The WSAWS Motif Is C-Hexosylated in a Soluble Form of the Erythropoietin Receptor†

Aleksandra Furmanek,‡ Daniel Hess,‡ Hélène Rogniaux,‡,§ and Jan Hofsteenge*,‡

Friedrich Miescher Institute, Novartis Research Foundation, Maulbeerstrasse 66, 4058 Basel, Switzerland, and INRA-URPVI, Rue de la Geraudiere, BP 71627, F-44316 Nantes, Cedex 3, France

Received January 21, 2003; Revised Manuscript Received May 7, 2003

ABSTRACT: The WSXWS motif is a highly conserved structural feature of the type I cytokine receptor family. It has previously been demonstrated that mutations in the ^{232}WSAWS^{236} motif in the erythropoietin receptor (EPOR) can result in strongly inhibited surface expression, due to defective intracellular transport [Hilton, D. J., et al. (1996) *J. Biol. Chem. 271*, 4699−4708]. Here we report that the first tryptophan in the motif of the recombinant extracellular domain of EPOR (sEPOR) expressed in HEK-EBNA cells carries a C-linked hexosyl residue. The S233A mutation completely abolished secretion of sEPOR, whereas the A234E mutation resulted in enhanced secretion. Comparison of the level of C-hexosylation in the wild-type protein and in the mutant proteins isolated from the conditioned medium and/or the cells suggested that C-hexosylation of the motif did not play a role in the correct intracellular transport of sEPOR.

Erythropoietin and its receptor (EPOR)[1] are essential for the late stages of formation of erythrocytes (*1*, *2*). The receptor belongs to the family of type I cytokine receptors, which is comprised mostly of cytokine receptors (for IL-2−IL-4, IL-6, IL-7, granulocyte colony stimulating factor, granulocyte macrophage colony stimulating factor, and leukemia inhibitory factor) in addition to receptors for prolactin, growth hormone, and leptin (*3*). The characteristic structural features of the family are four conserved cysteine residues in the N-terminal type III fibronectin domain (D1) and the membrane-proximal WSXWS motif in the C-terminal domain (D2) (*3*). By these criteria, and its recently determined three-dimensional structure, the β-chain of IL-12, a cytokine, also belongs to this family (*4*).

A number of studies have addressed the function of the WSXWS motif in the receptor for EPO, prolactin, IL-2, and granulocyte macrophage colony stimulating factor by site-directed mutagenesis, implicating it in ligand binding, receptor internalization, signal transduction, intersubunit interactions, and protein folding (*5*−*10*). The results obtained for the ^{232}WSAWS^{236} motif in EPOR were not always in agreement. For example, both Quelle et al. (*7*) and Hilton et al. (*8*) reported a lack of growth signal transduction in cells transfected with mutant EPOR, but their explanations differed. Quelle et al. reported unaltered surface expression of the W235F and W235S mutants in transfected FDC-P1 cells, attributing the signaling defect to involvement of the motif in EPO binding (*7*). On the other hand, Hilton et al. observed strongly inhibited or completely absent cell surface expression of these mutants in COS and Ba/F3 cells (*8*), caused by defective intracellular transport. It has been proposed that the motif has a purely structural role and that the mutations cause a defect in folding, resulting in accumulation of the proteins in the endoplasmic reticulum (*8*). This interpretation is in agreement with the three-dimensional structure of EPOR (*11*, *12*), which shows that the motif is structurally important. In particular, the two Trp residues are part of a π-cation system in which the indole moieties alternate with arginine side chains. The motif itself does not form part of the binding site for erythropoietin, but residues in the loop immediately preceding it are involved in ligand binding.

The WSXWS motif is not unique to the type I cytokine receptor family, since it also occurs frequently in proteins containing thrombospondin type 1 repeats (TSRs) (*13*). Interestingly, all TSR proteins that have been examined (C7, C8α, C8β, C9, properdin, platelet thrombospondin-1, and F-spondin) were found to be C-mannosylated on Trp residues in the motif (*14*−*17*). C-Mannosylation involves attachment of an α-mannopyranosyl residue to the C2 atom of the indole moiety through a C−C bond (*18*−*20*). The enzyme activity catalyzing this reaction has been found in organisms ranging from *Caenorhabditis elegans* to humans, with a broad expression in different tissues and cell lines (*21*, *22*). In addition to the TSR family proteins, this modification also

† This work was supported by the Novartis Research Foundation.

* To whom correspondence should be addressed: Friedrich Miescher Institute, Novartis Research Foundation, Maulbeerstrasse 66, 4058 Basel, Switzerland. Telephone: +41 61 697 47 22. Fax: +41 61 967 39 76. E-mail: jan.hofsteenge@fmi.ch.

‡ Novartis Research Foundation.

§ INRA-URPVI.

[1] Abbreviations: C7, C8, C9, complement factors 7, 8, 9, respectively; CID, collision-induced dissociation; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EPO, erythropoietin; EPOR, erythropoietin receptor; sEPOR-*κ*, soluble EPOR tagged with mouse c-*κ*; ESI-MS, electrospray ionization mass spectrometry; GST, glutathione *S*-transferase; FCS, fetal calf serum; HEK-EBNA, human embryonic kidney cells containing Epstein-Barr nuclear antigen; LC−MS, liquid chromatography coupled with mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; IL, interleukin; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; PNGase, peptide *N*-glycosidase; RNase, ribonuclease; SDS−PAGE, polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate; TFA, trifluoroacetic acid; TSP-1, thrompospondin-1; TSR, thrombospondin type I repeat.

