Identification of a cytoplasmic motif in the erythropoietin receptor required for receptor internalization

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Received 1 March 1998; revised version received 1 April 1998

Abstract Erythropoietin (EPO) promotes the viability, proliferation and differentiation of mammalian erythroid progenitor cells via its specific cell surface receptor. The EPO receptor (EPO-R) is a member of the cytokine receptor superfamily and is comprised of one identified subunit which homodimerizes upon ligand binding. To study the role of the intracellular domain of the EPO-R in the endocytosis of EPO, we compared the rate and extent of ^{125}I-EPO endocytosis by wild type (wt) EPO-R and five cytoplasmically truncated EPO-Rs: 1–251 EPO-R, 1–257 EPO-R, 1–267 EPO-R, 1–276 EPO-R and 1–306 EPO-R which contain 4, 10, 20, 29 or 59 amino acids of the cytoplasmic domain, respectively. We also studied an EPO-R mutant (PB) which lacks amino acid residues 281–300 of the cytoplasmic domain. The experiments were conducted in COS 7 cells transfected with the EPO-R cDNAs and in Ba/F3 cells stably expressing the wt EPO-R, 1–251 or 1–257 EPO-R. Cells expressing wt EPO-R, PB EPO-R (Δ281–300), 1–276 EPO-R or 1–306 EPO-R internalized approximately 50% of ^{125}I-EPO bound to the cell surface, while cells expressing 1–251, 1–257 or 1–267 EPO-R internalized only 25% of the bound ^{125}I-EPO. The steady-state expression levels of these latter receptors on the cell surface were typically 2–5-fold higher than wt EPO-R. Our data indicate that amino acid residues 267–276 (FEGLFTTHK) of the EPO-R cytoplasmic domain may have a role in receptor internalization. Metabolic labeling experiments suggest that in transiently transfected COS 7 cells most of the wt EPO-R and 1–257 EPO-Rs do not exit the ER and may be degraded there. The half-life of both receptors was essentially similar and was in the range of 1 h. In Ba/F3 cells the mature Golgi processed 1–257 EPO-R was more stable than the corresponding form of the wt EPO-R, possibly contributing to its higher cell surface expression.

Key words: Erythropoietin; Erythropoietin receptor; Endocytosis

1. Introduction

Proliferation of immature red blood cells and their differentiation into mature erythrocytes are mediated by erythropoietin (EPO). The erythropoietin receptor (EPO-R) [1] belongs to the superfamily of cytokine receptors which includes the receptors for interleukins (IL) 2, 3, 4, prolactin, growth hormone (GH) and leukemia inhibitory factor. These receptors all have one transmembrane domain, four cysteine residues similarly spaced and a common WSXWS sequence motif in their extracellular domain [2]. Unlike receptor tyrosine kinases, the cytoplasmic domain of cytokine receptors is devoid of kinase activity and is presumably phosphorylated by Janus kinases (JAKs) following ligand binding [3]. Thus far, only one subunit of the EPO-R has been identified, which homodimerizes upon ligand binding [4,5]. EPO-Rs which lack the entire cytoplasmic domain have been suggested to occur in bone marrow [6] and to function as dominant negative regulators of the EPO-R [7,8].

One striking property of the EPO-R is its low cell surface expression, suggesting that cell surface levels of EPO-R may be tightly controlled in order to regulate erythroid proliferation and differentiation in response to physiological levels of EPO under normal or hypoxic conditions. Both translational [9,10] and posttranslational [11] mechanisms probably control the steady-state levels of cell surface EPO-R. The latter may include for example protein folding [11], protein stability and receptor downregulation by internalization and ligand mediated endocytosis. Structural features of the EPO-R which regulate its cell surface expression are still unclear. Accumulating evidence suggests that efficient internalization of membrane receptors requires a specific sequence(s) in the cytoplasmic domain which may interact with auxiliary cellular molecules [12–14]. The sequences include D-leucine based motifs [15,16] and Tyr based motifs [13,17]. Identification of such sequences is of extreme importance in view of their possible roles in controlling surface as well as intracellular levels of the cell surface receptors. The contribution of the EPO-R cytosolic domain to cell surface EPO-R expression has not yet been resolved.

The cytosolic domain of the GH receptor (GHR) [18] as well as that of the prolactin receptor [19] contain sequence motifs which are required for ligand endocytosis. Recently, it was shown that the IL-2 β chain cytosolic domain contains sequences that designate it for degradation after endocytosis [20]. In this study we set forth to delineate cytoplasmic residues of the EPO-R that are involved in regulating cell surface expression of the EPO-R and receptor mediated endocytosis of EPO. We show that EPO-Rs containing at least 276 amino acids endocytose ligand similarly to the full length wild type (wt) EPO-R. On the other hand, EPO-Rs which are truncated at cytoplasmic residues 251, 257 or 267 are expressed at higher levels on the cell surface and internalize ^{125}I-EPO approximately two-fold less than full length EPO-R. These findings suggest that a motif required for receptor internalization may occur between residues 267 and 276 of the EPO-R cytoplasmic domain.

