Polar residues drive association of polyleucine transmembrane helices

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Although many polar residues are directly involved in transmembrane protein functions, the extent to which they contribute to more general structural features is still unclear. Previous studies have demonstrated that asparagine residues can drive transmembrane helix association through interhelical hydrogen bonding [Choma, C., Gratkowski, H., Lear, J. D. & DeGrado, W. F. (2000) *Nat. Struct. Biol.* 7, 161–166; and Zhou, F. X., Cocco, M. J., Russ, W. P., Brunger, A. T. & Engelman, D. M. (2000) *Nat. Struct. Biol.* 7, 154–160]. We have studied the ability of other polar residues to promote helix association in detergent micelles and in biological membranes. Our results show that polyleucine sequences with Asn, Asp, Gln, Glu, and His, residues capable of being simultaneously hydrogen bond donors and acceptors, form homo- or heterooligomers. In contrast, polyleucine sequences with Ser, Thr, and Tyr do not associate more than the polyleucine sequence alone. The results therefore provide experimental evidence that interactions between polar residues in the helices of transmembrane proteins may serve to provide structural stability and oligomerization specificity. Furthermore, such interactions can allow structural flexibility required for the function of some membrane proteins.

Transmembrane (TM) α-helices contain few strongly hydrophilic residues compared with the composition of proteins in general (1–4), which can be explained in part by the high energetic cost associated with burying polar side chains in a hydrophobic environment (5). Serine and threonine are found in TM helices more frequently than are other polar residues (N, D, Q, E, R, K, and H), partially because of their potential to form intrahelical hydrogen bonds to main-chain carbonyl oxygens (6). In fact, permitting S, T, Y, and C in TM regions seems to improve TM helix prediction (7). Interestingly, despite their rare presence in TM helices, strongly polar residues are highly conserved, especially in multispanning TM proteins, suggesting molecular interactions that either functionally or structurally favor these residues (2, 3). Functional roles of some polar residues are observed in structures of proteins, such as binding of prosthetic groups (H and E) in the photosynthetic reaction center (8), retinal binding (K) and proton transport (D) in bacteriorhodopsin (9, 10), binding of hemes (H) in cytochrome *c* oxidase (11) and the cytochrome bc_1 complex, and Ca^{2+} binding (N, D, E, and T) in the Ca^{2+} ATPase (12).

Structural contributions of polar residues in the membrane are less well understood. Interhelical polar interactions have been observed in some integral membrane protein structures available at high resolution. Ion pairs (E65/R185 and R70/E180) are suggested to have a role in the stabilization of the structure of the light-harvesting complex (13). The hydrogen bond formed between conserved E97 and H219 in subunit III of cytochrome *c* oxidase is thought to play a structural role (11). At least one hydrogen bond (main chain to side chain or side chain to side chain) exists between each pair of adjacent helices within the monomer of bacteriorhodopsin; some of the hydrogen bonds are bridged by water molecules (10).

Genetic and biophysical studies of TM proteins have identified critical polar residues that may participate in electrostatic interactions. The *Escherichia coli* lactose permease has only six irreplaceable residues for its function, all of which are polar and reside within TM helices (14–16). Each one is in proximity to one of the others, probably forming three pairs of hydrogen bonds or salt bridges (E126/R144, E269/H322, and R302/E325). These residues as well as a number of other polar ones are implicated in substrate binding and substrate-induced conformational changes. Voltage-gated Na^+, K^+, and Ca^{2+} channels have highly conserved polar residues in their TM helices S2–S4 (17). These residues in the Shaker K^+ channel are proposed to interact in two clusters (E283/R368/R371 and E293/D316/K374) and may be involved in voltage sensing and/or association of helices (18–20). In the inwardly rectifying K^+ channel, functionally conserved S95 of M1 and Q164 of M2 are interacting within the same subunit, presumably forming an interhelical hydrogen bond (21). In the *E. coli* F_1F_o ATP synthase, H^+-transporting residue D61 of subunit *c* is thought to functionally interact with R210 of subunit *a* during the protonation–deprotonation cycle that drives the c_{12} oligomer rotor and the subsequent ATP synthesis (22–25).

