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Insertion of an NPVY sequence into the cytosolic domain of the erythropoietin receptor selectively affects erythropoietin-mediated signalling and function

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EPO (erythropoietin), the major hormone regulating erythropoiesis, functions via activation of its cell-surface receptor (EPO-R) present on erythroid progenitor cells. One of the most striking properties of EPO-R is its low expression on the cell surface, as opposed to its high intracellular levels. The low cell-surface expression of EPO-R may thus limit the efficacy of EPO that is routinely used to treat primary and secondary anaemia. In a recent study [Nahari, Barzilay, Hirschberg and Neumann (2008) Biochem. J. **410**, 409–416] we have shown that insertion of an NPVY sequence into the intracellular domain of EPO-R increases its cell-surface expression. In the present study we demonstrate that this NPVY EPO-R insert has a selective effect on EPO-mediated downstream signalling in Ba/F3 cells expressing this receptor (NPVY-EPO-R). This is monitored by increased phosphorylation of the NPVY-EPO-R (on Tyr^{479}), Akt, JAK2 (Janus kinase 2) and ERK1/2 (extracellular-signal-regulated kinase 1/2), but not STAT5 (signal transducer and activator of transcription 5), as compared with cells expressing wild-type EPO-R. This enhanced signalling is reflected in augmented proliferation at low EPO levels (0.05 units/ml) and protection against etoposide-induced apoptosis. Increased cell-surface levels of NPVY-EPO-R are most probably not sufficient to mediate these effects as the A234E-EPO-R mutant that is expressed at high cell-surface levels does not confer an augmented response to EPO. Taken together, we demonstrate that insertion of an NPVY sequence into the cytosolic domain of the EPO-R confers not only improved maturation, but also selectively affects EPO-mediated signalling resulting in an improved responsiveness to EPO reflected in cell proliferation and protection against apoptosis.

Key words: apoptosis, erythropoietin, NPXY motif, phosphorylation, signal transduction.

INTRODUCTION

EPO (erythropoietin) is a 34 kDa cytokine required for the survival, proliferation and differentiation of committed erythroid progenitor cells [1]. EPO is produced mainly by the kidney and mediates its function via its receptor, EPO-R, a 62 kDa protein which belongs to the cytokine receptor superfamily [2,3]. EPO-R has no intrinsic kinase activity and therefore relies on the tyrosine kinase JAK2 (Janus kinase 2) to initiate downstream signalling. EPO binding to the receptor induces a conformational change in the EPO-R homodimer which initiates the activation of JAK2 [4–7]. Activated JAK2 phosphorylates the EPO-R at multiple cytoplasmic tyrosine residues, which results in the recruitment to the receptor of SH2 (Src homology 2)-containing proteins such as the STAT5 (signal transducer and activator of transcription 5) transcription factor [8–10], MAPK (mitogen-activated protein kinase) [11–13] and the p85 regulatory subunit of PI3K (phosphoinositide 3-kinase) [7,9,14]. Following the recruitment of p85 to phosphorylated Tyr^{479} of the EPO-R, the catalytic subunit of PI3K is activated, leading to the activation of Akt (also known as protein kinase B) which in turn triggers cell proliferation [15,16], maturation [14,17] and cell survival [18,19]. Recruitment of p85 to the activated receptor can also enhance EPO-R internalization [20].

One of the characteristics of EPO-R is its low cell-surface expression and its high levels in the ER (endoplasmic reticulum) [21]. Increasing the levels of EPO-R are attempted in order to achieve a better response to EPO that is routinely administered to elevate red blood cell counts in patients suffering from kidney failure and to cancer patients undergoing chemotherapy [22]. ER retention of the EPO-R was observed in fetal liver cells [23] and erythroid cell lines [24] that endogenously express the receptor. ER retention was also observed in cell lines such as Ba/F3 cells [25,26] and COS7 cells transfected with EPO-R cDNA [27]. The low surface expression levels of EPO-R are attributed to poor folding of its extracellular domain [27]. Accordingly, targeted mutations in the extracellular domain of the EPO-R were shown to facilitate [27–29] or inhibit [30,31] surface expression of the receptor. The contribution of the intracellular domain of EPO-R to receptor trafficking towards the cell surface is less clear. In that respect, JAK2 was shown to bind to the cytosolic domain of EPO-R and to promote cell-surface localization of the receptor [20,32]. Deletion of the EPO-R intracellular cytosolic domain was associated with higher surface levels of the receptor [33,34], further supporting the role of the EPO-R intracellular domain in its surface expression.

The NPXY motif is a well-characterized internalization signal responsible for clathrin-mediated endocytosis that is present

Abbreviations used: EPO, erythropoietin; EPO-R, EPO receptor; ER, endoplasmic reticulum; ERK1/2, extracellular-signal-regulated kinase 1/2; FCS, fetal calf serum; IL, interleukin; IRS-1, insulin receptor substrate-1; JAK2, Janus kinase 2; LDL, low-density lipoprotein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; PI, propidium iodide; PI3K, phosphoinositide 3-kinase; PTB, phosphotyrosine-binding; rHuEPO, recombinant human EPO; Shc, Src homology and collagen homology; STAT5, signal transducer and activator of transcription 5; wt, wild-type.

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in multiple membrane proteins such as LDL (low-density lipoprotein), insulin, transferrin and IGF-I (insulin-like growth factor I) receptors [35,36]. This motif was shown to play a role in the sorting of newly synthesized proteins to the basolateral membrane [37]. The NPXY motif is also important for protein–protein interactions since it serves as a recognition sequence for the PTB (phosphotyrosine-binding) domain, present in a wide array of proteins [38]. The biological function of the PTB domain is to drive recruitment of signalling adaptors such as IRS-1 (insulin receptor substrate-1) or Shc (Src homology and collagen homology) to NPXY [39,40]. Notably, the PTB domain was shown to bind both phosphorylated and non-phosphorylated NPXY [41].

