Articles

Role of Glycosylation on the Secretion and Biological Activity of Erythropoietin

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ABSTRACT: The erythropoietin (EPO) molecule contains four carbohydrate chains. Three contain N-linkages to asparagines at positions 24, 38, and 83, and one contains an O-linkage to a serine at position 126. We constructed human EPO variants that eliminated the three N-glycosylation sites by replacing the asparagines with glutamines singly or in combination. The O-linked carbohydrate chain was removed by replacing the serine with glutamine, valine, histidine, or alanine. A variant with a double mutation (Gln38,83) and another with a triple mutation (Gln24,38,83) were secreted poorly from COS1 and CHO cells even though RNA encoding these variants was present. All other variants with mutations in N-linked glycosylation sites were secreted normally. Removal of any of the N-glycosylation sites reduced the in vivo but not the in vitro biological activity of the EPO molecule. All the mutations at Ser126, the O-glycosylation site, were secreted normally. In vitro activity was also unaffected except for Ala126 which had a 50-fold decrease. The Val126 variant was tested in vivo, and its specific activity was only slightly less than that of the native EPO, which indicates that the O-linked carbohydrate is not essential for activity.

Erythropoietin (EPO) is produced in the kidneys and secreted into the blood stream to stimulate the differentiation of precursor cells into erythrocytes. EPO contains N-linked carbohydrate chains at positions 24, 38, and 83 and an O-linked carbohydrate chain at position 126. Recombinant EPO secreted by CHO cells was shown to contain carbohydrates with linkages and structures present in EPO isolated from human urine (Takeuchi et al., 1988). The major sugar chains were fucosylated tetraantennary complex type with smaller amounts of triantennary and biantennary sugar chains. A high degree of heterogeneity in the branching and the sialic acid distribution was observed within each N-linked glycosylation site as well as between each of the sites (Sasaki et al., 1987, 1988).

The function of the oligosaccharides on the EPO molecule is complex. EPO glycosylation has been implicated in the secretion, biological activity, and stabilization of the protein (Goto et al., 1988; Goochee et al., 1991; Takeuchi & Kobata, 1991; Kornfeld & Kornfeld, 1985; Rademacher et al., 1988). Several approaches have been used to determine the role of carbohydrates on the biological activity of EPO. All involved studying partially or fully deglycosylated EPO. Prevention of glycosylation has been accomplished by producing EPO in glycosylation-mutant cells (Wasley et al., 1991), using glycosylation inhibitors (Nielsen et al., 1987), removing glycosylation sites by in vitro mutagenesis (Dube et al., 1988), and treating native EPO with *N*-glycanase (Tsuda et al., 1990; Sytkowski et al., 1991; Takeuchi et al., 1990). In each case, undesired modifications result. *N*-Glycanase treatment leaves an aspartic acid in place of asparagine (Tarentino et al., 1985), EPO produced in CHO ldlD mutants bears corelike truncated N-linked oligosaccharides, and site-directed mutagenesis, by definition, results in a mutation. It is not clear what effects these modifications have on EPO structure. Furthermore, because purified urinary EPO is scarce, many of the studies on the role of glycosylation have been done using recombinant EPO produced in a variety of host cells. Not all mammalian cells produce a form of EPO that is biologically active in vivo (Goto et al., 1988). Finally, the presence of sialic acid on the carbohydrate chains is essential for the full in vivo, but not in vitro, biological activity of EPO (Takeuchi et al., 1989). Asialoglycoproteins are rapidly cleared from the circulation by hepatic cells which recognize the terminal galactose exposed upon removal of the sialic acid residue (Fukuda et al., 1989a,b; Morell et al., 1968, 1971; Spivack & Hogans, 1989; Goldwasser et al., 1974). Thus, the limitations inherent with each approach may have led to some of the conflicting results reported in the literature.

We have reexamined the function of carbohydrate on EPO. Our approach has been to eliminate each carbohydrate chain, individually or in combination by sited-directed mutagenesis, to look at the role of each of the oligosaccharide chains on the secretion and biological activity of EPO both in vitro and in vivo. To determine whether the EPO structure was affected by the mutations or by the absence of carbohydrates, an RIA was developed using anti-EPO antibodies whose immunoreactivity changes when the EPO conformation changes.