An interesting group of TM proteins with conserved polar residues belongs to members of the G-protein-coupled receptor superfamily, in particular the family of rhodopsin-like receptors, which has about 1,500 members known to date (26–29). A number of conserved polar residues in the TM helices are found to be vital to ligand binding and/or signal transduction; interactions among these residues are proposed to conformationally constrain the receptors in their inactive states in the absence of light or ligands (28, 30). Incorporating hydrogen bonding interactions between conserved polar residues as constraints has improved molecular modeling of the TM helix bundle of rhodopsin-like receptors (31). A number of such hydrogen bonds involving the most conserved polar residues (N55, N78, D83, E134, R135, and N302) are confirmed by the recent crystallographic structure of bovine rhodopsin in the ground state (32).

The identification of TM polar interactions may provide helpful structural insights into understanding disease mechanisms. The transformation activity of the bovine papillomavirus E5 protein is a result of TM helix association with platelet-derived growth factor β receptor, an interaction partly mediated by polar residues (33). A single mutation (V664E) in the TM helix of the *neu/erb-2* protooncogene leads to constitutive activation of the encoded tyrosine kinase receptor (34). It was found later that the glutamic acid is involved in interhelical hydrogen bonding interactions (35). However, it remains unclear

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Staphylococcal Nuclease Poly-Leucine Chimeras:

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Nuclease--ERVQLAHHFSEPEITLIIFGVMAGVIGTILLISYGIRRLI      GpA

                    a   d   a   d   a   d   a
                    RVLLLLLLNLLLLLLLVLLLLLLVLRRLI         VNVV

                    RLLLLLLLLLLLLLLLLLLLLLLLRRLI          L23
                    RLLLLLLLNLLLLLLLLLLLLLLLLRRLI         L7NL15
                    RLLLLLLLDLLLLLLLLLLLLLLLLRRLI         L7DL15
                    RLLLLLLLQLLLLLLLLLLLLLLLLRRLI         L7QL15
                    RLLLLLLLELLLLLLLLLLLLLLLLRRLI         L7EL15
                    RLLLLLLLHLLLLLLLLLLLLLLLLRRLI         L7HL15
                    RLLLLLLLSLLLLLLLLLLLLLLLLRRLI         L7SL15
                    RLLLLLLLYLLLLLLLLLLLLLLLLRRLI         L7YL15
                    RLLLLLLLTLLLLLLLLLLLLLLLLRRLI         L7TL15
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Synthetic Peptide:

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                    RVLLLLLLNLLLLLLLVLLLLLLVLRRLI         VNVV
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Fig. 1. Sequences of staphylococcal nuclease chimeras and synthetic peptide. TM sequences were fused to the C terminus of the nuclease via a linker sequence (ERVQLAHHFSEP). Flanking residues of TM sequences are also shown. Positions that vary from leucine are in boldface. The sequence of the synthetic peptide VNVV is identical to that of the TM region of the corresponding chimeric protein VNVV. The study of peptide VNVV and chimeras GpA, VNVV, and L_{23} (also known as LLLL) was reported previously (36).

whether such interactions directly cause dimerization and/or activation of the receptor.

It is obvious that polar residues in TM helices are important, both functionally and structurally. In many cases, however, these residues may be involved not only in binding of ligands/substrates, but also in inducing conformational changes or maintaining structural integrity crucial to the protein function. Such structure–function dependence complicates the interpretation of structural contributions of polar interactions—roles of individual polar residues can only be inferred from the activity of the whole protein. The extent to which these polar residues specify the arrangement of TM helices or stabilize helix–helix interactions is energetically unclear. Therefore, it would be desirable to develop more direct approaches to identifying polar interactions that define structural features of membrane proteins.

We reported previously that asparagine drives TM helix association, possibly by forming interhelical hydrogen bonds (36), which was demonstrated by examining synthetic peptide and chimeric proteins both in detergent and in biological membrane environments. An accompanying report drew comparable conclusions by studying similar peptides, with the use of analytical ultracentrifugation and fluorescence resonance energy transfer methods (37).

