Association of JAK2 and STAT5 with Erythropoietin Receptors

ROLE OF RECEPTOR PHOSPHORYLATION IN ERYTHROPOIETIN SIGNAL TRANSDUCTION*

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Cytokine receptors act at least partially by associating with Janus tyrosine protein kinases at the conserved box one motif of the receptor. These receptor-associated kinases then activate STAT transcription factors through phosphorylation. We found that the 78-kDa erythropoietin receptor (EPO), a highly modified form of the 62-kDa receptor which is abundant in HCD57 cells, was phosphorylated on serine residues without EPO stimulation. Coprecipitation experiments showed the 78-kDa EPO not but the more abundant 62-kDa EPO was associated with JAK2, a Janus kinase, in both the presence and absence of EPO. Solubilized 78-kDa EPO bound to purified, genetically engineered JAK2 better than the 62–76-kDa receptor proteins, and additional phosphorylation of tyrosine residues further increased the binding of the 78-kDa EPO to JAK2-agarose beads. STAT5 DNA binding was activated by 10–100-fold lower concentrations of EPO in HCD57 cells than in primary erythroid cells, and STAT5 associated with the EPO in an EPO-dependent manner. These data suggest that phosphorylation of either serine or tyrosine residues of the EPO can enhance the association of the receptor with JAK2, possibly increasing the sensitivity to EPO.

Erythropoietin is the glycoprotein hormone responsible for the primary regulation of the number of red blood cells that mature from progenitor cells and acts through receptors present on these immature erythroid cells to promote survival, proliferation, and differentiation (1–3). EPO1 belongs to the cytokine receptor superfamily that includes receptors for factor VIII, epidermal growth factor, and prolactin (4, 5). Not only do these receptors interact with members of the Janus family of tyrosine protein kinases that include JAK1, JAK2, JAK3, and TYK2 (6–8).

JAK2 kinase interacted in vitro with bacterial fusion proteins expressing cytoplasmic domains of the EPO, and JAK2 was tyrosine-phosphorylated in EPO-treated cells (9). The conserved box one motif of the cytokine receptors (membrane proximal cytoplasmic domain) is required for the association with the Janus tyrosine protein kinases (10–12); however, the nature of this binding and modifications of either receptor or kinase that affect this interaction have not been reported. Miura and co-workers showed that JAK2 interacted with the EPO only after EPO binding (7); however, Wakao and co-workers (13) showed JAK2 binding to the EPO in both the presence and absence of EPO. We have investigated possible explanations for these disparate findings, and report here that the constitutive phosphorylation of the EPO on serine residues appeared to enhance the association of JAK2 with the EPO in erythroid cells. In addition, EPO-induced tyrosine phosphorylation of the EPO appeared to additionally increase the affinity for JAK2. We report more extensive processing of the EPO in erythroid cells compared to other cells (26–29).

Recent information suggests that the STAT proteins are substrates of the Janus kinases bound to the cytokine receptors (14–16). We previously demonstrated that both STAT1 and STAT5 were tyrosine-phosphorylated, translocated to the nucleus, and activated to bind DNA in primary murine erythroid cells activated by EPO (17). Others have also shown that STAT5 was activated in response to EPO and cytokines related to EPO: prolactin, interleukin-2, interleukin-3, interleukin-5, and granulocyte-macrophage colony stimulating factor, granulocyte-macrophage colony stimulating factor, and factors not related such as epidermal growth factor (13, 18–24). In this study we demonstrate that in HCD57 erythroleukemia cells, STAT5 is activated to bind DNA at EPO concentrations 100-fold less than previously reported for primary erythroid cells and that STAT5 binds to the activated EPO.

MATERIALS AND METHODS

Cells—HCD57 cells, obtained from Sandra Ruscetti at the National Cancer Institute in Frederick, MD (25, 30), were cultured in the presence of 1.0 unit of EPO/ml in Iscove’s modified Dulbecco’s medium and 25% fetal calf serum for several days. In most experiments, the cells were further cultured overnight in the same medium devoid of EPO. As previously reported (25), this deprivation of EPO increased cell surface EPO.

