Glycosylation of the murine erythropoietin receptor

P. Mayeux, N. Casadevall, O. Muller and C. Lacombe

ICGM, Inserm U152, Pavillon G. Roussy, Hôpital Cochin, 27 rue du Fbg Saint Jacques, 75674 Paris Cedex 14, France

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Murine erythropoietin-responsive Rauscher Red 5-1.5 cells were used to determine the contribution of glycosylation to the size and function of the erythropoietin receptor. The half life of the receptors was determined to be 4 h. The number of receptors was not significantly decreased in cells treated for 48 h with inhibitors of glycosylation (tunicamycin, glucosamine or swainsonine) and their affinity was slightly enhanced in tunicamycin- or glucosamine-treated cells. Erythropoietin was cross-linked with two proteins of 104 and 86 kDa. Their molecular masses were not significantly reduced in cells treated with the glycosylation inhibitors. When immunoprecipitated cross-linked receptors were digested with endoglycosidases, the molecular masses of both proteins were only slightly modified giving values of 100 and 82 kDa. Thus we can conclude that the proteins cross-linked to erythropoietin are very weakly glycosylated.

Erythropoietin receptor; Erythroleukaemia cell; Glycosylation; Glycosylation inhibitor; Endoglycosidase

1. INTRODUCTION

Despite extensive studies, the Epo mode of action remains largely unknown. We [1,2] and others [3–6] have shown that Epo binds to high affinity membrane receptors on erythroid cells. Cross-linking experiments have shown that Epo associates with two proteins of near 100 and 85 kDa [1,5–7]. The relationship between these two proteins remains unknown but it has been suggested that the smaller component could be a proteolytic fragment of the larger one [7]. A cDNA coding for an Epo binding protein was recently cloned from murine erythroleukaemia cells [8]. It encodes a 55 kDa protein with a single hydrophobic domain located near the middle of the molecule. The relationship between the 100 and 85 kDa proteins evidenced by cross-linking experiments and the 55 kDa protein corresponding to the cloned cDNA remains to be established. As suggested by D'Andrea et al. who cloned this cDNA, the 85 and 100 kDa proteins could be derived from the 55 kDa protein by glycosylation [8]. We tested this hypothesis and we show in this paper that the 85 and 100 kDa proteins exhibit very little, if any, glycosylation.

2. MATERIALS AND METHODS

2.1. *Materials*

The glycohydrolases were from Boehringer Mannheim. The cell culture products were purchased from Gibco (Paisley, Scotland). All other reagents were from Sigma (St. Louis, MO). Human recombinant Epo (spec. act. $>10^5$ U/mg) was iodinated using Iodogen as described in [3].

Correspondence address: P. Mayeux, INSERM U152, Pavillon G. Roussy, Hôpital Cochin, 27 rue du Fbg Saint Jacques, 75674 Paris Cedex 14, France

Abbreviations: Epo, erythropoietin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate

2.2. *Cell culture and labeling*

The Rauscher erythroleukaemia cell line Red 5-1.5 kindly provided by Dr N. de Both was used. These cells differentiate under Epo action [9]. They were cultured in α-modification of Eagles medium containing 5% fetal calf serum. Epo labeling of the cells, Scatchard analysis and cross-linking with disuccinimidyl suberate were done as previously described [1,2].

2.3. *Immunoprecipitation of cross-linked Epo-receptor complexes and enzymatic deglycosylation*

The cells were solubilized in 25 mM Hepes buffer pH 7.8 containing 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1.5% Triton X100 using 1 ml of solution for 10^8 cells. After 15 min on ice, the mixture was centrifuged at 150000 × g for 30 min and the cross-linked Epo-receptor complex was immunoprecipitated using Protein A-Sepharose beads and a rabbit antiserum raised against human recombinant Epo. Immunoprecipitated materials bound on the beads were resuspended in 50 μl of 50 mM sodium phosphate buffer pH 6.00 containing 0.1% SDS, 80 mM dithiothreitol, 1 mM *O*-phenanthroline, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of aprotinin, leupeptin and pepstatin, 1% Triton X100 and 0.02% NaN_3. The suspension was incubated for 1 h at 37°C with 50 mU of neuraminidase from *Arthrobacter ureafaciens*, then 5 mU of endo-α-*N*-acetylgalactosaminidase and 300 mU of endoglycosidase F were added and the incubation was continued for 18 h. After the addition of Laemmli sample buffer, the preparation was boiled and analysed by SDS polyacrylamide gel electrophoresis according to Laemmli [10].