MATERIALS AND METHODS

EPO cDNA Clone. A human EPO cDNA clone was constructed from the mRNA derived from COS-1 cells transformed with a human genomic DNA clone (pSVgHuEPO) (Lin et al., 1985; Law et al., 1986). The cDNA was 1.8 kb. The coding region was recovered as an 810 bp *Bst*EII–*Bgl*II fragment and cloned into pDSVL (Lin et al., 1985) to produce pecl. pecl contains an SV40 promoter, an EPO 5′-untranslated leader, a SV40 terminater, and a DHFR gene for selection. This construction served as our positive control and was used to transfect COS and CHO cells.

Mutagenesis. The 810 bp fragment containing EPO-coding sequences was cloned into the *Bam*HI site of m13mp18. Oligonucleotide-directed mutagenesis was performed as previously described (Kunkel, 1985) except that single-stranded DNA was prepared by growing the phage in the *Escherichia*

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coli strain RZ1032 (dut⁻ ung⁻) to replace thymine with uracil. Synthetic DNA primers containing the desired changes were 21mers. The double and triple mutants were constructed by performing mutagenesis on single-stranded DNA containing single and double mutants, respectively. The presence of mutations was confirmed by DNA sequence analysis. The *Xho*II–*Bst*EII DNA fragment containing the desired mutation was isolated and ligated into a gel-purified, phosphatased *Bst*EII–*Bgl*II-digested vector (pec) for expression in COS and CHO cells.

Hybridization for Determining DNA and RNA Cell Copy Number. The DNA and RNA isolated from semiconfluent CHO cells transformed with the EPO variants were analyzed by liquid hybridization with a gene-specific probe as previously described (Zinn et al., 1983).

Cell Transfection. COS-1 and CHO cells were transfected with 2–10 μg of pec plasmids containing the desired EPO genes using the standard calcium phosphate/DNA microprecipitate method (Wigler et al., 1978). The COS-1 cells were transfected with circular plasmids for transient expression. Approximately 1×10^6 cells per 60-mm dish were plated the day before transfection. The conditioned media were collected and assayed for EPO content 4–5 days later. The CHO cells were transfected with the same plasmids as the COS cells except that the plasmids were linearized with *Pvu*I. The CHO transformants were selected for DHFR and amplified stepwise with 5, 10, and 30 nM methotrexate for 3–4 weeks at each step. The medium was replaced with fresh medium when the cells were approximately 70% confluent on 100-mm plates, but we did not attempt to standardize the number of cells. Ten milliliters of medium was conditioned for 4–7 days and assayed by RIA and EIA. The conditioned media which contained very low amounts of EPO were concentrated by ultrafiltration through a YM10 Amicon filter.

Removal of N-Linked Carbohydrates. COS-1 cell-conditioned media containing EPO variants were reacted with a polyclonal EPO antibody and immunoprecipitated with protein A–agarose. The immunoprecipitated samples were then digested with PNGaseF, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase (EC 3.5.1.52) (*N*-glycanase, Genzyme Corp.).

Twenty-five-microliter reaction mixtures containing the immunoprecipitated samples, 50 mM sodium phosphate, pH 7.5–8.0, 0.5% SDS, 50 mM β-mercaptoethanol, and 50 mM EDTA were denatured by boiling the samples for 5 min. Five microliters of a 7.5% NP-40 solution was added to the samples before digesting overnight, at 37 °C, with 1.5 μL of *N*-glycanase.

Immunoblots. Immunoprecipitated EPO variants were fractionated on 12.5% SDS–PAGE and blotted onto 0.2-μm nitrocellulose sheets using an electro-transfer apparatus (Scientific Idea, Inc., Minneapolis, MN) connected to a 12-V battery charger for 1 h in a transfer buffer composed of 25 mM Tris base, 200 mM glycine, and 20% methanol. The blots were blocked in 5% fetal bovine serum in PBS for 1 h and reacted with the EPO antibodies overnight at room temperature. The blots were washed 3 times with PBS and processed with a Vectastain kit (Vector Laboratories, Inc., Burlingame, CA).

