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Tyrosine 343 in the erythropoietin receptor positively regulates erythropoietin-induced cell proliferation and Stat5 activation

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While previous studies with truncated erythropoietin receptors (EpRs) have suggested that the tyrosine phosphorylation of the EpR does not play a role in Ep-induced proliferation, we have found, using a more subtle, full length EpR mutant, designated Null, in which all eight of the intracellular tyrosines have been substituted with phenylalanine residues, that Null cells require substantially more Ep than wild-type cells in order to proliferate as efficiently. A comparison of Ep-induced proliferation with Ep-induced tyrosine phosphorylation patterns, using wild-type and Null EpR-expressing cells, revealed that Stat5 tyrosine phosphorylation and activation correlated directly with proliferation. Moreover, studies with a Y343F EpR point mutant and various EpR deletion mutants revealed that both Ep-induced proliferation and Stat5 activation were mediated primarily through Y343, but that other tyrosines within the EpR could activate Stat5 in its absence.

Keywords: erythropoietin signaling/proliferation/Stat5 activation/tyrosine phosphorylation

Introduction

Erythropoietin (Ep), the principal *in vivo* stimulator of mammalian erythropoiesis (Krantz, 1991), exerts its action by binding to receptors on the surface of erythroid progenitors (Sawada *et al.*, 1990; Wognum *et al.*, 1990b, 1992). These Ep receptors (EpRs) are type 1 transmembrane proteins that belong to a family of hematopoietin receptors whose members are characterized by the presence of four conserved cysteines and Trp–Ser–X–Trp–Ser motifs in their extracellular domains and the absence of any known catalytic activity in their intracellular regions (D'Andrea *et al.*, 1989; Bazan, 1990). Nonetheless, although the EpR lacks tyrosine kinase activity, it, along with a number of other cellular proteins, becomes transiently phosphorylated on tyrosine residues within minutes of binding Ep (Carroll *et al.*, 1991; Miura *et al.*, 1991; Quelle and Wojchowski, 1991b; Damen *et al.*, 1992, 1993a; Dusanter-Fourt *et al.*, 1992; Komatsu *et al.*, 1992). Recent evidence suggests that this rapid phosphorylation is carried out, at least in part, by the cytoplasmic tyrosine kinase, Jak 2 (Harpur *et al.*, 1992; Witthuhn *et al.*, 1993; Miura *et al.*, 1994b). Following this phosphorylation of the EpR, a number of Src homology 2 (SH2)-containing proteins become associated with it (Damen *et al.*, 1993a,b; He *et al.*, 1993; Liu *et al.*, 1994; Miura *et al.*, 1994a; Klingmuller *et al.*, 1995; Tauchi *et al.*, 1995; Yi *et al.*, 1995). These proteins include Grb2 (Damen *et al.*, 1993a; Liu *et al.*, 1994), Shc (Damen *et al.*, 1993a; Liu *et al.*, 1994), HCP (Klingmuller *et al.*, 1995; Yi *et al.*, 1995), Syp (Tauchi *et al.*, 1995) and phosphatidylinositol 3-kinase (Damen *et al.*, 1993b; Miura *et al.*, 1994a). Interestingly, although several of these proteins have been implicated in mediating proliferation signals in other growth factor systems (Bennett *et al.*, 1994; Cheatham *et al.*, 1994; Chung *et al.*, 1994; Li *et al.*, 1994; Sasaoka *et al.*, 1994) and thus might be expected to play a role in Ep-induced proliferaton, there is evidence that the tyrosine phosphorylation of the EpR itself may not be required for Ep-induced mitogenesis. Specifically, several investigators have found that EpR deletion mutants containing only the membrane-proximal tyrosine, i.e. Y343 based on nomenclature for the mature protein and possessing no detectable tyrosine phosphorylation (Yi *et al.*, 1995), functioned as well as (Quelle and Wojchowski, 1991a; Miura *et al.*, 1994c) or better than (D'Andrea *et al.*, 1991) wild-type (WT) EpRs in mediating Ep-induced proliferation. However, it is possible that low level phosphorylation of this remaining tyrosine might be sufficient to induce the required signaling. Related to this, recent evidence suggests that SH2-containing intermediates are somewhat promiscuous and can bind to different tyrosine-phosphorylated regions in a hierarchical fashion [for example in the epidermal growth factor receptor (Batzer *et al.*, 1994)]. It is conceivable, therefore, that the retention of only one tyrosine within the EpR may be sufficient, once phosphorylated, to stimulate entry into S phase by binding (albeit with lower affinity) SH2-containing proteins that normally bind to other tyrosine-phosphorylated residues within the EpR.

To address this question, site-directed mutagenesis was used to generate a full length EpR mutant in which all eight intracellular tyrosines were substituted with phenylalanines. Using this mutant, designated Null, we found that the absence of EpR tyrosine phosphorylation substantially increased the concentration of Ep required to transmit a proliferative signal. Moreover, using both this mutant, various deletion mutants and a full length Y343F mutant, a direct correlation was observed between Ep-induced proliferaton and the tyrosine phosphorylation and activation of the SH2-containing protein, Stat5. This activation of Stat5 was shown to be mediated primarily through Y343 within the activated EpR, but other tyrosines could activate Stat5 in its absence.

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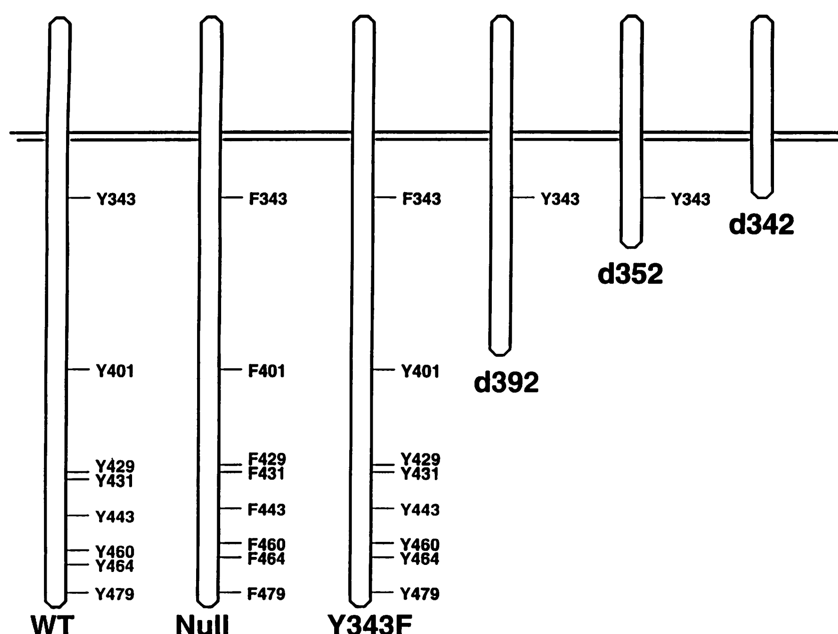

Fig. 1. Schematic representation of WT and mutant EpRs. All the EpRs shown have 223 amino acid extracellular and 24 amino acid transmembrane domains. The full length 483 amino acid WT, Null and Y343F EpRs possess 236 amino acid cytoplasmic regions. The deletion mutants are named on the basis of their total amino acid content.

Results

To resolve whether tyrosine phosphorylation of the EpR plays a significant role in Ep-induced proliferation, several EpR mutants, illustrated diagrammatically in Figure 1, were constructed. These included a full length Null EpR in which all eight intracellular tyrosines were exchanged for phenylalanines, another full length EpR mutant in which only the membrane-proximal tyrosine was replaced with a phenylalanine (i.e. Y343F) and three deletion mutants, designated d392, d352 and d342, which lacked the C-terminal 91, 131 and 141 amino acids, respectively.