A question that arises from the previous results is whether the interhelical hydrogen bond formation is a characteristic of other polar residues that can act as both hydrogen bond donors and acceptors. Here we report contributions by other polar residues to TM helix association through chimeras, in which each TM helix includes a single polar substitution (N, D, Q, E, H, S, T, or Y) in a polyleucine sequence. The ability of these chimeras to associate was tested in detergent micelles and in biological membranes, and the results were compared with those of the glycophorin A (GpA) chimeras.

Materials and Methods

Construction of Staphylococcal Nuclease (SN) Chimeras with TM Domain (Fig. 1). Plasmids pT7SN/GpA99, pT7SN/VNVV, and pT7SN/LLLL (renamed pT7SN/L_{23} here) were made previously (36, 38). The *Avr*II–*Bam*HI fragments for pT7SN/L_7XL_{15} (where X = N, D, Q, E, H, S, Y, and T) plasmids were made in a fashion similar to the way in which pT7SN/L_{23} was made (i.e., by annealing of two oligonucleotides and subsequent amplifica-

tion by polymerase chain reactions). One of the oligonucleotides is degenerate to create different amino acids at the variable position.

Construction of TOXCAT Chimeras. The construction of pccKAN and chimeras of L_{13} (previously LL), GpA, and GpA(G83I) has been described (36, 39). The *Avr*II-*Bam*HI fragments for TOX-CAT L_{19} and L_7XL_{11} chimeras (where X = N, D, Q, E, H, S, Y, or T) were amplified by polymerase chain reactions from corresponding pT7SN/L_7XL_{15} plasmids and inserted into pccKAN between the *Nhe*I and *Bam*HI sites. All TOXCAT constructs, except L_7QL_{11}, were obtained and confirmed by sequencing.

The cells transformed with ligation products of pccKAN and the L_7QL_{11} fragment consistently gave plasmids with identical in-frame deletions of 12 residues within the insert, including the glutamine. The resulting plasmids have only seven leucine residues inserted as the TM sequence and therefore are not useful for our experiments.

Expression and Purification of Nuclease Chimeras. Plasmids were transformed into *E. coli* HMS174(DE3) cells (Novagen). The procedures for cell growth, protein expression, and ion exchange purification were described previously (36). For the purpose of SDS/PAGE analysis, small amounts of purified chimeric proteins can also be obtained with spin columns. Buffers and ion-exchange resins were identical to those used for larger scale purification (36). Twenty to fifty milliliters of cell cultures in LB medium is sufficient to yield enough purified proteins (about 0.5–1 mg). Two to three milliliters of lysis buffer was used, and extraction/centrifugation steps were carried out in microcentrifuge tubes. Extracted samples were dialyzed in 10- to 100-μl or 0.5- to 3-ml Slide-A-Lyzers (Pierce). Resin binding, washing, and elution of the protein were done in microcentrifuge tube filters (0.5 ml, 0.45 μm; PGC Scientific, Gaithersburg, MD). Purified GpA chimera was kindly provided by Zimei Bu (National Institute of Standards and Technology).

Gel Electrophoresis. SDS/PAGE of nuclease chimeras was performed in homogeneous 20% PhastGels (Amersham Pharmacia) and stained with Coomassie brilliant blue. Samples were not boiled before electrophoresis. Heterooligomerization experiments required mixing of the VNVV peptide and purified chimeric proteins in SDS immediately before electrophoresis (36).

Expression of TOXCAT Chimeras and Chloramphenicol Acetyltransferase (CAT) Assays. Plasmids containing GpA, GpA(G83I), L_{13}, L_{19}, and L_7XL_{11} TOXCAT chimeras were transformed into *E. coli* NT326 (MalE$^-$) cells (kindly provided by H. Shuman, Columbia University), which constitutively express these chimeric proteins at low levels. Cell growth, Western blots, malE complementation, CAT, and disk diffusion assays were carried out as previously described (36, 39).

