Activation of the erythropoietin receptor by the gp55-P viral envelope protein is determined by a single amino acid in its transmembrane domain

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The spleen focus forming virus (SFFV) gp55-P envelope glycoprotein specifically binds to and activates murine erythropoietin receptors (EpoRs) coexpressed in the same cell, triggering proliferation of erythroid progenitors and inducing erythroleukemia. Here we demonstrate specific interactions between the single transmembrane domains of the two proteins that are essential for receptor activation. The human EpoR is not activated by gp55-P but by mutation of a single amino acid, L238, in its transmembrane sequence to its murine counterpart serine, resulting in its ability to be activated. The converse mutation in the murine EpoR (S238L) abolishes activation by gp55-P. Computational searches of interactions between the membrane-spanning segments of murine EpoR and gp55-P provide a possible explanation: the face of the EpoR transmembrane domain containing S238 is predicted to interact specifically with gp55-P but not gp55-A, a variant which is much less effective in activating the murine EpoR. Mutational studies on gp55-P M390, which is predicted to interact with S238, provide additional support for this model. Mutation of M390 to isoleucine, the corresponding residue in gp55-A, abolishes activation, but the gp55-P M390L mutation is fully functional. gp55-P is thought to activate signaling by the EpoR by inducing receptor oligomerization through interactions involving specific transmembrane residues.

Keywords: Epo receptor signaling/red cell formation/SFFV-gp55 env proteins/transmembrane coiled-coil interactions

Introduction

Activation of the erythropoietin receptor (EpoR) (D'Andrea *et al.*, 1989) by erythropoietin (Epo) is required

for survival, proliferation and maturation of erythroid progenitors into mature red cells (Wu *et al.*, 1995). The EpoR is activated by Epo-induced dimerization (Figure 1) (Watowich *et al.*, 1996; Syed *et al.*, 1998), reminiscent of the activation of the growth hormone receptor by growth hormone (De Vos *et al.*, 1992). Bivalent monoclonal antibodies directed to the extracellular domain of the EpoR and small non-covalently dimerized peptides also dimerize the EpoR and induce proliferation of Epo-dependent cell lines and formation of burst forming unit erythroid (BFU-E) colonies (Elliott *et al.*, 1996; Livnah *et al.*, 1996; Wrighton *et al.*, 1996). A point mutation, R129C, in the extracellular domain of the EpoR renders the receptor constitutively active (Yoshimura *et al.*, 1990b) due to formation of an intermolecular disulfide bond between membrane-proximal segments (Figure 1) (Watowich *et al.*, 1994). While activation of the EpoR by high levels of Epo (i.e. Epo transgenic animals) (Semenza *et al.*, 1990) results in erythrocytosis, it does not induce emergence of leukemic cells. Expression of the constitutively active EpoR (R129C) in early blood progenitors results in Epo-independent red cell formation and leukemias of different lineages (Longmore and Lodish, 1991; Longmore *et al.*, 1992, 1993). This suggests that constitutive activation of the EpoR leads to cell transformation.

The EpoR belongs to the type I superfamily of cytokine receptors. Upon dimerization it activates a number of intracellular signal transduction pathways which include JAK2, STAT5, protein tyrosine phosphatases SHP-1 and -2, PI-3' kinase, MAP-kinase and protein kinase C (Ihle *et al.*, 1995; Watowich *et al.*, 1996). The particular signals required for proliferation, survival and erythroid differentiation functions are not understood as all of these pathways are activated by many cytokine receptors (Socolovsky *et al.*, 1997) and growth factor receptor tyrosine kinases.

Both the polycythemic (P) and anemic (A) strains of the spleen focus forming virus (SFFV) induce erythroleukemia in adult mice (Friend, 1957; Mirand *et al.*, 1968; MacDonald *et al.*, 1980). SFFV-P also induces polycythemia (increased numbers of red cells), a condition reminiscent of human *Polycythemia vera*, while SFFV-A does not (Tambourin *et al.*, 1979). The only SFFV genes required for oncogenesis and red cell formation are those encoding the envelope proteins gp55-P and -A (Linemeyer *et al.*, 1982; Wolff and Ruscetti, 1985). gp55-P specifically binds to and activates murine erythropoietin receptors (EpoRs) (Li *et al.*, 1990), triggering abnormal proliferation of erythroid progenitors, most of which undergo normal erythroid differentiation. This initial stage of Friend disease, Epo-independent polyclonal erythroblastosis (Mirand *et al.*, 1968; Wolff and Ruscetti, 1985), is followed by the emergence of malignant clones of abnormally proliferating erythroid progenitors due to subsequent Spi-1 overexpression and mutations in genes such as p53

Fig. 1. Mechanisms of activation of the EpoR. The natural ligand, Epo, dimerizes the receptor and induces red cell formation. The receptor can be covalently dimerized and constitutively activated by the R129C point mutation in the extracellular domain (Yoshimura *et al.*, 1990b), which results in Epo-independent formation of red cells and in leukemias of multiple lineages. Coexpression in the same cell of the gp55-P viral envelope protein activates the EpoR (Li *et al.*, 1990) and is oncogenic only for the erythroid lineage since it requires expression of the EpoR. Cell-surface gp55-P is a dimer (Gliniak *et al.*, 1991); the present work demonstrates specific binding to the EpoR, mediated by the membrane-spanning domains, which induces EpoR oligomerization and Epo-independent activation.

(Moreau-Gachelin *et al.*, 1990). Intracellular signals induced by constitutively active forms of EpoR cooperate with the Spi-1 transcription factor to inhibit erythroid differentiation and promote transformation to erythroleukemia (Quang *et al.*, 1997).

Only gp55-P and not gp55-A can activate the murine EpoR to support proliferation of cell lines and to induce Epo-independent red cell formation from early erythroid progenitors. The human EpoR binds to but is not activated by gp55-P and cannot support Epo-independent proliferation of cell lines (Showers *et al.*, 1993; Hoatlin and Kabat, 1995).

We showed previously that gp55-A can activate the EpoR, albeit quantitatively or qualitatively weaker than gp55-P (Constantinescu *et al.*, 1998). Expression of gp55-P in day 12.5 fetal liver erythroid progenitors resulted in Epo-independent formation of erythroid colonies from both BFU-Es and CFU-Es, while expression of gp55-A promoted only CFU-Es but not BFU-Es to proliferate and differentiate into erythroid colonies (Constantinescu *et al.*, 1998). The effect of both gp55-P and -A was mediated through the EpoR, since EpoR^{-/-} fetal liver progenitors were not stimulated by expression of gp55 proteins, but did form erythroid colonies when the EpoR was expressed via retroviral transfection (Wu *et al.*, 1995; Constantinescu *et al.*, 1998). These data suggest that activation of the EpoR by gp55-A is different from that by gp55-P. Biochemical experiments have established that gp55 proteins do interact with the EpoR (Li *et al.*, 1990; Zon *et al.*, 1992). While ¹²⁵I-labeled Epo can become crosslinked to the EpoR and to gp55-P (Ferro *et al.*, 1993), it cannot be crosslinked to gp55-A, suggesting that gp55-A and -P interact differently with the EpoR (Tarr *et al.*, 1997).