10.1021/bi034112p CCC: $25.00
Published on Web 06/27/2003

Table 1: Primers Used for the Construction of the Expression Plasmid for sEPOR-κ and Its Mutants[a]

name		restriction site	sequence
IgL	5′	*Hin*dIII	ACGACG**AAGCTT**GCCGCCACCATGAGT
	3′	*Kpn*I	ACGACG**GGTACC**GTCACAACGCGTACC
c-κ	5′	*Sph*I	ACGACG**GCATGC**GCTGATGCTGCACCAACTGTA
	3′	*Xba*I	ACGACG**TCTAGA**TTACTAACACTCATTCCTGTTGAAGCT
EPOR ED	5′	*Kpn*I	ACGACG**GGTACC**GCACCTTCACCCAGC
	3′	*Sph*I	ACGACG**GCATGC***GGATCCACGTGGGACCAG*AGGGTCCAGGTCGCT
A234E	5′		GGATTCTGGAGTGAATGGTCTGAGCCCGCG
A234E	3′		CGCGGGCTCAGACCATTCACTCCAGAATCC
S233A	5′		GGATTCTGGGCCGCCTGGTCTGAGCCCGCG
S233A	3′		CGCGGGCTCAGACCAGGCGGCCCAGAATCC

[a] Restriction enzyme sites are shown in bold. In primers used for mutagenesis, the mutated site is underlined. The sequence encoding the thrombin cleavage site is shown in italics.

occurs in human RNase 2, in IL-12β, and most likely in a neuropeptide from stick insect (*18*, *23*, *24*).

These observations prompted us to examine whether the WSAWS motif in EPOR can be C-mannosylated, and to test whether the modification is important for intracellular transport. It was found that the c-κ-tagged, soluble extracellular domain of EPOR purified from the conditioned medium of transfected HEK-EBNA cells is approximately 50% C-hexosylated on Trp232. The wild-type protein purified from the cells and the nonsecreted S233A mutant were C-hexosylated to approximately the same degree. This strongly suggests that the modification is not essential for intracellular transport of sEPOR-κ.

EXPERIMENTAL PROCEDURES

DNA Constructs and Mutagenesis. The cDNA encoding full-length mouse EPOR cloned into the mammalian expression vector pXM was a kind gift from B. Groner (Georg-Speyer-Haus, Frankfurt, Germany). The extracellular domain (amino acids 25−249, counting from the start of translation, according to ref *25*) was recloned into the pRS7 vector (a generous gift from S. Geisse and R. Schmitz, Novartis Pharma, Basel, Switzerland), containing an IgL secretion signal sequence, a c-κ sequence tag, a thrombin cleavage site, and a multiple-cloning site. To obtain C-terminally tagged sEPOR-κ, the vector was rearranged as follows. The sequences encoding the secretion signal, the c-κ tag, and the thrombin cleavage site were removed by cleavage with *Hin*dIII and *Xba*I. In separate preparations, the fragments encoding the secretion signal and c-κ were generated by PCR using pRS7 as a template, and the oligonucleotides shown in Table 1 as primers. The sequence for the extracellular domain of EPOR was amplified from the pXM vector using the oligonucleotides shown in Table 1. The fragments were digested with appropriate restriction enzymes, joined together in the order, secretion signal, sEPOR, thrombin cleavage site, c-κ tag, using the Rapid Ligation Kit (Promega), and cloned into the pRS7 vector between the restriction sites for *Hin*dIII and *Xba*I (pRSsEPOR-κ).

Mutants of sEPOR-κ were generated by the polymerase chain reaction (QuickChange mutagenesis, Stratagene) using the degenerate oligonucleotides (Table 1). All constructs and mutations were verified by DNA sequencing.

Protein Expression and Purification. HEK-EBNA cells (Invitrogen) were seeded in 100 mm tissue culture dishes at a density of 10^6 cells/plate in DMEM containing 10% FCS, 10 mM glutamine, and 100 μg/mL G-418. Transfections were performed after 24 h with pRSsEPOR using the calcium phosphate method (*26*). Twenty-four hours post-transfection, the medium was changed to Optimem (Invitrogen). The conditioned medium was collected 72 h post-transfection, and sEpoR-κ expression was assessed by Western blotting with anti-κ antibody. The expression levels were in the range of 1−5 μg/mL of conditioned medium, as estimated from Western blots in comparison with the known amount of a standard, GST−c-κ fusion protein. All studied proteins were expressed and analyzed at least twice. Up to 750 mL of conditioned medium with added protease inhibitor cocktail (final protease inhibitor concentrations of 0.2 μg/mL benzamidine, 5 μg/mL pepstatin, 5 μg/mL leupeptin, 2 mM EGTA, and 40 μg/mL PMSF) was concentrated to 5−10 mL using Centricon filtering devices (Amicon), with a 10 kDa cutoff. The concentrate was dialyzed overnight at 4 °C against 20 mM Tris-HCl (pH 7.5) and 0.1 M NaCl (binding buffer) and added to CH-Sepharose beads (Pharmacia) containing goat anti-κ antibody (5 mg/mL; Southern Biotechnology Associates Inc., Birmingham, AL), and the mixture was rotated overnight at 4 °C. The beads were washed three times with binding buffer followed by four washes with 20 mM Tris-HCl (pH 7.5) and 0.5 M NaCl and transferred to a column. The protein was eluted with 0.1 M glycine (pH 2.5) into Eppendorf tubes containing 1 M Tris base for neutralization. The protein content of elution fractions was monitored by measuring A_{280}. Fractions containing protein were analyzed by SDS−PAGE (*27*). Duplicate gels were run and either stained with colloidal Coomassie (Invitrogen) for MS analysis, silver-stained (*28*), or processed for Western blotting with goat anti-κ primary antibody and anti-goat antibody conjugated to horseradish peroxidase. Blots were developed using an ECL kit (Pharmacia).

