Amino acid residues 268–276 of the erythropoietin receptor contain an endocytosis motif and are required for erythropoietin-mediated proliferation

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Received 11 February 2002; revised 3 April 2002; accepted 3 April 2002

First published online 16 April 2002

Edited by Felix Wieland

Abstract Erythropoietin (EPO) promotes viability, proliferation and differentiation of mammalian erythroid progenitor cells via its specific cell surface receptor (EPO-R). We have previously shown that truncated EPO-Rs containing 267 amino acids or less were defective in internalization of ^{125}I-EPO, whereas internalization via a receptor derivative containing 276 amino acids was unaffected, thus directing focus to the nine amino acid residues FEGLFTTHK at positions 268–276 [Levin, Cohen, Supino, Yoshimura, Watowich, Neumann, FEBS Lett. 427 (1998) 164–170]. Here, a panel of EPO-R mutants was generated to determine the role of these residues in EPO endocytosis, down regulation of cell surface receptors and EPO-mediated signaling. While linking amino acid residues 268–276 to a truncated EPO-R (Δ+9 EPO-R) conferred both ligand uptake and ligand-independent down regulation of the respective receptor from the cell surface, Phe 272 was crucial for EPO endocytosis but not for ligand-independent down regulation. Additional receptor motifs probably play a role in EPO endocytosis and receptor down-regulation, as these processes were not adversely impaired in Δ268–276 EPO-R. A central role of residues 268–276, in particular Phe, was demonstrated by the inability of Δ268–276 and F268,272A EPO-Rs to support EPO-mediated signal transduction. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Erythropoietin; Erythropoietin receptor; Endocytosis; Tyr-phosphorylation

1. Introduction

Proliferation of immature red blood cells and their differentiation into mature erythrocytes are mediated by binding of erythropoietin (EPO) to its cell surface receptor (EPO-R) a member of the superfamily of cytokine receptors [1]. Unlike receptor Tyr kinases, the cytosolic domain of cytokine receptors is devoid of kinase activity and is phosphorylated by Janus kinases (JAKs) following ligand binding [2]. The low cell surface expression of EPO-R suggests that a tight control mechanism operates to regulate erythroid proliferation and differentiation in response to physiological levels of EPO under normal or hypoxic conditions [3,4]. Receptor internalization and ligand-mediated endocytosis are pivotal processes, which regulate cell surface receptors. Accumulating evidence suggests that endocytosis of membrane proteins is mediated by specific sequence(s) in their cytosolic domains, which interact with auxiliary cellular molecules [5–7]. The sequences include di-Leu-based motifs [8–10], Tyr-based motifs [6,11] and a third group of sequences containing various features such as clustered acidic residues [12], or mono amino acid-based motifs [13].

The capacity of a series of cytoplasmic tail truncated EPO-R variants to internalize ^{125}I-EPO was measured by us [14] and others [15] demonstrating that a membrane proximal cytosolic domain of the EPO-R was the minimal region required for receptor-mediated ligand internalization. Ligand binding to the EPO-R was found to accelerate the rate of receptor internalization of cell surface receptor from $T_{1/2}$ of 3 h in the absence of ligand to 15 min in its presence [16]. The contribution of selected EPO-R cytosolic regions to ligand-independent internalization of the EPO-R is not known. The present study was designed to examine the involvement of the cytosolic sequence FEGLFTTHK (amino acid residues 268–276) in the internalization of EPO via cell surface EPO-R, in constitutive down regulation of surface receptors and in EPO-mediated signal transduction. The results showed that the addition of amino acid residues 268–276 to an EPO-R which is devoid of the cytosolic domain (Δ+9 EPO-R) improved the capacity of the respective receptor to endocytose ^{125}I-EPO as well as conferred its constitutive down regulation from the cell surface. Mutation of Phe to Ala at position 272 (Δ+9 (F272A) EPO-R) adversely affected receptor-mediated ligand endocytosis, but did not alter the $T_{1/2}$ of cell surface receptor in the absence of ligand, suggesting that these two processes require distinct structural features of the receptor. Excision of amino acids 268–276 from wild type (wt) EPO-R did not impair ^{125}I-EPO internalization via the receptor, nor alter the $T_{1/2}$ of cell surface receptor in the absence of ligand, yet it abrogated binding of the receptor to JAK2 and EPO-mediated proliferation of Ba/F3 cells. Phe residues within the segment 268–276 seem to play a major role in EPO-mediated signal transduction.

