An extracellular region of the erythropoietin receptor of the subterranean blind mole rat *Spalax* enhances receptor maturation

Orly Ravid*, Imad Shams†, Nathalie Ben Califa*, Eviatar Nevo†‡, Aaron Avivi†‡, and Drorit Neumann*‡

*Department of Cell and Developmental Biology, Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv 69978, Tel Aviv, Israel; and †Laboratory for Animal Molecular Evolution, Institute of Evolution, University of Haifa, Mount Carmel, Haifa 31905, Israel

Contributed by Eviatar Nevo, July 19, 2007 (sent for review February 10, 2007)

Erythropoietic functions of erythropoietin (EPO) are mediated by its receptor (EPO-R), which is present on the cell surface of erythroid progenitors and induced by hypoxia. We focused on EPO-R from *Spalax galili* (sEPO-R), one of the four Israeli species of the subterranean blind mole rat, *Spalax ehrenbergi* superspecies, as a special natural animal model of high tolerance to hypoxia. Led by the intriguing observation that most of the mouse EPO-R (mEPO-R) is retained in the endoplasmic reticulum (ER), we hypothesized that sEPO-R is expressed at higher levels on the cell surface, thus maximizing the response to elevated EPO, which has been reported in this species. Indeed, we found increased cell-surface levels of sEPO-R as compared with mEPO-R by using flow cytometry analysis of BOSC cells transiently expressing HA-tagged EPO-Rs (full length or truncated). We then postulated that unique extracellular sEPO-R sequence features contribute to its processing and cell-surface expression. To map these domains of the sEPO-R that augment receptor maturation, we generated EPO-R derivatives in which parts of the extracellular region of mEPO-R were replaced with the corresponding fragments of sEPO-R. We found that an extracellular portion of sEPO-R, harboring the N-glycosylation site, conferred enhanced maturation and increased transport to the cell surface of the respective chimeric receptor. Taken together, we demonstrate higher surface expression of sEPO-R, attributed at least in part to increased ER exit, mediated by an extracellular region of this receptor. We speculate that these sEPO-R sequence features play a role in the adaptation of *Spalax* to extreme hypoxia.

signal transduction | intracellular trafficking | hypoxia | glycosylation

Erythropoietin (EPO) promotes proliferation and differentiation of erythroid cell precursors via activation of its cell-surface receptor (EPO-R) (1, 2). EPO-R is a member of the cytokine-receptor superfamily, characterized by the presence of four conserved Cys residues and a "WSXWS" motif in its extracellular domain. The lack of enzymatic activity in the intracellular domain of these receptors necessitates complex formation of ligand-bound receptors with other signaling partners [i.e., Janus kinases (JAKs)] (3) to initiate signaling cascades. EPO-R is synthesized in the endoplasmic reticulum (ER) as a major 64-kDa form with a single endoglycosidase H (Endo H)-sensitive high-mannose oligosaccharide and as a nonglycosylated 62-kDa form. Forty to 60% of the 64-kDa EPO-R molecules undergo further glycan maturation to generate the 66-kDa Endo H-resistant EPO-R (4).

In contrast to other type 1 cytokine receptors (5–7), a most striking property of the EPO-R is its low expression on the cell surface (4, 8–10) despite its high intracellular levels. The majority of newly synthesized EPO-R remains sequestered intracellularly and is rapidly degraded ($t_{1/2} \approx 45$ min) (11). These metabolic features are similar in both transfected (4) and fetal liver cells that endogenously express the EPO-R (12), supporting the idea that structural features of the receptor molecule regulate its surface expression. Low surface-expression levels of EPO-R have been attributed, among other factors (13, 14), to poor folding of its extracellular domain (15). Hence, targeted mutations in the extracellular domain of EPO-R either facilitate (15) or inhibit (16–18) surface expression of the receptor. The low surface expression of EPO-R in various cell lines provides an attractive, important model for studying receptor maturation.

EPO and EPO-R production were shown to increase in response to tissue oxygen deficiency (19). The subterranean mole rat (*Spalax*) thus provides a unique natural model, because it has evolved adaptive strategies to cope with extreme underground conditions of hypoxic stress (20). The high tolerance of *Spalax* to hypoxia seems related to a wide array of respiratory and cardiovascular adaptations, as reflected in structural and functional properties of several hypoxia-related genes including EPO (21), EPO-R (22), VEGF (23, 24), HIF-1 (24, 25), and p53 (26, 27). The sequence of *Spalax* EPO-R (sEPO-R) exhibits unique features compared with EPO-R of other mammals (22).

Here, we show improved processing and surface expression of sEPO-R, mediated, at least in part, by the extracellular domain of the receptor. We further demonstrate that a unique segment of sEPO-R extracellular domain confers improved processing/cell-surface expression or secretion of the corresponding mouse/*Spalax* chimeric EPO-R.

Results

Higher Cell-Surface Expression and Improved Glycan Maturation of sEPO-R. We identified cell-surface EPO-R on BOSC-23T transiently expressing mouse or *Spalax* EPO-Rs by using flow cytometry analysis. To avoid possible different binding parameters of the anti-EPO-R N terminus antibody to the *Spalax* and mouse EPO-Rs, we introduced an HA tag at the N termini of both *Spalax* and mouse EPO-Rs [supporting information (SI) Fig. 6] (10) and used anti-HA antibody for detection. Whereas the profiles of mouse EPO-R (mEPO-R) and control cells were almost superimposed, that of sEPO-R-transfected cells was considerably right-shifted (Fig. 1), indicating higher cell-surface expression of sEPO-R.

