N-Glycosylation-defective receptor for erythropoietin can transduce the ligand-induced cell proliferation signal

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Abstract Erythropoietin receptor (EPOR) contains a single N-linked sugar in an extracellular domain. It has been suggested that an erythroleukemia cell line with high sensitivity to EPO expresses a high molecular mass form of EPOR, which appears to be a highly *N*-glycosylated form responsible for EPO-mediated signal transduction [Sawyer and Hankins (1993) Proc. Natl. Acad. Sci. USA 90, 6849–6853]. To examine the role of the N-linked sugar chain, we prepared EPO-dependent cell lines expressing the wild-type EPOR and *N*-glycosylation-defective EPOR. There was little difference in the expression of EPOR on the cell surface, EPO binding kinetics, and EPO-induced cell proliferation between the clones expressing the mutant EPOR and those expressing the wild-type EPOR.

Key words: Erythropoietin receptor; *N*-Glycosylation; Signal transduction

1. Introduction

Carbohydrates attached to proteins have been shown to play a role in multiple biochemical pathways including folding, stability, targeting and clearance of proteins, and cell recognition (see [1] for review). Most of the growth factor receptors are glycoproteins and the functions of the carbohydrates have been examined using several receptors [2–15]. In some receptors the carbohydrates have been implicated in ligand binding and the signal transduction pathway. Acquisition of the ligand-binding capacity by the epidermal growth factor receptor is glycosylation-dependent [6,7]. *N*-Glycosylation at a specific site of the insulin receptor β subunit appears to be important for the receptor activation and transmembrane signaling [4,5]. Deglycosylation of the basic fibroblast growth factor receptor results in the loss of the ligand-binding [8] and sialylation of the N-linked sugars in the somatostatin receptor is required for maintenance of the high-affinity binding state [9].

EPO transduces the proliferation and differentiation signal through its receptor on erythroid precursor cells and some other lineage cells [16,17]. By molecular cloning of murine [18,19] and human [20,21] EPOR, the mature 53-kDa EPOR has been predicted to consist of an extracellular 225–226 amino acid domain, 22 amino acid transmembrane region, and an intracellular 236 amino acid domain. A single *N*-glycosylation site is present in the extracellular domain in EPOR. Studies on the IL-3-dependent Ba/F3 cells that were stably transfected with the wild-type EPOR and became EPO-responsive indicated that the EPOR with 66 kDa was the mature glycosylated form exposed on the cell surface [22] and that the 72-kDa EPOR could be an EPO-induced phosphorylated form of 66-kDa EPOR [23,24]. BHK cells stably transfected with the wild-type EPOR cDNA and the *N*-glycosylation-defective mutant expressed 68-kDa and 66-kDa EPOR, respectively and the two forms of EPOR were not different in the ligand-binding assay [14]. More recently, Sawyer and Hankins [25] reported that an EPO-dependent murine erythroleukemia cell line, which was highly sensitive to EPO, expressed the 78-kDa high molecular mass form of EPOR and that the 78-kDa EPOR was a functional form of EPOR because it was correlated well with cell surface expression, endocytosis and EPO-induced phosphorylation. They also indicated that the 78-kDa EPOR resulted from high *N*-glycosylation rather than phosphorylation, suggesting the importance of *N*-glycosylation for expression of the functional EPOR or the ligand-sensitive EPOR. These findings of EPOR prompted us to examine the role of the receptor glycosylation using the *N*-glycosylation-defective EPOR. Herein, we prepared EPO-dependent cell lines expressing wild-type or mutant EPOR, and compared the ligand-saturation curves with respect to EPO binding and EPO-dependent cell proliferation.

2. Materials and methods

2.1. *EPO-dependent cells expressing mouse wild-type EPOR and N-glycosylation-defective EPOR*

The plasmid for expression of *N*-glycosylation-defective EPOR (Asn^{51}→Gln) was prepared [14] from the plasmid for expression of wild-type EPOR (pXM190) [18]. IL-3-dependent BaF-BO3 cells, a subclone of the mouse pro-B cell line (Ba/F3), were maintained in the medium consisting of RPMI1640, 10% fetal calf serum and 10% WEHI3B conditioned medium as a source of IL-3. To prepare BaF-BO3 cells expressing EPOR, 7×10^7 cells in 0.8 ml of RPMI1640 were co-transfected with 10 μg pKSV10neo and 100 μg of the wild-type or *N*-glycosylation-defective EPOR by electroporation. The transfected cells were cultured in a 96-well plate in the complete medium plus 1 mg/ml G418. Drug-resistant colonies were picked up and the resistant cells were cloned by limiting dilution. The cloned cells were examined specific binding with the radioiodinated EPO and finally we established four cells lines expressing wild-type EPOR and two cell lines expressing *N*-glycosylation-defective EPOR. They were maintained in the medium containing the WEHI3B conditioned medium.