Results

Helix Association in a Detergent Environment. We have investigated the ability of polar side chains (N, D, Q, E, H, S, Y, and T) to mediate TM helix association in the context of a polyleucine sequence fused to the C terminus of the staphylococcal nuclease via a flexible linker to form chimeric proteins (Fig. 1). All chimeras were expressed in *E. coli* and purified in the presence of detergent as described previously (36). The GpA chimera is known to associate as a dimer and was used as a positive control. The ability of chimeras to associate in detergent was tested by SDS/PAGE (Fig. 2). As previously observed, the GpA chimera is dimeric and L_{23} is mostly monomeric (see Fig. 1 for terminology). L_7NL_{15} and VNVV chimeras form similar amounts of dimers. Whereas L_7EL_{15} shows only a slight amount of dimer, L_7DL_{15} is primarily in the form of dimer with little monomer.
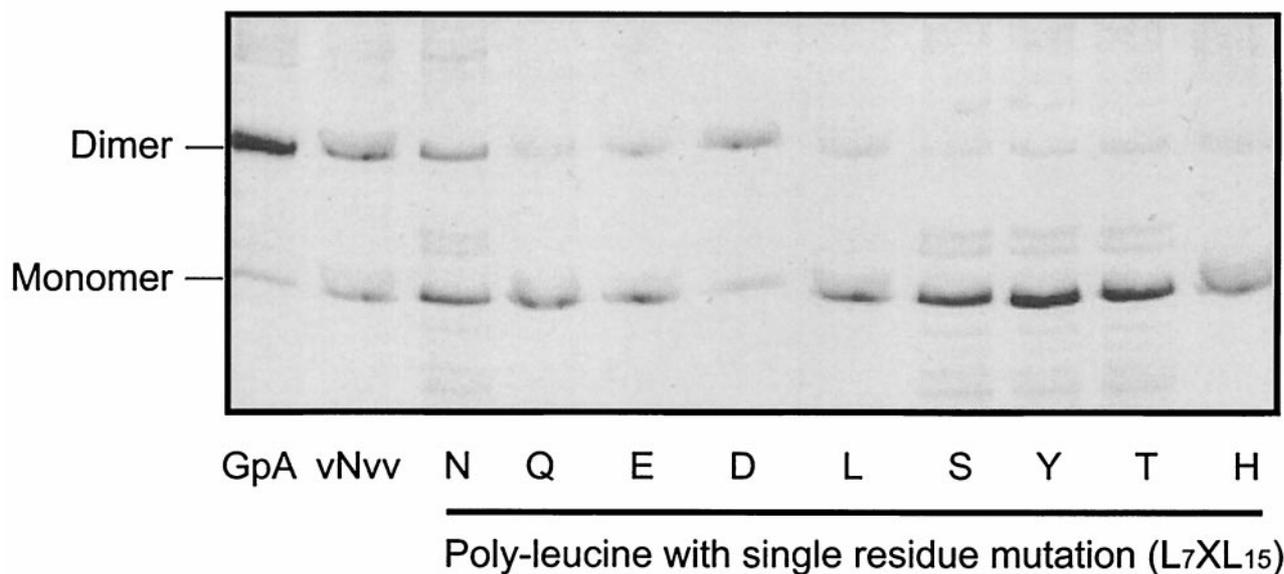
Fig. 2. SDS/PAGE analysis of purified SN chimeric proteins. Polyleucine chimeric proteins with single residue variations (L_7XL_{15}) were analyzed along with the GpA and VNVV chimeras. The L_{23} chimera is shown as L_7XL_{15}, where X = L. The positions of monomer and dimer are marked on the left.

There is no discernible difference between L_7QL_{15}, L_7HL_{15}, L_7SL_{15}, L_7YL_{15}, and L_7TL_{15} in SDS/PAGE, which, like L_{23}, all appear to be mostly monomeric. Therefore, in SDS micelles the propensity of nuclease chimeras to form a homodimer is GpA > L_7DL_{15} > L_7NL_{15} > L_7EL_{15} > L_7QL_{15}, L_7HL_{15}, L_7SL_{15}, L_7YL_{15}, L_7TL_{15}, and L_{23}.

