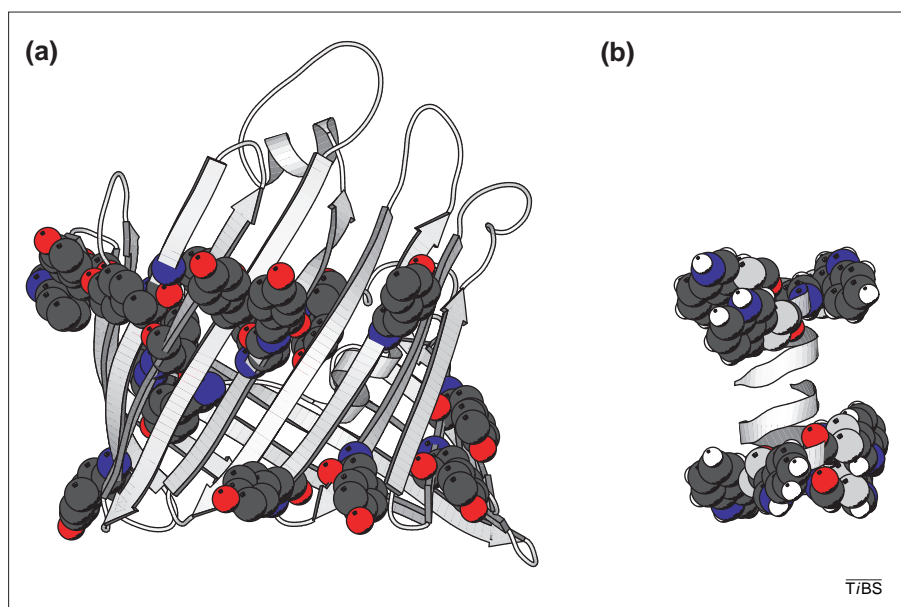It is well established that the interfacial region influences membrane interactions of water-soluble proteins and peptides. Interactions with the membrane–water interface can promote binding and folding of proteins and peptides, and determine their precise localization and orientation at the interface[1,2]. It is often less well appreciated that the membrane–water interface can also be important for the structure and function of transmembrane proteins. Residues that flank the hydrophobic membrane-spanning segments of membrane proteins might interact with the interface on each side of the membrane and thereby determine the precise interfacial positioning of these segments or influence

their orientation in the membrane. Because the strength and nature of such interfacial interactions in a given membrane will depend on the properties of the amino acid side chains involved, one would expect the interfacial region to be enriched for specific amino acids if such interactions are indeed functionally relevant.

What the 3D structures say

The first indication that parts of membrane proteins located at the membrane–water interface are enriched in particular amino acids came with the first high-resolution porin structures[3–5]. Although no lipid molecules were visible in these structures, a striking architecture was revealed in which a central hydrophobic section rich in aliphatic residues, presumably exposed to the lipid hydrocarbon chains, is bordered on both sides by 'aromatic belts', which were proposed to interact favorably with the lipid headgroups (Fig. 1a). Statistical analyses of helix-bundle membrane proteins such as cytochrome *c* oxidase later revealed a similar tendency, with exposed Trp and Tyr (but not Phe) concentrated in regions close to the membrane–water interface[6]. Both for the porins and the helix-bundle proteins, surface-exposed charged residues are found in quantity only outside the aromatic belts[6,7], suggesting a preference for the aqueous environment rather than the interface region.

Isolated lipid molecules, trapped within protein complexes, are visible in

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Figure 1
Porin from the outer membrane of *Rhodobacter capsulatus* (a) and the membrane-bound form of the gramicidin A dimer (b). Trp and Tyr residues are shown as spacefilling models. The figure was drawn using MolScript[31].

some of the more recently determined membrane protein stuctures[8–12], and their headgroups are located roughly where one would expect, given the location of the border between the hydrophobic and hydrophilic protein surfaces. In particular, a single lipopolysaccharide (LPS) molecule specifically bound to the outer membrane β-barrel protein FhuA has its glucosamine moieties placed slightly above the aromatic belt[8]. The headgroups of lipids bound to bacteriorhodopsin contact both aromatic and positively charged residues, with the aromatic residues located deeper in the membrane than the basic ones[9].

