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Biochemistry

The functional form of the erythropoietin receptor is a 78-kDa protein: Correlation with cell surface expression, endocytosis, and phosphorylation

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ABSTRACT **An abundant 70- to 78-kDa form of the erythropoietin receptor (EPOR) was observed in HC-D57 murine erythroleukemia cells deprived of erythropoietin (EPO). In contrast to the 64- and 66-kDa EPOR proteins, these high molecular mass forms of EPOR (hmm-EPOR) correlated well with the number of binding sites and endocytosis of EPO. The hypothesis that hmm-EPOR are more highly glycosylated forms of the EPOR, appear on the cell surface, and represent at least one component of the biologically active EPOR was tested. Consistent findings were as follows. (*i*) Only hmm-EPOR increased following withdrawal of EPO from HC-D57 cells, correlating with a 10-fold increase in binding of ^{125}I-labeled EPO. In addition, the EPO-dependent downregulation of ^{125}I-EPO binding and disappearance of hmm-EPOR occurred in parallel while the amount of 66-kDa EPOR did not change. (*ii*) The 78-kDa EPOR was detected in COS cells expressing EPOR cDNA. (*iii*) Probing of the intact surface of these cells with anti-NH_2-terminal antibody recovered only the 78-kDa EPOR. (*iv*) Enzymatic deglycosylation and dephosphorylation showed that hmm-EPOR apparently resulted from additional N-linked glycosylation of a 62-kDa EPOR. (*v*) The hmm-EPOR turnover in HC-D57 cells was accelerated 12-fold in the presence of EPO (half-life changed from 3 hr to 15 min). (*vi*) Anti-phosphotyrosine antiserum detected an EPO-dependent phosphorylation of the 78-kDa EPOR. The kinetics of tyrosine phosphorylation of a 97-kDa protein correlated with the occupancy and internalization of hmm-EPOR. In summary, we suggest that the 78-kDa EPOR is directly involved in the initial biological actions of EPO.**

Erythropoietin (EPO) is the glycoprotein hormone responsible for the primary control of the number of erythrocytes in mammals (1). The hormone interacts with erythroid progenitors in the bone marrow, spleen, or fetal liver to stimulate their viability (2), proliferation, and maturation to continually replenish the supply of red cells in circulating blood.

^{125}I-EPO binding studies revealed 100–7000 ligand-specific receptors on the primary erythroid precursors and erythroid cell lines (3). The recent molecular cloning of the murine EPO receptor (EPOR) cDNA (4) permitted prediction of the receptor's primary amino acid sequence, preparation of anti-receptor antibodies (5), and subsequent isolation of human cDNA and genomic EPOR clones (6).

Cloned EPOR cDNA encodes a 55-kDa polypeptide with an NH_2-terminal extracytoplasmic (exoplasmic) domain, a single membrane-spanning domain, and a cytoplasmic portion of approximately the same length as the exoplasmic domain. Several features in the exoplasmic domain—e.g., four conserved cysteines and a Trp-Ser-Xaa-Trp-Ser motif, put the EPOR in the recently recognized cytokine receptor superfamily which includes the receptors for interleukins 2, 3, 4, 5, 6, and 7, granulocyte-colony-stimulating factors, and others (1, 6).

Crosslinking studies have identified 90-kDa and 105-kDa proteins crosslinked to EPO (7, 8). Antibodies raised against the cloned receptor do not recognize either of these proteins (5, 9) but rather have identified what appear to be several forms of posttranslationally modified forms of the cloned EPOR. Immunoprecipitation and Western blotting with anti-EPOR peptide antiserum have identified 62-, 64-, and 66-kDa proteins which are progressively glycosylated as they transit the endoplasmic reticulum and Golgi apparatus (5, 10–14). EPO binding leads to phosphorylation of proteins ranging from 38 to 153 kDa (6, 11, 15–22). Since a 72-kDa phosphorylated protein was recognized by anti-EPOR antiserum, this protein was thought to be derived by extensive phosphorylation of the 66-kDa EPOR protein (10, 11).

