

## GALLEX, a Measurement of Heterologous Association of Transmembrane Helices in a Biological Membrane\*

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Whereas a variety of two-hybrid systems are available to measure the interaction of soluble proteins, related methods are significantly less developed for the measurement of membrane protein interactions. Here we present a two-hybrid system to follow the heterodimerization of membrane proteins in the *Escherichia coli* inner membrane. The method is based on the repression of a reporter gene activity by two LexA DNA binding domains with different DNA binding specificities. When coupled to transmembrane domains, heterodimeric association is reported by repression of  $\beta$ -galactosidase synthesis. The LexA-transmembrane chimeric proteins were found to correctly insert into the membrane, and reproducible signals were obtained measuring the homodimerization as well as heterodimerization of wild-type and mutant glycophorin A transmembrane helices. The GALLEX data were compared with data recently gained by other methods and discussed in the general context of heteroassociation of single TM helices. Additionally, the formation of heterodimers between the TM domains of the  $\alpha_4$  and the  $\beta_7$  integrin subunits were tested. The results show that both homo- and heterodimerization of membrane proteins can be measured accurately using the GALLEX system.

Specific protein-protein interaction is an important process in biology, not only in the formation of a stable quaternary structure but also in transient interactions such as those in signal transduction and control of gene expression. Although several organisms' complete genomes are available, understanding the functions of many gene products requires identifying interactions among the encoded proteins. A variety of approaches have been developed in recent years to study such interactions, including the development of the two-hybrid system in yeast (1). Whereas the two-hybrid approach is well established for soluble proteins, measuring the interaction of integral membrane proteins is more difficult, and it is only recently that useful approaches have emerged (2–5).

The finding by Kolmar *et al.* (6) that the transmembrane part of the transcription activator ToxR from *Vibrio cholerae* drives the dimerization of the DNA binding domains to activate the *ctx* promoter and that this TM domain can be functionally

replaced by any other oligomerizing protein or peptide led to the development of an *Escherichia coli* two-hybrid approach measuring the homo-oligomerization of single TM<sup>1</sup> helices in the inner membrane of *E. coli* (7, 8). Although these systems are very useful for the measurement of homo-oligomerization (7–13), they cannot report heterologous interactions.

Here we present a system to measure the heterologous association of transmembrane helices in *E. coli*. Recently, an *E. coli* two-hybrid system based on the LexA DNA binding domain was developed, and it was shown that coupling of this domain to any soluble protein can be used to measure homo-oligomerization of a soluble protein (14). LexA is a transcription factor with an N-terminal DNA binding domain containing about 70 amino acids and a C-terminal dimerization domain (for a review on LexA, see Ref. 15). LexA dimerization is required to repress transcription efficiently, and the LexA DNA binding domain does not contribute to the dimerization of the protein. The discovery of LexA DNA binding domain variants that bind to different DNA sequence enabled the development of a system to analyze heterodimerization of soluble proteins in *E. coli* (16).

To study the interaction of two different transmembrane domains, the GALLEX system presented here was developed. Two LexA DNA binding domains with different DNA sequence specificity were coupled to wild type (WT) and mutated glycophorin A (GpA) TM helices and the promoter/operator sequence contained one specific binding site for each. Association of two separately expressed chimeras that interact in the membrane represses the synthesis of a reporter gene,  $\beta$  galactosidase. The correct membrane insertion of the chimeric proteins was tested as well as the ability of the system to measure either homo- or heterodimerization of single TM helices. Using GALLEX, different interactions between WT and mutated glycophorin A TM helices were measured. By this method, it could be shown that the WT TM domain interacts with other investigated TM helices to a certain degree, and a strongly heterodimerizing pair of glycophorin A mutants was found. The heterodimerization of two TM helices in a glycophorin A-like manner was recently described as key to the formation of heterodimeric integrin complexes (17). Integrins are a family of single spanning membrane proteins that form  $\alpha/\beta$  heterodimers. The analysis of the interaction of the  $\alpha_4$  and the  $\beta_7$  integrin TM helices using GALLEX showed that both TM domains form weak homo- as well as hetero-oligomers. Although heterodimer formation results also from the interaction of a large extracellular domain (18), the role of the transmembrane domains for the formation of these heterodimers was recently discussed (17, 19).

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<sup>1</sup> The abbreviations used are: TM, transmembrane; WT, wild type; GpA, glycophorin A; IPTG, isopropyl-1-thio- $\beta$ -D-galactopyranoside; MBP, maltose-binding protein.

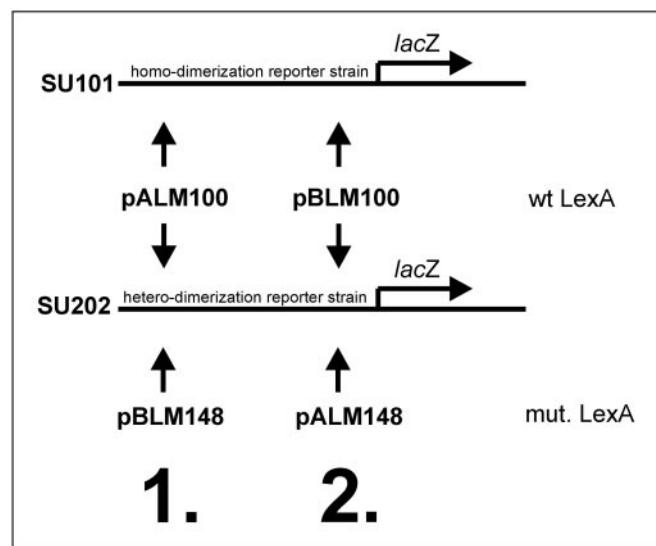


FIG. 1. Overview over the generated plasmids and the used reporter strains. A homodimerizing fusion protein from one of the WT LexA plasmids (pALM100 or pBLM100) will bind to the WT LexA promoter/operator and represses expression of *lacZ* in the genome of the reporter strain SU101. For monitoring heterodimerization, one subunit is expressed from one WT LexA plasmid, and the other one is expressed from the corresponding mutated (*mut.*) LexA plasmid (combination 1 or 2). A heterodimerizing fusion will bind to the hybrid LexA promoter/operator and represses the expression of *lacZ* in the genome of the reporter strain SU 202. The plasmids pALM are derived from pACYC184, and the pBLM plasmids are pBR322-based.