gp55 proteins are recombinants between envelope pro-

teins of xenotropic and ecotropic retroviruses (Amanuma *et al.*, 1983); an important feature is the absence of any cytosolic domain (Amanuma *et al.*, 1989), with the C-terminus coinciding with the end of the transmembrane domain (reviewed in Kabat, 1989). The differences between the biological activities of gp55-P and gp55-A are due to nine sequence differences between gp55-P and gp55-A in the C-terminal region, including five amino acid differences and the addition of two leucine residues in gp55-P within the transmembrane domain (Chung *et al.*, 1989). Thus, differences between the transmembrane domains of gp55-P and -A were suggested to be the cause of their different biological activities (Amanuma *et al.*, 1989; Chung *et al.*, 1989). While gp55-P is well expressed at the cell-surface, gp55-A is not (Ruscetti *et al.*, 1981; Amanuma *et al.*, 1989). The two additional leucine residues in the transmembrane domain of gp55-P were suggested to be the sequence responsible for its efficient cell surface expression (Amanuma *et al.*, 1989), but this di-leucine motif has not been examined in the absence of the other amino acid sequence differences between gp55-P and gp55-A or in the absence of other mutations. Differences in cell-surface expression can be important since only gp55 proteins that are expressed at the cell surface are able to stimulate the EpoR for mitogenesis (Ferro *et al.*, 1993; Li *et al.*, 1995). On the other hand, gp55 mutants that had deletions in transmembrane residues but that were expressed well on the cell surface have been isolated recently (Watanabe *et al.*, 1995). A possible interaction between transmembrane domains of gp55-P and EpoR is further suggested by data showing that chimeric Epo receptors in which the membrane-spanning domain was replaced by that of the IL-3 receptor were

not activated by gp55-P but responded normally to Epo (Zon *et al.*, 1992).

We hypothesized that the sequence of the transmembrane domain of gp55 proteins critically influences their biological activity (polycythemia versus anemia) by interacting differently with the transmembrane domain of the EpoR. Such distinct interactions may translate into different receptor activation mechanisms. Also, we hypothesized that the human EpoR is not activated by gp55-P because of distinct sequences in its transmembrane domain. Here we show that the human EpoR can indeed become activated by gp55-P if one transmembrane residue (Leu238) is mutated to serine (as in the mouse counterpart) and, conversely, the mouse EpoR can no longer be activated by gp55-P if Ser238 is mutated to Leu (as in the human counterpart). Using molecular dynamics simulations coupled to mutagenesis, we propose a model in which one face of the gp55-P transmembrane domain interacts with the EpoR transmembrane domain, exposing Ser238 in the interface. At one position of gp55, a methionine (in gp55-P) or leucine, but not an isoleucine (in gp55-A), supports EpoR activation, and our calculations predict that this methionine or leucine interacts specifically with Ser238 of the EpoR. Because gp55-P proteins are disulfide-linked dimers on the cell surface (Gliniak *et al.*, 1991), by interacting with one gp55-P dimer, two EpoRs can be brought in sufficient proximity to activate signaling.

Results

Mutations in the transmembrane domains of the murine and human EpoRs do not modify the receptor's ability to transduce signals when activated by Epo

The human EpoR binds to but is not activated by gp55-P (Zon *et al.*, 1992; Hoatlin *et al.*, 1995). Overall, the human and murine EpoRs are 82% identical in sequence (Jones *et al.*, 1990) and differ in only three of the 21 amino acids in the membrane-spanning segment. We generated all possible human→mouse and mouse→human EpoR mutations in this region. To test the biological activity of these mutants we utilized a bicistronic retroviral vector containing the encephalomyocarditis virus internal ribosome entry site (IRES), in which the mutant EpoR and green fluorescent protein (GFP) are translated from the same mRNA (Figure 2A) (Liu *et al.*, 1997). Vectors expressing the different EpoRs were transiently transfected into BOSC 23 packaging cells, and the released retroviruses used to infect IL-3-dependent Ba/F3 cells. The population of cells selected for growth in Epo expressed high levels of GFP (Figure 2B) and high constant levels of EpoR protein (Figure 2C). Expression of all wild-type or mutant EpoRs tested supported normal growth in response to Epo addition (Figure 3A and B); typical examples are shown in Figure 3C. In other experiments, infected cells growing in IL-3 were selected for the top 0.1% GFP fluorescence; all of these cells could grow in Epo and exhibited levels of the appropriate mutant EpoR and GFP, similar to those in the cell populations depicted in Figure 2B. All of the resultant pools of cells expressed the same numbers of EpoRs with the same affinity for Epo, as assayed by [¹²⁵I]Epo binding and Scatchard

analysis (data not shown). Thus, none of the mutations affected receptor biogenesis or activation by Epo.

A leucine to serine point mutation in the transmembrane sequence renders the human EpoR sensitive to activation by gp55-P

Cell populations expressing wild-type or mutant EpoRs were then assayed for the ability to support gp55-P-dependent proliferation in the absence of Epo; to this end, cells were infected with retroviruses encoding gp55-P (Constantinescu *et al.*, 1998). Cells expressing gp55-P and the wild-type human EpoR or mutants V236L and V239L were unable to proliferate in the absence of Epo. Importantly, cells expressing gp55-P and the human EpoR mutant L238S were able to grow in the absence of Epo; they did so at a rate similar to cells expressing gp55-P and the wild-type murine EpoR (Figure 3B and D).

A serine to leucine point mutation in the transmembrane sequence renders the murine EpoR resistant to activation by gp55-P

With one exception, cells expressing all of the mutant murine EpoRs tested (Figure 3A) were able to grow in the absence of Epo when gp55-P was coexpressed. The exception was mutant murine EpoR S238L, which did not support activation by gp55-P. Thus, Ser238 is crucial for the functional interaction between the EpoR and gp55-P. A hydroxyl group at position 238, however, is not essential: cells coexpressing gp55-P and mutant murine EpoR S238A do grow in the absence of Epo. The fact that a methyl side chain at position 238 suffices for activation by gp55 suggests that putative EpoR–gp55 interactions at this position involve stereochemical van der Waals packing and not the presence of a hydroxyl group on the EpoR. This is not surprising, since serine hydroxyl groups tend to hydrogen bond back to the i-4 carbonyl in transmembrane helices, resulting in a van der Waals surface similar to that of alanine. Mutation of the other hydroxyl-containing amino acids in the transmembrane domain of the murine EpoR (Thr229, Ser231 or Thr242) to alanine, or mutation of either threonine residue to valine did not affect activation of the EpoR by Epo or gp55-P (Figure 3A).