Protein Characterization. The amino acid residues in murine EPOR were numbered as described elsewhere (*25*). To confirm the identity of the purified protein and to obtain preliminary evidence for its C-mannosylation, analysis by SDS−PAGE and peptide mapping were performed. The Coomassie-stained band was cut out of the gel, reduced, carbamidomethylated, and digested with trypsin (Promega) as described previously (*29*). The resulting peptides were purified on a C4 ZipTip microcolumn (Millipore, Bedford, MA) and crystallized on the MALDI target using α-cyano-4-hydroxycinnamic acid as the matrix. MALDI-TOF MS was performed on a Micromass TofSpec 2E instrument in the positive ion reflectron mode with delayed extraction, using MassLynx 3.5 for data analysis.

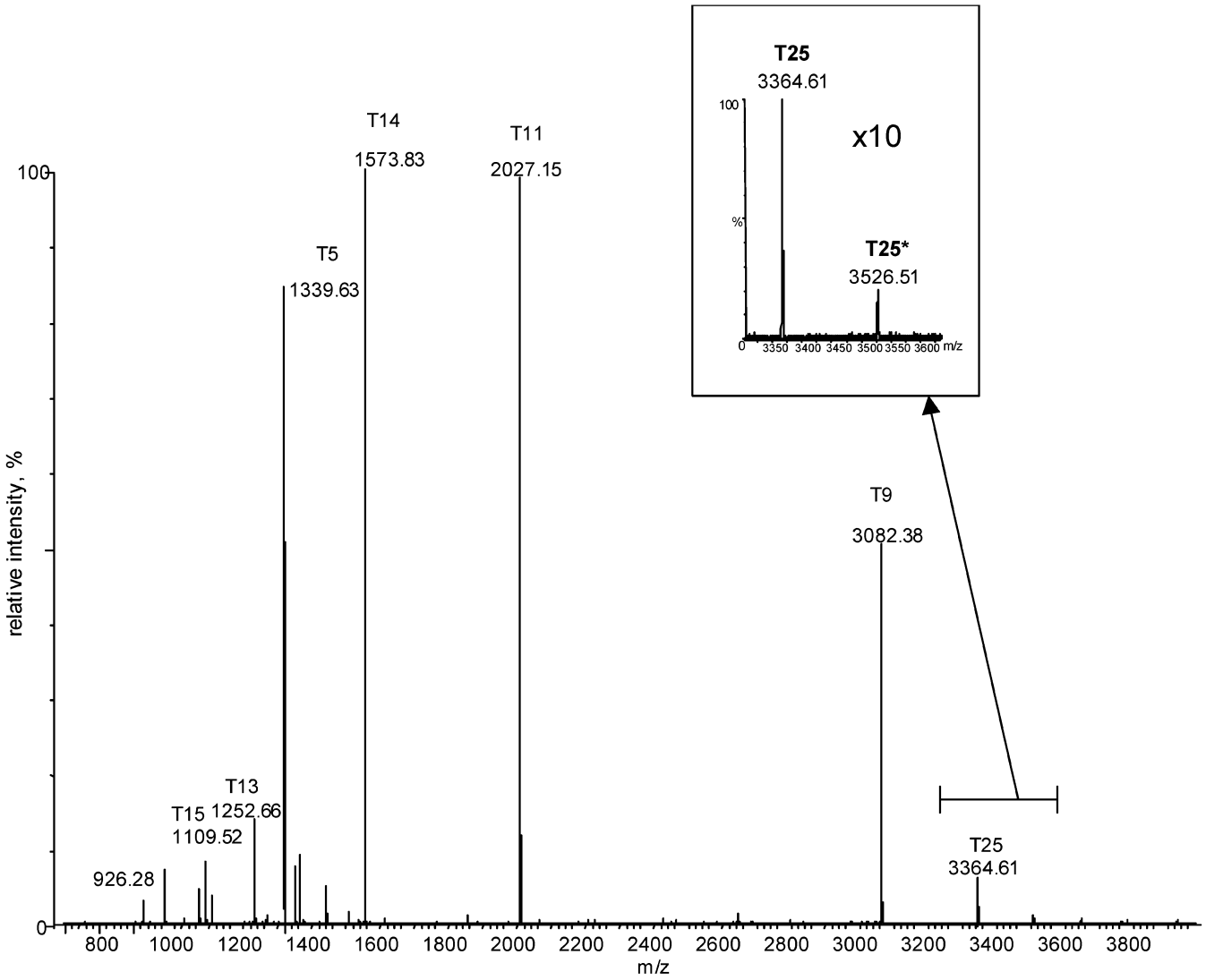

FIGURE 1: Mass spectrometric analysis of in-gel-digested sEPOR-*κ*. Tryptic peptides obtained by in-gel digestion of sEPOR-*κ* were analyzed by MALDI-TOF MS, using α-cyano-4-hydroxycinnamic acid as the matrix. The portion of the spectrum containing peptide T25 and its modified counterpart (T25*) is shown in the inset. The mass of the lowest isotope has been indicated.

For detailed characterization and peptide sequencing, the purified protein was precipitated (*30*), reduced, carbamidomethylated as previously described (*31*), and digested with trypsin [twice, 2 h and overnight at 37 °C in 50 mM Tris-HCl (pH 8.0) with 5 mM $CaCl_2$; enzyme:substrate ratio of approximately 1:50], or with trypsin (as above) followed by chymotrypsin (200 ng, 2 h, 37 °C). The digests were fractionated by C_{18} reversed phase LC−ESI-MS using a Vydac C_{18} column (1 mm diameter) and a 60 min gradient from 0 to 50% B (buffer A being 2% acetonitrile and 0.05% TFA, and buffer B 80% acetonitrile and 0.045% TFA). The eluate was monitored at 214 nm, and 10% of the sample was sprayed into a Sciex API 300 mass spectrometer. The fractions containing the peptides of interest were dried and sequenced by nanoelectrospray low-energy CID tandem MS as described elsewhere (*32*). Ratios of modified and unmodified peptides were determined by integrating the appropriate peaks in the UV trace.

