

# The Erythropoietin Receptor Transmembrane Domain Mediates Complex Formation with Viral Anemic and Polycythemic gp55 Proteins\*

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**Erythropoietin receptor (EpoR) activation is crucial for mature red blood cell production. The murine EpoR can also be activated by the envelope protein of the polycythemic (P) spleen focus forming virus (SFFV), gp55-P. Due to differences in the TM sequence, gp55 of the anemic (A) strain SFFV, gp55-A, cannot efficiently activate the EpoR. Using antibody-mediated immunofluorescence co-patching, we show that the majority of EpoR forms hetero-oligomers at the cell surface with gp55-P and, surprisingly, with gp55-A. The EpoR TM domain is targeted by gp55-P and -A, as only chimeric receptors containing EpoR TM sequences oligomerized with gp55 proteins. Both gp55-P and gp55-A are homodimers on the cell surface, as shown by co-patching. However, when the homomeric interactions of the isolated TM domains were assayed by TOXCAT bacterial reporter system, only the TM sequence of gp55-P was dimerized. Thus, homo-oligomerization of gp55 proteins is insufficient for full EpoR activation, and a correct conformation of the dimer in the TM region is required. This is supported by the failure of gp55-A→P, a mutant protein whose TM domain can homo-oligomerize, to fully activate EpoR. As unliganded EpoR forms TM-dependent but inactive homodimers, we propose that the EpoR can be activated to different extents by homodimeric gp55 proteins, depending on the conformation of the gp55 protein dimer in the TM region.**

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Binding of erythropoietin (Epo)<sup>1</sup> to the erythropoietin receptor (EpoR) is crucial for production of mature red cells. EpoR belongs to the homodimerizing subclass of the cytokine receptor superfamily. These receptors function as ligand-induced or ligand-stabilized homodimers (1). Epo binding triggers auto- or trans-phosphorylation of Janus kinase 2 (JAK2) bound to the receptor cytosolic (CT) domain (2), activating JAK kinase activity toward a variety of substrates, such as the receptors themselves, signal transducers and activators of transcription proteins, and a variety of cytosolic signaling molecules (3).

A number of studies have pointed to the possibility that the EpoR may form dimers prior to Epo binding (4–6). We have shown recently that in transfected HEK293-derived cells the majority of the EpoRs is present at the plasma membrane as preformed homo-oligomers (most likely dimers) that do not signal in the absence of Epo; this homodimerization was mediated by the EpoR transmembrane (TM) domain (6). Furthermore, we have shown that Epo binding to the extracellular (EC) domain is transmitted to pre-bound JAK2 via key juxtamembrane (JM) residues, some of which are responsible for binding JAK2 and others for its switching on (7, 8). Interestingly, the JM domain contains a conserved hydrophobic motif that is  $\alpha$ -helically oriented by the TM  $\alpha$ -helix (8). Therefore, the EpoR TM domain, shown to homodimerize in a sequence-specific manner (9, 10), seems to be involved in assembling ligand-independent homodimers, in keeping those inactive, and in orienting the JM domain of the ligand-bound EpoR properly for transmitting the signal to JAK2.

Strikingly, the EpoR TM domain seems also to be required for pathologic activation of the EpoR by co-expression of the gp55 envelope protein of the polycythemic strain of the spleen focus forming virus (SFFV) (11, 12). Two different strains of SFFV have been identified, the polycythemic (P) and the anemic (A) (13, 14). Both P and A strains trigger erythroleukemia (EL) (15, 16) via expression of their envelope proteins, gp55-P and -A, respectively (17–19). A major player in triggering

<sup>1</sup> The abbreviations used are: Epo, erythropoietin; CT, cytosolic; EC, extracellular; EL, erythroleukemia; EpoR, Epo receptor; G $\alpha$ M, goat anti-mouse IgG; G $\alpha$ R, goat anti-rabbit IgG; G $\alpha$ Rat, goat anti-rat IgG; IL3, interleukin 3; JAK2, Janus kinase 2; JM, juxtamembrane; MBP, maltose binding protein; PrlR, prolactin receptor; SFFV, spleen focus forming virus; TM, transmembrane; TpoR, thrombopoietin receptor; FITC, fluorescein isothiocyanate; HA, hemagglutinin; IRES, internal ribosome entry site; gp, glycoprotein; FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein; CAT, chloramphenicol acetyltransferase; A, anemic; P, polycythemic.

erythroid expansion by Friend virus infection is represented by a truncated form of the stem cell kinase receptor (sf-Stk) (20). SFFV-P induces EL and polycythemia (massive increase in the number of mature red cells), whereas SFFV-A induces EL and anemia, due to hemodilution (21). Because co-expression of gp55-P and EpoR in cytokine-dependent Ba/F3 cells, which lack sf-Stk, results in factor independence (22–24) in the absence of sf-Stk expression (25), it was proposed that induction of polycythemia associated with EL can occur through an alternative mechanism, involving EpoR activation by gp55-P. The close proximity of gp55-P to the EpoR was initially demonstrated by cross-linking of <sup>125</sup>I-Epo to gp55-P (26). gp55-P must be at the cell surface in order to activate the EpoR (27, 28). However, the putative cell-surface complex between EpoR and gp55-P has not yet been visualized.

The polycythemic effects of gp55-P are due to sequences at the 3' end of the gp55 gene, which encode the TM domain (29). Studies employing chimeric receptors showed that the TM domain of the EpoR is required for the polycythemic effects of gp55-P (11). The TM domains of gp55-P and -A differ by several point mutations and an insertion of two extra Leu residues (Leu<sup>396</sup> and Leu<sup>397</sup>) in gp55-P (29). gp55-P loses its ability to induce a polycythemic phenotype if Met<sup>390</sup> is mutated to Ile (the corresponding sequence in gp55-A) or if Leu<sup>397</sup> or Leu<sup>396</sup> and Leu<sup>397</sup> are deleted from its TM sequence (12, 30). Such mutants whose phenotypes are altered from P to A are designated gp55-P→A mutants. The activation of murine EpoR by gp55-P requires Ser<sup>238</sup> of the EpoR TM domain (12). Based on molecular dynamics simulations, we propose that a specific interaction between the TM domains requires an interface containing Ser<sup>238</sup> of the EpoR TM domain and Met<sup>390</sup> of the gp55-P TM domain (12).

The mechanism by which expression of gp55-A induces EL has remained unclear, because gp55-A does not render the proliferation of EpoR-expressing cells factor-independent (31), and only infection with SFFV-P, but not with SFFV-A, induces the formation of erythroid bursts from bone marrow (32). We have shown recently that gp55-A induces fetal liver colony-forming unit-erythroid progenitors to differentiate into red cell colonies in the absence of Epo and that this effect requires expression of the EpoR (33). Unlike gp55-P, gp55-A cannot stimulate earlier progenitors, burst-forming unit-erythroid, to survive, proliferate, and differentiate into mature red cell colonies (33). We proposed that the polycythemic phenotype may be related to the ability of gp55-P to expand early burst-forming unit-erythroid progenitors.

Here we show that complexes between EpoR and gp55-P can be visualized on the surface of live transfected cells by antibody-mediated immunofluorescence co-patching. The interaction between EpoR and gp55-P is specific because gp55-P did not oligomerize with other related receptors, such as thrombopoietin (TpoR) or prolactin (PrLR) receptors. By using chimeric EpoR-PrLR constructs containing either the EC, TM, or CT EpoR segments, we show that the EpoR TM domain is the target of gp55-P. Unexpectedly, gp55-A also formed heteromeric complexes with the cell-surface EpoRs, and the target of the gp55-A interactions was also the EpoR TM domain. By using TOXCAT genetic assays we show that the isolated TM sequence of gp55-P but not of gp55-A has homodimerizing ability, although both full-length gp55 proteins are present at the cell surface as homo-oligomers. Thus, gp55 homodimerization is not sufficient for full EpoR activation, as suggested also by the finding that insertion of two extra Leu residues in the TM sequence of gp55-A to resemble gp55-P (the mutant is designated gp55-A Ins Leu<sup>396</sup>, Leu<sup>397</sup>) resulted in a TM sequence which homo-oligomerized, but still failed to fully acti-

vate the EpoR. These results show that the dimeric EpoR TM domain, which normally maintains the unliganded EpoR in an inactive dimeric state, is the target of both oncogenic gp55-P and -A protein dimers.