DA-3 cells, which do not express any endogenous EpRs (Miura *et al.*, 1991), and Ba/F3 cells, which partially differentiate along the erythroid pathway (i.e. express β-globin mRNA) when infected with WT EpRs and stimulated with Ep (Liboi *et al.*, 1993; Krosl *et al.*, 1995), were retrovirally infected as described previously (Damen *et al.*, 1992) with the WT and Null EpR cDNA constructs, and clones were selected in G418 and interleukin (IL)-3. Preliminary analysis revealed that selection of Ba/F3 clones in the presence of Ep resulted in the appearance of clones with higher levels of endogenous EpRs than were normally present in these cells (Damen *et al.*, 1992). This was in agreement with previous studies (Yoshimura, 1994) and indicated that such a selection procedure would invalidate any efforts to establish the capabilities of introduced mutant EpRs. Thus, for all the studies presented herein, neither Ba/F3 nor DA-3 cells were exposed to Ep prior to the specific experiment described. In addition, we found that simply expressing higher numbers of WT EpRs on the surface of these two cell lines dramatically increased their sensitivity to Ep in proliferation assays, in keeping with previous findings in our laboratory (Damen *et al.*, 1992). This suggested that the intracellular signaling intermediates involved in mitogenesis were not limiting, at least within the 1000–10 000 EpRs/cell range used for our studies, and that, in order to compare Ep responsiveness of WT and mutant EpRs, it was critical to compare cell clones displaying the same EpR levels.

EpR tyrosine phosphorylation facilitates Ep-induced proliferation

Clones of WT- and Null EpR-expressing cells, displaying the same numbers of EpRs, as determined by biotinylated Ep/FACS (Wognum *et al.*, 1990b), Western analysis with anti-EpR antibodies (Damen *et al.*, 1995) and [^{125}I]Ep binding studies (D'Andrea *et al.*, 1991), were first analyzed for their mitogenic response to Ep. Using short-term assays (involving 15 h of exposure to different concentrations of Ep, followed by 2 h with [^{3}H]thymidine) to gain insight into the time of entry into the first S phase, having established this as the time at which cells enter their first S phase using both [^{3}H]thymidine pulse (data not shown) and propidium iodide/FACS studies (Krosl *et al.*, 1995), DA-3 cells expressing the Null EpR were consistently found to require ~5- to 10-fold higher concentrations of Ep to stimulate the same level of [^{3}H]thymidine incorporation as that obtained with WT EpR-expressing DA-3 cells (Figure 2A). To ensure this phenomenon was not cell type specific, Ba/F3 cells expressing the Null EpR were also tested and shown to require ~5-fold higher Ep levels to incorporate the same level of [^{3}H]thymidine as WT EpR-expressing Ba/F3 cells (Figure 2B).

The long-term growth of these cells was also examined in the presence of different concentrations of Ep. A typical result, shown with WT and Null DA-3 cells, expressing 10 000 EpRs/cell, revealed that the Null cells required 50- to 100-fold more Ep than the corresponding WT cells to grow at the same rate (Figure 2C). Null EpR-expressing Ba/F3 cells also grew far more poorly than their corresponding WT cells at low Ep concentrations and, as can be seen in Figure 2D with clones expressing 3000 EpRs/cell, died in the presence of 0.2 U/ml Ep.

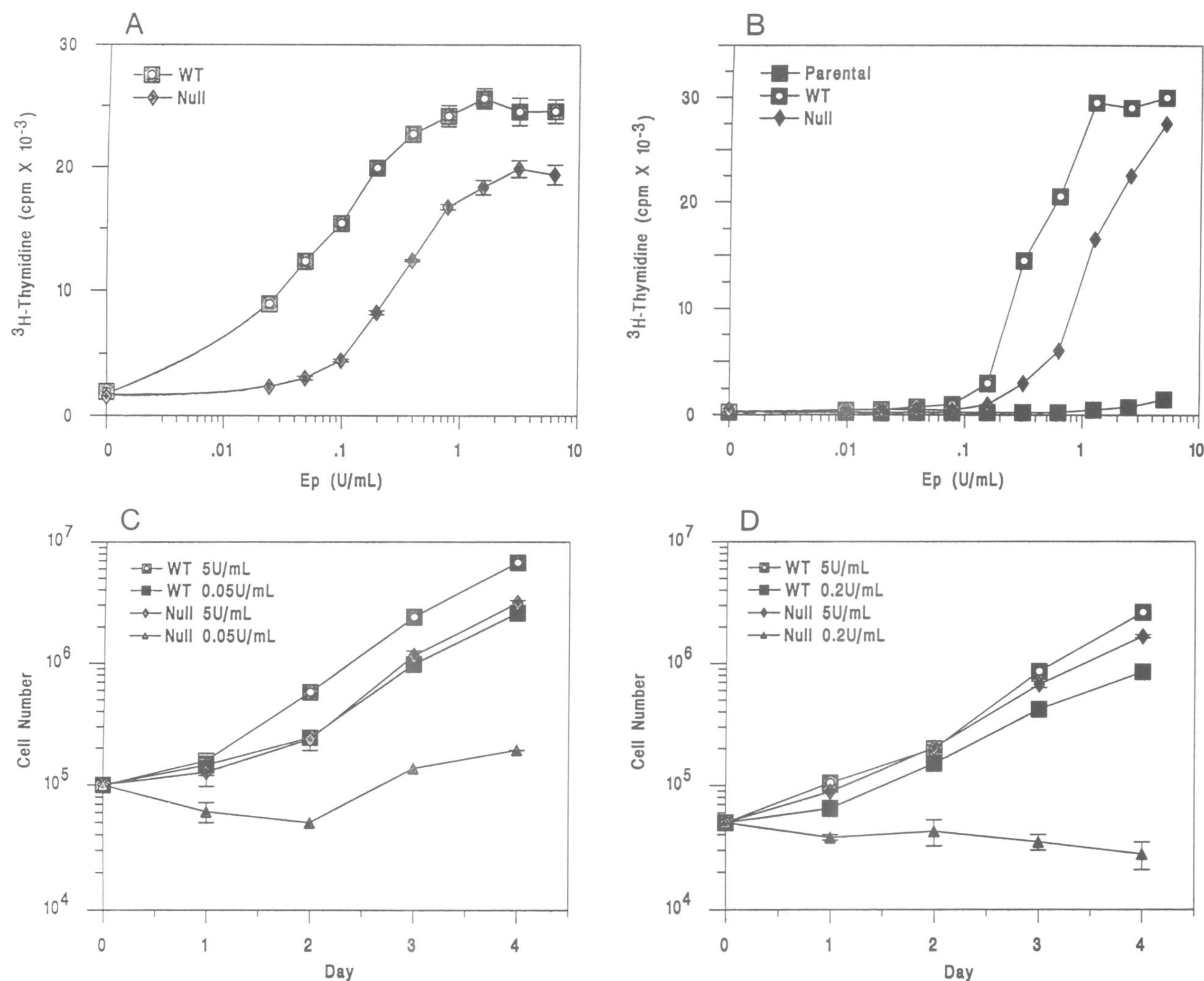

Fig. 2. EpR tyrosine phosphorylation facilitates Ep-induced proliferation in both DA-3 and Ba/F3 cells. Short-term [^{3}H]thymidine incorporation assays were carried out with (**A**) WT- and Null EpR-expressing DA-3 cells and with (**B**) WT- and Null EpR-expressing Ba/F3 cells, as described in Materials and methods. Included in (B) is the Ep-induced proliferation response of parental Ba/F3 cells for comparison. Each data point represents the mean ± SE of triplicate determinations. Error bars are not shown if smaller than the symbols. Long-term growth curves for WT- and Null-expressing DA-3 (**C**) and Ba/F3 (**D**) cells in the presence of the indicated concentrations of Ep were also carried out and data points represent the mean ± SE of duplicate determinations. Similar results were obtained in five separate experiments.

Tyrosine phosphorylation of the EpR is not required for Jak2 tyrosine phosphorylation but facilitates Shc tyrosine phosphorylation and activation of the Ras pathway

To determine, at the cell signaling level, what might be responsible for the difference in Ep sensitivity between WT and Null cells, we reasoned that there might be a signaling pathway that was active in WT cells but not Null cells at low Ep concentrations but that became activated in Null cells at high Ep concentrations. With this rationale in mind, we first investigated whether the EpR-associated tyrosine kinase Jak2 [shown to be critical for Ep-induced proliferation by Zhuang *et al.* (1994)] was tyrosine phosphorylated at lower Ep concentrations in WT cells than in Null cells. For these and subsequent cell signaling studies, we concentrated our efforts on EpR-infected DA-3 cells, since the presence of low levels of endogenous EpRs in Ba/F3 cells might make interpretation of the effects of exogenous EpRs difficult. Thus, DA-3 cells expressing identical levels of WT and Null EpRs were incubated for 5 min at 37°C, in the presence and absence of increasing concentrations of Ep, and lysates prepared from these cells were immunoprecipitated with anti-Jak2 antibodies. Western analysis of these samples, using anti-PY antibodies, revealed no difference in the tyrosine phosphorylation level of this early acting kinase (Figure 3A). A reprobing of this blot with anti-Jak2 antibodies confirmed equal loading of the samples (Figure 3A, lower panel).

