BJ www.biochemj.org

BJ Cell

Biochem. J. (2008) **410**, 409–416 (Printed in Great Britain) doi:10.1042/BJ20071297

multimedia

A transplanted NPVY sequence in the cytosolic domain of the erythropoietin receptor enhances maturation

Tal NAHARI*, Eran BARZILAY*, Koret HIRSCHBERG† and Drorit NEUMANN*[1]

*Department of Cell and Developmental Biology, Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv 69978, Israel, and †Department of Pathology, Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv 69978, Israel

Activation of the EPO-R [EPO (erythropoietin) receptor] by its ligand EPO promotes erythropoiesis. Low cell surface EPO-R levels are traditionally attributed to inefficient folding mediated by the receptor extracellular domain. In the present study, we addressed the role of the EPO-R intracellular domain in exit from the ER (endoplasmic reticulum) and surface expression. A fusion protein between the thermo-reversible folding mutant of VSVG (vesicular-stomatitis-virus glycoprotein) (VSVGtsO45) and the EPO-R cytosolic domain [VSVG-WT (wild-type)] displayed delayed intracellular trafficking as compared with the parental VSVGtsO45, suggesting that the EPO-R cytosolic domain can hamper ER exit. Although NPXY-based motifs were originally associated with clathrin binding and endocytosis, they may also function in other contexts of the secretory pathway. A fusion protein between VSVGtsO45 and the cytosolic portion of EPO-R containing an NPVY insert (VSVG-NPVY) displayed enhanced glycan maturation and surface expression as compared with VSVG-WT. Notably, the NPVY insert also conferred improved maturation and augmented cell surface EPO-R. Our findings highlight three major concepts: (i) the EPO-R cytosolic domain is involved in ER exit of the receptor. (ii) Sequence motifs that participate in endocytosis can also modulate transport along the secretory pathway. (iii) VSVG-fusion proteins may be employed to screen for intracellular sequences that regulate transport.

Key words: cytosolic domain, endoplasmic reticulum, erythropoietin receptor, Janus kinase, NPXY motif, vesicular-stomatitis-virus glycoprotein (VSVG).

INTRODUCTION

Targeting of newly synthesized proteins to their intracellular destination via the secretory pathway is a pivotal multistep process [1,2] crucial for most cell functions. The EPO-R [EPO (erythropoietin) receptor], present on erythroid progenitor cells [3,4], is the major regulator of erythropoiesis, via activation by its ligand, EPO. EPO-R is a member of the cytokine receptor superfamily, characterized by the presence of four conserved cysteine residues and a 'WSXWS' motif in their extracellular domain. The lack of enzymatic activity in the intracellular domain of these receptors necessitates complex formation of ligand-bound receptors with other signalling partners [i.e. JAKs (Janus kinases)] [5] to initiate the signalling cascade. In contrast with other cytokine receptors [e.g. GH (growth hormone) receptor [6]], most of the newly made EPO-R molecules remain sequestered intracellularly, presumably in association with the ER (endoplasmic reticulum) [7,8]. This metabolic profile is similar in both transfected cells [9] and in fetal liver cells endogenously expressing the EPO-R [10], thus suggesting that the structural features of the receptor molecule regulate its surface expression. Previous reports have attributed the low surface expression levels of the EPO-R to the poor folding of its extracellular domain [7]. Accordingly, targeted mutations [7] or certain sequence features [11] in the extracellular domain of the EPO-R were shown to facilitate [7,11] or inhibit [12,13] surface expression of the receptor.

The contribution of the EPO-R intracellular domain to its trafficking to the cell surface is not clear. In this respect, JAK2 was shown to bind to an EPO-R cytosolic domain and to promote cell surface localization of the receptor [8]. JAK2-mediated increase in surface receptors was also shown for the thrombopoietin receptor [14,15]. Notably, deletion of the EPO-R intracellular cytosolic domain was associated with higher surface levels of the receptor [16]. This could result from impaired endocytosis [17,18], retarded degradation and/or enhanced trafficking of the EPO-R to the cell surface. The ability to isolate the net contribution of the EPO-R cytosolic domain to surface expression of the receptor is hampered due to the inefficient folding of the EPO-R extracellular domain [7], which in itself is sufficient to retard the receptor molecules in the early secretory pathway [11,17,18].

The thermo-reversible folding mutant of VSVG (vesicular-stomatitis-virus glycoprotein) (VSVGtsO45) was extensively employed to examine ER-to-Golgi transport processes (e.g. [19–21]). When incubated at 40 °C, the cargo glycoprotein VSVGtsO45 is misfolded and accumulates within the ER, whereas upon temperature shift to 32 °C, VSVGtsO45 is refolded, released from the ER and directed to the plasma membrane [22]. VSVGtsO45 thus offers a valuable experimental system that enables synchronizing the release of newly synthesized molecules from the ER and tracking their journey to the plasma membrane. Using this experimental design, one can determine the potency of selected motifs to modify the trafficking kinetics of VSVGtsO45 when implanted into the parent molecule [23].

Tyrosine-based motifs were found to participate in intracellular targeting of proteins [24]. NPXY motifs were found to mediate binding to clathrin [25–27]; thus for example, the NPVY sequence in the cytoplasmic domain of the low-density lipoprotein receptor

Abbreviations used: BP, biotinylated proteins; endo H, endoglycosidase H; EPO, erythropoietin; EPO-R, EPO receptor; ER, endoplasmic reticulum; GFP, green fluorescent protein; HRP, horseradish peroxidase; IL-3, interleukin-3; JAK, Janus kinase; PBS^{2+}, PBS supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$; PNGase F, peptide N-glycosidase F; PTB domain, phosphotyrosine-binding domain; rHuEPO, recombinant human EPO; TCL, total cell lysates; TGN, *trans*-Golgi network; VSVG, vesicular-stomatitis-virus glycoprotein; WT, wild-type; YFP, yellow fluorescent protein.

[1] To whom correspondence should be addressed (email Histo6@post.tau.ac.il).

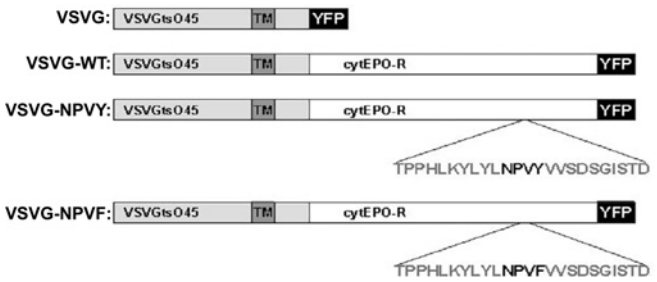

Figure 1 Schematic representation of the fusion proteins between VSVGtsO45 and the cytosolic portion of EPO-R

VSVGtsO45–YFP (VSVG), VSVGtsO45–cytEPO-R–YFP (VSVG-WT), VSVGtsO45–cytEPO-R (NPVY)–YFP (VSVG-NPVY) and VSVGtsO45–cytEPO-R (NPVF)–YFP (VSVG-NPVF) are shown.

is a major site of recognition for incorporation into clathrin-coated pits for sorting the receptor after internalization [25]. Phosphorylation of the tyrosine in the context of the NPEY sequence in the IR (insulin receptor) juxtamembrane region is required for insulin-induced receptor endocytosis [28]. It was also shown to play a role in sorting of the newly synthesized receptor to the basolateral plasma membrane [29]. Hence, the NPXY motif may also function in contexts of protein trafficking other than endocytosis.