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Abbreviations: EPO, erythropoietin; EPO-R, erythropoietin receptor; Endo H, endoglycosidase H; ER, endoplasmic reticulum

PII S0014-5793(98)00414-1

2. Materials and methods

2.1. Materials

Purified recombinant human EPO was a kind gift from Kirin, Japan. All other materials were obtained from sources previously listed [21].

2.2. Antibodies

Rabbit antibodies directed against the cytoplasmic domain [22] or the extracellular domain [23] of the murine EPO-R were used at a 1:500 dilution for immunoprecipitations, and at a dilution of 1:1000 for immunoblotting, as indicated.

2.3. Construction of mutant EPO-R cDNAs

1–251, 1–267 and 1–276 EPO-R were constructed using the PCR method to insert a stop codon after amino acid residue 251 or 267 or 276 respectively, followed by an *Eco*RI restriction site for convenient cloning into pXM [1]. The cDNAs of 1–251, 1–267 and 1–276 EPO-R were generated by the PCR method, using the primer 5′-GAGTCGGTACCTGAAGCTAGGGCTGC-3′ along with each of the following primers: 5′-GAGGAATTCCTACCGGCGGTGGGACTG-3′; 5′-GACGAATTCATCCGCTCTCTGGGC-3′; 5′-GAGGAATTCCTTGTGGGTGGTGAAGAGACC-3′ for 1–251, 1–267 and 1–276 EPO-R respectively. The structure of the plasmids was confirmed by DNA sequencing. 1–257 and 1–306 EPO-Rs have been described elsewhere [7]. The cDNA of PB EPO-R (Δ281–300) was a generous gift from Dr. J. Ihle (Memphis, TN).

2.4. Cell culture and transfection

COS 7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) (v/v). Cells were cultured to 60% confluence and were transiently transfected using the DEAE dextran/chloroquine method [24] with 5 μg of EPO-R cDNA cloned in pXM [1]. ^{125}I-EPO binding and internalization were determined 48 h after transfection.

Ba/F3 cell lines stably expressing 1–251 and 1–257 EPO-R were generated as described [7]. Ba/F3 cell lines stably expressing the wt EPO-R cDNA [25], 1–251 or 1–257 EPO-Rs in pXM were maintained in RPMI supplemented with 10% FCS (v/v) and 0.25 U/ml EPO for wt EPO-R or 10% conditioned medium from WEHI 3B cells as a source of IL-3 for the truncated EPO-Rs.

2.5. Iodination of EPO, binding and internalization

Recombinant EPO was labeled using the iodine monochloride method [26] and had a specific activity of 4×10^6 cpm/pmol. Briefly, 2.5 μl of a 1.25 mg/ml solution of EPO in PBS was labeled by the addition of 100 μl of 200 mM sodium phosphate, 0.02% (v/v) Tween 20 at pH 7.4 and 1 mCi of Na^{125}I. Two 5 μl aliquots of 200 mM ICl in 2 M NaCl were added at 1 min intervals while vortex mixing. Radioiodinated EPO (^{125}I-EPO) was separated from ^{125}I by gel filtration and cation exchange chromatography.

2.6. ^{125}I-EPO binding

Cells (10^7 COS 7 cells, lifted from the plates by treatment with 15 mM EDTA pH 8.0 in PBS, or 2×10^6 Ba/F3 cells, cultured in suspension) were washed twice in either DMEM or in RPMI, respectively. Cells were incubated with ^{125}I-EPO for 16 h at 4°C, in a final volume of 100 μl, in the appropriate medium containing 10% FCS, 20 mM HEPES pH 7.4 (binding medium). After sedimentation of cells through ice cold FCS, cell associated radioactivity and free ^{125}I-EPO were measured. Specific binding was determined by subtracting the cell associated radioactivity in the presence of unlabeled EPO (100 nM) from that associated with the cells in the absence of excess unlabeled ligand. To measure endocytosed ^{125}I-EPO, the cell pellets were further resuspended in 100 μl of binding medium and incubated for different time periods at 37°C. Subsequently, the cells were sedimented again through ice cold FCS, to remove dissociated ^{125}I-EPO. Cell pellets were then exposed to an acid wash (4% acetic acid in PBS) for 2.5 min at 4°C, and released, cell surface ^{125}I-EPO was separated from internalized ligand by a third centrifugation through an FCS cushion. Specific binding was defined as the difference between ^{125}I-EPO bound to the cells in the absence and in the presence of unlabeled EPO. Continuous uptake of ^{125}I-EPO was measured by incubating the cells with ^{125}I-EPO for 90 min at 37°C. Cell surface bound and internalized ^{125}I-EPO were determined as described.

