Saturation Mutagenesis of the WSXWS Motif of the Erythropoietin Receptor*

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The WSXWS motif in the extracellular domain defines members of the cytokine receptor family, yet its role in receptor structure and function remains unresolved. To address this question we have generated a panel of 100 mutants within the WSXWS motif of the erythropoietin receptor, which represents all single amino acid substitutions of these five amino acids. All mutants were synthesized at the same level; however, their passage from the endoplasmic reticulum to the Golgi apparatus differed. Because of this, expression of mutant receptors at the cell surface appeared varied more than 300-fold. The tolerance of the tryptophan and serine residues to substitution was quite narrow; as a result, most of these mutants were retained in the endoplasmic reticulum and showed no cell surface expression or reduced cell surface expression. Although many mutants with substitutions at the middle residue of the motif reached the cell surface, it was notable that one mutant, A234E, was processed more efficiently than the wild type receptor and was expressed in elevated numbers at the cell surface. Despite this variation, all mutant receptors that reached the cell surface appeared able to bind erythropoietin and transduce a proliferative signal normally. These results are discussed in terms of a general model for WSXWS function in which the motif contributes to efficient receptor folding.

Erythropoietin (Epo) plays a central role in the regulation of red blood cell formation. Epo exerts this effect by binding to receptors expressed on the surface of erythroid progenitors and stimulating these cells to proliferate and differentiate.

The Epo receptor cDNA has been isolated (1); its sequence reveals that the receptor is a member of the cytokine receptor family (2–5). This group of proteins includes the receptors for growth hormone, prolactin, interleukins (ILs) 2, 3, 4, 5, 6, 7, 9, 11, and 13, granulocyte and granulocyte-macrophage colony-stimulating factors, leukemia inhibitory factor, oncostatin-M, ciliary neurotrophic factor, thrombopoietin, and the p40 subunit of IL-12 and the IL-12 receptor (2, 5–31). At the primary sequence level, the most striking similarities between these receptors are present in the extracellular domain and include four cysteine residues, the spacing of which is conserved, a series of aromatic residues, and the five-amino acid motif Trp-Ser-Xaa-Trp-Ser (WSXWS). The receptors for interferon-α/β, interferon-γ, and IL-10 are more distantly related to the cytokine receptor family, as is the cell surface protein tissue factor (3). These molecules share one pair of conserved cysteines with the cytokine receptor family but lack a definitive WSXWS motif.

Using primary sequence alignments, Bazan (3) predicted that members of the cytokine receptor family would contain two, and in some cases four, domains similar to those present in members of the immunoglobulin superfamily. Each of the domains contains seven β-strands that form a barrel. Upon solution of the structure of the growth hormone/growth hormone receptor complex (32), the general details of Bazan’s predictions were confirmed. The organization of β-strands in the growth hormone receptor, however, more closely resembled the D2 domains of pap-D and CD-4 than immunoglobulins.

The crystal structure of the growth hormone receptor also revealed the position, and in certain cases suggested a function, for residues conserved among cytokine receptors. For example, the four cysteines and one conserved tryptophan are buried within the interior of the first β-barrel and presumably stabilize this domain. In contrast, although the homolog of the WSXWS motif in the growth hormone receptor, YGEFS, is close to a solvent-accessible surface, it is neither part of the hormone-binding nor the receptor dimerization surfaces (32). Thus, few clues could be found as to the function of the WSXWS motif.

In an attempt to determine the function of this region of cytokine receptors, several studies have been carried out in which residues of the WSXWS motif of the granulocyte-macrophage colony-stimulating factor receptor α-chain, IL-2 receptor β-chain, Epo receptor, growth hormone receptor, and prolactin receptor have been mutated (33–40). While the number of the mutations made in these studies were limited, no consistent picture emerged concerning the function subserved by this motif; rather, a wide spectrum of hypotheses were proposed, including roles in ligand binding, receptor internalization, signal transduction, intersubunit interactions, and protein folding (33–37). Despite these suggestions, it seems unlikely that a motif such as WSXWS, which is conserved among proteins with very little overall sequence similarity, will perform a radically different function in each receptor.

Here we examine in detail the structural restrictions placed on the murine Epo receptor WSXWS motif by the generation...
and analysis of all possible single amino acid substitutions of these five residues. The results are discussed in terms of a simple model in which the sequence WSXWS is a structurally important element of cytokine receptors and other proteins.