Only the mouse EpoR and the human EpoR L238S mutant are activated by gp55-P to support formation of erythroid colonies from CFU-Es in day 12.5 EpoR^{-/-} fetal liver cells

In order to assess activation of the EpoR by gp55-P in primary erythroid progenitors, we used fetal liver cells derived from EpoR^{-/-} embryos. These animals die at day 12.5 due to severe anemia but do contain erythroid progenitors that can be rescued by expression of the EpoR (Wu *et al.*, 1995). EpoR^{-/-} progenitors allow us to assess the capacity of mutant EpoRs to respond to either Epo or gp55-P expression in the absence of endogenous wild-type EpoR. To this end we generated bicistronic retroviral vectors in which cDNAs encoding mutant EpoRs and gp55-P were coexpressed from the same mRNA (Figure 4A). BOSC 23 cells transfected with similar bicistronic vectors showed similar levels of expression of both proteins (Liu *et al.*, 1997; Constantinescu *et al.*, 1998; data not shown). Viral titers were measured by incubating 5-fold dilutions of the packaged viruses with NIH 3T3

Fig. 2. Expression of EpoR mutants in Ba/F3 cells. (A) Schematic showing the bicistronic pMX-EpoR-IRES-GFP vectors encoding mutant EpoRs and GFP. (B) Ba/F3 cells growing in IL3 containing medium were infected with bicistronic retroviruses encoding the wild-type human EpoR (HuEpoR) or the indicated mutants. The efficiency of infection averaged 9–15%, as judged by the fraction of cells positive for GFP. Cells were washed and cultured in 0.5–1 U/ml Epo; >95% of cells growing for 2–3 days in Epo were positive for GFP. Similar results were obtained when cells were first grown in IL-3 and then sorted for the top 0.1% GFP fluorescence; these cells could grow in Epo and exhibited levels of the appropriate mutant EpoR and GFP similar to those depicted in this panel. (C) Western blot analysis using antibodies directed against the human EpoR, showing that pools of Ba/F3 cells expressing various mutant human EpoRs and selected for growth in Epo (B) contain similar numbers of Epo receptors.

cells and then analyzing for the expression of the gp55 protein by Western blotting. This assay is appropriate since all viruses contain the same IRES-gp55 sequence. As shown in Figure 4D, the titers of the MuEpoR-IRES-gp55-P and MuEpoR S238L-IRES-gp55-P viruses, encoding murine EpoRs, were the same, but were 4- to 5-fold higher than the titers of the two viruses encoding the HuEpoR, HuEpoR-IRES-gp55-P and HuEpoR L238S-IRES-gp55-P. This may be due to differences between the 5' untranslated sequences upstream of the murine and human EpoR cDNAs.

Day 12.5 EpoR^{-/-} fetal liver cells were infected by packaged retroviruses and subsequently plated in methylcellulose in the presence or absence of 3 U/ml Epo. Cells expressing gp55-P and either wild-type murine EpoR or MuEpoR S238L formed the same number of erythroid CFU-E colonies in the presence of Epo (Figure 4B, columns 2 and 4), confirming that the S238L mutation did not affect surface expression of the receptor or its ability to respond to Epo stimulation. When plated in the

absence of Epo, cells expressing the wild-type murine EpoR and gp55-P formed 5- to 6-fold more CFU-E colonies (Figure 4B, column 1) than cells infected with MuEpoR-IRES-GFP virus (Figure 4B, column 5) or with the control GFP virus, confirming the ability of gp55-P to activate the wild-type murine EpoR. Among the explanations for the observed lower numbers of CFU-E colonies induced by gp55-P versus Epo is that the gp55-P cDNA was cloned downstream of the IRES while the EpoR cDNA was cloned upstream of the IRES. Often, the level of expression of genes cloned downstream of the IRES is lower than those cloned upstream. Furthermore, the inherent intracellular signal induced by gp55-P is probably weaker than that induced by Epo, as also reflected by the smaller size of the colonies induced by gp55-P versus Epo (Constantinescu *et al.*, 1998).

Importantly, only background levels of CFU-E colonies were formed by cells coexpressing gp55-P and the mutant EpoR S238L in the absence of Epo (Figure 4B, compare columns 3 and 1). The difference between the ability of

Fig. 3. Activation by Epo and by gp55-P of EpoR mutants in Ba/F3 cells. Mutations introduced in the murine (A) and human (B) EpoR transmembrane domains and the effect of these mutations on the ability of receptors to support proliferation in the presence of Epo or upon coexpression of gp55-P and culture in the absence of Epo. Because, compared with the murine, the human EpoR receptor has one extra amino acid in its extracellular domain, the numbering of HuEpoR residues was adjusted to that of the murine for easy comparison. (C) Indistinguishable Epo sensitivity for proliferation of Ba/F3 cells expressing the wild-type human EpoR (HuEpoR), HuEpoR mutant L238S, and murine EpoR (MuEpoR). Ba/F3 cells growing in 1% WEHI (as a source of IL3) were infected with IRES–GFP bicistronic viruses encoding HuEpoR, HuEpoR L238S or MuEpoR. After 3 days, the cells were switched from medium containing WEHI to that with 1 U/ml Epo, as detailed in the legend to Figure 2, and in the Materials and methods. After 48 h growth, the cells were washed three times in RPMI and then cultivated in RPMI supplemented with 10% FBS and the indicated concentrations of Epo (0.001–10 U/ml). Cells expressing HuEpoR (□), HuEpoR L238S (◇) and MuEpoR (●) were counted at 72 h after addition of Epo (at the indicated concentrations) on a Coulter Cell Counter. Each point is the average of four replicates (±SD). (D) Epo-independent growth of pools of Ba/F3 cells coexpressing MuEpoR or HuEpoR L238S with gp55-P. As shown in (A) and (B), coexpression of gp55-P with MuEpoR or HuEpoR L238S results in Epo-independence. Two independent pools of cells coexpressing MuEpoR and gp55-P (● and △, respectively) or two independent pools of cells coexpressing HuEpoR L238S and gp55-P (□ and ◇, respectively) were incubated at a density of 75 000/ml in RPMI supplemented with 10% bovine calf serum but no other growth factor (i.e. IL3 or Epo) and counted on a Coulter Cell Counter at the indicated time points.

MuEpoR and MuEpoR S238L to support gp55-P induced colony formation (112.93 ± 17.23 CFU-Es per fetal liver, $n = 3$, versus 52.7 ± 11.86 CFU-Es per fetal liver, $n = 3$) is statistically significant (Student's *t*-test, $p < 0.005$). Importantly, when the infected cells were cultured in the absence of Epo, the MuEpoR-IRES–GFP virus induced only background level of colonies (column 5), showing that all colonies induced by MuEpoR-IRES-gp55-P virus were due to expression of gp55-P.

Similarly, cells expressing gp55-P and either wild-type human EpoR or HuEpoR L238S formed the same number of erythroid CFU-E colonies in the presence of Epo (Figure 4C, columns 2 and 4). The lower absolute numbers of colonies induced by Epo in cells infected with HuEpoR coding viruses (Figure 4C, columns 2 and 4) versus cells infected with murine EpoR-coding viruses (Figure 4B, columns 2, 4 and 6) were expected, given the lower titers of HuEpoR-coding viruses (Figure 4D). However, only cells coexpressing gp55-P and the mutant HuEpoR L238S formed colonies in the absence of Epo (Figure 4C, column 3); those expressing the wild-type human EpoR did not (Figure 4C, column 1). The difference between the ability of HuEpoR L238S and HuEpoR to support gp55-P-induced colony formation (66.76 ± 14.42 CFU-Es per fetal liver, $n = 5$, versus 24.76 ± 4.67 CFU-Es per fetal liver, $n = 5$) is statistically significant (Student's *t*-test, $p < 0.005$).