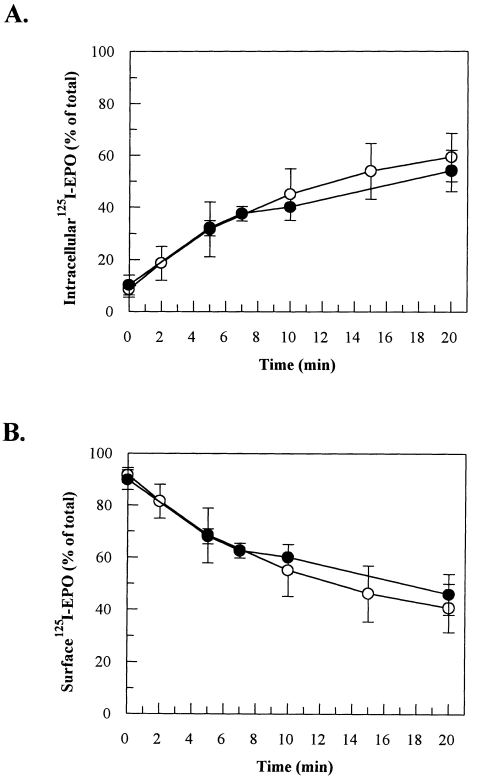

Fig. 1. ^{125}I-EPO internalization in Ba/F3 cells and in COS 7 cells expressing the EPO-R. Ba/F3 cells expressing wt EPO-R and COS 7 cells transfected with wt EPO-R cDNA were incubated at 4°C for 16 h in the appropriate binding medium containing ^{125}I-EPO. Unbound ligand was removed by centrifugation through FCS. Cells were then resuspended in binding medium and incubated at 37°C for different time periods (as indicated). Cells were then cooled to 4°C, and centrifuged through an FCS layer. To quantify endocytosed ^{125}I-EPO, the cell pellets were treated with PBS containing 4% acetic acid for 2.5 min, to dissociate surface bound ligand. Specific internalized (A) or surface bound (B) ^{125}I-EPO was determined by subtracting ^{125}I-EPO counts in the presence of 100 nM unlabeled EPO from the counts obtained in its absence. Values are presented as percent of total surface and intracellular ^{125}I-EPO at each time point. The values represent the mean ± S.D. of two different experiments, performed in triplicate.

2.7. Metabolic labeling, immunoprecipitation and endoglycosidase H (Endo H) digestion

Ba/F3 cells expressing the EPO-R or COS 7 cells transiently transfected with EPO-R cDNAs (2×10^6 cells for each time point) were labeled with [^{35}S]cysteine/methionine as previously described [25]. Subsequently cells were chased in the presence of non-labeled amino acids for the indicated periods of time. Solubilization of the cells, immunoprecipitation and digestion with Endo H were performed as previously described [21,22].

2.8. Western blot analysis

Western blot analysis was performed as described previously [25].

3. Results

3.1. Endocytosis of ^{125}I-EPO by wt EPO-R is similar in Ba/F3 and in COS 7 cells

A wealth of information has accumulated on signal transduction and structure-function relationship of the EPO-R expressed in the pro B cell line Ba/F3. When these cells are transfected with EPO-R cDNA, EPO can replace their need for interleukin 3 (IL-3), thus rendering this system useful for studying the EPO-R. Importantly, many properties of the

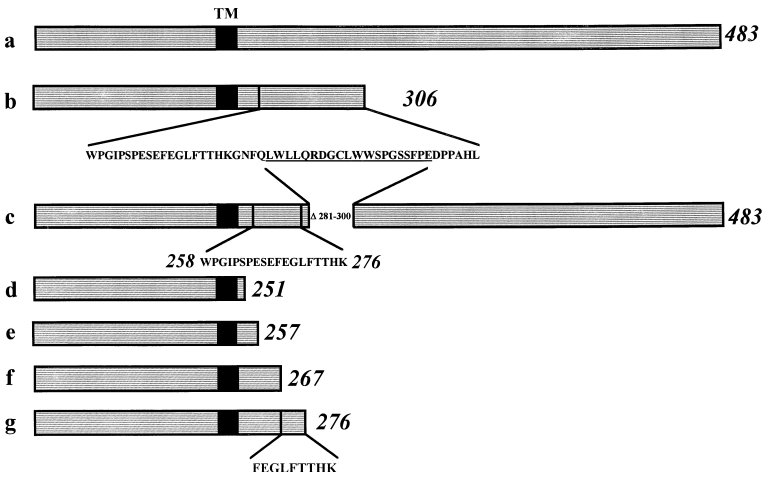

Fig. 2. Schematic representation of the EPO-R mutants used in this study. The EPO-R mutants employed in this study were (a) wt EPO-R, (b) 1–306 EPO-R, (c) PB EPO-R (Δ281–300), (d) 1–251 EPO-R, (e) 1–257 EPO-R, (f) 1–267 EPO-R and (g) 1–276 EPO-R. The transmembrane (TM) region is indicated.

