The Interface between Self-assembling Erythropoietin Receptor Transmembrane Segments Corresponds to a Membrane-spanning Leucine Zipper*

Weiming Ruan†, Verena Becker§, Ursula Klingmüller¶, and Dieter Langosch‡

From the †Lehrstuhl Chemie der Biopolymere, Technische Universität München, Weihenstephaner Berg 3, 85354 Freising, Germany and the §Max-Planck-Institut für Immunobiologie, Stäubeweg 51, 79108 Freiburg, Germany

Structural and functional studies recently indicated that the erythropoietin receptor exists as a preassembled homodimer whose activation by ligand binding requires self-interaction of its transmembrane segment. Here, we probed the interface formed by the transmembrane segments by asparagine-scanning mutagenesis in a natural membrane. We show that this interface is based on a leucine zipper-like heptad repeat pattern of amino acids. The strongest impact of asparagine was observed at position 241, suggesting the highest packing density around this position, which is in agreement with results obtained upon mutation to alanine. Interestingly, the same face of the transmembrane helix had previously been shown to enter a heterophilic interaction with the transmembrane segment of gp55-P, a viral membrane protein that leads to ligand-independent receptor activation in infected cells. Further, functional characterization of an erythropoietin receptor mutant with asparagine at position 241 in a hematopoietic cell line showed that this protein could still be activated by erythropoietin yet was not constitutively active. This suggests that forced self-interaction of the transmembrane segments does not suffice to induce signaling of the erythropoietin receptor.

Sequence-specific interactions between α-helical transmembrane segments (TMSs) support assembly of many integral membrane proteins (1–3). A recent example where TMS-TMS interaction has functional consequences is the erythropoietin receptor (EpoR), a single-span membrane protein (4, 5). The EpoR is a member of the hematopoietic cytokine receptor family. Signal transduction through the EpoR is crucial for the proliferation of infected erythroid progenitor cells that is due to constitutive mEpoR activation following complex formation with gp55-P (11, 12). Because association of both single-span proteins is sensitive to sequence variations within their TMSs (13–15), gp55-P is thought to activate mEpoR signaling upon heterophilic interaction of their TMSs.

Here, we have systematically analyzed self-interaction of the mEpoR TMS in the context of ToxR chimeric proteins. Anchored within the inner Escherichia coli membrane by heterologous TMSs, ToxR chimeric proteins self-assemble depending on the mutual affinity of the respective TMS as monitored by transcription activation of a reporter gene (16, 17). Taking advantage of the previous observation that asparagine residues within TMSs drive their interaction by hydrogen bond formation (18, 19), we determined the critical residue positions by asparagine-scanning mutagenesis. Interestingly, we find that the homophilic TMS-TMS interface is identical to the face previously (15) shown to interact with the gp55-P TMS. Further, we demonstrate that an asparagine residue at position 241 that induced strong homophilic interaction of mEpoR TMSs disrupts its self-assembling ability (11, 12). Because association of both single-span proteins is sensitive to sequence variations within their TMSs (13–15), gp55-P is thought to activate mEpoR signaling upon heterophilic interaction of their TMSs.

Experimental Procedures

Plasmid Constructs—Construction of plasmids pToxRmEpoR16 and its mutant L240G/L241P was described previously (5). Plasmids mEpoR3.16.1, mEpoR4.16, mEpoR5.15, and mEpoR6.14 were constructed by ligating synthetic oligonucleotide cassettes encoding the desired sequences into the plasmid pHToxR/Thm-sMalE (20) or ToxR IV (21) previously cut with Nhel and BamHI. Each site-directed mutant was constructed by the Kunkel method (22) using a Bio-Rad T7 mutagenesis kit according to the manufacturer’s instructions. All of the constructs were verified by dyeex sequencing. The retroviral expression vectors pMOWS-mEpoR and pMOWS-HA-mEpoR were described elsewhere (23). The construct pMOWS-L241N was established by PCR

* This work was supported by the Deutsche Forschungsgemeinschaft (Graduiertenkolleg "Molekulare und zelluläre Neurobiologie") and the Stiftung der Deutschen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany.

‡ The abbreviations used are: TMS, transmembrane segment; Epo, erythropoietin; EpoR, Epo receptor; HA, hemagglutinin; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptors.