The tyrosine phosphorylation of Shc in WT and Null cells in response to increasing concentrations of Ep was then examined by immunoprecipitating cell lysates with anti-Shc antibodies and carrying out Western analysis with anti-PY antibodies. As can be seen in Figure 3B, the p46 and p52 isoforms of Shc were clearly tyrosine phosphorylated in WT cells in response to 5 and 50 U/ml of Ep. However, these Shc isoforms were phosphorylated to a far lesser degree in Null cells at these Ep concentrations. A reprobing of this blot with anti-Shc antibodies confirmed equal loading of the samples (Figure 3B, lower panel). This suggested that the Ep-induced tyrosine phosphorylation of Shc, and perhaps the Ep-induced

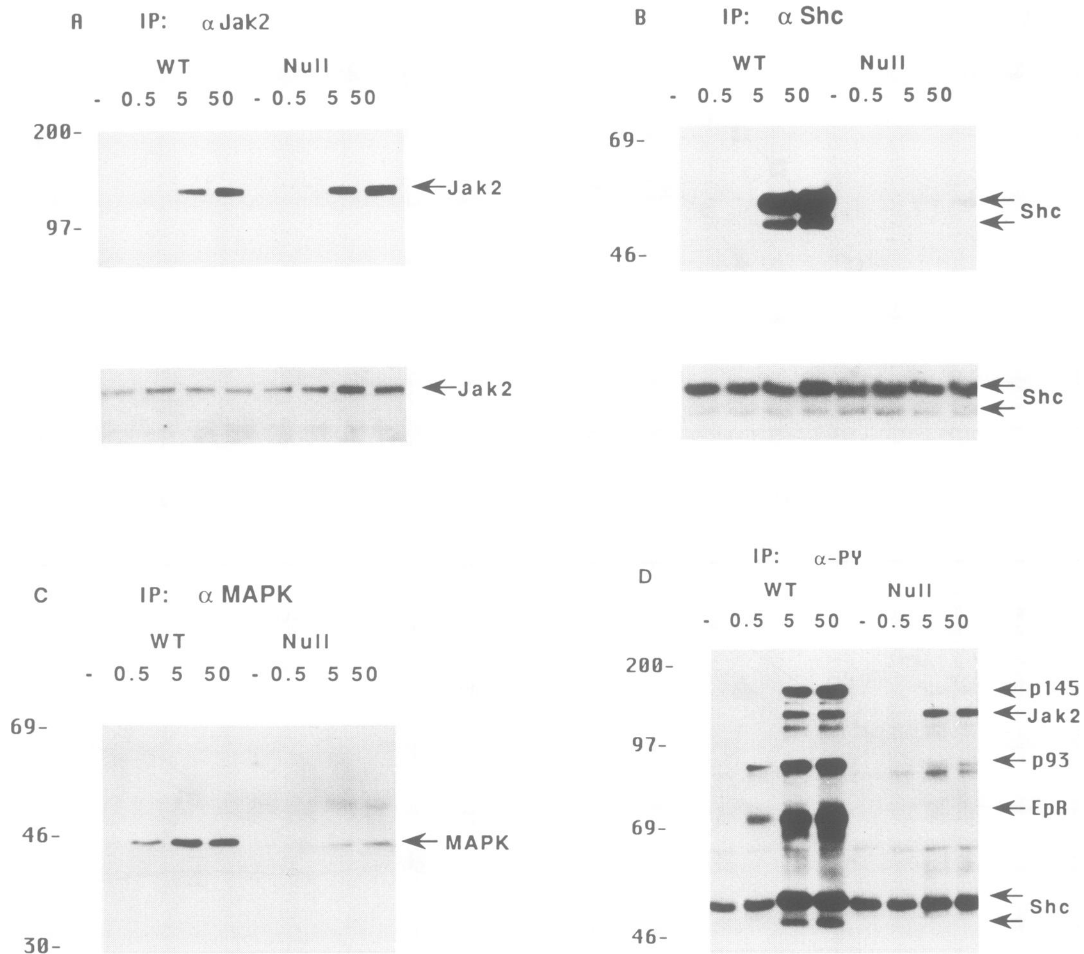

Fig. 3. Tyrosine phosphorylation of the EpR is not required for Jak2 tyrosine phosphorylation but facilitates Shc tyrosine phosphorylation and activation of the Ras pathway. (**A**) DA-3 cells expressing WT and Null EpRs were stimulated with the indicated concentrations of Ep (in U/ml) for 5 min at 37°C and cell lysates were subjected to immunoprecipitation with anti-Jak2 antibodies. Following Western analysis with anti-PY antibodies (upper panel), the blot was reprobed with anti-Jak2 antibodies to confirm equal loading (lower panel). (**B**) Other aliquots of the same cell lysates were immunoprecipitated with anti-Shc antibodies, and Western analysis was carried out with anti-PY antibodies (upper panel). The blot was reprobed with anti-Shc antibodies to confirm equal loading (lower panel). Aliquots from the same cell lysates were (**C**) boiled in SDS to denature the proteins (Miura *et al.*, 1994c) and then subjected to immunoprecipitation with anti-MAPK antibodies and Western analysis with anti-PY antibodies or (**D**) subjected to both immunoprecipitation and Western analysis with anti-PY antibodies.

activation of the Ras pathway (Carroll *et al.*, 1991), was greatly facilitated by EpR tyrosine phosphorylation. To investigate this further, the proteins in total cell lysates from WT and Null cells, incubated with different concentrations of Ep, were denatured with SDS and subjected to immunoprecipitation with anti-mitogen-activated protein kinase (MAPK) antibodies. Western analysis with anti-PY antibodies demonstrated that MAPK was strongly phosphorylated on tyrosine residues in response to Ep in WT cells, but was far less phosphorylated in Null cells, reflecting the relative Shc phosphorylation intensities seen with these two cell types (Figure 3C).

We next compared the levels of the other major tyrosine-phosphorylated proteins induced in WT and Null cells in response to increasing concentrations of Ep by subjecting lysates from these cells to immunoprecipitation and Western analysis with anti-PY antibodies. As can be seen in Figure 3D, of the seven major bands which typically appear in response to Ep (Miura *et al.*, 1991; Quelle and Wojchowski, 1991b; Damen *et al.*, 1992; Dusanter-Fourt *et al.*, 1992), corresponding to the Shc-associated p145 (Damen *et al.*, 1993a; Liu *et al.*, 1994), Jak2 (p130) (Miura *et al.*, 1994b), an as yet uncharacterized 93 kDa protein(s) (Quelle and Wojchowski, 1991b; Dusanter-Fourt *et al.*, 1992), the activated EpR (p72) (Miura *et al.*, 1991; Quelle and Wojchowski, 1991b; Damen *et al.*, 1992; Dusanter-Fourt *et al.*, 1992), p70 [which is most likely composed of HCP (Klingmuller *et al.*, 1995; Yi *et al.*, 1995) and Syp (Tauchi *et al.*, 1995)] and the p52 and p46 isoforms of Shc (Damen *et al.*, 1993a), only the 93 kDa and EpR bands were increased over background levels in WT cells at 0.5 U/ml of Ep. By 5 U/ml of Ep, all the bands typically seen in response to Ep were clearly evident in the WT cells. In contrast, only the 93 kDa band was increased above background levels in the Null cells at low Ep levels, albeit with far less intensity than that seen in WT cells. At 5 U/ml, the intensity of this band increased in Null cells and Jak2 appeared and was tyrosine-phosphorylated at the same level as that seen in WT cells (confirming the results shown in Figure 3A). In addition, there was no evidence of a tyrosine-phosphorylated EpR, as expected for a Null mutant. The correlation of the