Western Blot Analysis of Phosphotyrosine and EPO—The 62–78-kDa forms of the EPO were visualized after immunoprecipitation and Western blotting with anti-receptor, affinity-purified IgG raised against a synthetic peptide corresponding to the 15-terminal amino acids of the receptor (25). The EPO was detected on x-ray film by a light generating reaction catalyzed by peroxidase linked to a secondary antibody (ECL from Amersham). Tyr(P)-containing proteins were detected by the immunoprecipitation and Western blotting method described before (25) except monoclonal antibodies against Tyr(P) from either Transduction Laboratories (RC20H) or UBI (4G10) were also used. Polyclonal anti-Tyr(P) antibodies from Zymed were used to immunoprecipitate and concentrate the Tyr(P)-containing proteins before they were analyzed by Western blot. In many experiments, the blots were stripped of...
bound antibody and reprobed one or two more times with other antisera. The blot was stripped as described by the Amersham literature with the ECL kit. Briefly, the bound antibody was released in the presence of 2% SDS and reducing agents at 50 °C for 30 min.

Enzymatic Deglycosylation and Dephosphorylation of EPOR and Phosphotyrosine-containing Proteins—N-Linked carbohydrate from the immunoprecipitated EPOR was digested away with either 50 or 500 units of N-glycanase/ml for 20 h as described previously (25). Phosphate on all residues of the EPOR and Tyr(P)-containing proteins was eliminated by digestion of immunoprecipitates with 50 units of alkaline phosphatase/ml for 6 h at 37 °C in a pH 9.0 buffer as described previously (25, 26). In the experiment where deglycosylation and dephosphorylation were both done, alkaline phosphatase treatment of the immunoprecipitated EPOR was followed by dialysis, concentration in a Speedvac, and then N-glycanase treatment. Undigested controls were treated similar without enzymes.

In Vivo Labeling of the EPOR with 32Pi and Phosphoamino Acid Analysis—HCD57 cells were labeled with 10 mCi of 32Pi for 1 h in phosphate-free medium and dialyzed serum. The EPOR was immunoprecipitated and analyzed by SDS-PAGE, autoradiography, and Western blotting using anti-EPOR IgG. Afterwards, the radioactive bands comigrating with the 78-kDa EPOR were cut out of the nitrocellulose blot and placed directly in 6 N HCl and heated for 1.5 h at 100 °C under nitrogen. Products of this acid hydrolysis were analyzed on two-dimensional high voltage electrophoresis, first at pH 1.9, and then at pH 3.5, on thin layer plates as described by others (31). Phosphoamino acids were visualized by autoradiography and compared to standards visualized by staining with ninhydrin.

Copro precipitation of EPOR, JAK2, and STAT5—These studies were carried out in detergent extracts of HCD57 cells made in 1% digitonin lysis solution as described in the report by Miura and co-workers (7). Briefly, HCD57 cultured without EPO were pretreated with 0.5 mM Na3VO4, and either not treated or treated with 10 units of EPO for 10 min at 37 °C in the culture medium containing 25% fetal calf serum. The cells were diluted with an excess volume of ice-cold culture medium and washed one time. The pellets were suspended in the ice-cold digitonin lysis buffer for 5 min and insoluble material was removed by ultracentrifugation at 250,000 × g for 30 min. The supernatant was divided into equal aliquots and incubated with anti-EPOR IgG, anti-JAK2 antisera (UBI, Lake Placid, NY), or anti-Tyr(P) antibody. Following SDS-PAGE and Western blotting the blot was probed with either anti-Tyr(P) monoclonal antibody, anti-JAK2 antisera, anti-STAT5 monoclonal antibody (Transduction Laboratories), or the anti-EPOR IgG. For a control, nuclear proteins were extracted from parallel cells, by the method described previously (17, 24), and were also run on the same SDS-PAGE gel and blot and were probed with anti-STAT5 antiserum to indicate the position of activated STAT5 that was translocated to the nucleus in EPO-treated cells. These same techniques were used to measure the effect of EPO concentration on STAT5 tyrosine phosphorylation and nuclear translocation. STAT5 binding to radiolabeled DNA (prolactin inducible element) was done as described previously (17).

