Vol. 200, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1994 Pages 482-488

REFOLDING OF THE ISOLATED EXTRACELLULAR DOMAIN OF THE ERYTHROPOIETIN RECEPTOR[△]

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Received February 24, 1994

Refolding of the soluble recombinant binding-active extracellular domain of the murine erythropoietin receptor (sEPO-R) was achieved with greater than 90% recovery either from urea/NaCl (1.5/0.5 M) or guanidine HCl (1 M). An expression plasmid encoding the extracellular plasma region of the human EPO-R (sEPO-R) was transfected into COS cells, and the sEPO-R so produced was labelled with [^{35}S]methionine and purified by EPO-affinity chromatography on a EAH-Sepharose 4B-EPO column. Rapid rates of refolding were required for recovery of the native proteins. Refolding was evaluated by rebinding of the sEPO-R to the EPO affinity column.

Erythropoietin (EPO) is a 34-40 KD glycoprotein hormone synthesized in the kidney that induces proliferation and differentiation of erythroid progenitor cells (1). Recently, a cDNA encoding an EPO receptor (EPO-R) was cloned from a murine erythroleukemia cell line (MEL) cDNA library (2). As inferred from the cDNA sequence, the murine EPO-R is a 507 amino acid polypeptide (55 KDa) with a single membrane-spanning domain (2).

The human EPO-R has also been cloned and is 82% identical with the murine receptor (3). EPO-R is a member of a recently described cytokine

△ This research was supported by a grant from Amgen.

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Abbreviations: EPO, erythropoietin; EPO-R, erythropoietin receptor; sEPO-R, soluble form of EPO-R; MEL, murine erythroleukemia cell line; Gd.HCl, guanidine hydrochloride; PBS, 10mM phosphate buffered saline pH 7.4; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle media; PEG, polyethylene glycol (MW 5000); EDC, 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride.

0006-291X/94 $5.00

receptor superfamily (4,5). The EPO-R can be activated to signal cell proliferation by binding either EPO or gp55, the Friend spleen focus-forming virus glycoprotein (6). Also, a constitutively active mutant form of the EPO-R (Cys-129) has been reported (7). The mechanism of action of EPO is poorly understood.

Erythropoiesis is induced by the association of EPO with a specific cell surface receptor (like others glycoprotein hormones). Nakamura *et al.* (8) suggest that upon interaction with EPO, the receptor may transduce a signal to prevent apoptosis (programmed cell death) of developing erythrocites.

After transfection of the murine cDNA into COS cells, EPO can be cross-linked with two proteins of 105 and 65 KDa in COS cells (2). It was shown that EPO-R has both high and low-affinity receptor sites (2). Recently, ligand binding studies on the extracellular domain of the human and murine EPO-R have shown a single low affinity binding pattern (9,10). The structural determinants of EPO-R and/or sEPO-R on binding affinity are unknown. But studies on chimeric murine EPO-R, by point mutations of the extracellular domain, have shown that the two conserved disulfide bonds (C1-C2 and C3-C4), and the highly conserved W-S-X-W-S domain near the transmembrane region, cannot be mutated without losing the bioactivity (11,12).

We report herein the purification of the transfected murine sEPO-R by EPO-affinity chromatography on a EAH-Sepharose-4B-EPO-column and a high yield refolding procedure.

MATERIALS AND METHODS

Cells and cell cultures. COS cells were routinely mantained in DMEM supplemented with 10% (vol/vol) FCS in a humidified CO_2 (10% CO_2 and 90% air) incubator at 37°C.

COS cell transfection. 10 μg of PXM-plasmids containing the extracellular domain of the EPO-R (11) were transfected into COS cell monolayers grown on 10 cm tissue culture dishes. Transfection was a DEAE-dextran protocol modified by Oprian *et al.* (13). After 72 hrs of incubation, media was labelled with 300 μCi/5mL of [^{35}S]methionine and incubated for two hours at 37°C. The supernatant containing the sEPO-R was removed by aspiration, filtered through a 0.45 μm disposable filter, and stored at 4°C.

EPO-affinity column. 2 mL of EAH-4B sepharose affinity coupling gel (Pharmacia) were used to immobilize EPO by coupling it via the water-soluble carbodiimide EDC (N-ethyl-N'-(3-dimethyl-aminopropyl)carbodiimide hydrochloride) using standard procedures. The coupled polymer was then packed into a 1 mL tuberculin syringe in the presence of PBS, pH 7.4.