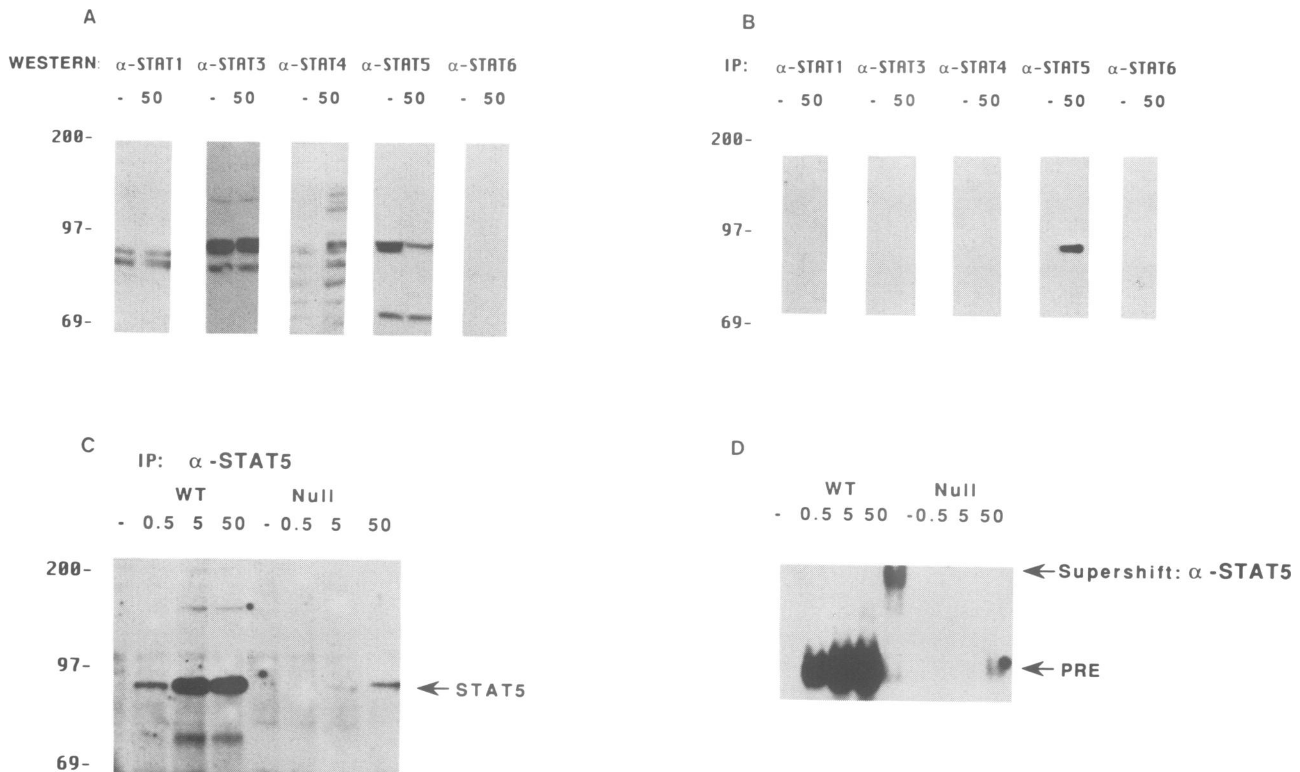

Fig. 4. Stat5 is tyrosine-phosphorylated and activated at lower Ep concentrations in WT cells than in Null cells. (**A**) Total cell lysates from WT EpR-expressing DA-3 cells, stimulated with 50 U/ml of Ep, were subjected to Western analysis with antibodies to the indicated Stat. (**B**) Cell lysates from WT EpR-expressing DA-3 cells, stimulated with 50 U/ml of Ep, were subjected to immunoprecipitation with antibodies to the Stats indicated, and Western analyses were carried out using anti-PY antibodies. (**C**) Lysates from WT and Null cells, stimulated with the indicated concentrations of Ep for 5 min at 37°C, were subjected to immunoprecipitation with anti-Stat5 antibodies and Western analysis with anti-PY antibodies. (**D**) Nuclear extracts from these same cells were subjected to electrophoretic mobility shift assays using ^{32}P-labeled PRE. Samples for the two unmarked lanes consist of nuclear extracts (from cells treated with 50 U/ml Ep) incubated with anti-Stat5 antibodies prior to electrophoresis.

intensity of the 93 kDa band with Ep-induced proliferation suggested that one or more of the proteins comprising it might play an important role in mitogenesis.

Ep-induced proliferation in WT and Null cells correlates with the tyrosine phosphorylation and activation of Stat5

Because of the potential importance of this protein, we sought to identify it using antibodies to candidate proteins. Since, from the recent literature, one obvious set of candidates was the Stat proteins, total cell lysates from WT cells, incubated in the presence and absence of 50 U/ml of Ep, were subjected to Western analysis with antibodies to Stats that had molecular masses ~93 kDa, i.e. Stat1, 3, 4, 5 and 6. These blots revealed that Stat1, 3 and 5 were clearly present in EpR-expressing DA-3 cells, while Stat4 and 6 were either not present or were below the detection limit of the assay (Figure 4A). Interestingly, a comparison of the anti-Stat5 lanes suggested that, after stimulation with Ep, there was a significant decrease in the level of Stat5, especially the lower molecular weight Stat5B isoform (Mui *et al.*, 1995), consistent with its translocation into the nucleus (i.e. the nuclei were removed in the preparation of our total cell lysates). Aliquots from these same cell lysates were also subjected to immunoprecipitation with these anti-Stat antibodies, and Western analysis with anti-PY antibodies revealed that only Stat5 was tyrosine phosphorylated in response to Ep (Figure 4B). A reprobing of this blot with the appropriate anti-Stat antibody demonstrated that Stat3, 4, 5 and 6 were immunoprecipitable (data not shown). This revealed that Stat 4 and 6 were indeed expressed in DA-3 cells but were not present at sufficient levels to be detected in unconcentrated cell lysates.

Since Stat5 appeared to be the only Stat that was tyrosine-phosphorylated in response to Ep in these cells, we then investigated whether its level of phosphorylation correlated with Ep-induced proliferation by subjecting anti-Stat5 immunoprecipitates from WT and Null cells, stimulated with different concentrations of Ep, to Western analysis with anti-PY antibodies. As can be seen in Figure 4C, Stat5 co-migrated with the 93 kDa band and showed a similar intensity pattern to that of the 93 kDa band seen in anti-PY immunoprecipitates (Figure 3D). To determine whether the tyrosine phosphorylation of Stat5 correlated with its activation, nuclear extracts from these same cells were subjected to electrophoretic mobility shift assays using ^{32}P-labeled prolactin response element (PRE). As can be seen in Figure 4D, Ep induced the appearance of a PRE binding protein at both low and high Ep concentrations in WT cells but only at high Ep concentrations in Null cells. Moreover, this binding protein was specifically supershifted by anti-Stat5 antibodies, confirming that Stat5 specifically associated with this oligonucleotide.

Stat5 tyrosine phosphorylation and activation is mediated by Y343 at low Ep concentrations

Having established that tyrosine phosphorylation of the EpR lowers the concentration of Ep required for inducing