Thus, both in cell lines and in primary erythroid cells, the presence of a small amino acid—either serine or alanine—at one position in the center of the transmembrane sequence of the EpoR is crucial for efficient activation by gp55-P, but not for activation by Epo.

Ser238 of the EpoR and Met390 of gp55-P may lie in the interface of interacting EpoR-gp55-P transmembrane helices

A computational search strategy (Adams *et al.*, 1995, 1996; Surti *et al.*, 1998) was used to identify low energy 'clusters' of interacting EpoR-gp55-P transmembrane helices. The structures generated from this global search can be evaluated on the basis of the mutational and biochemical data. As described in the Materials and methods, the search calculates low energy structures using molecular dynamics and energy minimization protocols at different rotational orientations and interaxial spacings of transmembrane helices. The lowest energy structure found ($\Delta G = -71$ kcal/mol) places Ser238 of the murine EpoR in the dimer interface interacting with Met390 of gp55-P. This dimer structure is stabilized by interactions along the length of the interface (Figure 5A and D) and provides a direct explanation for the observed mutational results, since Ser238 is critical for interaction of the murine EpoR with gp55-P, and Met390 is one of the six transmembrane residues that differ between gp55-P and gp55-A (Figure 6).

Fig. 4. gp55-P expression activates the wild-type murine EpoR and a mutant HuEpoR L238S to promote erythroid colony formation from day 12.5 EpoR^{-/-} fetal liver CFU-Es. (A) Schematic diagram showing the bicistronic vectors encoding mutant EpoRs and gp55-P. (B) The number of CFU-E colonies generated in the absence of Epo (-Epo, hatched columns) or in the presence of 3 U/ml Epo/ml (+Epo, solid columns) in day 12.5 EpoR^{-/-} fetal liver CFU-Es after infection by viruses encoding the wild-type or S238L mutant murine Epo receptors (MuEpoR) and gp55-P, wild-type MuEpoR and GFP in place of gp55-P, or only GFP (GFP-Control). Fetal liver cells were infected with different viruses having similar titers [as assayed by Western blot analysis for gp55 expression on infected NIH 3T3 cells, (D)] for 4 h at 37°C. Cells were plated in methylcellulose and CFU-Es were scored by staining with benzidine 72 h after plating. Results are expressed as colonies induced per fetal liver in the presence or absence of Epo. Data represent the mean of four assays (columns 1–4 and 6) or three assays (columns 5 and 7) ± 1 SD. All EpoR-encoding viruses induced the same number of CFU-E colonies when the infected cells were cultured in the presence of Epo (columns 2, 4 and 6). In contrast, the number of CFU-E colonies induced by the MuEpoR-IRES-gp55-P virus when the infected cells were cultured in the absence of Epo (column 1) was significantly greater (Student's *t*-test, *p* < 0.005) than those induced by the MuEpoR S238L-IRES-gp55-P virus (column 3) or by the MuEpoR-IRES-GFP virus which expresses GFP in place of gp55-P (column 5). (C) The number of CFU-E colonies formed in the absence of Epo (-Epo, hatched columns) or in the presence of 3 U/ml Epo/ml (+Epo, solid columns) in day 12.5 EpoR^{-/-} fetal liver CFU-Es after infection by viruses encoding the wild-type or L238S mutant human Epo receptors (HuEpoR) and gp55-P, or only GFP (GFP-Control). Infection and detection of CFU-E colonies were as in (B); results are expressed as colonies induced per fetal liver in the presence or absence of Epo. Data represent the mean of five independent assays ± 1 SD. Both EpoR-encoding viruses induced the same number of CFU-E colonies when the infected cells were cultured in the presence of Epo (columns 2 and 4). In contrast, the number of CFU-E colonies induced by the HuEpoR L238S-IRES-gp55-P virus when the infected cells were cultured in the absence of Epo (column 3) was significantly greater (Student's *t*-test, *p* < 0.005) than those induced by the wild-type HuEpoR-IRES-gp55-P virus (column 1). The latter figure was not significantly different from the number of CFU-E colonies induced by the control GFP virus (column 5). (D) Expression of gp55 protein in NIH 3T3 cells infected with virus encoding MuEpoR-IRES-gp55-P, MuEpoR S238L-IRES-gp55-P, HuEpoR-IRES-gp55-P and HuEpoR L238S-IRES-gp55-P. Typical titration experiments were performed by incubating 5-fold dilutions of packaged virus with NIH 3T3 cells (50 000 cells/well) for 8 h in the presence of 8 µg/ml polybrene. Cells were counted after 72 h and analyzed by Western blotting with an anti-gp55 antibody. Shown is a typical example of an infection with undiluted virus.

Fig. 5. Modeling of transmembrane interactions between the murine EpoR and gp55-P proteins using molecular dynamics simulations and energy minimization. (A) EpoR–gp55-P interactions. Energy contributions of EpoR residues 226–249 calculated for the complex between the transmembrane domains of the EpoR and gp55-P proteins. The average structure shown in (D) is the lowest energy complex of eight clusters generated in the conformational search. The interaxial distance was fixed at 10.5 Å. (B) EpoR–gp55-P interactions. Energy contributions of gp55-P residues 382–404 calculated for the complex shown in (D) between the transmembrane domains of the EpoR and gp55-P proteins. Comparison of panels (A) and (B) with the structure shown in (D) indicates that the low energy contribution of Thr383 results from hydrogen-bonding interactions with Ser231 of EpoR. In contrast, Ser238 of EpoR hydrogen-bonds back to Leu234 rather than across the dimer interface. The energy contributions of Ser238 and Met390 (on gp55-P) results predominantly from van der Waals interactions. (C) gp55-P–gp55-P interactions. Energy contributions of gp55-P residues 382 to 404 calculated for the homodimer complex shown in (E). The average structure shown in (E) is the lowest energy complex of 10 clusters generated in the conformational search. The interaxial distance was fixed at 10.5 Å. Conformational searches with interaxial separations of 9.5 and 10.0 Å led to fewer clusters of structures. The lowest energy clusters had the orientation of gp55-P helices seen in (E) and an increased packing contribution for Gly391. (D) Details of the predicted interaction between the transmembrane domains of the murine EpoR and gp55-P; Met390 of gp55-P is predicted to interact at the interface with Ser238 and Leu234 of the murine EpoR. (E) Predicted structure of the homodimer of the gp55-P transmembrane domain; Gly391 is placed in the interface of the gp55-P homodimer. The face of the gp55-P helix predicted to interact with the murine EpoR is opposite to that predicted to form the homodimer.

Importantly, no low energy clusters were obtained for interactions between the transmembrane domains of murine EpoR and gp55-A (not shown). A computational search on the gp55-P homodimer revealed one very low energy structure that is stabilized by interhelical interactions involving a different face of the gp55-P helix than that predicted to interact with the EpoR (Figure 5D and E). We hypothesize that each of the transmembrane domains in a cell surface gp55-P dimer (or higher oligomer) binds specifically to the transmembrane segment of one EpoR, dimerizing and activating the EpoR.