#### EXPERIMENTAL PROCEDURES

**Generation of EpoR Mutant Plasmids**—The cDNA encoding SFFV gp55 proteins, all cloned into SFFV cDNA, was kindly provided by Dr. Sandra K. Ruscetti, NCI, Frederick, MD. SFFV<sub>AP-L</sub>, which exerts effects identical to SFFV-P, was denoted gp55-P and is in fact gp55-APP, as described (33). The mutant gp55-P M390I, in the same vector, was described (12). The cDNAs coding for mutant gp55 proteins have been cloned in SFFV as described (12). For epitope-tagging gp55-P and gp55-A, DNA sequences encoding the HA (YPYDVPDY) or Myc (EQKLISEEDL) tags were inserted by PCR just downstream of the signal peptidase cleavage site predicted by the SignalP program (34). The murine EpoR cDNA was cloned in the pMX-IRES-GFP 1.1 bicistronic retroviral vector upstream of the IRES as described (12). The level of GFP expression from these vectors is proportional over a 50-fold range to the level of expression of the protein encoded by the cDNA placed upstream of IRES (35). The rabbit PrLR cDNA and the cDNA coding the CHI PrLR-EpoR chimeric construct (the EC domain of PrLR fused to the TM and CT domains of the murine EpoR) cloned in MSCV (36) were kindly provided by Dr. Isabelle Dusanter-Fourt, Hôpital Cochin, Paris, France. They were tagged by replacing the signal peptide with a signal peptide from pFLAG-CMV-1 (Eastman Kodak Co.) followed by the sequence encoding two FLAG epitopes. The tagged receptors were cloned in pMX-IRES-GFP 1.1. In the resulting FLAG-tagged CHI PrLR-EpoR construct, the PrLR EC domain, ending in <sup>206</sup>FTMKD<sup>210</sup>, is followed by the EpoR TM (starting with Leu<sup>226</sup>) and CT segments, as described for the original PrLR-EpoR chimeric construct (36). We designate this construct FLAG-PEE, following nomenclature relating to the receptors (P, PrLR; E, EpoR) from which the EC, TM and CT segments, in that order, were derived (see Fig. 2). The precise junctions in the different chimeric receptors (FLAG-PEP, FLAG-PPE, and untagged EPP) have been described (6). The cDNA of FLAG-tagged murine TpoR (also known as c-mpl), cloned in pcDNA3, was a gift from Amgen.

**Antibodies**—Rat monoclonal 7C10 IgG against gp55 was a generous gift from Dr. Sandra Ruscetti. Goat anti-gp55 polyclonal antibodies (National Cancer Institute repository) were kindly made available by Quality Biotech Inc. Rabbit C-189 antibodies against the EC domain of murine EpoR (anti-EpoR) were described previously (37). Rabbit antibodies against the murine TpoR (anti-TpoR) were from Amgen. Mouse monoclonal anti-FLAG M2 IgG (anti-FLAG) was from Sigma. Rabbit HA.11 against the HA tag (anti-HA) and mouse monoclonal 9E10 anti-Myc tag were from Covance. Biotinylated goat anti-rat IgG (GaRat) minimized for cross-reactivity against mouse serum proteins, FITC-coupled donkey anti-mouse IgG minimized against rat serum proteins, FITC-conjugated goat anti-rabbit IgG (GaR), Cy3-goat anti-mouse IgG (GaM), and Cy3-streptavidin were from Jackson ImmunoResearch. Rabbit antibodies to maltose-binding protein (anti-MBP) were from New England Biolabs.

**Immunofluorescence Co-patching of Cell-surface Proteins**—To measure heteromeric complex formation between EpoR and gp55 proteins, or homomeric complex formation of gp55 proteins directly at the cell surface, we employed antibody-mediated immunofluorescence co-patching of antigenically distinct membrane proteins, as we described recently (38–40). BOSC23 cells grown on glass coverslips were transiently co-transfected using calcium phosphate with gp55-P and gp55-A or mutants together with either EpoR, FLAG-PrLR, chimeric constructs of the two receptors, differently tagged gp55 constructs, or other control cDNAs in mammalian expression vectors. After 48–72 h, live cells were incubated with normal goat IgG (200 µg/ml, 45 min, 4 °C, in HBSS containing 20 mM HEPES, pH 7.4, and 1% bovine serum albumin) to block nonspecific IgG binding. In experiments on heterocomplex formation between a gp55 protein and a receptor (for antibody labeling in gp55 homo-oligomerization studies, see Fig. 4), this was followed by successive incubations (4 °C, 45 min each, with 3 washes between incubations, all carried out in the cold to avoid internalization and enable exclusive cell surface labeling) with the following: (a) rat anti-gp55 7C10 IgG (50 µg/ml) together with a primary antibody against the co-transfected receptor (rabbit anti-EpoR, 50 µg/ml; mouse anti-FLAG, 20 µg/ml; or rabbit anti-TpoR, 5 µg/ml); (b) biotinylated GaRat (20 µg/ml, to enhance patching of gp55) together with FITC-conjugated secondary antibodies (30 µg/ml) to label and patch the co-transfected receptor (FITC-GaR for primary rabbit antibodies or FITC-donkey anti-

mouse IgG for the mouse anti-FLAG to label FLAG-tagged proteins); (c) Cy3-streptavidin (1.3  $\mu\text{g}/\text{ml}$ ) to label and further patch the biotinylated G $\alpha$ Rat bound to the 7C10 IgG, whose patching capability is lower. The protocol results in red-labeled gp55, whereas the co-expressed receptors are labeled with green fluorescence. After washing, the cells were fixed in methanol (5 min,  $-20^\circ\text{C}$ ) and acetone (2 min,  $-20^\circ\text{C}$ ), thereby denaturing GFP and eliminating its fluorescence, and mounted in Slow-Fade (Molecular Probes). Fluorescence digital images were recorded using a CCD camera as described (40). The FITC and Cy3 images were exported in TIFF format to Photoshop (Adobe) and superimposed. The numbers of red, green, and yellow (superimposed red and green) patches were counted on the computer screen, in each case counting at least 100 patches per cell on 10–15 cells.