In Vitro Binding of EPOR to JAK2 Beads—Purified JAK2 protein expressed in the S9 cells-baculovirus system that was conjugated to agarose beads was obtained commercially from UBI. HCD57 cells that were deprived of EPO overnight were pretreated with 0.5 mM Na3VO4, for 50 min, and the cells were either treated with nothing or 10 units of EPO/ml for 10 min at 37 °C. Following this incubation, the cells were then lysed at 4 °C in a 1% Triton X-100 solution used previously as the binding buffer to demonstrate the interaction of JAK2 with fusion proteins containing domains of the EPOR (9). The unsolvulubalized material was removed by centrifuging at 25,000 × g for 60 min and the extracts were incubated with 33 μl of the JAK2 beads for 4 h at 4 °C. The beads were then washed 5 times with ice-cold binding solution to remove any soluble EPOR trapped in the pellet. The JAK2 resin and associated EPOR proteins were then exposed to SDS-PAGE sample buffer at 100 °C to release bound protein, and the forms of EPOR bound were analyzed by SDS-PAGE and Western blotting using anti-EPOR IgG.

RESULTS

Two Forms of EPOR, 72 and 78 kDa, Were Phosphorylated on Tyrosine Residues after EPO Treatment—We previously demonstrated the 78-kDa form of the EPOR and an unidentified 95-kDa protein were phosphorylated on tyrosine residues following treatment of HCD57 erythroid cells with EPO (25). This experiment was repeated with monoclonal anti-Tyr(P) antibody instead of the polyclonal antiserum. Fig. 1 shows an experiment comparing the effects of increasing EPO concentration on Tyr(P) proteins in HCD57 cells determined with a monoclonal anti-Tyr(P) (Fig. 1, upper panel) and the polyclonal antiserum used previously (25) (lower panel). The polyclonal anti-Tyr(P) was effective in recognizing a phosphorylated 95-kDa protein and weakly detecting the 78-kDa EPOR, but the monoclonal antiserum strongly recognized the EPOR and number of proteins ranging from 47 to 200 kDa. We have determined that the predominant band at 78 kDa in EPO-treated cells is the phosphorylated EPOR as previously reported (Fig. 2). This was shown by the appearance of the 78-kDa band in the anti-EPOR immunoprecipitate (Fig. 2, lane D) and the corresponding anti-Tyr(P) immunoprecipitate when reprobed with anti-EPOR IgG (lane G). However, prolonged exposure to film revealed another Tyr(P)-containing band of 72 kDa in the anti-EPOR immunoprecipitate. Reprobing this blot with anti-EPOR IgG revealed that the 72-kDa band, immunoprecipitated by anti-Tyr(P), was also a form of the EPOR. This 72-kDa band reacted with both anti-COOH and anti-NH2 anti-EPOR IgG, indicating that it was not a truncated form of receptor. Therefore, both a major 78-kDa EPOR and less abundant 72-kDa EPOR were phosphorylated in a EPO-dependent manner.

The Largest Form of EPOR, 78 kDa, Was Glycosylated and Phosphorylated on Serine Residues in the Absence of EPO—Previous studies showed that the Tyr(P)-containing 72-kDa form of the EPOR resulted from the shift of a 66-kDa EPO to 72 kDa because of phosphate interference in SDS-PAGE (7,
26–28). However, the 78-kDa EPOR was present before EPO treatment. Fig. 3 shows that the Tyr(P)-containing 78-kDa EPOR was shifted to 72 kDa by enzymatic dephosphorylation. Control experiments in which the alkaline phosphatase activity was inhibited with vanadate (not shown) verified that the 6-kDa shift of the EPOR in apparent molecular mass was due to phosphate on the protein and was not the result of contaminating proteinase activity.

Because the 78-kDa EPOR was also present in cells not treated with EPO, the possibility that the 78-kDa EPOR was phosphorylated before EPO-induced tyrosine phosphorylation was tested. Fig. 4, lane 7, shows that sequential deglycosylation and dephosphorylation completely converted all the forms of EPOR (62–78 kDa) found in HCD57 cells in the absence of EPO into the 62-kDa unprocessed peptide (lane 7). This indicates that both N-linked glycosylation and phosphorylation is contributed to the larger molecular weight forms of the receptor because deglycosylation alone generated a subset of receptors with a molecular mass of 68 kDa (Fig. 4, lanes 4 and 6) and dephosphorylation alone shifted the total receptors from 78 to 72 kDa. These data are consistent with the 78-kDa EPOR shifted in migration on SDS-PAGE both due to extensive glycosylation (10-kDa shift) and phosphorylation in the absence of EPO (6-kDa shift) such that EPO-induced phosphorylation on tyrosine residues did not additionally retard the migration of the protein on SDS-PAGE.