receptor are retained in transiently transfected COS 7 cells (e.g. ligand binding affinity and kinetics), thereby providing a system in which the EPO-R can be readily analyzed [11]. Initially we demonstrated that ^{125}I-EPO was internalized to a similar extent and with similar kinetics by wt EPO-R, whether stably expressed in Ba/F3 cells, or transiently expressed in COS 7 cells (Fig. 1). Cells were incubated overnight with ^{125}I-EPO at 4°C. Unbound ligand was removed the following day, and the cells were further incubated at 37°C, for 0–20 min as indicated (Fig. 1). As can be observed, the rate and extent of ^{125}I-EPO endocytosis, as well as the reduction of cell surface bound ^{125}I-EPO, were essentially similar in Ba/F3 cells and in COS 7 cells. Significant advantages of COS 7 cells are their endurance of temperature shifts (4°C to 37°C), and much higher expression levels of cell surface EPO-Rs as compared to Ba/F3 cells. We thus compared the endocytosis of ^{125}I-EPO by EPO-R mutants in transiently transfected COS 7 cells.

3.2. *^{125}I-EPO internalization mediated by truncated EPO-Rs*

A schematic representation of EPO-R mutants employed in this study is depicted in Fig. 2. EPO-Rs truncated in their cytoplasmic domain were constructed in order to identify regions in this domain, involved in receptor internalization. In addition, PB EPO-R (Δ281–300) [27], which lacks the JAK 2 binding domain, was employed.

^{125}I-EPO binding and internalization were measured in COS 7 cells transiently transfected with each EPO-R mutant (Fig. 3A). To initially characterize EPO internalization by the mutants, cells were incubated at 37°C in the presence of ^{125}I-EPO for 90 min. Here, continuous internalization of ^{125}I-EPO was measured, unlike the experiments described in Figs. 1 and 4, which depict only one wave of ^{125}I-EPO internalization. Cell surface and internalized ^{125}I-EPO were determined as described in Section 2. This experiment revealed that for three truncated EPO-Rs (1–267, 1–257 and 1–251 EPO-R) the ratio of ^{125}I-EPO specifically internalized to that bound on the cell surface over a 90 min incubation was about 0.3. This ratio in wt EPO-R, PB (Δ281–300), 1–276 and 1–306 was about 1. Noteworthy is the fact that the cell surface expression of these three endocytosis deficient EPO-Rs (1–251, 1–257 and 1–267 EPO-R) under these conditions was typically 2–5-fold higher than that of the wt EPO-R, 1–306 and 1–276 EPO-R. Surface levels of PB EPO-R (Δ281–300) were usually 1.5–3-fold higher than that of wt EPO-R. A similar pattern of ^{125}I-EPO binding and internalization was observed in Ba/F3 cells stably expressing wt, 1–251 and 1–257 EPO-Rs (Fig. 3B). In agreement with Quelle et al. [28], we observed a significant

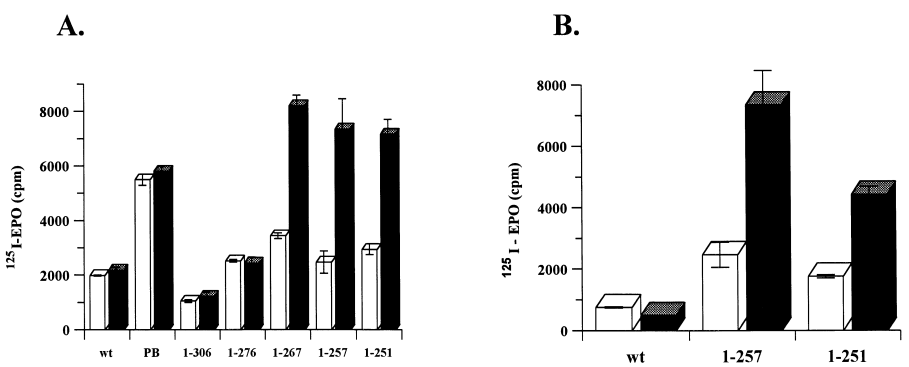

Fig. 3. Binding and internalization of ^{125}I-EPO by wt EPO-R and EPO-R mutants. COS 7 cells (A) or Ba/F3 cells (B) expressing wt EPO-R or EPO-R mutants were incubated in the presence of ^{125}I-EPO for 90 min at 37°C. Subsequently cells were separated from non-bound radioactive ligand. Cell surface bound ^{125}I-EPO was removed by acid wash. Specific binding was determined by subtracting the measured counts in the presence of cold ligand from those in its absence. The filled and empty bars represent cell surface bound and internalized ^{125}I-EPO respectively. Values represent the mean ± S.D. of triplicates. The figure represents one representative experiment out of six experiments.