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Abbreviations: EPO, erythropoietin; EPO-R, erythropoietin receptor; JAK2, Janus kinase 2

PII: S0014-5793(02)02691-1

2. Materials and methods

2.1. Materials

Purified recombinant Human EPO was a kind gift from Kirin Inc., Japan. All other materials were obtained from sources previously listed [17].

2.2. Antibodies

Rabbit antibodies directed against the extracellular domain of the murine EPO-R [18] were used at 1:500 and 1:2000 dilutions for immunoprecipitations and Western blot analysis, respectively, of EPO-R cloned in pXM and expressed in COS 7 or Ba/F3 cells. Antibodies against JAK2 and EPO-R from Upstate Biotechnology were used for analysis of Ba/F3 cells stably transfected with EPO-R cDNAs cloned in the bicistronic retroviral vector, pMX-IRES-GFP. Anti-P-Tyr antibody (4G10), was obtained from Upstate Biotechnology.

2.3. Construction of mutant EPO-R cDNAs

1–257, 1–267 and 1–276 EPO-Rs were previously described [14]. The cDNAs of Δ268–276 or Δ256–267 EPO-Rs were generated using the Altered SitesII in Vitro Mutagenesis System (Promega) [19] and the following two oligonucleotides, respectively: (A) 5′-ACGCTGCAGCAGCCACAGCTGGAAGTTACCCTCGCTCTCTGGGCTTGGGATGCCAGGCCA-3′; (B) 5′-CTTGTGGGTGGTGAAGAGACCCTCAAACTGCTGCAGAGTCCGGCGGTGGGACAG-3′. EPO-R mutants (Δ+9, Δ+9 (F272A), 1–276 (F272A)) were prepared by the PCR methodology, using the forward primer 5′-GAGTCGGTACCTGAAGCTAGGGCTGC-3′, which contains a *Kpn*I site in its 5′-end. Δ+9 EPO-R (an EPO-R truncated at amino acid 255 onto which the sequence motif 268–276 was attached) was generated using Δ256–267 EPO-R as a template and the reverse primer 5′-ATGCGGAATTCCTACTTGTGGGTGGTGAAGAGACC-3′. Δ+9(F272A) and 1–276 (F272A) EPO-Rs were generated using Δ+9 and 1–276 EPO-Rs as templates, respectively and the reverse primer 5′-ATGCGGAATTCCTACTTGTGGGTGGTGGCGAGACCCTCAAAC-3′. Both reverse primers contained an *Eco*RI site for subcloning into the pXM vector.

F268,272A EPO-R was generated by using the forward primer 5′-AGATCTGGCCTGGCATCCCAAGCCCAGAGAGCGAGGCTGAGGGTCTCGCCA-3′ along with the reverse primer from the EPO-R sequence 5′-GGCTGGATCCCTAGGAGCAGGCCAC-3′ to generate a *Bgl*II–*Eco*RI PCR fragment which was exchanged with the corresponding region of the wt EPO-R cDNA in pXM.

Wt EPO-R, Δ268–276 and Δ256–267 EPO-Rs were also cloned in the pMX-IRES-GFP bicistronic retroviral vector upstream of the internal ribosome entry site (IRES) [20,21]. All constructs were verified by sequencing.

2.4. Cell culture and transfection

COS 7 cells were grown and transfected as previously described [22].

Ba/F3 cell lines stably expressing Δ268–276 or Δ256–267 EPO-Rs cloned in pXM were generated as described [23]. Ba/F3 cell lines stably expressing wt or Δ268–276 or Δ256–267 EPO-Rs and green fluorescent protein (GFP) were obtained as described [24]. It was previously shown [20,21] that over a 50-fold range there is correlation between the level of GFP (monitored by FACS) and that of the EPO-R. Hence, cells were sorted by FACS to a similar amount of GFP, indicating that they express the corresponding EPO-R mutants to a similar level.

Ba/F3 cell lines stably expressing wt EPO-R cDNA were maintained in RPMI supplemented with 10% FCS (v/v) and 0.25 U/ml EPO or 10% conditioned medium from WEHI 3B cells as a source of interleukin (IL)-3 for mutant EPO-Rs.