To determine whether the higher surface expression of sEPO-R is also reflected in its improved glycan maturation, we

Author contributions: O.R. and I.S. contributed equally to this work; O.R., A.A. and D.N. designed research; O.R., I.S., and N.B.C. performed research; O.R., I.S., E.N., A.A., and D.N. contributed new reagents/analytic tools; O.R., I.S., N.B.C., and D.N. analyzed data; and O.R., A.A., and D.N. wrote the paper.

The authors declare no conflict of interest.

Abbreviations: EPO, erythropoietin; EPO-R, EPO receptor; mEPO-R, mouse EPO-R; sEPO-R, *Spalax* EPO-R; ER, endoplasmic reticulum; Endo H, endoglycosidase H; sol, soluble; sExmIn, a chimera of the extracellular domain of sEPO-R and of transmembrane and intracellular domains of mEPO-R.

‡To whom correspondence may be addressed. E-mail: histo6@post.tau.ac.il, nevo@research.haifa.ac.il, or aaron@research.haifa.ac.il.

This article contains supporting information online at www.pnas.org/cgi/content/full/0706777104/DC1.

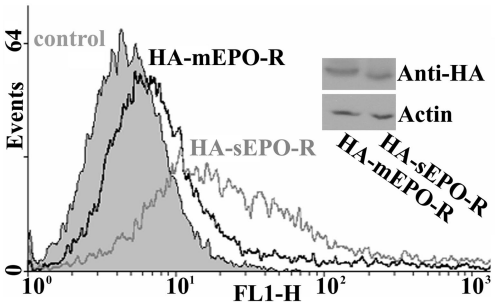

Fig. 1. Surface expression of HA-tagged full-length EPO-Rs. BOSC-23T cells transiently expressing HA-tagged mEPO-R or sEPO-R were labeled with anti-HA antibodies and analyzed for EPO-R surface expression by flow cytometry. Open histograms represent the profile of cells transfected with the indicated EPO-R; the filled histogram represents the profile of control, background FITC fluorescence. Histograms display cell number (events) as a function of EPO-R surface staining [FL1-Height (FL1-H)] on a logarithmic scale. (*Inset*) Equivalent EPO-R expression levels in cell lysates subjected to Western blot analysis with anti-HA and anti-actin antibodies. Data depict one of three similar experiments.

compared *Spalax* and mouse EPO-Rs empolying digestion with Endo H, which cleaves N-linked high-mannose-containing oligosaccharides on the immature EPO-R (4). EPO-R expression in the IL-3-dependent Ba/F3 cells allows a reliable comparison between EPO-R derivatives, both in terms of receptor processing (4, 18) and capacity to elicit EPO-mediated signaling cascades (16, 28–32). Of note, despite the sequence differences between *Spalax* and mouse EPO-Rs, EPO as a single growth factor was sufficient to promote proliferation of Ba/F3 cells expressing sEPO-R (SI Fig. 7), pointing to the functionality of the sEPO-R in a murine cell background. Western blot analysis of lysates from EPO-R-expressing Ba/F3 cells (Fig. 2*A*) showed Endo H sensitivity of the majority of mEPO-R molecules, whereas sEPO-R disclosed less Endo H-sensitive receptor forms. N-glycanase digest of both mouse and *Spalax* EPO-Rs yielded a single lower molecular weight band representing the completely deglycosylated EPO-Rs.

To determine whether the extracellular domain of sEPO-R confers increased cell-surface expression of the receptor, we generated truncated mouse and *Spalax* EPO-Rs lacking the cytosolic domain. In transiently transfected BOSC-23T cells, glycan maturation exhibited a higher dominance of Endo H-resistant truncated sEPO-R species than that of the corresponding mEPO-R (Fig. 2*B*). Of note, the higher prominence of full-length Endo H resistant sEPO-R as compared with Endo H-resistant truncated sEPO-R, may either reflect the different cell lines or suggest that the extracellular domain of the EPO-R is only partially responsible for enhanced maturation of sEPO-R. Moreover, the surface level of truncated HA-tagged sEPO-R was higher than in the respective mEPO-R derivative (SI Fig. 8).

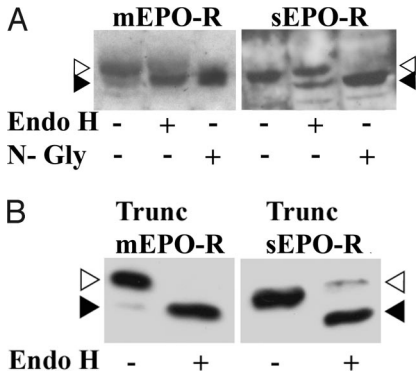

Fig. 2. Endo H susceptibility of full-length and truncated EPO-Rs. Lysates from Ba/F3 cells expressing full-length sEPO-R and mEPO-R (*A*) and BOSC-23T cells expressing truncated mEPO-R or sEPO-R (*B*) were incubated in the presence (+) or absence (−) of Endo H or N-glycanase (N-Gly) and followed by Western blot analysis with anti mEPO-R N terminus antibody. Filled and open arrows indicate Endo H-sensitive and Endo H-resistant EPO-Rs, respectively.