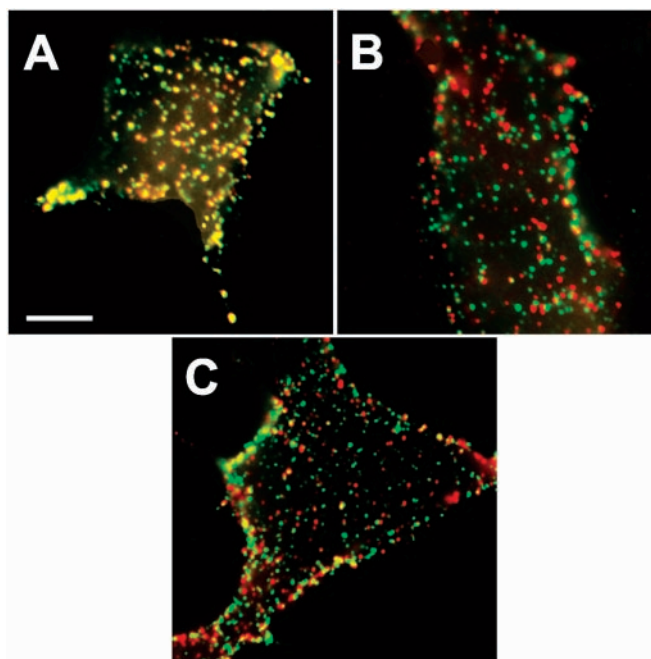
**Co-immunoprecipitation Experiments**—BOSC23 cells grown in 10-cm dishes were transiently co-transfected with vectors encoding gp55-A or gp55-P together with either FLAG-PrIR, FLAG-PEP, Myc-EpoR, or Myc-EPP using the Profection kit (Promega). After 48 h, cells were washed with phosphate-buffered saline and lysed on ice (1 h) in lysis buffer containing 1% Brij97 (Sigma). Immunoprecipitation of gp55 proteins was carried out using anti-gp55 antibodies bound to protein G-Sepharose (Sigma). Samples were separated on 12% SDS-PAGE, transferred to nitrocellulose, and incubated with anti-FLAG or anti-Myc IgG (5  $\mu\text{g}/\text{ml}$ ) followed by peroxidase-coupled goat anti-mouse antibody (Amersham Biosciences); detection was by enhanced chemiluminescence (Amersham Biosciences).

**Infection of Ba/F3 Cells and Epo-dependent Proliferation Assays**—For infection of Ba/F3 cells with retroviruses, high titer replication-defective retroviral supernatants were generated as described (35). First, Ba/F3 cell pools expressing the murine EpoR (Ba/F3-EpoR) were isolated by infecting interleukin 3 (IL3)-dependent Ba/F3 cells with retroviruses encoding EpoR in the pMX-IRES-GFP1.1 vector. Infection was on cells growing in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum, antibiotics (100  $\mu\text{g}/\text{ml}$  streptomycin, 100 units/ml penicillin), and 5% supernatant of WEHI cell line as a source of IL3. EpoR-expressing cells were isolated after washing the cells 4 times in RPMI 1640 and selection by growing (7–9 days) in medium without IL3 but with 1 unit/ml Epo. The EpoR-expressing cells were subjected to a second round of retroviral infection as above, this time with retroviruses generated using gp55 constructs cloned into SFV cDNA. After washing 4 times with RPMI 1640, they were assayed for growth factor independence by growing 4 days in the absence of both IL3 and Epo in RPMI with 10% fetal calf serum, as described (33). High expression of gp55 proteins was demonstrated by Western blotting of cell lysates using a goat anti-gp55 antibody, as described (12, 33).

**TOXCAT Assays**—TOXCAT chimeras containing TM sequences corresponding to the gp55-P TM residues Leu<sup>385</sup>-Leu<sup>403</sup> (LISTIIGSLIILLIILLIILL), the gp55-A TM residues Leu<sup>385</sup>-Leu<sup>401</sup> (LVSTIIGSLIILLIILLIILL), or the TM residues of the gp55-A Ins Leu<sup>396</sup>, Leu<sup>397</sup> mutant, which is gp55-A with an insertion of two extra Leu residues to resemble gp55-P (Leu<sup>385</sup>-Leu<sup>403</sup>, LVSTIIGSLIILLIILLIILL), were generated by PCR amplification of oligonucleotides encoding the TM regions. The PCR products were cloned into pccKAN (41) as *NheI/DpnII* fragments. The resulting plasmids were transformed into NT326 cells (42) and grown as described previously. TOXCAT chimeras with TM sequences derived from glycophorin A or its G83I mutant (41) were used as controls. Expression levels of the chimeras were assayed using anti-MBP immunoblotting. Membrane insertion of the chimeras was verified by survival on M9-maltose medium (not shown), indicating periplasmic localization of the MBP domain. Chloramphenicol acetyltransferase assays of cell extracts were performed using the Quan-T-CAT kit (Amersham Biosciences).

## RESULTS

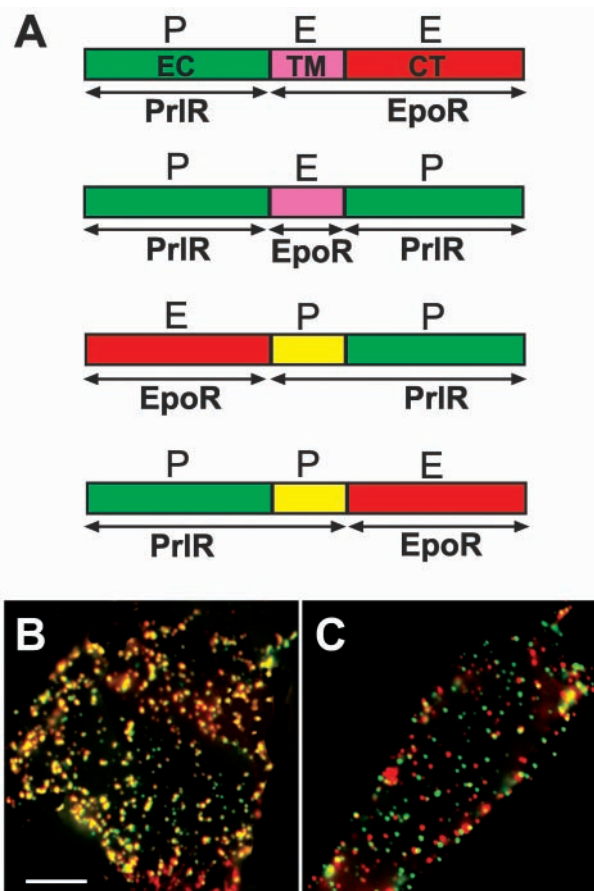
**Cell-surface EpoR and gp55-P Form Heteromeric Complexes**—In order to detect the formation of heteromeric complexes between the murine EpoR and gp55-P at the surface of live cells, we co-expressed these proteins in HEK293-derived BOSC23 cells and employed the immunofluorescence co-patching method. In this method (detailed in Ref. 40), two membrane proteins recognized by different primary antibodies from different animal species are co-expressed at the surface of live cells. One protein is forced into micropatches by a double layer of bivalent IgGs using a fluorescent secondary antibody. The co-expressed protein, which is antigenically distinct and recognized by a different antibody, is patched and labeled by primary antibodies from another species and secondary antibodies



**FIG. 1. Immunofluorescence co-patching to explore heteromeric EpoR-gp55-P complexes at the cell surface.** BOSC23 cells were co-transfected transiently with a retroviral expression vector encoding gp55-P together with vectors encoding either murine EpoR (A), FLAG-TpoR (B), or FLAG-PrIR (C) as described under “Experimental Procedures.” Live cells were consecutively labeled in the cold (to avoid antibody internalization) by a series of antibodies to mediate patching and fluorescent labeling of the co-expressed proteins at the cell surface (see “Experimental Procedures”). The labeling protocol employed results in Cy3-labeled (red fluorescence) gp55-P, whereas the co-expressed receptors (EpoR, FLAG-TpoR, or FLAG-PrIR) are labeled by FITC (green). Fluorescence images were taken by a CCD camera using selective filter sets for FITC or Cy3 and superimposed. Bar, 10  $\mu\text{m}$ . A, EpoR (green) and gp55-P (red) exhibit a high degree of co-patching (yellow). B, FLAG-TpoR (green) and gp55-P (red) show a low level of co-patching; most patches are either red or green, and only a minority is yellow. C, FLAG-PrIR (green) and gp55-P (red) exhibit a low degree of co-patching.

coupled to another fluorophore, or by biotinylated secondary IgG followed by a fluorescent streptavidin derivative. Membrane proteins residing in mutual oligomers will be swept into the same micropatches. If one uses red (e.g. Cy3) and green (FITC) fluorophores, mutual patches appear yellow when the two images are overlapped. Fig. 1 shows typical results of co-patching experiments aimed at analyzing the oligomerization state of the EpoR and gp55-P; the average data from many such experiments are depicted in Fig. 3. Fig. 1A demonstrates that the majority ( $\sim 80\%$ ; see Fig. 3) of cell-surface EpoR and gp55-P are present in mutual (yellow) patches. This co-patching is specific because gp55-P did not co-patch effectively with the related TpoR or PrIR, with most patches being either red or green (Fig. 1, B and C). These low levels of co-patching (25–30%; see Fig. 3) presumably represent the basal level of co-patching due to the cumulative contribution of factors other than specific oligomeric interactions (e.g. accidental overlap of patches, co-localization due to mutual association within specialized subcellular or membrane domains, or nonspecific interactions). As shown in Fig. 1A and quantified in Fig. 3, about 80% of the patches contain both EpoR and gp55-P. This high percentage suggests that the majority of the EpoR and gp55-P proteins present at the cell surface reside in mutual complexes. This interaction is also validated by co-immunoprecipitation of EpoR with gp55-P (see Fig. 7).