2.5. Iodination of EPO, binding and internalization

Recombinant EPO labeling and internalization were determined as described [22,25], respectively. $T_{1/2}$ of cell surface EPO-R was determined essentially according to [16,26]. COS 7 cells transfected with EPO-R cDNAs were incubated in the presence of 100 μg/ml cycloheximide (CHX) at 37°C. Cells were collected at different time points after CHX addition and incubated over night at 4°C with ^{125}I-EPO to determine the levels of cell surface EPO-Rs.

2.6. Metabolic labeling, immunoprecipitation and Western blot analysis

COS 7 cells transiently transfected with EPO-R cDNAs (2×10^6 cells for each time point) were labeled with [^{35}S]Cys/Met for 30 min, solubilized and subjected to immunoprecipitation as described [27]. Western blot analysis was performed as described [24].

2.7. EPO-dependent proliferation

Cell proliferation in response to EPO was determined by methylthiazol–tetrazolium analysis [28].

3. Results

3.1. ^{125}I-EPO internalization mediated by EPO-R mutants

A panel of EPO-R mutants was constructed to determine whether amino acid residues (FEGLFTTHK) at position 268–276 are sufficient to mediate internalization of EPO and whether individual amino acid residues in this sequence are required for this process (Fig. 1A).

We have previously shown that 1–267 EPO-R (Fig. 1A) is

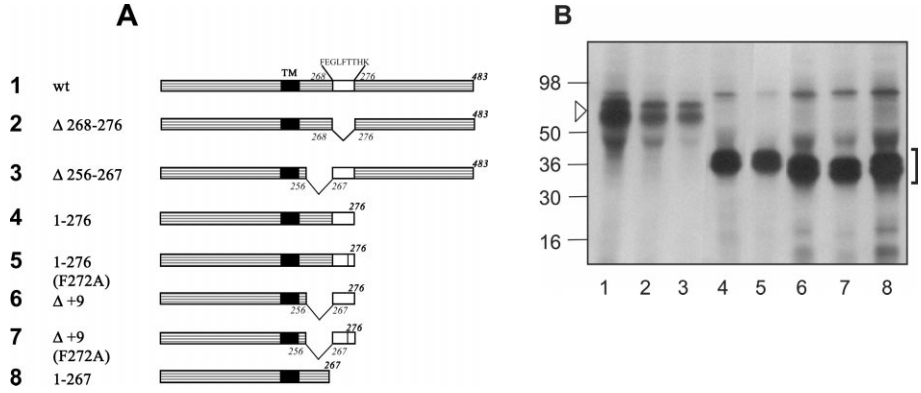

Fig. 1. EPO-R variants and their expression in COS 7 cells. A: Schematic representation of EPO-Rs. The EPO-R mutants employed in this study were 1– wt EPO-R, 2– Δ268–276 EPO-R, 3– Δ256–267 EPO-R, 4– 1–276 EPO-R, 5– 1–276 (F272A) EPO-R, 6– Δ+9 EPO-R, 7– Δ+9 (F272A) and 8– 1–267 EPO-R. The transmembrane (TM) region is indicated. Amino acids at positions 268–276 are depicted as a white box, the F to A substitution at position 272 is indicated as a vertical line, deletions are indicated. B: Expression of EPO-Rs in COS 7 cells. COS 7 cells transiently transfected with EPO-R cDNAs were pulse labeled for 30 min with [^{35}S]Cys/Met and 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. Subsequently, samples were separated by 10% SDS–PAGE, and autoradiographed. Molecular weight markers in kDa are depicted on the left. The EPO-R derivatives in lanes 1–8 correspond to mutants 1–8, depicted in (A). The bracket points at truncated EPO-R derivatives (lanes 4–8), the arrowhead points at wt, Δ268–276 and Δ256–267 EPO-Rs (lanes 1–3).