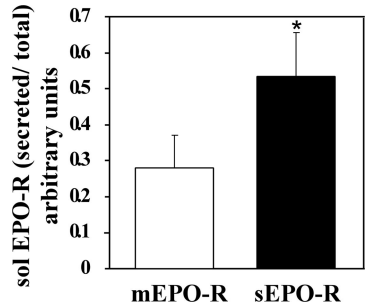

Fig. 3. Secretion of sol EPO-Rs. Quantification of EPO-R secretion ($n = 3$) (SI Fig. 9 depicts a representative experiment). Open and filled bars indicate mean ± standard error of secreted sol mEPO-R and sol sEPO-R, respectively, each divided by the sum of its secreted plus intracellular levels (total). *, $P < 0.05$.

Our data suggest that the sEPO-R extracellular and/or transmembrane domains harbor sequence features which endow it with improved maturation. The ability to detect Endo H-resistant truncated sEPO-Rs in BOSC-23T cells highlights the robust effect of the sEPO-R extracellular domain on receptor maturation; mEPO-R expressed in BOSC and COS 7 cells rarely demonstrates Endo H-resistant species (15, 33, 34).

The Extracellular Region of sEPO-R Confers Increased Transport of the Receptor to the Cell Surface. To focus on the extracellular domain of EPO-R, we used soluble (sol) EPO-Rs lacking both cytosolic and transmembrane domains. Sol EPO-R derivatives were transiently transfected into BOSC-23T cells, and the intracellular and secreted sol EPO-Rs were measured. Intracellular sol *Spalax* and mouse EPO-R molecules were both Endo H-sensitive (SI Fig. 9*A*), indicating their prevalence in an early compartment of the secretory pathway. Notably, the levels of secreted sol sEPO-R were ≈2-fold higher than sol mEPO-R (Fig. 3 and SI Fig. 9*B*). We thus postulate that the extracellular region of sEPO-R mediates increased deposition at the cell surface of the respective receptor as compared with that of mEPO-R.

The Extracellular Region of sEPO-R Accelerates EPO-R Maturation. Higher surface levels of EPO-R derivatives that contain the extracellular portion of sEPO-R raised the question of whether these *Spalax* sequences also enhance the kinetics of receptor maturation. We thus used pulse-chase analysis to compare the kinetics of glycan maturation of mouse and *Spalax* EPO-Rs and a chimeric EPO-R that harbors the extracellular region of sEPO-R and the transmembrane and intracellular regions of the mEPO-R (sExmIn) expressed in Ba/F3 cells (Fig. 4). At 30 min of chase, Endo H-resistant forms of sEPO-R and sExmIn comprised >50% of the newly synthesized molecules, whereas Endo H resistant forms of mEPO-R were still barely discernible. At 90 min of chase, approximately all sEPO-R and sExmIn molecules acquired resistance to Endo H digestion, whereas mEPO-R was still partially sensitive to Endo H. The contribution of the EPO-R intracellular domain to trafficking of the receptor to the cell surface is not yet clear; however, it may inhibit trafficking to the cell surface (10, 35). In that respect, enhanced glycan maturation of both *Spalax* and sExmIn EPO-Rs indicates

CELL BIOLOGY

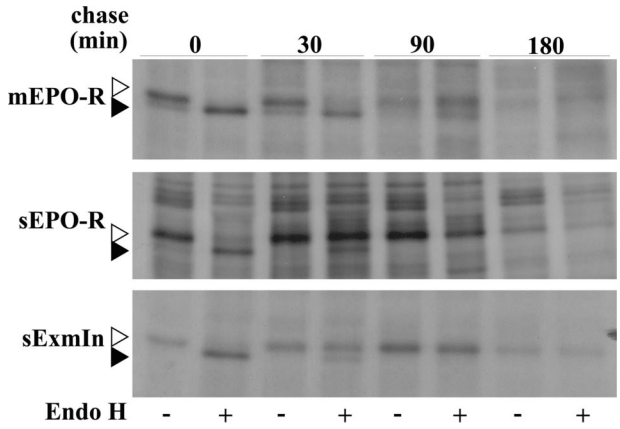

Fig. 4. Maturation kinetics of mouse, *Spalax*, and sExmIn EPO-Rs. Ba/F3 cells stably expressing mEPO-R, sEPO-R, or sExmIn were pulse-labeled for 30 min in the presence of [^{35}S]Cys-Met and chased in nonradioactive medium. EPO-Rs were immunoprecipitated with anti mEPO-R N terminus antibody followed by Endo H digestion, separation by SDS/PAGE, and autoradiography. Filled and open arrows indicate Endo H-sensitive and resistant EPO-Rs, respectively.

that the extracellular domain of sEPO-R can mediate maturation of the corresponding full-length receptor, overriding possible inhibitory effects of the intracellular domain.

Extracellular Segments of sEPO-R Confer Enhanced Maturation. To map extracellular regions of sEPO-R that confer enhanced maturation, we created chimeric receptors in which parts of the extracellular region of mEPO-R were replaced with the corresponding sEPO-R regions (Fig. 5*A*, *SI Methods*, and SI Table 1). It is worth noting that Ba/F3 cells expressing the chimeric EPO-Rs maintained their ability to proliferate when EPO was the sole added growth factor (data not shown), suggesting that the chimeras meet basic structural requirements for ligand activation (36). EPO activation of these cells resulted in Tyr phosphorylation of both EPO-R and STAT5, indicating the functionality of these EPO-Rs (SI Fig. 10).