Here, we asked whether the cytosolic domain of the EPO-R plays a role in intracellular retention of the EPO-R. To this end, we utilized the YFP (yellow fluorescent protein)-tagged VSVGtsO45 [23]. Our results demonstrate that (i) attachment of the cytosolic domain of the EPO-R to VSVGtsO45 retards maturation of the corresponding fusion protein and (ii) attachment of an EPO-R cytosolic domain containing a transplanted NPVY sequence motif improved glycan maturation of the VSVGtsO45–EPO-R fusion protein. Increased surface expression of the respective EPO-R molecule, which harbours the NPVY insertion (NPVY-EPO-R), suggests that this is a valid experimental approach for screening sequences that may introduce novel modes of regulating receptor surface expression.

EXPERIMENTAL

Reagents

Materials were obtained from previously listed sources [19,30,31].

Antibodies

Rabbit polyclonal antibodies directed against the N-terminus of the EPO-R were previously described [32]. Anti-GFP (green fluorescent protein) antibodies (Roche Diagnostics) were used at a dilution of 1:1000 for biochemical detection of VSVGtsO45 (VSVG) and the fusion proteins of VSVGtsO45 on to which the cytosolic portion of the EPO-R variants was attached (Figure 1). Mouse monoclonal antibodies directed to the N-terminal 26 amino acids of rHuEPO (recombinant human EPO; R&D Systems) were used at a dilution of 1:1000 for Western-blot analysis, as described in [19–21].

Plasmids and generation of fusion proteins and mutants

The thermo-reversible folding mutant of VSVG fused to YFP (VSVGtsO45–YFP) was employed [33]. The cytosolic domain of EPO-R was amplified by the PCR method and the fragment obtained was inserted in frame between VSVGtsO45 and YFP using SacII and EcoRI restriction sites. Amplification of the cDNA encoding the EPO-R cytosolic domain was performed by using the following primers: CCGGAATTCTGTCCCACCGCCGGA (sense primer) and TCCCCGCGGGGAGCAGGCCACAT (antisense primer).

The NPVY and NPVF sequences were inserted into the EPO-R cDNA in pXM [34], by using the QuikChange® site-directed mutagenesis kit (Stratagene), thus generating NPVY-EPO-R and NPVF-EPO-R. The primers used for the NPVY insertion were: GAAGTACCTATACCTTAACCCCGTCTATGTGGTGTCCGATTCTGG (sense) and CCAGAATCGGACACATAGACGGGGTTAAGGTACTTC (antisense). The primers used for the NPVF insertion were: GAAGTACCTATACCTTAACCCCGTCTTTGTGGTGTCCGATTCTGG (sense) and CCAGAATCGGACACAAAGACGGGGTTAAGGTACTTC (antisense). All cDNA constructs were verified by sequencing.

Cell culture and transfection

COS7 and BOSC-23T cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) fetal calf serum. COS7 cells cultured to 60 % confluence were transiently transfected, by using FuGENE™ 6 (Roche Diagnostics) according to the manufacturer's instructions. BOSC-23T cells cultured to 60 % confluence were transiently transfected by using the calcium phosphate method [35]. Ba/F3 cells stably transfected with EPO-R cDNAs were generated as previously described [32] and were cultured in RPMI 1640 medium supplemented with 10 % conditioned media from WEHI3B cells as their source of IL-3 (interleukin-3) or with 0.5 unit/ml rHuEPO (Eprex; Janssen Cilag).

Cell lysis

Cells (10^5) were lysed in 30 μl of PBS containing 1 % (v/v) Triton X-100, 0.5 % (w/v) deoxycholate and 5 mM EDTA supplemented with protease inhibitors (Complete™ protease inhibitors; Roche Diagnostics).

endo H (endoglycosidase H) digestion

Cells lysates obtained from 10^5 cells were denatured in 0.5 % (w/v) SDS for 5 min at 100 °C prior to the addition of 3 μl of 0.5 M sodium citrate and incubation with or without 500 units of endo H (New England Biolabs) for 1 h at 37 °C. Samples were then resolved on SDS/PAGE, prior to Western-blot analysis, as described in [32].

PNGase F (peptide N-glycosidase F) digestion

Cell lysates obtained from 10^5 cells were denatured in 0.5 % (w/v) SDS at 100 °C for 5 min. Subsequently, samples were incubated with or without 0.6 unit of PNGase F (Roche Diagnostics) for 2 h at 37 °C, prior to Western-blot analysis, as described in [32].

VSVG trafficking and confocal microscopy

COS7 cells transiently expressing VSVGtsO45–YFP or VSVGtsO45–EPO-R fusion proteins, cultured on glass coverslips (Nunc), were incubated for 20 h at 40 °C. Cells were subjected to a temperature shift to 32 °C for the indicated time periods. Coverslips were fixed for 30 min in 3 % (w/v) formaldehyde solution before visualization in a Zeiss LSM PASCAL laser-scanning confocal microscope with a 514 nm laser line.

Cell surface biotinylation

BOSC-23T cells (2.5×10^6) were transfected with the VSVGtsO45-containing cDNA constructs. Immediately following transfection, the cells were incubated for 20 h at 40 °C and then shifted to 32 °C. Cells were rinsed twice in PBS supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS^{2+}) and incubated with sulfosuccinimidobiotin (Pierce) at 0.5 mg/ml for 30 min on ice. The cells were rinsed three times in PBS^{2+} and lysed in 1 ml of lysis buffer [PBS containing 1 % (v/v) Triton X-100, 0.5 % (w/v) deoxycholate and 5 mM EDTA supplemented with protease inhibitors (Complete™ protease inhibitors; Roche Diagnostics)]. Aliquots of the lysates [10 % of the TCL (total cell lysates)] were subjected to Western-blot analysis with anti-GFP antibodies. Precipitation of BP (biotinylated proteins) was performed with streptavidin–agarose beads (Sigma) on the remaining 90 % of the lysate. Precipitates were washed three times with PBS and eluted into sample buffer. Samples were resolved on SDS/PAGE and immunoblotted with anti-GFP antibodies.

FACS analysis

Surface EPO-R levels in Ba/F3 cells (10^6) stably expressing WT (wild-type), NPVY or NPVF EPO-R were determined by FACS (Becton Dickinson), using anti-EPO-R antibodies (1:100) followed by Cy2-conjugated secondary antibodies (Jackson Immuno Research Laboratories).

Biochemical analysis of EPO and anti-EPO-R antibody binding to cells

Surface EPO-R levels in Ba/F3 cells (10^6 cells) stably expressing WT, NPVY or NPVF EPO-R were also determined by biochemical binding analysis. Cells were starved for 1 h in a medium deprived of serum prior to incubation for 1 h at 4 °C with 20 units/ml rHuEPO or with anti-EPO-R serum diluted 1:50. Cells were washed to remove unbound rHuEPO or unbound rabbit anti-EPO-R antibodies. Cell lysates were resolved on SDS/PAGE and immunoblotted with anti-EPO antibodies or with anti-rabbit antibodies.