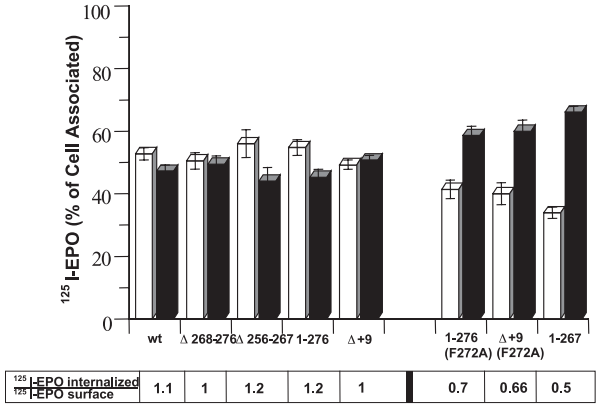

Fig. 2. Binding and internalization of ^{125}I-EPO by EPO-Rs. COS 7 cells expressing EPO-R variants were incubated in the presence of ^{125}I-EPO for 75 min at 37°C. Specific binding was determined by subtracting the measured counts in the presence of cold ligand from those in its absence. The filled and empty bars represent the ratio of cell-surface bound and internalized ^{125}I-EPO respectively, to the total amount of ^{125}I-EPO, which was associated with the cells. The ratio of internalized to surface ^{125}I-EPO for each EPO-R represents the mean of at least three identical experiments, performed in triplicates.

defective in its capacity to endocytose EPO, whereas 1–276 EPO-R (Fig. 1A) can internalize EPO to a similar extent as wt EPO-R [14]. To address whether these nine amino acids confer receptor-mediated ^{125}I-EPO internalization we generated an EPO-R mutant (Δ+9 EPO-R; Fig. 1A), truncated at amino acid 255 onto which amino acid residues 268–276 were ligated. The rationale for preparing this mutant was double: (a) to rule out that impaired endocytosis by 1–267 EPO-R was merely due to the extent of truncation of the EPO-R; (b) to establish whether amino acids 268–276 on their own, were sufficient to confer receptor-mediated ^{125}I-EPO internalization. Even though 1–267 EPO-R was defective in its capacity to internalize ^{125}I-EPO [14], it was necessary to establish that the membrane proximal cytosolic amino acids located N-terminus to residue 267 are not required for EPO endocytosis in context of the full-length EPO-R. For that purpose, we constructed Δ256–267 EPO-R (Fig. 1A). It also served as a control for Δ+9 EPO-R, since both receptors (Δ256–267 EPO-R and Δ+9 EPO-R) lacked residues 256–267; in Δ256–267 EPO-R the remaining intact cytosolic domain was attached whereas in Δ+9 EPO-R only the nine amino acid residues FEGLFTTHK were attached. To determine whether F272 contributes to the internalization process, 1–276 (F272A) and Δ+9 (F272A) EPO-Rs were generated (Fig. 1A). In addition, Δ268–276 EPO-R (Fig. 1A) was engineered to resolve the contribution of amino acids 268–276 to EPO endocytosis and signaling in context of the full-length receptor.

EPO endocytosis via the EPO-R was essentially similar in different cellular systems [14,15], supporting the notion that this process relies mainly on intrinsic properties of the receptor molecule and not on the cell type in which it is expressed. We have previously shown [14] that while the kinetics of ^{125}I-EPO internalization by EPO-R and truncated EPO-Rs were similar, the level of maximum endocytosis was lower in the truncated, endocytosis-deficient EPO-R mutants. Thus, there was a correlation between the general profile of maximum endocytosis when one wave of endocytosis was measured and when the experiment was performed for 75 min at 37°C. Thus, here we measured continuous internalization of ^{125}I-EPO in COS 7 cells transfected with the panel of EPO-Rs depicted in Fig. 1A. Expression of the corresponding receptors was verified by immunoprecipitation of metabolically labeled EPO-Rs (Fig. 1B).

COS 7 cells transiently transfected with the panel of EPO-R mutants (Fig. 1A) were incubated at 37°C in the presence of ^{125}I-EPO for 75 min and the amount of internalized versus cell surface-associated ^{125}I-EPO was determined. Wt and 1–276 EPO-Rs were employed as positive controls in comparison to 1–267 EPO-R, which served as a reference for an endocytosis-defective receptor. Results are plotted as the percent of cell surface associated or intracellular ^{125}I-EPO, with respect to the total amount of cell-associated (surface plus intracellular) ^{125}I-EPO. As shown in Fig. 2 the ratio of internalized ^{125}I-EPO to that bound on the cell surface was approximately 1 for wt, Δ268–276, Δ256–267, 1–276 and Δ+9 EPO-Rs, whereas the ratio ranged from 0.5 to 0.7 for 1–257 (data not shown and [14]), 1–267, Δ+9 (F272A), and 1–276 (F272A) EPO-Rs.