We have shown previously that a synthetic peptide VNVV is able to form heterooligomers with chimeric protein VNVV (36). To examine possible heterooligomeric interactions between different polar residues, the VNVV peptide was mixed with the L_7XL_{15} and VNVV chimeras and analyzed by SDS/PAGE. Fig. 3 shows the chimeras either alone (Fig. 3A) or mixed with the VNVV peptide (Fig. 3B). It is clear that the peptide is able to interact strongly with VNVV, L_7NL_{15} (not shown), L_7QL_{15}, L_7EL_{15}, and L_7DL_{15} to form heterooligomers. L_7HL_{15}, L_{23}, and other L_7XL_{15} chimeras (L_7SL_{15}, L_7YL_{15}, and L_7TL_{15}, not shown) interact only weakly with the peptide, which can be attributed to interactions between leucines. The heterooligomers observed appear to comprise 1-peptide/1-chimera heterodimers, 2-peptide/1-chimera heterotrimers (1), and 1-peptide/2-chimera heterotrimers (2), the last one often being poorly resolved from the chimera homodimers. The heterodimer of the L_7QL_{15} chimera and VNVV peptide indicates hydrogen bonding between glutamine and asparagine. Interestingly, not only do these polar residues interact with Asn, but they do so differently—each pair of interactions yields different relative amounts of heterodimer or trimers. The bias in oligomerization states suggests inherent interaction preferences among these polar residues. By matching preferred partners, polar residues may provide weak specificity in addition to stability in the association of TM helices. However, a single polar residue still may not be sufficient to define a unique oligomerization state; additional polar or van der Waals interactions are likely to be required to further define the association interface and oligomerization state. Furthermore, not all polyleucine TM helices with a polar residue heterooligomerize in SDS/PAGE, because the L_7HL_{15}, L_7SL_{15}, L_7YL_{15}, and L_7TL_{15} chimeras do not interact with the VNVV peptide, at least not differently from the L_{23} chimera. In addition, pairing among L_7HL_{15}, L_7SL_{15}, and L_7QL_{15} chimeras does not produce heterooligomers in SDS/PAGE (results not shown). However, it should be noted that SDS/PAGE analyses might not show true interac-
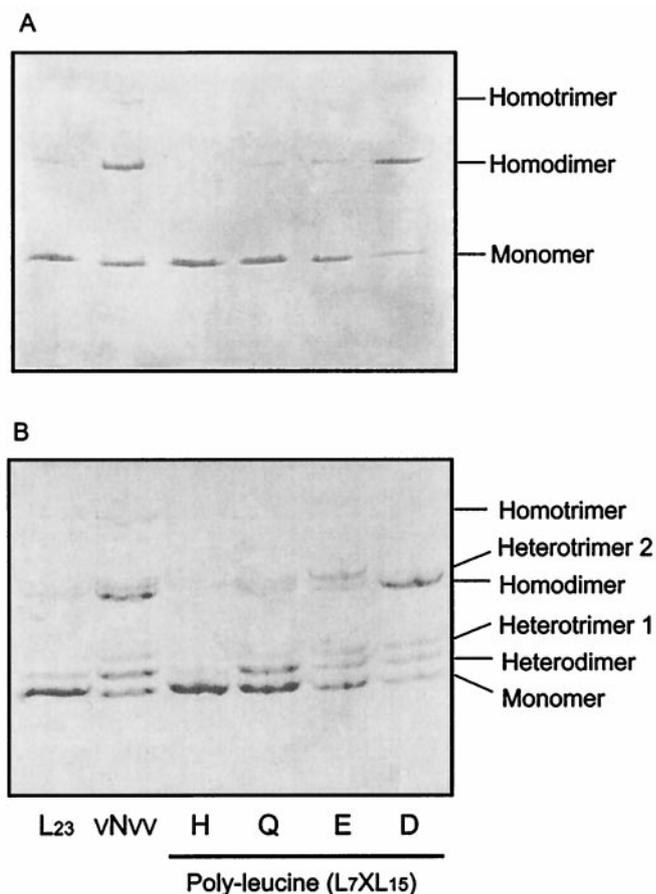

Fig. 3. Heterooligomeric interactions with peptide VNVV. Purified SN chimeras L_{23}, VNVV, L_7HL_{15}, L_7QL_{15}, L_7EL_{15}, and L_7DL_{15} were mixed with peptide VNVV at a peptide/chimera ratio of about 1:5. Samples were tested for heterooligomeric interactions in SDS/PAGE. Positions of monomers, homodimers, and homotrimers of chimeras and heterodimers and heterotrimers (1 and 2) of chimera and peptide are marked on the right. (A) The chimeras without peptide VNVV. (B) The chimeras after the addition of the peptide.