Assays. (*A*) *RIA.* Radioimmunoassays were performed according to standard procedures using a rabbit anti-EPO polyclonal antibody (Egrie et al., 1986). In some cases, a rabbit polyclonal antibody directed against the first 20 amino acids of human EPO was used (Egrie & Lane, 1984). To determine whether the mutations altered protein folding, RIAs were performed with 9G8A, a monoclonal antibody raised against recombinant human EPO which recognizes denatured or unfolded EPO more effectively than native, folded, EPO.

(*B*) *EIA.* EPO levels in conditioned medium were also quantitated by Elisa (EIA) using standard methods. The plates were coated with either D11 or F12 monoclonal antibodies which recognize different epitopes on the EPO molecule. The combination of methods and antibodies used to quantitate the variants was necessary because of the possibility that some of the mutations might affect the binding of the antibody to its epitope and give erroneous values. All antibodies used in the immunoblots, RIAs, and EIAs described above were produced at Amgen and will be described elsewhere.

(*C*) *In Vitro.* ^{59}Fe uptake by erythroid precursors was used as a measure of EPO activity. The assay was performed as described previously (Goldwasser et al., 1974) using rat bone marrow cells freshly collected.

In vitro activity was also measured with the erythroid colony-formation assay using human bone marrow cells. The assays were performed aseptically as described by Iscove et al. (1974). The only difference was that the cells were first fractionated on a 10-mL cushion of Ficoll-paque (Pharmacia, catalog no. 17-0840-02) in a 15-mL tube. The light, mononucleated cells which banded at the interface between the Ficoll cushion and the medium were collected, washed and resuspended at 1×10^6 cells/mL in Iscove medium, and used in the assay. The erythroid colonies were counted after about 8–10 days of incubation at 37 °C in 100% humidity and 10% CO_2 atmosphere.

(*D*) *In Vivo.* The in vivo assay was performed in polycythemic mice as previously described (Cotes & Bangham, 1961).

RESULTS AND DISCUSSION

Secretion

We used the site-directed mutagenesis to study the role of glycosylation on secretion. We replaced one, two, or three of the N-linked asparagines with glutamines, thereby preventing glycosylation. Secretion of the EPO variants from both CHO and COS cell-conditioned media was measured by RIA, EIA/F12, and EIA/D11. F12 and D11 are monoclonal antibodies which recognize different epitopes on EPO. RIA, EIA/F12, and EIA/D11 values were within experimental error for all the EPO variants. This suggests that the concentrations determined with these assays reflect true concentrations. Average values for the various assays are shown in Table I.

The amount of EPO secreted depends on where the gene was inserted in the genome, on the number of cells present on the plate, on the DNA copy number, and on whether the cells are semiconfluent and rapidly growing or confluent and growth-arrested. The amount of EPO accumulated in the medium will also depend on the plate size, the volume of medium bathing the cells, and on how long the medium has been conditioned.

N-Glycosylation Variants. We subjected conditioned media containing each N-linked glycosylation variant to SDS–PAGE to see the effect of carbohydrate chain removal on the apparent molecular weight. As expected, prevention of N-glycosylation at the mutated sites reduced the apparent molecular weight of the EPO variants. In agreement with previous reports that the oligosaccharide chain at position 24 is mostly biantennary while those at positions 38 and 83 are mostly tetraantennary (Sasaki et al., 1988), the shift in the mobility of pec8 (Gln38)