Received for publication, August 22, 2003, and in revised form, November 4, 2003
Published, JBC Papers in Press, November 5, 2003, DOI 10.1074/jbc.M309311200

Printed in U.S.A.
Erythropoietin Receptor Transmembrane Segment Interface

mutagenesis using pMX-mEpoR as template and 5'-GCGATACGTGC-GACCACTGGAACATTCTG-3' as 5'-primer and 5'-GCCGATCTG-TCGCTGACAGCTCGGCGGGCAGGGCCAGAACCCGAT-3' as 3'-primer, and the fragment was subcloned into the SalI and BglII restriction sites of pBabe-mEpoR (24), yielding pBabe-L241N. pMOWS-L241N was generated by subcloning the XhoI and EcoRI fragment from pBabe-L241N into the corresponding sites of pMOWS-mEpoR. The construct pMOWS-HA-L241N was established by subcloning the PmlI and EcoRI fragment of pMOWS-L241N into the corresponding sites of pMOWS-HA-mEpoR. To generate pMOWS-R129C, a XhoI and EcoRI fragment of pMX-R129C (5) was subcloned into the corresponding sites of pMOWS-mEpoR. All of the constructs were verified by dye-excision sequencing.

ToxR Activity Assay—For pHKToxR(TM+)-MaIE vector constructs, plasmid-transformed FHK12 cells were grown for 24 h at 37 °C under shaking in the presence of 2% (w/v) maltose, 0.4 mM isopropyl-1-thio-β-D-galactopyranoside, and 33 μg/ml chloramphenicol. For ToxR vector constructs, plasmid-transformed FHK12 cells were grown for 24 h at 37 °C under shaking in the presence of 2% (w/v) glucose, 0.4 mM isopropyl-1-thio-β-D-galactopyranoside, 10 μg/ml kanamycin, and 0.02-0.15% (w/v) L-arabinose as indicated in the figure legends. β-Galactosidase activities of cell-free extracts were determined as described previously (16) and are given in Miller units.

Western Blotting and Growth Assays—Western blotting was done as described (16) with an antiseminer recognizing the MaIE moiety of the ToxR proteins. Functional complementation of MaIE deficiency of PD28 cells by the periplasmic MaIE domain of correctly integrated ToxR chimeric proteins was done as described (17) by measuring the growth kinetics of expressing cells in minimal medium with maltose as the only carbon source. Growth assays with proteins encoded by ToxR vectors were done without induction because arabinose was found to inhibit uptake of maltose. MaIE complementation in these experiments thus rests on basal expression levels.

Immunoprecipitation and Immunoblotting—An equivalent of 1 × 10^7 BaF3 cells stably expressing HA-tagged versions of the wild-type mEpoR or the mutant L241N were lysed in Nonidet P-40 buffer (0.5% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM NaF, 0.5 mM Na3VO4, 1 mM dithiothreitol, 1 mM aprotinin (Sigma), 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (Sigma) and subjected to immunoprecipitation with anti-EpoR antibody (Santa Cruz Biotechnology). After solubilization with sample buffer, the immunoprecipitates were resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The proteins were detected using anti-EpoR antibody (Santa Cruz Biotechnology) as a primary antibody and protein A coupled to horseradish peroxidase (Amersham Biosciences) and enhanced chemiluminescence system (Amersham Biosciences). Quantification was performed with the Lumi-Imager F1 (Roche Applied Science).

Cell Surface Expression—BaF3 cells expressing HA-tagged versions of the wild-type mEpoR or of the mutant L241N were incubated with rat anti-HA (Roche Applied Science) as primary antibody and with the secondary antibody anti-rat IgG coupled to Cy5 (Dianova, Hamburg, Germany) and analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany).

Proliferation Assay—BaF3 cells stably expressing the wild-type mEpoR or the mutants L241N or R129C were washed three times into RPMI 1640 (Invitrogen) and plated at densities of 4 × 10^4 cells/well in 24-well plates in the presence of Epo concentrations ranging from 0.1 to 1 unit/ml or in medium without Epo. After 4 days, the cell numbers were determined using a Coulter counter and expressed as the percentage of growth obtained in a parallel well containing 5% Walter and Eliza Hall Institute conditioned medium as a source for interleukin-3 instead of Epo.