RESULTS

Attenuated maturation conferred by the EPO-R cytosolic domain

We raised the question of whether the EPO-R cytosolic domain contributes to intracellular retention of the receptor. To dissociate intracellular retention of the EPO-R conferred by the EPO-R extracellular domain, we attached the EPO-R intracellular, cytosolic domain to VSVGtsO45 tagged with YFP [23]. The resulting fusion protein is thus subjected to thermal regulation for its luminal folding, conferred by the luminal portion of VSVGtsO45. The underlying premise is that the cytosolic sequences of the EPO-R will provide additional modules that may regulate the exit of VSVGtsO45 from the ER as well as its processing and transport through the Golgi to the plasma membrane. Figure 1 represents the VSVGtsO45–YFP-containing proteins that were used in the present study.

Fluorescence analysis

First, we compared the trafficking of VSVGtsO45 (Figure 1, VSVG) with that of the VSVGtsO45 on to which the cytosolic portion of the EPO-R was attached (Figure 1, VSVG-WT) by using laser-scanning microscopy. COS7 cells were transfected with the cDNAs of VSVG and VSVG-WT and were cultured at 40 °C (the restrictive temperature) for 20 h before shifting them to 32 °C (the permissive temperature) [36]. Fixation was then carried out prior to (zero time) and after 1 and 5 h incubation at 32 °C (Figure 2). In addition, we performed live cell image analysis (see Supplementary Movies at http://www.BiochemJ.org/bj/410/bj4100409add.htm). At 40 °C, the cellular distribution of the VSVG-WT showed an ER pattern resembling that of the VSVG parent molecule. Following shift to the permissive temperature (32 °C), VSVG-WT trafficked through the secretory pathway (ER–Golgi–cell surface), albeit with slower kinetics than the parental VSVG molecule. Thus, 5 h after the shift to 32 °C, while most of the VSVG molecules decorated the cell surface, only a fraction of the VSVG-WT was detected at the cell surface. The remaining intracellular VSVG-WT molecules were predominantly concentrated in the Golgi complex, as revealed by co-localization with galactosyltransferase (results not shown). The advantage of this experimental approach is thus the ability to 'pulse' the fusion proteins in the ER and then to 'chase' their cellular distribution with respect to the time following their release from the ER.

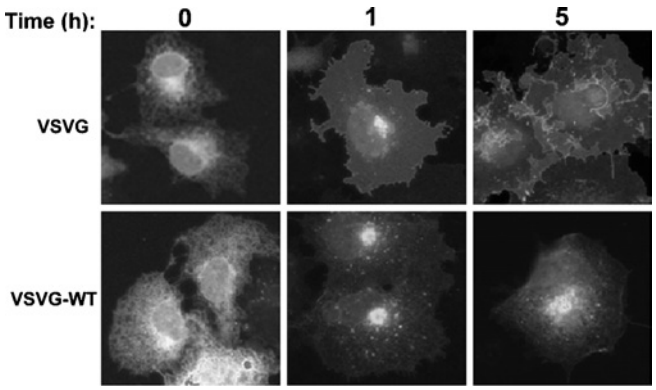

Figure 2 Delayed intracellular transport of VSVG-WT

COS7 cells transfected with VSVG and VSVG-WT were incubated for 20 h at 40 °C to trap the newly synthesized proteins in the ER. Subsequently, cells were shifted to 32 °C, the permissive temperature for ER exit, for 1 and 5 h. Cell fluorescence was detected by laser-scanning confocal microscopy. This Figure is representative of one of four experiments displaying similar results.

Glycan maturation

Acquisition of resistance to cleavage by endo H provides a quantitative measurement of glycan maturation of proteins within the Golgi complex. Hence, it provides an indirect means for assessing exit of the glycoproteins from the ER [32]. To quantify the extent of glycan processing of VSVG and VSVG-WT fusion protein, we determined their sensitivity to cleavage with endo H as a function of time following release of the cells from the restrictive temperature. BOSC-23T cells were transfected with VSVG and VSVG-WT cDNAs (Figure 3). Immediately following transfection, the cells were incubated for 20 h at 40 °C to trap the newly synthesized proteins in the ER. Subsequently, cells were shifted to 32 °C, the permissive temperature for ER exit [36]. Cell lysates were subjected to digestion with endo H at the indicated time points following shift of the cells to 32 °C and then subjected to Western-blot analysis with anti-GFP antibodies. As shown in Figure 3, after 1 h at 32 °C, VSVG began to gain resistance to endo H digestion, whereas endo H-resistant VSVG-WT was not detected. After 3 h at 32 °C, while more than 50 % of the VSVG molecules were endo H-resistant, endo H-resistant forms of the VSVG-WT were still barely discerned. Hence, the VSVGtsO45 moiety directs transport to the cell surface, whereas the EPO-R

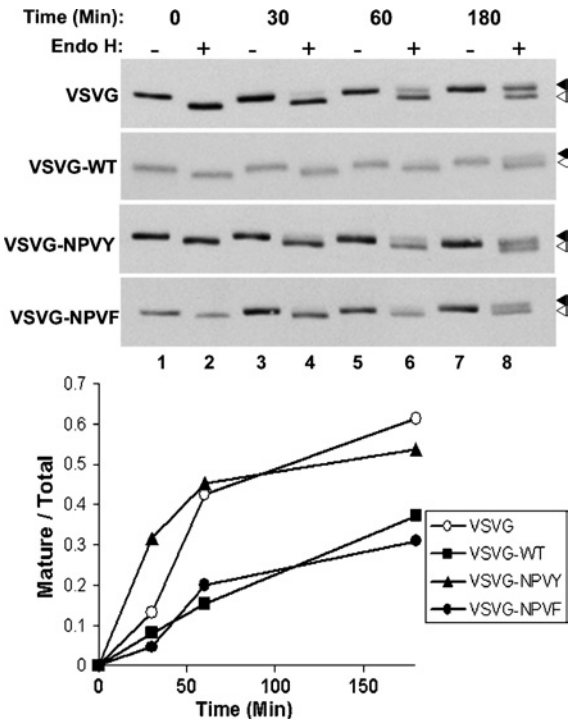

Figure 3 Insertion of an NPVY sequence to the EPO-R cytosolic domain improves ER exit of the corresponding VSVG–EPO-R

BOSC-23T cells transfected with VSVG, VSVG-WT, VSVG-NPVY and VSVG-NPVF cDNA constructs were incubated for 20 h at 40 °C. Subsequently, cells were shifted to 32 °C for the indicated time periods. Cell lysates were subjected to endo H digestion prior to Western-blot analysis with anti-GFP antibodies. Full and empty arrowheads indicate endo H-resistant and endo H-sensitive forms of the VSVG and VSVG fusion proteins respectively. The graph represents the fraction of endo H-resistant proteins with respect to their total levels as a function of time following transfer to 32 °C. This Figure is representative of one of four experiments displaying similar results.

cytosolic domain retards this transport. Taken together, Figures 2 and 3 demonstrate for the first time that the EPO-R cytosolic domain inhibits the intracellular transport along the secretory pathway. We next attempted to determine whether modification within the cytosolic domain of EPO-R could improve glycan maturation.