2.2. *Western blotting of EPOR expressed in BaF-BO3*

BaF-BO3 cells expressing EPOR were solubilized with 100 μl of 1% Triton X-100 and the solubilized EPOR was affinity purified by an EPO-conjugated Sepharose gel column [26]. After subjected to SDS-polyacrylamide gel electrophoresis, EPOR was detected with anti-EPOR N-terminal sequence antiserum using a peroxidase-labeled anti-rabbit IgG and ECL kit (Amersham) [26].

2.3. *EPO dependency of cell proliferation*

For the assay of EPO dependency of cell proliferation, the cells were

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Abbreviations: EPO, erythropoietin; EPOR, erythropoietin receptor; BHK, baby hamster kidney; IL-3, interleukin 3; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

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washed extensively with RPMI 1640 before assay. Cells at a density of 30,000/well were seeded in 96-well microtiter plates in the presence of various concentrations of EPO and cultured for 40 h. Viable cells were measured colorimetrically by the use of the cleavage of MTT [27].

2.4. EPO binding

Recombinant human EPO [28,29] was radioiodinated at a specific activity of 1.11 MBq/μg protein [30]. Binding of EPO was assayed as described previously [19] except that incubations for binding were done at 4°C for 30 min using 2×10^6 cells. Specific binding was defined as the difference in bound radioactivity between samples incubated in the absence and the presence of 100-fold unlabeled EPO.

3. Results and discussion

We established four BaF-BO3 cell lines expressing wild-type EPOR, termed ER1–4, and two cell lines expressing *N*-glycosylation-defective EPOR, termed ERM1 and -2. Parental BaF-BO3 cells required IL-3 for their survival and proliferation, whereas all of these colonies expressing EPOR could proliferate in the presence of EPO instead of IL-3 as shown later. However, these clones were never maintained with EPO because culture with EPO might result in commitment to erythroid cells and in expression of the endogenous EPOR [31]. First, we analyzed EPOR by Western blotting (Fig. 1). EPOR was undetectable in the parental BaF-BO3 cells, while all of the EPOR-transfected cells lines expressed EPOR. The molecular sizes of 66 kDa for the wild-type EPOR and 62 kDa for the *N*-glycosylation-defective EPOR were consistent with those of EPOR with and without N-linked sugar, respectively [14].

Next, the binding properties of EPO were examined using radioiodinated recombinant EPO. There was no specific binding of EPO on the parental BaF-BO3 cells and the specific binding of the cells expressing EPOR was completely abrogated when the radioactive EPO was preincubated with excess soluble-EPOR [26] capable of binding with the ligand (data not shown). As Fig. 2 shows, the Scatchard plots of ligand saturation curves of all BaF-BO3 clones expressing EPOR are straight lines, indicating that they have a single affinity for EPO. Dissociation constants were 530, 470, 430, and 560 pM for four clones (ER1–4) expressing wild-type EPOR, respectively (Fig. 2A), and those for two clones (ERM1 and -2) expressing the mutant EPOR were 690 pM (Fig. 2B); there was not much difference in the dissociation constant of the two types of EPOR. The total numbers of the wild-type EPOR (ER1–4) were 2700, 440, 3200, and 3000 sites/cell and those of the mutant EPOR (ERM1 and -2) were 340 and 1700. This

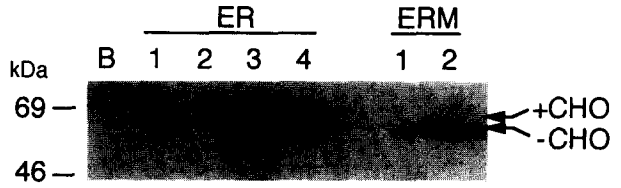

Fig. 1. Western blot analysis of EPOR. B = parental BaF-BO3 cells; ER1–4 = clones expressing the wild-type EPOR; ERM 1 and 2 = clones expressing the *N*-glycosylation-defective EPOR. In all cases, 10^7 cells were used except that 3×10^7 cells were used for ER3. Solubilization and purification of EPOR were as described in section 2. Proteins were fractionated with 6% SDS-polyacrylamide gel electrophoresis and EPOR was detected. +CHO and −CHO indicate EPOR with and without N-linked sugar, respectively.