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TOXCAT Poly-Leucine Chimeras:

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ToxR--RASLIIFGVMAGVIGT.......ILIN--MBP      GpA
        RASLIIFGVMAIVIGT.......ILIN          GpA(G83I)
        RASLLLLLLLLLLLLLL.......ILIN          L13

        a   d   a   d   a   d
        RARLLLLLLLLLLLLLLLLLLGILIN            L19
        RARLLLLLLLNLLLLLLLLLLLGILIN           L7NL11
        RARLLLLLLLDLLLLLLLLLLLGILIN           L7DL11
        RARLLLLLLLELLLLLLLLLLLGILIN           L7EL11
        RARLLLLLLLHLLLLLLLLLLLGILIN           L7HL11
        RARLLLLLLLSLLLLLLLLLLLGILIN           L7SL11
        RARLLLLLLLYLLLLLLLLLLLGILIN           L7YL11
        RARLLLLLLLTLLLLLLLLLLLGILIN           L7TL11
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Fig. 4. Sequences of the TM region of TOXCAT chimeras. TM sequences were inserted between the ToxR transcription activation domain (ToxR) and the maltose binding protein domain (MBP). The flanking residues (RAS and ILIN) of the inserted TM sequences are also shown. The mutation in the GpA sequence (G83I) is underlined. The varying position in the polyleucine sequences is in bold. GpA, GpA(G83I), and L_{13} (also known as LL) were previously reported in refs. 36 and 39.

Table 1. Disk diffusion assay

Peptide	ZOI, mm
GpA	31–32
GpA (G83I)	36–37
L_{13}	33–34
L_{19}	34–35
L_7NL_{11}	30–31
L_7DL_{11}	29–30
L_7EL_{11}	30
L_7HL_{11}	31–32
L_7SL_{11}	34–35
L_7YL_{11}	33–34
L_7TL_{11}	34

Values represent diameter measurements of circular zones of inhibition (ZOI) around the chloramphenicol disk devoid of bacterial growth. The concentration of chloramphenicol in the solid medium diminishes with increasing distance from the disk. Small ZOIs indicate chloramphenicol-resistant bacteria and higher CAT activity.

tions between polar residues, because polar head groups of detergent molecules can modulate these interactions (36, 39, 40).

Helix Association in a Biological Membrane. To determine whether the association propensity of L_7XL_{15} chimeras in SDS micelles reflects their ability to interact in a biological membrane, these TM helices (including L_{23}) were subcloned from their nuclease chimeras to form TOXCAT chimeras (39). Only 19 of the 23 TM residues were transferred, giving rise to chimeras L_7XL_{11} and L_{19} (Fig. 4). GpA and L_{13} chimeras are controls as used previously (36). GpA (G83I) is a disruptive mutant of GpA that destabilizes the dimer (40, 41). Subcloning of the L_7QL_{11} chimera was not successful, despite repeated attempts.

In the TOXCAT system, the level of CAT activity (representing the amount of CAT expression) in cell lysates has been shown to correlate with the extent of TM helix association (39). Fig. 5 shows the CAT activity of cells expressing L_7XL_{11} chimeras relative to that of GpA. Chimeras L_7NL_{11}, L_7DL_{11}, and L_7EL_{11} induced CAT expression marginally more than GpA, and induction by L_7HL_{11} is similar to that by GpA. Polyleucine chimeras (L_{13} and L_{19}) produced much lower CAT activities, although these were still higher than that produced by the GpA (G83I) chimera. Induction by L_7SL_{11}, L_7YL_{11}, and L_7TL_{11} is not

much different from induction by L_{19}. Thus these data confirm the finding that in SDS/PAGE all polyleucine TM helices associate to a certain extent, especially at high concentration. These CAT activities are in agreement with the measurements by disk diffusion assays (Table 1). Variations in chimera expression levels were insignificant as analyzed by Western blots, and membrane insertion of the chimeras was confirmed by malE complementation assays (results not shown). In general, the TOXCAT results are consistent with those from SDS/PAGE, although L_7EL_{11} and L_7HL_{11} show relatively stronger associations in a biological membrane than in detergent. This discrepancy may be a result of interactions between the polar side chains and detergent molecules as previously discussed (36, 39, 40). The results also indicate that these TM helix associations can occur in a parallel orientation in biological membranes. A heterooligomerization assay has not yet been developed in membranes to confirm the interactions between the chimeras and peptide observed in detergent.