Table I: Expression of EPO Variants in COS Cells

plasmid	mutation	Wt aa	RIA/EIA (units/mL)	RIA/9G8A[a] (units/mL)	activity (units/mL) in vitro	
					^{59}Fe	CFUe
pec1	wt	wt	110	137		147
pec1	wt	wt	75	53		83
pec1	wt	wt	25	16		27
pec2	Val126	Ser	57	74	37	52
pec2	His126	Ser	35	52	21	33
pec4	Glu126	Ser	86	45	55	
pec4	Glu126	Ser	34		52	54
pec5	Ala126	Ser	25	202	0.3	
pec6	Thr126	Ser	9	10	18	11
pec8	Gln38	Asn	30	246	41	66
pec8	Gln38	Asn	20	60		34
pec9	Gln8	Asn	51	107	90	132
pec9	Gln8	Asn	10	11		20
pec10	Gln24	Asn	38	15		60
pec10	Gln24	Asn	10	5		28
pec10	Gln24	Asn	21	17		55
pec10	Gln24	Asn	87	246	215	
pec40	Gln24,38	Asn	62	170	54	77
pec40	Gln24,38	Asn	7	14		9
pec41	Gln24,83	Asn	50	190	52	30
pec41	Gln24,83	Asn	13	41		13
pec41	Gln24,83	Asn	2.0	3.4		4.2
pec42	Gln38,83	Asn	0.5	6		
pec43	Gln24,38,83	Asn	1	13		
EPO produced in *E. coli*			191000	7600000		
EPO produced in *E. coli*			207000	8300000		
purified rHuEPO			134000	90000		120000
N-glycanase-treated rHuEPO			42800	1680000		25000

[a] EIA/9G8A used 9G8A monoclonal antibody which reacts strongly with unfolded or denatured EPO.

and pec9 (Gln83) on SDS–PAGE was much more pronounced than that of pec10 (Gln24), indicating that the carbohydrate chain at position 24 is, on average, smaller than at positions 38 or 83, although it should be noted that less pec8 and pec9 were loaded on the gel. Consistent with removal of two N-glycosylation sites, the double mutants Gln24,38 and Gln24,83 had similar mobilities and migrated faster than the single mutants (Figure 1A).

N-Linked glycosylation has been reported to affect the secretion of glycoproteins (Rademacher et al., 1988; Kornfeld & Kornfeld, 1985) and surface membrane proteins (Guan et al., 1988). To see if removal of N-linked glycosylation sites affected secretion of EPO, one, two, or three of the N-glycosylated asparagines were replaced with glutamines. In different experiments, we have found that secretion levels varied from 10 (not shown) to 110 units/mL for COS-1 cells transformed with pec1 (native EPO). Therefore, secretion of 10 or more units of EPO variants per milliliter of conditioned medium was considered normal (Table I). In separate experiments, COS-1 secretion was 20 and 30 units/mL for pec8 (Gln38) and 10 and 51 units/mL for pec9 (Gln83). That of pec10 (Gln24) was 10, 21, 38, and 87 units/mL, indicating that none of the N-linked chains was essential for secretion. pec40 (Gln24,38) secreted 7 and 62 units/mL and pec41 (Gln24,83) 50, 13, and 2 units/mL. These results demonstrated that glycosylation at either Asn38 or Asn83 was sufficient for normal secretion while glycosylation at Asn24 only, as seen in pec42 (0.5 unit/mL), was not. Replacing all three glycosylated asparagines with glutamines (pec43) prevented secretion also (1 unit/mL). pec42 (Gln38,83) and pec43 (Gln24,38,83) were transfected into COS cells in several other experiments, and the secretion was always less than 1 unit/mL each. Detection of as much as 1 unit/mL of EPO in media conditioned by pec43 as shown in Table I occurred only once. This suggests that some carbohydrate is required for secretion and that the chain remaining on pec42, at position 24, by itself, does not efficiently allow secretion.