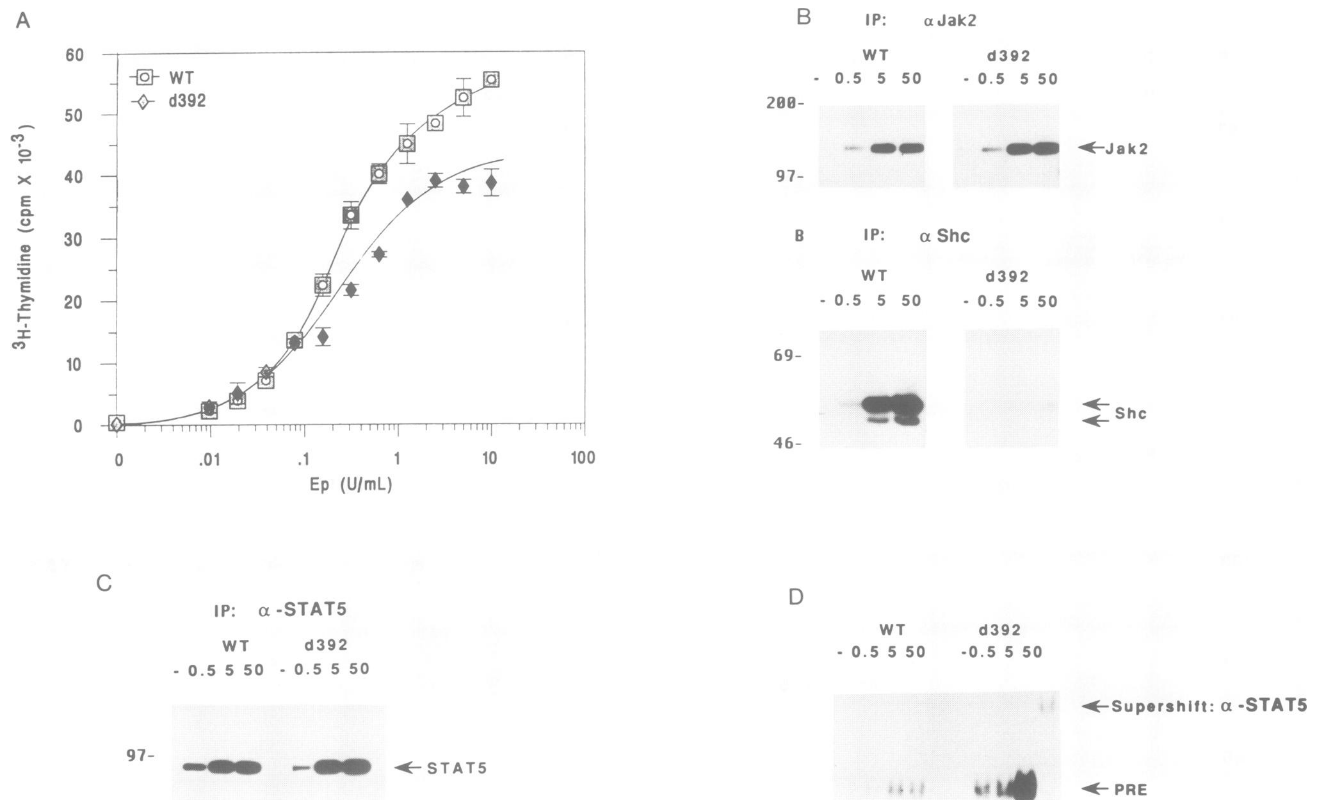

Fig. 5. The d392 EpR stimulates the tyrosine phosphorylation and activation of Stat5 as well as the WT EpR. (**A**) WT- and d392-expressing DA-3 cells, expressing the same number of cell surface EpRs, were assayed in short-term [^{3}H]thymidine assays. Data points represent the mean ± SE of triplicate determinations. Similar results were obtained in three separate experiments. (**B**) Lysates from WT- and d392 EpR-expressing DA-3 cells, stimulated with the indicated concentrations of Ep for 5 min at 37°C, were subjected to immunoprecipitation with anti-Jak2 antibodies and Western analysis with anti-PY antibodies. Reprobing with anti-Jak2 antibodies confirmed equal loading (data not shown). In the lower panel, other aliquots of the same cell lysates were immunoprecipitated with anti-Shc antibodies, and Western analysis was carried out with anti-PY antibodies. Reprobing with anti-Shc antibodies confirmed equal loading (data not shown). (**C**) Aliquots of the same lysates were subjected to immunoprecipitation with anti-Stat5 antibodies, and Western analysis was carried out with anti-PY antibodies. (**D**) Nuclear extracts from these same cells were subjected to electrophoretic mobility shift assays using ^{32}P-labeled PRE. Samples for the two unmarked lanes consist of nuclear extracts (from cells treated with 50 U/ml Ep) incubated with anti-Stat5 antibodies prior to electrophoresis.

both proliferation and the tyrosine phosphorylation of Stat5 and Shc, we set out to determine which tyrosines within the EpR might be capable of mediating these events. In terms of Ep-induced proliferation, one obvious candidate was the membrane-proximal Y343, since cells expressing truncated EpRs possessing only this tyrosine have been reported to proliferate as well as (Quelle and Wojchowski, 1991a; Miura *et al.*, 1994c) or better than (D'Andrea *et al.*, 1991) WT EpR-expressing cells. We therefore tested the Ep sensitivity of DA-3 cells expressing an EpR deletion mutant (d392), which lacked all the intracellular tyrosines except for Y343, in short-term proliferation assays. These assays revealed that d392 cells incorporated [^{3}H]thymidine as well as did WT cells at low Ep concentrations but slightly less well at higher Ep levels (Figure 5A). Long-term proliferation assays corroborated these findings (data not shown). An anti-PY immunoblot of anti-Jak 2 and anti-Shc immunoprecipitates from WT and d392 cells, stimulated with increasing concentrations of Ep, demonstrated that Jak2 was tyrosine-phosphorylated to the same degree in both cell types (Figure 5B) while Shc was poorly tyrosine-phosphorylated in d392 cells (Figure 5B, lower panel), reminiscent of the results we observed with Null cells. An anti-Stat5 immunoprecipitation and anti-PY immunoblot of these same samples demonstrated that Stat5 was phosphorylated to the same degree in WT and d392 cells (Figure 5C). Similarly, anti-PY immunoblots of anti-PY immunoprecipitates showed that the tyrosine phosphorylation levels of the 93 kDa band were the same in WT and d392 (data not shown), suggesting perhaps that this band was composed, to a large extent, of Stat5. Mobility shift assays with nuclear extracts from these cells confirmed that Stat5 was activated consistently in d392 either to the same degree or to a slightly higher degree than in WT-expressing DA-3 cells in response to Ep (Figure 5D). Thus, of the eight tyrosines within the cytoplasmic domain of the EpR, the C-terminal seven tyrosines appear to facilitate maximal Shc phosphorylation and to be required for maximal proliferation of DA-3 cells (J.E.Damen and G.Krystal, in preparation). The remaining tyrosine, i.e. Y343, on the other hand, appears to be both capable and sufficient to stimulate Stat5 and the proliferative response to low levels of Ep.

Stat5 activation can be mediated by other phosphorylated tyrosines within the EpR

Having established with the Null mutant that tyrosine phosphorylation of the EpR is required for Stat5 activation at physiological levels of Ep and that a deletion mutant