RESULTS

Optimizing the Orientation of the mEpoR TMS within the ToxR Protein—Previously, stretches of 16 residues representing parts of the predicted TMSs of the murine and of the human EpoR have been shown to self-interact as assessed by the ToxR system (4, 5). In the present study, we initially determined the optimal orientation of the interacting face of the TMS relative to the DNA-binding ToxR domain. This orientation influences the coupling of TMS-TMS interaction to transcription activation as has been found in earlier studies with other TMSs (Ref. 16 and results not shown). Therefore, we determined the effect of inserting the mEpoR TMS at different phases into ToxR chimeric proteins. These initial studies were performed using the original ToxR system where expression is driven by a constitutively active promoter (16). Assuming α-helicity of the TMS, stepwise insertion of four additional residues at its N terminus concurrent with stepwise deletion of four residues at its C terminus rotates the potential TMS-TMS interface by up to 4 × 100 = 400°, i.e. more than a full helical turn, relative to the ToxR domain. Accordingly, ToxR activities of four different constructs harboring 20 TMS residues each were compared with the mEpoR16 protein and its nondimerizing L240G/L241P mutant (5) (Fig. 1A). As shown in Fig. 1B, construct mEpoR4.16 that contained four additional N-terminal residues elicited a stronger ToxR activity than the original mEpoR16 construct and the other constructs with 20 TMS residues. Thus, the phase of the TMS appears to be optimal within mEpoR4.16, and this construct was therefore used for further analysis.

Control experiments ascertained that the different ToxR proteins were expressed at comparable levels as tested by Western blotting of whole cell lysates (Fig. 1C). Further, we tested whether their concentrations in inner bacterial membranes were comparable by determining their ability to complement the deficiency in maltose-binding protein (MaIE) of an E. coli deletion strain (PD28). This strain cannot grow in minimal medium with maltose as the only carbon source unless the C-terminal MaIE-domain of expressed ToxR chimeric proteins is successfully translocated to the periplasmic space (17). Because expression of all ToxR constructs tested here allowed for similar growth kinetics, i.e. maltose uptake (Fig. 1D), we excluded the possibility that the degrees of transcription activation are significantly influenced by differences in membrane integration of the ToxR proteins. A cytoplasmically localized construct where the TMS had been deleted (ΔT) served as negative control.

Identification of Interfacial TMS Residues by Asparagine-scanning Mutagenesis—In the absence of high resolution structures, protein-protein interactions are frequently mapped by investigating the effects of site-directed point mutations on the degree of interaction. Alanine-scanning mutagenesis is usually used for this purpose because exchange of most residue types to alanine creates voids resulting in incremental reductions of protein-protein affinity, and thereby the critical residues can be identified. Because most mutations to alanine, as tested here, reduced self-interaction of the mEpoR TMS only slightly (see Fig. 3), we developed an alternative approach. This novel method is based on recent findings demonstrating that asparagine residues located within TMSs drive their self-interaction in apolar environments like a lipid membrane. This is due to the formation of strong hydrogen bonds between their side chains when water molecules are not available as alternative hydrogen-binding partners (18, 19). We therefore reasoned that systematic replacement of the mEpoR TMS residues by asparagine would result in different enhancements of TMS-TMS affinity depending on whether the mutated position was closely juxtaposed to its counterpart within the helix-helix interface or not.

Accordingly, we performed asparagine-scanning mutagenesis of the TMS within mEpoR4.16 and measured the resulting signal increases. For these analyses, chimeric ToxR proteins were expressed from the pToxRIV plasmid where transcription is under control of the arabinose promoter. The concentration of ToxR proteins within the bacterial membrane can thus be regulated by the concentration of arabinose in the culture medium (21). Because TMS-TMS interaction exhibits concentration dependence like any reversible protein-protein interaction, we tested the effect of the mutations at two different expression levels (Fig. 2A) where mEpoR4.16 elicited less transcription activation than upon constitutive expression (Fig. 1B).
paring the wild-type sequence with the point mutants revealed that the different TMS positions responded to exchange for asparagine with different signal increases. Within the N-terminal six positions (Leu\textsuperscript{228}–Ile\textsuperscript{233}) only S231N showed a slightly increased signal (induction by 0.05\% arabinose; Fig. 2).