**MATERIALS AND METHODS**

DNAs and the Generation of WSXWS Mutants—The sequence of the oligonucleotides used in this study are shown in Table I. The oligonucleotides were synthesized by Research Genetics (Huntsville, AL). Briefly, the cDNA encoding the wild type Epo receptor (1) was subcloned into the KpnI site of a derivative of pBluescript KS+, pBS, in which the restriction sites of the multiple cloning site, other than KpnI, had been deleted. The resulting plasmid was termed pBSwtEpoR. The 668-base pair BbsI/BglII fragment of pBSwtEpoR was replaced by the homologous fragment from the mutant R129C (41) to generate pBS5CepoR. The 69-base pair BssHl/NheI fragment of pBS5CepoR was then replaced with the annealed oligonucleotides WS1 and WS2 (Table I) to yield pBSWS. The Epo receptor cDNA from pBSWS was subcloned into the mammalian expression vector from which the NcoI site had been deleted to give pcMWS. The NcoI/MluI fragment of pcMWS was replaced with pairs of annealed oligonucleotides (XSAWS1 and -2, WXAWS1 and -2, WSXWS1 and -2, and WSAWX1 and -2) (Table 1) to generate pools of plasmids containing mutations at positions Trp229, Ser233, Ala234, Trp235, and Ser236, respectively. The pools of mutants were transfected into Escherichia coli, and the DNA from individual colonies was sequenced using the primer 600f (Table I) by the dyeoxy chain termination method (42). Large preparations of DNA were purified on QIagen columns according to the manufacturer’s instructions and were also sequenced.

**COS Culture and Transfection—** COS cells (43) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS). The interleukin-3-dependent cell lines 32-D1.2 and Ba/F3 (44) and BA/F3 (45) were maintained as described previously (46, 47). In semisolid culture experiments, 300 Ba/F3 cells were cultured in 0.3% (v/v) fetal calf serum, 0.2% (w/v) sodium dodecyl sulfate, and 1.0% (w/v) sodium deoxycholate, and were then transfected with plasmid DNA at a concentration of 0.1 to 1 pg/μl. Cultures were then incubated for 7 days at 37°C in a fully humidified incubator containing 10% CO2 in air, after which colonies of cells were counted using a dissecting microscope.

COS cells were transiently transfected with 1 μg of DNA using the DEAE-dextran method (48). The transfected cells were incubated for 2 days at 37°C in a fully humidified incubator containing 5% CO2 in air and were then used for binding assays or metabolic labeling experiments. Stable transfection of hematopoietic cells was achieved by electroporation as described (49).

**Binding Studies—** Pure recombinant human Epo was the generous gift of Drs. A. M. de Vos, A. A. Kossiakoff, and Dr. J. Sponer of the GenBank and EMBL databases and were aligned by eye using highly conserved regions such as the WSXWS motif as “anchor points.” The atomic coordinates of the growth hormone receptor were generously provided by Drs. A. M. de Vos, A. A. Kossiakoff, and colleagues at Genentech Inc. (South San Francisco, CA).

**RESULTS**

Generation of WSXWS Point Mutants of the Epo Receptor—The WSXWS motif is important in defining members of the cytokine receptor family. The majority of members, including the murine Epo receptor, contain the definitive sequence at this position, while several contain variants. To determine which structural features within this region are important for receptor function, all possible single amino acid substitutions were generated in the WSXWS region of the Epo receptor cDNA. This was accomplished by cassette mutagenesis, using pools of oligonucleotides encoding all possible amino acid substitutions for a particular residue in the WSXWS motif. Individual clones were sequenced at random to find all of the amino acid substitutions at a particular codon. The representation of each codon
Transfected COS cells provide an excellent system for rapidly assaying the expression of Epo receptor mutants, as well as for examining characteristics of Epo binding to cell surface receptors. However, a relatively small proportion of the receptor synthesized in COS cells reaches the cell surface. In contrast, Epo receptors expressed by the factor-dependent hematopoietic cell line Ba/F3 are processed more efficiently; these cells, therefore, allow comparisons to be made between the intracellular processing of various Epo receptor mutants. Fig. 1B shows that in stably transfected Ba/F3 cells, both wild type and mutant Epo receptors are initially synthesized as an endo-H-resistant glycoprotein with an apparent molecular weight of 64,000. After an hour, approximately 25% of wild type Epo receptors had become endo-H-resistant, confirming previous findings (49, 52) that about 20–25% of newly made Epo receptors exit the endoplasmic reticulum to the Golgi. In contrast, endo-H-resistant forms of the mutants W232Y, S233Y, S233F, S233A, and A234W were undetectable, suggesting that all of these receptor molecules are retained in the endoplasmic reticulum. Endo-H-resistant forms of the mutant S233G were also difficult to detect at any time point of chase, even though this mutant was detected at the cell surface by iodinated Epo binding (Fig. 1B, Fig. 3, and data not shown). Remarkably, one mutant, A234E, appeared to be processed more efficiently than wild type, with 80–90% of the newly synthesized molecules showing resistance to endo-H resistance after 1 h. These differences were confirmed by more extensive pulse-chase experiments in which endo-H-resistant wild type and A234E Epo receptors were detectable after a 15-min chase and reached a plateau level of 25–30% and 70–80% of total pulse-labeled receptor after 30 min of chase, respectively (53). The balance of newly made wild type and mutant receptors were degraded without exiting the endoplasmic reticulum (49, 52).