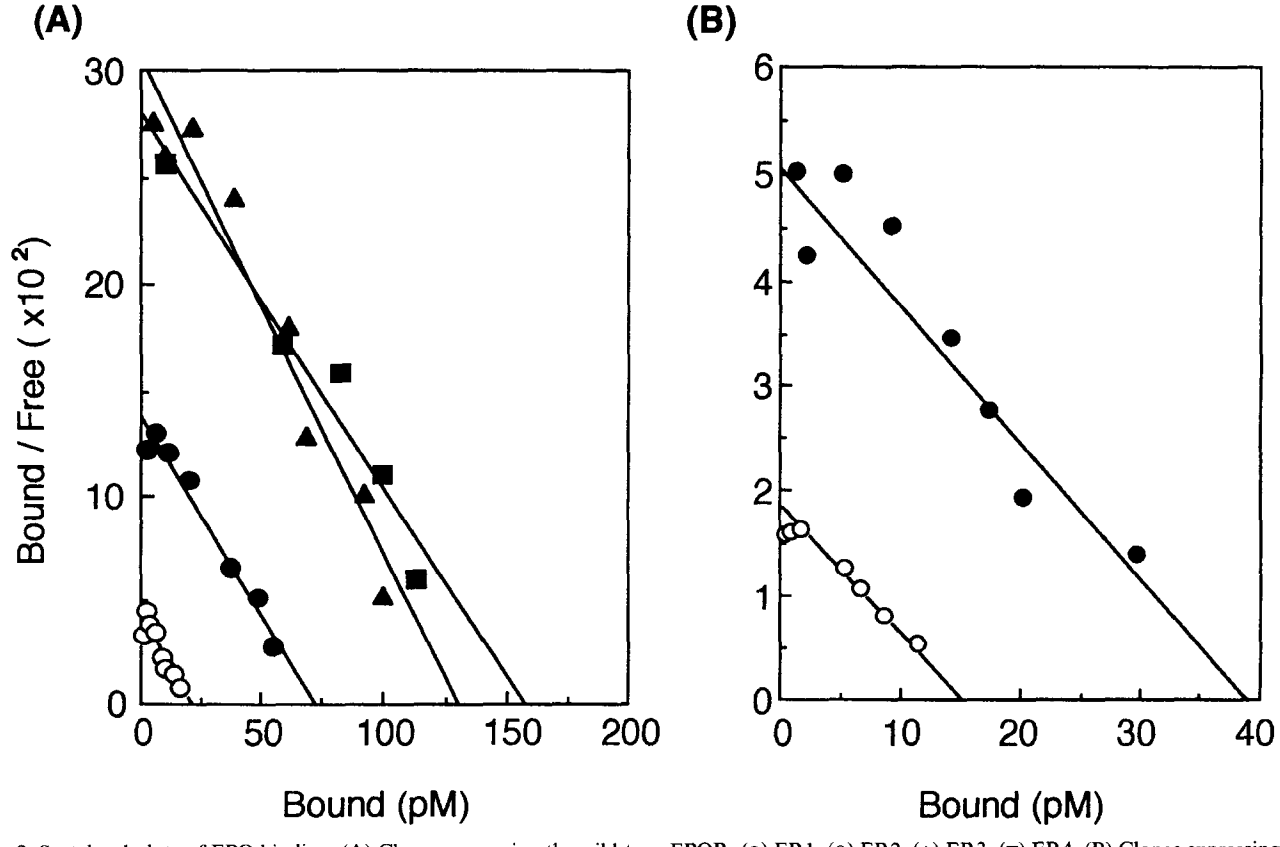

Fig. 2. Scatchard plots of EPO binding. (A) Clones expressing the wild-type EPOR. (●) ER1, (○) ER2, (▲) ER3, (■) ER4. (B) Clones expressing the *N*-glycosylation-defective EPOR. (○) ERM1, (●) ERM2. Each point indicates the average of triplicate assays.