Fig. 1. Identifying the optimal orientation of the mEpoR TMS within ToxR chimeric proteins. A, alignment of the predicted TMS with the previously investigated sequence containing 16 residues, its non-dimerizing L240G/L241P mutant, and sequences containing 20 residues in four different phases relative to the ToxR domain. The dots represent wild-type residues. B, degrees of self-assembly of the TMSs shown in A as revealed by β-galactosidase activities (in Miller units, means ± S.E., n = 12) determined upon expression of the respective ToxR chimeric proteins in the reporter strain FHK12. C, protein expression levels were comparable as confirmed by Western blot. D, growth kinetics of PD28 cells expressing the ToxR constructs in minimal medium with maltose were similar, thus indicating similar concentrations of the ToxR proteins in the membrane. A construct where the TMS had been deleted (ΔTM) served as negative control.

Fig. 2. Identification of the mEpoR TMS-TMS interface by asparagine-scanning mutagenesis. A, self-assembly of asparagine mutants as compared with the wild-type construct mEpoR\textsubscript{4.16}. All of the constructs were encoded by pToxRIV plasmids and analyzed at two different inducer concentrations (0.02\% or 0.05\% arabinose). Residue positions that respond to exchange for asparagine with high β-galactosidase (β-gal) activities (means ± S.E., n = 12) follow a heptad repeat pattern and are labeled accordingly. B, protein expression levels were comparable as confirmed by Western blot as shown for induction by 0.05\% arabinose. The order of samples corresponds to that in A. C, growth kinetics of PD28 cells expressing the ToxR constructs in minimal medium with maltose were similar, thus indicating similar densities of the ToxR proteins in the membrane. A construct where the TMS had been deleted (ΔTM) served as negative control. The cell densities observed upon 24 or 48 h were significantly below those shown in Fig. 1D because the growth assay shown here was done without induction by arabinose.
proline residues, and residue Ser238 that is central for interaction with gp55-P is in bold type (Fig. 3). Much stronger signal increases were seen upon mutating residues Leu234 → Ala245, and the strongest signals were of the order L241N > A245N > S238N > L234N. These residues are likely to constitute the mEpoR TMS-TMS interface. The mutation L241N was especially striking because it increased the signal by up to 22-fold and may correspond to a hot spot where packing density is maximal. Although the absolute signal strengths increased at higher arabinose concentration, the relative differences between the mutants and the wild type decreased. This suggests that induction by 0.05% arabinose shifts the concentration of ToxR proteins in the membrane toward saturation of the TMS-TMS binding isoform. Therefore, we inserted a flexible linker sequence (KGSSGSGSK) between the ToxR domain and the mEpoR4.16 TMS and reinvestigated the effect of all asparagine mutations (result not shown), we conclude that the orientation of the TMS interface is comparable in both constructs.

As noted above, stimulation of TMS-TMS interaction was most pronounced for asparagines located from positions 234 to 245. We experimentally tested two potential explanations of this effect. First, we asked whether the low impact of asparagine mutations was due to steric hindrance of TMS-TMS interaction by the adjacent ToxR domains. Therefore, we inserted a flexible linker sequence (KGSSGSGSK) between the ToxR domain and the mEpoR4.16 TMS and reinvestigated the effect of all asparagine mutations in the presence of the linker. We found that the distribution of β-galactosidase activities elicited by the different asparagine mutants was virtually identical to that seen in Fig. 2B (results not shown). Therefore, the insensitivity to asparagine mutation of the N-terminal six positions appears not to be related to steric hindrance of TMS-TMS interaction by the ToxR domains. Second, the observation that exchanging Leu241 to asparagine had a stronger effect than all other mutations suggested that the packing density at this position might be higher than elsewhere in the TMS-TMS interface. To test this possibility, we examined the effects of mutating residues at different α and β angles to alanine. In other words, we tested whether the orientation of interacting TMS faces relative to ToxR domains is similar between mEpoR4.16 and the previously analyzed mEpoR16 construct (5) where roughly one N-terminal helical turn is missing. To
the different TMS mutations did not affect expression levels (Fig. 4C) and membrane integration (results not shown). Thus, the TMS appears to self-interact over its entire length, and position 241 may indeed be the site with the highest packing density within the interface.