Purification of the sEPO-R. [^{35}S]Labelled supernatant from transfected COS cells was passed through the column at a rate of 120 λ/min and washed exhaustively with PBS buffer pH 7.4. The column was then washed with low concentrations (0.3 M urea/0.05 M NaCl of denaturants to remove non-specific

protein binding and very low affinity EPO-binding of the sEPO-R, followed by PBS washing. The protein was then released from the column by adding the minimal concentration of denaturant (urea/NaCl 1.5 M/0.5 M or Gd.HCl 1 M) in PBS/0.04% BSA buffer pH 7.4, required to remove more than 90% of the receptor. The column was washed with 6M Gd.HCl to release the remaining attached receptors and then with 10-15 mL of PBS buffer to regenerate the EPO column. Fractions containing the sEPO-R were detected by a gamma-counter (Beckman LS 6800).

Refolding of the sEPO-R. 100 λ of a fraction (40,000 counts) containing the denatured sEPO-R was refolded by dilution from 1M Gd.HCl to 0.01M Gd.HCl or from 1.5 M urea/0.5 M NaCl to 0.15 M urea/0.05 M NaCl at 4°C at a rate of 200-250 λ per minute. The refolding solution was composed of 0.4 mg/mL of fat free BSA (Sigma) in PBS buffer. After 20 minutes of equilibration, the refolded solution was passed through the EPO-column, as described (rate of 120 λ per minute), then PBS was passed through the column to wash out all remaining unfolded sEPO-R. When no further counts were observed, a solution of 6M Gd.HCl in PBS was passed through the EPO-column.

$$\% \text{ of refolding} = \frac{\text{Number of counts in 6M Gd.HCl}}{\text{Total counts}} \times 100$$

where total counts is the number of counts of the refolded solution (after being passed through the EPO-column), including those counts washed out with PBS, plus the number of counts in 6M Gd.HCl.

Gel chromatography (PAGE). 20 λ of the transfected supernatant was mixed with 20 λ of sample buffer and electrophoresed on a PAGE GEL (10% SDS) (14). Dried gels were autoradiographed by exposure to Kodak XAR film with intensifying screens.

RESULTS

Low concentrations of denaturants (1M Gd.HCl or 1.5 M urea/0.5 M NaCl were sufficient to displace more than 86% of the truncated receptor bound to the EAH-Sepharose 4B EPO-column (Tables 1 and 2).

Table 1

Percent[a] of counts detached from the EAH-Sepharose-4B-EPO-column with increasing concentrations of Guanidine HCl[b]

	Gd.HCl[c] (M)					
	0.01	0.5	1.0	1.5	2.0	6.0
Expt. No. 1	11		82		4.0	3.0
Expt. No. 2		87	6.0			3.0
Expt. No. 3				93		7.0

[a] % represents the amounts eluted at a given Gd.HCl concentration.
[b] The column was previously washed exhaustively with PBS till no counts were eluted.
[c] In 10 mM PBS pH 7.4 with 0.04% fat free BSA.

Table 2

Percent of counts detached from the EAH-Sepharose-4B-EPO-column with increasing concentrations of Urea[a]

Urea/NaCl[b] (M)	% detached
0.3/0.05	14
1.5/0.5	80
3/0.5	2
6 M Gd.HCl[c]	4

[a] The column was previously washed exhaustively with PBS till no counts were eluted.
[b] In 10 mM PBS pH 7.4 with 0.04% fat-free BSA.
[c] Final wash.

Low folding recoveries (24-41%) were obtained by refolding the receptor slowly (300 min.) in the presence of PBS/BSA buffer pH 7.4 from 6M to 0.1M Gd.HCl. The yield was not improved by addition of 3% of Polyethylen glycol, MW 5000 (PEG) as a cosolvent (15) (Table 3). When the refolding experiments were performed by dilution from 1.5 M to 0.1 M or 0.01 M Gd.HCl, the recovery increased to 60% and 83%, respectively, in PBS/BSA/PEG buffer (Table 3). Matrix bound EPO-columns have been previously reported (9,10).

In urea, high rates of refolding (94%) were obtained when the sEPO-R was diluted from 0.3 M Urea to 0.03 M in PBS/BSA/PEG buffer (Table 3). Washing the EPO-column with a low concentration of Gd.HCl, resulted in the release of 11% of the protein (Table 1). Refolding the remaining sEPO-R at higher speed (30 min.) from 1 M to 0.1 or 0.01 M Gd.HCl afforded 76% and 94% of the folded protein, respectively (Table 3). Without the presence of 3% PEG, the refolding yield decreased from 94% to 80% (Table 3).