RESULTS

sEPOR-κ Is C-Hexosylated on Trp232. In contrast to N- and O-glycosylation, C-mannosylation cannot be inferred from shifts in molecular mass on an SDS−PAA gel. Until now, the degree of C-mannosylation has been determined by analyzing peptide maps from purified, soluble proteins by a combination of ESI-MS and Edman degradation. Nevertheless, it would be convenient to be able to obtain evidence for this modification from material separated by SDS−PAGE. To examine this, the two bands just below 40 kDa, identified by Western analysis as containing sEPOR-*κ*, were excised from a Coomassie-stained gel, and its tryptic peptides were examined by MALDI-TOF MS. This analysis confirmed the identity of the protein. The potential C-mannosylation site in sEPOR-*κ* occurs in the tryptic peptide (T25), which consists of residues 223−249 of EPOR followed by the sequence LVPR of the thrombin cleavage site (^{223}MAEPSFSGF**WSAW**SEPASLLTASDLDP^{247}LVPR). Peptide masses corresponding to the singly C-hexosylated (3526.5 Da; T25*) and non-C-hexosylated (3364.6 Da; T25) forms of the peptide were detected (Figure 1), strongly suggesting that the modification was present. No mass corresponding to a peptide containing two C-hexosylated tryptophan residues was observed. It should be noted that the area of the peak for T25* in the MALDI-TOF spectrum

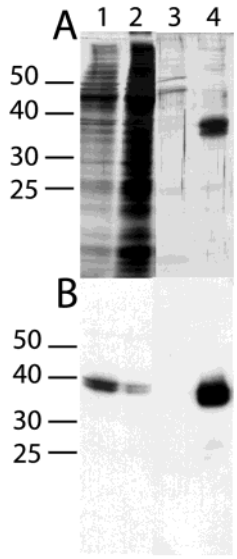

FIGURE 2: Purification of recombinant sEPOR-κ. HEK-EBNA cells (9×10^7) were transiently transfected with a cDNA encoding sEPOR-κ. Conditioned medium was collected after 3 days. The soluble receptor was isolated by immunoaffinity chromatography using anti-κ antibodies. An aliquot of the concentrated and dialyzed conditioned medium (lane 1), the pool of unbound protein (lane 2), the wash fractions (lane 3), and the pool eluted with 0.25 M glycine-HCl (pH 2.5) (lane 4) were analyzed by SDS−PAGE. (A) Silver-stained gel with 30 μL of each sample loaded. (B) Western blot probed with anti-κ antibodies in which 10 μL of each sample was loaded. The upper band of the two in lane 4 disappeared upon treatment with PNGase F, with a concomitant increase in the intensity of the lower one (data not shown).

is much smaller than that of T25. However, quantification by HPLC using UV absorption showed the two peptides to be present in approximately equal amounts (see below). Because of differences in ionization efficiency and suppression effects, peak intensity in MALDI-TOF does not necessarily reflect the amount of a particular peptide present in a mixture.

Table 2: Modification of the Tryptic Peptide from sEPOR-κ Containing the C-Mannosylation Motif, As Determined by LC−ESI-MS

			average mass (Da) of peptide T25 modified with a C-hexosyl residue		modified/total ratio	
protein	secreted	intracellular	expected	found	average	range
wild type	+		3528	3528	0.5	0.4−0.6 ($n = 2$)
		+	3528	3529	0.5	0.3−0.7 ($n = 2$)
S233A	+		3512	nd[a]	−	
		+	3512	3481[b]	0.5	− ($n = 1$)
A234E	+		3586	3585	0.3	0.3 ($n = 2$)
		+	3586	3586	0.5	0.5−0.6 ($n = 2$)

[a] No secreted protein was detected. [b] In addition to the S233A mutation, the peptide contained the M223T substitution.

A larger amount of sEPOR-κ was purified to near homogeneity from the supernatant of transfected HEK-EBNA cells (Figure 2), and its tryptic peptides were fractionated by reverse phase LC−MS (Figure 3). To locate the elution position of peptide T25, the data were extracted for its theoretical mass, taking into account the possible presence of none, one, or two hexoses. This revealed an unmodified peptide eluting at 60.9 min ($[M + 2H]^{2+}$ at m/z 1684, $[M + 3H]^{3+}$ at m/z 1122) and the modified one at 58.6 min ($[M + 2H]^{2+}$ at m/z 1765, $[M + 3H]^{3+}$ at m/z 1177). The ratio of modified to unmodified peptide was calculated from integration of the areas under the two peaks in the UV trace to be 0.4−0.6 (Table 2).