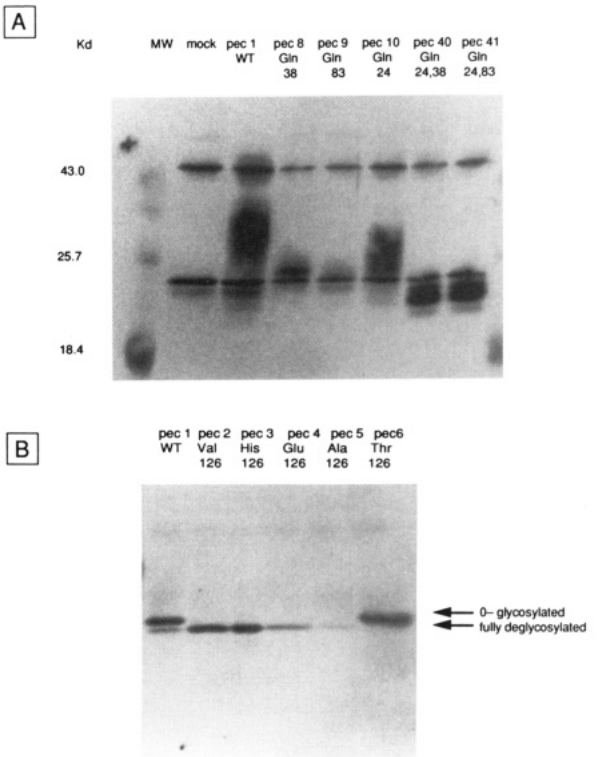

FIGURE 1: Analysis of N- and O-linked oligosaccharides in EPO variants. Media conditioned by COS-1 cells transfected with the indicated EPO variants were immunoprecipitated, electrophoresed on 12.5% SDS–PAGE, and then subjected to Western analysis using the monoclonal antibody 9G8A as described under Materials and Methods. (A) Untreated N-glycosylation mutants immunoprecipitated with a monoclonal antibody against native EPO. The two bands visible in the mock lane are due to the monoclonal antibody used in the immunoprecipitation. (B) O-Glycosylation mutants immunoprecipitated with a polyclonal antibody against native EPO and digested with *N*-glycanase to remove the N-linked oligosaccharides.

Table II: Expression of EPO Variants in CHO Cells

plasmid	mutation	wt aa	RIA/EIA (units/mL)	activity (units/mL) in vitro CFUe/^{59}Fe	activity (units/mL) in vivo
pec1	wt	wt	4.8	3.7	2.75
pec1	wt	wt	2.3		1.7
pec1	wt	wt	7.8	8.5	5.1
pec2	Val126	Ser	2.7	1.6	1.2
pec8	Gln38	Asn	2.3	3.2	0.6
pec8	Gln38	Asn	2.3		0.25
pec8[a]	Gln38	Asn	330.0	425.0	52.0
pec9	Gln83	Asn	1.7	2.2	0.6
pec9	Gln83	Asn	1.0		0.27
pec9[a]	Gln83	Asn	378.0	363.0	87.0
pec10	Gln24	Asn	20.3	43.0	3.2
pec10	Gln24	Asn	14.0	51.0	
pec40	Gln24,38	Asn	1.7		<0.34
pec40[a]	Gln24,38	Asn	9.0	6.5	<0.25
pec41	Gln24,83	Asn	1.0		<0.2
pec41[a]	Gln24,83	Asn	6.0		2.3
pec41	Gln24,83	Asn	1.7	2.0	
pec42	Gln38,83	Asn	<0.07		
pec43	Gln24,38,83	Asn	<0.07		

[a] Concentrated samples.

To see if the structures of pec42 and -43 differed in any way from the other EPO variants, RIA/9G8A assays were done. RIA/9G8A values for pec42 and pec43 were 12- and 13-fold higher (respectively) than the EIA/F12 or EIA/D11 values, indicating that the proteins had differences in conformation compared with native EPO. Higher than normal RIA/9G8A values were also observed for pec8, -40, and -41 (Table I) which indicates that these mutations also affected EPO conformation. However, the effect is not as great as that of pec42 and -43.

Although CHO cells secreted less EPO than COS cells, similar trends were observed. CHO cells transformed with pec1 (native EPO), in three different experiments, secreted 2.3, 4.8, and 7.8 units of EPO/mL of conditioned medium. Secretion of less than 1 unit of EPO variant/mL of conditioned medium is considered poor secretion for either COS or CHO cells. CHO pec8 secreted 2.3 units/mL, pec9 1.0 and 1.7 units/mL, pec10 14.0 and 20.3 units/mL, pec 40 1.7 and 9.0 units/mL, and pec41 1.0, 1.7, and 6.0 units/mL. On average, the secretion of pec8 (Gln38), pec9 (Gln83), pec10 (Gln24), pec40 (Gln24,38), and pec41 (Gln24,83) was normal. As with COS-1 cells, secretion of pec42 (Gln38,83) and pec43 (Gln24,38,83) was below normal and was below the sensitivity of our RIA and EIA assays (70 milliunits/mL) (Table II).