We questioned whether the inserted sEPO-R segments confer enhanced maturation to the respective full-length EPO-R chimeras. Endo H susceptibility of the full-length chimeric EPO-Rs revealed that the *Spalax* domain harboring the N-glycosylation site (s46–136) conferred an increase in mature receptor species (Fig. 5*B*). The shorter *Spalax* motifs, s46–57 and s58–136, each enhanced receptor maturation, but to a lower extent. The segment sATG-45 that includes the *Spalax* signal peptide did not improve maturation of the respective EPO-R chimera. It is worth noting that the higher ratio of mature versus immature EPO-R in sExmIn as compared with the s46–136 chimera suggests that other extracellular determinants in sEPO-R may also contribute to this phenotype. These results are corroborated by experiments using the truncated membrane-bound and soluble forms of the chimeric EPO-Rs (Fig. 5 *C* and *D*), respectively. The sol EPO-R chimeras were generated with an HA tag (Fig. 5*D*) to enable convenient means for detecting EPO-Rs in the absence of specific antibodies to the extracellular domain of sEPO-R (37). In both cases, as observed for the full-length mouse/*Spalax* EPO-R chimeras, s46–136 improved maturation/secretion of the respective chimera more than segment s46–57 or s58–136.

Discussion

Our study demonstrates increased levels of sEPO-R expression at the cell surface. The underlying mechanism, at least in part, involves improved ER exit of sEPO-R mediated by an extracellular region of the receptor.

ER Exit and Intracellular Trafficking. A chimera of the extracellular domain of sEPO-R and of the transmembrane and cytosolic domains of mEPO-R (sExmIn) displayed improved glycan processing at steady state and accelerated glycan maturation compared with mEPO-R. These findings imply that (*i*) the extracellular domain of sEPO-R overrides putative cytosolic motifs (in mEPO-R or sEPO-R) that may confer retention/degradation in early compartments of the secretory pathway (10, 35), or (*ii*) the cytosolic segment of sEPO-R is inert with respect to receptor maturation. Truncated mEPO-R lacking the cytosolic region was found at higher levels at the cell surface (13), although the majority of the receptor is retained intracellularly (38). In addition to its role in endocytosis of surface receptor (38, 39), the EPO-R intracellular domain acts in several pathways that determine surface expression, including binding to auxiliary molecules (10) and directing to ER-associated degradation (35).

In both fetal liver cells endogenously expressing the EPO-R (12) and in EPO-R-transfected cells (15), the majority of the newly made EPO-Rs is retained intracellularly, and only a small fraction of receptor molecules is expressed on the plasma membrane (4, 11, 35, 40, 41). In EPO-dependent 32D cells, endogenously expressed EPO-Rs exhibited higher surface-expression as compared with the 32D cells, despite comparable intracellular EPO-R levels (42). Similarly, overexpression of mEPO-R in UT7 cells led to replacement of the endogenous human EPO-R on the cell surface, suggesting that chaperones and/or auxiliary molecules may be a limiting factor for surface deposition (43). In that respect, overexpression of exogenous EPO-Rs in other cell lines, including Ba/F3 cells, still results in its low surface expression (4, 44). Improved glycan processing and cell-surface expression of sEPO-R and EPO-R derivatives containing sEPO-R extracellular sequences in both Ba/F3 and in BOSC-23T cells suggest that the role of these domains extends beyond a particular cell type.

Successful export from the ER does not guarantee trafficking and functional expression of EPO-Rs at the cell surface, because cargo transport from the Golgi and stability at the plasma membrane are also governed by strict quality control processes; yet, it is an imperative first step (37). Inefficient folding and processing of EPO-R in the ER may represent one of the mechanisms responsible for controlling the levels of surface EPO-R (15). Long-lived interactions of chaperones with newly made polypeptides usually indicate protein misfolding in the ER. Binding of the ER-associated chaperone BiP to newly made full-length and sol mEPO-R (45), and the inability of the Endo H-sensitive ER form of the EPO-R to bind EPO, indicates that it is not fully folded (16, 18). We envisage more, as yet undiscovered, molecular players in the EPO-R folding pathway that may differ between mEPO-R and sEPO-R. The underlying premise is that the extracellular sequences of sEPO-R modulate molecular interactions that regulate the exit of EPO-R from the ER as well as its processing and transport through the Golgi to the plasma membrane, yet the contribution of EPO-R intracellular domains cannot be excluded.

Glycosylation. The extracellular portion (residues 46–136) of sEPO-R confers enhanced maturation to the EPO-R. The N-glycosylation site *per se* is not sufficient to provide this advantage, because residues s46–57 containing the N-glycosylation site did not improve EPO-R maturation to the same extent. In that respect, impaired N-glycosylation of EPO-R (46, 47) had no measurable effect on cell-surface EPO-R levels, EPO binding kinetics, and EPO-induced cell proliferation. Carbohydrates attached to proteins play a role in multiple biochemical pathways including folding, stability, targeting, and clearance of proteins (48). Although we cannot rule out N-glycan differences between sEPO-R and mEPO-R, glycan variations do not seem to play a significant role in this process.