Improved maturation conferred by addition of an NPXY motif

NPXY-based motifs are involved in receptor endocytosis from the cell surface. We questioned whether this motif might also play a role in exocytosis, namely transport along the secretory pathway. NPXY motifs are often found within medium-length cytosolic domains ranging from ~40 to ~200 amino acid residues. Proteins have either a phenylalanine or a tyrosine residue, two positions upstream of the asparagine [F/Y]XNPXY [37].

The retarded intracellular transport of VSVG-WT as compared with that of the parental VSVG moiety thus provides a unique experimental tool to determine whether an NPXY motif can enhance trafficking along the secretory pathway. Accordingly, we introduced an NPVY amino acid insert in the cytosolic domain of EPO-R, between amino acid residues 432 and 433, two positions downstream of Tyr^{431}. VSVGtsO45 on to which the cytosolic portion of the EPO-R containing a transplanted NPVY sequence was attached is represented as VSVG-NPVY (Figure 1). To test whether functionality of the NPVY insert in the context of receptor maturation is phosphorylation-dependent, we replaced the tyrosine residue within this motif with phenylalanine residue, thereby yielding the VSVG-NPVF fusion protein (Figure 1).

First, we planned to test whether the NPVY insert could modify the exit of VSVG-WT from the ER. We thus quantified the extent of glycan processing of VSVG-NPVY as compared with that of the VSVG–WT fusion protein. As shown in Figure 3, endo H-resistant forms of VSVG-NPVY were more prominent than those of VSVG-WT and VSVG-NPVF (Figure 3, lanes 4, 6 and 8). Thus introduction of an NPVY sequence into the EPO-R cytosolic domain improved glycan maturation of the respective fusion protein, as compared with that of VSVG-WT, suggesting that the NPVY insert may play a role in ER exit. The possibility that tyrosine residue phosphorylation of this motif may play a role in this process is inferred from the lower glycan maturation of VSVG-NPVF.

Improved cell surface expression conferred by the NPVY insert

Improved glycan maturation of VSVG-NPVY as compared with that of VSVG-WT raised the question whether this fusion protein is also expressed at higher levels on the cell surface. Towards this end, we measured the levels of the surface biotinylated VSVG, VSVG-WT, VSVG-NPVY and VSVG-NPVF proteins (Figure 4). BOSC-23T cells were incubated at 40 °C immediately following transfection. On the following day, the cells were transferred to 32 °C for the indicated time periods and subjected to cell surface biotinylation [31]. Cell lysates were subjected to precipitation with streptavidin–agarose beads to isolate the BP, followed by Western-blot analysis with anti-GFP antibodies (Figure 4A, BP). Surface-biotinylated VSVG and VSVG–EPO-R fusion proteins were quantified (Figure 4A, graph) by normalizing the levels of the surface-biotinylated VSVG proteins (Figure 4A, BP) to their respective levels in aliquots of the cell lysates, as detected by anti-GFP antibodies (Figure 4A, TCL). The results of this experiment demonstrate that addition of an NPVY sequence to the fusion protein improved its cell surface expression at all time periods following transfer of the cells to 32 °C. This improvement requires the presence of the tyrosine residue, as the NPVF sequence was by far less potent in conferring surface expression of the respective VSVG fusion protein. The results of this experiment thus correlate with the endo H susceptibility of the VSVG constructs, as depicted in Figure 3. However, whereas the addition of an NPVY sequence to the VSVG–EPO-R fusion protein improved its maturation levels to those of the native VSVG molecule, the surface expression of VSVG-NPVY was still at a lower level than that of VSVG. The higher surface expression of VSVG as demonstrated by the biotinylation assay is in line with the fluorescence imaging illustrated in Figure 2.

We next questioned what step(s) of the secretory pathway might be facilitated by the presence of the NPVY insert. To address this issue, our strategy was to accumulate the proteins in different intracellular compartments, by using temperature blocks. A 40 °C block was used for retaining the proteins in the ER [22], as performed in the previous analyses (Figures 2, 3 and 4A), and a 20 °C block was used for accumulation in the TGN (*trans*-Golgi network) [38]. BOSC-23T cells were incubated at 40 °C immediately following transfection. On the following day, the cells were transferred to 20 °C for 2 h, in order to accumulate the proteins in the TGN. The cells were then transferred back to 40 °C for 30 min and subjected to cell surface biotinylation. The latter transfer to 40 °C was performed in order to selectively track TGN-associated proteins, without the interference of newly synthesized molecules originating in the ER (Figure 4B). The results of this experiment demonstrate that addition of the NPVY sequence also improved the trafficking of the fusion protein from

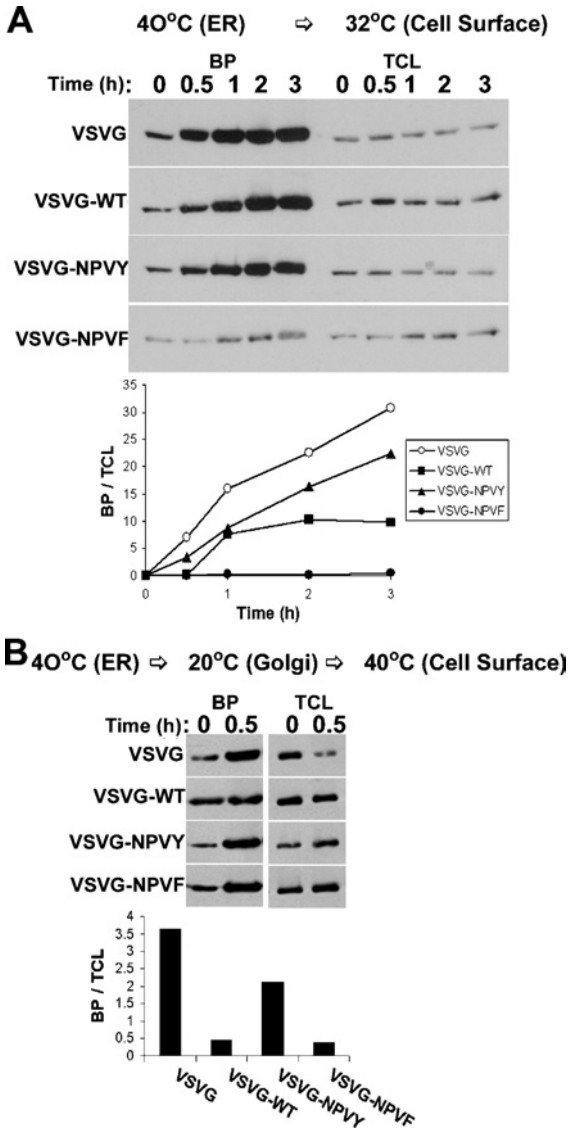

Figure 4 The NPVY cytosolic insert confers increased cell surface expression of the corresponding VSVG–EPO-R

BOSC-23T cells were transfected with VSVG, VSVG-WT, VSVG-NPVY and VSVG-NPVF cDNA constructs. Immediately following transfection, the cells were incubated for 20 h at 40 °C. (**A**) Cells were shifted to 32 °C for the indicated time periods and subjected to cell surface biotinylation. (**B**) Cells were transferred to 20 °C for 2 h in order to accumulate the proteins in the TGN. The cells were then transferred back to 40 °C for 30 min and subjected to cell surface biotinylation. Cell lysates (**A**, **B**) were subjected to precipitation with streptavidin–agarose beads (BP). The streptavidin precipitates and 10 % aliquots of the TCL were subjected to Western-blot analysis with anti-GFP antibodies to determine the levels of VSVG and VSVG–EPO-R fusion proteins. The graphs represent the ratios of the levels of surface-biotinylated VSVG and VSVG–EPO-R fusion proteins to their total levels in the cell. This Figure is representative of one of three experiments displaying similar results.

the TGN to the cell surface. This improvement required the presence of the tyrosine residue within the NPXY motif.