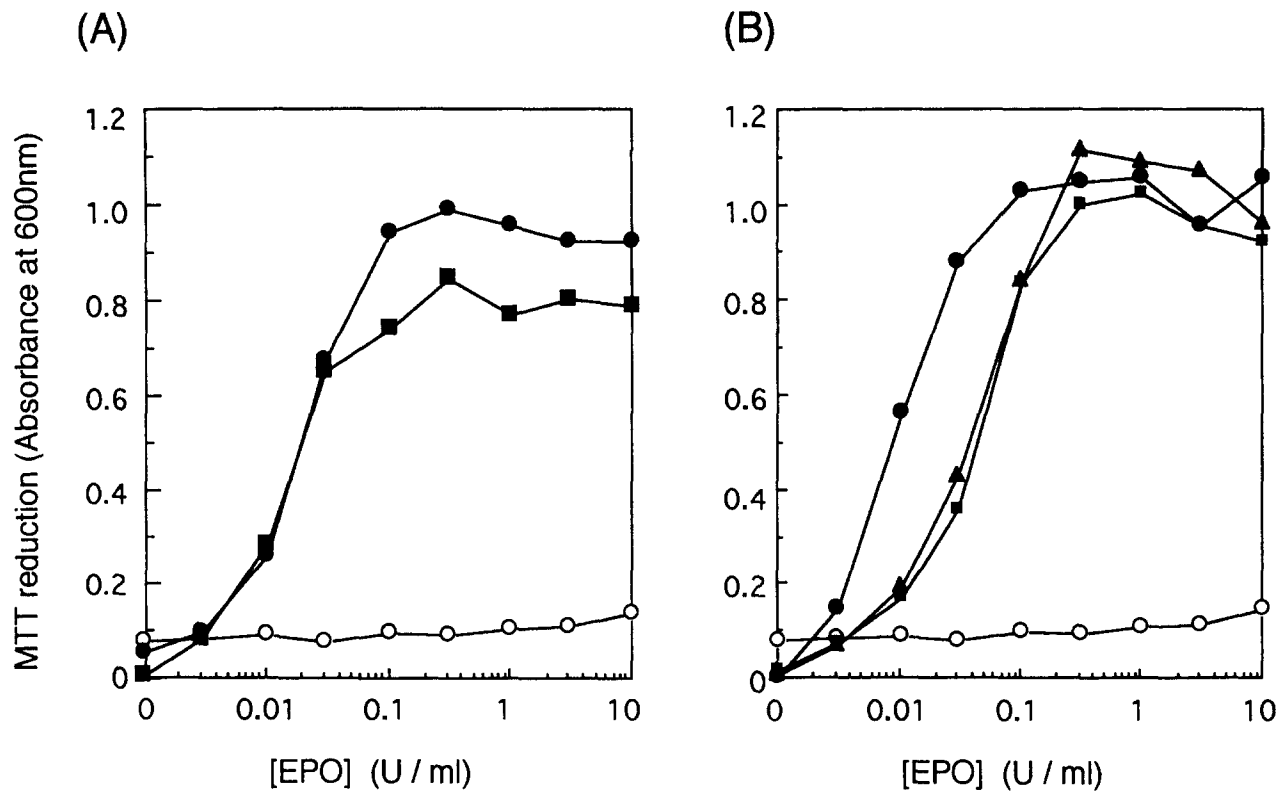

Fig. 3. EPO-dependent proliferation. (A) Clones expressing the *N*-glycosylation-defective EPOR. ● = ERM1, ■ = ERM2, ○ = parental cells. (B) Clones expressing the wild-type EPOR. ▲ = ER1, ● = ER2, ■ = ER3, ○ = parental cells. Each point is the average of triplicate assays.

indicates that *N*-glycosylation is not essential for EPOR to be transported onto the cell surface but *N*-glycosylation appears to somewhat facilitate the transport because the wild-type EPOR is usually expressed more abundantly on the cell surface than the mutant counterpart. This tendency was also observed when the wild-type and *N*-glycosylation-defective EPOR were expressed in BHK cells [14].

We next examined whether the *N*-glycosylation-defective EPOR could transduce the EPO signal for cell proliferation or not. As Fig. 3 shows, parental BaF-BO3 did not respond to EPO. The BaF-BO3 clones expressing the mutant EPOR (Fig. 3A) proliferated in the presence of EPO with a dose–response similar to the clones expressing the wild-type EPOR (Fig. 3B).

In conclusion, *N*-glycosylation of EPOR is not essential for its expression on the cell surface nor for the ligand binding, which is consistent with our previous findings obtained using EPO-independent BHK cells [14] and those obtained from the treatment of EPO-responsive cells with glycosylation inhibitors [15]. The *N*-glycosylation-defective EPOR can transduce EPO-induced cell proliferation signal and *N*-glycosylation of EPOR does not facilitate the signal transduction. Thus the high response of 78 kDa EPOR to EPO found by Sawyer and Hanks [25] on erythroleukemia cells would not be due to *N*-glycosylation but might be due to other modifications which deserve further analyses to elucidate the molecular mechanism of EPO-induced signal transduction.

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