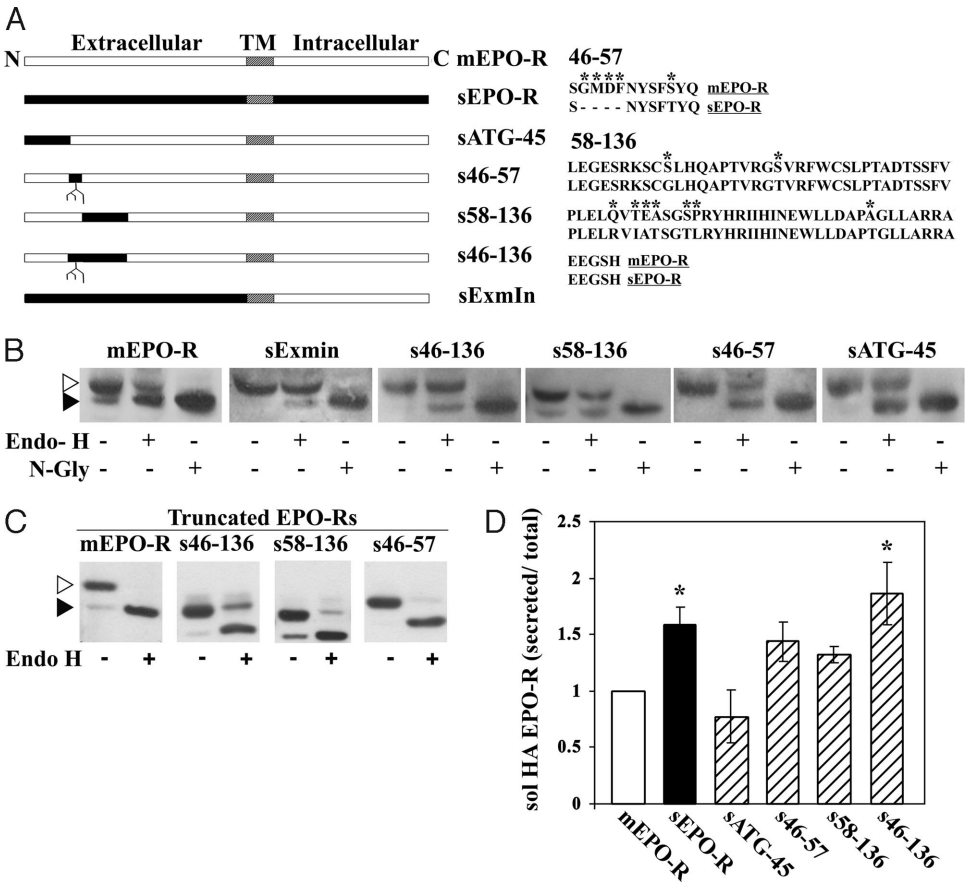

Fig. 5. *Spalax*/mouse chimeric EPO-Rs. (*A*) Schematic representation of *Spalax*/mouse chimeric EPO-Rs. mEPO-R is presented in white, sEPO-R is presented in black, and the chimeric EPO-Rs are presented with the relevant amino acid substitutions. Transmembrane domain, the putative oligosaccharide, and amino acid sequence alignment between residues 46 and 136 of the *Spalax* and mouse EPO-Rs are depicted. (*B*) Endo H susceptibility of full-length chimeric EPO-Rs. Lysates of Ba/F3 cells expressing full-length *Spalax*/mouse EPO-R chimeras were subjected to Endo H or N-glycanase (N-Gly) digestion and Western blot analysis with anti mEPO-R C terminus antibody. Endo H-sensitive and -resistant EPO-Rs are indicated by filled and open arrows, respectively. (*C*) Endo H susceptibility of truncated chimeric EPO-Rs. Lysates of BOSC-23T cells transiently expressing the truncated forms of *Spalax*/mouse EPO-R chimeras were subjected to Endo H digestion and Western blot analysis with anti-mEPO-R N terminus antibody. Endo H-sensitive and -resistant EPO-Rs are indicated by filled and open arrows, respectively. (*D*) Secretion of sol HA-tagged chimeric EPO-Rs. Aliquots of the media and whole cell lysates from BOSC-23T cells transiently expressing sol *Spalax*/mouse EPO-R chimeras were subjected to Western blot analysis with anti-HA antibodies. Graphed results depict the mean ± standard error of secreted sol HA-EPO-R divided by its total levels (secreted and intracellular) normalized to the ratio of sol HA-mEPO-R secretion ($n = 3$). Open, filled, and striped bars represent sol HA-mEPO-R, sol HA-sEPO-R, and chimeric sol HA-EPO-Rs, respectively. *, $P < 0.05$.

EPO-R Secretion. Secretion of sol EPO-R simulates arrival at the cell surface while discarding processes of membrane deposition and down-regulation by endocytosis or any influences of the EPO-R cytosolic domain. The effects of substitutions from mouse to *Spalax* in the extracellular part of EPO-R on intracellular transport originally observed in membrane-bound EPO-R were essentially preserved in the secreted sol EPO-R chimeric derivatives. The low amounts of secreted sol mEPO-R (Fig. 3 and ref. 41) could be due to inefficient transport of sol mEPO-R from the ER or the instability of the mature forms. Accumulation of the s46–136 sol EPO-R chimera at higher levels in the medium as compared with s46–57 and s58–136 chimeras, implies that the amino acid stretch 46–136 of sEPO-R confers enhanced transport to the cell surface and secretion, disregarding the contribution of endocytosis. Amino acids 46–57 and 58–136 differ significantly between mouse and *Spalax* EPO-Rs and were shown to be in proximity in the crystal structure of ligand-bound human EPO-R (49). Moreover, the more prominent negative charge of residues 46–136 in mEPO-R may dictate a unique profile of interacting chaperones (50).