Enhanced maturation of EPO-R conferred by insertion of an NPVY sequence to the cytosolic domain

We hypothesized that the extent of glycan maturation and cell surface expression of the VSVG–EPO-R fusion proteins would reflect the capacity of the corresponding EPO-R cytosolic sequences to modulate maturation and surface expression of the respective

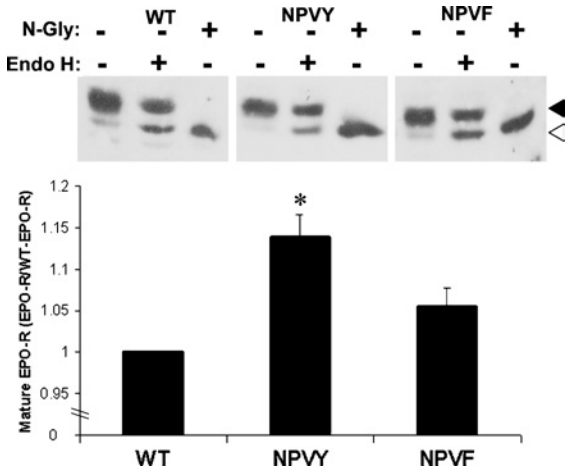

Figure 5 Increased levels of endo H-resistant EPO-R conferred by the NPVY insert

Lysates of Ba/F3 cells expressing WT-EPO-R, NPVY-EPO-R or NPVF-EPO-R at steady state were subjected to endo H or PNGase F (N-Gly) digestion prior to Western-blot analysis with anti-EPO-R antibodies. Full and empty arrowheads indicate endo H-resistant and endo H-sensitive EPO-Rs respectively. The histogram represents the ratio of mature EPO-R to the total EPO-R in proportion to the ratio of mature WT-EPO-R to the total EPO-R ($n = 3$). $^*P < 0.03$, glycan maturation of NPVY EPO-R as compared with that of WT and with that of NPVF EPO-Rs. This Figure is representative of one of three experiments displaying similar results.

EPO-Rs. We thus set forth to determine whether the NPVY cytosolic insert would confer improved maturation and surface expression on the corresponding EPO-R molecule. Towards this goal, we generated EPO-R mutants containing NPVY or NPVF inserts in their cytosolic domain (NPVY-EPO-R and NPVF-EPO-R respectively). The respective EPO-Rs were stably transfected into the Ba/F3 cell line. The choice of the Ba/F3 proB-cell line was based on the wealth of information that has accumulated on signal transduction and structure–function relationship of the EPO-R and other cytokine receptors expressed in these cells. Upon transfection of the Ba/F3 cells with the EPO-R cDNA, EPO can replace their need for IL-3, thus rendering this system most useful for studying the EPO-R and EPO-mediated EPO-R signalling [39]. It is noteworthy that Ba/F3 cells expressing the NPVY-EPO-R and NPVF-EPO-R are capable of growing in EPO as a substitute for IL-3 (results not shown), thereby assuring that the NPVY and NPVF insertions did not impair the ability of the respective EPO-Rs to confer EPO-mediated proliferation.

Glycan maturation

To determine glycan maturation of Ba/F3 cells expressing WT-EPO-R, NPVY-EPO-R or NPVF-EPO-R at steady state (Figure 5), cell lysates were subjected to digestion with endo H or with PNGase (to remove the entire glycan chain). This analysis confirms the higher abundance of endo H-resistant NPVY-EPO-R, as compared with endo H-resistant WT-EPO-R species and NPVF-EPO-R (Figure 5, solid arrowhead). The results are quantified as the ratio of mature endo H-resistant EPO-R to the total EPO-R (endo H-resistant and endo H-sensitive). This result lends support to the premise that the NPVY insert confers enhanced maturation of the EPO-R through the early secretory pathway, as reflected by improved glycan maturation. This enhancement requires the tyrosine residue in the NPXY motif, which might suggest the involvement of tyrosine phosphorylation. Our results are in line with the observed improved ER exit of the VSVG-NPVY fusion protein as compared with that of VSVG-WT and VSVG-NPVF (Figure 3).

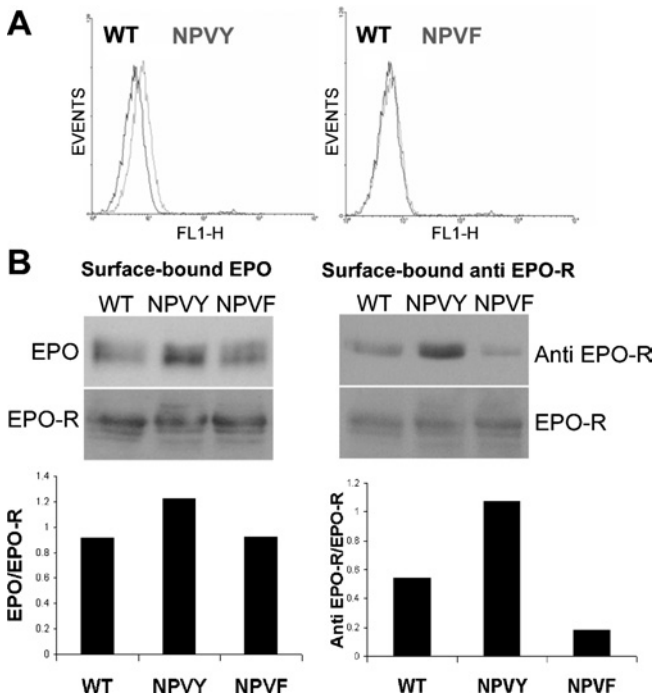

Figure 6 Elevated cell surface expression of EPO-R conferred by the NPVY insert

(**A**) Surface EPO-R levels in Ba/F3 cells stably expressing WT-EPO-R, NPVY-EPO-R or NPVF-EPO-R were determined by FACS analysis by using anti-EPO-R antibodies followed by Cy2-conjugated secondary antibodies. (**B**) Surface EPO-R levels in these cells were also determined by biochemical binding analysis. Cells were incubated for 1 h at 4 °C with 20 units/ml rHuEPO (left) or with anti-EPO-R antibodies (right). Cells were washed to remove unbound ligand or antibody, followed by Western-blot analysis with anti-EPO antibodies or with HRP-labelled goat anti-rabbit antibodies. Histograms represent the ratio of surface-bound EPO to the total amount of cellular EPO-R (left) and the ratio of anti-EPO-R antibodies to the total amount of cellular EPO-R (right). The FACS and biochemical analyses each represent one of five experiments displaying similar results.