We have recently demonstrated that insertion of an NPVY sequence into the cytosolic domain of EPO-R, in a region downstream to box2, leads to enhanced maturation and increased surface expression of the EPO-R [42]. In contrast, insertion of an NPVF sequence to the EPO-R cytosolic domain had no affect on maturation and cell-surface levels of EPO-R [42]. In the present study we examined the effect of the NPVY insert on EPO-mediated signalling and cellular responses, as well as the relationship between EPO-R cell-surface levels and downstream signalling.

MATERIALS AND METHODS

Antibodies

The antibodies against phosphorylated STAT5, Akt, ERK1/2 (extracellular-signal-regulated kinase 1/2) and JAK2 were purchased from Cell Signaling Technology. Anti-phospho-EPO-R ($pTyr^{479}$) antibodies were purchased from Santa Cruz Biotechnology and anti-actin antibodies were purchased from Chemicon. Anti-EPO antibodies were purchased from R&D systems.

Plasmids and generation of mutants

The NPVY, NPVF and AAAA sequences were inserted into the EPO-R cDNA in pXM as described previously [42], using the QuikChange® site-directed mutagenesis kit (Stratagene). All constructs were verified by sequencing.

Cell culture and stable transfection

Ba/F3 cells were stably transfected with 40 μg of cDNAs of wt-EPO-R (wild-type EPO-R) or EPO-R mutants by electroporating the cells at 300 V (1500 μF) as described previously [42]. Following transfection, the cells were cultured in RPMI 1640 medium containing 10 % (v/v) FCS (fetal calf serum) and 0.5 units/ml of rHuEPO (recombinant human EPO; Janssen Cilag) in order to generate stable cell lines. As a control for the proliferation and apoptosis experiments, the cell lines were transferred a day prior to the experiment from the EPO-containing medium to RPMI medium containing 10 % FCS and supplemented with 10 % conditioned media from WEHI3B cells as source of IL (interleukin)-3.

EPO-induced EPO-R activation

Ba/F3 cells expressing EPO-Rs (3×10^6 cells for each time point) were starved for 1 h at 37 °C by incubating the cells in RPMI medium without supplements. Subsequently, rHuEPO (50 units/ml) was added to the cells for different time points. Cells were then lysed at 4 °C in 200 μl of lysis buffer [50 mM Tris/HCl (pH 7.4), 1 % Triton X-100, 5 mM iodoacetic acid, 5 mM EDTA and 150 mM NaCl] containing phosphatase (2 mM zinc chloride, 2 mM vanadate, 50 mM sodium fluoride and 20 mM sodium pyrophosphate) and protease inhibitors (Complete Protease Inhibitor Cocktail, Roche Diagnostics). Cell lysates were then centrifuged at 20 800 ***g*** for 10 min and the supernatants were collected, separated on SDS/PAGE gels (7.5 % gel) and subjected to Western blot analysis [43].

EPO binding assay

Surface EPO-R levels in Ba/F3 cells stably expressing wt-EPO-R, NPVY-EPO-R, NPVF-EPO-R, AAAA-EPO-R or A234E-EPO-R were determined by biochemical binding analysis. Cells were starved for 1 h in a medium deprived of EPO and serum prior to incubation for 1 h at 4 °C with 20 units/ml rHuEPO. Cells were then washed three times to remove the unbound EPO. Cell lysates were resolved on SDS/PAGE (7.5% gel) and subjected to Western blot analysis with anti-EPO antibodies (R&D Systems).

Apoptosis assay

Ba/F3 cells expressing EPO-Rs were treated with 50 μM etoposide (Sigma) for 16 h in the presence of 10 % FCS and 0.5 units/ml rHuEPO. Cells were then collected and assayed for Annexin V binding using the Mebcyto apoptosis kit according to the manufacturer's instructions (MBL International). Following incubation for 15 min with anti-Annexin V antibodies and PI (propidium iodide), the cells were sorted by flow cytometry.

Cell-cycle analysis

Ba/F3 cells expressing the EPO-R were cultured for 16 h in the presence of 10 % FCS and rHuEPO at 0.5, 0.05 or 0.01 units/ml. Subsequently, the cells were collected and washed in PBS. Cells were then stained with PI (in PBS containing 0.1 % Triton X-100 and 0.1 % sodium citrate) for 1 h and subjected to flow cytometry analysis.

Proliferation assay

To determine the proliferation rate, 3×10^3 cells per well were grown in 96-well plates in the presence of rHuEPO (0.5 or 0.05 units/ml) or 10 % WEHI-conditioned media (a source of IL-3) for 1–5 days. Viability was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] colorimetric assay. Absorbance at 570 nm was measured after 4 h incubation.