In previous studies, several EPO-dependent erythroid cell lines (HC cells) that exhibited extreme sensitivity to EPO were isolated (23, 24). In an attempt to monitor metabolism of the EPOR, a 10-fold increase in the number of binding sites was noted when HC cells were deprived of EPO for 24 hr.

The present study was undertaken as a result of an unexpected finding when the EPOR in these EPO-starved cells was analyzed by Western blotting with an affinity-purified anti-EPOR antibody. The result was that the 10-fold increase in EPO binding was accompanied by the appearance of a heretofore unrecognized species of EPOR which had an apparent mass of 78 kDa. Readdition of EPO not only stimulated phosphorylation and proliferation but also led to the rapid disappearance of the 78-kDa protein. We therefore tested the hypothesis that the 78-kDa protein is the cell surface form of the EPOR gene product, is phosphorylated upon EPO binding, and represents at least one component of the EPO–receptor complex which mediates the biological actions of this hormone. Our results provide evidence that this hypothesis is correct and are the subject of this report. The data also demonstrate the endocytosis and degradation of the receptor which mediates the apparent downregulation of EPORs.

MATERIALS AND METHODS

Cell Lines. A subclone of EPO-dependent murine erythroleukemia HC cells, HC-D57, was cultured as described (23, 24). COS-1 cells were transfected with EPOR cDNA (from Alan D'Andrea, Dana–Farber Cancer Institute) by the calcium phosphate method.

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Abbreviations: EPO, erythropoietin; EPOR, erythropoietin receptor; hmm-EPOR, high molecular mass forms of EPOR.

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Affinity-Purified Anti-EPOR IgG. Antisera against the EPOR were prepared in rabbits against synthetic peptides corresponding to the COOH-terminal (amino acids 487–505) and NH_2-terminal (amino acids 25–38) regions of the EPOR. Affinity-purified monospecific IgG was prepared by using peptides coupled to Bio-Gel beads (Bio-Rad).

The EPOR can be clearly detected by Western blotting after concentrating the receptor by immunoprecipitation. Anti-COOH-terminal IgG (2–4 μg) was added to soluble extract [1% (vol/vol) Triton X-100/20 mM Tris·HCl, pH 7.2/1 mM EDTA/1 mM phenylmethanesulfonyl fluoride] from $\geq 10^7$ cells. The immunoprecipitate collected with protein A-agarose was analyzed by SDS/PAGE and Western blotting using anti-COOH-terminal antibody (2 μg/ml) and alkaline phosphatase-conjugated secondary antibody.

To detect cell surface EPOR, COS cells expressing EPOR or mock-transfected cells were incubated with affinity-purified anti-NH_2-terminal IgG (50 μg/ml of growth medium) for 4 hr at 0°C. The EPOR–IgG immune complexes were recovered in the protein A-agarose pellet and analyzed by SDS/PAGE and Western blotting as described above.

Detection of Phosphotyrosine-Containing Proteins. HC-D57 cells were lysed in the buffer described above but containing 0.1 mM orthovanadate. Two micrograms of anti-phosphotyrosine IgG (Zymed Laboratories) was added to 1 ml of cell extract and incubated for 4 hr at 0°C. The immune complexes were recovered on protein A-agarose and analyzed by SDS/PAGE and Western blotting. Anti-phosphotyrosine IgG was used to detect the phosphotyrosine-containing proteins by using the procedures described above and an enhanced chemiluminescence system (ECL, Amersham). To detect phosphotyrosine on the EPOR, the EPOR was immunoprecipitated with anti-COOH-terminal IgG, and Western blotting was carried out with anti-phosphotyrosine IgG.

^{125}I-EPO Binding and Endocytosis. EPO was iodinated with Iodo-Gen (Pierce), and binding and internalization of ^{125}I-EPO were assayed as described (25). In brief, the radioactivity released into a high-salt, pH 2.5 wash was considered to be cell surface-bound EPO, while the remaining radioactivity was considered to be internal EPO.