Biological Functionality of the mEpoR Mutant L241N—To assess whether an increase in self-interaction of the TMS by the mutation L241N could constitutively activate the mEpoR, we generated a mutant receptor harboring the L241N mutation in the TMS (mEpoR L241N). First, we examined expression of the mutant receptor in comparison with the wild-type mEpoR and analyzed BaF3 cells stably expressing HA-tagged versions of the wild-type mEpoR or the mutant mEpoR L241N by immunoprecipitation and immunoblotting with anti-EpoR antibody (Fig. 5A, left panel). Quantification of the immunoblot revealed that expression of full-length mEpoR L241N is 8-fold increased in comparison with the wild-type mEpoR, whereas the amount of the C-terminal degradation products (27) was reduced 2-fold. Despite this increase in the total amount of the mutant receptor, flow cytometric analysis showed that cell surface expression of mEpoR L241N (mean fluorescence intensity = 5.54 ± 0.26) is reduced by about 45% in comparison with the wild-type mEpoR (mean fluorescence intensity = 10.42 ± 0.46) (Fig. 5A, right panel). These data indicate that the TMS mutation renders the receptor more stable against proteolytic degradation and results in increased intracellular accumulation.

Second, we evaluated the potential constitutive activation of the mutant receptor by comparing the abilities of the wild-type mEpoR, of the mutant mEpoR L241N, and of the previously established constitutively active mutant mEpoR R129C (28) to support proliferation of the factor-dependent pro B cell line BaF3 in the absence or presence of Epo. Parental BaF3 cells and BaF3 cells expressing the wild-type mEpoR or the mutant receptors L241N or R129C were cultured in the presence of Epo concentrations ranging from 0 to 1 unit/ml for 4 days. As expected, BaF3 cells expressing the wild-type mEpoR showed Epo-dependent proliferation, whereas BaF3 cells expressing mEpoR R129C exhibited a substantial degree of Epo-independent growth (Fig. 5B). By comparison, BaF3 cells expressing mEpoR L241N did not grow in the absence of Epo. At a low (0.1 unit/ml) Epo concentration, proliferation of L241N-BaF3 cells was reduced by up to 50% in comparison with cells expressing the wild-type mEpoR. This reduced activity could be attributed to the reduced cell surface expression of the mutant receptor in comparison with the wild-type mEpoR (Fig. 5A, right panel). At higher Epo concentrations, proliferation of cells expressing the wild-type mEpoR or mEpoR L241N was comparable.

Assuming that the L241N mutation increased noncovalent self-interaction of the mEpoR TMS in the BaF3 cell membranes, we conclude that strengthening TMS-TMS interaction by hydrogen bond formation is not sufficient to constitutively activate the mEpoR.

**DISCUSSION**

**Structural Implications—**Our results suggest that mEpoR TMS residues Ser231, Leu234, Ser238, Leu241, and Ala245 occupy α and δ positions of an (abcdefg)₇ heptad repeat motif. This type of repeat pattern is characteristic of soluble (25) and membrane-spanning (26) leucine zipper interaction domains where side chains at α and δ positions tightly interdigitate, whereas e and g positions are located at the periphery of the helix-helix interface. This "knobs-into-holes" type of side chain packing results in positive crossing angles, i.e., left-handed helix-helix pairs. Accordingly, our data suggest that mEpoR TM helices cross each other at a positive angle. Asparagine at some b, c, e, f, and g positions also elicited significant signal increases relative to the wild-type sequence. In the framework of a leucine zipper, the distance between these positions would be too large for hydrogen bond formation to occur (Fig. 3B). Conceivably, the helices may adjust by local distortion or by rotation around their long axes, thus forming alternative, albeit energetically less favorable, interfaces with juxtaposed asparagines. Our data are compatible with this possibility because signals elicited by asparagines at the original e and g positions were above those at adjacent b, c, and f positions whose juxtaposition would require more drastic reorientations.

A leucine zipper type of side chain packing has also been found to drive TMS-TMS interactions of phospholamban (29)
and of the influenza M2 proton channel (30). In contrast to leucine zippers, the TMSs of some other membrane proteins interact via residue patterns of the type (abcd)ₙ, where a and d residues are interfacial. Examples of the latter include glycophasrin A (31), the M13 major coat protein (32), and SNARE proteins (33, 34). Computer modeling (35) and NMR studies (36, 37) showed that these TMSs adopt negative crossing angles.