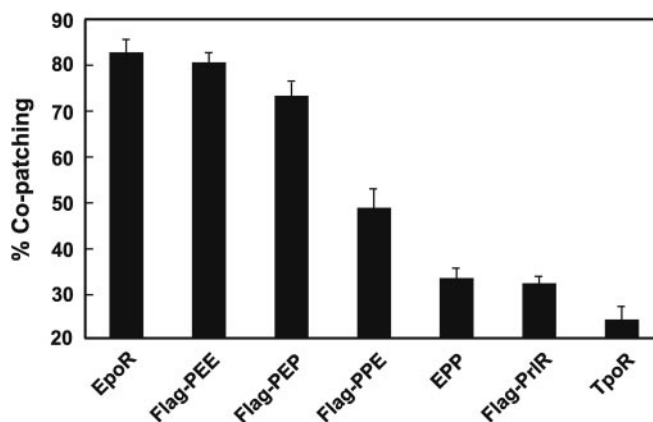
**The Transmembrane Domain of the EpoR Mediates the Interaction with gp55-P**—To investigate the role of the different



**FIG. 2. The EpoR TM domain enables PrIR-EpoR chimeras to co-patch with gp55-P.** A, schematics of PrIR-EpoR chimeric constructs. E, EpoR-derived domains; P, PrIR-derived domains. The domain order is given as EC/TM/CT. Shown are the chimeric constructs PEE, PEP, EPP, and PPE. Junctions between EpoR and PrIR domains are described under “Experimental Procedures.” B, FLAG-PEP (green) and gp55-P (red) exhibit a high degree of co-patching (yellow). Transfection and co-patching were performed as described under “Experimental Procedures” and in Fig. 1. Bar, 10  $\mu$ m. C, EPP (green) and gp55-P (red) show only low levels of co-patching.

EpoR domains (EC, TM, and CT) in heterocomplex formation with gp55-P, we employed a series of chimeric receptors composed of specific domains derived from the EpoR and the PrIR, taking advantage of the fact that the PrIR does not co-patch with gp55-P above the basal level (Fig. 1C). We examined the ability of FLAG-PrIR chimeric constructs containing specific EpoR domains to co-patch with gp55-P. The chimeras are designated by three-letter codes (see Fig. 2A) denoting the origin of the domain sequences, where E indicates EpoR and P indicates PrIR (for example, the construct PEE consists of the EC domain of FLAG-PrIR and the TM and CT domains of EpoR). Representative images of these experiments are depicted in Fig. 2B, and the average results from many images are given in Fig. 3. These studies clearly demonstrate that hetero-oligomerization of the EpoR with gp55-P is driven mainly by the TM domain of the EpoR. This is evident from the high degree of co-patching measured between gp55-P and either FLAG-tagged PEE or PEP chimeric receptors (Figs. 2B and 3). The latter is a chimeric receptor containing only the EpoR TM domain flanked by the PrIR EC and CT domains. Its ability to confer high co-patching levels with gp55-P demonstrates that the EpoR TM domain is sufficient for association with gp55-P; this conclusion is supported also by the ability of PEP to co-precipitate with gp55 proteins (see Fig. 7).

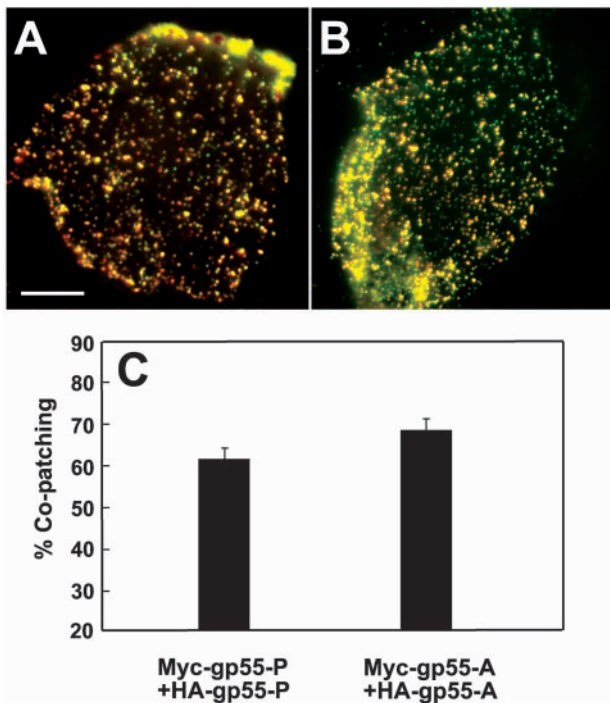
The averaged results of such measurements on many cells



**FIG. 3. Quantification of co-patching between gp55-P and EpoR or PrIR-EpoR chimeras at the surface of live cells.** Immunofluorescence co-patching experiments were performed as in Figs. 1 and 2 on BOSC23 cells expressing one of the receptors depicted below the bars (EpoR, FLAG-PrIR, FLAG-TpoR, or PrIR-EpoR chimeras). Superimposed red and green images were analyzed by counting the numbers of green (G), red (R), and yellow (Y) patches (counting at least 100 patches per cell on 10–15 cells in each case). The % co-patching (percentage of a given receptor in mutual patches with gp55-P) is given by  $100 \times (Y/Y + R)$  for the red-labeled gp55-P proteins and by  $100 \times (Y/Y + G)$  for the various receptors, which are all green-labeled (40). Because in all cases the values of  $Y/Y + R$  and  $Y/Y + G$  were very close to each other, only one value (mean  $\pm$  S.E.) is depicted for each receptor-gp55-P pair.

are depicted in Fig. 3. Although a high level of co-patching (70–80%) was observed between gp55-P and either PEE or PEP, only basal co-patching levels were seen between gp55-P and EPP, a chimeric receptor containing the EpoR EC domain fused to the TM and CT domains of the PrIR. This shows that the EpoR EC domain lacks significant affinity for the EC domain of gp55-P. Interestingly, a small but noticeable increase in co-patching was seen when gp55-P was co-expressed with PPE, which contains only the EpoR CT domain. Although small, this increase may suggest that the N terminus of the EpoR CT domain is able to interact to some extent with the very short (4 amino acids) CT domain of gp55-P.