Enzymatic Deglycosylation and Phosphorylation. Immunoprecipitated EPOR was denatured in 0.5% SDS and released from the protein A-agarose and then digested for 20 hr with N-Glycanase (6 units/ml; Genzyme) in 100 mM phosphate buffer, pH 8.5/0.1% SDS/1% (vol/vol) Nonidet P-40. To remove O-linked carbohydrate the immunoprecipitate was incubated with neuraminidase in 50 mM phosphate buffer (pH 7.2) for 4 hr, followed by 10 units of *O*-Glycanase for 18 hr at 37°C. For complete deglycosylation, *N*-Glycanase was also added in addition to *O*-Glycanase during the 18-hr incubation. To remove phosphate, the immunoprecipitated EPOR was boiled and incubated for 6 hr at 37°C in the presence of 50 units of calf intestinal alkaline phosphatase (11).

RESULTS

Recognition of High Molecular Mass Forms of EPOR (hmm-EPOR) in EPO-Starved Cells. In preliminary studies of an EPO-dependent erythroid cell line, HC-D57, the number of binding sites and the affinity for ^{125}I-EPO were investigated. These HC-D57 cells are dependent on EPO for proliferation and die after 2 or 3 days in the absence of the hormone; however, the cells were deprived of EPO overnight to see whether the level of EPOR was downregulated by continual exposure to EPO. This EPO deprivation resulted in a 10-fold increase in the number of binding sites for EPO (increase 600 to 6000 binding sites per cell), yet the affinity (500 pM) was unchanged.

Initial attempts to define the physical form and location of the EPOR required antibody against the receptor. Therefore, affinity-purified anti-peptide IgG directed against the EPOR was developed. The anti-COOH-terminal antibody was effective in both immunoprecipitation and Western blotting of EPOR in transfected cells; however, the rare EPOR in erythroid cell lines and primary erythroid cells was detected only when the EPOR was concentrated by immunoprecipitation from large numbers of cells and then detected by Western blotting.

When the structure of EPOR was investigated in the HC-D57 cells which were deprived of EPO overnight, the predominant forms of the receptor were previously unrecognized forms of 70–78 kDa, with the 78-kDa band predominating (Fig. 1, lane B). When EPO was added to these cells or cells were taken directly from culture in the presence of EPO, these hmm-EPOR were almost absent (Fig. 1, lane A). In sharp contrast, the previously recognized 62- to 66-kDa forms of EPOR did not change as a result of exposure to EPO. This unexpected finding suggested that these hmm-EPOR proteins, rather than the 66-kDa EPOR, might represent the cell surface form of receptor through which the hormone exerts its biological actions. In addition to finding 78-kDa EPOR in these HC-D57 cells, we detected 70- to 78-kDa EPOR in erythroblasts from Friend virus-infected mice (up to 50% of total EPOR), HC-D33 erythroleukemia cells and ME-26 cells (26) deprived of EPO (50–75% of total EPOR), and MEL-745 cells (2% of total EPOR).

hmm-EPOR Are Highly Glycosylated Products of the Cloned EPOR Gene. The correlation of hmm-EPOR appearance with EPO binding led us to seek more information about this heretofore undetected species. The bands of 70–78 kDa detected by anti-EPOR IgG were determined to be antigenically related to the cloned EPOR by the following experiments (*i*) EPOR proteins were immunoprecipitated by three different antisera (NH_2 terminus, amino acids 180–197, and COOH terminus) in the presence and absence of competing peptide and analyzed by SDS/PAGE and Western blotting using the anti-COOH-terminal antibody. All of the 62- to 78-kDa bands were immunoprecipitated in the absence of competing peptide and completely eliminated by the presence of peptide in the immunoprecipitation phase of the experiment (data not shown). (*ii*) Preimmune IgG and immune IgG which did not absorb to the peptide affinity column did not recognize the 62- to 78-kDa bands. (*iii*) COS cells transiently expressing the cDNA encoding the receptor had the 64- and 66-kDa receptors expected as well as a minor species of receptors up to 78 kDa (see Fig. 5). Mock-transfected cells had no 62- to 78-kDa bands either by immunoprecipitation or by Western blotting. (*iv*) Analysis of the following nonerythroid mouse tissues did not reveal any 62- to 78-kDa immunoreactive proteins: normal spleen, liver, kidney, heart, and brain. Enzymatic deglycosylation converted hmm-EPOR to the previously identified 62-kDa unprocessed form of EPOR (Fig. 1). We therefore con-