A similar situation is observed for single-spanning membrane proteins. Also here, a statistical preference of Trp and Tyr for the interface region is observed, which is even more pronounced than in multi-spanning membrane proteins[13,14], and, again, the charged residues extend further into the polar region. A striking example of the enrichment of Trp at the interface of small membrane proteins is provided by the channel-forming peptide gramicidin A. This peptide contains four Trp residues, all located near its C terminus. The 3D structure of the peptide in a lipid bilayer is known to atomic detail[15] (Protein Data Bank entry 1MAG). The peptide spans the bilayer as an N-to-N-terminal dimer, so that the four Trp residues of each monomer are located at the interface (Fig. 1b). Crystal structures of this peptide, obtained from organic solvent, show a completely different fold. It is now widely recognized that it is a specific preference of Trp for the interfacial environment that is responsible for inducing the functionally active conformation of the channel, as observed in lipid bilayers[16].

Chemical properties of interfacially located amino acids

What chemical properties would be responsible for a preferred localization of aromatic and charged amino acid side chains near the interfacial region? For Trp, the indole side chain appears ideally suited for interacting with the polar–apolar interface. It has a large hydrophobic, fused aromatic ring, which might be preferentially buried in the hydrophobic part of the bilayer. Attached to this aromatic ring is an amide group that can be expected to localize preferentially in the more polar environment at the interface. The amide group gives the side chain polarity and a considerable dipole moment, and can act as a hydrogen bond donor. Tyr, which is also

enriched in interfacial regions of membrane proteins, has roughly comparable properties but a smaller ring system. Phe, on the other hand, although aromatic, is completely hydrophobic and is found in the transmembrane rather than in interfacial parts of membrane proteins[6]. It thus does not appear to have a special affinity for the interface.

Also the positively charged amino acids Lys and Arg might be expected to have a special interaction with the interface. These amino acids have a relatively long aliphatic side chain with a positively charged amine or guanidinium group at the end. The aliphatic part might prefer a localization in the hydrophobic part of the bilayer, whereas the positively charged end would prefer localization in the more polar part, where it can interact, for example, with negatively charged phosphate groups. This behavior has been described as 'snorkeling'[17,18]. Negatively charged residues have only a small side chain and are repelled by negatively charged phosphate groups. Therefore, they are not expected to interact favorably with the lipid–water interface.

Studies on interfacial model peptides

Much insight about interactions between specific amino acid side chains and the membrane–water interface has been obtained from studies of small water-soluble peptides. White and Wimley[1,19] analysed the interfacial affinity of different amino acid side chains by incorporating various amino acids in host peptides that are sufficiently small, so that they cannot form secondary structures. The partitioning of these host peptides between water and the interface of lipid bilayers was measured and compared with partitioning in octanol or cyclohexane. In this way, the energetics of the interface interaction of several side chains was characterized in detail. It was found that in particular Trp and Tyr have a special affinity for the interface.

To obtain insight into the physical basis underlying the preference of Trp for the interface, studies with indole and modified indole analogs were also carried out[20,21]. Based on these studies, it was suggested that contributions, if any, from H-bonding and polarity to the preferred interfacial positioning were only minor, and that other factors, like size, rigidity and aromaticity, might be more important[1,21]. No indication for a favorable interaction with the interface was found for any of the charged residues,

suggesting that the side chains in these small peptides do not 'snorkel'. However, it should be realized that the situation might be different for transmembrane protein segments or for longer interfacially localized peptides, where other types of interactions might allow the backbone C_α atom of Lys or Arg to become localized deeper into the membrane, in or near the hydrophobic acyl chain region. Indeed, 'snorkeling' of charged amino acid side chains was first proposed based on studies with larger amphipathic helical peptides[17,22]. Such peptides can be buried completely in the large interfacial area, more or less parallel to the lipid–water interface[2], with hydrophilic side chains extending towards the aqueous phase and hydrophobic side chains penetrating into the hydrophobic part of the membrane. Under such conditions, the C_α backbone position of Lys or Arg residues can become localized in the hydrophobic part of the helix near the polar–apolar interface, while the long and flexible positively charged side chains snorkel towards the more polar region of the interface[17,22].