RESULTS

An NPVY insert in the cytosolic domain of EPO-R selectively affects EPO-induced signalling

We have recently demonstrated that insertion of an NPVY, and not an NPVF, sequence into the EPO-R cytosolic domain led to improved maturation of EPO-R and increased its cell-surface levels [42]. In the present study we set out to examine whether the NPVY insert can affect EPO-mediated downstream signalling and cell proliferation mediated by the receptor. Ba/F3 cells expressing wt-EPO-R, NPVY-EPO-R or NPVF-EPO-R (Figure 1) were starved from serum and from EPO for 1 h, followed by exposure to 50 units/ml rHuEPO for the time periods indicated in Figure 2(A). Cell lysates were subjected to Western blot analysis with anti-pEPO-R ($pTyr^{479}$) antibodies, and downstream EPO-mediated

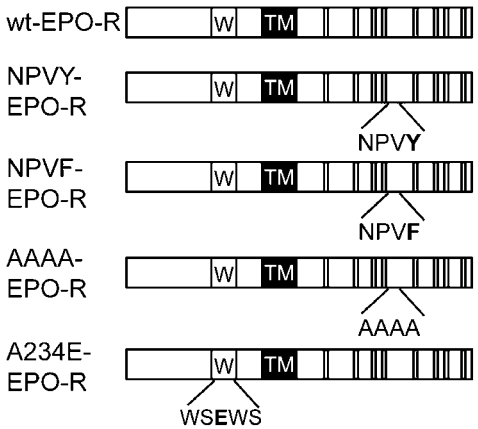


Figure 1 Schematic representation of wt-EPO-R and EPO-R mutants

Depicted are wt-EPO-R and EPO-R mutants used in the present study. The schemes present the extracellular domains including the WSXWS motif (W), the transmembrane domain (TM) and the intracellular domain. The eight tyrosine residues in the intracellular domain are marked by grey lines.

signalling was analysed by assessing the levels of phosphorylated STAT5, JAK2, ERK1/2 and Akt.

As shown in Figure 2(A), insertion of the NPVY sequence into EPO-R led into a selective increase in EPO-induced phosphorylation of EPO-R, JAK2, Akt and ERK1/2, but not of STAT5. The EPO-R containing an NPVF sequence was used in order to test the effect of the tyrosine residue within the motif on EPO signalling. The results showed that NPVF-EPO-R led to a phosphorylation pattern similar to NPVY-EPO-R, and not to that of wt-EPO-R (Figure 2A).

In order to assess the correlation between EPO-R surface levels and EPO-mediated downstream signalling we quantified surface EPO-R levels by EPO binding. As shown in Figure 2(B), NPVY-EPO-R demonstrates a 1.4-fold increase as compared with wt-EPO-R, whereas NPVF-EPO-R cell-surface levels are statistically unchanged from wt-EPO-R. The phosphorylation levels of EPO-R, STAT5, JAK2, ERK1/2 and Akt following 5 min of EPO stimulation were quantified and divided by the cell-surface levels of the respective receptors (Figure 2C). The values indicated in Figure 2(C) thus represent EPO-R signalling normalized for cell-surface levels of the respective receptors. As can be seen, insertion of the NPVY sequence into EPO-R led to increased EPO-driven phosphorylation of EPO-R on Tyr^{479}, as well as to an increased phosphorylation of Akt, JAK2 and ERK1/2 compared with cells expressing wt-EPO-R (Figures 2A and 2C). In contrast, there was no statistical difference in STAT5 phosphorylation mediated by wt-EPO-R and NPVY-EPO-R. Similar effects on signalling were also observed at lower EPO concentrations (5 units/ml) and were displayed by two independent clones of Ba/F3 cells stably expressing NPVY-EPO-R (results not shown). These results suggest that the cytosolic NPVY insert enhances EPO-induced intracellular signalling involving Akt and ERK pathways. However, insertion of the NPVY sequence did not seem to affect STAT5 phosphorylation, suggesting that the insert has a selective effect on EPO-R signalling.

To test whether the effect of the NPVY insert into EPO-R can be attributed to a conformational change caused by insertion of four amino acid residues, a mutant in which the NPVY motif was replaced by four alanine residues (AAAA-EPO-R) (Figure 1) was employed. The AAAA-EPO-R demonstrated comparable surface levels with those of the wt-EPO-R (Figure 3A, left-hand panel). EPO-driven downstream signalling of the AAAA-EPO-R was similar to that of the wt-EPO-R (Figure 3B). This similarity was maintained when the phosphorylation levels were normalized for cell-surface levels of AAAA-EPO-R and wt-EPO-R (Figure 3D). It thus appears that the enhanced signalling of