The WSXWS Motif. Mutations in the WSXWS motif result in ER retention of the EPO-R (18). Strikingly, one EPO-R mutant, which harbored the A234E mutation in this motif, was exported more efficiently from the ER and was expressed at higher levels at the cell surface, suggesting that it folded more efficiently (15). This mutant bound and internalized EPO similarly to the WT EPO-R and was capable of transducing EPO-mediated proliferation (18). Both human and mouse EPO-Rs have an Ala residue at the middle position of the WSXWS motif (51, 52), suggesting that selection pressure during evolution operated to maintain the poor folding and surface transport of the EPO-Rs (15). The preservation of this Ala residue in sEPO-R supports the concept that sEPO-R harbors unique sequence features (residues s46–136) that improve its maturation, overriding this evolutionary trait. Amino acid residues s46–136 span across both seven-stranded D1 and D2 fibronectin III-like domains identified for the human EPO-R (49, 53, 54).

Prospects. Beyond the results presented in this study, the variations in signaling cascades downstream of the mouse and *Spalax* EPO-Rs deserve attention; notably, sEPO-R lacks two of the eight Tyr residues (22). Importantly, the capacity of sEPO-R to support EPO-mediated proliferation of Ba/F3 cells (SI Fig. 7) implies that the murine background of key signaling molecules

in Ba/F3 cells is sufficient for signaling via sEPO-R. Moreover, all of the chimeric *Spalax*/mouse EPO-Rs displayed EPO-mediated signaling (SI Fig. 10), supporting the notion that EPO binding was generally unharmed. Although we could not detect differences in the proliferative response to EPO of the mouse and *Spalax* EPO-Rs expressed in Ba/F3 cells, slight differences may become apparent in the animal, where the hematocrit level is tightly controlled. These data are consistent with the inability to detect differences in proliferation between cells expressing the WT EPO-R and the A234E EPO-R, which displays enhanced surface expression (15).

Spalax evolved 40 million years ago as an old rodent offshoot, whereas mice and rats are only ≈15 million years old (55). Evolution of *Spalax* was to underground hypoxic situations, whereas that of mice and rats was to above-ground life. We speculate that, as one of the adaptive mechanisms to cope with hypoxia, *Spalax* erythroid progenitor cells express a high level of EPO-Rs on their surface, thus contributing to the higher hematocrit and hemoglobin levels of *Spalax* (56). The current study highlights sEPO-R as an additional possible unique adaptive mode to cope with hypoxia, with significance beyond this evolutionary aspect. Here, we measured EPO-R surface expression under normoxic conditions; these data now call for analyzing the effect of cell stress/hypoxia on surface EPO-R. Our approach in using the sEPO-R and delineating its improved processing may also be implemented to overcome low levels of cell-surface EPO-Rs, which currently present a major challenge in experimental designs. Additionally, it may open new avenues to elucidate the functional implications of the unique structural features of EPO-R that may be instrumental in designing treatment modalities for conditions of hypoxia extending beyond the hematopoietic system.

Materials and Methods

Antibodies. Rabbit polyclonal antibodies to the N and C termini of EPO-R were described in refs. 12 and 41. Anti-actin (MAB1501; Chemicon,Temecula, CA) and anti-HA (MMS-101R; BAbCO, Richmond, CA) monoclonal antibodies were used for Western blot analysis. Anti-HA (F-7; Santa Cruz Biotechnology, Santa Cruz, CA, catalog number sc-7392) and FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) served for flow cytometry analysis.

Plasmids. *Spalax galili* (21), one of the four Israeli species of *Spalax ehrenbergi* superspecies (57), and mouse (28) EPO-R cDNAs were used (GenBank accession nos. AM039929 and AM193090, respectively). sEPO-R cDNA was a clonal variant, a result of polymorphism common in wild populations, displaying Lys at position 348 instead of Glu, similar to the sequence of the *Rattus* (21). All EPO-R constructs were digested by KpnI–EcoR1 and cloned into pcDNA3 or pXM.

HA EPO-Rs were generated by introducing an HA tag (YPYDVPDYA) between L30 and P31 in mEPO-R, and between L31 and Q32 in sEPO-R by two PCRs. The first PCR amplified the 5′ fragment using T7 as a forward primer and a reverse primer containing the HA tag; the second PCR amplified the 3′ fragment using a forward primer with HA, and SP6 as the reverse primer. Overlapping fragments were assembled by PCR with T7 and SP6 primers and cloned into pcDNA3 as described above.

Truncated sEPO-R is naturally found in *Spalax* species (GenBank accession no. AM039933) (22). Truncated mEPO-R (1–257) has been described in ref. 38.

Sol EPO-R was generated by a PCR-introduced stop codon after T242 in sEPO-R or after the corresponding S245 in mEPO-R (41).