Studies on transmembrane model peptides

A first indication that Lys might snorkel also in transmembrane segments came from biophysical studies on the phage M13 coat protein. The protein was labeled at specific sites with spin-labeled and fluorescent groups and incorporated in model membranes[23,24]. From properties of these different labels, such as mobility and accessibility to the aqueous and/or hydrophobic phase, it was concluded that the backbone C_α atom of at least one Lys close to the interface was still in the hydrophobic region, suggesting that its side chain snorkels to the interface.

Recently, a biophysical approach was developed that, for the first time, allows a direct comparison between the interfacial interactions of different amino acid side chains in transmembrane peptides[18]. For these studies model peptides were used, consisting of a sequence of variable hydrophobic length of alternating Leu and Ala residues, flanked by either Trp (WALP peptides) or Lys (KALP peptides) at both ends (Fig. 2a). These peptides were incorporated into pure lipid model membranes, and the interfacial interactions of both types of residues were first compared by analysing the effects of progressively shortening the hydrophobic length of the peptides relative to the bilayer

thickness on lipid organization (Fig. 2b). If the side chains of the peptides prefer to maintain their interaction with the interfacial region, this can be expected to affect lipid organization as soon as the peptides become too short to fit into a bilayer. Indeed, it was found that upon decreasing the relative hydrophobic length of the peptides, the lipids could adopt nonlamellar phases. This adequately relieves the hydrophobic mismatch, because the acyl chains of the lipids in these nonlamellar phases are more disordered, and, hence, their effective hydrophobic length is smaller. When comparing the effects of WALP and KALP peptides on lipid organization, one striking difference was observed: KALP peptides appeared to have a shorter effective length than WALP peptides; that is, a 23-amino-acid KALP peptide (KALP23) behaved exactly the same as a 21-amino-acid WALP peptide (WALP21).

How can we explain this? In these relatively short peptides, the C_α carbons of Lys and Trp will be buried in the hydrophobic part of the bilayer. Molecular modeling indicates that when the Lys side chains in KALP23 'snorkel' and extend upwards towards the aqueous phase, their positively charged NH_3 group can reach a position approximately 3.4 Å further away from the bilayer center than the indole NH of the Trp side chains in WALP21. This corresponds well with the difference between the positions of the carbonyl group and the phosphate group in a lipid bilayer[25]. Thus, the results suggest that Lys is located with its charged NH_3 moiety close to the lipid phosphate group at the interface and the Trp side chain with its indole NH close to the lipid carbonyl ester. Localization of these groups deeper into the hydrophobic part of the membrane is unfavorable and will result in a different lipid organization.