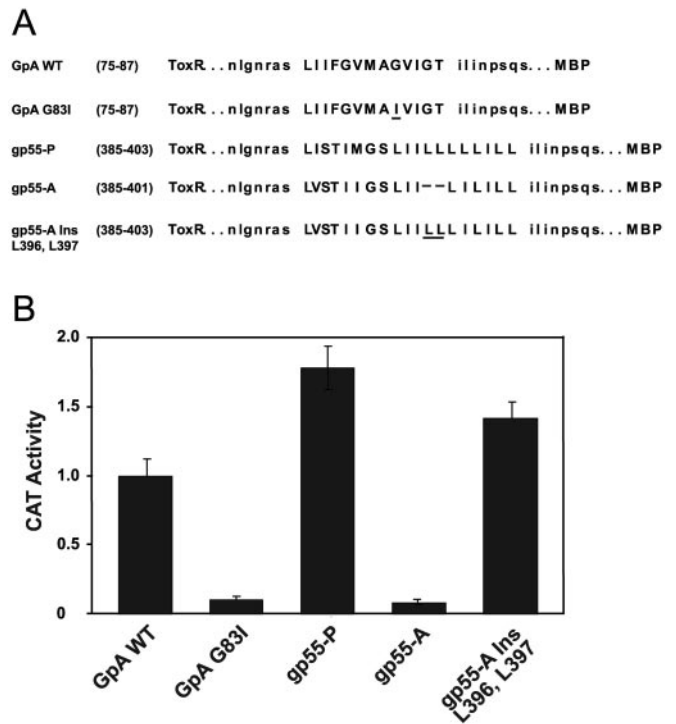
*Full-length gp55-P and gp55-A Form Homodimers/Oligomers at the Cell Surface but Only the Isolated TM Sequence of gp55-P Is a Homodimer in TOXCAT Assays*—The finding that the EpoR TM domain interacts with gp55-P suggests that the TM domain of the latter is involved in these interactions. Such TM domain interactions could also lead to homodimerization of the gp55-P proteins, as observed earlier for EpoR homodimerization (6, 10). Moreover, the differences between the TM sequences of gp55-P and gp55-A may lead to different tendencies for homo-oligomerization or to different conformation of the dimerized TM regions; this in turn could influence their association with the EpoR and their ability to activate it. We therefore compared the oligomerization status of full-length gp55-P and gp55-A at the cell surface with the ability of their isolated TM sequences to homodimerize. Fig. 4 depicts immunofluorescence co-patching experiments on BOSC23 cells co-transfected with vectors encoding two versions (HA-tagged and Myc-tagged) of the same protein (gp55-P or gp55-A), enabling studies on homo-oligomerization using patching by distinct anti-tag antibodies. These studies demonstrated that both full-length gp55 proteins exhibit a high tendency to form homo-oligomers. It should be noted that the percent co-patching measured (60% for gp55-P and 65% for gp55-A; Fig. 4C) is suggestive of near-complete homodimerization. As discussed by us in detail earlier (6, 40), this percentage is very close to the statistical prediction of 66.6% (2/3) co-patching expected for a pure homodimeric population. This is due to the fact that unlike heteromeric



**FIG. 4. Co-patching studies reveal that full-length gp55-P and gp55-A form homo-oligomers at the cell surface.** BOSC23 cells were co-transfected as in Fig. 1 with Myc- and HA-tagged gp55-P (A) or gp55-A (B). Co-patching was performed as described under “Experimental Procedures” and Fig. 1, except that the antibodies employed (all at 20  $\mu\text{g/ml}$ ) were mouse anti-Myc together with rabbit HA.11 for the first incubation, followed by FITC-GaR together with Cy3-GaM (secondary antibodies). This protocol results in *green*-labeled HA-tagged proteins and *red*-labeled Myc-tagged proteins. Bar, 10  $\mu\text{m}$ . Quantification of the % co-patching (C) was as described in Fig. 3; the % co-patching is given as mean  $\pm$  S.E. of measurements on 10–15 cells in each case.

co-patching, where all the heterocomplexes must contain the two different proteins, homodimers containing two receptors with the same tag may also form but would not be swept into mutual patches with similar receptors carrying the other tag (40). The fraction of “same tag” complexes for a dimer is 1/3, leaving 2/3 of the dimers containing two different tags. Thus, 60–65% co-patching would reflect nearly complete homodimerization. Interestingly, oligomerization of gp55-P proteins has been suggested to occur via disulfide bonds between the EC domains (43) and was proposed to be essential for gp55-P transport to the cell surface (44).

To explore the ability of the isolated TM domain sequences of gp55-P and gp55-A to undergo homo-oligomerization, we employed the TOXCAT assay (41), which measures the association between TM helices in the *Escherichia coli* inner membrane. The assay exploits a dimerization-dependent activator of transcription, called ToxR (45). The TM sequence of interest is expressed in the bacteria as a chimeric protein flanked by ToxR and by the maltose-binding protein (MBP). TM domain-mediated oligomerization results in ToxR-activated expression of a reporter gene encoding chloramphenicol acetyltransferase (CAT). The level of CAT activity is correlated to the strength of association (41). The results obtained in the TOXCAT assay for sequences derived from the gp55-P and gp55-A TM domains are depicted in Fig. 5. These studies demonstrate that the gp55-P TM sequence has a strong tendency to form homo-oligomers. Unexpectedly, the gp55-P TM sequence gave a significantly higher signal in this assay than the glycoprotein A TM sequence (Fig. 5B), which has been demonstrated to possess a strong tendency for homodimerization (46, 47). For comparison, the signal obtained with a glycoprotein A TM sequence



**FIG. 5. Homo-oligomerization of gp55-P and gp55-A isolated TM sequences.** The TOXCAT assay was used to measure homo-oligomerization of gp55-P or gp55-A TM sequences in the *E. coli* inner membrane. A, sequences of the TM region of TOXCAT chimeras. Shown are glycoprotein A (*GpA* WT or the *GpA* G83I mutant) TM sequences 75–87, gp55-P TM residues Leu<sup>385</sup>–Leu<sup>403</sup>, gp55-A TM residues Leu<sup>385</sup>–Leu<sup>401</sup>, and gp55-A Ins Leu<sup>396</sup>, Leu<sup>397</sup> (*gp55Ains* L396, L397) TM residues Leu<sup>385</sup>–Leu<sup>403</sup>. The TM domain sequences (*capital letters*) were inserted between the ToxR transcriptional activation domain (*ToxR*) and the MBP domain. Sequences that flank the inserted TM domains are shown in *lowercase letters*. Mutations in the *GpA* and *gp55-A* TM sequences are *underlined*. B, cells expressing the TOXCAT chimeras were lysed and assayed for CAT activity. Data are shown as mean  $\pm$  S.D. values of at least three independent measurements. Chimera expression levels were determined by immunoblotting the periplasmic MBP domain (not shown); the variations in expression levels were insignificant.

containing a mutation that destabilizes the dimer (G83I) (41) is shown. Interestingly, the TM domain of gp55-A had a very low homodimerizing capability (Fig. 5B). This TM domain has several differences from that of gp55-P (3), including being shorter by two Leu residues, Leu<sup>397</sup> and Leu<sup>397</sup> (Fig. 4A and Table I). These Leu residues are important for homodimerization of the isolated TM sequences, because their insertion into the gp55-A TM sequence restored its homodimerization (Fig. 5B). Furthermore, the gp55-P $\rightarrow$ A mutant M390I, which induces anemic effects and cannot activate proliferation of Epo-dependent cells (12, 30), oligomerizes in TOXCAT assays (Table I) like gp55-P or gp55-A Ins Leu<sup>396</sup>, Leu<sup>397</sup>. In conclusion, both gp55 proteins are homodimers/oligomers on the cell surface, but the conformations of these dimers must be different because only the isolated TM sequences of gp55-P homodimerize, implying close proximity of the TM domains.