Both forms of peptide T25 were subjected to low-energy CID tandem MS, which confirmed their identity (data not shown). In the case of the modified peptide, the 120 Da loss, characteristic for C-linked sugar residues (*18*), was observed. However, the results did not allow unequivocal assignment

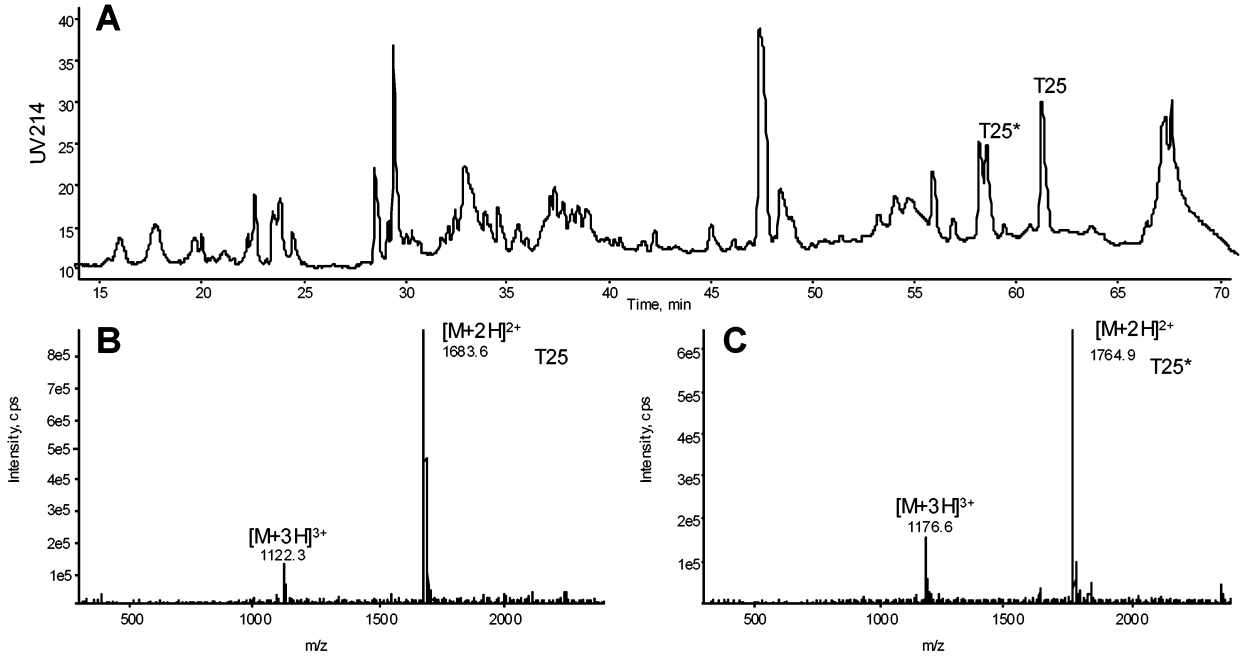

FIGURE 3: Isolation of the C-hexosylated peptide from sEPOR-κ. (A) Reduced and carbamidomethylated sEPOR-κ was cleaved with trypsin. The resulting peptides were fractionated by reversed phase LC−ESI-MS using a C18 column. Peptide T25 was found in modified (T25*) and unmodified (T25) forms. (B and C) ESI-MS spectra of peptides T25 and T25*, respectively.

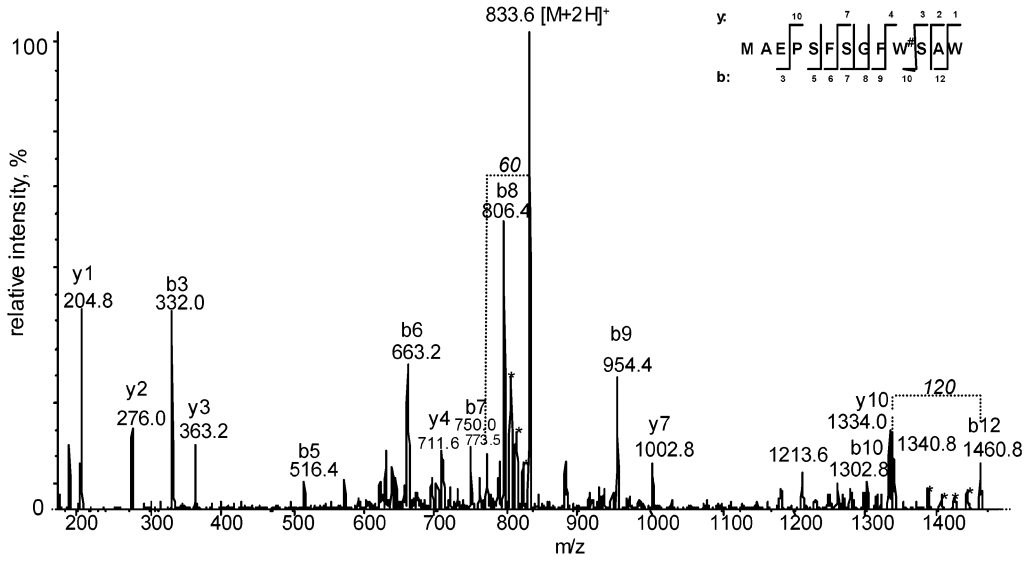

FIGURE 4: Low-energy CID tandem ESI-MS of C-hexosylated peptide T25*-Ch. The experiment was performed on an API 300 triple-quadrupole instrument, operating in the nanospray mode. The 120 Da loss, typical for aromatic C-glycosides, has been indicated with *120* and *60* for singly and doubly charged ions, respectively. W# represents C-hexosylated Trp.

of the attachment site, because of overlap of the relevant fragment ions with doubly charged b or y ions. Therefore, peptide T25* was quantitatively cleaved with chymotrypsin, which yielded only one C-hexosylated peptide with a mass of 1664.8 Da (T25*-Ch) upon LC−MS analysis. The purified peptide was subjected to low-energy CID tandem MS. From the presence of the y_{1-3}, b_{10}, and b_{12} fragment ions (Figure 4), it was concluded that Trp232 and not Trp235 was modified. The observed 120 Da losses from the parent and b_{12} ion provided evidence for the C-glycosidic linkage.