The effects of the two types of peptides were also compared upon increasing the relative hydrophobic peptide length with respect to the bilayer thickness[26] (Fig. 2c). In this case, if the flanking groups maintain their interaction with the interface, the surrounding lipids can stretch their acyl chains and increase the hydrophobic thickness of the bilayer. This is exactly what was found for WALP peptides, again indicating that the Trp side chains anchor at a well-defined position at the interface. By contrast, KALP peptides with corresponding length showed hardly any effect on bilayer thickness[18]. This suggests that the
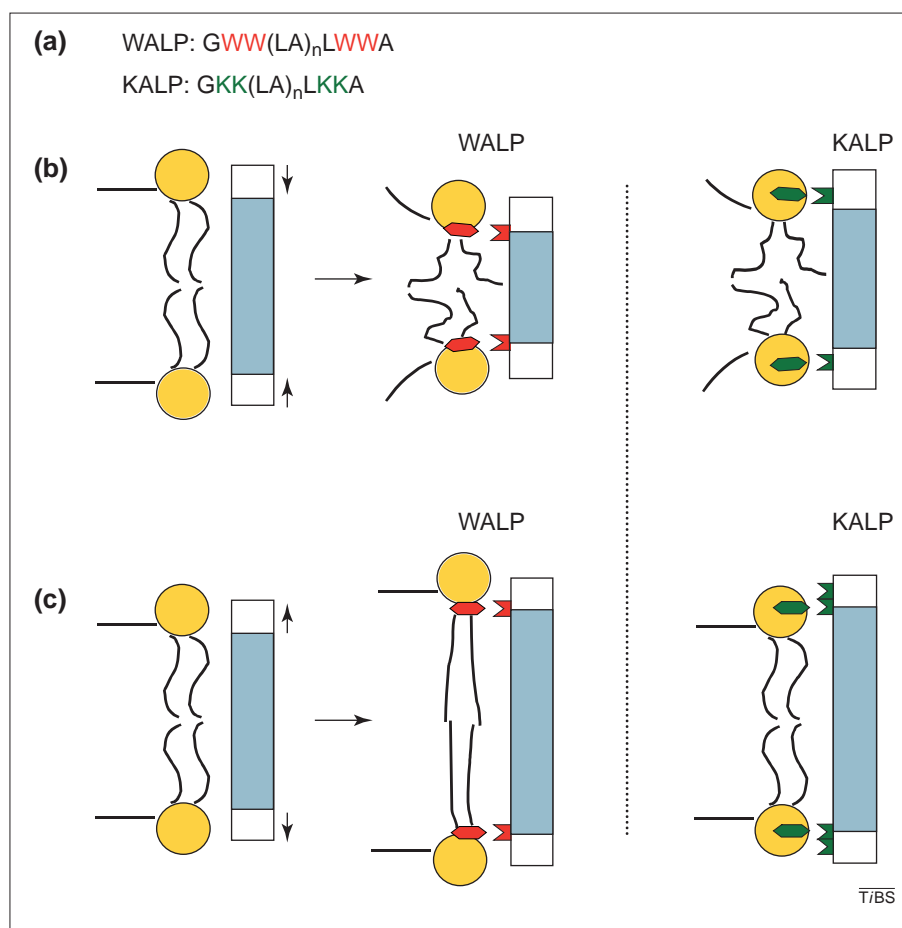
Figure 2
(a) Amino acid sequences of WALP and KALP peptides. The N termini are acetylated and the C termini are blocked with either ethanolamine or amide. The flanking residues W and K are highlighted in red and green, respectively. **(b)** Model of the effect of decreasing the relative length of WALP and KALP peptides. The peptides are drawn as rectangles in which the gray areas represent the hydrophobic Leu-Ala stretch. The putative interaction sites between the side chains and the lipid–water interface are represented by red symbols for the Trp interaction sites and by green symbols for the Lys interaction sites. Incorporation of too-short peptides will result in the formation of nonlamellar phases, in which the interface is curved and the acyl chains are more disordered, thereby relieving the hydrophobic mismatch. WALP peptides behave in the same way as slightly longer KALP peptides, because the latter anchor in a more polar region closer to the aqueous phase. **(c)** Model of the effect of increasing the relative length of WALP and KALP peptides. The defined anchoring position of the Trp side chains causes a stretch of the lipid acyl chains upon increasing the length of WALP peptides. Upon increasing the length of KALP peptides, the long and flexible Lys side chains can either interact with other sites closer to the aqueous phase (e.g. position of 'outer' green symbols, closest to the termini of the peptide), or they can partially bend backwards and still have the positively charged ends interacting with a preferred site in the head-group region, without a need for stretching the acyl chains (position of 'inner' green symbols on the peptide).

Lys side chains either stick out in the more polar part of the interfacial region, or that they partially bend backwards with their hydrophobic part towards the more hydrophobic region of the interface and their positively charged end interacting with the polar head group region. Note that this type of snorkeling is slightly different from the previous situation for relatively short hydrophobic segments, where an extended conformation of the Lys side chain is required to stretch out sufficiently to reach the interfacial region.

In any case, the results on the effects of the two types of flanking residues in situations where the hydrophobic length of the peptides is relatively short or long strongly suggest that Trp is anchored rather rigidly to the interface, whereas Lys is much more flexible and can be accommodated in a larger range of bilayer thicknesses than Trp.