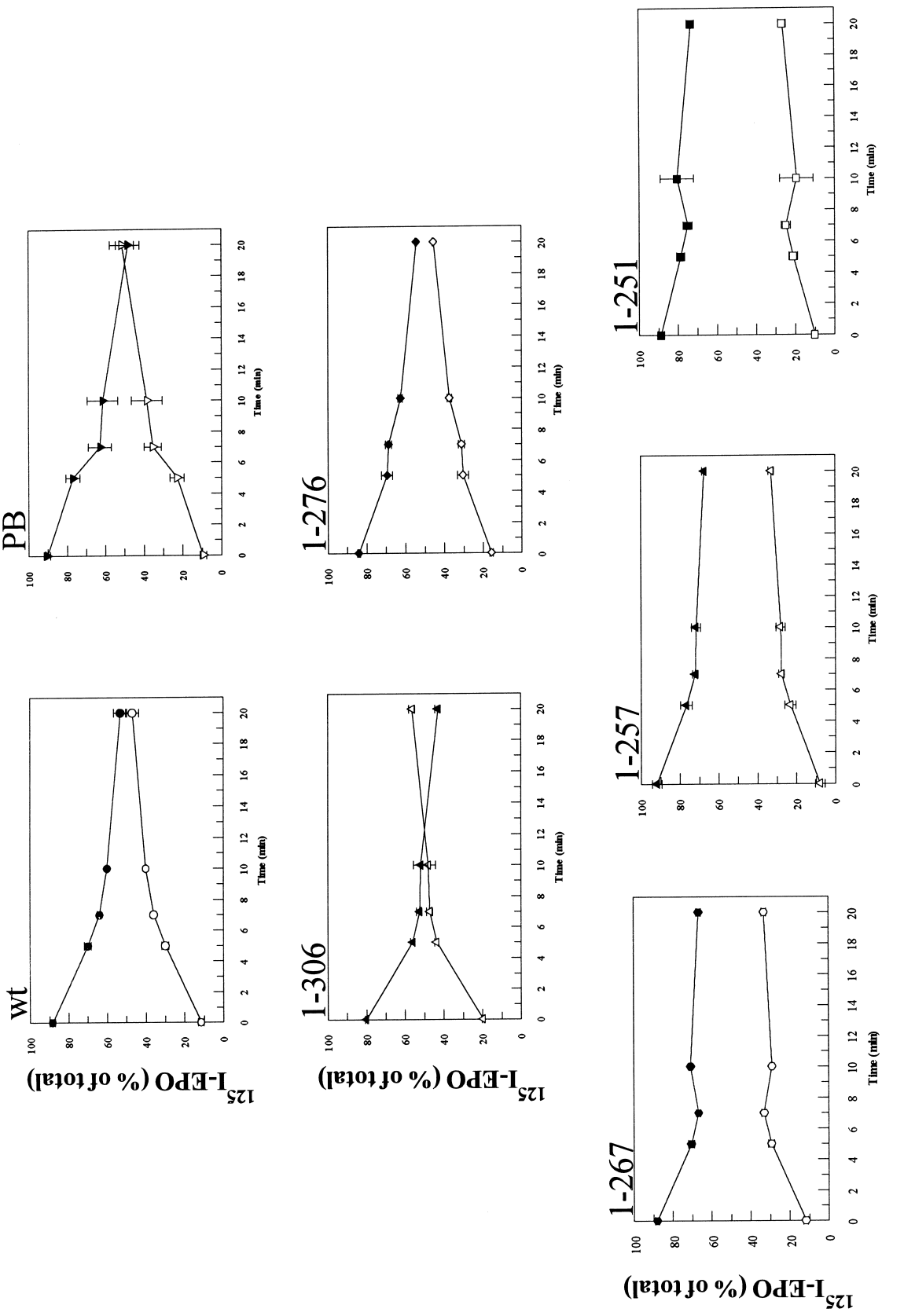

Fig. 4. Internalization of surface bound ^{125}I-EPO by wt EPO-R and EPO-R mutants. COS 7 cells transfected with wt EPO-R, PB (Δ281–300) EPO-R, 1–306 EPO-R, 1–276, 1–267, 1–257, 1–251, cDNAs, were incubated in binding medium containing ^{125}I-EPO for 16 h at 4°C. Cells were treated and tested for specific binding as described in Section 2. Values were calculated as in Fig. 1 and represent the mean ± S.D. of at least three experiments performed in triplicate. Filled and open symbols represent percent cell surface and internalized ^{125}I-EPO respectively.