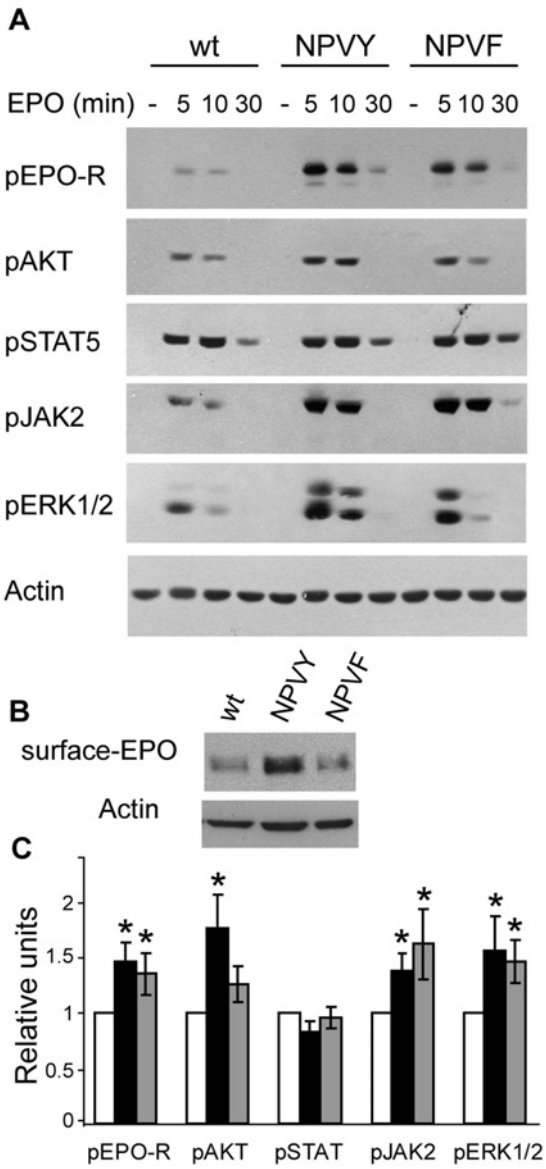
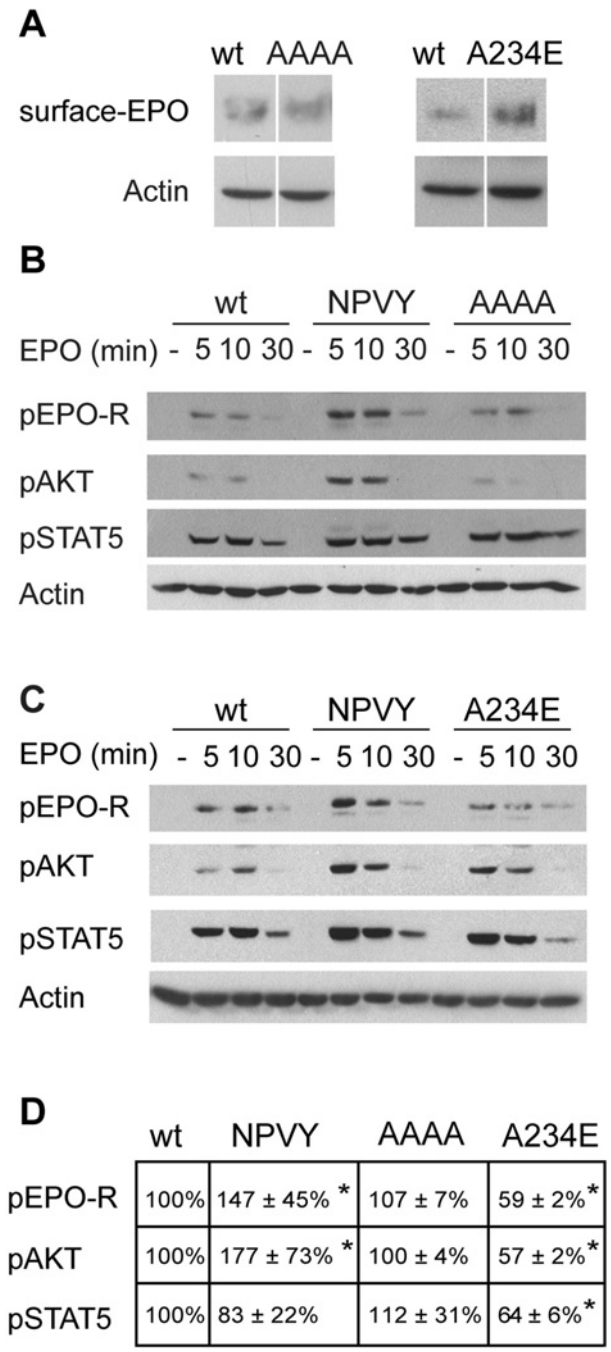


Figure 2 NPVY insertion into EPO-R enhances receptor signalling

Ba/F3 cells expressing wt-EPO-R, NPVY-EPO-R or NPVF-EPO-R were deprived of serum and EPO for 1 h. Cells were then incubated with or without rHuEPO (50 units/ml) for the time points indicated. (**A**) Representative Western blot analysis of cell lysates probed with antibodies against $pTyr^{479}$ EPO-R (pEPO-R), pAKT, pSTAT5, pJAK2, pERK1/2 and actin as a loading control. (**B**) Surface EPO-Rs were assessed by EPO binding to the cells. Ba/F3 cells expressing wt-, NPVY- or NPVF-EPO-Rs were incubated with 20 units/ml EPO on ice for 1 h. Cells were washed three times at 4 °C to remove free EPO. Cells lysates were resolve by SDS/PAGE and bound EPO was detected by immunoblot analysis with anti-EPO antibodies. A representative blot of four independent experiments is depicted. (**C**) Histogram summarizing the phosphorylation levels at 5 min of EPO stimulation, divided by cell-surface levels of the indicated receptor as measured by EPO binding. Phosphorylation levels are expressed as the fold increase compared with cells expressing wt-EPO-R. White bars represent wt-EPO-R-, black bars represent NPVY-EPO-R- and grey bars represent NPVF-EPO-R-expressing cells. $n = 6$ independent experiments, $^*P < 0.05$, compared with wt-EPO-R.



	wt	NPVY	AAAA	A234E
pEPO-R	100%	147 ± 45% *	107 ± 7%	59 ± 2%*
pAKT	100%	177 ± 73% *	100 ± 4%	57 ± 2%*
pSTAT5	100%	83 ± 22%	112 ± 31%	64 ± 6%*

Figure 3 Increased cell surface levels are not sufficient to elevate EPO-induced signalling

(**A**) Cell-surface levels of wt-EPO-R compared with either AAAA-EPO-R (left-hand panel) or A234E-EPO-R (right-hand panel). Ba/F3 cells expressing wt-EPO-R, or the indicated mutants, were exposed to EPO at 4 °C. EPO-R cell-surface levels were monitored by anti-EPO antibodies as described in the legend to Figure 2. Representative blots of four independent experiments are shown. (**B**) Phosphorylation levels of EPO-R, Akt and STAT5 in EPO-stimulated Ba/F3 cells expressing wt-EPO-R, AAAA-EPO-R or NPVY-EPO-R. (**C**) Phosphorylation levels of EPO-R, Akt and STAT5 in EPO-stimulated Ba/F3 cells expressing wt-EPO-R, A234E-EPO-R or NPVY-EPO-R. (**D**) Summary of phosphorylation levels at 5 min, expressed as the fold increase from wt-EPO-R and divided by EPO-R cell-surface levels in six independent experiments for wt-EPO-R and NPVY-EPO-R and at least three experiments for A234E-EPO-R and AAAA-EPO-R. *$P < 0.05$.