As a control in the experiment shown in Fig. 4, HCD57 cells were exposed to EPO for 24 h or longer such that the 70–78-kDa EPOR were down-regulated through destruction of the cell surface receptors (Fig. 4, lane 1). The remaining 64–69-kDa receptors in these cells were completely converted to the 62-kDa unprocessed protein by N-glycanase, which demonstrated that the digestion with N-glycanase was complete (lanes 1 and 3). As a further control, 10-fold more N-glycanase did not diminish the 68-kDa material that remained after enzymatic deglycosylation (compare lane 6 with lanes 3 and 4). The combined digestion with alkaline phosphatase and N-glycanase did not generate fragments of EPOR that would be expected if a contaminating proteinase had cleaved 6 kDa from the receptor in Fig. 3, indicating the absence of proteolytic artifacts.

To examine the nature of non-tyrosine phosphorylation on the EPOR in the unstimulated state more directly, the EPOR was immunoprecipitated from 32P-labeled HCD57 cells untreated or treated with EPO. As illustrated in Fig. 5, radioactive bands comigrating with the 78-kDa EPOR were seen in cells treated with both EPO and untreated cells (lanes A and B). The radioactivity was increased after EPO treatment by 2–3-fold compared to untreated cells, but the band was not shifted on SDS-PAGE. A control Western blot with anti-Tyr(P) antiserum showed that no Tyr(P) was detected in the 78-kDa EPOR as a result of EPO treatment was reduced to an apparent molecular mass of 72 kDa after enzymatic dephosphorylation. Tyr(P)-containing proteins were recovered by immunoprecipitation with either untreated HCD57 cells (A and D) or cells treated with EPO for 10 min at 37°C (B, C, E, and F). The immunoprecipitated proteins were then incubated in either buffer only (A, B, D, and E) or buffer and alkaline phosphatase for 6 h at 37°C as described under “Materials and Methods” (lanes C and F). Following SDS-PAGE and Western blotting, the blot was first probed with anti-Tyr(P) antibody to visualize the Tyr(P)-containing proteins and then the blot was stripped of bound antibody and reprobed with anti-EPOR IgG to visualize the tyrosine-phosphorylated EPOR (lane E) and the dephosphorylated form of this subset of EPOR (lane F).
After washing at 4°C to remove unincorporated $^{32}$P, the cellswere
10 units of EPO/ml (absence and presence of EPO (Fig. 7)). JAK2 was associated with the EPOR in both the
appearance of $^{32}$P in Tyr(P) in the band at 78 kDa. EPO
treatment also increased the incorporation of $^{32}$P into
phosphoserine residues of the EPOR in this experiment. These
studies indicate that the 78-kDa EPOR was shifted in molecular
mass by the constitutive serine phosphorylation as well as
N-linked carbohydrate, such that additional phosphorylation
on tyrosine residues do not retard the protein on SDS-PAGE.

