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

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INTRODUCTION

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 Tyr479), 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.

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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 NPYX 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 NPYX [39,40]. Notably, the PTB domain was shown to bind both phosphorylated and non-phosphorylated NPYX [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 (pTyr479) 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 rHuEPO. Cells were then stained with PI (in PBS containing 0.1% Triton X-100 and 0.1% sodium citrate) to improve maturation of EPO-R and increased its cell-surface expression [42]. In contrast, insertion of an NPVF, sequence into the EPO-R cytosolic domain led to improved maturation of EPO-R and increased 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 EPO-R were cultured for 16 h in the presence of 10% FCS and rHuEPO. Cells were then stained with PI (in PBS containing 0.1% Triton X-100 and 0.1% sodium citrate) to improve maturation of EPO-R and increased its cell-surface expression [42]. In contrast, insertion of an NPVF, sequence into the EPO-R cytosolic domain led to improved maturation of EPO-R and increased 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, 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 stained with PI (in PBS containing 0.1% Triton X-100 and 0.1% sodium citrate) to improve maturation of EPO-R and increased cell-surface levels [42]. In contrast, insertion of an NPVF, sequence into the EPO-R cytosolic domain led to improved maturation of EPO-R and increased 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-EPO antibodies (R&D Systems).

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 stained with PI (in PBS containing 0.1% Triton X-100 and 0.1% sodium citrate) to improve maturation of EPO-R and increased cell-surface levels [42]. In contrast, insertion of an NPVF, sequence into the EPO-R cytosolic domain led to improved maturation of EPO-R and increased 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-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 Mecyclo apotosis 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 × 10^6 cells perwell 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-2H-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-EPO antibodies, and downstream EPO-mediated
A cytosolic NPVY insert affects erythropoietin receptor-mediated signalling

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 to 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 Tyr479, 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...
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, pTyr479 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 Tyr479 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 × 10⁵ 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
A cytosolic NPVY insert affects erythropoietin receptor-mediated signalling

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 at 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-G1 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,
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 differ from cells expressing wt-EPO-R when grown in 0.05 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-G1 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 less sensitive with the NPVF-EPO-R, suggesting an important role for the tyrosine residue within this motif in conferring the improved responsiveness.
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 Tyr479 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. The ability of the NPVF, but not AAAA, EPO-R mutant in augmenting the sensitivity of the cells to stimulation by EPO is known to activate EPO-R and initiate survival signals mainly by phosphorylation of EPO-R Tyr479 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 NPVF-EPO-R (and to a certain extent NPVF-EPOR) 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. Dorrit Neumann and Miriam Souroujon planned and analysed experiments, and wrote the paper.

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