Cell Surface Expression of Receptors—Preliminary experiments revealed that the amount of 125I-Epo bound at steady state by COS cells expressing the wild type Epo receptor decreased as the temperature was increased from 32 to 37°C. This effect appeared to be due to an increase in the number of receptors present at the cell surface at the lower temperature. Thus, the ability of COS cells transiently expressing WSXWS mutant Epo receptors to specifically bind 125I-Epo was measured at both 32 and 37°C in order to identify any mutants with a temperature-dependent phenotype. Binding was carried out using 3–5 independent transfections of each mutant at both temperatures, often in two or more independent experiments. The amount of Epo bound by COS cells expressing mutant Epo receptors varied over at least 2 orders of magnitude. In each case, however, binding was 3–5-fold higher at 32 than 37°C (Figs. 2, 3, and 4).

The number and type of substitutions tolerated differed at each position of the WSXWS motif. The position most sensitive to change was Trp232 (Fig. 2). COS cells expressing the mutant W232F bound approximately 100-fold less 125I-Epo than cells expressing the wild type receptor, while the capacity of cells expressing W232Y to bind Epo was below the limit of detection. COS cells expressing Epo receptors with substitutions of Trp232 to aliphatic hydrophobic residues, to polar residues, or to charged residues bound no detectable 125I-Epo. Mutants of Trp235 exhibited a similar pattern of 125I-Epo binding to those of Trp232 (Fig. 2), although substitution to other aromatic hydrophobic residues was slightly better tolerated in the former. Binding of Epo to COS cells expressing the mutants W235F and W235Y was 14- and 100-fold lower than to cells expressing the wild type receptor. Again, expression of receptors containing other substitutions at position Trp235 did not yield detectable Epo binding.

Within a pool of mutants did not deviate significantly from the expected value (data not shown).

Biovivn of Receptors—Initially, the synthesis of the Epo receptor mutants was examined by transient expression in COS cells. COS cells, transfected with each of the mutant Epo receptor cDNAs, were metabolically labeled for 60 min, after which proteins were immunoprecipitated with an antisera directed against the N terminus of the mature Epo receptor and resolved by SDS-polyacrylamide gel electrophoresis or 10% gel. The gel was then dried, fluorographed, and exposed to autoradiography. The one-letter amino acid code above the autoradiograph identifies the mutants of the Epo receptor at Ser236 transfected into COS cells. Am, amber stop codon; A, untransfected COS cells; wt, COS cells transfected with the wild type Epo receptor; tr, COS cells transfected with a truncated Epo receptor containing amino acids 1–271. B, 5 × 105 Ba/F3 cells, stably transfected with the designated Epo receptor mutants, were pulse-labeled for 15 min (P) or pulse-labeled for 15 min and then chased for 60 min (C). Proteins were then extracted and immunoprecipitated with an antisera directed against the N terminus of the mature Epo receptor; half of each sample was treated with endo-M (+), while the remainder was left untreated (−), prior to resolution by SDS-polyacrylamide gel electrophoresis on a 10% gel and autoradiography.