Cell surface expression

To determine the cell surface expression of EPO-Rs, we measured the binding of anti-EPO-R antibodies to Ba/F3 cells expressing WT, NPVY or NPVF EPO-Rs, by using flow cytometry. The results (Figure 6A) demonstrate increased cell-surface-bound anti-EPO-R antibodies in the NPVY-EPO-R-expressing Ba/F3 cells as compared with Ba/F3 cells expressing the WT or NPVF EPO-Rs. To further validate these results, we also subjected the EPO-R-expressing Ba/F3 cells to anti-EPO-R antibody binding or EPO binding, at 4 °C (Figure 6B). Non-bound anti-EPO-R antibodies and ligand (EPO) were removed by washing with cold PBS. Cell lysates were prepared and subjected to Western-blot analysis with HRP (horseradish peroxidase)-labelled goat anti-rabbit antibodies or anti-EPO antibodies respectively. For comparison, total cellular levels of EPO-R are depicted. Quantification of this analysis is presented as the ratio of surface-bound anti-EPO-R or anti-EPO antibodies to the cellular levels of EPO-R (Figure 6B). In accordance with the VSVG–EPO-R fusion proteins, an EPO-R containing the NPVY insertion is expressed at higher levels on the cell surface as compared with WT and NPVF EPO-Rs.

Taken together, we demonstrate for the first time that the EPO-R cytosolic domain hampers maturation of the receptor and that the NPVY cytosolic insert improves maturation and cell surface expression of the EPO-R. These results are in line with the premise that VSVG–EPO-R fusion proteins can reflect intracellular maturation and transport of the respective EPO-R derivatives. We thus propose that the VSVG–EPO-R fusion proteins offer a unique experimental approach to screen motifs that regulate intracellular trafficking as well as to screen sequences within the EPO-R that are involved in regulating its intracellular trafficking.

DISCUSSION

Little is known about the identity of ER export signals and how they are used to regulate the number of proteins on the cell surface. Retention of most of the EPO-R molecules in early compartments of the secretory pathway [9] has been attributed to inefficient folding of the receptor extracellular domain. The present study addressed the role of the EPO-R intracellular domain in ER export. We employed a unique experimental approach, which was based on fusion of the cytosolic domain of the EPO-R to VSVGtsO45. On the one hand, this experimental design enables the identification of sequences within the cytosolic domain of the EPO-R that regulate ER exit and cell surface expression, and on the other hand, it may provide a useful screen for targeted modifications in the EPO-R cytosolic domain that can modulate EPO-R trafficking.

Export of VSVG from the ER as well as from the TGN was shown to rely on particular sequence motifs within the VSVG cytosolic domain [40,41]. The VSVG–EPO-R fusion proteins are transported along the secretory pathway as the parental VSVGtsO45 molecule (Figure 2), yet their intracellular trafficking and subsequent deposition at the surface are delayed. These results suggest that the EPO-R cytosolic domain can override the endogenous plasma-membrane-targeting motifs of VSVG. The advantage of using BOSC-23T cells for these experiments is the delayed maturation of VSVGtsO45 [19], thereby enabling a broader timeframe for determining the maturation kinetics of the VSVG–EPO-R fusion proteins.

The steady-state levels of surface membrane receptors are governed by a combination of factors including ER exit, protein stability and endocytosis. In that respect, it was shown that surface expression can be augmented by chemical compounds that inhibit degradation [42] or enhance folding [43,44]. Here, we demonstrate that insertion of an NPVY sequence to the cytosolic domain of the EPO-R downstream of the box2 motif [45] increases the surface expression of the EPO-R. Although the NPXY motif was originally associated with clathrin binding and endocytosis, it may also function in other contexts of protein traffic, including ER exit and post-Golgi trafficking [46]. Sequence motifs that govern exit from the ER and deposition at the cell surface appear to be degenerate, with no known universal consensus sequences or features [37,47]. Nevertheless, it is intriguing to speculate that these signals may be similar to signals that specify inclusion in clathrin-coated vesicles at the plasma membrane [37]. Such signals are known to interact with well-characterized adaptor complexes [48,49]. It is possible that adaptor proteins, which participate in sorting of proteins into the cell [50], may also play a role in other positions along the secretory pathway. NPXY motifs are also recognized by proteins containing a PTB domain (phosphotyrosine-binding domain) [51]. The PTB domain can bind the NPXY motif in a phosphorylation-independent manner and also in a phosphorylation-dependent manner [52]. It is thus tempting to speculate that the NPXY motif can also serve as an ER export signal that interacts with proteins containing PTB domains and together, by using appropriate adaptor complex(es), mediate ER export.

VSVGtsO45–EPO-R fusion proteins thus provide valuable experimental tools that enable the dissection of molecular determinants within the intracellular portion of the EPO-R that

regulate its intracellular trafficking. The preservation of thermal sensitivity of the fusion proteins enables the accumulation of proteins in the ER and allows tracking their localization upon shift to the permissive temperature [23]. This approach overcomes the inherent difficulty in monitoring the dynamic segregation of the newly synthesized EPO-R. It also enables us to follow two different critical stages of the maturation: (i) exit from the ER and (ii) cell surface expression, while the parental molecule, VSVGtsO45, serves as a reference. The NPVY insert confers improved glycan processing on the respective VSVG-NPVY fusion protein. This could be attributed, for instance, to enhanced glycan processing within the Golgi apparatus, or to facilitated ER exit, or intra-Golgi transport. The first possibility is less likely, as it was recently demonstrated that for VSVG, the multienzymatic process of N-glycan modification does not comprise a rate-limiting step for its Golgi efflux [53]. The NPVY insert also improved cell surface expression of the VSVG-NPVY fusion protein, as compared with VSVG-WT, albeit the cell surface expression of both EPO-R-containing VSVG fusion proteins was less efficient than that of VSVG (Figure 4).

The steady-state level of cell surface EPO-R is a result of a number of mechanisms: trafficking through the secretory pathway, internalization and degradation. The contribution of each pathway to the increased surface expression of NPVY-EPO-R warrants further investigation. Addition of the EPO-R cytosolic domain to VSVGtsO45 inhibited the exit of the corresponding fusion protein (VSVG-WT) from the ER, as judged by its reduced ability to acquire endo H resistance (Figure 3). In this respect, VSVG and VSVG-NPVY were similar in their susceptibility to endo H. Yet, the lower surface expression of VSVG-NPVY as compared with that of VSVG implies that there are other points of regulation of trafficking in which these two molecules differ. Fitting the experimental data of endo H resistance of VSVG–EPO-R derivatives (Figure 3) to a simple first-order saturation equation [$y = A(1 - e^{-kt})$] showed that VSVG-NPVY acquired endo H resistance at levels 1.5–2-fold faster than VSVG-WT. Maintenance of improved endo H resistance by the NPVY motif, both in the context of the EPO-R (Figure 5) and in the context of the VSVG–EPO-R fusion proteins (Figure 3), emphasizes the relevance and validity of the approach described herein. Improved glycan maturation conferred by the NPVY motif is more pronounced in the context of VSVG as compared with its effect on the EPO-R. This is probably due to the presence of the EPO-R extracellular domain, which contributes to ER retention of the receptor [7,11].