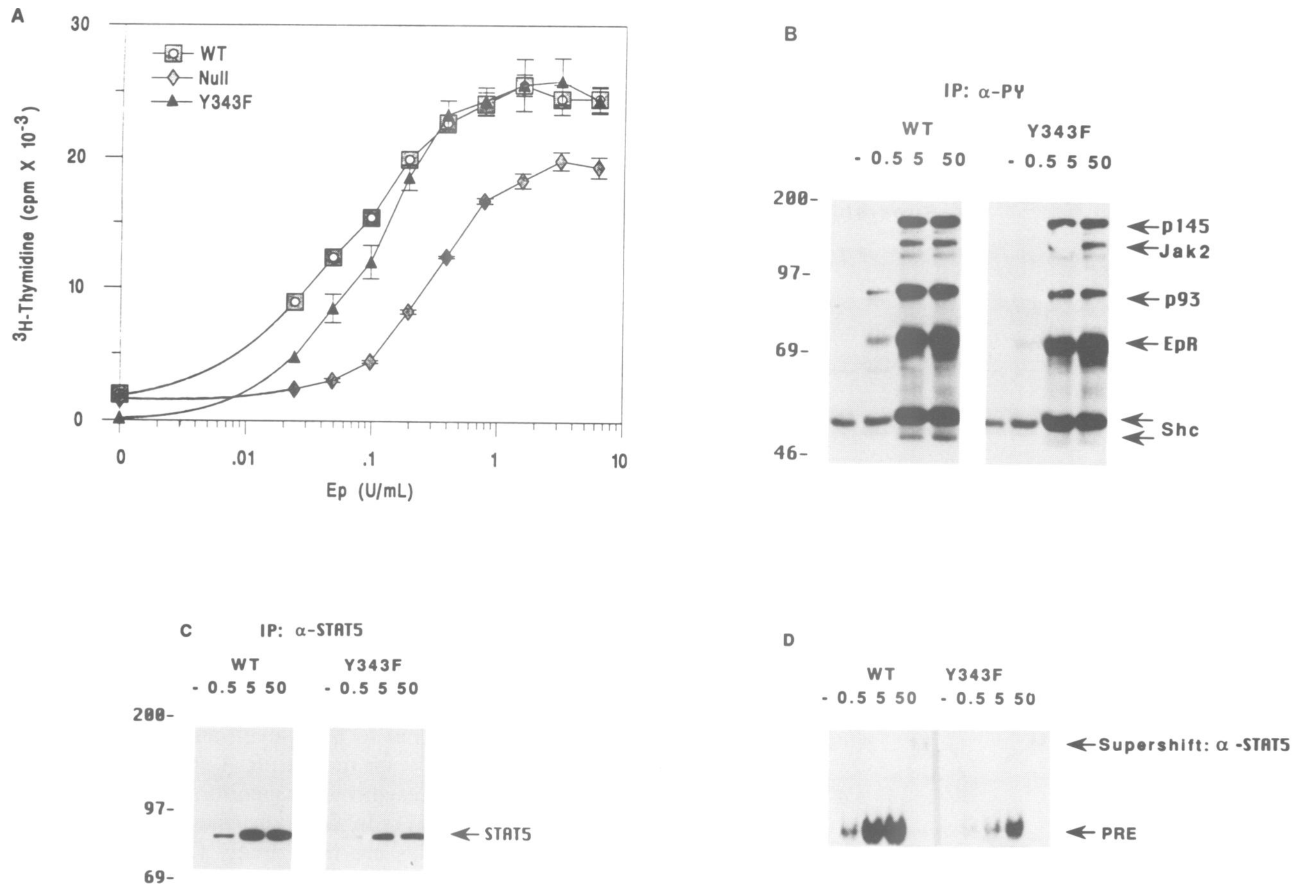

Fig. 6. Stat5 can be activated by other tyrosines within the activated EpR at intermediate Ep concentrations. (**A**) WT-, Y343F- and Null EpR-expressing DA-3 cells, displaying the same number of cell surface EpRs, were assayed in [^{3}H]thymidine incorporation assays. Data points represent the mean ± SE of triplicate determinations. Similar results were obtained in three separate experiments. (**B**) Lysates from WT- and Y343F EpR-expressing DA-3 cells, stimulated with the indicated concentrations of Ep for 5 min at 37°C, were subjected to both immunoprecipitation and Western analysis with anti-PY antibodies. (**C**) Other aliquots of the same cell lysates were subjected to immunoprecipitation with anti-Stat5 antibodies and Western analysis with anti-PY antibodies. (**D**) Nuclear extracts from these same cells were subjected to electrophoretic mobility shift assays using ^{32}P-labeled PRE. Samples for the two unmarked lanes consist of nuclear extracts (from cells treated with 50 U/ml Ep) incubated with anti-Stat5 antibodies prior to electrophoresis.

retaining only Y343 is capable of mediating this event, we next investigated whether other phosphorylated tyrosines within the EpR were capable of activating Stat5 in the absence of Y343 by comparing a DA-3 cell clone expressing a Y343F full length EpR with WT and Null cells in short-term proliferation assays. As can be seen in Figure 6A, when clones displaying identical cell surface EpRs were tested, the Y343F-containing cells incorporated less [^{3}H]thymidine at low Ep concentrations but the same level as WT cells at high Ep concentrations. Null cells, shown for comparison, incorporated less than Y343F cells at low Ep concentrations and never reached the level of incorporation seen with WT cells. Long-term proliferation assays confirmed these findings (data not shown). A comparison of the tyrosine phosphorylation patterns stimulated by different concentrations of Ep in WT and Y343F cells revealed a striking similarity, except for the 93 kDa band which was noticeably reduced in Y343F cells (Figure 6B). A reprobing of this anti-PY immunoblot with anti-Shc antibodies indicated that Shc was tyrosine-phosphorylated in response to Ep to the same degree in both cell types, as expected (data not shown), and this may account, at least in part, for the ability of Y343F-expressing DA-3 cells to proliferate as well as WT cells at high Ep concentrations. The reduction of the 93 kDa band seen in Figure 6B was mirrored in anti-PY Western blots of anti-Stat5 immunoprecipitates from these same cell samples (Figure 6C), i.e. tyrosine-phosphorylated Stat5 was not observed at 0.5 U/ml of Ep and was slightly less intense in Y343F cells than in WT cells at high Ep concentrations. Mobility shift assays of nuclear extracts from these same cells revealed that Stat5 activation (Figure 6D) mirrored its tyrosine phosphorylation pattern. This suggested that Y343 was the major site responsible for Stat5 activation at low Ep concentrations but that one or more of the remaining seven tyrosines within the cytoplasmic domain of the EpR could substitute for Y343 in activating Stat5 at intermediate Ep concentrations.

Lastly, we sought to determine the mechanism by which Stat5 was being activated at high Ep concentrations in the Null mutant. We hypothesized that this was mediated either through low affinity binding directly to Jak2 or to a non-tyrosine-phosphorylated region of the EpR. To explore this, two further EpR deletion mutants, d352 and d342, were tested in mobility shift assays. Comparing d352 with WT EpRs, we found, as we did with the longer d392, that Y343 was sufficient for Stat5 activation at low Ep concentrations and, in addition, that the 40 amino

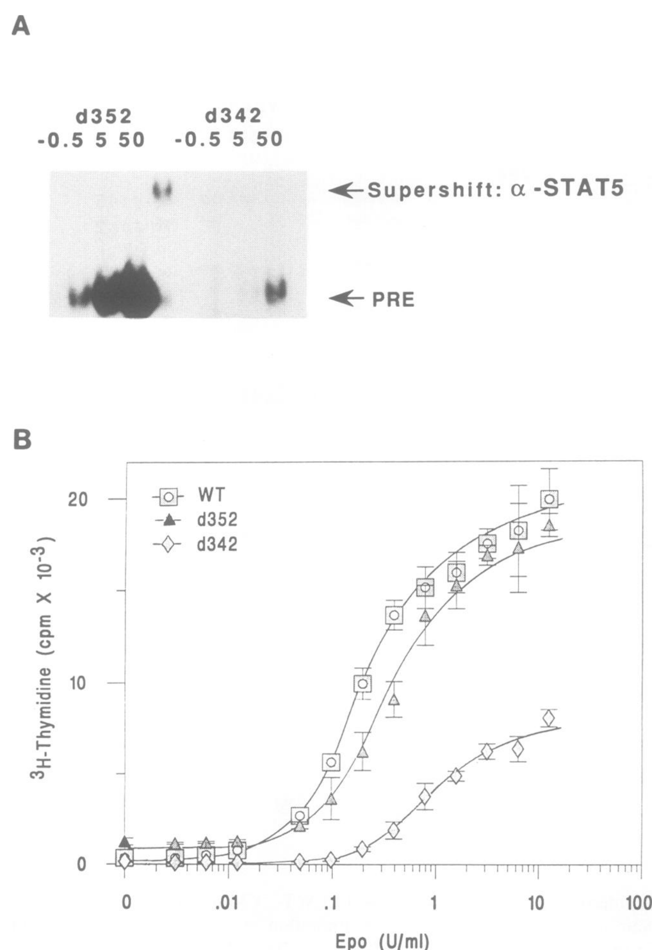

Fig. 7. d352 EpR-expressing DA-3 cells are similar to WT cells in Ep-induced Stat5 activation while d342 EpR-expressing DA-3 are similar to Null cells. (**A**) Nuclear extracts from WT-, d352- and d342 EpR-expressing DA-3 cells expressing the same cell surface receptor numbers were subjected to electrophoretic mobility shift assays using ^{32}P-labeled PRE. Samples for the unmarked lanes consist of nuclear extracts (from cells treated with 50 U/ml Ep) incubated with anti-Stat5 antibodies prior to electrophoresis. (**B**) The same cell clones were assayed in short-term [^{3}H]thymidine incorporation assays and data points represent the mean ± SE of triplicate determinations. Similar results were obtained in three separate experiments.

acids downstream of d352 were not required for this activation (Figure 7A). However, DA-3 cells expressing the EpR mutant, d342, which is only 10 amino acids shorter and lacks Y343, did not stimulate Stat5 activation in response to low levels of Ep, as expected, but did activate Stat5 at high Ep concentrations and did so to an extent similar to that seen with the Null mutant. This suggested that Stat5 activation at high Ep concentrations was mediated through direct binding to Jak2 or through binding to the unphosphorylated EpR at a region upstream of amino acid 342, i.e. within the membrane-proximal 95 amino acids.

Short-term [^{3}H]thymidine incorporation assays were also carried out with DA-3 cells expressing these two deletion mutants and revealed that the d352 cells grew similarly to d392 cells while the d342 cells grew even less well than Null cells (Figure 7B). This was confirmed in long-term growth assays and demonstrated that the loss of the 10 amino acids spanning Y343 dramatically reduced cell proliferation. This further substantiated a model of EpR signaling in which Y343 within the EpR mediates Stat5 activation and cell proliferation in response to physiological levels of Ep.