#### MATERIALS AND METHODS

**Plasmid Construction**—Molecular cloning was carried out using standard techniques described in Ref. 20. All enzymes used for PCR and cloning and plasmids were purchased from New England Biolabs.

The N-terminal part (corresponding to residues 1–87) of the WT *lexA* gene from *E. coli* (21) was amplified by PCR, introducing an *NdeI* site at the 5'-end and an *SacI* site at the 3'-end. The restriction-digested PCR fragment was ligated to the *NdeI/SacI* restriction-digested plasmid pMal-p2, resulting in the plasmid pLEX. A fragment from the plasmid pccGpA (8), carrying the TM region of GpA C-terminally fused to the maltose-binding protein (MBP) domain, was amplified by PCR, introducing an *XbaI* site at the 3'-end of the fragment. After digestion with *SacI* and *XbaI*, the fragment was ligated into the *SacI/XbaI*-digested plasmid pLEX, resulting in the plasmid pLGM. In construction of pLKM, for cloning reasons, a  $K_m^R$  cassette was amplified by PCR and ligated to the *SacI/BamHI* restriction-digested vector pLGM, replacing the GpA TM region and introducing a *SpeI* site at the 5'-end of the *malE* gene.

For the measurements of heteroassociation, a two-plasmid system was created, allowing the simultaneous low level expression of two different chimeric proteins. The plasmid pLKM was restriction-digested with *ScaI* and *PvuII*, and the resulting termini were filled by T4 DNA polymerase to generate blunt ends. The plasmid pBR322 was restriction-digested with *SspI/AvaI*, and the termini of the resulting 2743-bp fragment were filled with T4 DNA polymerase. The plasmid pACYC184 was restriction-digested with *AvaI/PvuII*, and the termini of the resulting 2423-bp fragment were filled with T4 DNA polymerase. The fragment originating from pLKM was cloned into the fragments of pBR322 and pACYC184, finally producing the plasmid pBLM100 and pALM100, respectively. These plasmids can be used for the measurement of hetero- as well as homoassociation of single TM helices. For generating plasmids expressing a chimera with a mutated LexA protein (LexA408), the mutated gene was amplified by PCR from the plasmid pSR659 (21). All cloning steps were identical as described above for the WT gene. The fragment originating from pLKM(*mut*) was cloned into the fragments of pBR322 and pACYC184, finally producing the plasmids pBLM148 and pALM148, respectively. These plasmids can be used to express a chimeric protein with the mutated LexA DNA binding domain, and, in combination with pALM100 or pBLM100, they can be used to measure heteroassociation of TM helices. The plasmids and *E. coli* strains used for testing the homo- and heteroassociation capacity of given TM helices are shown in Fig. 1.

All further pBLM- and pALM-based constructs were made by ligat-

ing synthetic oligonucleotide cassettes, which encode for the TM sequences of interest, into the *SpeI/SacI* restriction-digested vectors.

**$\beta$ -Galactosidase Assay**—The association capacity of different chimeric proteins was measured as the repression of reporter gene ( $\beta$ -galactosidase) activity in the *E. coli* SU101 (homoassociation) and SU202 (heteroassociation) indicator strains. The genotypes of these strains are described previously (16). To measure dimerization, overnight cultures of freshly transformed SU101 cells were grown in the presence of various IPTG concentrations, diluted to an  $A_{600} = 0.1$  and finally grown to an  $A_{600} = 0.6$  in LB medium containing the appropriate antibiotics and IPTG. 100  $\mu$ l of cells were used for the measurement of the  $\beta$ -galactosidase activity as described in Ref. 21.

**Test for Insertion and Orientation**—For maltose complementation assays, a single colony of NT326 cells expressing the LexA-TM-MalE chimeric proteins was cultured on M9 agar plates containing 0.4% maltose, 1% ion agar, 0.02% IPTG, and 200  $\mu$ g/ml ampicillin. Plates were incubated at 37  $^{\circ}$ C for 3 days. Only if the MBP portion of the chimeric protein is present in the periplasm will the cells be able to use maltose as the carbohydrate source, since this strain is deficient in endogenous MBP.