Mutagenesis of the gp55-P and gp55-A transmembrane domains reveals which of the differing transmembrane residues are important for EpoR activation

These computational searches suggest that the transmembrane domain of gp55-P has a stronger interaction with the EpoR transmembrane domain than does gp55-A. This would be a possible explanation for the poor ability of

gp55-A to activate EpoR signaling and for the well established results showing that the transmembrane sequence of gp55-P is crucial for inducing polycythemia *in vivo* (Amanuma *et al.*, 1989; Chung *et al.*, 1989; Watanabe *et al.*, 1995). While these molecular models are a provocative way to imagine a possible interaction, they do not provide sufficient detail to suggest compensatory mutations in the EpoR and gp55. However, the biology of our system allowed us to explore further the precise sequences required for productive transmembrane interactions, since sequence differences between gp55-P and gp55-A must be responsible for the different biological effects of these proteins. The membrane-spanning segment of gp55-P differs from that of gp55-A at six positions, and also contains two leucine residues (L396 and 397) 'deleted' in gp55-A (Figure 6A). To test our model, we generated all possible gp55-P→gp55-A mutations, as well as gp55-P mutants missing one (Del L397) or both (Del L396, 397) of the 'extra' leucine residues. As determined by the ability to induce Epo-independent growth of

Fig. 6. Identification of the precise transmembrane sequences of gp55-P which differ from gp55-A that are involved in activation of the EpoR signaling. (A) Sequence alignment between the transmembrane sequences of gp55-P and -A. (B) The indicated gp55-P mutants were tested in proliferation assays for their capacity to induce Epo-independent growth in murine EpoR-expressing Ba/F3 cells. Except for M390L and G391L all mutations convert residues in gp55-P to those in gp55-A.

Ba/F3 cells expressing the murine EpoR, only gp55-P mutants M390I, Del L396, L397, Del L397 and L399I could not activate the EpoR (Figure 6B). Both M390 and L397 are on the face of the gp55-P transmembrane helix predicted to interact with the EpoR, forming a left-handed coiled-coil. However, two other amino acids, G391 and L399, are not predicted to be on this face of the transmembrane helix (Figure 5D and E; see below).

The position of a methyl group at position 390 of the gp55-P transmembrane domain determines the polycythemic versus anemic phenotype

As noted, an Ile at position 390 renders gp55-P unable to activate the EpoR. Ile has a β -branched side chain, lacks rotational freedom, and is known to have drastic effects on packing of α -helical coiled coils (Harbury *et al.*, 1993). We therefore predicted that, unlike isoleucine, introduction of a Leu at position 390 (gp55-P M390L) would allow gp55-P to activate the murine EpoR. Indeed, the gp55-P M390L mutant was fully able to activate the murine EpoR (Figure 6B). The position of one methyl group, at carbon γ or at carbon β is all that differs between leucine and isoleucine. These results thus support the notion that the transmembrane segments of the two proteins interact in a highly specific manner. According to our model (Figure 5D), Met390 of gp55-P binds specifically to the murine EpoR in the region of Ser238. On the opposite face of the gp55-P transmembrane helix, a mutation of L399 to I abolishes activation. A residue common to both gp55-P and -A, G391, immediately following M390 in gp55-P, seems to be important for activation since the gp55-P G391L mutant cannot activate the EpoR (Figure 6B). However, these results can only be interpreted correctly if these mutant gp55 proteins are expressed similarly on the cell surface. This issue is critical, since gp55-A is poorly expressed on the cell surface (Ruscetti *et al.*, 1981; Amanuma *et al.*, 1989).

Cell surface expression of gp55 mutant proteins and their biological activity

Intracellular processing and cell-surface expression of gp55 proteins expressed in NIH 3T3 cells can be used as

a metric for the intrinsic ability of these proteins to be transported to the plasma membrane (Ruscetti *et al.*, 1981; Amanuma *et al.*, 1989; Watanabe *et al.*, 1990, 1995). In order to determine the capacity of the various gp55 mutants to be expressed at the cell surface, we generated stably expressing NIH 3T3 cell lines by infection with high titer SFFV virus encoding gp55 proteins. The different cell lines were analyzed by Western blotting with goat anti-Rauscher gp55 antibodies and shown to express similar levels of gp55 proteins (not shown). Cells were then analyzed by immunofluorescence microscopy using a triple sandwich (monoclonal anti-gp55 antibody 7C10, biotinylated goat anti-rat IgG and Cy3-streptavidin) as described in Materials and methods. All labeling steps were performed on live cells at 4°C in order to exclusively label the cell surface. This generates a patchy labeling pattern, as the antibody-labeled proteins at the surface of live, unfixed cells are swept into micropatches by cross-linking via the secondary antibodies and Cy3-streptavidin, as previously described (Henis *et al.*, 1994).

As shown in Figure 7, gp55-P was very well expressed at the cell surface while gp55-A was not, thus confirming results of others (Ruscetti *et al.*, 1981; Amanuma *et al.*, 1989). Mutants gp55-P M390I and G391L were equally well expressed at the cell surface but, importantly, both fail to activate the EpoR. Moreover, two other mutants which are also unable to activate the MuEpoR, gp55-P Del L396, L397 and gp55-P Del L397, are expressed significantly at the cell surface, albeit at lower levels. Therefore, deleting both extra leucines, L396 and L397, in gp55-P or L397 alone does not abolish cell-surface expression, while it does abolish activation of the EpoR. In a previous study (Amanuma *et al.*, 1989), a mutant gp55-like molecule which lacked the two extra leucines was found to be absent on the cell surface. In that case, however, additional mutations reminiscent of the Friend murine leukemia virus envelope protein were also present in the transmembrane domain (i.e. S392P).

Interestingly, the gp55-P L399I mutant failed to express at detectable levels on the cell surface (Figure 7) and also failed to activate the EpoR. L399 was predicted to be on the opposite face of the transmembrane helix from that involved in interaction with the EpoR (Figure 5D and E) and thus would not have been expected to influence activation of the EpoR. Its lack of cell-surface expression explains its inability to activate the EpoR (Figure 6B). These data show clearly that sequence specificity is required for activation of the EpoR by gp55 proteins, as a number of gp55 mutants that cannot activate the EpoR do reach the cell surface. Mutation of G391 (the residue following M390) also abolishes activation; G391 is predicted to allow the gp55-P and EpoR transmembrane helices to cross in close contact. In neu* (activated form of the neu protein through a V→E transmembrane mutation), the glycine residue (G665) following E664 is also critical for activation of neu, as glycine residues are found with high frequency in a number of transmembrane helices to allow close packing/crossing between helices.