What happens *in vivo*?

Although biophysical studies such as those discussed above have the obvious advantage that detailed and fairly direct
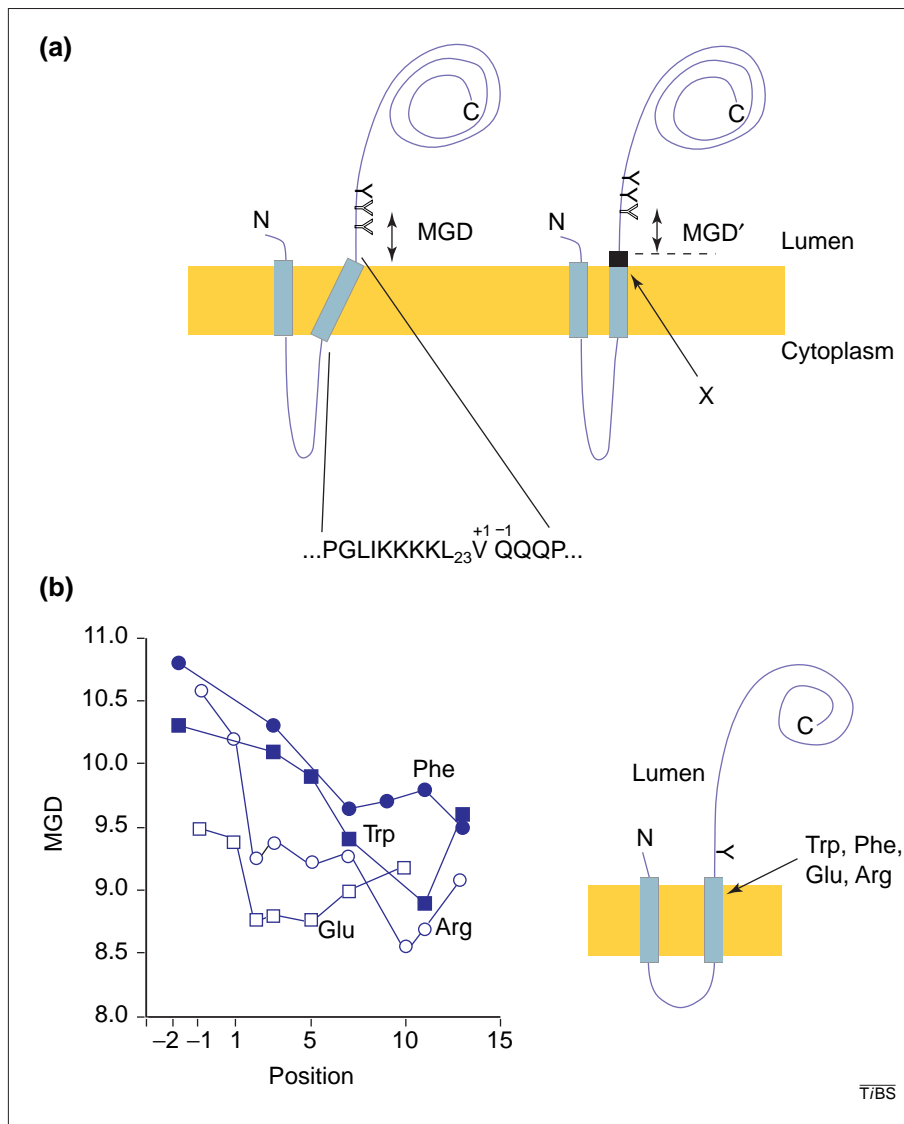
Figure 3
(a) The 'minimal glycosylation distance' (MGD) is determined by placing potential glycosylation acceptor sites (Asn-Ser-Thr) in a series of positions downstream of a transmembrane helix and analysing the constructs by *in vitro* translation in the presence of microsomes (left). A mutation (X) can cause a part of the helix (black) to be expelled from the membrane and a corresponding change in the MGD (right). Glycosylated (Y) and nonglycosylated (Y̶) Asn-Ser-Thr acceptor sites are shown. **(b)** MGD values for Phe, Trp, Arg and Glu mutations in different positions relative to the C-terminal end of a 23-residue poly-Leu transmembrane helix engineered into the *E. coli* inner-membrane protein leader peptidase. Position +1 is the most C-terminal residue in the poly-Leu stretch and counting is in the C→N terminal direction [see (a)].