Finally, we demonstrate that although NPXY motifs often act as internalization signals at the cytoplasmic tail of membrane receptors, addition of an NPVY sequence increased glycan maturation of the EPO-R (Figure 5) and the number of receptor molecules on the cell surface (Figure 6). Hence, the NPVY sequence might also act as a maturation (or ER export) signal. Furthermore, we have shown that modification of the cytosolic domain of the EPO-R is a feasible experimental approach to improve the surface expression of the EPO-R. The attachment of EPO-R cytosolic sequences to VSVGtsO45 can be implemented, on the one hand, to define sequences within the cytosolic domain of the EPO-R that are involved in intracellular retention, and on the other hand, to screen for EPO-R cytosolic sequences that can promote maturation and cell surface expression of the respective EPO-R molecules. The premise is that the activity of these sequences displayed in the VSVG–EPO-R fusion proteins will also be reflected in the EPO-R molecule.

We expect that this experimental approach may also be implemented for the modulation of glycan processing and surface expression of other membrane proteins/receptors.

We thank Professor Ronit Sagi-Eisenberg, Dr Ruth Orkin and Liron Yosha (Department of Cell and Developmental Biology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel) for critically reviewing this paper before its submission. This work was supported by grants from the Israel Science Foundation administered by the Israel Academy of Sciences and Humanities to D.N. (643/06) and K.H. (650/02). This work was carried out in partial fulfilment of the requirements for the Ph.D. degree for T.N. from the Sackler Faculty of Medicine, Tel Aviv University.

REFERENCES

1 van Vliet, C., Thomas, E. C., Merino-Trigo, A., Teasdale, R. D. and Gleeson, P. A. (2003) Intracellular sorting and transport of proteins. Prog. Biophys. Mol. Biol. **83**, 1–45
2 Olkkonen, V. M. and Ikonen, E. (2000) Genetic defects of intracellular-membrane transport. N. Engl. J. Med. **343**, 1095–1104
3 Wu, H., Liu, X., Jaenisch, R. and Lodish, H. F. (1995) Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell **83**, 59–67
4 Mulcahy, L. (2001) The erythropoietin receptor. Semin. Oncol. **28**, 19–23
5 Frank, S. J. (2002) Receptor dimerization in GH and erythropoietin action – it takes two to tango, but how? Endocrinology **143**, 2–10
6 Strous, G. J., van Kerkhof, P., Verheijen, C., Rossen, J. W., Liou, W., Slot, J. W., Roelen, C. A. and Schwartz, A. L. (1994) Expression of functional growth hormone receptor in a mouse L cell line infected with recombinant vaccinia virus. Exp. Cell Res. **211**, 353–359
7 Hilton, D. J., Watowich, S. S., Murray, P. J. and Lodish, H. F. (1995) Increased cell surface expression and enhanced folding in the endoplasmic reticulum of a mutant erythropoietin receptor. Proc. Natl. Acad. Sci. U.S.A. **92**, 190–194
8 Huang, L. J., Constantinescu, S. N. and Lodish, H. F. (2001) The N-terminal domain of Janus kinase 2 is required for Golgi processing and cell surface expression of erythropoietin receptor. Mol. Cell **8**, 1327–1338
9 Yoshimura, A., D'Andrea, A. D. and Lodish, H. F. (1990) Friend spleen focus-forming virus glycoprotein gp55 interacts with the erythropoietin receptor in the endoplasmic reticulum and affects receptor metabolism. Proc. Natl. Acad. Sci. U.S.A. **87**, 4139–4143
10 Cohen, J., Altaratz, H., Zick, Y., Klingmuller, U. and Neumann, D. (1997) Phosphorylation of erythropoietin receptors in the endoplasmic reticulum by pervanadate-mediated inhibition of tyrosine phosphatases. Biochem. J. **327**, 391–397
11 Ravid, O., Shams, I., Ben-Califa, N., Nevo, E., Avivi, A. and Neumann, D. (2007) An extracellular region of the erythropoietin receptor of the subterranean blind mole rat Spalax enhances receptor maturation. Proc. Natl. Acad. Sci. U.S.A. **104**, 14360–14365
12 Yoshimura, A., Zimmers, T., Neumann, D., Longmore, G., Yoshimura, Y. and Lodish, H. F. (1992) Mutations in the Trp-Ser-X-Trp-Ser motif of the erythropoietin receptor abolish processing, ligand binding, and activation of the receptor. J. Biol. Chem. **267**, 11619–11625
13 Hilton, D. J., Watowich, S. S., Katz, L. and Lodish, H. F. (1996) Saturation mutagenesis of the WSXWS motif of the erythropoietin receptor. J. Biol. Chem. **271**, 4699–4708
14 Royer, Y., Staerk, J., Costuleanu, M., Courtoy, P. J. and Constantinescu, S. N. (2005) Janus kinases affect thrombopoietin receptor cell surface localization and stability. J. Biol. Chem. **280**, 27251–27261
15 Tong, W., Sulahian, R., Gross, A. W., Hendon, N., Lodish, H. F. and Huang, L. J. (2006) The Membrane-proximal region of the thrombopoietin receptor confers its high surface expression by JAK2-dependent and -independent mechanisms. J. Biol. Chem. **281**, 38930–38940
16 Motohashi, T., Nakamura, Y., Osawa, M., Hiroyama, T., Iwama, A., Shibuya, A. and Nakauchi, H. (2001) Increased cell surface expression of C-terminal truncated erythropoietin receptors in polycythemia. Eur. J. Haematol. **67**, 88–93
17 Beckman, D. L., Lin, L. L., Quinones, M. E. and Longmore, G. D. (1999) Activation of the erythropoietin receptor is not required for internalization of bound erythropoietin. Blood **94**, 2667–2675
18 Levin, I., Cohen, J., Supino Rosin, L., Yoshimura, A., Watowich, S. S. and Neumann, D. (1998) Identification of a cytoplasmic motif in the erythropoietin receptor required for receptor internalization. FEBS Lett. **427**, 164–170
19 Barzilay, E., Ben-Califa, N., Hirschberg, K. and Neumann, D. (2005) Uncoupling of Brefeldin A-mediated coatomer protein complex-I dissociation from Golgi redistribution. Traffic **6**, 794–802
20 Hirschberg, K., Miller, C. M., Ellenberg, J., Presley, J. F., Siggia, E. D., Phair, R. D. and Lippincott-Schwartz, J. (1998) Kinetic analysis of secretory protein traffic and characterization of Golgi to plasma membrane transport intermediates in living cells. J. Cell Biol. **143**, 1485–1503