An unexpected observation made in this study was that asparagine had a much stronger impact on mEpoR TMS-TMS interaction when located in the central versus the terminal regions. This finding is likely to result from two overlapping effects. First, the central region around Leu₂₃₁ may be more efficiently packed than the termini. This is supported by the fact that the disruptive effect of mutation to alanine was more pronounced here than elsewhere in the sequence. This explanation does not, however, resolve why alanines at positions 231, 234, and 238 have similar impacts, whereas asparagine exerts a much weaker impact at position 231 compared with the other a and d positions. Second, therefore, we suggest that the ability of asparagine to promote TMS-TMS assembly may be influenced by a polarity gradient in the lipid bilayer. As outlined above, enhancement of binding affinity between TMSs by asparagine results from formation of strong hydrogen bonds within the apolar/polar regions of the lipid bilayer. Because hydrogen bonds are essentially electrostatic in nature, their strength critically depends on the polarity of the immediate environment. Consequently, a polarity gradient across the inner bacterial membrane may partially be responsible for the differential effect of asparagines on TMS-TMS assembly. Indeed, electron paramagnetic resonance spectroscopy previously revealed a respective polarity gradient in model membranes (38). Specifically, a membrane composed of mono-unsaturated phospholipids is highly nonpolar only at a central slab of its acyl chain region, and polarity increases toward peripheral regions. A similar picture emerged from neutron diffraction studies where it was found that the concentration of the strongly hydrophobic molecule hexane dissolved in a bilayer composed of unsaturated lipids peaked at its center and decreased toward its boundaries (39). The E. coli inner membrane with approximately equal fractions of saturated and mono-unsaturated phospholipids (40) may exhibit a similar polarity gradient. Accordingly, TMSs are thought to experience the lowest polarity at their central regions, which in turn could account for increased strength of hydrogen bonding between asparagine residues from positions 234 to 245. In line with this, we detected an unequal distribution of the impact of asparagine also on self-assembly of a membrane-spanning oligoleucine sequence. This distribution was similar to the one seen here but not identical because it peaked exactly at the middle of the oligoleucine TMS at a site equivalent to mEpoR Ser₂₃₈ (41). Thus, the position-specific ability of hydrogen bond-forming side chains to drive TMS-TMS interactions is not restricted to the mEpoR TMS. This conclusion is supported by a recent study addressing the effect of asparagine on self-assembly of a designed hydrophobic leucine zipper (42). By analytical ultracentrifugation of corresponding synthetic peptides in detergent micelles, these authors showed that asparagine residues within the apolar region of the peptide provide a significantly larger driving force than an asparagine near the apolar/polar interface of the micelle. In sum, an increased density of side chain packing around Leu₂₄₁ and a polarity gradient across the acyl chain region of the lipid bilayer may both contribute to the observed different impact of asparagine at different a and d positions along the TMS-TMS interface.

Functional Implications—One interesting implication of the present study is that the same face of the mEpoR TM helix may alternatively mediate homophilic interaction or heterophilic binding to the TMS of gp55-P in cells infected with polycythemic Friend spleen focus forming virus. The disulfide-linked single-span homodimeric membrane protein gp55-P binds and activates the mEpoR, but not its human homolog, in an Epo-independent way. Constantinescu et al. (15) have shown that functional interaction between gp55-P and the mEpoR depends on the TMS sequences of both proteins. Specifically, this heterophilic interaction requires Ser²₃₈ within the mEpoR TMS and Met²₄⁶ of the gp55-P TMS. Therefore, both residues appear to be localized within the gp55-P-mEpoR TMS-TMS interface. Based on these experimental results, computational searching of low energy structures and model building provided a three-dimensional model of this interface and predicted that it corresponds to a left-handed leucine zipper stabilized by van der Waals’ interactions (15). Interestingly, residues Ser²₃₁, Leu²₃₄, Ser²₃₈, Leu²₄₁, and Ala²₄₅ that account for homophilic assembly of the mEpoR TMS as identified in the present study correspond precisely to the residues contacting the gp55-P TMS in this model. Thus, homophilic interaction of the mEpoR TMSs may compete with its heterophilic interaction with the gp55-P TMS in the membrane of an infected erythroid cell. How this heterophilic TMS-TMS interaction induces constitutive mEpoR activity is presently not clear.

Further, we assessed whether enhanced TMS-TMS interaction would lead to constitutive mEpoR activation. When we characterized the proliferative activity of different variants of full-length mEpoR in a hematopoietic cell line, we found that the mutation L2₄₁N that had the strongest impact on TMS-TMS assembly in bacterial membranes did not induce Epo-independent receptor activity. Control experiments showed that the observed lack of constitutive activation is not due to reduced stability of the mutant receptor. The TMS mutation rendered the receptor more resistant to proteolytic degradation in the cell but resulted in reduced surface expression of the mutant receptor. It has been shown previously that cell surface expression is already quite inefficient for the wild-type receptor whose majority is retained intracellularly. We show that the mutant receptor is fully functional despite its decreased cell surface expression because it mediates wild-type level BaF3 cell proliferation at Epo concentrations of 0.5 or 1 unit/ml. Thus, the TMS mutation did not reduce stability of the receptor or its capability to convert Epo binding to intracellular signaling.