*The EpoR TM Domain Mediates Formation of Hetero-oligomers with gp55-A and with gp55-P $\rightarrow$ A TM Mutants*—In view of the inability of the TM sequence of gp55-A to homo-oligomerize in the TOXCAT assay, we investigated whether gp55-A can form heteromeric complexes on the cell surface with EpoR and with PrIR-EpoR chimeras. BOSC23 cells were co-transfected with the corresponding cDNAs, and immunofluorescence co-patching studies were conducted to determine the extent of complex formation. As shown in Fig. 6, the majority of the

TABLE I  
gp55-EpoR hetero-oligomerization and gp55 homo-oligomerization are not sufficient for induction of proliferation of EpoR-expressing Ba/F3 cells

Epo-dependent Ba/F3 cell pools expressing the murine EpoR (Ba/F3-EpoR) were generated as described under "Experimental Procedures." The cells were infected with retroviruses containing the cDNA encoding one of the gp55 proteins. Listed are sequences Leu<sup>385</sup>-Ser<sup>409</sup> of gp55-P TM and sequences Leu<sup>385</sup>-Ser<sup>407</sup> of gp55-A TM domain. Residues at positions 390, 396, 397, and 404 (numbered as in the gp55-P sequence) are in boldface. Ba/F3-EpoR cell proliferation was measured 4 days after removing Epo from the growth medium of the infected cells ("Experimental Procedures"). + represents proliferation comparable with or higher than 80% of the proliferation induced by 1 unit/ml Epo in Ba/F3-EpoR cells that were not infected with gp55 viruses. Heterocomplex formation was measured by immunofluorescence co-patching between a given gp55 protein and the EpoR at the cell surface. Homo-oligomerization of the TM sequences of various gp55 proteins was measured by the TOXCAT assay. ND, not determined.

gp55 protein	TM sequence	Ba/F3-EpoR proliferation	gp55-EpoR co-patching	TOXCAT gp55 homo-oligomerization
gp55-P	LISTIMGSLIILLLLLLILLIWTLHS	+	+	+
gp55-A	LVSTIIGSLII-LILILLIWTLHS	-	+	-
gp55-A Ins Leu <sup>396</sup> , Leu <sup>397</sup>	LVSTIIGSLIILLLILILLIWTLHS	-	ND	+
gp55-P Del Leu <sup>396</sup> , Leu <sup>397</sup>	LISTIMGSLII-LLLLLLIWTLHS	-	+	ND
gp55-P M390I	LISTIIGSLIILLLLLLILLIWTLHS	-	+	+
gp55-P Del Ile <sup>404</sup>	LISTIMGSLIILLLLLLILL-WTLHS	-	ND	ND
gp55-P Del Leu <sup>397</sup> Ins Ile <sup>404</sup>	LISTIMGSLIIL-LLLLLLIWTLHS	-	ND	ND

cell-surface EpoR and gp55-A were in mutual co-patches (70–75%), very close to the level obtained for co-patching of gp55-P with the EpoR. The specificity of the co-patching is demonstrated by the failure of the PrIR to interact with gp55-A (Fig. 6). Next, we tested whether an inactive gp55-P→A mutant (gp55-P M390I), where Met<sup>390</sup> is replaced by Ile (the corresponding residue in gp55-A), can co-patch with the EpoR. As shown in Fig 6C, high levels of co-patching were observed between these two proteins. Therefore, the ability of either gp55-A or the gp55-P→A mutant gp55-P M390I to form hetero-oligomers with cell-surface EpoRs is similar to that of gp55-P. In line with this conclusion, another gp55-P→A mutant (gp55-P Del Leu<sup>396</sup> and Leu<sup>397</sup>), where the two extra Leu residues in the TM sequence of gp55-P were deleted, was also able to co-patch (55–60%) with the EpoR.

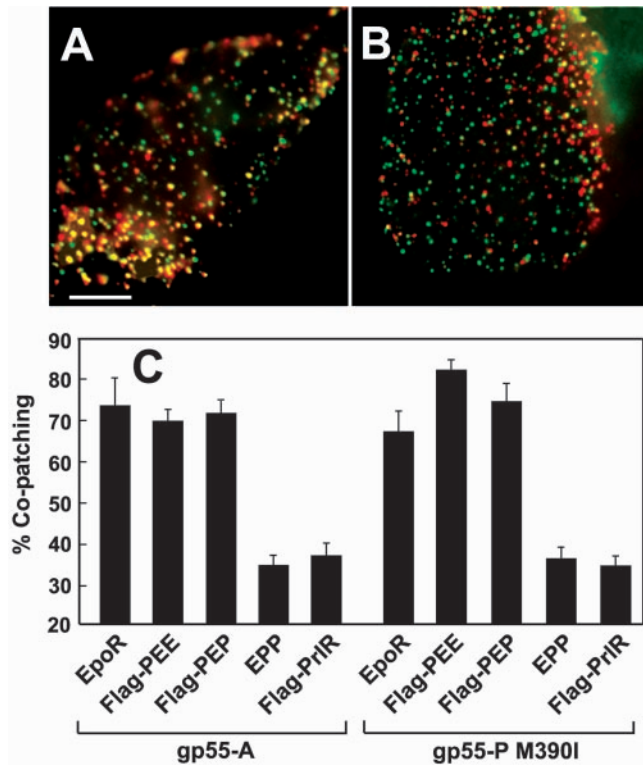
Because specific gp55-P and EpoR TM sequences were required for EpoR activation (12, 29), we proceeded to explore whether the interactions of the activation-defective gp55-A or gp55-P M390I with the EpoR are still mediated via the EpoR TM domain. To this end, we measured the co-patching between these gp55 proteins and PrIR-EpoR chimeras (Fig. 6B). Whereas only basal co-patching levels were observed between either gp55-A or gp55-P M390I and the PrIR, high co-patching levels were detected between these proteins and the PEE chimeric receptor, resembling the high co-patching between gp55-P and PEE. Furthermore, gp55-A and gp55-P M390I co-patched strongly with the PEP chimera, suggesting that gp55-A and gp55-P M390I interact with the EpoR TM domain (Fig. 6C). The ability of gp55-P M390I to co-patch with PEP also demonstrates that the critical gp55-P→A M390I mutation (12, 30) does not impair binding of the gp55 TM domain to the EpoR TM domain. Importantly, neither gp55-A nor gp55-P M390I co-patched with EPP (Fig. 6C), suggesting that an interaction between the EC domains of the EpoR and gp55-A (or gp55-P M390I) is not sufficient for complex formation. Because our studies employed gp55 proteins with almost identical EC domains (gp55-P is in fact gp55-APP, whereas gp55-A is gp55-AAA, as defined in Ref. 33), this result is consistent with a crucial role for gp55 TM sequences in EpoR activation.

In order to obtain further support for the interactions between gp55 proteins and the EpoR TM domain, we employed co-immunoprecipitation studies. To validate the critical observation that gp55-P and gp55-A interact with the EpoR TM domain, we compared the co-precipitation of FLAG-PEP and FLAG PrIR (control) with gp55-P and gp55-A. As shown in Fig. 7A and quantified in Fig. 7C, the PrIR did not co-precipitate

with either gp55 protein, whereas replacement of the PrIR TM domain with that of the EpoR resulted in co-precipitation of the chimera (PEP) with both gp55 proteins. This finding reiterates that the EpoR TM domain is required and sufficient to confer complex formation with gp55-P and gp55-A. To validate further that the interactions are not mediated via the EC domains, Fig. 7 (B and C) demonstrates that although the full-length EpoR co-precipitates with gp55-P, the EPP chimera (containing the EpoR EC domain) fails to do so.

The data presented here suggest that the TM domains of both gp55-A and gp55-P exhibit sequence-specific affinity for the EpoR TM domain. To our knowledge this is the first study to identify the EpoR TM domain as the target of the gp55-A interaction. Because gp55-A does induce EL *in vivo* in adult mice (13, 15), and because the effect of gp55-A on fetal liver erythroid progenitors requires expression of the EpoR (33), our results may offer a molecular explanation for these gp55-A effects in cells lacking sf-Stk.