Fig. 1. Synthesis of Epo receptors with substitutions in the WSXWS motif. A, 1 × 10⁶ COS cells were transfected transiently with cDNAs encoding every possible single amino acid substitution of the Epo receptor at position 236. Cells were metabolically labeled for 60 min, after which proteins were immunoprecipitated with an antisera directed against the N terminus of the mature Epo receptor and resolved by SDS-polyacrylamide gel electrophoresis on a 10% gel. The gel was then dried, fluorographed, and exposed to autoradiography. The one-letter amino acid code above the autoradiograph identifies the mutants of the Epo receptor at Ser236 transfected into COS cells. Am, amber stop codon; A, untransfected COS cells; wt, COS cells transfected with the wild type Epo receptor; tr, COS cells transfected with a truncated Epo receptor containing amino acids 1–271. B, 5 × 10⁵ Ba/F3 cells, stably transfected with the designated Epo receptor mutants, were pulse-labeled for 15 min (P) or pulse-labeled for 15 min and then chased for 60 min (C). Proteins were then extracted and immunoprecipitated with an antisera directed against the N terminus of the mature Epo receptor; half of each sample was treated with endo-M (+), while the remainder was left untreated (−), prior to resolution by SDS-polyacrylamide gel electrophoresis on a 10% gel and autoradiography.

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The middle residue of the WSXWS motif of the Epo receptor is alanine. COS cells expressing all mutants at this position, except A234W, bound 125I-Epo. Not all mutants of Ala234, however, were equivalent (Fig. 4). As we recently described, cells expressing A234E reproducibly bound 3-5-fold more 125I-Epo than cells expressing the wild type Epo receptor (53). A234E was, indeed, the only mutant of the 100 examined that exhibited such a phenotype. Cells expressing receptors carrying substitutions of Ala234 to other polar and charged residues bound 125I-Epo at levels similar to wild type, and in general, hydrophobic residues were less well tolerated at position 234 than hydrophilic residues. An increase in the hydrophobicity of the side chain at position 234 was correlated with a decrease in the amount of 125I-Epo bound. For example, cells expressing the mutants A234W, A234F, and A234V bound, respectively, less than 0.5%, 0.6-0.9%, and 2-3% of the 125I-Epo bound by cells expressing the wild type Epo receptor, while for the mutants A234I, A234L, and A234V the levels were 2-3%, 6-8%, and 20%, respectively.

To determine whether mutant receptors that failed to bind Epo were expressed at the cell surface, we attempted to detect Epo receptors using a polyclonal antiserum raised against a peptide from the N terminus of the mature Epo receptor. Initial experiments using immunofluorescence microscopy on transiently transfected COS cells suggested that the mutant receptors that were unable to bind Epo were not expressed at the cell surface. Such data, however, could not be quantitated (data not shown). To overcome this problem, we purified, using protein A, the IgG fraction of antisera directed to the N-terminal and C-terminal peptides of the Epo receptor. The IgG fraction was then radioiodinated and used in a binding assay. No specific binding of the N-terminal or C-terminal antibodies could be detected to untransfected COS cells. Binding of the labeled anti-N-terminal IgG but not the anti-C-terminal IgG was observed to transfected COS cells. Binding of the labeled anti-N-terminal IgG but not the anti-C-terminal IgG was observed to COS cells transiently expressing the wild type Epo receptor. This binding was specific, since it was inhibited by unlabeled IgG from the N-terminal antiserum but not by IgG purified from antisera to the C-terminal peptide or an irrelevant antigen (data not shown).

Using this technique we examined the cell surface expression of a number of WSXWS mutant Epo receptors. Although each mutant was synthesized at an equivalent level (Fig. 1A and data not shown), the amount of receptor detected at the cell surface varied at least 10-fold. The mutants A234T, S233T, S236T, and S236G were, for example, expressed on the surface at similar levels to the wild type receptor, while A234V, A234L, S236A, S236C, and W235F were 3-20-fold lower and A234I, A234F, and A234V the levels were 2-3%, 6-8%, and 20%, respectively.

Like the tryptophan residues, relatively few mutants containing substitutions at the serine residues of the WSXWS motif were able to bind Epo when expressed transiently in COS cells (Fig. 3). Cells expressing the mutants S233T and S233G bound 2- and 50-fold less 125I-Epo than those expressing the wild type receptor. Mutation of Ser233 to other residues resulted in receptors that displayed no ability to bind Epo. Substitutions at position Ser236 were less deleterious than the corresponding mutants of Ser233 (Fig. 3). For example, COS cells expressing Epo receptors with the changes S236T and S236G bound 125I-Epo at levels comparable with wild type, while those expressing S236C and S236A were also functional, at 25 and 12% of wild type, respectively. Cells synthesizing S236V also bound 125I-Epo, although this binding was approximately 30- to at least 100-fold lower than wild type. In common with mutations at Ser233, no 125I-Epo binding could be detected to COS cells expressing mutants containing substitutions of Ser236 to charged or hydrophobic residues.