Discussion

Our key findings can best be summarized by a molecular model (Figure 1): each subunit of a dimeric plasma

Fig. 7. Cell-surface expression of gp55 mutant proteins. NIH 3T3 cells stably expressing various gp55 mutants at similar levels (as judged by Western blot using anti-gp55 goat serum, not shown) were processed for immunofluorescence microscopy as described under Materials and methods. All labeling steps were performed on live cells at 4°C, in order to label exclusively cell-surface proteins. All photographs were taken in the same session, under identical conditions and exposure times, and the film rolls were developed together and printed under identical conditions to enable direct comparison. Bar = 10 μ m. For each cell line the percentage of fluorescent-labeled cells was evaluated by counting 500 cells in several fields and scoring cells visibly labeled above the background level. Samples with 20–30% labeled cells were marked ‘++’, those with 10–15% labeled cells were designated ‘+’; and samples with 2% or fewer labeled cells were marked ‘-’.

membrane gp55-P binds, through its membrane-spanning segment, a single EpoR polypeptide, dimerizing the receptor and thus activating EpoR signal transduction pathways. A more detailed molecular model for the specific interactions between the transmembrane domains of the EpoR and gp55-P proteins (Figure 5D) was derived from computational searches. The convergence of these unbiased searches on a low energy structure consistent with the experimental data was striking. First, serine 238 of the murine EpoR is predicted to be at the interface with gp55-P; a leucine residue is at the same position in the human EpoR, which is not activated by gp55-P. Mutation of this one amino acid in the human EpoR to its murine counterpart (HuEpoR L238S) results in its ability to be activated by gp55-P. Conversely, mutating the serine in

the murine EpoR to its human counterpart (MuEpoR S238L) abolishes activation by gp55-P. The computational searches of EpoR-gp55-P transmembrane interactions yielded a low energy structure in which S238 of the EpoR transmembrane domain interacts specifically with M390 of gp55-P. Support for this low energy structure was provided by mutational studies analyzing the differences between gp55-A and gp55-P. In gp55-A, which is much less effective than gp55-P in activating the murine EpoR, the residue at position 390 is isoleucine. We show that mutation of Met390 in gp55-P to its counterpart in gp55-A (M390I) results in its inability to activate the murine EpoR. In contrast, replacing this methionine with a leucine allows full biological activity. Thus, the EpoR can be activated by a non-related protein through a novel mechan-

ism which involves highly specific interactions between residues within the transmembrane domains.

Activation of the EpoR by dimerization

EpoR can be activated by Epo-induced dimerization (Watowich *et al.*, 1994), by dimerization induced by small mimetic peptides (Wrighton *et al.*, 1996), by bivalent monoclonal antibodies directed to the EpoR, or by a point mutation in the extracellular domain which constitutively dimerizes the receptor (Yoshimura *et al.*, 1990b; Watowich *et al.*, 1992). The three dimensional structure of the complex of two EpoR extracellular domains bound to Epo shows that receptor orientation by ligand is critical for the efficiency of signaling (Syed *et al.*, 1998). A different orientation of the extracellular domains of two EpoR monomers is induced by peptide mimetics (Livnah *et al.*, 1996). Both peptide- and Epo-induced EpoR dimer assemblies differ from that of the human growth hormone receptor bound by its ligand (De Vos *et al.*, 1992; Livnah *et al.*, 1996; Wrighton *et al.*, 1996; Syed *et al.*, 1998). It is likely that in the R129C EpoR mutant, which is a disulfide-linked dimer, the two EpoR extracellular domains will have a yet another orientation, as will those dimerized by bivalent anti-receptor antibodies. While the efficiency of signaling (the ratio between the amount of substance required for cell growth and the K_d for binding) may be dependent on receptor orientation (Syed *et al.*, 1998), these data show that more than one mode of receptor dimerization can induce signal transduction, cell proliferation and red cell differentiation. Recently, the crystal structure of the extracellular domain of the EpoR in its unliganded form has unexpectedly also revealed a preformed dimer in which the individual membrane-spanning and intracellular domains would be too far apart to permit transphosphorylation of JAK2 molecules (Livnah *et al.*, 1999). An *in vivo* fragment complementation assay further supports a model in which ligand binding induces a conformational change that allows JAK2 to be brought into closer proximity and thus activated (Remy *et al.*, 1999). Assuming that this model were correct, our results would suggest that binding of a gp55-P dimer to the preformed EpoR inactive dimer is able to induce a conformational change or to further move the EpoR monomers in close proximity in such a way that JAK2 activation is possible. Indeed, we have shown (data not published) that gp55-P and gp55 mutants that induce Epo-independence also induce constitutive activation of JAK2.

Specific interactions between the EpoR and gp55-P transmembrane domains

The residues in the murine EpoR and gp55-P transmembrane domains that we identified experimentally as crucial for biological activity are the ones that are shown computationally to stabilize the lowest energy complex between the EpoR and gp55-P transmembrane domains. The productive binding involves one face of the gp55-P transmembrane domain which recruits one face of the transmembrane domain of the mouse EpoR (Figures 1 and 5D). The combined approach of computational searches and mutagenesis allows us to begin to see the details of this specific interaction.

The human EpoR is not activated by gp55-P (Showers *et al.*, 1993; Hoatlin *et al.*, 1995); the murine and human

EpoRs differ only in three positions in the transmembrane domain and are 82% identical overall (Jones *et al.*, 1990). Strikingly, mutation of Leu238 to Ser in the transmembrane domain rendered the human EpoR sensitive to activation by gp55-P. Conversely, mutation of Ser238 of the murine EpoR to Leu abolished activation by gp55-P. Mutation of Ser238 to Ala did not abolish activation, nor did mutation of other hydroxyl-containing amino acid residues to Ala or Thr (Figure 3A and B). The key position seems to be in the middle of the transmembrane domain as mutation of Ile227 or Leu228 to Ser did not have any effect on activation by gp55-P or by Epo (data not shown).

Activation of the EpoR by gp55-P was also assayed in primary fetal liver erythroid progenitors isolated from EpoR^{-/-} embryos. Epo receptors with mutations in their transmembrane domains were equivalent in their ability to respond to Epo and induce erythroid colony formation from CFU-Es. Only the murine EpoR and the mutant HuEpoR L238S could support CFU-E differentiation when activated by gp55-P. Like the human EpoR, the murine EpoR S238L mutant was not activated by gp55-P but responded normally to Epo. Therefore, both in cell lines and in primary erythroid progenitors, the presence of a small amino acid—either serine or alanine—at one position in the center of the transmembrane sequence of the EpoR is crucial for efficient activation by gp55-P but not for activation by Epo.