The fact that ^{125}I-EPO internalization via Δ+9 EPO-R was essentially similar to wt EPO-R implies that the membrane proximal cytosolic amino acid stretch 268–276 harbors a sequence or structure motif that confers receptor-mediated ligand endocytosis. Impaired endocytosis of ^{125}I-EPO via Δ+9 (F272A) points out that Phe 272 contributes to this process. Deletion of residues 268–276 from the full-length EPO-R did not abrogate ^{125}I-EPO internalization, suggesting the presence of additional endocytosis motifs in the EPO-R cytosolic domain.

3.2. Down regulation of cell surface EPO-Rs in the absence of ligand

To determine whether amino acids 268–276 are also involved in constitutive ligand-independent down regulation of cell surface EPO-R, we measured down regulation of cell surface EPO-Rs in the absence of ligand under conditions in which protein synthesis is blocked [16] (Fig. 3). It should be noted that these experimental conditions do not account for

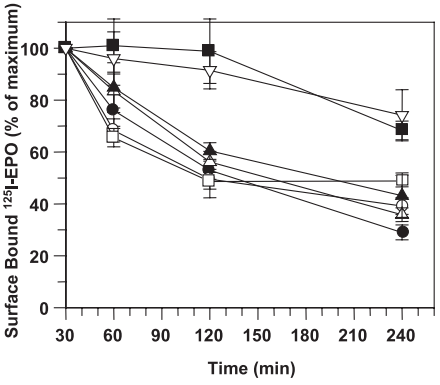

Fig. 3. Constitutive down regulation of cell surface EPO-Rs. COS 7 cells transfected with EPO-R cDNAs were incubated in the presence of CHX (100μM) at 37°C for the duration of the experiment. Cells were collected 30, 60, 120 and 240 min (8×10^6 cells for each time point) following addition of CHX. Cells were subjected to ^{125}I-EPO binding at 4°C. The graph represents the means (±S.E.M.) of three identical experiments. Filled squares, triangles, circles and empty inverted triangles represent 1–267, Δ+9 (F272A), wt and 1–257 EPO-Rs, respectively. Empty squares, triangles and circles represent 1–276, Δ+9 and Δ268–276 EPO-Rs, respectively.

differences between the receptor mutants, which may include the rate of their arrival to the cell surface or later sorting. Transfected COS 7 cells were incubated with CHX, and cells were collected 30, 60, 120 and 240 min thereafter. Binding of ^{125}I-EPO was performed at 4°C for at least 10 h, thereby reaching saturation of cell surface receptors. The results of this experiment (Fig. 3) demonstrate that the rate of down regulation is most aberrant for 1–257 and 1–267 EPO-Rs, which once at the cell surface appear to be rather stable. These data are in line with the higher cell surface levels of these EPO-R derivatives [14]. Cell surface 1–257 and 1–267 EPO-Rs declined by 30% after 4 h of CHX treatment, probably reflecting 'bulk internalization', whereas all the other EPO-Rs declined by 50–60%. Since down regulation of cell surface Δ+9 EPO-R was similar to that of wt EPO-R, we suggest that amino acid residues 268–276 may also contribute to ligand-independent down regulation. The profile of Δ268–276 EPO-R down regulation implies that additional determinants of the EPO-R are involved in this process. Notably, cell surface Δ+9 (F272A) EPO-R was down regulated with similar kinetics as wt EPO-R. Therefore, unlike its contribution to the ligand-mediated endocytosis, F272 does not seem essential for ligand-independent EPO-R down regulation.

3.3. Involvement of amino acids 256–276 in EPO-mediated signal transduction

To resolve whether amino acid residues 268–276 of the EPO-R are essential for signal transduction via the receptor, the IL-3-dependent pro-B cell line Ba/F3 was employed. When these cells are transfected with the EPO-R cDNA they can respond to EPO with faithful signal transduction, proliferation and partial erythroid differentiation [29]. This experimental system has been extensively utilized for studying the biochemistry, trafficking and signal transduction via the EPO-R. Ba/F3 cells were stably transfected with the cDNAs of Δ256–267, Δ268–276 and wt EPO-Rs in pXM. Cell lines were generated from each transfection, and subjected to biochemical analysis. Both Δ256–267 and Δ268–276 EPO-Rs expressing Ba/F3 cells grew in WEHI containing media, (data not shown) yet their proliferation in EPO was abrogated (Fig. 4A). Cell surface levels of the EPO-R derivatives on the Ba/F3 cells were similar (data not shown), as were their total cellular levels (Fig. 4B).