The upper panel of the WS motif of the Epo receptor was equivalent level (Fig. 1A and data not shown), the amount of receptor detected at the cell surface varied at least 10-fold. The mutants A234T, S233T, S236T, and S236G were, for example, expressed on the surface at similar levels to the wild type receptor, while A234V, A234L, S236A, S236C, and W235F were 3-20-fold lower and A234I, A234F, and A234V the levels were 2-3%, 6-8%, and 20%, respectively.

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not shown). The mutant A234E again proved exceptional in that it was expressed on the surface at 1.5–3-fold higher levels than wild type (see also Ref. 53). Although immunodetection proved to be about 10–20-fold less sensitive than 125I-Epo binding in detecting cell surface receptors, there was an excellent correlation between the two methods (Fig. 5). In summary, the mutant A234E was expressed at higher levels on the cell surface than the wild type Epo receptor and mutants such as S233T and S236T, while cell surface expression of other mutants was reduced or undetectable. These results further support the notion that the primary effect of mutating the WSXWS motif is to affect receptor folding and, as a consequence, transport of the receptor from the endoplasmic reticulum and its expression at the cell surface.

Equilibrium, Steady-state, and Kinetic Analyses of Epo Binding—Our initial results suggested that reduced cell surface expression contributed to differences in the ability of mutants to bind 125I-Epo (Fig. 5); however, variation in the affinity of Epo for each mutant might also contribute. To test this possibility, saturation binding experiments were performed (Fig. 6A). COS cells transiently expressing representative mutants were incubated at 4 °C with concentrations of 125I-Epo between 30 pM and 4 nM. After 16 h the amount of 125I-Epo specifically bound to the cells was determined. The results of the saturation binding isotherms are illustrated in Fig. 6 in the form of Scatchard analyses. The affinity of Epo for each of the WSXWS mutant receptors expressed in COS cells was indistinguishable from that of the wild type ($K_D = 400–750$ pM). In
contrast, the average number of cell surface Epo receptors varied from less than 10,000/transfected COS cell for W235Y to more than 630,000/transfected COS cell for A234E. There was an excellent correlation between the amount of 125I-Epo bound to cells expressing mutant receptors and the amount of cell surface receptors detected by the anti-receptor antisera (Fig. 5).

A similar relationship was observed in Ba/F3 and 32-D cells, although the numbers of receptors expressed by these cell lines were far lower than for COS cells. In both factor-dependent hematopoietic cell lines the affinity of wild type and mutant receptors for Epo varied from 450 to 1,100 pM; however, while there were ~1000 binding sites at the cell surface of Ba/F3 cells expressing the wild type Epo receptor, there were 3,000–7,000 sites on cells expressing the A234E mutant but only 100–200 on cells expressing the S233G mutant (data not shown).

The characteristics of Epo binding by WSXWS mutant receptors expressed by COS cells were further explored by analyzing the kinetics of Epo association and dissociation as well as the ability of each receptor to internalize Epo. The kinetic association constant for the complex between Epo and each of the mutant receptors was between 0.02 and 0.04 min⁻¹ at 4°C, the same as for the wild type receptor (data not shown). The curves depicting the progress of Epo binding to equilibrium were also similar, suggesting that the kinetic association rate constant governing the formation of Epo-Epo receptor complexes at 4°C was also the same for each of the mutant receptors (data not shown). Finally, for each mutant receptor examined, the ratio of 125I-Epo specifically internalized to that bound at the cell surface was 2.5–3.5 at 37°C under steady state conditions (Fig. 6B), indicating that receptor endocytosis and degradation of bound ligand was not altered for these mutants.