The face of the gp55-P transmembrane domain (containing Met390 and Leu397) predicted to interact with the mouse EpoR is different from that predicted to form the gp55-P homodimer (Figure 5E). Sequence specificity is demonstrated further by our results using gp55 mutant proteins, in which residues that differ between the polycythemic and anemic strains were changed to the corresponding sequences of the anemic strain. Previously it has been suggested that gp55-A does not activate the EpoR because it is poorly expressed on the cell surface (Ruscetti *et al.*, 1981; Amanuma *et al.*, 1989). Here (Figure 7), we show that gp55-A is indeed not detected by our triple-sandwich immunofluorescence on the cell surface while gp55-P is well expressed. Notably, several mutant gp55 proteins that do not activate the EpoR were expressed at high levels on the cell surface (gp55-P M390I and gp55-P G391L) while others were expressed at lower but significant levels (gp55-P Del L396, L397 and gp55-P Del L397). The one mutation (L399I) that would not have been predicted to inhibit activation of the EpoR was not expressed on the surface, thus explaining its lack of activation of EpoR. These results are consistent with those reported by Watanabe *et al.* (1995), which showed that deletion of three residues at a time from the transmembrane domain of gp55-P can abolish activity but without affecting cell-surface expression. Although the gp55-P mutant that lacks both extra-leucine residues (Del L396, L397) is expressed at slightly reduced levels on the cell surface, we suggest that this is not the reason why this mutant is inactive. A previous study (Amanuma *et al.*, 1989) reported the isolation of a gp55-like molecule which did not have the two extra leucine residues and was not expressed significantly on the cell surface. As this mutant gp55 contained additional amino acid changes we suggest that the two leucines are crucial for biological activity and not for cell-surface expression. Insertion of the two extra

leucines into gp55-A does not rescue the cell-surface expression defect (Y.I.Henis, personal communication). Taken together, our data clearly show that stringent transmembrane sequence specificity is required for activation of the EpoR by gp55 proteins. More importantly, replacing the methionine at position 390 in gp55-P with leucine (M390L) does allow full activation of the EpoR, thus excluding a unique role for the sulfur atom of the methionine. Since an isoleucine at that position in gp55 (M390I) leads to a protein unable to activate the EpoR, the ability of gp55-P to activate the EpoR depends on whether the methyl group is attached to the β (Ile) or γ (Leu) carbon atom of the aliphatic amino acid at position 390. Ile is present at this position in gp55-A (Figure 6A), which does not activate the EpoR to promote Ba/F3 cell proliferation. Such specificity is consistent with the notion that Met390 (or Leu390) in gp55-P mediates activation through specific van der Waals packing interactions with EpoR in the region of Ser238, possibly a left handed coiled-coil arrangement of transmembrane helices.

Such subtle changes causing dramatic biological effects are not without precedent. In other coiled coils, such as the GCN4 leucine zipper, replacement of Leu with Ile at the hydrophobic positions of the heptad repeat changes the oligomerization status of the protein from dimer to trimer or tetramer (Harbury *et al.*, 1993). Met and Leu were also shown to be compatible at a critical position in transmembrane helix 6 of rhodopsin, where Met is involved in a specific interaction with transmembrane helix 7 by which rhodopsin signaling is kept off in the dark. However, Met257 cannot be changed to any residue other than Leu without a loss of basal 11-*cis*-retinal induced inhibition of signaling in the dark (Han *et al.*, 1998).

Unique features of EpoR/gp55-P transmembrane domain interactions

The interaction between the transmembrane domains of murine EpoR and gp55-P is translated into an array of biological activities. On the structural level, we suggest that this interaction is novel since it involves a stereochemical fit between hydrophobic amino acids of two unrelated membrane proteins with single membrane-spanning helices. While there are a number of examples where charged and/or polar residues are determinants of interactions between membrane domains, only in the case of glycoporphin is the homodimerization of two transmembrane domains generated entirely by van der Waals interactions.

The *neu* tyrosine kinase receptor can be activated by a Val to Glu point mutation in the transmembrane domain (Bargmann *et al.*, 1986), which dimerizes and constitutively activates the receptor (Bargmann *et al.*, 1986; Burke *et al.*, 1997). Achondroplasia, the most common genetic form of dwarfism, results from a Gly to Arg substitution in the transmembrane domain of the fibroblast growth factor receptor 3 (FGFR3) (Rousseau *et al.*, 1994; Shiang *et al.*, 1994) causing constitutive activation of the receptor (Webster and Donoghue, 1996). Transmembrane helical interactions are crucial for the assembly of the T-cell receptor (TCR); specific pairs of TCR chains (i.e. TCR α and CD-3 δ) assemble due to interactions between specific charged sequences in their transmembrane domains (Manolios *et al.*, 1990). Complex formation between

class II MHC α and β chains relies on residues with opposite charge at the N-terminus of the membrane-spanning domains and on a structural motif involving several glycines on the same face of the putative helices (Cosson and Bonifacio, 1992).

The ability of hydrophobic packing interactions to stabilize helix association has previously been seen in the glycoporphin A transmembrane helix dimer (Furthmayr and Marchesi, 1976; MacKenzie *et al.*, 1997). The glycoporphin A transmembrane domain forms a stable homodimer through packing interactions involving a seven-residue motif, LIxxGVxxGVxxT. Interestingly, the polar side chain of the threonine residue does not stabilize the dimer through interhelical hydrogen bonding, but rather the threonine contributes to dimerization through non-polar interactions involving the β -methyl group (MacKenzie *et al.*, 1997). As predicted in the EpoR for the β -hydroxyl group of Ser238, the threonine β -hydroxyl group hydrogen bonds back to the i-4 carbonyl of the same helix. The interaction between the murine EpoR and gp55-P transmembrane domains is unique, however, since it involves a stereochemical fit between hydrophobic amino acids of two unrelated proteins.

Finally, many viruses encode small single-spanning membrane proteins, which activate specific growth factor receptors and may participate in cell transformation and/or stimulate virus growth (Drummond-Barbosa and Di Maio, 1997; Petti *et al.*, 1997). The fibropapillomavirus protein E5 forms a stable complex with and activates specifically the PDGF β receptor (Petti *et al.*, 1997). The determinants for specific activation of the PDGF β receptor are charged and polar sequences in the extracellular and transmembrane domains of the receptor. The interaction results in dimerization and constitutive activation of the receptor (Petti *et al.*, 1997). This interaction, like that between gp55-P and the EpoR, may prove to be a general mechanism by which unrelated protein oligomers function to dimerize cell-surface receptors via interactions within transmembrane α -helices.

Materials and methods

Generation of EpoR mutants

The murine and human EpoR cDNAs were cloned in the pMX-IRES-GFP bicistronic retroviral vector upstream of the IRES (Liu *et al.*, 1997; Constantinescu *et al.*, 1998). The translation of the two proteins is tightly linked in that expression of GFP is proportional over a 100-fold range to the level of expression of the protein encoded by the cDNA placed upstream of the IRES (Liu *et al.*, 1997). All mutant Epo receptor constructs were generated by PCR overlap extension using vector- and IRES-derived sequences as external primers. Because the human EpoR receptor has, compared with the murine, one extra amino acid in its extracellular domain, the numbering of HuEpoR residues was adjusted to that of the murine for easy comparison. After sequencing, plasmid DNAs were used to transfect retroviral packaging cells in order to generate high titer retroviruses. gp55-P cDNA, kindly provided by Dr Sandra Ruscetti (National Cancer Institute, Frederick, MD) was used as a template to generate gp55-P to -A mutants by PCR overlap extension.

Generation of retroviral supernatants

High titer replication free retroviral supernatants were generated by transient transfection of the BOSC packaging cell line (Pear *et al.*, 1993). Of each retroviral construct, 5 μ g of DNA was transfected by the calcium phosphate method (Constantinescu *et al.*, 1998). After collection of viral supernatants, expression in BOSC cells of various EpoR or gp55 mutants was measured by Western blots as described (Constantinescu *et al.*, 1998), while expression of GFP was measured

by fluorescence-activated cell sorting (FACS). Viral titers were measured by FACS as described previously (Constantinescu *et al.*, 1998).