NPVY-EPO-R compared with wt-EPO-R cannot be explained by the mere addition of four amino acids to the cytosolic domain of the receptor.

Elevated cell-surface EPO-R levels are not sufficient to augment EPO-mediated signalling

The observed increase in EPO-mediated signalling by NPVY-EPO-R does not seem to result solely from increased cell-surface levels of this receptor. Notably the NPVF-EPO-R cell-surface levels are similar to those of wt-EPO-R (Figure 2B) [42], yet it displays selectively increased EPO-mediated signalling (Figure 2A) which is also maintained after normalization for surface receptor levels (Figure 2C). Like NPVY-EPO-R, NPVF-EPO-R leads to elevated phosphorylation of EPO-R, JAK2 and ERK1/2, but unlike NPVY-EPO-R, Akt phosphorylation mediated by NPVF-EPO-R is not statistically different from that mediated by the wt-EPO-R. This suggests that the increased EPO-driven phosphorylation of NPVY-EPO-R or NPVF-EPO-R as compared with wt-EPO-R cannot be solely attributed to their cell-surface levels.

To further investigate the contribution of cell-surface EPO-R levels to EPO-induced cellular signalling we compared EPO-mediated signalling conferred by wt-EPO-R with EPO-mediated signalling conferred by A234E-EPO-R. The A234E-EPO-R harbours a mutation in the WSXWS motif and was shown to be expressed approx. 2-fold higher than the wt-EPO-R on the cell surface [27,29] (see Figure 3A, right-hand panel). In order to assess whether elevated surface EPO-R levels are sufficient to account for increased EPO mediated signalling, we compared EPO-induced phosphorylation of EPO-R, Akt and STAT5 in Ba/F3 cells expressing either wt-EPO-R, NPVY-EPO-R or A234E-EPO-R. Cells were stimulated with 50 units/ml rHuEPO, and EPO-mediated signalling was analysed by Western blotting as described above. As can be seen, in contrast with the augmented signalling by NPVY-EPO-R, the signalling profile of cells expressing A234E-EPO-R was similar to that of cells expressing wt-EPO-R (Figure 3C). Moreover, after normalization for surface levels of the receptors, it seems that the extent of signalling per molecule of A234E-EPO-R is even lower than that of the wt-EPO-R (Figure 3D). These observations are in agreement with data by Hilton et al. [29] demonstrating that despite a 2-fold increase in surface levels of A234E-EPO-R, EPO-mediated proliferation by cells expressing this receptor was identical with that of cells expressing wt-EPO-R. Taken together, our results imply that elevated cell-surface EPO-R is not sufficient to enhance EPO-induced downstream signalling cascades.

Insertion of NPVY into EPO-R confers protection against apoptosis

The increased EPO-driven phosphorylation of EPO-R in cells expressing the NPVY-EPO-R and NPVF-EPO-R, which was also observed after correction for EPO-R surface levels, may also be reflected in biological functions controlled by EPO-R. In that respect, pTyr^{479} was shown to be associated with activation of Akt [18,44], which is associated with pro-survival signals [17,19]. As described above, EPO stimulation resulted in enhanced phosphorylation of Tyr^{479} of the EPO-R and of Akt in the NPVY-EPO-R. Hence, the NPVY insert may lead to improved protection against apoptosis. Ba/F3 cells expressing either wt-EPO-R, NPVY-EPO-R or NPVF-EPO-R were grown in 0.5 units/ml rHuEPO (5×10^5 cells per plate) and incubated with or without 50 μM etoposide for 16 h. In order to ensure that the effects on apoptosis were mediated by EPO-mediated signalling, as a control cells were grown in IL-3 (WEHI-conditioned medium) enabling the Ba/F3 cells expressing EPO-R to signal via their endogenous IL-3 receptor and not via their transfected EPO-R. The next day cells were collected and stained for Annexin V and PI. Wt-EPO-R-expressing cells exposed to etoposide showed a