Analyses of Signaling in Hematopoietic Cells—The properties of mutant Epo receptors expressed in 1L-3-dependent Ba/F3 cells and 32-D cells paralleled those observed in COS cells, with the major difference between mutants being the extent of intracellular processing and as a consequence the number of receptors expressed at the cell surface. Factor-dependent cell lines also allow us to examine the capacity of Epo receptor mutants to transduce a proliferative signal. Similar results were obtained for both 32-D and Ba/F3, and those for the latter are discussed in detail. Stable transfectants of Ba/F3 cells were initially selected for their ability to grow in IL-3, and G418, after which their proliferation was compared in response to IL-3, various doses of Epo, or the absence of growth factor. A dramatic difference was observed in each culture condition (Fig. 7, A–C). Cells died rapidly (data not shown) and failed to produce colonies in semisolid cultures in the absence of IL-3 or Epo (Fig. 7, A–C), irrespective of which WSXWS mutant they expressed. These results indicated that unlike other mutations of the Epo receptor (e.g., R129C, E132C, and E133C; Refs. 41 and 47), no single point mutation in the WSXWS motif examined was capable of conferring to cells the ability to proliferate in the absence of growth factor. Likewise, the ability of Ba/F3 cells expressing each of the mutant Epo receptors to proliferate in response to IL-3 was indistinguishable from parental Ba/F3 cells and those cells expressing wild type Epo receptors (data not shown).
Ba/F3 cells expressing various WSXWS mutants responded differently to Epo. Like parental Ba/F3 cells (Fig. 7A), those lines expressing mutant Epo receptors that did not reach the cell surface (i.e. W232F, W232Y, S233Y, S233F, and A234W) did not proliferate in response to Epo at concentrations from 0.005 to 10 units/ml (Fig. 7C and data not shown). These cells were also unable to survive with Epo as the sole growth factor (data not shown). Ba/F3 cells expressing the mutants A234E and S233G exhibited 3000–7000 and 100–200 receptors per cell, compared with cells expressing wild type Epo receptors that exhibited 1500–2000 receptors/cell. Despite differences in the expression of cell surface receptors, each cell line exhibited a similar dose-dependent ability to proliferate in response to Epo (Fig. 7B).

**DISCUSSION**

WSXWS mutants differ in their ability to exit the endoplasmic reticulum. The results described in this paper represent the first systematic study of the relationship between the structure of a cytokine receptor WSXWS motif and its function. A series of 100 point mutations representing all possible single amino acid substitutions in this region were created in the WSXWS motif of the murine Epo receptor. The salient property of the mutants generated in this study was that they differed in their ability to exit the endoplasmic reticulum and as a consequence to be expressed at the cell surface. This is most likely due to a difference in the ability of the mutants to fold into a normal conformation since endo-H-sensitive forms of the wild type receptor are unable to bind Epo, while endo-H-resistant forms are able to bind (37). Thus the endoplasmic reticulum-localized forms of the wild type receptor are unable to bind Epo, while endo-H-resistant forms are able to bind (37). Thus the endoplasmic reticulum-localized forms of the wild type receptor are in various stages of folding and appear to acquire the native conformation just prior to or coincident with transport through the medial Golgi, where resistance of oligosaccharides to endo H is acquired. While the majority of mutants showed a defect in transport through the secretory pathway and presumably in folding, one, A234E, was transported and folded more efficiently than wild type. Importantly, the mutants that were transported to the cell surface had properties indistinguishable from the wild type receptor: each bound Epo with normal affinity, internalized Epo at the normal rate and, where tested, were capable of transducing a normal proliferative signal.

We examined the fate of WSXWS mutants of the Epo receptor expressed transiently in COS cells and stably in the factor-dependent hematopoietic cell line, Ba/F3. Although newly synthesized wild type receptor is processed to very different extents in the two cell types, the effect of mutations in the WSXWS motif appears analogous. In COS cells, the Epo receptor is transiently expressed at very high levels. The vast majority of Epo receptor synthesized, however, never exits the endoplasmic reticulum and is degraded. Despite inefficient processing, extremely high levels of Epo receptor synthesis ensure that a large number of receptors are expressed at the surface of the transfected COS cells. Differences in receptor processing in COS cells are therefore best assessed indirectly by measuring Epo receptors at the cell surface. In COS cells each mutant was synthesized at a similar level to the wild type receptor; however, cell surface expression levels varied over 300-fold, with the mutant A234E being expressed at 3–5-fold higher levels than wild type receptor and others being expressed at levels similar to or lower than the wild type.

We have shown previously that the endogenous Epo receptor expressed in murine fetal liver cells is inefficiently processed and transported from the endoplasmic reticulum. In addition, Epo receptors ectopically expressed in a variety of hematopoietic cell lines, including Ba/F3 cells, are poorly processed, independent of the level of receptor expression (53). The extent of Epo receptor processing and transport through the endoplasmic reticulum is, however, more efficient in hematopoietic cells than in COS cells, although the overall levels of receptor expression are lower (data not shown). In Ba/F3 cells 25% of newly synthesized Epo receptors exit the endoplasmic reticulum and acquire endo-H-resistant carbohydrates (Fig. 1, B and C, and Refs. 49 and 52). In Ba/F3 cells it is therefore possible to monitor the effect of mutations directly on intracellular receptor processing. Strikingly, the mutant (A234E) that was expressed at higher levels on the surface of COS cells was also more efficiently processed in Ba/F3 cells, with 80–90% of newly synthesized Epo receptors exiting the endoplasmic reticulum within an hour and 2–3-fold more receptors being present at the cell surface (53). Likewise we showed that there was an excellent correlation between reduced cell surface expression of mutants such as S233G and W232F in COS cells and a reduction in the efficiency of receptor processing, measured by endo-H sensitivity, and cell surface expression in Ba/F3 cells. The cell type-independent nature of the phenotype of WSXWS mutants suggests that this motif plays an important role in generating a correctly folded protein.