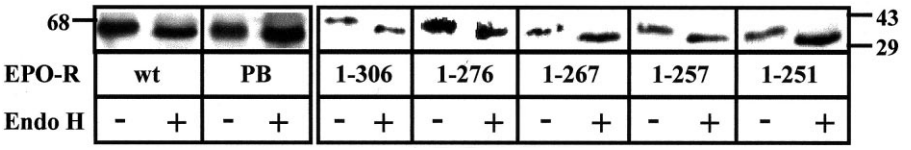

Fig. 5. Western blot analysis of wt EPO-R and EPO-R mutants in COS 7 cells. COS 7 cells expressing wt EPO-R were lysed in 500 μl PBS containing 1% Triton, 0.5% deoxycholate and 5 mM EDTA, in the presence of protease inhibitors. Cell lysates were immunoprecipitated with antibodies against the extracellular domain of the EPO-R, followed by the addition of protein A Sepharose. Each sample was divided into two aliquots which were incubated overnight at 37°C in the presence or absence of Endo H. Subsequently, samples were separated by 10% SDS-PAGE, and blotted onto nitrocellulose membrane filter. The blot was probed with anti-EPO-R extracellular antibodies.

amount of ^{125}I-EPO which was internalized by the cytoplasmically truncated mutants (1–251, 1–257 and 1–267 EPO-R). However, the ratio which we observed between the internalized and cell surface EPO was typically lower for these truncated mutants than for the wt EPO-R, suggesting that their ability to internalize EPO is impaired.

To further characterize ^{125}I-EPO internalization, we examined the kinetics of ^{125}I-EPO internalization by wt EPO-R and EPO-R mutants (Fig. 4). COS 7 cells were transiently transfected with EPO-R cDNA, and 48 h later subjected to binding and internalization of ^{125}I-EPO. Binding of ^{125}I-EPO was performed at 4°C for at least 10 h, thereby reaching saturation of the surface receptors under conditions where virtually no endocytosis occurs. Following removal of free unbound ligand, internalization of ^{125}I-EPO was performed at 37°C, for time intervals between 0 to 20 min. These conditions are more sensitive for detecting differences in the rate and extent of endocytosis between the various mutants than analysis of steady-state endocytosis, because one is able to analyze the initial steps of endocytosis independently of the later stage of ligand internalization and degradation. The differences in the binding levels of ^{125}I-EPO at 4°C, between all the mutants tested, maintained the profile which was observed in Fig. 3. Namely, 1–251, 1–257 and 1–267 EPO-R typically bound 2–5-fold more ^{125}I-EPO at the cell surface (data not shown). In general, the rate of ^{125}I-EPO endocytosis was similar for all the mutants. However, EPO-R mutants which contained 4, 10 or 20 amino acids of the intracellular domain (1–251, 1–257 and 1–267 EPO-R respectively), internalized less ^{125}I-EPO after 20 min of incubation at 37°C as compared to wt EPO-R (Fig. 4). In cells expressing wt, 1–306, or PB EPO-R (Δ281–300), 50% of surface bound ^{125}I-EPO was internalized after 20 min, as compared to 25% in those expressing 1–251, 1–257 or 1–267 EPO-R. Hence, our data indicate that the sequence residing between amino acid residues 276 and 267 may be important for internalization of the EPO-R.

3.3. Expression and metabolism of EPO-Rs

Expression of the EPO-R mutants was examined by Western blot analysis (Fig. 5). The EPO-Rs were immunoprecipitated from lysates of transiently transfected COS 7 cells and subjected to digestion with Endo H as indicated. All truncated EPO-Rs, similarly to wt EPO-R and PB EPO-R (Δ281–300), were poorly processed and their great majority was sensitive