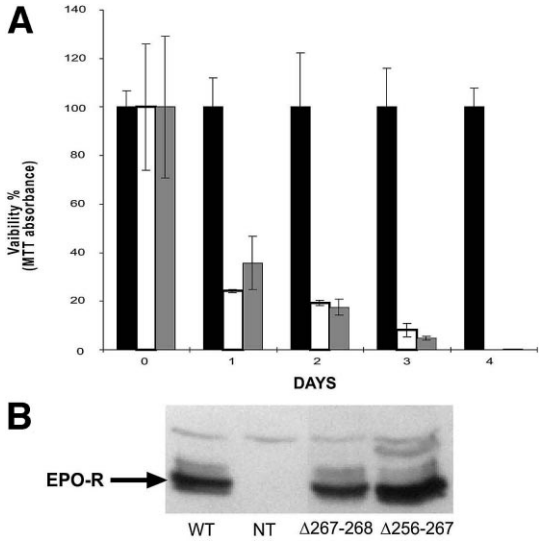

Fig. 4. Residues 268–276 of the EPO-R are required for EPO-mediated proliferation. Ba/F3 cells expressing wt, Δ256–267 or Δ268–276 EPO-Rs cloned in pXM were cultured in the presence of EPO and proliferation was determined by the methylthiazol–tetrazolium method (A) [43]. The white and gray bars represent proliferation of Ba/F3 cells expressing Δ256–267 or Δ268–276 EPO-Rs, respectively, as calculated with respect to the proliferation of Ba/F3 cells expressing wt EPO-R, referred to as 100% (black bars). Receptor expression was verified by Western blot analysis with antibodies directed against the extracellular portion of the EPO-R (B). Non-transfected Ba/F3 cells (NT) are depicted as control.

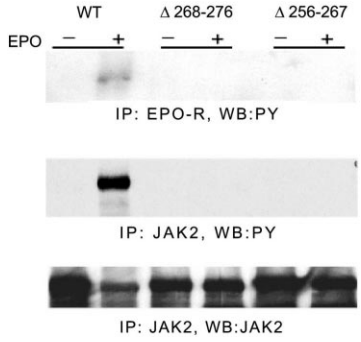

Fig. 5. Residues 256–276 of the EPO-R are required for EPO-mediated activation of JAK2. Ba/F3 cells expressing wt or mutant EPO-Rs, cloned in the pMX-IRES-GFP bicistronic retroviral vector, were depleted of IL-3 for 4 h prior to stimulation with 100 U/ml EPO for 7 min. Cell lysates were immunoprecipitated with antibodies against EPO-R or JAK2 followed by Western blot analysis using anti-P-Tyr (PY) or anti-JAK2 antibodies as indicated.

The amino acid deletion in Δ256–267 EPO-R spans the majority of box 1 [30], required for JAK2 activation, thereby explaining its inability to support EPO-mediated proliferation. Amino acid residues 268–276 are located penultimate to box 1 in the segment bridging box 1 and box 2. This segment also contains Trp at position 282, which is crucial for EPO-mediated signal transduction [31]. The inability of Δ268–276 EPO-R to support EPO-mediated proliferation of Ba/F3 cells points out the involvement of this segment in EPO-mediated signaling. This is, in addition to amino acids 280–300, the deletion of which was found to block EPO-mediated signal transduction via the respective receptor (Δ280–300 EPO-R (PB)) [32].

As a first step towards characterizing the molecular block in signal transduction via Δ256–267 and Δ268–276 EPO-Rs we examined the ability of these EPO-Rs to activate JAK2. Using bicistronic retrovirus vectors, we stably expressed wt, Δ256–267 and Δ268–276 EPO-Rs in Ba/F3 cells. These vectors contain the IRES and GFP downstream; the level of expression of GFP (monitored by FACS) and that of the EPO-R are strongly correlated [21,24]. Ba/F3 cells expressing wt, Δ256–267 or Δ268–276 EPO-Rs were sorted by flow cytometry and subjected to stimulation with 100 U/ml of EPO for 7 min (Fig. 5). Cell lysates were immunoprecipitated with anti-EPO-R or anti-JAK2 antibodies. The immunoprecipitates were subjected to Western blot analysis with anti-P-Tyr antibodies (upper two panels), or anti-JAK2 antibodies (lower panel). As shown in Fig. 5, stimulation of Ba/F3 cells expressing Δ256–267 or Δ268–276 EPO-Rs did not result in Tyr phosphorylation of either EPO-R (Fig. 5, upper panel) or of JAK2 (Fig. 5, middle panel). The Western blot of JAK2 immunoprecipitates was subsequently probed with anti-JAK2 antibodies (Fig. 5, lower panel). Hence, the inability of ligand-bound Δ256–267 and