**Sensitivity to Substitution versus Familial Divergence—The ability of Epo receptors bearing substitutions in the WSXWS motif to fold correctly, to exit the endoplasmic reticulum, and to be expressed at the cell surface can be summarized as follows:**

- For positions Trp<sup>233</sup> and Trp<sup>235</sup>, Trp > Phe > Tyr > remainder; for position Ser<sup>233</sup>, Ser, Thr > Gly > remainder; for position Ser<sup>236</sup>, Ser, Thr > Gly, Cys > Ala > Tyr > remainder; for
position A234E, Glu > polar > hydrophobic. Within the cytokine receptor family, the WSXWS motif is not absolutely conserved. The degree to which substitution has occurred during evolution shows a remarkable parallel to the tolerance of the Epo receptor WSXWS motif to mutagenic substitution. While 28/36 of human and murine cytokine receptors do have the definitive WSXWS motif, variants are observed, notably WSXWG, WGXWS, LXSXW, YSXFS, and YGFXS. Moreover, the middle residue, while variable, is often glutamic acid and is in most cases, polar. These parallels predicate that the WSXWS motif plays a similar role in the function of each member of the family.

A Structural Role for the WSXWS Motif?—Since the structures of the growth hormone receptor and prolactin receptors have been solved (32, 54), what does the position of the sequence YGEFS and WSXWS in these receptors tell us about the function of the WSXWS motif?

The motif is located on a distorted region at the top of strand G in the second β barrel. This region of the receptor is not directly involved in binding growth hormone, nor is it implicated in the formation of receptor homodimers (Fig. 8A; Ref. 32). Importantly, this observation argues against theoretical predictions that have suggested that the WSXWS motif lies on the “floor of the binding crevice” (3) and that have been previously used to assess the function of the WSXWS motif in cytokine receptors (33–35).

In addition to suggesting that the WSXWS motif does not participate directly in ligand binding or receptor homodimerization, the structure of the growth hormone receptor permits the residues that interact with the YGEFS sequence to be identified. This process is illuminating since it raises the possibility that the WSXWS motif might primarily act in a structural role.

The Tryptophan Residues—The aromatic side chains of the growth hormone receptor YGEFS sequence present on strand G (Tyr222 and Phe225) and a third aromatic residue on strand C (Trp186) are co-planar and interact with the methylene groups of a series of neighboring polar residues, Glu173, Glu175, Glu179, Glu209, Arg211, Arg213, and Lys215, to form a “hydrophobic sheath” (32). A similar arrangement is observed in the prolactin receptor, which contains a definitive WSXWS motif (54). Thus, the pattern of residues that were tolerated in place of the tryptophan residues of the Epo receptor fits very well with their proposed structural role. Given a requirement of the tryptophan residues of the motif to stack and to interact with a series of methylene groups from the side-chains of neighboring polar residues, the accommodation, though poor, of aromatic residues and the deleterious effect of mutations to aliphatic hydrophobic residues is explicable.

In addition to the presumptive stabilizing role of the WSXWS motif, the hydrophobic interactions involving the WSXWS motif of the growth hormone receptor provide a scaffold for a series of solvent-accessible polar and charged residues. A striking pattern is observed in the growth hormone receptor, in which one face of this surface contains exposed oxygen atoms and the other exposed nitrogens. The position of these residues is conserved...
served within the cytokine receptor family; however, their identity varies (Fig. 8A). This pattern of solvent-exposed charged and polar residues, supported by the hydrophobic scaffold provided by the WSXWS motif, represents an ideal surface for intermolecular interaction. As this surface is apparently uninvolved in either cytokine binding or homodimerization, it is attractive to speculate that it might be important in the formation of ternary receptor complexes. Substitution of these polar residues with residues of different charge (e.g. E173Q or E173K, K215E or K215Q in the growth hormone receptor) may be illuminating in determining the function of these residues and the veracity of this hypothesis.