**JAK2 Associated with EPOR in the Presence and Absence of
EPO, and STAT5 Associated with the EPOR Only in EPO-
treated Cells—**Proteins coprecipitating with the EPOR were
examined in HCD57 cells lysed in 1% digitonin, as shown in
Fig. 7. Proteins coprecipitating with JAK2 were also analyzed.
The anti-Tyr(P) blot (lanes A-D) showed that the tyrosine-
phosphorylated 78-kDa EPOR was immunoprecipitated from
EPO-treated cells by anti-EPOR IgG and JAK2 (135 kDa) was
coprecipitated with EPOR (lane B). Tyrosine-phosphorylated
JAK2 was immunoprecipitated from EPO-treated cells with
anti-JAK2 antiserum and the 78-kDa EPOR was coprecipitated
with JAK2 (lane D). In addition, a 95-kDa Tyr(P)-containing
band was present in both immunoprecipitates from EPO-
treated cells but was severalfold more abundant in the anti-
EPOR immunoprecipitate. This blot was stripped of bound
anti-Tyr(P) antibody and reprobed with anti-JAK2 antiserum
(lanes E-H). JAK2 was associated with the EPOR in both the
absence and presence of EPO (lanes E and F). Furthermore,
approximately half of the JAK2 protein in the cell appeared to
be associated with the EPOR. The blot was then stripped of
bound JAK2 antibody and reprobed with anti-STAT5 mono-
clonal antibody. The 95-kDa Tyr(P)-containing protein copre-
icipitated with the EPOR in EPO-treated cells appeared to be
STAT5 because a 95-kDa band was seen in anti-EPOR immu-
noprecipitates when probed with anti-STAT5, but only in immu-
noprecipitates from EPO-treated cells (lane J). A many fold
the JAK2 protein in the cell appeared to
be associated with the EPOR. The blot was then stripped of
bound JAK2 antibody and reprobed with anti-STAT5 mono-
clonal antibody. The 95-kDa Tyr(P)-containing protein copre-
icipitated with the EPOR in EPO-treated cells appeared to be
STAT5 because a 95-kDa band was seen in anti-EPOR immu-
noprecipitates when probed with anti-STAT5, but only in immu-
noprecipitates from EPO-treated cells (lane J). A many fold
weaker 95-kDa band was also seen in the anti-JAK2 immu-
noprecipitates from EPO-treated cells, but this is likely STAT5
bound to the coprecipitated EPOR in these immune complexes.
For comparison, nuclear extracts from parallel HCD57 cells not
treated or treated with EPO (Fig. 7, lanes M and N; respec-
tively) were run on the same gel and probed with anti-STAT5
antibody. STAT5 was translocated to the nucleus in an EPO-
dependent fashion as we have demonstrated previously in
other erythroid cells (17). This authentic STAT5 comigrated
with the 95-kDa Tyr(P)-containing band and STAT5 coprecipi-
tated with the EPOR. It was estimated that approximately 2%
of the STAT5 activated, as quantified by the amount translo-
cated to the nucleus in EPO-treated cells, was bound to the
EPOR in this experiment.

**JAK2 Selectively Coprecipitated the 78-kDa EPOR—**The
forms of EPOR that coprecipitated with JAK2 were examined.
The blot used in Fig. 7 did not give a clear result when stripped
and reprobed with anti-EPOR IgG. Therefore, a new blot was
prepared from the same cell extracts, and this blot was probed
with anti-EPOR IgG as shown in Fig. 8. In addition to immuno-
precipitates using anti-EPOR (Fig. 8, C and D) and anti-JAK2
antibodies (E and F), anti-Tyr(P) antibodies recovered Tyr(P)-
containing EPOR (A and B). All these immunoprecipitates
were Western blotted and probed for EPOR proteins. Confirm-
ing the data from Fig. 2, the 78-kDa form of the EPOR was
phosphorylated on the tyrosine residues in EPO-treated cells
(Fig. 8, lanes A and B), a minor 72-kDa form was selected with
longer film exposures, and there was no apparent shift in
molecular mass of the EPOR forms ranging from 62 to 78 kDa
with EPO treatment (lanes C and D). Interestingly, only the
78-kDa form of the EPOR was detected associated with JAK2
as shown in Fig. 8, lanes E and F. As expected from the data in
Fig. 7, the 78-kDa EPOR was coprecipitated with JAK2 in both
unreated and EPO-treated cells.

The 78-kDa EPO Selectively Associated with Genetically Engineered JAK2 Protein Compared to the 62-76-kDa Form s of EPO—To rule out the possibly that the unique association of JAK2 with the 78-kDa EPO resulted from circumstances other than the direct interaction of these proteins, solubilized HCD57 cellular extracts, 1% Triton X-100, were incubated with purified JAK2 conjugated to agarose beads. The forms of EPO in the detergent extract that associated with these JAK2 beads were analyzed by Western blotting with anti-JAK2 antiserum (lanes A, C, and E) and anti-EPOR IgG (lanes B, D, and F) control extracts (lanes A, C, and E) were immunoprecipitated with anti-Tyr(P) antisera (lanes A and B), anti-EPOR IgG (lanes C and D), and anti-JAK2 antisera (lanes E and F). After SDS-PAGE and Western blotting, the blot was probed with anti-EPOR IgG. Arrows indicate the 62-78-kDa range of EPO forms present in these cells.