We investigated the possibility that pec42 (Gln38,83) and pec43 (Gln24,38,83) genes were not expressed in CHO cells. Hybridization experiments were designed to quantitate the number of copies of mRNA per cell carrying the EPO variant genes. The CHO cells transfected with pec1, the native gene, carried 1 copy of DNA and 11 copies of mRNA per cell. This particular pec1 clone secreted 8.0 units/mL. pec42 (Gln38,-83) and pec43 (Gln24,38,83) contained 10 and 16 copies of EPO mRNA per cell, respectively, indicating that these EPO variant genes were expressed (Table III). Thus, the secretion defect for pec42 and pec43 must be posttranscriptional and is probably posttranslational.

Others also reported that replacing all three N-linked asparagines with glutamines prevented secretion (Dube et al., 1988); however, in contrast to their findings, we found that removing a single N-linked chain at a time did not affect secretion. Preventing N-linked glycosylation by treating EPO-expressing cells with tunicamycin also prevented secretion of EPO, suggesting that the N-linked carbohydrates play an important role in secretion (Nielsen et al., 1987). Interestingly, truncated N-linked core-type sugars are sufficient for EPO secretion (Wasley et al., 1991).

Table III: EPO DNA and RNA Copy Number in CHO Cells

plasmid	mutation	EPO DNA	EPO RNA	EIA (units/mL)
pec1	native	1	11	8.0
pec2	Val126	9	34	2.5
pec8	Gln38	44	193	6.7
pec9	Gln83	1	53	5.6
pec10	Gln24			
pec40	Gln24,38	2	11	1.7
pec41	Gln24,83	1	40	1.7
pec42	Gln38,83	1	10	<0.01
pec43	Gln24,38,83	9	16	<0.01

O-Glycosylation Variants. To examine the role of O-glycosylation on secretion, we constructed five variants in which we replaced Ser126 with valine (pec2), histidine (pec3), glutamine (pec4), alanine (pec5), or threonine (pec6). To observe the effect of altering the O-glycosylation site at position 126, the O-glycosylation variants and the native EPO were digested to completion with *N*-glycanase (Figure 1B). The lower band of the *N*-glycanase-treated native EPO represents completely deglycosylated EPO (data not shown). As expected, pec6 was O-glycosylated since threonine is a substrate for O-glycosylation. Interestingly, pec6 (Thr126) was fully O-glycosylated while pec1 (Ser126), the native gene, was only about 60–80% O-glycosylated, suggesting that threonine was a better acceptor of O-linked carbohydrate chains at position 126 (Figure 1B). Since elimination of the O-glycosylation site at position 126 (pec2, pec3, pec4, and pec5) resulted in one band which comigrated with unglycosylated native EPO, we confirmed previous reports that EPO bears only one O-linked carbohydrate chain (Sasaki et al., 1987; Lai et al., 1986).

Secretion of the Val126, His126, Glu126, and Ala126 variants ranged from 25 to 86 units/mL in COS cell-conditioned medium (Table I). Contrary to reports that removal of the O-linked site prevents secretion (Dube et al., 1988), we found, in agreement with others (Wasley et al., 1991), that the mutations did not affect secretion. This indicates that O-glycosylation of Ser126 is not significantly involved in the secretion of EPO.

In Vitro Biological Activity

The EPO in vitro assays give a measure of how well EPO interacts with its receptor on erythroid precursor cells. A change in the native conformation or in the amount of carbohydrate branching can affect the interaction (Takeuchi et al., 1989).

A standard curve relating the number of erythroid colonies formed per milliunits of EPO standard was used to determine the amount of EPO variant present in the samples. A fully active sample will give values very close to the values obtained by EIA or RIA analysis. The conditioned media from COS and CHO cells transfected with each of the EPO variants were tested for in vitro biological activity.