structural interpretations can often be made, they are quite far removed from the complexities of a *bona fide* biological membrane, and their relevance for understanding what happens *in vivo* can be questioned. However, the available methods for studying membrane proteins *in vivo* rarely allow measurements to be made with the precision required for locating individual amino acids relative to the membrane–water interface.

One novel approach that appears to provide sufficient spatial resolution for such studies is the so-called glycosylation mapping technique (Fig. 3a). The idea is to place potential glycosylation acceptor sites (Asn-Ser-Thr) at a series of positions downstream of a transmembrane helix and to use the lumenally exposed endoplasmic reticulum enzyme oligosaccharyl transferase to glycosylate these sites. In this way, it is possible to determine the 'minimal glycosylation distance' (MGD); that is, the number of residues required to span the distance between a reference residue at the end of the transmembrane helix and the first acceptor site that can be glycosylated by the oligosaccharyl transferase. Single point mutations can then be introduced

at various sites in the transmembrane helix, and their effect on the position of the helix in the membrane can be inferred from their effect on the MGD[27]. MGD values can be determined by *in vitro* transcription–translation of the mutant proteins in the presence of dog pancreas microsomes (i.e. under conditions that closely mimic those in the intact cell).

This technique has so far been used to analyse the effects of all the charged residues (Asp, Glu, Arg, Lys), as well as of the aromatic residues Trp and Phe on the position of a 24-residue poly-Leu transmembrane segment in the microsomal membrane[28,29]. Interestingly, the effects of the different residues are quite distinct (Fig. 3b) and largely consistent with the biophysical measurements discussed above.

When Glu (or Asp) is placed at the C-terminal end of the transmembrane helix (position +1), the MGD is reduced by roughly one residue, compared with Arg, Trp and Phe, corresponding to the expulsion of a part of the transmembrane segment into the lumen. In fact, there is already a drop in the MGD when Glu is placed next to the hydrophobic stretch (position −1), suggesting that it pulls the transmembrane segment towards the aqueous phase. By contrast, Arg (or Lys) only decreases the MGD when placed two or more residues into the hydrophobic stretch, and the drop in MGD values is smaller than for Glu. These results are consistent with the 'snorkel' model discussed above: the long Arg and Lys side chains are largely hydrophobic, with a charged moiety at the end, and will be able to reach up into the membrane–water interface, even from positions located quite far into the transmembrane segment.

Phe and Trp also have different effects, which, again, are consistent with their biophysical properties. Trp has a similar effect as that caused by Arg and Lys, except that it only decreases the MGD substantially when placed more than five residues from the C-terminal end of the transmembrane segment, suggesting that it does not need to reach as far up into the membrane–water interface regions as do Arg and Lys. Phe has the weakest effect of all. This is precisely the behavior expected from the biophysical studies discussed above, where Lys was shown to reach near the phosphate groups, whereas Trp only reached into the carbonyl region.

In summary, there is a reassuring similarity between the results on the

interactions of Trp and Lys residues with the bilayer interface obtained with biophysical and biochemical approaches, lending strong support to the notion that these effects are biologically relevant.

Conclusions and outlook

Among the amino acids that can be expected to interact with the interface, Trp and Lys have been most extensively studied to date. Trp appears to have a strong preference for a well-defined position near the lipid carbonyls, whereas Lys, thanks to its long and flexible side chain, can interact over a wider interfacial region. The side chains of both Lys and Arg can 'snorkel' either by bending backwards, with the hydrophobic part of the side chain dipping into the hydrophobic core of the membrane, or, if the position of the backbone C_α atom is localized deeper within the membrane environment, by fully extending the side chain towards the aqueous phase.