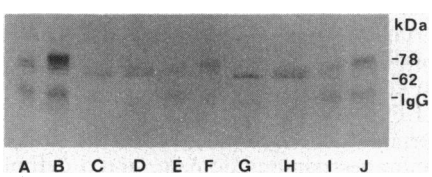

FIG. 1. Appearance and characterization of the 78-kDa EPOR in HC-D57 cells deprived of EPO. EPOR was immunoprecipitated from HC-D57 cells fed EPO (lanes A, C, E, G, and I) or starved for EPO overnight (lanes B, D, F, H, and J) and either not treated (lanes A and B) or treated with *N*-Glycanase (lanes G and H), neuraminidase plus *O*-Glycanase (lanes E and F), neuraminidase plus *O*- and *N*-Glycanase (lanes C and D), or 50 units of alkaline phosphatase (lanes I and J). EPOR was detected by Western blotting.

cluded that these 62- to 78-kDa proteins were products of the EPOR gene.

The experiments above suggested that hmm-EPOR were modified forms of the cloned gene product. We therefore explored the nature of modification by enzymatic deglycosylation and enzymatic dephosphorylation. All forms of the EPOR appear to result from differential N-linked glycosylation of a 62-kDa EPOR rather than phosphorylation (Fig. 1). However, the results are not absolute, since a small fraction of hmm-EPOR was reduced in mass to only 66 kDa by *N*-Glycanase treatment.

Kinetics of Appearance and Disappearance of hmm-EPOR Correlate with Binding and Internalization of ^{125}I-EPO. Endocytosis of ^{125}I-EPO was studied in HC-D57 cells to determine whether the reduction in cell surface receptors correlated with the loss of hmm-EPOR when the cells were exposed to EPO. When binding was assayed at 15°C to prevent endocytosis, 51,000 ± 1000 cpm of ^{125}I-EPO was bound on the cell surface at equilibrium (4 hr). The surface-bound ^{125}I-EPO was maximum by 10 min after addition of the hormone to cells at 37°C and then rapidly fell during the next 20 min, reaching a level about a third of maximal (Fig. 2). The radioactivity in the cell interior (resistant to a pH 2.5 wash) reached a maximum at 30 min and then declined, whereas the total radioactivity bound was maximal by 10 min and then declined after 30 min of exposure to ^{125}I-EPO.

The time course of the loss of the EPOR was determined by treating HC-D57 cells with EPO and determining the abundance of the 66-kDa EPOR and hmm-EPOR by immunoprecipitation/Western blotting technique. The amount of hmm-EPOR was constant for 5–10 min following treatment with EPO and then decreased rapidly, reaching 25% of the starting value in 60 min (Fig. 3). When the data for the disappearance of cell surface receptors (^{125}I-EPO binding) and hmm-EPOR are plotted on the same graph (Fig. 3*A*), the curve for the loss of hmm-EPOR is very similar to the loss of cell surface EPOR but delayed 10 min. It is tempting to speculate that this 10 min is consumed by internalization and degradation of hmm-EPOR. In contrast, the level of 66-kDa EPOR did not change appreciably in the presence of EPO.

In the converse set of experiments, the increase in the number of binding sites for ^{125}I-EPO and the amount of hmm-EPOR in HC-D57 cells were compared following the withdrawal of EPO. Parallel increases (10-fold) in hmm-