To prove that the chimeric proteins are associated with the membrane, cells were extracted with NaOH as described in detail in Ref. 22. After extraction, the pellet, containing stably associated membrane proteins, was resuspended in SDS sample buffer. The proteins in the supernatant fraction, which contains cytoplasmic, periplasmic, and peripheral membrane proteins, were precipitated by 10% trichloroacetic acid and finally resuspended in SDS sample buffer. Proteins were separated on 10% SDS-gels and blotted on nitrocellulose membranes for Western analysis. Western analysis was performed using anti-MBP antibodies (New England Biolabs), and blots were finally developed using goat anti-rabbit alkaline phosphatase-conjugated antibody (Bio-Rad) with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

#### RESULTS

**Outline of the Strategy**—A schematic of the system to measure heterologous association of transmembrane helices in the *E. coli* inner membrane is shown in Fig. 2. The basic idea is to detect association by suppression of  $\beta$ -galactosidase when two versions of LexA domains bind to two adjacent, different DNA sequences. For initial testing and optimization of the system, GpA TMs were used, because the self-association of this transmembrane helix is very well characterized (8, 23–25). Each of the individual single transmembrane fragments has an N-terminal LexA DNA binding domain and a C-terminal MBP domain from *E. coli*. The hydrophobicity of the transmembrane domain functions as a membrane insertion signal, placing the LexA domain in the cytoplasm and the MBP domain in the periplasm of *E. coli*. If the transmembrane domains interact, the LexA cytoplasmic domains are in close proximity and can bind to the operator region, repressing the expression of  $\beta$ -galactosidase. The expression of chimera with noninteracting TMs results in a high  $\beta$ -galactosidase activity due to the lack of repression. To measure heteroassociation, two chimeras were simultaneously expressed from the plasmid pair pALM148 and pBLM100. Whereas the pALM plasmids originate from the plasmid pACYC184 and contain a tetracycline resistance gene, the pBLM plasmids are derived from pBR322 and contain the gene for  $\beta$ -lactamase, resulting in ampicillin resistance. In these plasmids, the expression of the chimeric proteins is driven by the inducible  $P_{tac}$  promoter, and until IPTG is added the transcription is strongly repressed by the *lacI* gene encoding the Lac repressor.

*E. coli* strain SU101 was used to measure the homo-oligomerization capacity of a given TM helix. In this strain, the *lacZ* gene is under the control of the wild-type LexA recognition sequence (*op+*) stably integrated into the *E. coli* genome.

In *E. coli* SU202, the *lacZ* reporter gene is placed under the control of a *op408/op+* hybrid operator. This asymmetric promoter is composed of half of the wild-type promoter plus an altered half (16) (Fig. 2) and allows the binding of a LexA heterodimer composed of one wild-type LexA DNA binding

FIG. 2. The GALLEX assay for measuring TM helix-helix interaction in a biological membrane. The TM domain anchors the chimera in the cytoplasmic membrane of *E. coli* with the C-terminal MBP domain located in the periplasm and the LexA DNA binding domain in the cytoplasm. Interaction of the TM domains leads to the formation of LexA heterodimers, which can bind to the operator region. The binding of the LexA dimer results in repression of the reporter gene (*lacZ*) activity.

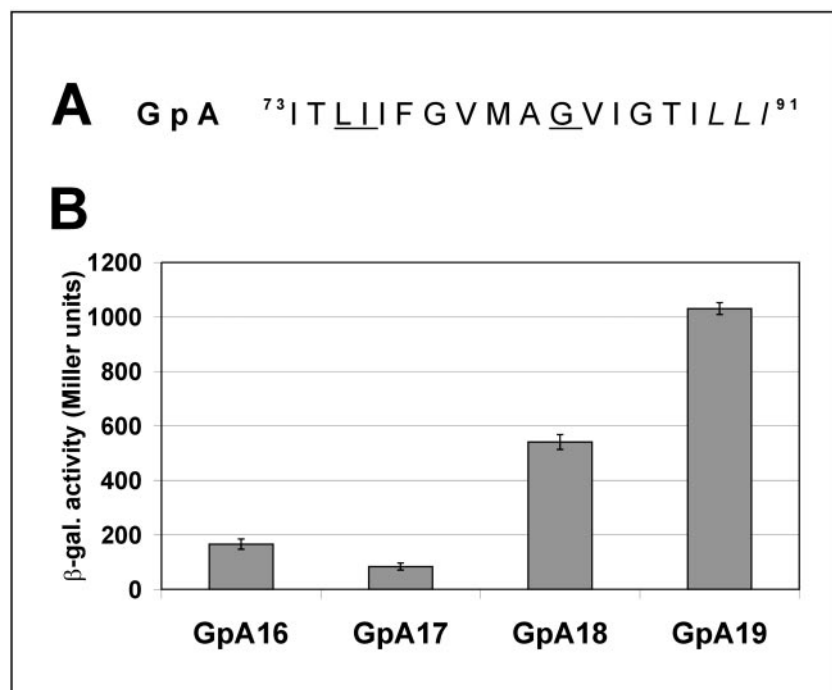
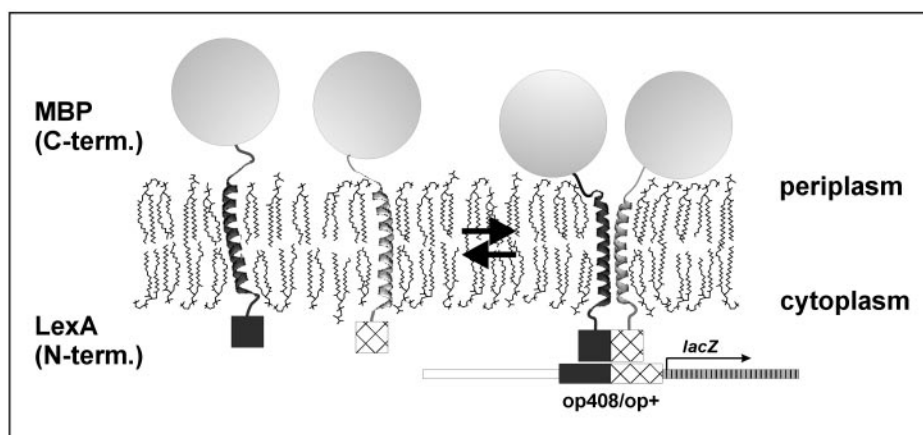