What is the biological relevance of these findings? From a functional point of view, the results imply that Trp (and, possibly, Tyr) as flanking residues of transmembrane segments might influence the precise interfacial positioning of membrane proteins. This will be important for accessibility of defined sites near the interface of, for example, receptor proteins or channel-forming proteins to enzymes, substrates or ligands involved in regulation of the activity of these proteins. Very subtle changes in position might influence the accessibility of these sites both from the aqueous phase and the membrane phase.

The nature of anchoring interactions might also be important for the precise orientation of transmembrane segments within a membrane. For example, strong interfacial anchoring interactions at both sides of a membrane might cause a relatively long helix to adopt a relatively tilted orientation with respect to the bilayer normal. Therefore, the strength of interfacial interactions might influence the conformational flexibility of multi-spanning membrane proteins. Relatively rigid proteins that require no or only very subtle structural changes for functioning, such as gramicidin or porins, might be conveniently and stably anchored by Trp. If functional properties require changes in tilt or conformation, more flexible anchors, at least on one side of the membrane, might be more suitable. This would be in agreement with the recent suggestion for single-span membrane proteins that Trp fulfills
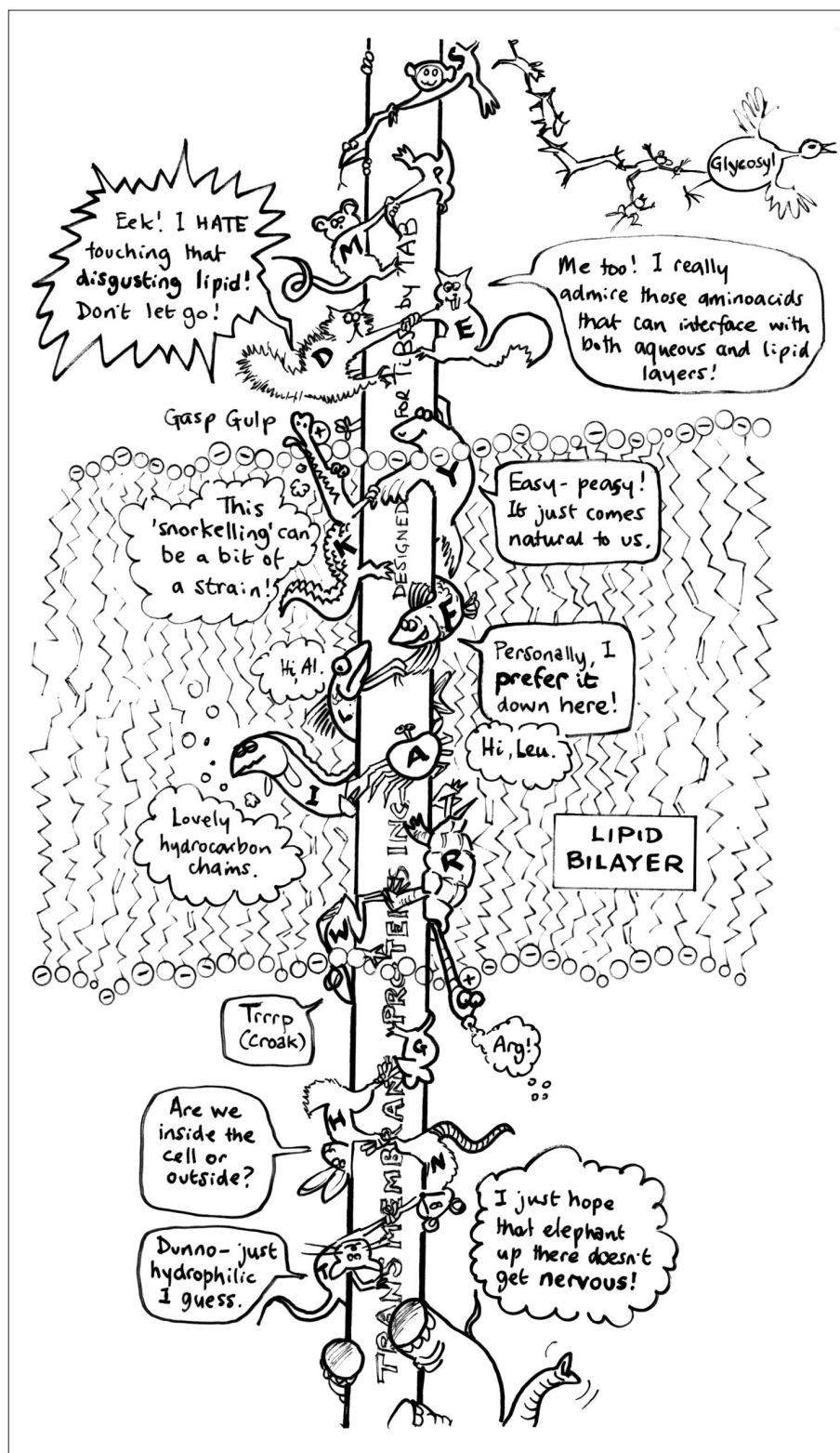
a stabilizing role as interfacial anchoring residue, in particular at the *trans*-side of the membrane, whereas Lys, as topological determinant, remains preferentially at the *cis*-side, where it can act as a flexible anchor[30].