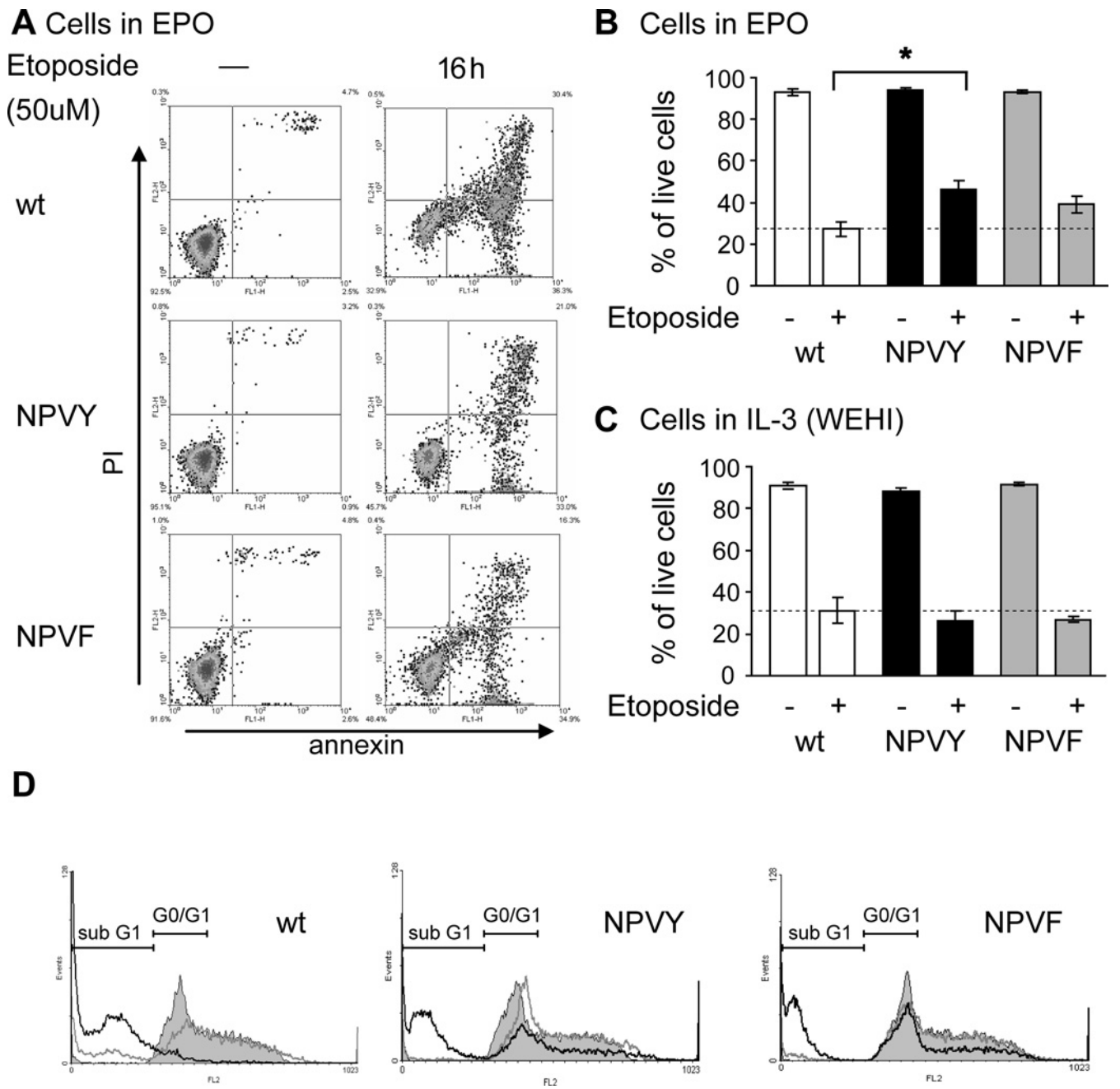


Figure 4 Increased protection against apoptosis in Ba/F3 cells expressing NPVY-EPO-R

Ba/F3 cells expressing wt-EPO-R, NPVY-EPO-R or NPVF-EPO-R were exposed to 50 μM etoposide for 16 h in the presence of 10 % FCS and 0.5 units/ml rHuEPO. Apoptosis was analysed by flow cytometry using Annexin V and PI. (**A**) Representative density plot in which the *y*-axis represents PI staining and the *x*-axis represents Annexin V staining. Early-apoptotic cells are Annexin-V-positive, whereas late-apoptotic cells are positive for both Annexin V and PI. (**B** and **C**) Histograms summarizing the percentage of live cells in untreated and etoposide-treated cells grown in the presence of EPO (**B**) or WEHI (**C**). White bars represent wt-EPO-R, black bars represent NPVY-EPO-R and grey bars represent NPVF-EPO-R. Data are obtained from four independent experiments. (**D**) Cell-cycle analysis of Ba/F3 cell expressing wt-EPO-R, NPVY-EPO-R or NPVF-EPO-R. The cells were cultured for 16 h in 0.5 units/ml of EPO (solid grey), 0.05 units/ml of EPO (grey line) or 0.01 units/ml of EPO (black line). Subsequently, cells were stained with PI, and subjected to flow cytometry analysis. Apoptotic cells appear in sub-G_1 phase.

dramatic decrease in the number of live cells, with a viability of 27 ± 6 % of the cells. On the other hand, NPVY-EPO-R-expressing cells that were exposed to etoposide demonstrated fewer apoptotic cells and a higher portion of live cells (46 ± 8 %) (Figures 4A and 4B). Ba/F3 cells expressing NPVF-EPO-R and exposed to etoposide showed a somewhat lower protective effect, displaying 39 ± 5 % live cells (Figures 4A and 4B). The difference between wt-EPO-R and the NPVY/NPVF-EPO-R mutants was not observed in control cells grown in IL-3 (31 ± 6 %, 26 ± 5 % and 27 ± 1 % live cells in wt-EPO-R-, NPVY-EPO-R- and NPVF-EPO-R-expressing cells respectively) (Figure 4C). These data indicate that insertion of NPVY or NPVF into EPO-R confers an increase in EPO-mediated protection of cells against apoptosis. These results were further supported by cell-cycle analysis. Cells expressing wt-EPO-R, NPVY-EPO-R and NPVF-EPO-R were cultured for 16 h at three different concentrations of EPO (0.5,