Because these results showed that both the C-hexosylated and nonmodified protein were secreted, it was also of interest to determine the degree of modification of sEPOR-κ that stayed within the HEK-EBNA cells. Tryptic peptides obtained from the purified protein were analyzed in the same way as described above. Peptide T25 was also found to be C-hexosylated, and the level of modification was similar to that of the secreted form (Table 2).

C-Hexosylation of sEPOR-κ that Has Been Mutated in the WSXWS Motif. The effects of mutations in the ^{232}WSAWS^{236} motif on intracellular transport were originally observed in membrane-bound EPOR (*8*), but it is not known whether they have the same effect in sEPOR-κ. Therefore, one mutation that completely blocked surface presentation (S233A) and one that improved it 2−3-fold (A234E) were also introduced into sEPOR-κ. The relative amount of secreted protein was estimated by SDS−PAGE and Western blotting using anti-κ antibodies (Figure 5). Compared to wild-type sEPOR-κ, the A234E mutant exhibited a clearly increased level of secretion, whereas no sEPOR-κ was detected in the conditioned medium of the S233A mutant. Both mutants were detected in the total cell lysates in comparable amounts.

C-Hexosylation of the two mutant proteins purified from the conditioned medium and from the cell lysate (A234E) or from the cell lysate only (S233A) was analyzed by LC−MS of their tryptic peptides. The modified peptides from both mutants were detected and sequenced by low-energy CID tandem MS as described above. Both mutants were C-hexosylated in peptide T25, and the degree of modification was similar to that of the wild-type receptor (Table 2). The

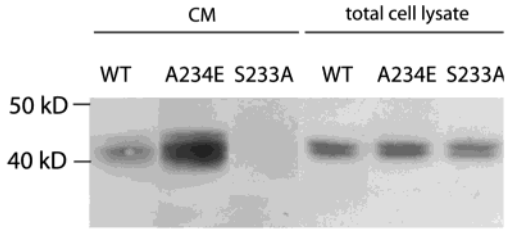

FIGURE 5: Secretion of sEPOR-κ with mutations in the WSXWS motif. HEK-EBNA cells (1×10^6) were transiently transfected with a cDNA encoding sEPOR-κ. Conditioned medium (CM) was collected after 3 days, and equal aliquots were separated by SDS−PAGE. The corresponding cells were lysed as described in Experimental Procedures, and 1% of the sample was analyzed. Proteins were blotted onto nitrocellulose and probed with anti-κ antibodies. WT stands for wild type.

analysis further revealed that a second mutation (M223T) had inadvertedly been introduced into the S233A mutant.

DISCUSSION

The WSXWS motif is evolutionarily conserved in nearly all type I cytokine receptors, and has been implicated in various aspects of receptor biology (see the introductory section). The results presented here add another possible reason for its conservation, i.e., C-hexosylation of the first Trp residue. Although we did not determine the type of hexose present, it most likely is mannose, because this sugar was found in all 50 previously examined cases. Together with the cytokine IL-12β, EPOR is the second member of the type I cytokine receptor family that can undergo this kind of glycosylation. This raises the possibility that other members of the family are also modified, in particular because a similar motif in TSRs is C-mannosylated in all cases that have been examined (*14*−*17*). An uncertainty remains, however, with respect to membrane-bound receptors. Unfortunately, attempts to analyze C-mannosylation in full-length EPOR failed, because of insufficient quantities of pure protein. In principle, it is possible, however, that natural EPOR undergoes this modification as well. First, we have previously demonstrated that RNase 2 from erythrocytes

is C-mannosylated (*20*), which strongly suggests that erythrocyte precursors contain C-mannosyltransferase activity. Second, although all previously reported C-mannosylated proteins were soluble ones (*33*), it has recently been found that a membrane-bound protein can be C-hexosylated in a variety of mammalian cell lines (*34*).

Using COS and Ba/F3 cells, Hilton et al. (*8*) have provided compelling evidence that the WSXWS motif plays a role in the proper folding of EPOR, and thereby indirectly in intracellular transport. Our results with mutants of sEPOR-κ in HEK-EBNA cells are in agreement with their observations; i.e., mutant S233A was not secreted at all, whereas the extent of secretion of mutant A234E was increased (Figure 5). The results differ, however, from those of Quelle et al., who used FDC-P1 cells and found that mutations in the motif do not affect surface presentation, but rather reduce the affinity for EPO (*7*).

We found partial C-hexosylation of secreted sEPOR-κ, indicating that the modification was not crucial for intracellular transport. It could, however, facilitate this process, in which case intracellular accumulation of the unmodified form could be expected. Given that sEPOR-κ purified from the conditioned medium and sEPOR-κ purified from the cells themselves had very similar degrees of C-hexosylation (Table 2), we conclude that the modification does not play a role in the secretory process of sEPOR-κ. This is corroborated by the observation that the nonsecreted mutant, S233A, was also approximately 50% modified, and that the more strongly secreted mutant A234E did not show an increase in the extent of C-hexosylation (Table 2). Although this conclusion cannot be applied to the membrane-bound form, it is most likely relevant for the naturally occurring sEPOR that is formed as a result of alternative splicing, and is capable of binding EPO (*35*, *36*).