Chimeric EPO-Rs were generated by exchanging mEPO-R cDNA fragments with the corresponding sEPO-R fragments (SI Table 1 and *SI Materials and Methods*).

Cell Culture and Transfection. BOSC-23T cells were transiently transfected by using calcium phosphate (58). Ba/F3 cells stably expressing EPO-R (59) were cultured in RPMI medium 1640 containing either 10% conditioned medium from WEHI3B cells or EPO (0.5 units/ml).

Glycosidase Treatment. Endo H digestion was performed as described in ref. 60. For N-glycanase (PNGase F) digestion, denatured lysates (60) were incubated with 0.6 units of PNGase F (Roche Diagnostics, Indianapolis, IN) for 18 h at 37°C. Samples were resolved on 7.5% SDS/PAGE before Western blot analysis.

Flow Cytometry. Surface HA-EPO-Rs were analyzed by standard two-step flow cytometry by using anti-HA and FITC-conjugated secondary antibodies (*SI Materials and Methods*).

Metabolic Labeling and Immunoprecipitation. Labeling of cells with [^{35}S]Cys-Met and further processing were performed as described (11).

Sol EPO-R Secretion. BOSC-23T cells expressing sol EPO-R cDNA (41, 61) were cultured in 6-cm plates. Confluent cell cultures were incubated at 37°C for 3 h in 1 ml of DMEM containing 0.2% FCS. Cells were lysed in 0.5 ml of sample buffer X2 and boiled for 15 min. Aliquots of media (40 μl) and cell lysates (20 μl) were subjected to Western blot analysis (61).

We thank Prof. Ronit Sagi-Eisenberg for critically reviewing the manuscript and Ms. Alma Joel for technical assistance. This work was supported by Israel Science Foundation Grant 643/06 (to D.N.), and the Ancell Teicher Research Foundation for Molecular Genetics and Evolution (E.N.). This work was carried out in partial fulfillment of the requirements for O.R.'s PhD from the Sackler Faculty of Medicine, Tel Aviv University.

1. Wu H, Liu X, Jaenisch R, Lodish HF (1995) *Cell* 83:59–67.
2. Mulcahy L (2001) *Semin Oncol* 28:19–23.
3. Frank SJ (2002) *Endocrinology* 143:2–10.
4. Yoshimura A, D'Andrea AD, Lodish HF (1990) *Proc Natl Acad Sci USA* 87:4139–4143.
5. Strous GJ, van Kerkhof P, Verheijen C, Rossen JW, Liou W, Slot JW, Roelen CA, Schwartz AL (1994) *Exp Cell Res* 211:353–359.
6. Yang N, Huang Y, Jiang J, Frank SJ (2004) *J Biol Chem* 279:20898–20905.
7. He K, Wang X, Jiang J, Guan R, Bernstein KE, Sayeski PP, Frank SJ (2003) *Mol Endocrinol* 17:2211–2227.
8. Sawyer ST, Hankins WD (1993) *Proc Natl Acad Sci USA* 90:6849–6853.
9. Hilton CJ, Berridge MV (1995) *Growth Factors* 12:263–276.
10. Huang LJ, Constantinescu SN, Lodish HF (2001) *Mol Cell* 8:1327–1338.
11. Neumann D, Wikstrom L, Watowich SS, Lodish HF (1993) *J Biol Chem* 268:13639–13649.
12. Cohen J, Altaratz H, Zick Y, Klingmuller U, Neumann D (1997) *Biochem J* 327:391–397.
13. Motohashi T, Nakamura Y, Osawa M, Hiroyama T, Iwama A, Shibuya A, Nakauchi H (2001) *Eur J Haematol* 67:88–93.
14. Walrafen P, Verdier F, Kadri Z, Chretien S, Lacombe C, Mayeux P (2005) *Blood* 105:600–608.
15. Hilton DJ, Watowich SS, Murray PJ, Lodish HF (1995) *Proc Natl Acad Sci USA* 92:190–194.
16. Yoshimura A, Zimmers T, Neumann D, Longmore G, Yoshimura Y, Lodish HF (1992) *J Biol Chem* 267:11619–11625.
17. Quelle DE, Quelle FW, Wojchowski DM (1992) *Mol Cell Biol* 12:4553–4561.
18. Hilton DJ, Watowich SS, Katz L, Lodish HF (1996) *J Biol Chem* 271:4699–4708.
19. Maiese K, Li F, Chong ZZ (2005) *J Am Med Assoc* 293:90–95.
20. Savic IR, Nevo E (1990) *Prog Clin Biol Res* 335:129–153.
21. Shams I, Nevo E, Avivi A (2005) *FASEB J* 19:307–309.