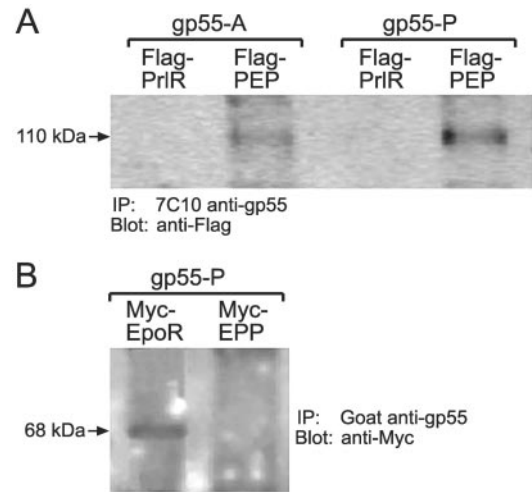
*Functionality of gp55 Proteins in Supporting Proliferation of Cells Expressing EpoR*—gp55-P is able to activate the EpoR to induce proliferation in hematopoietic cell lines. To explore whether such activation is related to the ability of gp55 proteins to form homomeric complexes and/or heteromeric complexes with the EpoR, we compared the ability of various gp55 proteins and mutants to support survival and proliferation of Ba/F3 cells expressing the murine EpoR (Table I). gp55-P, our positive control, which exhibits a high degree of both homo- and hetero-oligomerization with the EpoR, was highly effective in this biological assay, in agreement with former studies (22). On the other hand, gp55-A, our negative control, which forms heteromeric complexes with the EpoR but whose isolated TM sequence is defective in homomeric complex formation (Figs. 5 and 6), was ineffective in inducing survival/proliferation of EpoR-expressing Ba/F3 cells (Table I) (31). Interestingly, insertion of the two Leu residues missing in the gp55-A TM domain (the gp55-A Ins Leu<sup>396</sup>, Leu<sup>397</sup> mutant), which restored homo-oligomerization capacity to the gp55-A TM sequence (Fig. 5), was unable to activate the EpoR to support survival and proliferation of these cells (Table I). Yet these residues are important for the activity of gp55-P, as deletion of the two Leu residues from gp55-P (the gp55-P Del Leu<sup>396</sup>, Leu<sup>397</sup> mutant) is sufficient to switch gp55-P to an anemic phenotype (12). Conversely, the gp55-P→A M390I mutation does not inhibit oligomerization of the isolated TM domain in TOXCAT assays (Table I) but abolishes the ability of gp55-P to induce a polycythemic phenotype (12, 30). Therefore, the homomeric inter-



**FIG. 6. Immunofluorescence co-patching demonstrates that gp55-A and gp55-P M390I interact with the EpoR TM domain.** BOSC23 cells were co-transfected with vectors encoding one of the following receptors (*EpoR*, *Flag-PEE*, *Flag-PEP*, *EPP*, or *Flag-PrIR*) together with either gp55-A or the gp55-P→A mutant gp55-P M390I. Labeling with antibodies and co-patching were as described in Figs. 1 and 2. *Bar*, 10  $\mu$ m. *A*, a typical cell co-expressing EpoR (green) and gp55-A (red); a high degree of co-patching (yellow) is observed. *B*, a cell co-expressing FLAG-PrIR (green) and gp55-A (red); only low level of co-patching (yellow) is observed. *C*, quantification of the co-patching results. The % co-patching (% of a given receptor in mutual patches with a gp55 protein) was calculated as in Fig. 3 and is given as mean  $\pm$  S.E. of measurements on 10–15 cells in each case. The results demonstrate a high level of co-patching between either gp55-A and gp55-P M390I with chimeric receptors containing the EpoR TM domain, suggesting that the interactions driving heterocomplex formation are mediated through the EpoR TM domain.

actions leading to the homodimerization capability of the isolated gp55 TM domain reflect a conformation that is required but is not sufficient *per se* to induce EpoR activation. The above gp55 mutants (Del Leu<sup>396</sup>, Leu<sup>397</sup>, and M390I), as well as gp55-A, are still capable of forming heterocomplexes with the EpoR (Fig. 6C), suggesting that binding of gp55 to the EpoR (heterocomplex formation) is not sufficient for activation.

One striking aspect of gp55-P TM sequence requirements is the fact that eliminating one residue (Leu<sup>397</sup>), which shifts all the remaining residues by one step, results in loss of gp55-P activity, although at that position another Leu (Leu<sup>398</sup>, which advances to position 397) takes its place (12). We constructed two gp55-P mutants (see Table I): (i) gp55-P Del Ile<sup>404</sup>, where the TM sequence is intact up to Ile<sup>404</sup> but beyond this residue resembles gp55-P Del Leu<sup>397</sup> (because all residues from Trp<sup>405</sup> and on upshift one position); (ii) gp55-P Del Leu<sup>397</sup> Ins Ile<sup>404</sup>, where an additional Ile was inserted right after Ile<sup>403</sup> (which was at position 404 in gp55-P, but shifted one position due to the deletion of Leu<sup>397</sup>), thus re-shifting the sequence from Ile<sup>404</sup> and on back to that of intact gp55-P. Interestingly, both mutants were inactive (Table I), suggesting that important interactions occur both before and after Ile<sup>404</sup> between the gp55-P TM domains, and that the sequence-specific interaction with the EpoR TM domain involves an extended portion of the



**FIG. 7. Co-immunoprecipitation of chimeras containing the EpoR TM domain with gp55-P and gp55-A.** BOSC23 cells were transiently co-transfected with DNAs encoding gp55-A or gp55-P together with either FLAG-PrIR (control), FLAG-PEP, Myc-EpoR, or Myc-EPP. After lysis and immunoprecipitation (IP) with anti-gp55 antibodies, Western blotting was performed using anti-FLAG or anti-Myc IgG to detect co-precipitated epitope-tagged receptor constructs (see “Experimental Procedures”). All lanes were loaded with immunoprecipitates derived from the same amount of cell lysates (one 10-cm dish). *A*, FLAG-PEP but not FLAG-PrIR co-precipitate with gp55-P and gp55-A. *B*, Myc-EpoR but not Myc-EPP co-precipitate with gp55-P.

TM domains. These data indicate that although many gp55 TM sequences can bind to the EpoR, stringent requirements must be fulfilled to enable the gp55 TM sequence to fully activate the EpoR.

#### DISCUSSION

Our main observation is that both viral oncogenic proteins gp55-A and gp55-P form heteromeric complexes with the murine EpoR at the surface of live transfected cells. These interactions are specific because neither gp55 protein interacts with other related cytokine receptors, such as the PrIR or the TpoR. The ability of both gp55-P and gp55-A to form heterocomplexes with the EpoR is further substantiated by the similar interaction of several gp55-P→A mutants, which were shown to have acquired anemic properties, with the EpoR (Figs. 3, 6, and 7). Unexpectedly, not only gp55-P but also gp55-A targeted the TM domain of the EpoR. For gp55-P it was known from studies that employed chimeric receptors that the TM domain of the EpoR was required for activation by gp55-P (11). Only PrIR-EpoR chimeras containing the EpoR TM domain oligomerized on the cell surface with gp55 proteins (Fig. 6C). However, whereas the related TM sequences of either gp55-P or gp55-A are able to interact specifically with the EpoR TM domain, only the gp55-P TM sequence is capable of inducing full activation of the EpoR. This suggests that the gp55 TM sequence has to meet additional stringent requirements in order to fully activate the EpoR.

Our experiments focused on the interaction between the EpoR and gp55 proteins and did not address the interaction between the gp55-P-EpoR complex with the truncated transmembrane tyrosine kinase sf-Stk. Expression of this protein has been identified as the molecular determinant of the Friend virus susceptibility locus, Fv2, providing a landmark achievement for understanding the pathogenesis of Friend erythroleukemia (20). Whereas sf-Stk is required for the Epo-independent expansion of infected erythroid cells (48), in other cells lacking sf-Stk (such as Ba/F3 cells) gp55 proteins can bind to the EpoR and activate it without sf-Stk (22, 25). Furthermore, a deletion mutant of gp55-P (BB6) can overcome Fv2 resistance (49),

although it fails to interact covalently with the EC domain of sf-Stk (25); yet this mutant strongly activates the EpoR and induces EL in Fv2r mice (49). Therefore, our current results are relevant to the mechanism of EpoR activation by gp55 proteins in the absence of sf-Stk.