If C-mannosylation is not important for secretion, what could its function be? One possibility is that it has a structural role. In the three-dimensional structure of (non-C-hexosylated) recombinant sEPOR from *Escherichia coli*, the WSXWS motif is part of a π-cation system, in which the Trp residues form a scaffold that interacts with Arg residues in the second β-strand of domain D2 (*11*, *12*). This forms a stack of alternating basic and aromatic residues at the surface of the molecule. The question of what influence C-mannosylation would have on the arrangement of the π-cation system, and thereby on the structure of the cytokine-binding domain, arises. The WSXWS motif is in the proximity of the N-terminal α-helix (*12*), located in the elbow between domains D1 and D2 (Figure 6A). A hexosyl residue on C2 of Trp232 would be expected to be on the surface of the protein in close contact with the side chains of Arg45 in the helix, and of Arg220 and Arg222, which interact with the two Trp residues of the motif, as well as Phe231 (Figure 6B). It has been proposed that the interactions of the WSXWS motif with the N-terminal helix and the β-sheet in D2 are biologically important in stabilizing the folded EPOR (*12*). The presence of a hexosyl residue attached to the first Trp could provide further stabilizing interactions. Such a structural role has been found in other glycoproteins. For example, NMR studies have shown that the single fucose O-linked to Thr9 of PMP-C, a small proteinase inhibitor, causes a decrease in the number of dynamic fluctuations of the molecule (*37*), whereas O-fucosylation of the EGF-like

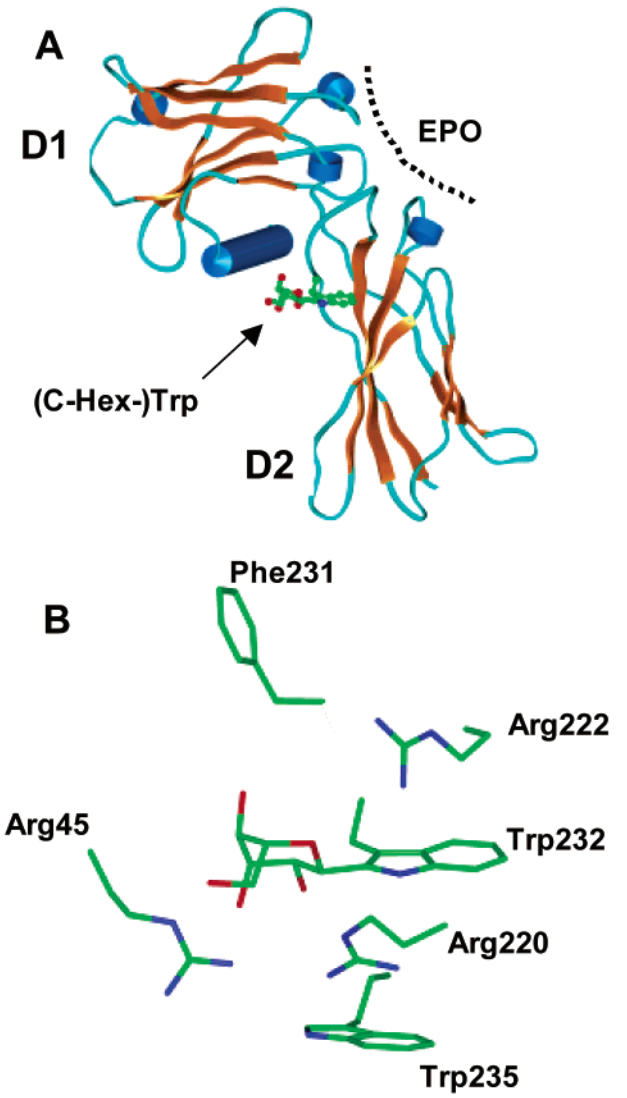

FIGURE 6: (C-Hex-)Trp in the three-dimensional structure of sEPOR. An α-mannopyranosyl residue in the $^4C_1$ conformation was incorporated into the model of the human EPOR−EPO complex (PDB entry 1EER), and its position was adjusted manually. (A) Schematic putative structure of the C-hexosylated sEPOR monomer. The approximate position of the ligand has been indicated (EPO). D1 and D2 denote the N- and C-terminal domains, respectively. (B) Amino acid side chains that are within van der Waals distance of the sugar residue (except Trp235). The numbering of mouse EPOR has been used for consistency.

domain in factor V indirectly limits the flexibility of the loop connecting two β-strands (*38*).

Alternatively, the WSXWS motif and the C-hexosyl residue could interact with other macromolecules, although at present such molecules are not known. Interestingly, a very similar arrangement of Trp and Arg residues, as found in type I cytokine receptors, has recently been observed in recombinant TSR2 and -3 from thrombospondin-1 (*39*). TSP-1 from platelets is C-mannosylated, and model building suggests that the sugar residues protrude into solution and potentially form part of a surface involved in macromolecular interactions. This section of the TSR module is known to interact with CD36, gp120, and glycosaminoglycans (*13*).

In summary, we have shown that the WSXWS motif in the soluble form of a cytokine receptor can be C-hexosylated, but as yet, no function can be linked to this modification.

However, since all structures of type I cytokine receptors that have been determined to date have used non-C-mannosylated proteins, the results presented here warrant further three-dimensional structural studies on C-mannosylated EPOR and other receptors of this family. This may ultimately lead to defining a function for this form of glycosylation in an important family of receptor proteins.

ACKNOWLEDGMENT

We thank Mr. Dominique Klein for help with molecular biological techniques, Ms. Ragna Sack for help with MALDI-TOF data analysis, and Drs. Jeremy Keusch and Guillaume de Sampaio for critical reading of the manuscript and useful comments.