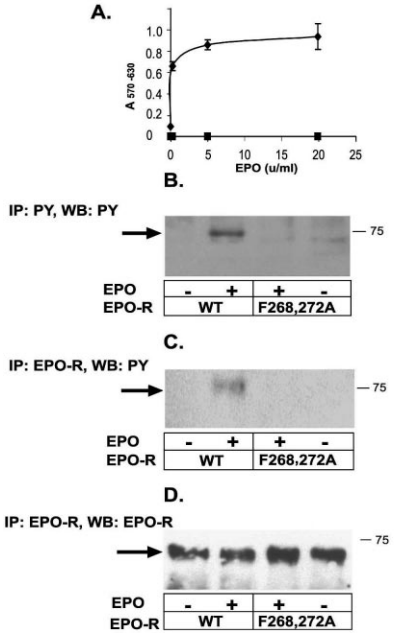

Fig. 6. F268,272A EPO-R does not support proliferation of Ba/F3 cells in EPO. A: Ba/F3 cells expressing F268,272A or wt EPO-Rs cloned in pXM (squares and circles, respectively; 0.8×10^6 cells/ml) were incubated in the presence of increasing concentrations of EPO for 24 h. Cell proliferation was determined by the methylthiazol–tetrazolium method. The experiment represents one of four identical experiments performed in triplicate. Ba/F3 cells expressing F268,272A or wt EPO-Rs were treated with EPO (100 U/ml) for 10 min. Cell lysates were immunoprecipitated with anti-PY (B), or anti-EPO-R (C,D) antibodies, and separated on 10% SDS–PAGE gel, followed by immunoblot analysis with anti-PY (B,C) or anti-EPO-R (D) antibodies.

Δ268–276 EPO-Rs to mediate JAK2 Tyr phosphorylation as well as their inability to undergo EPO-induced Tyr phosphorylation was consistent with their inability to proliferate in EPO (Fig. 4).

To pinpoint individual amino acid residues within the segment 268–276 of the EPO-R which are crucial for signal transduction, we mutated both Phe residues 268 and 272 of the EPO-R to Ala. Ba/F3 cell lines stably expressing F268,272A EPO-R in pXM were generated, and their proliferation in a wide range of EPO concentrations as well as EPO-induced Tyr phosphorylation of the receptor was determined (Fig. 6). As shown in Fig. 6A, F268,272A EPO-R did not support proliferation of Ba/F3 cells in EPO. EPO-mediated Tyr phosphorylation of cellular proteins (Fig. 6B) as well as EPO-mediated Tyr phosphorylation of F268,272A EPO-R was not detected (Fig. 6C). These data support the notion that Phe residues 268 and/or 272 are essential for EPO-mediated signal transduction.

4. Discussion

Previous studies by us [14] and by others [15], employing truncated EPO-Rs have identified a membrane proximal intracellular motif, which is essential for EPO internalization. In this study we examined the capacity of this membrane proximal motif (amino acids 268–276) to confer EPO endocytosis and down regulate cell surface EPO-R and demonstrated its requirement for EPO-mediated signal transduction.

The amino acid stretch 268–276 harbors an endocytosis motif as shown by its capacity to augment ^{125}I-EPO internalization when attached to a truncated EPO-R (Δ+9) compared to ligand internalization mediated by 1–267 EPO-R. Di-Leu-based motifs [33,34], as well as Phe-based motifs have been documented to participate in endocytosis [35]. Hence, amino acid residues Leu–Phe (positions 271, 272) within the amino acid stretch 268–276 may constitute an endocytosis motif [36]. Impaired ligand internalization via Δ+9 (F272A) and 1–276 (F272A) EPO-Rs suggests that Phe 272 is crucial for ligand-mediated endocytosis, conferred by this motif. It has been shown that Ser phosphorylation upstream of di-Leu motifs may contribute to the capacity of cell surface receptors to internalize the ligand [33]. The question of whether the two Ser residues located upstream to amino acid residues 268–276 are phosphorylated remains to be addressed. Tyr-based motifs probably do not play a role in EPO endocytosis via the receptor, since an EPO-R in which all cytosolic Tyr residues were replaced by Phe endocytosed the ligand to the same extent as wt EPO-R [15]. Furthermore, 1–276 EPO-R, which endocytoses EPO to a similar extent as wt EPO-R, does not contain any Tyr residues [14]. Thus, it appears that endocytosis of EPO via the receptor occurs even with constructs which do not elicit signaling [15], as was also shown for the growth hormone receptor [37].