The 78-kDa EPO was the predominant receptor form that bound to the JAK2 beads while the most abundant 66-kDa form of EPO was bound to a lesser extent. In contrast to the earlier coprecipitation experiments showing equal association of JAK2 with the EPO in the presence and absence of EPO, the binding of the 78-kDa EPO to the JAK2 beads was increased 10-fold in extracts from EPO-treated cells compared to untreated cells. This experiment was carried out in a stringent 1% Triton X-100 detergent solution rather than mild digitonin lysis buffer such that JAK2, STAT5, and probably other associated proteins would be dissociated from the EPO (shown by the experiment in Fig. 2). This reduced the possibility that JAK2 or other proteins binding the EPO facilitated or interfered with the binding of the receptor to the JAK2 beads. We greatly reduced the possibility that the increased affinity of the 78-kDa EPO from EPO-treated cells resulted from a conformational change of the EPO induced by EPO binding rather than EPO-induced tyrosine phosphorylation by determining that virtually all the EPO bound to EPO was released during this experiment. These results are consistent with the hypothesis that the post-translational modification of the EPO by either constitutive phosphorylation on serine residues or EPO-induced phosphorylation on tyrosine residues enhanced the interaction of the EPO with JAK2.

Time Course and EPO Concentration Dependence of EPOR, JAK2, and STAT5 Activation and Effect of the Phosphatase Inhibitor, Orthovanadate—The time course and concentration of EPO needed for phosphorylation for total cellular proteins, EPOR, JAK2, and STAT5 were determined. As shown in Figs. 1 and 10, the tyrosine phosphorylation of the 78-kDa EPO was detected with 0.01 unit of EPO/ml (less than the physiological concentration of 20-40 milliunits/ml of plasma) and was maximum at 1 unit of EPO/ml in a 10-min treatment, time of peak kinase activity. Fig. 10 also shows that the 135-kDa band that coprecipitates with the EPO, shown to be JAK2 in Fig. 7, is substantially tyrosine-phosphorylated at 100 milliunits of EPO/ml and near maximally phosphorylated at 1 unit of EPO/ml. The tyrosine phosphorylation of STAT5, nuclear translocation of STAT5, and activation of STAT5 DNA binding activity were more sensitive to low concentrations of EPO than the phosphorylation of EPO or JAK2, becoming maximal at 100 milliunits of EPO/ml. Indeed, direct comparison of the EPO dose-response in HCD57 cells compared to primary proerythroblasts from mice infected with the anemia strain of Friend virus, FVA cells, showed that the activation of STAT5-like DNA binding activity was 10-100-fold more sensitive to EPO in the HCD57 cells (shown in Fig. 11).
Our unpublished experiments\(^2\) found that less than 10% of the 78-kDa EPOR molecules were phosphorylated on tyrosine in EPO-treated HCD57 cells; however, when the cells are pre-treated with 0.5 mM orthovanadate, EPO induced phosphorylation on tyrosine residues of nearly all the 78-kDa EPOR molecules (Figs. 2 and 8). Fig. 12 shows that pretreatment with vanadate both increased the tyrosine phosphorylation of the 78-kDa EPOR and most other phosphorylated proteins by 10–20-fold and changed the kinetics of phosphorylation following EPO induction, but did not significantly change the population of Tyr(P)-containing proteins (compare Figs. 1 and 3 without vanadate to Figs. 2 and 12 with vanadate treatment). In the absence of vanadate, the Tyr(P) content of the 78-kDa EPOR in EPO-treated HCD57 cells was constant for 60 min as previously reported (25). However, in the presence of vanadate, the tyrosine-phosphorylated 78-kDa EPOR was diminished after 10 min of treatment with EPO in parallel with the EPO-dependent down-regulation of the cell surface EPOR previously reported (25). Since vanadate is an inhibitor of phosphatase activity, it is surprising that treatment of HCD57 cells with it accelerated the disappearance of Tyr(P). However, this decline in Tyr(P)-containing proteins likely reflects the EPO-dependent destruction of the signaling molecules and other means to deactivate signaling more than dephosphorylation by phosphatase. JAK2, STAT5, and other Tyr(P)-containing proteins also declined after 10 min.