REFERENCES

1. Constantinescu, S. N., Ghaffari, S., and Lodish, H. F. (1999) *Trends Endocrinol. Metab. 10*, 18−23.
2. Mulcahy, L. (2001) *Semin. Oncol. 28*, 19−23.
3. Bazan, J. F. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6934−6938.
4. Yoon, C., Johnston, S. C., Tang, J., Stahl, M., Tobin, J. F., and Somers, W. S. (2000) *EMBO J. 19*, 3530−3541.
5. Doshi, P. D., and DiPersio, J. F. (1994) *Blood 84*, 2539−2553.
6. Miyazaki, T., Maruyama, M., Yamada, G., Hatakeyama, M., and Taniguchi, T. (1991) *EMBO J. 10*, 3191−3197.
7. Quelle, D. E., Quelle, F. W., and Wojchowski, D. M. (1992) *Mol. Cell. Biol. 12*, 4553−4561.
8. Hilton, D. J., Watowich, S. S., Katz, L., and Lodish, H. F. (1996) *J. Biol. Chem. 271*, 4699−4708.
9. Yoshimura, A., Zimmers, T., Neumann, D., Longmore, G., Yoshimura, Y., and Lodish, H. F. (1992) *J. Biol. Chem. 267*, 11619−11625.
10. Rozakis-Adcock, M., and Kelly, P. A. (1992) *J. Biol. Chem. 267*, 7428−7433.
11. Livnah, O., Stura, E. A., Johnson, D. L., Middleton, S. A., Mulcahy, L. S., Wrighton, N. C., Dower, W. J., Jolliffe, L. K., and Wilson, I. A. (1996) *Science 273*, 464−471.
12. Syed, R. S., Reid, S. W., Li, C., Cheetham, J. C., Aoki, K. H., Liu, B., Zhan, H., Osslund, T. D., Chirino, A. J., Zhang, J., Finer-Moore, J., Elliott, S., Sitney, K., Katz, B. A., Matthews, D. J., Wendoloski, J. J., Egrie, J., and Stroud, R. M. (1998) *Nature 395*, 511−516.
13. Adams, J. C., and Tucker, R. P. (2000) *Dev. Dyn. 218*, 280−299.
14. Hofsteenge, J., Blommers, M. M., Hess, D., Furmanek, A., and Miroshnichenko, O. (1999) *J. Biol. Chem. 274*, 32786−32794.
15. Hartmann, S., and Hofsteenge, J. (2000) *J. Biol. Chem. 275*, 28569−28574.
16. Hofsteenge, J., Huwiler, K. G., Macek, B., Hess, D., Lawler, J., Mosher, D. F., and Peter-Katalinic, J. (2001) *J. Biol. Chem. 276*, 6485−6498.
17. Gonzalez de Peredo, A., Klein, D., Macek, B., Hess, D., Peter-Katalinic, J., and Hofsteenge, J. (2002) *Mol. Cell. Proteomics 1*, 11−18.
18. Hofsteenge, J., Müller, D. R., de Beer, T., Löffler, A., Richter, W. J., and Vliegenthart, J. F. G. (1994) *Biochemistry 33*, 13524−13530.
19. de Beer, T., Vliegenthart, J. F. G., Löffler, A., and Hofsteenge, J. (1995) *Biochemistry 34*, 11785−11789.
20. Löffler, A., Doucey, M.-A., Jansson, A. M., Müller, D. R., de Beer, T., Hess, D., Meldal, M., Richter, W. J., Vliegenthart, J. F. G., and Hofsteenge, J. (1996) *Biochemistry 35*, 12005−12014.
21. Krieg, J., Gläsner, W., Vincentini, A., Doucey, M.-A., Löffler, A., Hess, D., and Hofsteenge, J. (1997) *J. Biol. Chem. 272*, 26687−26692.
22. Doucey, M.-A. (1998) Ph.D. Thesis, Université Louis Pasteur de Strassbourg, Strassbourg, France.
23. Doucey, M.-A., Hess, D., Blommers, M. J., and Hofsteenge, J. (1999) *Glycobiology 9*, 435−441.
24. Gäde, G., Keller, R., Rinehart, K. L., and Proefke, M. L. (1992) *Biochem. Biophys. Res. Commun. 189*, 1303−1309.
25. D'Andrea, A. D., Lodish, H. F., and Wong, G. G. (1989) *Cell 57*, 277−285.
26. Jordan, M., Schallhorn, A., and Wurm, F. M. (1996) *Nucleic Acids Res. 24*, 596−601.
27. Laemmli, U. K. (1970) *Nature 227*, 680−685.
28. Blum, H., Beier, H., and Gross, H. J. (1987) *Electrophoresis 8*, 93−99.
29. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Anal. Chem. 68*, 850−858.
30. Wessel, D., and Flugge, U. I. (1984) *Anal. Biochem. 138*, 141−143.
31. Hofsteenge, J., Servis, C., and Stone, S. R. (1991) *J. Biol. Chem. 266*, 24198−24204.
32. Wilm, M., and Mann, M. (1996) *Anal. Chem. 68*, 1−8.
33. Furmanek, A., and Hofsteenge, J. (2000) *Acta Biochim. Pol. 47*, 781−789.
34. Keusch, J., and Hofsteenge, J. (2002) *Glycobiology 12*, 674 (abstract).
35. Barron, C., Migliaccio, A. R., Migliaccio, G., Jiang, Y., Adamson, J. W., and Ottolenghi, S. (1994) *Gene 147*, 263−268.
36. Schimmenti, L. A., Blechert, G., Harris, K. W., and Winkelmann, J. C. (1995) *Exp. Hematol. 23*, 1341−1346.
37. Mer, G., Hietter, H., and Lefevre, J.-F. (1996) *Nat. Struct. Biol. 3*, 45−52.
38. Kao, Y. H., Lee, G. F., Wang, Y., Starovasnik, M. A., Kelley, R. F., Spellman, M. W., and Lerner, L. (1999) *Biochemistry 38*, 7097−7110.
39. Tan, K., Duquette, M., Liu, J. H., Dong, Y., Zhang, R., Joachimiak, A., Lawler, J., and Wang, J. H. (2002) *J. Cell Biol. 159*, 373−382.

BI034112P