N-Glycosylation Variants. As shown in Table I, removal of any one of the N-glycosylation sites (pec8, -9, -10) did not reduce the in vitro activity of EPO as determined by incorporation of ^{59}Fe into hemoglobin and differentiation of precursor cells into erythroid cells in in vitro bioassays. The in vitro activity was not affected by the removal of two N-linked chains at positions 24 and 83 (pec41) or positions 24 and 38

(pec40), indicating that glycosylation at position 38 or 83 was sufficient for EPO to maintain in vitro biological activity. We were unable to assay the biological activity of pec42 (Gln38,-83) or pec43 (Gln24,38,83) due to poor secretion and stability of these variants.

Since pec42 (Gln24,38,83) was not available for in vitro biological activity assays, we pursued our study of deglycosylated EPO using human EPO produced in *E. coli*. *E. coli* produced EPO is not glycosylated. We were unable to accurately determine the absolute specific activity of *E. coli* expressed EPO because of poor stability of the molecule and a tendency for the material to precipitate. However, we were able to conclude that properly refolded and soluble *E. coli* EPO has in vitro biological activity (data not shown), indicating that the carbohydrates are not involved in the interaction between EPO and its receptor. However, the carbohydrates do appear to give EPO stability and affect the protein conformation. The RIA/9G8A values of *E. coli* EPO were consistently about 40-fold higher than the normal RIA, EIA/F12, or EIA/D11 values (Table I), suggesting that the conformation of EPO is altered when the carbohydrates are removed. Similarly, with the N-glycosylation variants, we observed a 12-fold increase in the 9G8A values for pec42 (Gln38,83) and a 13-fold increase for pec43 (Gln24,38,83). This suggests that the increased 9G8A immunoreactivity in pec42 (Gln38,83) and pec43 (Gln24,38,83) is due to the absence of carbohydrate and not simply to amino acid substitutions. Our results are in agreement with reports that enzymatic removal of N-linked carbohydrates did not affect in vitro biological activity (Tsuda et al., 1990; Sytkowski et al., 1991; Higuchi et al., 1992) in contrast to another report in which removal of N-linked sugars by glycanase digestion resulted in the loss of in vitro activity. Since the stability of unglycosylated EPO is reduced compared to that of native EPO, it is possible that differences in preparation, storage, and handling of deglycosylated EPO in different laboratories result in the discrepancies noted in the literature regarding the in vitro biological activity of deglycosylated EPO. We have found that freeze/thaw cycles of the conditioned media result in conformational changes and reduce the in vitro biological activity of EPO variants. Similarly, others have reported that deglycosylated EPO was less thermally stable than glycosylated EPO (Tsuda et al., 1990).

O-Glycosylation. Elimination of the O-glycosylation site by replacing Ser126 with valine, histidine, or glutamic acid (pec2, -3, and -4, respectively) had little effect on the in vitro activity. In contrast, replacing Ser126 with alanine (pec5) reduced the in vitro biological activity by over 50-fold. Replacing Ser126 with glycine also severely reduced the in vitro biological activity (Dube et al., 1988).

Studies of protein conformation of the O-glycosylation sites have revealed that, with very few exceptions, O-glycosylation occurred in a predicted β-turn (Aubert et al., 1976; Fiat et al., 1980). In EPO, nearby prolines at positions 121, 122, and 129 bracket the sequence Asp123, Ala124, Ala125, Ser126, Ala127, and Ala128. Prolines are known to introduce breaks in regular secondary structures. Two prolines in succession tend to favor a conformation which is extended but cannot form β-sheet (Richardson & Richardson, 1989). On the basis of the above information, we propose that the structure around the O-glycosylation site is not normally α-helix or β-sheet. Since a succession of four alanines has been reported to favor α-helix formation (Chou & Fasman, 1978), we investigated the conformational changes resulting from replacing Ser126 with alanine, thus creating a stretch of five alanines (pec5).