FIG. 3. Optimal length of the GpA TM domain. **A**, amino acid sequence of the GpA TM domain. The length of the helix was altered by successive removal of a residue from the C-terminal, shown in *italics*. The *underlined residues* were mutated as described. **B**, repression of the β-galactosidase activity mediated by the dimerization of the expressed constructs in *E. coli* SU101. The bars represent data combined from three independent measurements. Expression of the chimeric proteins was induced by the addition of 0.5 mM IPTG.

domain and one altered domain (LexA408). It is reported that homodimers do not recognize this hybrid operator so that heterodimeric proteins alone efficiently repress the reporter gene activity (16). Therefore, the capacity for heterodimerization should be measurable even if the individual proteins homodimerize.

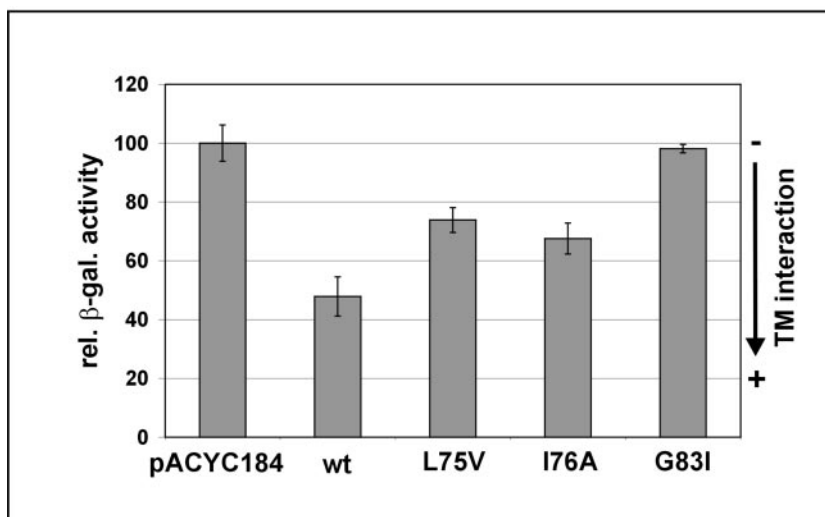
**Homodimerization of Glycophorin A**—To test the system's ability to measure the homodimerization of a given TM, the strongly dimerizing GpA WT sequence and the nondimerizing G83I sequence (8, 24) were fused to the LexA DNA binding domain and the MBP domain as outlined under "Materials and Methods." In GALLEX, the IPTG-inducible *lacUV5* promoter drives the expression of the chimeric proteins. The ability of the fusion proteins to repress the reporter gene's activity was determined at different levels of protein expression obtained upon varying the IPTG concentration (data not shown). In the range between 0.005 and 0.01 mM IPTG, a 50–80% difference was measured between the β-galactosidase activity of the strains expressing either the GpA WT or the GpA G83I chimera. Based on these results, an IPTG concentration of 0.005 mM was used for all further experiments testing for homodimerization in *E. coli* SU101 (if not indicated differently). Although higher IPTG concentration can result in lower β-galactosidase activities, the difference in the β-galactosidase activity between interacting

and weakly interacting TMs (GpA WT *versus* G83I) becomes reduced. It should be noted that the absolute difference in the β-galactosidase activity between the GpA WT and G83I chimeric constructs also depends on the plasmid used for the expression: using pALM100 as the parental plasmid, a difference in the β-galactosidase activity of about 50% was measured, whereas the use of pBLM100 led to differences of up to 80%. This observed discrepancy is most probably explained by the different copy numbers of the plasmids.

The optimal length of the incorporated TM for effective gene repression was determined. For this purpose, GpA TM segments of 16, 17, 18, and 19 residues comprising dimerization motifs (23) were cloned into the plasmid pBLM100, and the reporter gene activity was measured in the *E. coli* strain SU101 after induction of protein expression by the addition of 0.5 mM IPTG. As shown in Fig. 3, insertion of a TM of 17 residues led to the strongest repression. Remarkably, the repression of the β-galactosidase activity was about 10-fold stronger than for the TM helix of 19 amino acids.

To measure the relative homodimerization of the WT GpA TM and its mutants at interfacial residues (L75V, I76A, and G83I), the TM domains were expressed from pALM100, and the β-galactosidase activity was measured in *E. coli* SU101. As a control, the parental plasmid pACYC184 was transformed in *E.*

**FIG. 4. Homodimerization of GpA WT and mutated TM sequences.** As a control, the parental plasmid pBR322 was transformed into *E. coli* SU101, and the  $\beta$ -galactosidase activity was set to 100%. Introduction of the G83I mutation leads to a complete loss of the interaction capacity while the other sequences interact. Bars represent the  $\beta$ -galactosidase activities of three independent measurements, and activities are shown relative to the strain harboring the parental plasmid. The bars represent data combined from three independent measurements.