Taken together, therefore, strengthening TMS-TMS interaction appears not to be sufficient for constitutive activation of the receptor. This notion is in agreement with the previous observation that exchange of the mEpoR TMS for that of the strongly homodimerizing glycophasrin A resulted in a receptor that can be activated by Epo but is also not constitutively active (10).

Several functionally related growth factor receptors were previously shown to be constitutively activated by TMS mutations in different hereditary diseases. For example, the neu tyrosine kinase receptor is activated by a substitution of Val₆₆⁴ within its TMS for a glutamic acid residue (43) that appears to induce permanent receptor dimerization by interhelical hydrogen bond formation (44). The tyrosine kinase activity of fibroblast growth factor receptor 3 is activated by a glycine to arginine exchange within its TMS in patients suffering from achondroplasia (45). Further, mutating Ser⁴₉⁸ of the thrombopoietin receptor TMS to asparagine rendered this receptor constitutively active (46), and mutation of Thr⁴₂⁷ to asparagine within the granulocyte colony-stimulating factor receptor TMS as found in patients with acute myeloid leukemia conferred growth factor independence in expressing cells (47). Thus, stabilization of TMS-TMS interaction by hydrogen bond-forming residues may cause constitutive activation of some, but not all,
types of growth factor receptors, pointing at subtle differences in activation mechanisms.

Acknowledgment—We thank Dr. A. Ridder for critical comments on the paper.