22. Shams I, Nevo E, Avivi A (2005) *FASEB J* 19:1749–1751.
23. Avivi A, Resnick MB, Nevo E, Joel A, Levy AP (1999) *FEBS Lett* 452:133–140.
24. Avivi A, Shams I, Joel A, Lache O, Levy AP, Nevo E (2005) *FASEB J* 19:1314–1316.
25. Wiener CM, Booth G, Semenza GL (1996) *Biochem Biophys Res Commun* 225:485–488.
26. Ashur-Fabian O, Avivi A, Trakhtenbrot L, Adamsky K, Cohen M, Kajakaro G, Joel A, Amariglio N, Nevo E, Rechavi G (2004) *Proc Natl Acad Sci USA* 101:12236–12241.
27. Avivi A, Ashur-Fabian O, Joel A, Trakhtenbrot L, Adamsky K, Goldstein I, Amariglio N, Rechavi G, Nevo E (2007) *Oncogene* 26:2507–2512.
28. Sokol L, Prchal JF, D'Andrea AD, Rado TA, Prchal JT (1994) *Exp Hematol* 22:447–453.
29. Miller BA, Barber DL, Bell LL, Beattie BK, Zhang MY, Neel BG, Yoakim M, Rothblum LI, Cheung JY (1999) *J Biol Chem* 274:20465–20472.
30. Gaffen SL, Lai SY, Longmore GD, Liu KD, Goldsmith MA (1999) *Blood* 94:74–86.
31. Halupa A, Chohan M, Stickle NH, Beattie BK, Miller BA, Barber DL (2005) *Exp Cell Res* 309:1–11.
32. Barber DL, Beattie BK, Mason JM, Nguyen MH, Yoakim M, Neel BG, D'Andrea AD, Frank DA (2001) *Blood* 97:2230–2237.
33. Flint-Ashtamker G, Eisen-Lev R, Cohen J, Jun-shen Huang L, Neumann D (2002) *FEBS Lett* 518:189–194.
34. Cohen J, Oren-Young L, Klingmuller U, Neumann D (2004) *Biochem J* 377:517–524.
35. Supino-Rosin L, Yoshimura A, Altaratz H, Neumann D (1999) *Eur J Biochem* 263:410–419.
36. Kubatzky KF, Liu W, Goldgraben K, Simmerling C, Smith SO, Constantinescu SN (2005) *J Biol Chem* 280:14844–14854.
37. Lu M, Echeverri F, Moyer BD (2003) *Traffic* 4:416–433.
38. Levin I, Cohen J, Supino-Rosin L, Yoshimura A, Watowich SS, Neumann D (1998) *FEBS Lett* 427:164–170.
39. Beckman DL, Lin LL, Quinones ME, Longmore GD (1999) *Blood* 94:2667–2675.
40. D'Andrea AD, Zon LI (1990) *J Clin Invest* 86:681–687.
41. Neumann D, Yuk MH, Lodish HF, Lederkremer GZ (1996) *Biochem J* 313:391–399.
42. Migliaccio AR, Migliaccio G, D'Andrea AD, Baiocchi M, Crotta S, Nicolis S, Ottolenghi S, Adamson JW (1991) *Proc Natl Acad Sci USA* 88:11086–11090.
43. Hermine O, Dubart A, Porteux F, Mayeux P, Titeux M, Dumenil D, Vainchenker W (1996) *Blood* 87:1746–1753.
44. Verdier F, Walrafen P, Hubert N, Chretien S, Gisselbrecht S, Lacombe C, Mayeux P (2000) *J Biol Chem* 275:18375–18381.
45. Murray PJ, Watowich SS, Lodish HF, Young RA, Hilton DJ (1995) *Anal Biochem* 229:170–179.
46. Nagao M, Morishita E, Hanai Y, Kobayashi K, Sasaki R (1995) *FEBS Lett* 373:225–228.
47. Mayeux P, Casadevall N, Muller O, Lacombe C (1990) *FEBS Lett* 269:167–170.
48. Varki A (1993) *Glycobiology* 3:97–130.
49. Syed RS, Reid SW, Li C, Cheetham JC, Aoki KH, Liu B, Zhan H, Osslund TD, Chirino AJ, Zhang J, *et al.* (1998) *Nature* 395:511–516.
50. Jorgensen CS, Heegaard NH, Holm A, Hojrup P, Houen G (2000) *Eur J Biochem* 267:2945–2954.
51. Jones SS, D'Andrea AD, Haines LL, Wong GG (1990) *Blood* 76:31–35.
52. Winkelmann JC, Penny LA, Deaven LL, Forget BG, Jenkins RB (1990) *Blood* 76:24–30.
53. Livnah O, Stura EA, Middleton SA, Johnson DL, Jolliffe LK, Wilson IA (1999) *Science* 283:987–990.
54. Stroud RM, Wells JA (2004) *Sci STKE* 2004:re7.
55. Brodsky LI, Jacob-Hirsch J, Avivi A, Trakhtenbrot L, Zeligson S, Amariglio N, Paz A, Korol AB, Band M, Rechavi G, Nevo E (2005) *Proc Natl Acad Sci USA* 102:17047–17052.
56. Arieli R, Heth G, Nevo E, Hoch D (1986) *Experientia* 42:441–443.
57. Nevo E, Ivanitskaya E, Beiles A (2001) *Adaptive Radiation of Blind Subterranean Mole Rats* (Backhuys, Leiden, The Netherlands).
58. Chen CA, Okayama H (1988) *BioTechniques* 6:632–638.
59. D'Andrea AD, Yoshimura A, Youssoufian H, Zon LI, Koo JW, Lodish HF (1991) *Mol Cell Biol* 11:1980–1987.
60. Barzilay E, Ben-Califa N, Hirschberg K, Neumann D (2005) *Traffic* 6:794–802.
61. Barzilay E, Ben-Califa N, Supino-Rosin L, Kashman Y, Hirschberg K, Elazar Z, Neumann D (2004) *J Biol Chem* 279:6847–6852.