Assay for Epo- and gp55-P-dependent proliferation

IL-3-dependent Ba/F3 cells growing in RPMI medium supplemented with 10% fetal bovine medium, antibiotics and 5% WEHI supernatant (as a source of IL-3) were infected with bicistronic viruses encoding GFP and different EpoRs (murine, human, wild type or mutants) for 4 h in the presence of 4 µg/ml polybrene. Infected cells were scanned by FACS for GFP fluorescence; typically infection efficiencies were 9–15%. For every infected construct, cells were either selected directly in Epo (0.5–1 U/ml) for proliferation by removing IL3 or, alternatively, cells were first sorted for the top 0.1% GFP fluorescence and then placed in Epo in the absence of IL3. The capacity to support proliferation in Epo was measured for each transduced EpoR construct; both selected and sorted cells gave similar results for each construct. All experiments were performed on pools of cells and not on clones. The expression of the EpoR was confirmed by Western blot analysis using antibodies directed to the murine EpoR (C-187; Yoshimura *et al.*, 1990a) and human EpoR (C-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Cells expressing different EpoRs were infected with viruses encoding gp55-P as described previously (Constantinescu *et al.*, 1998), and the ability of transduced gp55-P to induce Epo-independent proliferation of cells was assayed by removing Epo or WEHI medium and placing cells in 24-well plates in medium lacking growth factor, as described previously (Constantinescu *et al.*, 1998). Epo-independent cells were expanded and tested for gp55-P expression; high levels of gp55-P were demonstrated in cells growing in the absence of Epo by Western blot analysis using an anti-Rauscher-gp70 goat antibody (National Cancer Institute serum repository) recognizing the xenotropic-derived region of gp55 proteins or using a monoclonal anti-gp55 antibody (7C10, a gift of Dr Sandra Ruscetti; data not shown).

Erythroid colony formation in primary fetal liver cells

Day 12.5 homozygous EpoR^{-/-} fetal liver cells were harvested and single cell suspensions were prepared as described (Wu *et al.*, 1995; Constantinescu *et al.*, 1998). Cells were infected with various bicistronic retroviruses in the presence of 4 µg/ml polybrene for 4 h in Iscove's modified Dulbecco's medium (IMDM) containing 20% fetal bovine serum (FBS). For colony forming unit-erythroid (CFU-E) assays, 10⁵ nucleated progenitors were plated in semisolid 0.1% methylcellulose medium (MethoCult 3230, Stem Cell Technologies, Vancouver, British Columbia, Canada) containing 20% FBS as described (Wu *et al.*, 1995; Constantinescu *et al.*, 1998) in the presence or absence of 3 U/ml Epo. Colonies generated by CFU-E progenitors were scored 72 h after plating by staining with diaminobenzidine (Sigma, St Louis, MO). The efficiency of infection for a particular set of bicistronic retroviruses was represented by the number of colonies induced by Epo, since EpoR^{-/-} progenitors absolutely require the EpoR to differentiate into red cells (Wu *et al.*, 1995). As described by Constantinescu *et al.* (1998), when viruses encoding both EpoR and GFP were used to infect parallel aliquots of fetal liver progenitors, which were cultured in suspension in the presence of Epo and scanned by FACS for GFP expression 36 h after infection, 10–15% infection efficiencies were typically obtained.

Computational searches

The computational search strategy has been described previously by Brünger and colleagues (Treutlein *et al.*, 1992; Nilges and Brünger, 1993; Lemmon *et al.*, 1994; Adams *et al.*, 1995, 1996). The method is applicable to either homo- or heterodimers of interacting helices. Canonical α -helices were generated from the transmembrane sequences of gp55-P (residues 382–404), gp55-A (residues 382–404) and the EpoR (residues 226–249). The calculations on gp55-A were carried with and without the insert of Leu396 and Leu397, present in the gp55-P sequence. Heterodimer searches were carried on the EpoR-gp55-P and EpoR-gp55-A helices, while homodimer searches were carried out on the gp55-P and gp55-A helices. Low energy conformations were identified by rotating each helix in the helix dimer through rotation angles j1 and j2 from 0–360°. At 45° increments, molecular dynamics simulations were performed using simulated annealing of all atomic coordinates. The structures were energy minimized before and after the molecular dynamics simulations. The parameters used for the molecular dynamics simulations and energy minimization were the same as those used by Adams *et al.* (1995). The starting geometries included both left handed (+25°) and right handed (–50°) crossing angles. The crossing angles, as well as the rotation angles j1 and j2, were allowed to vary during the simulation. This allows the helices to adopt low-energy coiled-coil geometries. In individual simulations,

however, the distance between the two helix axes was held fixed at 10.0, 10.5 or 11.0 Å. There was no translational offset between the helices. Four different molecular dynamics simulations were carried out for each starting geometry. During the cycle of molecular dynamics and minimization, the helix dimers migrate from their initial geometries and can group together to form well defined clusters of structures. A cluster is defined as a group of at least 10 structures, where the root mean square deviation of the atom positions is <1 Å between any given structure in the cluster and the next most similar structure. For each cluster, the individual dimer structures are averaged and then energy minimized to yield a single low energy dimer structure. This analysis leads to a small pool of possible structures that can then be evaluated on the basis of experimental data.

Immunofluorescence microscopy

NIH 3T3 cells stably expressing different gp55 mutants were washed twice with Dulbecco's PBS supplemented with 1% bovine serum albumin (Sigma), and incubated in the same buffer (45 min, 4°C) with goat γ -globulin (200 µg/ml) to block nonspecific staining. This was followed by successive incubations (4°C, 1 h each, with three washes between incubations) with: (i) 7C10 rat monoclonal antibody (ascites) (1:200 dilution); (ii) biotinylated goat anti-rat IgG (20 µg/ml); and (iii) Cy3-streptavidin (2 µg/ml). The 7C10 antibodies were a generous gift from Dr Sandra K. Ruscetti (National Cancer Institute, Frederick, MD), while the biotinylated goat anti-rat IgG and Cy3-streptavidin were from Jackson Immuno-Research Laboratories (West Grove, PA). All incubations with antibodies were carried out on live cells in the cold; under these conditions the antibodies cannot penetrate the cells and only cell-surface proteins exposed to the extracellular medium can be labeled. The cells were then washed and fixed successively in methanol (–20°C, 5 min) and acetone (–20°C, 2 min), and mounted with Prolong antifade mounting solution (Molecular Probes, Eugene, OR). Fluorescence digital images were acquired with a Nikon Eclipse 800 microscope (X100 oil immersion objective) coupled to a CCD camera (Micromax, Princeton Instruments), using OpenLab (by ImproVision) software. All images were taken under identical conditions, exported in TIFF format to Adobe Photoshop™ and printed.

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While this manuscript was being processed, a paper by Fang *et al.* was published (*Virology*, 1998, **252**, 46–53). This paper showed that two of the gp55-P to gp55-A transmembrane mutants we studied (M390I and Del L396, L397) cannot induce polycythemia in mice and are deficient in activation of the EpoR.