Discussion

Hydrogen Bonding as a General Driving Force for TM Helix Association. The results of L_7XL_{15} chimeras in SDS micelles and L_7XL_{11} chimeras in biological membranes clearly show that Asn is not the only polar residue that can drive strong helix association. In fact, residues with similar chemical properties (i.e., Asp, Gln, and Glu) can induce comparable or even stronger helix associations. The polar mutants of polyleucine TM helices tested for oligomerization can be divided into two groups. One group includes Asn, Asp, Gln, Glu, and His, which mediate homo- or heterooligomeric helix associations. The second group includes Ser, Thr, and Tyr, which provide little assistance in the association of TM helices. The ability of a polar residue to promote helix association appears to be related to the side chain's potential to be both a good hydrogen bond donor and good acceptor, in the cases of Asn, Asp, Gln, Glu, and His. Interhelical polar interactions between Ser, Tyr, and Thr or between these residues and backbone oxygens are probably insignificant; Ser and Thr may prefer to adopt intrahelical hydrogen bonding to the backbone. Therefore, side chain–side chain, rather than side chain–backbone, hydrogen bonding may be more important in stabilizing helix association. Our results provide evidence that interhelical hydrogen bonds between glutamic acids can drive TM helix association and thus may be the mechanism of constitutive activation of the neu/erbB-2 receptor (35). Although the testing of interhelical polar interactions done so far has not been exhaustive, the heterooligomerization of some polar residue-containing polyleucine TM helices suggests that other com-
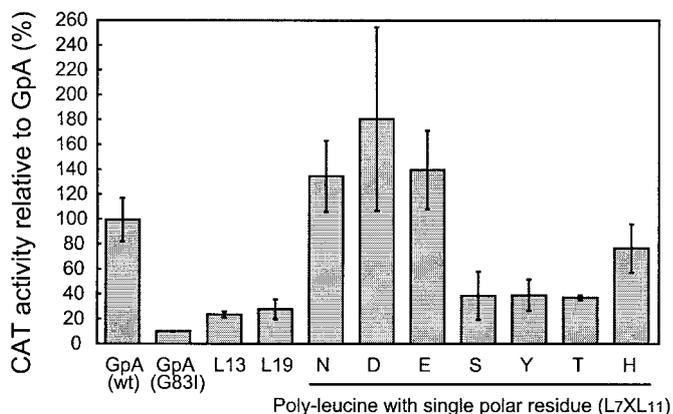

Fig. 5. TOXCAT assay of polyleucine chimeras. Cells expressing TOXCAT chimeras were lysed and assayed for CAT activity (shown relative to that of GpA wild type). All values are the average of at least three independent samples; the error bar shows the estimated standard deviation. The variations in expression levels were insignificant.

binations of polar residues may also drive TM helix association. Our results are in agreement with a similar study of polar residues in hydrophobic peptides by SDS/PAGE and analytical ultracentrifugation (42), where it is reported that, compared with Leu, Asn, Gln, Asp, and Glu strongly promote homotrimerization of these peptides, whereas Ser, Thr, Val, Ala, and Lys do not. Furthermore, both studies found that Asp induces the strongest homooligomeric association. It would be interesting to measure heterooligomerization mediated by interactions between acidic and basic polar residues, such as that between Asp and Lys or Arg suggested in the TM helix association of the α-chain of the T cell receptor and the CD3 δ-chain (43).