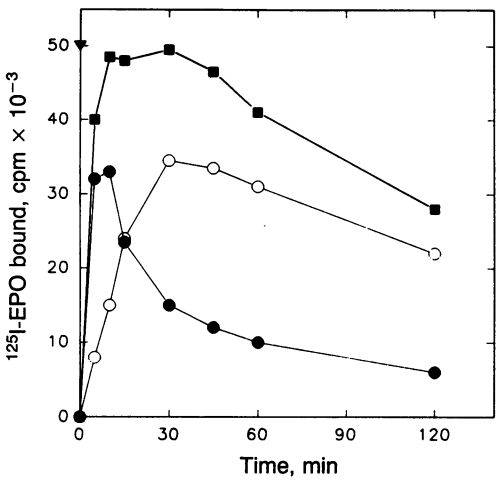

FIG. 2. Binding and endocytosis of ^{125}I-EPO in HC-D57 cells. Cells (2 × 10^6) were deprived of EPO for 20 hr, ^{125}I-EPO (10 units/ml) was added at zero time, and total bound (■), surface-bound (●), and internal (○) EPO were determined. In addition, total surface-bound EPO was determined without endocytosis, at 15°C (▼). Data are the mean of triplicates and the maximum standard deviation was 1500 cpm (not shown). A maximum of 6700 receptors per cell were occupied.

EPOR and binding sites for ^{125}I-EPO were observed during a 12-hr period following withdrawal of EPO (Fig. 3*B*). A 2-fold or less increase in the 66- and 62-kDa receptors also occurred. Blocking of protein synthesis with cycloheximide completely prevented the increase in ^{125}I-EPO binding. hmm-EPOR did not increase in the presence of cycloheximide and absence of EPO. These data suggest either that synthesis of hmm-EPOR occurs *de novo* or that protein synthesis may be required for the 66-kDa EPOR to be additionally glycosylated and reach the cell surface. Examination of the mRNA levels in HC-D57 cells by Northern blot analysis indicated a single size of message for EPOR, which was slightly (33%) decreased following withdrawal of EPO for 18 hr (data not shown). Together these results suggest that all forms of the EPOR gene product appear to be in equilibrium in the presence of EPO but that the 78-kDa EPOR accumulates during hormone withdrawal, due to lack of internalization and degradation.

Half-life of hmm-EPOR but Not 66-kDa EPOR Is Shortened by EPO. The hypothesis that the 78-kDa EPOR is internalized and degraded predicts that the half-life of hmm-EPOR should be shorter in the presence of hormone than in its absence. To test this idea, the effect of EPO on the turnover rate of the 66-kDa, 62-kDa, and hmm-EPOR species was investigated in the absence of protein synthesis (treated with cycloheximide). The half-life of hmm-EPOR and 66-kDa receptor in the absence of EPO was 3 hr, and that of the 62-kDa EPOR was 45 min (Fig. 4). However, when saturating levels of EPO were present, the degradation rate of the hmm-EPOR was accelerated 12-fold, so that the half-life was 15 min. The turnover rates of both the 66-kDa and the 62-kDa EPOR species were unaffected by the presence of EPO. This confirms the previous finding that cycloheximide did not allow further processing of the 62- to 64-kDa receptors. The destruction of the hmm-EPOR following the binding of EPO was shown by these studies.

Kinetics of EPO-Dependent Phosphorylation Correlates with Binding and Internalization of EPO. The experiments above suggested that the time course of the initial EPO response should be finite and short, since the hormone and receptor were quickly internalized and degraded within minutes of binding. Therefore, we attempted to establish a temporal correlation of receptor metabolism with receptor function. A major protein of 97 kDa (pp97) and a minor 78-kDa protein were phosphorylated on tyrosine in an EPO-dependent fashion (Fig. 3*C*). When the time course of the phosphorylation of pp97 was plotted on the same graph as the cell surface EPOR and the hmm-EPOR (Fig. 3*A*), all three disappeared with parallel curves. This strongly suggests that hmm-EPOR interact with a protein-tyrosine kinase after the binding of EPO and that internalization and destruction of hmm-EPOR turn off EPO-dependent phosphorylation.