Using a panel of truncated EPO-R mutants others [15] and we [14] have suggested the existence of endocytosis motifs in the membrane proximal intracellular region of the EPO-R. Both studies however did not address the activity of this segment on its own and in context of the full-length receptor, points which are tackled here. Although residues 268–276 harbor a motif, which mediates endocytosis of EPO and confers down regulation of cell surface receptors, deletion of this region from the intact EPO-R did not inhibit endocytosis of EPO or down regulation of the receptor from the cell surface. These data suggest that other cytosolic domains in the EPO-R are involved in ligand endocytosis and down regulation of cell surface receptor and function either in conjunction to 268–276 or assume activity upon excision of 268–276 from the receptor. The uninterrupted endocytosis of EPO via Δ268–276 EPO-R is in line with other experimental systems in which excision of an internalization determinant from the intact receptor did not impair ligand internalization, pointing to the presence of more than one major signal for internalization [9].

Amino acids 268–276 comprise a portion of the segment, which bridges the box 1 and the box 2 regions of the EPO-R. The contribution of the cytosolic region flanking box 1 and box 2 of the EPO-R for JAK2 activation has previously been suggested [32,38]. Excision of amino acid residues 280–300, or amino acid residues 267–293 penultimate to box 2, abolished EPO-mediated proliferation [32,38] and EPO-induced JAK2 binding and Tyr phosphorylation of the EPO-R [32].

The finding that a truncated EPO-R which contains box 1 and the domain flanking box 1 and box 2, yet lacks box 2, possesses EPO-mediated mitogenic activity [39] supports the notion that the segment 268–276 is essential for signal transduction. The lack of response to EPO of Ba/F3 cells expressing F268,272A EPO-R demonstrates the participation of these Phe residues in EPO-driven signal transduction. Our data are in line with the recent demonstration that residues 259–284 harbor essential motifs for JAK2 binding and activation [40].

Amino acid residues 268–276 also conferred ligand-independent down regulation from the cell surface of an EPO-R,

which lacks the vast majority of the cytosolic domain (Δ+9 EPO-R). As excision of this segment did not impair constitutive down regulation of the respective receptor molecule (Δ268–276 EPO-R) we postulate that additional receptor segments participate in this process. Unlike the case for receptor-mediated internalization of ^{125}I-EPO, mutation of F272 to A did not abrogate constitutive down regulation of cell surface Δ+9 EPO-R (F272A) or 1–276 (F272A) (data not shown), suggesting the involvement of distinct structural requirements and/or intracellular pathways in constitutive versus ligand-mediated receptor internalization. One may speculate that Phe 272 participates in the generation of a signaling competent conformation of the EPO-R. This conformation may also be required for receptor-mediated ligand endocytosis but not for constitutive down regulation of cell surface EPO-Rs.

For the growth hormone and IL-2 receptors, two other members of the cytokine receptor family, it has been demonstrated that ligand internalization may occur via detergent-resistant membrane domains [41,42]. These studies and our data direct future attempts to determine the route of EPO endocytosis and to compare the intracellular fate of EPO, which is internalized by the various EPO-R mutants.

Acknowledgements: We thank Prof. R. Sagi-Eisenberg for critically reviewing the manuscript, Lia Supino-Rosin for many helpful suggestions as well as helping in many practical aspects and Prof. Harvey F. Lodish for enlightening discussions. We gratefully acknowledge the assistance of Eran Barzilay and Mazal Reyna. This work was supported by a Grant from the Israel Science Foundation administered by the Israel Academy of Sciences and Humanities (574/99 18.2) and the Israel Cancer Research Fund (RCDA) to D.N. L.J.H. holds a postdoctoral fellowship from the National Institutes of Health. This work was performed in partial fulfillment of the requirements of an M.Sc. degree for G.F.A. and of Ph.D. degrees for R.E.-L. and J.C.

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