Discussion

In this study, we have demonstrated, using short-term proliferation assays, that Ep-induced tyrosine phosphorylation of the EpR facilitates entry into the first S phase following exposure to Ep. The cumulative effect of this difference in Ep responsiveness, as assessed by long-term growth assays, is that Null cells require ~50- 100-fold more Ep in order to proliferate as rapidly as WT cells. This most probably is an underestimate of the influence on proliferation of the positive regulators that normally bind to the activated EpR, since Klingmuller *et al.* (1995) elegantly demonstrated recently that 32D cells expressing mutant EpRs which lack Y429, and thus are incapable of binding the negative regulator, HCP, are 5- to 10-fold more sensitive to Ep in proliferation studies than WT EpR-expressing cells. In terms of the potential *in vivo* significance of the phosphorylation of the EpR on tyrosine residues, it should be borne in mind that Ep sensitivity is directly proportional to EpR level, and the cells used in our studies express far more EpRs (~1000–10 000 EpRs/cell) than native erythroid progenitors (100–1000 EpRs/cell; Krantz, 1991). In addition, the Ep concentrations used in our studies are most likely far higher than that within the normal marrow microenvironment, given that typical serum concentrations of Ep are in the range of 5–40 mU/ml (Wognum *et al.*, 1990a). Taken together, and given that we see no proliferation of Null cells below 50 mU/ml of Ep (see Figure 2A), it is likely that tyrosine phosphorylation of the EpR is essential for normal erythroid progenitor cell proliferation *in vivo*.

We have also demonstrated, by comparing various mutant EpR-expressing cells with WT cells at different Ep concentrations, that Stat5 tyrosine phosphorylation and activation correlate with Ep-induced proliferation. This transcription factor, which was identified originally in mammary gland cells of lactating animals and thus called mammary gland factor (MGF) (Schmitt-Ney *et al.*, 1991; Wakao *et al.*, 1994), has been shown recently to be activated not only in response to prolactin (Gouilleux *et al.*, 1994), but to Ep, IL-2 (Wakao *et al.*, 1995), IL-3, granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-5 (Mui *et al.*, 1995) and growth hormone (Gouilleux *et al.*, 1995) as well. Stat5 is one of seven Stats that have been cloned to date and shown to be involved in cytokine signaling (reviewed in Briscoe *et al.*, 1994; Darnell *et al.*, 1994; Taniguchi, 1995). Specifically, it is thought that ligand-induced cytokine receptor dimerization leads to intermolecular autophosphorylation and activation of its associated Jak. The activated Jak then phosphorylates the receptor on specific tyrosine residues and this, in turn, attracts specific Stats, via their SH2 domains, to the receptor (i.e. the specificity of the Jak–Stat pathway is conferred by tyrosine motifs within the cytokine receptors). The Stats are then phosphorylated on tyrosine residues, most likely by the Jaks, and then form homo- or heterodimers, in which the phosphotyrosine residue of each Stat interacts with the SH2 domain of its

partner (Heim *et al.*, 1995) and migrates into the nucleus to bind to promoter elements.

Experiments with the Null mutant established that tyrosine phosphorylation of the EpR facilitates both Ep-induced proliferation and Stat5 activation. Our results with the deletion mutant, d392, extended this observation by showing that an EpR retaining only Y343 is capable of transmitting, at low Ep levels, proliferation and Stat5 activation signals as efficiently as the WT EpR. This is consistent with a previous report from Wojchowski's laboratory showing that a region within the EpR between amino acids 329 and 372 (which is downstream of the Jak2 binding site) is critical for Ep-induced mitogenesis (He *et al.*, 1994). In addition, our results with the d392 EpR mutant strongly suggest that Y343 is tyrosine-phosphorylated to some extent, even though this is not detected using currently available anti-PY antibodies. With regard to the proliferation of d392-expressing cells, we have found, as others have (D'Andrea *et al.*, 1991), that Ba/F3 cells expressing this type of truncated EpR, which does not trigger the tyrosine phosphorylation of Shc or activate the Ras pathway (Miura *et al.*, 1994c), is 'hypersensitive' to Ep in proliferation assays when compared with its WT counterpart (data not shown). However, we did not observe this phenomenon with d392-expressing DA-3 cells (see Figure 5A). Interestingly, when these same 'hypersensitive' d392 EpR-expressing Ba/F3 cells were grown in synthetic serum without insulin, instead of in fetal calf serum (FCS), they proliferated far less well than their WT counterparts and this appears to be attributable to factors in FCS that stimulate the Ras pathway (J.E.Damen and G.Krystal, in preparation). Moreover, we have evidence that the absence of the 'hypersensitive' phenotype in d392-expressing DA-3 cells may be due to a lower basal Ras activity (J.E.Damen and G.Krystal, in preparation). Since cells expressing these truncated EpRs might be expected to be hypersensitive to Ep, as they lack the Y429 binding site for the negative regulator, HCP (Klingmuller *et al.*, 1995), the fact that they are not (when grown in synthetic serum without insulin) makes it likely that the Ras pathway contributes substantially to Ep-induced proliferation. Support for this notion comes from work carried out by Sakamaki and Yonehara (1994) who showed that FCS could compensate for the lack of Ras activation by truncated GM-CSF receptors and that antisense to c-*raf* blocked DNA synthesis of Ba/F3 cells. Also, more recently, Kinoshita *et al.* (1995) demonstrated that Ba/F3 cells expressing C-terminally truncated GM-CSF receptors, which do not activate the Ras pathway, die via an apoptotic pathway under serum-free conditions but can be rescued by an activated form of Ras.

Results obtained with the Y343F mutant corroborate our findings with the Null and d392 mutants and suggest that while Y343 is most probably the primary site responsible for Stat5 activation, other tyrosines within the EpR could substitute in its absence to carry this out. It is of interest, in this regard, that the amino acids on the carboxy side of Y343 (i.e. Y343LVL) are very similar to those of Y431 (i.e. Y431LVV). It is conceivable, therefore, that in cells expressing a Y343F mutant EpR, Y455 may be the tyrosine responsible for stimulating Stat5 activation. Our results with the Y343F mutant also suggest that, apart from Stat5, Y343 is not involved in the Ep-induced phosphorylation of the major tyrosine-phosphorylated proteins (Figure 6B). Lastly, our results with the deletion mutants d352 and d342 confirm that the loss of Ep-induced Stat5 activation coincides closely with the loss in Ep-induced cell proliferation and also suggest that activation of Stat5 in Null cells at high Ep levels may be mediated directly through Jak2.

As this work was being completed, Ohashi *et al.* (1995) reported that Ep might be stimulating the activation of Stat1 and 3 in murine erythroleukemic HCD-57 cells. We therefore carried out mobility shift assays in which we compared the ability of the sis-inducible element (SIE), which is a binding site for Stat1, 2 and 3, with the PRE to bind to our nuclear extracts. Using these two oligonucleotides, ^{32}P-labeled to the same specific activity, we found far less binding to the SIE and the little binding that did occur could be supershifted with anti-Stat5. This suggested that the binding observed to the SIE by Ohashi *et al.* (1995) might be through Stat5 (which they did not test). This possibility is consistent with our not seeing Stat1 or 3 becoming tyrosine-phosphorylated in our cells in response to Ep and is also consistent with the absence of a Stat3 consensus sequence (i.e. YXXQ; Stahl *et al.*, 1995) within the cytoplasmic domain of the EpR. However, the apparent discrepancy in our results may also be due to our using different hemopoietic cell lines. Interestingly, with regard to Stat binding sites, we have carried out co-immunoprecipitation studies with anti-EpR and anti-Stat5 antibodies and binding studies with an immobilized glutathione *S*-transferase (GST) fusion protein containing the SH2 domain of Stat5, and our preliminary results suggest that Stat5 may not bind to the activated EpR. This is in contrast to Stat studies in other systems (Taniguchi, 1995), but consistent with Stat5 results obtained with IL-3Rs (Mui *et al.*, 1995). As a result, we must tentatively postulate the existence of an upstream intermediate that binds with high affinity to Y343 and, perhaps, with low affinity directly to Jak2. Further studies will be required to elucidate completely the Jak2–Stat5 pathway in Ep-induced cells.

In summary, we have demonstrated that the tyrosine phosphorylation of the EpR plays a critical role in Ep-induced proliferation and Stat5 activation. Our studies also suggest that very high Ep levels, coupled with high levels of EpR expression, can override this requirement, most probably via direct binding and phosphorylation by Jak2. Indeed, some investigators have concluded, based on results obtained with very high cytokine levels, that receptor tyrosine phosphorylation is not required for proliferation or Stat activation (Taniguchi, 1995; Wang *et al.*, 1995). In addition, our results suggest, in contrast to what has been reported for the IL-2R system (Taniguchi, 1995), that Stat activation may be involved in proliferation, at least in the EpR system.