The 78-kDa Form of EPOR Is Phosphorylated in Response to EPO. From the anti-phosphotyrosine experiment, a 78-kDa band appeared to be phosphorylated in addition to the 97-kDa protein. To test whether this phosphorylated protein was the EPOR, we analyzed anti-EPOR immunoprecipitates by Western blotting with anti-phosphotyrosine. The 78-kDa band was recognized by antibody against EPOR (Fig. 3*C*). However, it is interesting that only the 78-kDa band was phosphorylated. It is possible that the tyrosine kinase recognizes only the 78-kDa form of the hmm-EPOR or that only this form is part of the active EPO–receptor complex. We did not see the coprecipitation of the 97-kDa phosphoprotein with the 78-kDa EPOR or see a 130-kDa phosphoprotein. Both 97- and 130-kDa proteins have been suggested to be associated with the EPOR (11, 19).

The turnover of 78-kDa phosphoprotein is slower than that of the hmm-EPOR. Recent experiments have verified that this phosphorylated 78-kDa protein immunoprecipitated by anti-phosphotyrosine was also recognized by anti-EPOR

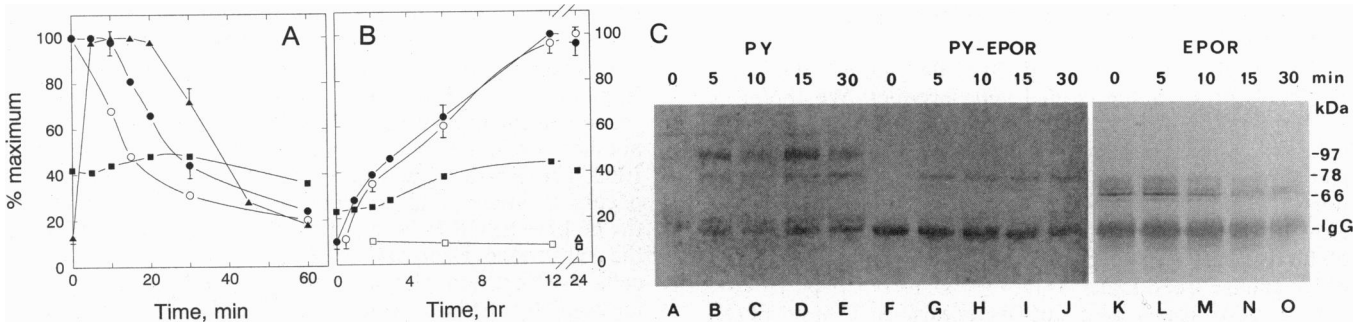

FIG. 3. Time course of the disappearance of cell surface EPOR, loss of hmm-EPOR, pp97 phosphorylation, and EPOR phosphorylation following EPO treatment as well as reappearance of cell surface EPOR and hmm-EPOR following EPO withdrawal. (*A*) HC-D57 cells were deprived of EPO and then treated with EPO (10 units/ml) for the indicated time. Cell surface binding sites from Fig. 2 are shown (○) in comparison to the hmm-EPOR (●), 66-kDa EPOR (■), and pp97 phosphorylation (▲). Data from *C* and other experiments were analyzed by densitometry. (*B*) HC-D57 cells in the presence of EPO were deprived of EPO at zero time and the total binding of ^{125}I-EPO (○), hmm-EPOR (●), and 66-kDa EPOR (■) was determined at the indicated time. In addition, the binding of ^{125}I-EPO (□) and hmm-EPOR (△) in cells which were treated with cycloheximide (1 μg/ml) at zero time is presented. Data are the mean of duplicate or triplicate determinations (error bar shows the standard deviation). (*C*) Time course of total phosphotyrosine phosphorylation, 78-kDa EPOR phosphorylation, and EPOR species following EPO treatment of HC-D57 cells. HC-D57 cells deprived of EPO overnight were exposed to EPO (10 units/ml) for the indicated time. Extracts were first analyzed by immunoprecipitation using anti-phosphotyrosine (PY) antibody lanes (A–E) or anti-EPOR IgG (lanes F–O). Then phosphotyrosine-containing proteins (lanes A–J) or EPOR proteins (lanes K–O) were visualized by Western blotting. The EPOR proteins in lanes K–O were visualized by stripping and reprobing the same blot (shown in lanes F–J) previously probed with anti-phosphotyrosine antibody.