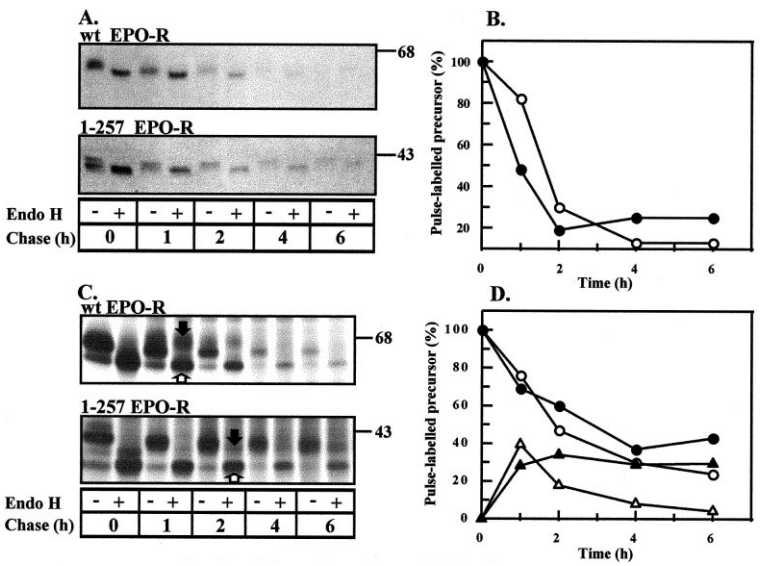

Fig. 6. Maturation and degradation of 1–257 EPO-R and wt EPO-R. COS 7 cells (A,B) or Ba/F3 cells (C,D) expressing wt EPO-R or 1–257 EPO-R were pulse labeled with [^{35}S]methionine/cysteine (30 min or 15 min for COS 7 and Ba/F3 cells, respectively), and then chased for the indicated periods of time in medium, as indicated. Detergent extracts of the cells were immunoprecipitated with an antiserum directed against the EPO-R COOH terminus (for wt EPO-R), or against the extracellular domain (for 1–257 EPO-R). The immunoadsorbed material was divided into two aliquots which were incubated overnight with either no addition (−) or with Endo H (+). Samples were then subjected to electrophoresis using SDS-PAGE, fluorographed and subjected to autoradiography. Panels B and D represents densitometric scans of the autoradiograms depicted in A and C respectively. Panel B represents bands corresponding to Endo H sensitive EPO-R in A, normalized to the amount of Endo H sensitive EPO-R present at 0 h of chase, Endo H sensitive wt EPO-R (empty circles), and Endo H sensitive 1–257 EPO-R (full circles). Panel D represents bands corresponding to Endo H sensitive wt EPO-R (empty circles), Endo H sensitive 1–257 EPO-R (full circles), Endo H resistant EPO-R (empty triangles) and Endo H resistant 1–257 EPO-R (full triangles).

to Endo H treatment (Fig. 5), corroborating the notion that they were preferentially localized in the endoplasmic reticulum (ER) [11]. Endo H-resistant EPO-R species were rarely detected, as previously noted for the wt EPO-R expressed in COS 7 cells [11]. Thus, the higher cell surface expression of 1–251, 1–257 and 1–267 EPO-Rs in COS 7 cells is not accompanied by the presence of detectable Golgi-processed EPO-Rs.

Increased metabolic stability of 1–251, 1–257 and 1–267 EPO-Rs may also contribute to their high cell surface expression. To address this possibility we compared the degradation of one representative EPO-R mutant, 1–257 EPO-R, to that of wt EPO-R. This experiment was performed both in transiently transfected COS 7 cells and in Ba/F3 cells stably expressing these receptors. Cells were pulse labeled with [^{35}S]cysteine/methionine and chased in the presence of non-labeled amino acids. At different times of the chase, cells were lysed and the EPO-Rs immunoprecipitated with antibodies directed against the extracellular domain of the EPO-R (for 1–257 EPO-R) or antibodies directed against the cytoplasmic domain of the EPO-R (for wt EPO-R). This experiment revealed that in COS 7 cells (Fig. 6A), similarly to the Western blot analysis (Fig. 5), the Endo H sensitive species of both wt EPO-R and 1–257 EPO-R were the predominant EPO-R species present during all time points of the chase. The half-lives of 1–257 EPO-R and of wt EPO-R were virtually identical, and were in the range of 1 h, as previously demonstrated for the wt EPO-R [25,29]. In Ba/F3 cells stably expressing the wt EPOR or 1–257 EPO-R, both Endo H sensitive and Endo H resistant EPO-R species were detected (Fig. 6C). The half-lives of the Endo H sensitive wt and 1–257 EPO-Rs were similar, whereas the Endo H resistant 1–257 EPO-R was more stable than the Endo H resistant wt EPO-R (Fig. 6D). The increased metabolic stability of Endo H resistant 1–257 EPO-R may also contribute to the increased cell surface expression of 1–257 EPO-R.

4. Discussion

This study demonstrates that cytoplasmically truncated EPO-Rs containing, 4, 10 or 20 amino acids of the cytoplasmic domain, are expressed at high levels on the cell surface and are deficient in their capacity to internalize membrane bound ^{125}I-EPO. While the kinetics of ^{125}I-EPO internalization by 1–251, 1–257 and 1–267 EPO-Rs were similar to wt EPO-R, the level of internalized ligand by these mutants was two-fold lower than that of wt EPO-R, PB EPO-R (Δ281–300), 1–306 EPO-R or 1–276 EPO-R. Hence the sequence FEGLFTTHK, flanked by amino acid residues 267 and 276, may participate in downregulation of cell surface EPO-R. 1–306 and 1–276 EPO-Rs endocytosed EPO similarly to wt EPO-R, suggesting that the Tyr residues in the EPO-R cytoplasmic domain do not affect the kinetics of EPO endocytosis. In agreement with this result, an EPO-R mutant in which all cytoplasmic tyrosine residues were replaced by phenylalanines displayed ^{125}I-EPO endocytosis with a similar kinetics to that of the wt EPO-R (data not shown).