Washing the receptor attached to EPO-column with 0.3 M urea/0.05 M NaCl removed 14% of the radioactive protein (Table 2). Releasing the remaining sEPO-R (80%) with 1.5 M urea/0.5 M NaCl and then refolding it in 2 or 30 minutes, with or without the presence of PEG in PBS/BSA buffer, resulted in 95% of recovery of the folded receptor.

Refolding was evaluated by rebinding the refolded sEPO-R to the EAH-Sepharose 4B EPO-column. The identity of the sEPO-R was achieved by radioautography of the bound receptor (Fig. 1). An apparent M.W. of 30,000 was

Table 3

Rates of Refolding[a] of sEPO-R in PBS/0.04% BSA buffer, pH 7.4, under different experimental conditions

(Molar)	Time[b] (min)	PEG (3 g/%)	Folding (%)
Gd.HCl from (M) to (M)			
	300	-	24
6 - 0.1	300	-	24[c]
	300	+	41
1.5 - 0.1	50	+	60
1.5 - 0.01	100	+	83
1 - 0.1[d]	30	+	76
1 - 0.01[d]	30	-	80
1 - 0.01[d]	30	+	94
Urea/NaCl[e]			
3/0.5 - 0.03/0.05	30	+	94
1.5/0.5 - 0.15/0.05[c]	30	+	95
1.5/0.5 - 0.15/0.05[c]	2	-	95

[a] Based on 100% of sEPO-R released with 6 M Gd.HCl.
[b] Total time for dilution.
[c] Without BSA.
[d] sEPO-R bound to the column was previously washed with 0.01 M Gd.HCl.
[e] sEPO-R bound to the column was previously washed with 0.3 M Urea/0.05 M NaCl.

observed, as previously reported (11). Denatured sEPO-R did not bind to the column.

DISCUSSION

It has been demonstrated that high rates of refolding of the binding-active extra cytoplasmatic domain of the murine recombinant erythropoietin receptor (sEPO-R) yield nearly quantitative amounts of the native product (95%). These high rates of refolding were obtained by rapid dilution of the protein in urea or Gd.HCl at 4°C in PBS/BSA buffer (Table 3).

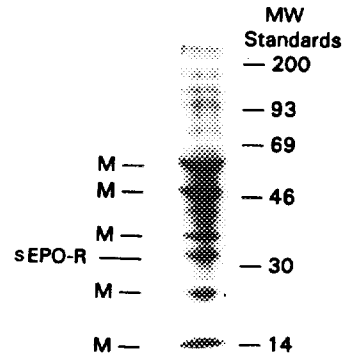

Fig. 1. 10% SDS-PAGE analysis of COS cells (supernatant) transfected with the plasmid encoding the extracellular domain of EPO-R (→). The proteins were eluted with 6 M Gd.HCl from the EAH-Sepharose-4B:EPO column. After SDS-Page, the gel was fixed, soaked with Amplify (Amersham) for 30 minutes, dried, and autoradiographed. The M's represent proteins which bind to the column with and without transfection (mock proteins).

These short periods of renaturation probably indicate that the sEPO-R refolds almost spontaneously, even though two disulphide bridges are present in the native protein. The presence of a redox buffer (0.5 or 0.1 mM of oxidized and 0.1 or 0.02 mM reduced glutathione, respectively) and EDTA (1 mM) in the refolding solution limited the rate of refolding (data not shown).

Studies on the ligand binding properties of the human or murine sEPO-R demonstrated a low affinity binding constant for this truncated receptor (9,10). This finding rationalizes the fact that sEPO-R bound to EPO-column can be effectively removed with low concentration of 1.5 M urea/0.5 M NaCl or 1 M Gd.HCl as denaturants (Table 1 and 2). With very low concentrations of 0.30 Urea/0.05 M NaCl or 0.01 M Gd.HCl, approximately 12% of the bound receptor was detached. The full EPO receptor expressed in COS cells contains about 15% of non-specific binding (2).

This fully folded sEPO-R should be a useful model for physicochemical studies on the Ligand-Receptor interaction. Such studies will hopefully shed light on other members of the cytokine-receptor superfamily.

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