**DISCUSSION**

This study extends our previous discovery and preliminary characterization of the 78-kDa form of EPOR in erythroid cells (25). The hypothesis that these serine-phosphorylated and highly glycosylated 78-kDa EPOR molecules transduced an intracellular signal better than the less modified EPOR molecules was tested. Supporting data are as follows. 1) the 78-kDa form of EPOR was selectively coprecipitated with JAK2. 2) The 78-kDa EPOR was phosphorylated on tyrosine residues in EPO-treated cells while only a minor 72-kDa form of EPOR containing 5% or less of the Tyr(P) of the 78-kDa EPOR was also observed. 3) Approximately half of the total cellular JAK2 was associated with this 78-kDa EPOR in both the absence and presence of EPO. 4) In vitro binding experiments showed that the soluble, serine-phosphorylated 78-kDa EPOR bound to genetically engineered JAK2 better than other forms of EPOR;
Although our data show that the 78-kDa EPOR was associated with JAK2 in the presence and absence of EPO. The results presented here also suggest that this interaction may depend on post-translational modification of the EPOR. The interaction of the STAT5 protein with the activated EPOR is likely STAT5 that is not yet phosphorylated and/or phosphorylated STAT that had not been approached by a dimerization partner.

The result of the experiment shown in Fig. 2 clarifies the controversial observation that a 78-kDa form of EPOR was phosphorylated on tyrosine residues in EPO-treated erythroid cells (25) while others observed only a 72-kDa form of phosphorylated EPOR in cells expressing the EPOR cDNA (7, 26–28). This observation of both 72- and 78-kDa forms of Tyr(P)-containing EPOR eliminates the possibility that the 72- and 78-kDa forms are the same EPOR assigned different molecular weights in different laboratories. This report also clarifies the unusual observation that the Tyr(P) content of EPOR molecules was constant over time while the majority of cell surface receptors were destroyed in EPO-treated cells (25). In the absence of vanadate (a tyrosine phosphatase inhibitor) only a minority of cell surface receptors were phosphorylated after EPO treatment; however, exposure to vanadate resulted in virtually every cell surface 78-kDa EPOR becoming phosphorylated on tyrosine residues. Thus, the tyrosine-phosphorylated EPOR molecules were then downregulated in the presence of EPO in parallel with EPO binding sites and total cell surface receptors. In the absence of vanadate, there appears to be a steady state equilibrium of tyrosine-phosphorylated EPOR molecules in EPO-treated cells that reflect the dephosphorylation by phosphatase, degradation of occupied EPO, continual phosphorylation by JAK2, and possibly other events. The physiological consequences of this is not clear. However, HCD57 cells were increased in sensitivity to EPO by vanadate treatment (40) which suggests that the level of receptor phosphorylation is directly related to signal transduction.

Although vanadate can further increase the sensitivity of HCD57 cells to EPO, these cells are remarkably sensitive to EPO in the absence of vanadate, being 10–100-fold more sensitive than most cells (40). This increased sensitivity is apparent in this study of the activation of STAT5 DNA binding activity in HCD57 cells by 10–100-fold less EPO compared to the activation of STAT5 in primary FVA erythroblasts (Fig. 11). Cells expressing EPOR cDNA may be manipulated to become hypersensitive to EPO through truncation of the COOH-terminal domain of the receptor (41) or with the disruption of the ability of a tyrosine phosphatase to interact with the
Activated cytokine receptors before becoming substrates for the JAK2 kinase suggests that there may be as yet unknown physiological control of EPO-mediated signal transduction. This report also shows that serine phosphorylation of the EPOR influences its association with JAK2. The 78-kDa EPOR had a higher affinity for the JAK2 proteinkinase another cellularformsoftheEPOR, and was post-translationally modifiedformofEPOR in signaltransduction.

In summary, these data demonstrate a major role of the most post-translationally modified form of EPOR in signal transduction. The 78-kDa EPOR had a higher affinity for the JAK2 protein kinase than other cellular forms of the EPOR, and was the preferred substrate for the JAK2 kinase that was bound to this receptor in the unactivated state. It is likely that the constitutive phosphorylation of this 78-kDa EPOR on serine residues enhances the ability of this receptor to interact with JAK2. The increased affinity of the tyrosine-phosphorylated 78-kDa EPOR for JAK2 may contribute to prolonged signal transduction following binding of EPO. The strong possibility that serine phosphorylation of the EPOR influences its association with JAK2 suggests that there may be as yet unknown physiological control of EPO-mediated signal transduction through serine protein kinases. This report also shows that STAT5 associates with the EPOR only after EPO binding. This supports the notion that STAT proteins must first bind to activated cytokine receptors before becoming substrates for the Janus protein kinase(s) associated with the receptor.

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