Polar Interactions Can Provide Stability and Specificity to Helix Association. It is remarkable that interhelical polar interactions are universal yet vary in strength. The lack of detailed structural information in the data precludes rigorous elucidation of these interactions. The chemical properties of Asn, Asp, Gln, and Glu may partially explain the differential hydrogen bonding strengths. Because Asp and Glu are most likely protonated in the membrane, hydrogen bonds formed are either N-H···O=C (in the case of Asn or Gln) or O—H···O=C (in the case of Asp or Glu). The observed stronger interaction of the latter may be explained by the greater electronegativity of oxygen. These residues may form either one or two hydrogen bonds across the helix interface, depending on the conformation of the interacting side chains, and consequently stabilize TM helix association differently. Variation in side-chain size between Asn (or Asp) and Gln (or Glu) may also contribute to their distinct oligomerization stabilities and specificities, which may be further influenced by the packing of nearby side chains across the helix–helix interface. Interactions between different residues offer even more diversity in terms of strength and specificity. Therefore, it is important to recognize these differences, even when making conservative substitutions, because these residues may not be equivalent.

There are cases in which conservative substitutions compromise structural integrity or functions of the TM proteins. For example, the side-chain size of conserved N111 in TM-III of angiotensin AT_1/AT_2 receptors plays a crucial role in constraining the receptor in its inactive state in the absence of ligands (44). The structural and functional difference between the carboxylic and amide side chains is also documented. Mutant D130N in TM III of the β_2 adrenergic receptor exhibits marked structural instability and altered activity, highlighting the importance of the conserved DRY motif in the rhodopsin subfamily of G protein-coupled receptors, among which 99% have D/E130 (45). In the recently published high-resolution structure of rhodopsin, the Glu (equivalent to D130) and Arg in this motif are involved in a network of hydrogen bonds between TM III and TM VI (32).

Interhelical Hydrogen Bonds May Constrain Protein Conformations. In addition to improving stability and specificity of TM helix association, interhelical hydrogen bonds may play a dynamic role in a protein's structure and function. Some TM helices are thought to be loosely packed but associated with other helices in the protein only by interhelical hydrogen bonds, for instance, in the lactose permease (15) and Ca^{2+} ATPase (12). In some cases, the hydrogen bonding partners may be partially accessible to the aqueous environment (12). This study, together with the work of Gratkowski *et al.* (42), has experimentally demonstrated that a range of polar side chains can mediate TM helix association, presumably through formation of interhelical hydrogen bonds. The free energy contribution from Asn, Asp, Gln, and Glu relative to nonpolar residues ($\Delta\Delta G$) calculated by Gratkowski *et al.* is in the range of 1–2 kcal/mol (42), comparable to the destabilization energy ($\Delta\Delta G$) of GpA mutants (L75A, I76A, and G83I), which is about 1–3 kcal/mol (41, 46, 47). Therefore, such polar interactions may potentially provide all of the association energy necessary to constrain a helix in a certain conformation relative to the helices with which it associates. This constraint may be released if the hydrogen bonds are disrupted by external forces or by the presence of alternative hydrogen bonding partners (such as a ligand, substrate, ion, or water molecule). Relaxation of such constraints may result in global conformational changes of the integral membrane protein as the helices rearrange to find new minimum-energy states. Such induced helix rearrangement may be a switching mechanism underlying the activation/inactivation of TM receptors or the opening/closing of ion channels/transporters. For example, proteins in the G protein-coupled receptor family, especially the rhodopsin subfamily, are thought to be constrained in their inactive states by interhelical interactions such as hydrogen bonds (27–30). Upon light absorption or ligand binding, the polar residues may no longer participate in the original hydrogen bonds, releasing the constraints and allowing receptor conformational changes and, thus, signal transduction activation. Domain movement of Ca^{2+} ATPase (12) and channel gating of the Shaker K^+ channel (20) may also involve rearrangement of interhelical hydrogen bonds. The conformational changes of the lactose permease during transport may be due to the reversible pairing of polar residues (14, 15). In the F_1F_o ATPase, the TM helix bearing residue D61 is proposed to rotate as D61 makes alternating interactions with residues in the *a* and *c* subunits during its protonation–deprotonation cycle (23–25). In these cases, constraints by van der Waals interactions may not be desirable, because the energy needed for constraining helices would demand very specific and extensive helix–helix packing interactions. Switching between two (or more) functional states requires structural flexibility and conformational changes that are readily reversible upon external stimulation. It is unclear whether packing interactions alone would suffice. Nevertheless, helices loosely packed but closely associated by hydrogen bonds may present a solution, where breaking/reforming of hydrogen bonds induced by external forces may be the triggering factor in the conformational changes.

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