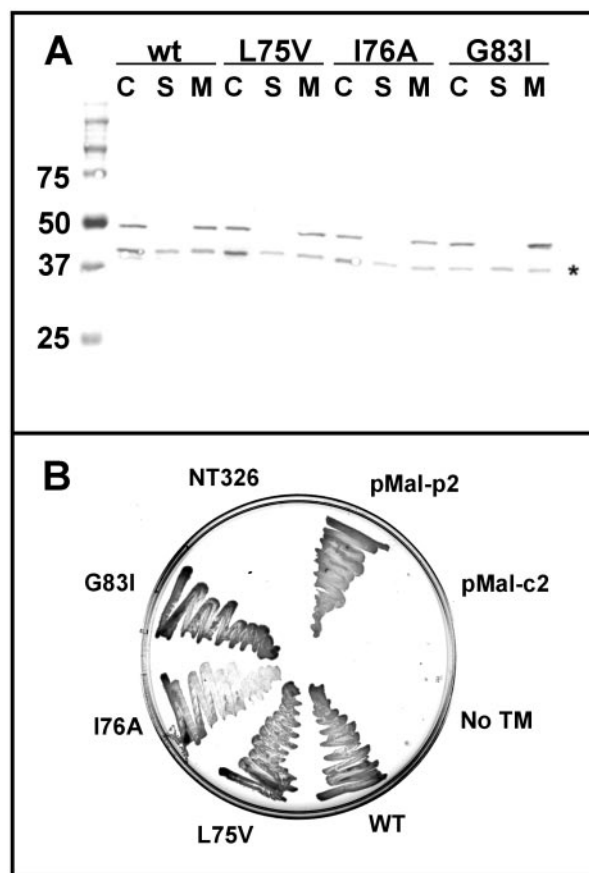
*coli* SU101, and the maximal  $\beta$ -galactosidase activity measured in this strain was set to 100%. The results shown in Fig. 4 demonstrate that the GpA TM domain with the G83I point mutation interacts very weakly. The different GpA TM helices interact in the order WT > I76A > L75V  $\gg$  G83I.

*The Chimeric Proteins Are Located in the Membrane and Have the Designed Topology*—Membrane localization of the chimeric proteins was shown by NaOH extraction. This method was used to separate membrane proteins from cytoplasmic and periplasmic proteins (26). After rigorous extraction of the cells with NaOH, the expressed chimeras are only found in the membrane protein fraction (Fig. 5A), so almost all of the chimeric protein is bound to the cytoplasmic membrane and not localized in the cytoplasm or periplasm.

The topology of the chimeric proteins was examined by complementation assays on M9 minimal medium. The *E. coli* strain NT326 lacks endogenous MBP, resulting in the inability of the cells to transport maltose into the cytoplasm. If the chimeric proteins are inserted into the cytoplasmic membrane with the MBP portion localized in the periplasm, the MBP domain will compensate for this deficiency, and the cells will be able to grow on minimal medium with maltose as the only carbon source. The results shown in Fig. 5B demonstrate that all chimeric proteins with a TM domain can complement the endogenous MBP deficiency, consistent with the correct insertion of the protein into the *E. coli* cytoplasmic membrane with the MBP domain located in the periplasm. And since the assay shows repression, the LexA domains are located in the cytoplasm, and the topology is established.

*Heterodimerization of WT Glycophorin A and Mutants*—For initial testing and optimization of the system to measure heterodimerization, the TM domains of GpA WT and GpA G83I were cloned into the vector pair pALM148/pBLM100 (see Fig. 1). After transformation, strains were obtained that measure the heterodimerization of the chimeric proteins (with respect to the LexA DNA binding domains) driven by the homodimerization of the GpA WT TM region. The other strain expresses the GpA G83I TM domain from both plasmids, which should result in little association. As expected, when the GpA WT chimeric proteins were co-expressed from the two different plasmids, the reporter gene was highly repressed at 0.01 mM IPTG, in contrast to the strain expressing the GpA G83I chimeric proteins (data not shown).

Although the GpA TM domain strongly self-dimerizes, which adds some complexity to the measurement, this TM was used for a more thorough characterization and optimization of the system, since the interaction of these helices is already well



**FIG. 5. Test for insertion and orientation of the chimeric proteins in *E. coli*.** A, Western analysis of *E. coli* cell extracts after NaOH extraction. C, whole cells; S, supernatant after NaOH extraction (soluble proteins); M, pellet after NaOH extraction (membrane proteins). The expressed chimeric proteins with a molecular mass of 54 kDa are found solely in the membrane protein fraction (pellet). \*, proteolyzed chimera. B, *malE* complementation assay to test for LexA(TM)MBP orientation. *E. coli* NT326 were transformed with various constructs and cultivated on M9 agar containing 0.4% maltose and 0.02% IPTG. The GpA TM sequences inserted were WT, L75V, I76A, and G83I. The MBP expression from pMAL-p2 results in periplasmic location of the MBP domain, whereas after expression from pMAL-c2, the MBP is located in the cytoplasm. No TM, pBLM100-based plasmid encoding for a non-TM-spanning domain.

documented (8, 23, 25). Further, it is desirable that the system should be able to measure heteroassociation of other TMs that strongly homodimerize.