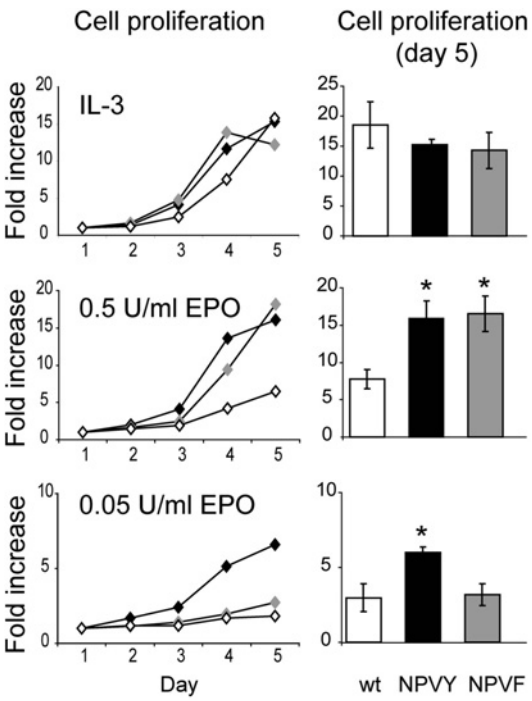


Figure 5 Enhanced cell proliferation in Ba/F3 cells expressing NPVY-EPO-R

Ba/F3 cells expressing wt-EPO-R, NPVY-EPO-R or NPVF-EPO-R were grown for 1–5 days in the presence of 0.05 units/ml EPO, 0.5 units/ml EPO or 10 % WEHI-conditioned medium (IL-3). Cell viability was measured daily using the MTT method. Left-hand panels: representative experiment. White, black and grey symbols represent wt-EPO-R-, NPVY-EPO-R- and NPVF-EPO-R-expressing cells respectively. Right-hand panel: cell viability at day 5 expressed as the fold increase from day 1. Data obtained from three independent experiments. $^*P < 0.05$, compared with wild-type.

0.05 and 0.01 units/ml). Subsequently, the cells were washed, stained with PI and analysed by flow cytometry. As can be seen in Figure 4(D), wt-EPO-R-, NPVY-EPO-R- and NPVF-EPO-R-expressing cells demonstrate similar repartition patterns when cultured at 0.5 units/ml EPO. However, when the cells were cultured at a lower concentration of EPO (0.01 units/ml), most of the wt-EPO-R-expressing cells were at sub-G_1, whereas NPVY-EPO-R- and NPVF-EPO-R-expressing cells were either at the sub-G_1 or at the G_0/G_1 stage. Cells cultured at 0.05 units/ml of EPO presented an intermediate profile (Figure 4D). These data imply that NPVY-EPO-R- and NPVF-EPO-R-expressing cells are more resistant to stress exerted by low EPO concentrations or by the apoptosis inducer, etoposide.

Insertion of NPVY into EPO-R enhances EPO-mediated proliferation of Ba/F3 cells

To further understand the effect of NPVY insertion into EPO-R on biological functions mediated by EPO-R in response to EPO, we measured EPO-induced cell proliferation. Ba/F3 cells expressing wt-EPO-R, NPVY-EPO-R or NPVF-EPO-R were plated in 96-well plates and incubated in the presence of rHuEPO (0.5 units/ml or 0.05 units/ml). Cell viability was measured daily for 5 consecutive days and proliferation rate of cells grown in the presence of IL-3 (WEHI) was used as a control, as described above. NPVY-EPO-R-expressing cells displayed an increased dose-dependent proliferation rate compared with cells expressing wt-EPO-R. At day 5, the response to 0.5 units/ml and 0.05 units/ml EPO of cells expressing NPVY-EPO-R was 2-fold higher than the response of cells expressing wt-EPO-R (Figure 5, lower and middle panels). There was no statistical difference between the proliferation rates of these two cell lines when they were grown in the presence of IL-3 (Figure 5, top panel). This result suggests that insertion of NPVY into EPO-R improves EPO-R signalling, thereby leading to enhanced proliferation. NPVF-EPO-R-expressing cells present an intermediate proliferation profile as they proliferate at an increased rate compared with wt-EPO-R-expressing cells when grown in 0.5 units/ml rHuEPO, but do not differ from cells expressing wt-EPO-R when grown in 0.05 units/ml rHuEPO. It thus appears that both NPVY and NPVF inserts improve EPO-R responsiveness to EPO. However, when cultured at a low EPO concentration (0.05 units/ml rHuEPO), the NPVY-EPO-R is more potent in transmitting signals as compared with the NPVF-EPO-R, suggesting an important role for the tyrosine residue within this motif in conferring the improved responsiveness.

DISCUSSION

One of the hallmarks of EPO-R is its low cell-surface expression and high intracellular levels, particularly in the ER [21,24,27]. The low surface levels of EPO-R are mostly attributed to poor folding of its extracellular domain during maturation. Yet the factors that regulate EPO-R maturation and surface expression are not fully resolved. Our recent publication [42] demonstrated that insertion of an NPVY sequence into the cytosolic domain of EPO-R leads to enhanced maturation and increased surface expression of the receptor, implying that manipulating the cytosolic domain of EPO-R can affect receptor maturation. In the present study we demonstrate that the NPVY insert has a specific effect on EPO-mediated signal transduction of EPO-R, as shown by enhanced phosphorylation levels of selected downstream effectors. This activity translates to enhanced proliferation, increased protection against apoptosis and a reduced level of cells in sub-G_1 in response to low EPO levels. We show that these cellular activities are not necessarily correlated with the higher expression levels of NPVY-EPO-R on the plasma membrane.

The NPXY motif has been shown to mediate rapid internalization of type I integral membrane proteins, including the LDL receptor, amyloid precursor protein and integrin β [36,45,46]. In addition, the NPXY motif was shown to play a role in sorting of proteins to the basolateral plasma membrane [47] and in transmission of growth and survival signals [35,48]. We have recently shown that insertion of an NPVY motif to EPO-R improves its exit from the ER and increases its cell-surface levels [42], while in the present study we describe its selective effect on signalling. We demonstrate that EPO-mediated phosphorylation levels of EPO-R and its downstream effectors Akt, JAK2 and ERK1/2 were all significantly elevated, whereas STAT5 phosphorylation was not increased in cells expressing NPVY-EPO-R compared with wt-EPO-R-expressing cells. A recent study [49] presenting a mathematical model for STAT5 signalling suggests that the average amount of STAT5 units activated per activated receptor remains very similar (approx. 100 units) for a wide range of EPO concentrations [49]. This may explain why STAT5 phosphorylation was less sensitive to small differences in the magnitude of EPO-R activation, and may explain the inability to detect differences in STAT5 phosphorylation between wt- and NPVY-EPO-R.