IgG; however, the phosphorylated receptor was ≥10% of the total 78-kDa EPOR. This result suggests either that the phosphorylation of the EPOR preserved it from endocytosis and/or degradation or that additional 78-kDa EPOR became phosphorylated following degradation of the phosphorylated receptor.

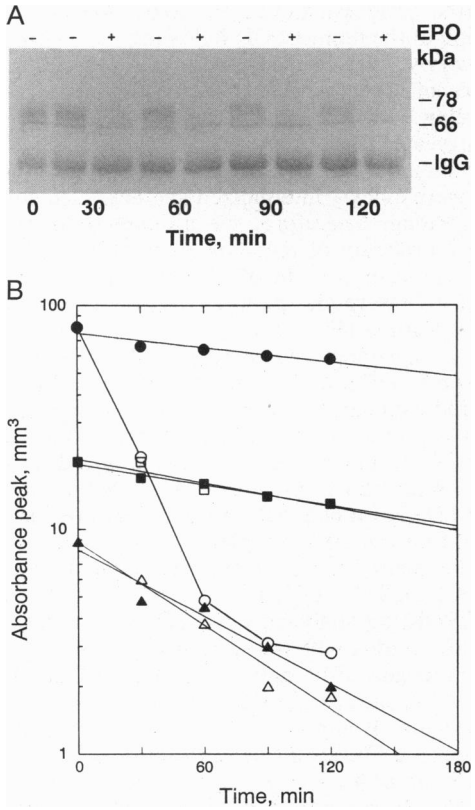

FIG. 4. Turnover of EPOR in HC-D57 cells. Cells were deprived of EPO for 20 hr, preincubated with cycloheximide (1 μg/ml) for 30 min, and the hmm-EPOR (○, ●), 66-kDa EPOR (□, ■), and 62-kDa EPOR (△, ▲) were determined at the indicated time in the presence of EPO (10 units/ml added zero) time (open symbols) or in the absence of EPO (filled symbols). (*A*) Western blot (*B*) Data obtained by densitometric analysis of the blot.

The 78-kDa EPOR Is on the Cell Surface Whereas the 62- to 66-kDa EPOR Is Cytoplasmic. The above experiments implicated a divergent pathway of posttranslational modification of the EPOR gene product which results in a more glycosylated cell surface form of EPOR and smaller intracellular forms. This hypothesis was tested in COS cells expressing cDNA coding for the EPOR. When intact COS cells expressing EPOR were incubated with antibody specific for the NH_2 terminus of EPOR, the antibody bound to the same hmm-EPOR protein implicated as the cell surface EPOR in HC-D57 cells (Fig. 5). The 78-kDa EPOR on the surface of HC-D57 was qualitatively demonstrated by this method (data not shown); however, only small amounts of 78-kDa EPOR were recovered due to the low affinity of the anti-NH_2-terminal antibody and the lower numbers of cell surface EPORs in HC-D57 cells (6000) compared with COS cells expressing receptor cDNA (10^6 EPORs).

DISCUSSION

We began with the investigation of the metabolism of EPOR in an EPO-dependent erythroid cell line. These cells undergo a 10-fold increase in binding sites for EPO when deprived of the hormone overnight. In an attempt to confirm this increase at the receptor protein level, we immunoprecipitated the EPOR and then performed Western blotting on the immu-

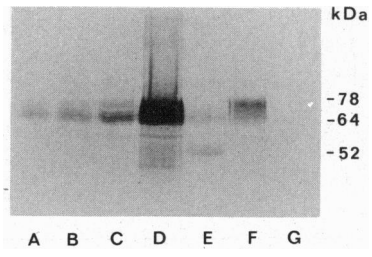

FIG. 5. Cell surface EPOR in COS cells expressing EPOR cDNA. COS cells transfected with EPOR cDNA (lanes A–D, and F) or mock transfected (lanes E and G) were used. Intact cells were treated with anti-NH_2-terminal EPOR antibody to detect EPOR on the cell surface (lanes F and G) or cell lysates to detect EPOR in the cell interior (lanes D and E). Samples consisting of 1/200th, 1/100th, and 1/50th (lanes A–C, respectively) of the total cell extract were analyzed by Western blot for comparison. Cell surface and internal EPORs were recovered from the cell extract on protein A-agarose.

noprecipitate with antibody to the COOH terminus of EPOR. We did not see an increase in the 62-, 64-, or 66-kDa receptor in EPO-deprived cells, but rather discovered the appearance of a previously unrecognized form of the cloned EPOR gene product with a mobility corresponding to 78 kDa in SDS/PAGE.