Truncated EPO-Rs (1–251, 1–257 and 1–267 EPO-Rs) were expressed at higher levels than wt EPO-R 1–306 and 1–276 EPO-R, on the surface of transiently transfected COS 7 cells, as monitored by surface bound ^{125}I-EPO at 37°C. A similar pattern of cell surface EPO-R levels was also observed at 4°C. These results suggest that 1–251, 1–257 and 1–267 EPO-Rs are deficient not only in receptor mediated endocytosis of the ligand, but also in ligand independent, constitutive internalization of the receptors. Interestingly, PB EPO-R (Δ281–300) was also expressed at higher levels (1.5–3-fold) at the cell surface compared to wt, 1–306 and 1–276. Yet, its ability to endocytose EPO was not impaired (Figs. 3 and 4), indicating that high cell surface expression of the receptor per se, does not necessarily predict impaired ligand endocytosis. Furthermore, since the level of surface PB EPO-R (Δ281–300), was similar to that of the endocytosis deficient EPO-R mutants, the decrease in the ability of these mutants to endocytose ligand is not due to saturation of the endocytic pathway, resulting from increased expression of the receptors. Western blot analysis of the EPO-R mutants expressed in COS 7 cells indicated that all the mutants employed in this study were poorly processed, as previously noted for the wt EPO-R [11]. Thus, the higher cell surface expression of the truncated EPO-R mutants and of PB EPO-R (Δ281–300) in COS 7 cells was not accompanied by a detectable increase in the levels of Golgi processed Endo H resistant EPO-R species. Increased cell surface expression of 1–257 EPO-R does not result from its faster transport to the membrane, since when cell surface EPO-Rs were removed by trypsin treatment at 4°C, the kinetics of recovery of wt and 1–257 EPO-R on the cell surface was identical (data not shown).

Increased metabolic stability of EPO-R may also contribute to increased cell surface expression of the EPO-R mutants. In transiently transfected COS 7 cells, only the Endo H sensitive forms of the EPO-Rs were detected (Fig. 6) probably due to poor processing of the receptors [11]. In metabolic labeling experiments, Endo H sensitive wt EPO-R and 1–257 EPO-R displayed similar metabolic stabilities. This was also the case for 1–251, 1–267 and PB (Δ281–300) EPO-R expressed in COS 7 cells (data not shown). In Ba/F3 cells approximately 40% of both wt and 1–257 EPO-Rs acquired resistance to Endo H. Endo H resistant 1–257 EPO-R was more stable than Endo H resistant wt EPO-R, suggesting that cytoplasmic residues may participate in receptor degradation, similarly to the IL-2 β chain [20]. Hence, at this point we cannot exclude the possibility that other receptor turnover processes may also contribute to the increased cell surface expression and impaired ligand internalization by the EPO-R mutants which lack the 9 amino acid motif.

The current study raises several intriguing questions. Do the truncated receptors (1–251, 1–257 and 1–267 EPO-Rs) direct internalized EPO to intracellular degradation by the same pathway as wt EPO-R? We have shown that the sequence FEGLFTTHK is necessary for EPO internalization, however the question of whether it is sufficient to mediate internalization of membrane proteins which are devoid of endogenous internalization signals [15,30], remains to be addressed. The phenylalanine (F) residues in the above sequence are probably not involved in endocytosis since internalization of ^{125}I-EPO was not impaired in a full length EPO-R mutant in which the two F residues were replaces by alanines (data not shown). Studies are under way to determine the contribution of individual amino acid residues in this sequence to endocytosis of EPO.

Acknowledgements: We thank Dr. J. Ihle for kindly providing the cDNA of PB (Δ281–300), Ms. Hideko Ohgusu for the preparation of 1–267 EPO-R and Profs. Yoav Henis and Ronit Sagi-Eisenberg

for critically reviewing the manuscript. This work was supported by a grant from the Ministry of Science, Israel, within the corporation program with the Science and Technology of Japan, by grants from the Israel Science Foundation administered by the Israel Academy of Sciences and Humanities, the Israel Cancer Research Fund (RCDA), and the Israeli Ministry of Science and Technology to D.N. and by a grant-in-aid from the American Heart Association, Texas Affiliate, Inc. (#96G-1616) to S.S.W. This work was performed in partial fulfillment of the requirements of an M.Sc. degree for I.L. and of Ph.D. degrees for J.C. and L.S.-R.

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