The effects of NPVY insertion into EPO-R cannot simply be explained by the addition of four amino acids to the cytosolic domain of the receptor, since a mutant in which an AAAA sequence had been inserted at the same position of EPO-R did not significantly affect EPO-mediated downstream signalling of

EPO-R. To further analyse the NPVY-mediated enhanced EPO-R signalling we employed the NPVF-EPO-R mutant. This mutant enables us to assess the contribution of the tyrosine residue to the observed effect of NPVY. We found that a similar amplitude of downstream signals was elicited in cells expressing NPVF-EPO-R and NPVY-EPO-R. However, the enhanced proliferation of cells expressing NPVY-EPO-R grown at a low EPO concentration (0.05 units/ml) was not observed in cells expressing the NPVF-EPO-R mutant, suggesting that the tyrosine residue plays a role in augmenting the sensitivity of the cells to stimulation by EPO. The ability of the NPVF, but not AAAA, EPO-R mutant to affect the signalling of EPO-R can be explained by the ability of both the NPVY and NPVF motifs to bind the PTB domain [45]. Since NPVY is a known substrate for PTB-containing proteins it is likely that NPVY-EPO-R can recruit a variety of signalling proteins, such as IRS-1 and Shc. The NPVF motif can also bind PTB-containing proteins, but to a lesser extent than NPVY. This feature can explain the improved, yet limited, responsiveness of NPVF-EPO-R to EPO [38,45].

By inserting the NPVY sequence we may have imposed a conformational change in the EPO-R. This supposition is supported by the fact that the NPXY motif is structurally one of the most highly favoured sequences for forming β turns [38]. However, secondary structure prediction that we performed using the PSIPRED protein structure prediction server was not able to shed light on the conformational change that this motif may have imposed on EPO-R. Elucidation of this issue would require X-ray crystallography of the EPO-R cytosolic domain which is not yet available. Thus we cannot rule out the possibility that insertion of NPVY at a different location would act differently.

In addition to affecting EPO-induced signal transduction, insertion of the NPVY sequence had an effect on cellular functions in which EPO-R is involved. EPO that is known to activate EPO-R and initiate survival signals mainly by phosphorylation of EPO-R Tyr^{479} was shown to confer protection against apoptosis [15,18,50,51]. In the present study we show that in the presence of EPO, the NPVY-EPO-R was able to confer increased protection against etoposide-induced apoptosis as compared with wt-EPO-R. In addition, insertion of NPVY enhanced EPO-mediated cell proliferation, even at low EPO concentrations. These activities of NPVY-EPO-R (and to a certain extent NPVF-EPOR) at low levels of EPO were also manifested in a higher viability of the cells and in a lower percentage of cells in sub-G_1.

Insertion of NPVY into EPO-R increased its cell-surface levels. It was thus reasonable to assume that these increased surface levels of the receptor are responsible for the enhanced responsiveness of NPVY-EPO-R-expressing cells to EPO and its effect on cellular activities. It seems, however, that there is no direct correlation between increased surface levels of EPO-R and enhanced EPO-mediated signalling. This lack of correlation is demonstrated by the observation that, despite the higher surface levels of NPVY-EPO-R compared with NPVF-EPO-R, these two EPO-R mutants confer similar effects on EPO-driven ERK1/2, JAK2 and STAT5 phosphorylation, when normalized to surface EPO-R levels. Moreover, we demonstrate that another EPO-R mutant, A234E-EPO-R, which is known to present high cell-surface levels [27], induced EPO-mediated signals of similar magnitudes to wt-EPO-R (Figure 3C). This is in agreement with the observation that A234E-EPO-R mediates cell proliferation rates similar to those of wt-EPO-R [27]. These data further support the supposition that high cell-surface levels of EPO-R may not be sufficient to confer elevation in EPO-R-mediated downstream signals and function.

In the present study we show that insertion of an NPVY sequence into the cytosolic domain of EPO-R specifically affects its response to EPO and its downstream signalling. These findings should be considered in other experimental systems in which transplanted foreign sequences may have unforeseen effects on receptor functions, in addition to their well-established roles. Insertion of the NPVY sequence into EPO-R also resulted in enhanced cell proliferation and in increased protection against apoptosis. We demonstrate that the effect of the NPVY insert cannot be attributed to the simple addition of four amino acid residues to the cytosolic domain of the receptor. However, by comparing NPVY-EPO-R with NPVF-EPO-R we show that the tyrosine residue in NPVY plays a role in enhancing the sensitivity to stimulation by EPO. Moreover, we demonstrate that the higher surface levels of NPVY-EPO-R compared with wt-EPO-R is not sufficient to confer elevation in EPO-R-mediated downstream signalling and activity. The latter observation is of utmost importance since attempts are made to increase EPO-R surface levels in order to achieve a better response to EPO which is routinely administered to elevate red blood cell counts in patients suffering from kidney failure and to cancer patients undergoing chemotherapy. Our data suggest that improving EPO-R maturation and increasing EPO-R cell-surface levels would not necessarily be sufficient to achieve enhanced EPO-mediated signalling and cellular activity.

AUTHOR CONTRIBUTION

Tamar Liron planned, performed and analysed experiments, and wrote the paper. Tal Nahari planned and performed experiments. Drorit Neumann and Miriam Souroujon planned and analysed experiments, and wrote the paper.

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