Since the 78-kDa EPOR (and minor species from 70 to 78 kDa) correlated with EPO binding sites, we tested the hypothesis that the 78-kDa protein might be the active form of EPOR. We gained supportive evidence that the 78-kDa form of receptor is (*i*) found on the cell surface, (*ii*) found only in cells known to bind EPO, (*iii*) a more glycosylated form of the 62-kDa EPOR, glycosylated through N-linked carbohydrate, (*iv*) accumulated upon EPO starvation, (*v*) lost as bound EPO is endocytosed and degraded, (*vi*) turned over 12 times faster in the presence of EPO than in the absence of the hormone, while the half-life of the 62- to 66-kDa EPOR is not affected, (*vii*) immediately phosphorylated on tyrosine residues following EPO binding, and (*viii*) correlated with the EPO-dependent tyrosine phosphorylation of a 97-kDa protein.

The finding of a posttranslationally modified product of the EPOR gene that correlates with the cell surface receptor for EPO explains previous paradoxical findings and focuses attention to new possibilities. Previous experiments with cells expressing EPOR cDNA found that the number of binding sites for EPO did not correlate with EPOR mRNA or the 62- to 66-kDa forms of EPOR. In light of the current findings, these cells seem to lack the ability to posttranslationally modify and transport to the surface the large number of receptors translated and thus accumulate disproportionate amounts of the 66-kDa intracellular EPOR. Further, the discovery of unphosphorylated 70- to 78-kDa forms of the EPOR forces one to reconsider the idea suggested by Yoshimura and Lodish (11) that the 72- to 75-kDa phosphorylated EPOR forms result from extensive phosphorylation of the 66-kDa EPOR protein. The 75-kDa protein identified by those authors in HC-D57 cells may be the 78-kDa EPOR identified in this study; however, this study and unpublished preliminary data strongly suggest that the 78-kDa EPOR which is phosphorylated on tyrosine residues is also 78 kDa when not phosphorylated. Extensive phosphorylation of the 66-kDa EPOR may occur in Ba/F3 cells expressing receptor cDNA, but additional studies are required (11, 22).

Recent studies have used nonerythroid cells transfected with EPOR cDNA and progenitor-like cell lines which respond to a variety of cytokines to identify EPO-dependent phosphorylation of a number of proteins ranging from 38 to 150 kDa (6, 11, 15–22). The small number of proteins phosphorylated, 78-kDa receptor and pp97, as well as the low abundance of phosphotyrosine on the proteins (as judged by very weak reactivity to anti-phosphotyrosine antibody) suggests a highly selective, EPO-dependent phosphorylation in these EPO-dependent erythroid cells.

The EPO-dependent phosphorylation response lasts <1 hr, but erythroid cells require prolonged exposure (hours to days) to EPO to complete differentiation (2), and the cell cycle is on the order of 8–12 hr. It is interesting to ponder the relationship of the kinetics of the EPO-dependent phosphorylation to other temporal events in the life of a developing red cell.

We have demonstrated a correlation between the endocytosis of ^{125}I-EPO and the destruction of the EPOR. The 12-fold decrease in the half-life of the 78-kDa EPOR protein in the presence of EPO indicates that hmm-EPOR are degraded following endocytosis rather than recycled to the cell surface. Since EPO is degraded in the lysosomes following endocytosis (25), it is very likely that both hormone and receptor are simultaneously degraded.

This study defined two components which have been missing from the EPOR life story. One is the receptor's maturity (in size, location, and function) and the other is the death of the receptor. The finding of the active cell surface 78-kDa EPOR should provoke additional experiments into the mechanism by which EPO stimulates cells.

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