REFERENCES

1. White, S. H., and Wimley, W. C. (1999) *Ann. Rev. Biophys. Biomol. Struct.* **28**, 319–365
2. Popot, J.-L., and Engelman, D. M. (2000) *Ann. Rev. Biochem.* **69**, 881–922
3. Langosch, D., Lindner, E., and Gurezka, R. (2002) *JUBMB Life* **54**, 1–5
4. Gurezka, R., Laage, R., Brosig, B., and Langosch, D. (1999) *J. Biol. Chem.* **274**, 9265–9270
5. Kabatzyk, K. F., Ruan, W., Gurezka, R., Cohen, J., Ketteler, R., Wastowich, S. S., Neumann, D., Langosch, D., and Klingmüller, U. (2001) *Curr. Biol.* **11**, 110–115
6. Constantinescu, S. N., Ghaffari, S., and Lodish, H. F. (1999) *Trends Endocrinol. Metab.* **10**, 18–23
7. Wilson, I. A., and Juliffe, L. K. (1999) *Curr. Opin. Struct. Biol.* **9**, 696–704
8. Livnah, O., Stura, E. A., Middleton, S. A., Johnson, D. L., Juliffe, L. K., and Wilson, I. A. (1999) *Science* **283**, 987–990
9. Remy, I., Wilson, I. A., and Michnick, S. W. (1999) *Science* **283**, 990–993
10. Constantinescu, S. N., Keren, T., Socolovsky, M., Nam, H. S., Henis, Y. I., and Lodish, H. F. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 4379–4384
11. Li, J.-F., D’Andrea, A. D., Lodish, H. F., and Baltimore, D. (1990) *Nature* **343**, 762–764
12. Tarr, K., Wastowich, S. S., and Longmore, G. D. (1997) *J. Biol. Chem.* **272**, 9099–9107
13. Chung, S.-W., Wolff, L., and Ruscinetti, S. K. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 7957–7960
14. Zon, L. I., Moreau, J.-F., Kos, J.-W., Mathey-Prevot, B., and D’Andrea, A. D. (1992) *Mol. Cell. Biol.* **12**, 2949–2957
15. Constantinescu, S. N., Liu, X., Beyer, W., Fallon, A., Shekar, S., Henis, Y. I., Smith, S. O., and Lodish, J. F. (1999) *EMBO J.* **18**, 3334–3347
16. Langosch, D. L., Brosig, B., Kolmar, H., and Fritz, H.-J. (1996) *J. Biol. Chem.* **263**, 525–530
17. Brosig, B., and Langosch, D. (1998) *Protein Sci.* **7**, 1052–1056
18. Zhou, F. X., Cocco, M. J., Russ, W. P., Brunger, A. T., and Engelman, D. M. (2000) *Nat. Struct. Biol.* **7**, 154–160
19. Choma, C., Gratkowski, H., Lear, J. D., and DeGrado, W. F. (2000) *Nat. Struct. Biol.* **7**, 161–166
20. Kolmar, H., Fritsch, C., Kleeman, G., Gotze, K., Stevens, F. J., and Fritz, H. J. (1994) *Biol. Chem. Hoppe-Seyler* **375**, 61–69
21. Gurezka, R., and Langosch, D. (2001) *J. Biol. Chem.* **276**, 45580–45587
22. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367–382
23. Swamye, I., Muller, T. G., Timmer, J., Sandra, O., and Klingmüller, U. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 1028–1033
24. Klingmüller, U., Bergelson, S., Hsiao, J. G., and Lodish, H. F. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 8324–8328
25. Lupas, A. (1996) *Trends Biochem. Sci.* **21**, 375–382
26. Langosch, D., and Heringa, J. (1998) *Proteins Struct. Funct. Genet.* **31**, 150–160
27. Neumann, D., Wikstrom, L., Wastowich, S. S., and Lodish, H. F. (1993) *J. Biol. Chem.* **268**, 13639–13649
28. Wastowich, S. S., Yoshimura, A., Longmore, G. D., Hilton, D. J., Yoshimura, Y., and Lodish, H. F. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 2140–2144
29. Zimmermann, H. K. B., Kohayashi, Y. M., Autry, J. M., and Jones, L. R. (1996) *J. Biol. Chem.* **271**, 5941–5946
30. Pinto, L. H., Dieckmann, O. R., Gandhi, C. S., Papworth, C. G., Braman, J., Shaugnessy, M. A., Lear, J. D., Lamb, R. A., and DeGrado, W. F. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 11301–11306
31. Leeminen, M. A., Flanagan, J. M., Treutlein, H. R., Zhang, J., and Engelmann, D. M. (1992) *Biochemistry* **31**, 12719–12725
32. deBer, C. M., Khan, A. R., Zoumi, L., Joenssen, C., Gilbovicza, M., and Wang, J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 11648–11652
33. Laage, R., and Langosch, D. (1997) *Eur. J. Biochem.* **249**, 540–546
34. Laage, R., Rohde, J., Brosig, B., and Langosch, D. (2000) *J. Biol. Chem.* **275**, 17481–17487
35. Fleming, K. G., and Engelman, D. M. (2001) *Protein Sci.* **45**, 313–317
36. MacKenzie, K. R., Prestegard, J. H., and Engelman, D. M. (1997) *Science* **276**, 131–133
37. Smith, S. O., Song, D., Shekar, S., Groesbeck, M., Ziliox, M., and Aimoto, S. (2001) *Biochemistry* **40**, 6553–6558
38. Subczynski, W. K., Wisniewska, A., Yin, J.-J., Hyde, J. S., and Kusumi, A. (1994) *Biochemistry* **33**, 7670–7681
39. White, S. H., King, G. L., and Cain, J. E. (1981) *Nature* **290**, 161–163
40. Neidhardt, F. C., Curtiss, R., and Lin, E. C. (eds) (1996) *Escherichia coli and Salmonella*, Vol. 1, ASM Press, Washington, D. C.
41. Ruan, W., Lindner, E., and Langosch, D. (2003) *Protein Sci.*, in press
42. Lear, J. D., Gratkowski, H., Adamian, L., Liang, J., and DeGrado, W. F. (2003) *Biochemistry* **42**, 6400–6407
43. Weiner, D. B., Liu, J., Cohen, J. A., Williams, W. V., and Greene, M. I. (1989) *Nature* **339**, 230–231
44. Smith, S. O., Smith, C. S., and Bornmann, B. J. (1996) *Nat. Struct. Biol.* **3**, 252–258
45. Webster, M., and Donoghue, J. (1996) *EMBO J.* **15**, 520–527
46. Onishi, M., Mui, A. L. F., Morikawa, Y., Cho, L., Kinoshita, S., Nolan, G. P., Gorman, D. M., Miyajima, A., and Kitamura, T. (1996) *Science* **276**, 1033–1037
47. Forbes, L. V., Gale, R. E., Pirzey, A., Pouwels, K., Nathwani, A., and Linch, D. C. (2002) *Oncogene* **21**, 5981–5989