Materials and methods

Cells and proliferation assays

The murine IL-3 dependent cell lines, DA-3 (generously provided by Dr J.Ihle, St Jude Children's Research Hospital, Memphis, TN) and Ba/F3 were retrovirally infected with a JZenTKneo vector, as described previously (Damen *et al.*, 1992), containing either the WT murine EpR cDNA or a site-directed mutant of the murine EpR, designated Null, in which all the eight cytoplasmic tyrosines were converted to phenyl-

alanines (see below). These cells were also retrovirally infected with another full length mutant of the murine EpR cDNA in which Y343 was replaced with a phenylalanine (Y343F) and with C-terminal truncated mutants lacking 91 (d392), 131 (d352) and 141 (d342) amino acids. Clones expressing similar levels of cell surface EpRs, based on FACS analysis with biotinylated Ep (Wognum *et al.*, 1990), were selected and grown in RPMI containing 10% FCS, 1.8 mg/ml G418 and 5 ng/ml COS cell-derived mouse IL-3.

For short-term proliferation assays, the various EpR-infected cells were grown to near confluence with IL-3, washed once with RPMI, resuspended in RPMI containing 10% FCS, aliquoted into 96-well U-bottom microtiter plates (Linbro, ICN, Mississauga, Ontario) to give a final volume of 100 µl/well and the plates were incubated for 15 h at 37°C in a humidified atmosphere of 5% CO_2, 95% air. Twenty µl of a 50 µCi/ml solution of [^{3}H]thymidine in RPMI were then added to each well to give a final concentration of 1 µCi/well. After another 2 h at 37°C, the contents of the well were harvested onto filtermats and counted using an LKB Betaplate harvester and liquid scintillation counter (LKB Wallac, Turku, Finland).

For long-term proliferation assays, the various EpR-infected DA-3 and Ba/F3 cells were washed with RPMI, starved in RPMI plus 0.1% bovine serum albumin (BSA) for 5 h at 37°C and then resuspended at 1×10^5 cells/ml in RPMI containing 10% FCS and aliquoted into Falcon 3047, 24-well flat bottom plates at a final volume of 1 ml/well. Viable cell counts were performed daily and the cells were diluted back to 1×10^5 cells/ml when they reached 5×10^5 cells/ml.

Generation of EpR mutants

The EpR mutants of the mEpR were constructed using site-directed mutagenesis. To construct the Null mutant, the initial template was a 1.7 kb *Kpn* fragment of the Y343F mEpR cDNA that had been cloned into M13mp19. Using this template, the mutations Y443F and Y479F were created by double mutagenesis. Using the Y343F/Y443F/Y479F mutant as a template, a triple mutagenesis was performed to add the mutations Y401F, Y429F, Y431F and Y464F, and, using the resulting template containing seven mutated tyrosines, the final mutation, Y460F, was inserted. To generate the deletion mutants, d392, d352 and d342, the initial template was a 1.7 kb *Kpn* fragment of the parental mEpR cDNA cloned into M13mp19. All of the mutagenesis reactions were performed using a modification of the method of Kunkel (1985). Briefly, uracil-containing single-stranded DNA template was generated by growth of the phage in *Escherichia coli* strain CJ236 [*dut, ung, thi, relA*; pCJ105(Cm^r)] and 0.3 pmol was annealed to 6 pmol of the mutagenic oligonucleotide prior to synthesis of the complementary strand with unmodified T7 DNA polymerase and ligation with T4 DNA ligase to form covalently closed circular DNA. For the deletion mutants, the mutagenic oligonucleotide consisted of a 30mer sequence, the 5′ 15 bp being homologous to the terminal sequences of the specific deletion mutant and the 3′ 15 bp being homologous to the stop codon and 3′ sequences of the mEpR cDNA. The conditions for the extension and ligation reactions were 23 mM Tris, pH 7.9, 5 mM $MgCl_2$, 35 mM NaCl, 1.5 mM dithiothreitol (DTT), 0.4 mM dATP, dCTP, dGTP and dTTP, 0.75 mM ATP, 1 unit T7 DNA polymerase (Pharmacia) and 2 units T4 DNA ligase (BRL) at 23°C for 4 h in a final volume of 13 µl. The mutagenesis mixture was then diluted with water to an appropriate level for transformation into *E.coli* strain MV1190 [(Δ*lac-pro* AB), *thi*, *sup*E(Δ*sr*1-*rec*A) 306:Tn10(tet^r)(F′:*tra*D36, *pro*AB, *lac*I^{q}Z ΔM15)]. Plaques were picked and phage minipreps prepared and screened for mutants by dot blot hybridization with ^{32}P-labeled mutagenic oligonucleotide. All mutations were verified by DNA sequencing, and the complete coding region of the cytoplasmic domains sequenced to verify that no adventitious mutations had occurred during the mutagenesis procedures.

Immunoprecipitations and Western blot analysis

DA-3 and Ba/F3 cells expressing the WT and mutant EpRs were starved in RPMI, 0.1% BSA for 6 h at 37°C and then incubated with or without various concentrations of Ep for 5 min at 37°C. The cells were washed once with phosphate-buffered saline (PBS), solubilized at 2×10^7 cells/ml with 0.5% NP-40 in 4°C phosphorylation solubilization buffer (PSB), i.e. 50 mM HEPES, pH 7.4, 100 mM NaF, 10 mM NaPPi, 2 mM Na_3VO_4, 4 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin and 2 µg/ml aprotinin and subjected to immunoprecipitation as described previously (Cutler *et al.*, 1993; Damen *et al.*, 1993a) with either anti-Jak2 (Upstate Biotechnology Inc, Lake Placid, NY), anti-Shc, anti-ISGF3 (anti-Stat1, cat. #G16930) (Transduction Laboratories, Lexington, KY), anti-Stat3 (cat. #sc-483), anti-Stat4 (cat. #sc-485) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Stat5 (generated in a rabbit immunized with the N-terminal 120 amino acids of sheep Stat5; Wakao *et al.*, 1994) or anti-phosphotyrosine (anti-PY) antibodies (4G10, UBI). For anti-Stat6 (anti-IL-4Stat, Santa Cruz, cat. # sc-621) and anti-MAPK (UBI, Cat. #06-182) immunoprecipitations, cell lysates in hypotonic lysis buffer (Damen *et al.*, 1992) were first boiled in 1% SDS and then diluted 10-fold with PSB containing 1% NP-40, similarly to as described by Miura *et al.* (1994).

Following SDS–PAGE using 7.5 or 10% polyacrylamide gels, proteins were electrophoretically transferred onto Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, MA) using 500 mA for 90 min at 23°C and 25 mM Tris, 192 mM glycine, 0.05% SDS, 20% methanol. Blots were blocked, incubated with anti-PY antibodies and then with horseradish peroxidase-conjugated second antibody (Jackson Immunoresearch, West Grove, PA) before adding ECL substrate solution (Amersham Corp, Arlington Heights, IL) and exposing to Kodak X-Omat film (Eastman Kodak, Rochester, NY) (Cutler *et al.*, 1993; Damen *et al.*, 1993a). In some experiments, blots were stripped with 62.5 mM Tris–Cl, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol at 50°C for 30 min, reblocked, washed and reprobed with either anti-Shc, anti-Jak2 or anti-Stat antibodies. The anti-EpR antibody, used in immunoblots to assess EpR levels in the various clones, was generated by immunizing rabbits with a GST fusion protein containing the C-terminal 18 amino acids of the EpR and was kindly provided by Drs Dwayne Barber and Alan D'Andrea (Dana Farber Cancer Institute, Boston, MA).

Electrophoretic mobility shift assays

Nuclear extracts were prepared as described previously (Mui *et al.*, 1995) and 8 µg added to a final volume of 20 µl containing 12 mM HEPES (pH 7.9), 10 fmol of a ^{32}P-labeled double-stranded oligonucleotide corresponding to the PRE in the bovine β-casein promoter (40 000 c.p.m.), 5% glycerol, 75 mM NaCl, 0.1% NP-40, 1 mg/ml BSA, 1 mM EDTA and 1 µg of poly(dI,dC). The mixture was incubated at 23°C for 30 min and 5 µl were loaded onto 5% polyacrylamide gels in 0.25× TBE as described previously (Mui *et al.*, 1995; Wakao *et al.*, 1995). For supershift experiments, 10 µg of anti-Stat5 antiserum, raised in chickens against a GST fusion protein containing a region of ovine Stat5 comprising amino acids 6–129 (Wakao *et al.*, 1995), were added to the binding reaction.

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