The RIA using antibody 9G8A was used to estimate the degree of unfolding caused by the mutations. pec1 (native EPO), pec2 (Val126), pec3 (His126), pec4 (Glu126), and pec6 (Thr126) reacted in the same way with RIA/9G8A as with EIA/F12 or EIA/D11, indicating that these mutations did not cause serious conformational changes. However, pec5 (Ala126) reacted 8-fold more strongly with RIA/9G8A than it did with EIA/F12 or EIA/D11, suggesting that creating a stretch of five alanines resulted in a conformational change, perhaps a short helix (Table I). In a similar experiment, Dube et al. (1988) reported that replacing Ser126 with glycine abolished in vitro biological activity. Although glycine is often considered a helix breaker, it can be found in the middle of helical structures. Glycine and alanine are both small amino acids and can often be substituted for each other without seriously changing the existing conformation of a protein (Aubert et al., 1976). This suggests that glycine and alanine have similar effects on EPO structure. Therefore, removal of the O-linked carbohydrate chain per se does not dramatically affect the in vitro activity of EPO, as others had concluded (Dube et al., 1988), but rather the local conformation of EPO is important in maintaining biological activity. Our results are in agreement with those of others (Higuchi et al., 1992).

In Vivo Biological Activity

Measuring biological activity in vivo adds parameters not present in vitro. For glycosylated, circulating proteins, one of the complications is the effect of clearing by hepatic cells (Fukuda et al., 1989a,b; Morell et al., 1968, 1971; Spivak & Hogans, 1989). Another complication is the extent of branching of the N-linked carbohydrate chains. EPO bearing highly branched and highly sialated N-linked carbohydrates has a longer half-life in the circulating blood which results in a higher apparent in vivo biological activity than a lesser branched version (Takeuchi et al., 1989).

N-Glycosylation. While the relative in vitro biological activities found in the conditioned media from COS and CHO cells were similar, the relative in vivo biological activities were different due to the differences in the carbohydrates added by COS and CHO cells. The biological activities are calculated relative to the EIA values. Conditioned medium from COS cells transfected with pec1 (native EPO) was only 16% active in the in vivo assay (data not shown). Medium conditioned by CHO cells transformed with pec1 was 56–74% as active as our EPO standard (Table II). We therefore focused our efforts on CHO-conditioned media.

EPO from crude conditioned medium is highly heterogeneous in the branching of the carbohydrate chains and in the number of sialic acid molecules on the chains (Sasaki et al., 1987, 1988), while the EPO standard used in the assays was more homogeneous and more active in vivo. As a result, the crude conditioned medium appears to be less biologically active in vivo than would be expected from the EIA values.

The removal of the carbohydrate chain at position 24 (pec10) was assayed once in vivo and was about 16% active (Table II). pec8 (Gln38) was 10–28% active in vivo, and pec9 (Gln83) was 24–35% active in vivo. These results indicate that N-glycosylation is important and that each chain contributes to in vivo biological activity. We were unable to determine the absolute in vivo activity for double mutants because their activities were below the level of detection in these assays. However, pec40 (Gln24,38) was less than 4% and less than 20% active in vivo in two experiments, while pec41 (Gln24,-83) ranged from less than 20% to 38% active.

O-Glycosylation. In agreement with others (Wasley et al., 1991; Higuchi et al., 1992), removal of the O-linked carbo-

hydrate chain had little effect on the in vivo biological activity. pec2 (Val126) was assayed once in vivo. In that experiment, the in vivo biological activity of pec2 was 44%, slightly lower than pec1 (native EPO) which ranged between 57 and 74% of the value of the EIA (Table II). Because the in vitro biological activity of CHO pec2 was also slightly lower than the EIAs, the slightly reduced in vivo activity may be due to an effect on both the in vitro and the in vivo activities.

CONCLUSION

Site-directed mutagenesis is a powerful tool in the study of the effect of glycosylation on secretion and biological activity. One is faced, however, with having to choose a replacement amino acid for the O- or N-linked amino acid. Aside from affecting the DNA sequence, which in itself can affect the transcription process, changing the amino acid sequence can affect both protein folding and local protein conformation. The interpretation of the results becomes complicated when the mutation abolishes the biological activity. We have succeeded, however, in reconciling some conflicting results found in the literature. In agreement with others using different approaches (Tsuda et al., 1990; Sytkowski, 1991; Higuchi et al., 1992), we found that glycosylation is not necessary for the in vitro biological activity of EPO as long as the native conformation is not drastically altered in the process of removing glycosylation sites. N-Glycosylation is essential for full biological activity in vivo, but O-glycosylation does not appear to be required for in vivo biological activity.

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