Finally, the results have implications for prediction methods involving membrane proteins. Detailed characterization of interfacial interactions of flanking residues, as described here, will help to improve optimizing predictions of the

precise length of transmembrane regions and their interfacial positioning. Moreover, this type of information could help in optimizing predictions of inter- or intramolecular helix–helix association of proteins in lipid bilayers. For example, an implication of the specific interaction of Trp with lipids near the interface is that flanking Trp residues might play a role in orienting transmembrane helices with respect to each other in multi-spanning membrane proteins.

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Indeed, in cytochrome *c* oxidase interfacially localized Trp have been found mostly in lipid-exposed locations[6]. Further studies using amino acid analogs and bilayers with different lipid composition will be needed to pinpoint the precise physical reasons for these interfacial interactions.

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TRANSCRIPTION SERIES

The Rel/NF-κB family: friend and foe

Neil D. Perkins

The members of the Rel/NF-κB family of transcription factors form one of the first lines of defense against infectious diseases and cellular stress. These proteins initiate a highly coordinated response in multiple cell types that effectively counteracts the threat to the health of the organism. Conversely, disruption of the regulatory mechanisms that control the specificity and extent of this response, which results in aberrant activation of NF-κB, can be one of the primary causes of a wide range of human diseases. Thus, targeting NF-κB might lead to the development of new pharmaceutical reagents that could provide novel treatments for many inflammatory diseases and cancer.

THAT NF-κB WAS likely to play an important role as a regulator of the immune response was apparent from its

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discovery as a constitutively nuclear transcription factor in mature B cells that bound to an element in the kappa immunoglobulin light-chain enhancer (from which its name, nuclear factor κB, was derived)[1]. Shortly afterwards, it was found that NF-κB consisted of a complex of two subunits with molecular weights of 50 kD (p50) and 65 kD (p65), and was present in most cell types in an inactive cytoplasmic form bound to an inhibitor protein termed IκB (Ref. 2). Further research confirmed its involvement in the immune response with the discovery that treatment with the inflammatory cytokines tumor necrosis factor α (TNFα) and interleukin 1 (IL-1) released NF-κB from IκB inhibition, thus allowing it to translocate to the nucleus[2,3]. Furthermore, other NF-κB target genes were identified, including cytokines, chemokines, cytokine and immuno-receptors, adhesion molecules, acute-phase proteins, stress-responsive genes and human immunodeficiency virus 1 (HIV-1)[3]. A definitive indication that NF-κB might have a wider role within cells came from the isolation of the gene encoding the p50 subunit[2]. The first surprise was that this gene actually encoded a 105-kD protein that required proteolytic cleavage to generate p50. The second was that the DNA-binding and dimerization domain of p50 was highly homologous to the viral oncoprotein v-Rel, its cellular counterpart c-Rel and the *Drosophila melanogaster* developmental protein