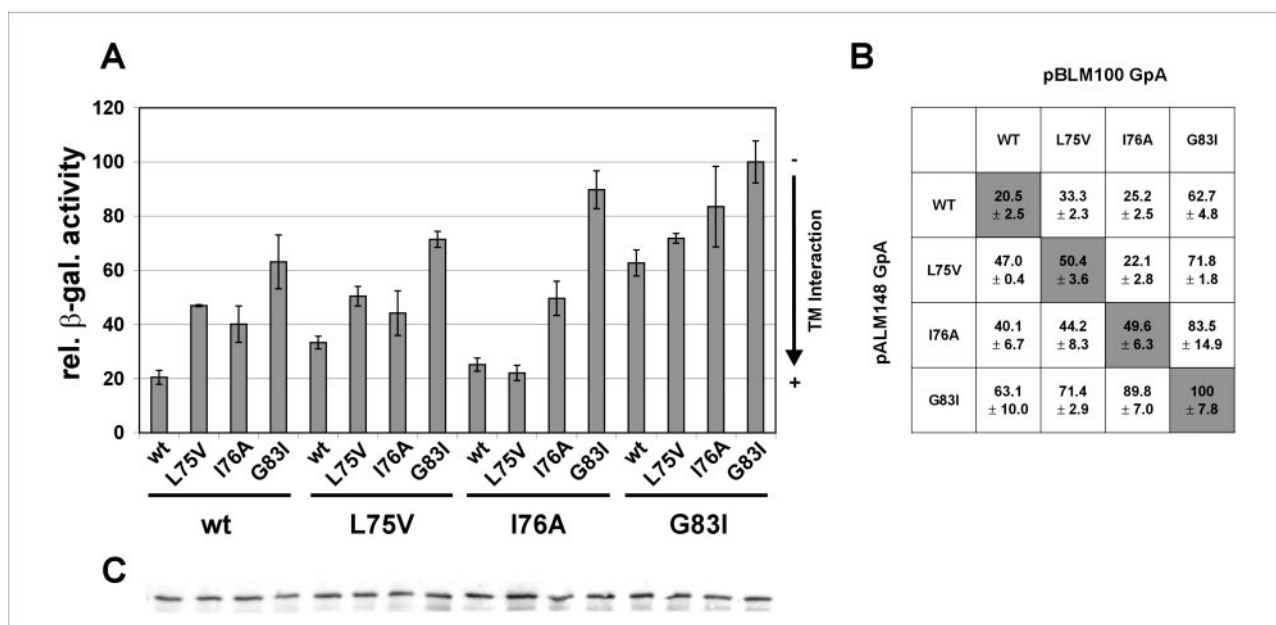


FIG. 6. Heterodimerization of GpA WT and mutated TM helices measured in *E. coli* SU202. Bars in A represent the  $\beta$ -galactosidase activities of three independent measurements and activities are shown relative to the strain expressing the G83I chimera from both plasmids. GpA TM sequences shown under the black bar are expressed from pBLM100; the ones above the bar are expressed from pALM148. In B, the values of A are summarized in a table. C, expression level of the chimera in each strain tested by Western analysis. Antibodies were directed against the MBP domain.

The relative heterodimerization of the WT GpA TM and its mutants at interfacial residues (L75V, I76A, and G83I) was measured in *E. coli* SU202. The results are shown in Fig. 6. The data obtained using the heterosystem show that the WT GpA TM helix is able to form heterodimers with each mutation in the order WT > I76A > L75V > G83I.

The GpA WT sequence forms strongly interacting homodimers, resulting in a high repression of the reporter gene (about 80% repression). Interestingly, the WT sequence also forms heterodimers with each of the other tested sequences, resulting in low  $\beta$ -galactosidase activity. The relative activities are shown in Fig. 6, A and B. Also, the L75V and I76A sequences form homodimers as well as heterodimers with the other TM helices. Besides the formation of heterodimers between GpA WT and any mutant, the results also show that the two mutants L75V and I76A homodimerize to a significant extent. If GpA I76A is expressed from pBLM100 and GpA L75V is expressed from pALM148, the repression reaches about the level of the GpA WT homodimer (22%), whereas if the TMs are expressed in the other orientation, the activity is only repressed to 44%. Nevertheless, in this case, the *lacZ* repression is almost as high as the GpA WT/I76A interaction, and the general lower  $\beta$ -galactosidase activity seems to be caused by the intrinsic asymmetry of the GALLEX system as discussed below. The amino acid substitution G83I is highly disruptive, resulting in high  $\beta$ -galactosidase activities, and none of the other mutations formed a strong dimer with the G83I TM, resulting in high  $\beta$ -galactosidase activities for each heterodimer studied (Fig. 6, A and B).

**Heteroassociation of  $\alpha$  and  $\beta$  Integrin TM Helices**—To analyze whether an GpA-like interaction of the  $\alpha$  and  $\beta$  integrin TM helices can contribute to the formation of the heterodimeric  $\alpha/\beta$  integrin as suggested in Ref. 17, the sequences encoding the two integrin  $\alpha_4$  and  $\beta_7$  TM helices were cloned, and the tendency to form homo- and heterodimers was measured with the GALLEX system. The membrane insertion and orientation of the expressed chimera was tested as described above for the GpA TM helices. The results of the GALLEX measurements shown in Fig. 7 indicate that each integrin TM helix has a tendency to form