Although considerable progress has been made in understanding the folding and packing of TM domains (50), little is known about the exact sequences required for distinct TM domains to interact with each other. Several genetic approaches exist to probe the ability of a particular TM sequence to homodimerize/oligomerize (9, 41, 42). The prototype homodimerizing interaction for single-span TM proteins is glycoporphin A (46, 47, 51), where the homodimer interface containing the Gly and Val residues resembles a "ridges-in-grooves" arrangement (52). Recently, such genetic assays were used to identify new motifs that induce homodimerization of TM domains (42, 53–55). Furthermore, a novel two-hybrid system (GALLEX) that follows the hetero-dimerization of membrane proteins in the *E. coli* inner membrane was reported (56). However, probing specific interactions between different distinct TM sequences in a eukaryotic cell membrane is much harder. To probe specific interactions between distinct TM sequences in mammalian cell membranes one would need a genetic system that allows cell survival/proliferation and selection only after hetero-oligomerization of TM sequences. We suggest that the specific interaction between the TM sequences of gp55 proteins and EpoR may constitute the basis for such an assay.

In the current study, we employed the TOXCAT assay to probe the homodimerizing/oligomerizing ability of isolated gp55 TM sequences, and we found that the gp55-P TM sequence homo-oligomerizes strongly whereas the TM sequence of gp55-A does not (Fig. 5). These results are distinct from those obtained in co-patching studies on the homo-oligomerization of the full-length gp55 proteins situated in the plasma membrane, where both gp55-P and gp55-A exhibited a high degree of homomeric complex formation (Fig. 4). These findings suggest that the organization of the TM regions within gp55-P and gp55-A dimers is different. Addition of the two extra Leu residues (Leu<sup>396</sup> and Leu<sup>397</sup>) found in gp55-P to the gp55-A isolated TM sequence (gp55-A Ins Leu<sup>396</sup>, Leu<sup>397</sup>) restored its oligomerizing ability (Fig. 5) but failed to restore full activation of the EpoR (Table I). Along the same lines, the gp55-P→A mutant M390I does not induce the polycythemic phenotype, but its isolated TM sequence can still undergo homo-oligomerization (Table I). These data suggest that gp55 homo-oligomerization is not sufficient to endow it with the ability to activate the EpoR. At the same time, all the gp55 variants and chimeras tested (gp55-P, gp55-A, gp55-P M390I, gp55-P Del Leu<sup>396</sup>, Leu<sup>397</sup>) were able to form heteromeric complexes with the EpoR to similar degrees (Figs. 3 and 6), but only gp55-P could fully activate its signaling (Table I). These findings indicate that gp55-EpoR heterocomplex formation, although necessary, is not sufficient for complete EpoR activation. Moreover, because our studies with the PrIR-EpoR chimeras clearly demonstrate that the EpoR TM domain is required for heterocomplex formation with all the gp55 proteins (including gp55-A and gp55-P M390I), they support the concept that the biological activity of gp55-P and the weaker incomplete biological effects of gp55-A or gp55-P M390I (Refs. 12 and 30; see below) also require interaction with the TM domain of the EpoR.

These results led us to examine the stringent sequence requirements of the gp55-P TM domain that enable activation of the EpoR (Table I). In cell lines gp55-A co-immunoprecipitates with the EpoR but does not activate it for proliferation (31). As we have described previously, several minimal mutations in

the gp55-P TM sequence result in a loss of the ability to fully activate the EpoR, with the gp55-P→A mutants gp55-P Del Leu<sup>396</sup>, Leu<sup>397</sup>, or gp55-P M390I inducing weak activation of the EpoR similar to gp55-A (12) (Table I). We have shown previously that Ser<sup>238</sup> in the TM domain of the murine EpoR is crucial to the activation by gp55-P and is likely to interact with Met<sup>390</sup> of gp55-P (12). Met<sup>390</sup> is indeed very important and, as stated before, its mutation to the equivalent residue of gp55-A, Ile, abolishes activation of the EpoR in cell lines (12). The geometry induced by Ile at position 390 is likely to induce a different conformation of the EpoR, resulting in anemia. This model is supported by two sets of data. First, the mutant gp55-P M390L where Met<sup>390</sup> was mutated to the non- $\beta$ -branched (non-rigid) isomer of Ile, Leu, was fully active (12). Second, the gp55-P M390I mutant is not completely inactive and was shown to induce EL with anemia *in vivo* in adult mice (30).

What would be then the mechanism by which gp55 proteins activate the EpoR in the absence of sf-Stk? Part of the answer may come from the unliganded conformation of the EpoR. In the absence of ligand, the EpoR is an inactive dimer on the cell surface; the unliganded dimer requires the TM sequence for cell-surface dimerization (6). Binding of a TM sequence of another protein (*e.g.* gp55) to the TM domain of the receptor dimer can potentially disrupt an inhibitory conformation. This may allow different degrees of receptor activation, depending on the specific nature of the TM sequence of the activating protein. Thus, although both gp55-P and gp55-A form homodimers, the conformation of the TM domains within the dimers is most likely different, as only the isolated TM sequence of gp55-P homodimerizes. Notably, the EpoR is not the only membrane receptor that dimerizes in the absence of ligand. Other examples include the following. (i) Ligand-independent growth hormone receptor dimerization occurs in the endoplasmic reticulum, possibly via TM interactions (57). (ii) The single TM domain of ErbB (neu/HER2) receptors self-associates in membranes in the absence of any mutation (58). (iii) The TM sequence of the constitutively active mutant of ErbB2 (neu\*), which has a Val → Glu mutation, dimerizes less well than the wild type ErbB2 TM sequence, suggesting that the mutation shifts the receptor dimer from an inactive to an active conformation rather than simply bringing two monomers together (58).

We suggest that sequence-specific interactions between the TM domains of gp55-A or of chimeric gp55-P/A proteins and the EpoR destabilize the EpoR TM dimerization (which maintains the EpoR inactive) allowing weak/incomplete levels of receptor activation. Such constitutive weak activation is sufficient to promote colony-forming unit-erythroid differentiation (33) and to synergize with Epo *in vivo* to trigger erythroblastosis, but is not sufficient to induce Epo-independent proliferation of cell lines or to promote burst-forming unit-erythroid differentiation in the absence of Epo (32, 33). In contrast, binding of the gp55-P TM sequence to the EpoR preformed dimer induces a receptor conformational change closely resembling that induced by Epo. This can result in full activation of the receptor. The Epo-induced conformational change at the EC region of the EpoR (59) is normally transmitted via the TM domain to the cytosolic JM region (7, 8). That a precise conformation is required for productive signaling by EpoR is emphasized by the distinct biological activity of EpoR mutants, which differ by the orientation of their JM regions. Insertion of one (EpoR 1A) or four (EpoR 4A) extra alanines into the JM region changes its  $\alpha$ -helical orientation and inactivates the receptor, whereas insertion of three extra alanines (EpoR 3A) does not interfere with EpoR activity, because the  $\alpha$ -helical orientation of the JM region is

preserved (8). Furthermore, the crystal structure of the EC domain of EpoR bound to an antagonist peptide suggests that dimerization can also be induced by antagonists and that in such inactive complexes the orientation between receptor molecules is altered relative to active complexes (60).

Taken together, our results demonstrate that the related but distinct TM sequences of the viral gp55 proteins recognize specifically the TM sequence of the EpoR. We suggest that the interactions between the EpoR and gp55 proteins may help in the design of a novel genetic system to study sequence-specific assembly between distinct TM sequences.

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