It is interesting to note that there is a second extensive family of proteins that contain a WSXWS motif - those that contain a thrombospondin type I repeat (Fig. 8B). This family includes thrombospondin, F-spondin, the terminal components of complement C6, C7, C8, and C9, properdin, and the thrombospondin-related surface proteins of various parasites (4, 55-58). Like the WSXWS motif in the cytokine receptor family, the residues in the thrombospondin motif are not absolutely conserved, with phenylalanine and tyrosine substituting for tryptophan and with threonine, glycine, or alanine replacing serine. As discussed, members of the cytokine receptor family contain a series of alternating hydrophobic and positively charged residues upstream of the WSXWS motif. This pattern is not observed upstream of the WSXWS motifs found in members of the thrombospondin family; rather, a similar set of residues is present the same distance toward the C terminus and may act in an analogous manner to stabilize the molecule and produce a surface for intermolecular interaction (compare Fig. 8A with Fig. 8B).

The Serine Residues—The region of strand G surrounding the WSXWS motif does not form typical hydrogen bonds with the adjacent β-strand, strand F. Rather, the side-chain of the serine residues of the WSXWS motif of the growth hormone and prolactin receptors faces the interior of the β-barrel (32) and form hydrogen bonds with adjacent main chain amides. In the growth hormone receptor, Ser226 interacts with Val212, while the glycine residue found in place of the usual serine residue at position 223 cannot hydrogen bond. In the prolactin receptor and presumably other cytokine receptors, like the Epo receptor, which contain a definitive WSXWS motif, both serine residues hydrogen bond with the adjacent β-strand (54).

The importance of a hydrogen bond between the serine hydroxyl group and the adjacent main chain amine is highlighted by the substitutions at positions 233 and 236 described in this study. Serine and threonine are well tolerated at both positions, while mutant receptors containing cysteine or tyrosine at position 236, which are also expected to form hydrogen bonds, also exit the endoplasmic reticulum and are expressed at the cell surface, although at far reduced numbers compared with the wild type receptor. Additionally, the tolerance, albeit less well, of small residues such as glycine and, in the case of Ser236, alanine, may reflect their ability to be accommodated in this region of the receptor without markedly distorting local structure.

The Middle Residue, Alanine—The side chain of the middle residue of the Epo receptor WSXWS motif is alanine. The equivalent position in the growth hormone and prolactin receptors is glutamic acid; in both cases the side chain of the glutamic acid residue is exposed to solvent. The substitution A234E of the Epo receptor is particularly interesting since it was the only mutant to exit the endoplasmic reticulum more efficiently and be expressed at the cell surface in higher numbers than the wild type receptor. Solvation of a glutamic acid residue at this position presumably enhances receptor folding.

Reconciliation of a Structural Role for the WSXWS Motif with Previous Mutagenic Studies—In previous studies of the WSXWS motif, mutagenesis was used to support the notion that these residues play a role in a variety of processes, including cytokine binding, receptor internalization, and signal transduction (33-35). Yet, as we have proposed, the primary function of the WSXWS is structural, with the serine residues forming hydrogen bonds with an adjacent β-strand and the tryptophan residues forming a hydrophobic scaffold that supports a series of polar residues (Fig. 8, A and B). How can these views be reconciled? As one example, Miyazaki et al. (34) showed that the mutant W194G/S195G of the IL-2 receptor β-chain was expressed at the cell surface but failed to bind ligand. This led to the proposition that the WSXWS motif may participate in the interaction between receptor and ligand. It is now known, however, that the binding of IL-2 by the β-chain requires interaction with the γ-chain (29). Thus, the phenotype of the W194G/S195G mutant is also consistent with a purely structural role of the WSXWS motif that we have proposed, that these substitutions alter the disposition of the polar residues that are supported by the hydrophobic scaffold generated by the WSXWS motif and in turn the ability of the β-chain to stably associate with the γ chain and, therefore, to bind IL-2.

The results presented in this study support the notion that the conserved WSXWS motif plays a pivotal role in the generation of a properly folded cytokine receptor. The details of this role in the overall folding process might be further addressed in vivo by comparing the interactions of resident endoplasmic reticulum proteins such as BiP and protein disulfide isomerase with wild type and mutant Epo receptors and by examining the structure of receptors retained in the endoplasmic reticulum using monoclonal antibodies to conformational epitopes. In addition the folding of the Epo receptor might also be followed in vitro. Finally, if the structural model of the WSXWS motif proposed in this paper proves generally applicable, then the interactions made by the motif in each cytokine receptor should closely resemble the interactions made by the sequence YGEFS in the growth hormone receptor. The solution of the structures of other members of the cytokine receptor family will enable such comparisons to be made.

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