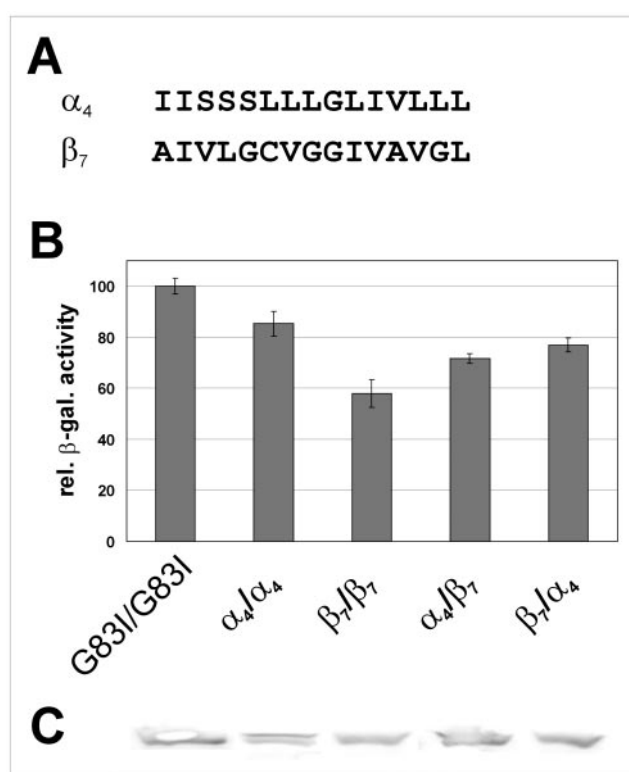


FIG. 7. Homo- and heterodimerization of the integrin  $\alpha_4$  and  $\beta_7$  TM domains measured in *E. coli* SU202. A, the sequences of the TM domains used for the GALLEX measurements. B, association capacity of integrin  $\alpha_4$  and  $\beta_7$  TM domains. Bars, the  $\beta$ -galactosidase activities of three independent measurements. To test for homodimerization, one subunit was expressed from both plasmids, and the capacity to form heterodimers was tested by expressing the two TM domains simultaneously from both plasmids in either orientation. C, expression level of the chimera in the reporter strain tested by Western analysis.

dimers. Whereas the  $\alpha_4$  TM helix shows a slight tendency to form homodimers, the TM helix of the  $\beta_7$  integrin homodimerizes more strongly. The measurement of the formation of  $\alpha/\beta$  heterodimers

shows that the two TM helices tend to form heterodimers, although these are relatively unstable compared with the GpA WT homodimer as shown above.

#### DISCUSSION

**Advantages of the System**—The homodimerization of TM helices has been intensively studied in recent years using reporters of homo-oligomerization in the *E. coli* inner membrane (7, 8, 27). The ToxR-based systems have been used to measure the homo-oligomerization of given TM domains (8–10) and to select for sequence motifs that drive homo-oligomerization from random libraries (11, 28). Although useful, these systems are limited to the investigation of homo-oligomerization. The published systems are based on the formation of a homodimeric ToxR DNA binding domain from *V. cholerae*, which is driven by the interaction of two identical TM helices. The basic principles used are similar to the GALLEX system presented here. Although with GALLEX, the repression of a reporter gene is measured in contrast to its activation, the activity of the reporter gene (*lacZ*) is directly related to the formation of LexA (hetero-) dimers driven by the association of the TM segments. In contrast to the ToxR-based systems, this system can measure homo-oligomerization of TM helices (using *E. coli* SU101) as well as hetero-oligomerization (using *E. coli* SU202) even if one or both of these helices homo-oligomerize. It was recently shown that the expression of chimeric soluble proteins from different plasmids can result in conflicting results (21) and that the rate of repression seems to depend on the plasmid from which each TM helix is expressed. Since controlling the level of protein expression from the two different plasmids (pALM and pBLM) simultaneously is difficult and the slightly different expression levels can obviously cause some variations, comparison of experiments with the reciprocal fusions of the TM helices is always recommended.

Other possible limitations of the system include the potential inhibition of DNA binding of the LexA DNA binding domain caused by its fusion to nonnative proteins as discussed in Ref. 29. Also, the length of the TM helices and the localization of the oligomerizing interface seem to be a critical factor. The results shown in Fig. 3 suggest that the relative orientation of the soluble domains is important. Whereas a high  $\beta$ -galactosidase activity is seen for the GpA TM helices of 18 or 19 residues, the TM helices of 16 or 17 residues seem to place the two DNA binding domains in the right orientation for optimal DNA binding. The 10-fold difference in the  $\beta$ -galactosidase activity of the different TM lengths seen in Fig. 3 is not to be directly compared with the 2-fold changes observed in the later experiments (Figs. 4, 6, and 7), since a higher concentration of IPTG was used for induction (0.5 versus 0.005 mM).

An optimized orientation of the interaction helical surface relative to the soluble domains was also found in earlier studies with the ToxR DNA binding domain coupled to the GpA TM helix (7). The observation of an optimized spatial orientation of the interaction helical surface relative to the soluble domains may make it necessary for an individual experiment to optimize the length of a TM helix and the orientation of the interacting interface relative to the soluble domains.

The analysis of the GpA TM helices that exhibits both homo- and heteroassociation introduces an additional level of complexity to the system, since multiple equilibria must be taken into account. If two single TM helices do not homodimerize, the observed degree of  $\beta$ -galactosidase activity directly reflects the level of heterodimerization of the two TM helices. If one or both helices homodimerize, the observed level of interaction depends on the individual strengths of dimerization, since the tendency of each TM to form homodimers influences the concentration of free monomers for heterodimer formation. In such a case, the

measured values of heterodimerization cannot be taken absolutely but demonstrates the ability of two given TM helices to heterodimerize. In any case, the simultaneous expression of the identical TM from both plasmids should always be done to check for homodimerization and to evaluate the results for the heterodimers properly. In the case of the analysis of the heteroassociation of one or two homodimerizing TM helices, the observed strength of the interaction can be taken not as absolute but as a trend.

**Heterodimerization of Glycophorin A**—The dimerization of glycophorin A is one of the best characterized TM helix-helix interactions to date. The plethora of data available on the homodimerization of GpA WT and mutants made it possible to optimize the GALLEX system and to compare the results with data obtained by other methods. The results shown in Figs. 4 and 6 clearly show that GpA WT and the two GpA TM domain mutants I76A and L75V strongly homodimerize in the *E. coli* inner membrane in contrast to the GpA G83I TM helix when tested in *E. coli* SU101 or SU202.