21 Mironov, A. A., Mironov, Jr, A. A., Beznoussenko, G. V., Trucco, A., Lupetti, P., Smith, J. D., Geerts, W. J., Koster, A. J., Burger, K. N., Martone, M. E. et al. (2003) ER-to-Golgi carriers arise through direct en bloc protrusion and multistage maturation of specialized ER exit domains. Dev. Cell **5**, 583–594
22 Bergmann, J. E. (1989) Using temperature-sensitive mutants of VSV to study membrane protein biogenesis. Methods Cell Biol. **32**, 85–110
23 Cole, N. B., Ellenberg, J., Song, J., DiEuliis, D. and Lippincott-Schwartz, J. (1998) Retrograde transport of Golgi-localized proteins to the ER. J. Cell Biol. **140**, 1–15
24 Kirchhausen, T. (1999) Adaptors for clathrin-mediated traffic. Annu. Rev. Cell Dev. Biol. **15**, 705–732
25 Chen, W. J., Goldstein, J. L. and Brown, M. S. (1990) NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. J. Biol. Chem. **265**, 3116–3123
26 Kibbey, R. G., Rizo, J., Gierasch, L. M. and Anderson, R. G. (1998) The LDL receptor clustering motif interacts with the clathrin terminal domain in a reverse turn conformation. J. Cell Biol. **142**, 59–67
27 Cohen, J. C., Kimmel, M., Polanski, A. and Hobbs, H. H. (2003) Molecular mechanisms of autosomal recessive hypercholesterolemia. Curr. Opin. Lipidol. **14**, 121–127
28 Smith, R. M., Zhang, S., White, M. F. and Jarett, L. (1996) The role of receptor kinase activity and the NPEY960 motif in insulin-accelerated receptor-mediated insulin internalization. J. Recept. Signal Transduct. Res. **16**, 339–355
29 Yokode, M., Pathak, R. K., Hammer, R. E., Brown, M. S., Goldstein, J. L. and Anderson, R. G. (1992) Cytoplasmic sequence required for basolateral targeting of LDL receptor in livers of transgenic mice. J. Cell Biol. **117**, 39–46
30 Neumann, D., Wikstrom, L., Watowich, S. S. and Lodish, H. F. (1993) Intermediates in degradation of the erythropoietin receptor accumulate and are degraded in lysosomes. J. Biol. Chem. **268**, 13639–13649
31 Supino-Rosin, L., Yoshimura, A., Yarden, Y., Elazar, Z. and Neumann, D. (2000) Intracellular retention and degradation of the epidermal growth factor receptor, two distinct processes mediated by benzoquinone ansamycins. J. Biol. Chem. **275**, 21850–21855
32 Neumann, D., Yuk, M. H., Lodish, H. F. and Lederkremer, G. Z. (1996) Blocking intracellular degradation of the erythropoietin and asialoglycoprotein receptors by calpain inhibitors does not result in the same increase in the levels of their membrane and secreted forms. Biochem. J. **313**, 391–399
33 Nichols, B. J., Kenworthy, A. K., Polishchuk, R. S., Lodge, R., Roberts, T. H., Hirschberg, K., Phair, R. D. and Lippincott-Schwartz, J. (2001) Rapid cycling of lipid raft markers between the cell surface and Golgi complex. J. Cell Biol. **153**, 529–541
34 D'Andrea, A. D., Lodish, H. F. and Wong, G. G. (1989) Expression cloning of the murine erythropoietin receptor. Cell **57**, 277–285
35 Chen, C. A. and Okayama, H. (1988) Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. BioTechniques **6**, 632–638
36 Doms, R. W., Ruusala, A., Machamer, C., Helenius, J., Helenius, A. and Rose, J. K. (1988) Differential effects of mutations in three domains on folding, quaternary structure, and intracellular transport of vesicular stomatitis virus G protein. J. Cell Biol. **107**, 89–99
37 Bonifacino, J. S. and Traub, L. M. (2003) Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu. Rev. Biochem. **72**, 395–447
38 Matlin, K. S. and Simons, K. (1983) Reduced temperature prevents transfer of a membrane glycoprotein to the cell surface but does not prevent terminal glycosylation. Cell **34**, 233–243
39 Li, J. P., D'Andrea, A. D., Lodish, H. F. and Baltimore, D. (1990) Activation of cell growth by binding of friend spleen focus-forming virus gp55 glycoprotein to the erythropoietin receptor. Nature **34**, 233–243
40 Nishimura, N. and Balch, W. E. (1997) A di-acidic signal required for selective export from the endoplasmic reticulum. Science **277**, 556–558
41 Nishimura, N., Plutner, H., Hahn, K. and Balch, W. E. (2002) The delta subunit of AP-3 is required for efficient transport of VSV-G from the *trans*-Golgi network to the cell surface. Proc. Natl. Acad. Sci. U.S.A. **99**, 6755–6760
42 van Kerkhof, P., Vallon, E. and Strous, G. J. (2003) A method to increase the number of growth hormone receptors at the surface of cells. Mol. Cell. Endocrinol. **201**, 57–62
43 Sato, S., Ward, C. L., Krouse, M. E., Wine, J. J. and Kopito, R. R. (1996) Glycerol reverses the misfolding phenotype of the most common cystic fibrosis mutation. J. Biol. Chem. **271**, 635–638
44 Loo, T. W., Bartlett, M. C., Wang, Y. and Clarke, D. M. (2006) The chemical chaperone CFcor-325 repairs folding defects in the transmembrane domains of CFTR-processing mutants. Biochem. J. **395**, 537–542
45 Jiang, N., He, T. C., Miyajima, A. and Wojchowski, D. M. (1996) The box1 domain of the erythropoietin receptor specifies Janus kinase 2 activation and functions mitogenically within an interleukin 2 beta-receptor chimera. J. Biol. Chem. **271**, 16472–16476
46 Rodriguez-Boulan, E. and Musch, A. (2005) Protein sorting in the Golgi complex: shifting paradigms. Biochim. Biophys. Acta **1744**, 455–464
47 Sevier, C. S., Weisz, O. A., Davis, M. and Machamer, C. E. (2000) Efficient export of the vesicular stomatitis virus G protein from the endoplasmic reticulum requires a signal in the cytoplasmic tail that includes both tyrosine-based and di-acidic motifs. Mol. Biol. Cell **11**, 13–22
48 Hirst, J. and Robinson, M. S. (1998) Clathrin and adaptors. Biochim. Biophys. Acta **1404**, 173–193
49 Bonifacino, J. S. and Dell'Angelica, E. C. (1999) Molecular bases for the recognition of tyrosine-based sorting signals. J. Cell Biol. **145**, 923–926
50 Ohno, H., Fournier, M. C., Poy, G. and Bonifacino, J. S. (1996) Structural determinants of interaction of tyrosine-based sorting signals with the adaptor medium chains. J. Biol. Chem. **271**, 29009–29015
51 Smith, M. J., Hardy, W. R., Murphy, J. M., Jones, N. and Pawson, T. (2006) Screening for PTB domain binding partners and ligand specificity using proteome-derived NPXY peptide arrays. Mol. Cell. Biol. **26**, 8461–8474
52 Rajala, R. V. and Chan, M. D. (2005) Identification of a NPXY motif in growth factor receptor-bound protein 14 (Grb14) and its interaction with the phosphotyrosine-binding (PTB) domain of IRS-1. Biochemistry **44**, 7929–7935
53 Vasserman, G., Magal, L. G., Shepshelovich, J., Elifaz, E. and Hirschberg, K. (2006) Processing of VSVG protein is not a rate-limiting step for its efflux from the Golgi complex. Biochem. Biophys. Res. Commun. **351**, 689–694

Received 20 September 2007/5 November 2007; accepted 12 November 2007
Published as BJ Immediate Publication 12 November 2007, doi:10.1042/BJ20071297