The general trend of homodimerization described under “Results” (WT > I76A > L75V  $\gg$  G83I) (Figs. 4 and 6) is identical to the data obtained using the TOXCAT system (8). Nevertheless, it should be mentioned that the relative strength of the interaction of the two mutants I76A and L75V is higher than measured with TOXCAT. In GALLEX, even weaker interactions seem to cause a strong response (repression) of the reporter gene. Although the relative stabilities measured by different methods vary, the hierarchy of the stability for GpA sequence variants is conserved, and it was recently shown that this hierarchy is conserved, even if the hydrophobic environment of the TM helices is altered (25).

The different conditions for measuring the homoassociation in *E. coli* SU101 and measuring the heteroassociation of the DNA binding domains driven by the homoassociation of the TM domains (expression of the same TM domain from two plasmids) allow only a qualitative comparison of the strength of the interaction but do demonstrate whether heteroassociation exists. The absolute degrees of repression should not be compared directly between different experimental setups.

Using GALLEX, the heterodimerization of GpA WT with each of the mutant TMs was also observed, although to a lesser extent than the WT-WT interaction. The GpA L75V and the GpA I76A chimeric proteins seem to interact more strongly with some of the other GpA TM helices than with themselves. Specifically, the pair GpA L75V/GpA I76A shows an interaction that is remarkably strong. In earlier work, the heterodimer formation between L75V and I76A was already observed on SDS gels (23). Using SDS-PAGE for the identification of heterodimerizing GpA pairs did not lead to the observation of any pair besides L75V/I76A, whereas an interaction between virtually all of the GpA variants with the exception of the G83I TM is seen in the present assay. The fact that GALLEX measures the interaction in a biological membrane, in contrast to the relatively harsh conditions of SDS-PAGE, may explain why the other interactions could not be observed on SDS gels.

As shown already in the case of the L75I/I76V pair, a point mutation on one TM can be partly compensated by another point mutation on the second GpA TM (23). In previous studies with the GpA TM domain, the effect of single point mutations on the homodimerization was monitored (7, 8, 23). The results presented here indicate that the disruptive effects on homodimer formation of the investigated mutations L75V, I76A, and G83I can be reduced by formation of heterodimers with the WT sequence or with other, more stably associating helices. Therefore, some of the stabilizing interactions disrupted in the homodimer of the three mutant GpA TM helices can be com-

compensated to a certain degree by heterodimer formation. This observed compensatory effect is of particular importance, since the interaction of two different TM helices in a GpA-like manner is proposed to play an important role in the formation of heterodimeric complexes (17, 30). The destabilizing effect of amino acids on the homodimerization of one TM could be partly compensated by the formation of a heterodimer with an other TM helix. By this mechanism, the formation of homodimeric complexes can be repressed, favoring the formation of the more stable heterodimers.

**Heterodimerization of the  $\alpha_4$  and  $\beta_7$  Integrin TM Helices—**Integrins mediate cell-cell and cell-matrix interactions by forming  $\alpha/\beta$  heterodimers. Each subunit of the heterodimer consists of a large extracellular domain, a single transmembrane helix, and a short cytoplasmic domain (reviewed in Ref. 31). Although it has been shown that the formation of heterodimers can be driven by interactions of the large cytoplasmic extracellular domains (18), the role of the transmembrane domains in the formation of hetero-oligomers has recently been discussed as an additional factor (17, 19). Computational studies showed that the TM domains of the  $\alpha_{IIb}/\beta_3$  heterodimer are likely to have a structure similar to the GpA homodimer (17). Almost all integrin TM domains show a GG4-like motif similar to GpA, and a global search of helix-helix interactions indicated that this motif could mediate the heterodimer formation of  $\alpha/\beta$  integrins. Protein fragments encompassing the transmembrane and the cytoplasmic domains of the  $\alpha_{IIb}$  and  $\beta_3$  integrins showed little or no tendency to form heterodimers; instead, the  $\alpha_2$  fragment was found to form a homodimer, and the  $\beta_3$  fragment was found to form a homotrimer (19). The analysis presented here shows that, although the  $\alpha_4$  and  $\beta_7$  TM domains have a tendency to form homodimers, the integrin TM domains also form heterodimers. Each of these dimers is relatively unstable compared with the GpA homodimer. It has been suggested that the relative orientation of the TM helices to each other may change during signal transduction (17). If such a mechanism occurs, very strong association of two TM helices would probably prevent such a reorientation. The observed homodimerization of the single TM helices is most likely due to the fact that in this assay no attention was paid to the extracellular domain of the integrins, a region that is known to mediate interaction and that might control the specificity of the heterodimer formation. The results presented here suggest that the interaction of the transmembrane parts of the integrins could contribute to the formation and/or stabilization of heterodimeric  $\alpha/\beta$  integrins.

**Conclusions—**The GALLEX system presented in this study provides a new approach to investigating helix-helix interactions in a biological membrane. By using two versions of the LexA DNA binding domain and a promoter/operator with two different sequences, the association of GpA WT and mutated TM helices was detected using a reporter gene,  $\beta$ -galactosidase.

Also, the formation of heterodimers between the integrin  $\alpha_4$  and  $\beta_7$  TM domains as well as homodimer formation of the two integrin TM helix alone was shown. In principle, the interaction of any two parallel TM helices should be assessable. As applications emerge, GALLEX should expand our understanding of the chemistry of membrane protein folding and oligomerization.

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