3. RESULTS AND DISCUSSION

3.1. *Effect of glycosylation inhibitors on Epo binding*

Our goal was to determine the effects of glycosylation inhibitors on the Epo binding ability of the receptors and on their molecular weight. It was therefore necessary to determine the half life of the receptor to

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ascertain that all the receptors present on the cells after incubation with the glycosylation inhibitors were synthesized during this incubation time. For this purpose, protein synthesis was blocked by treating the cells with 0.5 mM cycloheximide for various times and the remaining Epo specific binding sites were measured (Fig. 1). Half life of the receptor was so determined to be 4 h in Red 5-1.5 cells. Because all the experiments were made by preincubating the cells 48 h with the glycosylation inhibitors, all the Epo receptors present on the cells were synthesized in the presence of the inhibitors. Tunicamycin and glucosamine which inhibit N-linked glycosylation and swainsonine which inhibits N-linked oligosaccharide processing were used. Tunicamycin was highly toxic at the doses commonly used, so we determined the minimal dose able to block glycosylation (tested by mannose incorporation into trichloracetic precipitable material) with minimal effects on overall protein synthesis (tested by methionine incorporation). At the chosen concentration (0.2 μg/ml), tunicamycin inhibited mannose incorporation by 90% and methionine incorporation by 50%. Lower concentrations only partly inhibited mannose incorporation whereas higher concentrations strongly inhibited protein synthesis (data not shown). Glucosamine (10 mM) inhibited mannose incorporation by 95% and methionine incorporation by 30% whereas swainsonine (1 μg/ml) did not change methionine or mannose incorporations. Fig. 2 shows that the glycosylation inhibitors did not inhibit Epo binding. The number of Epo receptors per cell was 870, 890, 735 and 505 in control cells and in cells treated with swainsonine, glucosamine or tunicamycin, respectively. The decreases observed in tunicamycin (42%) and in glucosamine (16%) treated cells was of the same order as the overall protein synthesis inhibition. Interestingly, the glycosylation inhibitors increased the apparent affinity of the receptors since the K_d changed from 340–350 pM in control and swainsonine-treated cells to 220 and 204 pM in tunicamycin- and glucosamine-treated cells, respectively. Whether this effect was due to lack of glycosylation of the receptor itself or of other proteins surrounding the receptor remains to be determined but it is interesting to notice that the removal of sialic acid from Epo itself increases its affinity for the receptor [4]. It is conceivable that the presence of negatively charged groups both on the Epo molecule and on the receptor itself or in the vinicity of the receptor decreases the affinity of Epo for its receptor.

3.2. *Contribution of glycosylation to the molecular masses of the Epo cross-linked proteins*

As can be seen in Fig. 3A Epo could be cross-linked with two proteins of M_r ~85 and 100 kDa. This result confirms previously published observations [1,5–7]. Cell incubation with tunicamycin or glucosamine did not modify the molecular masses of these proteins showing that they did not contain N-linked carbohydrates. To ascertain this result and to test the presence of *O*-linked carbohydrates, we submitted cross-linked products to endoglycosidase action. Initial attempts to do this using all the solubilized products were unsuccessful because of the presence of proteases which degraded the cross-linked complexes. Therefore we used immunoprecipitated complexes. Fig. 3B shows that the molecular masses of both cross-linked com-

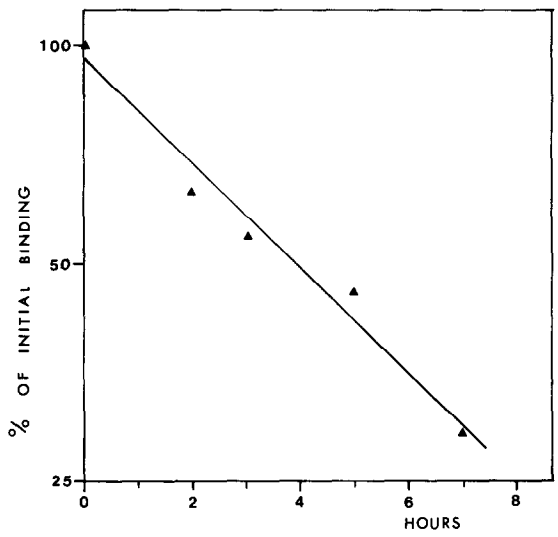

Fig. 1. Determination of the half life of Epo receptors. Cells were incubated with 0.5 mM cycloheximide for the indicated times. Epo specific binding was determined by adding 800 pM ^{125}I-Epo during the last 45 min of incubation.

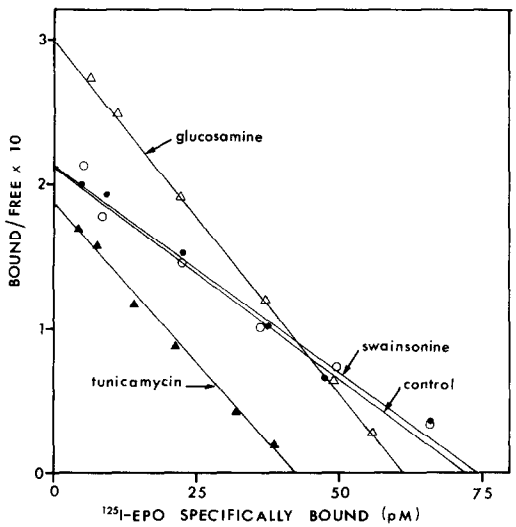

Fig. 2. Scatchard analysis of Epo binding to cells incubated with glycosylation inhibitors. Cells were cultured for 48 h with 0.2 μg/ml tunicamycin (closed triangles), with 10 mM glucosamine (open triangles), with 1 μg/ml swainsonine (open circles) or without inhibitor (closed circles). Scatchard analyses were then carried out in the presence of the inhibitors as previously described [1,2].

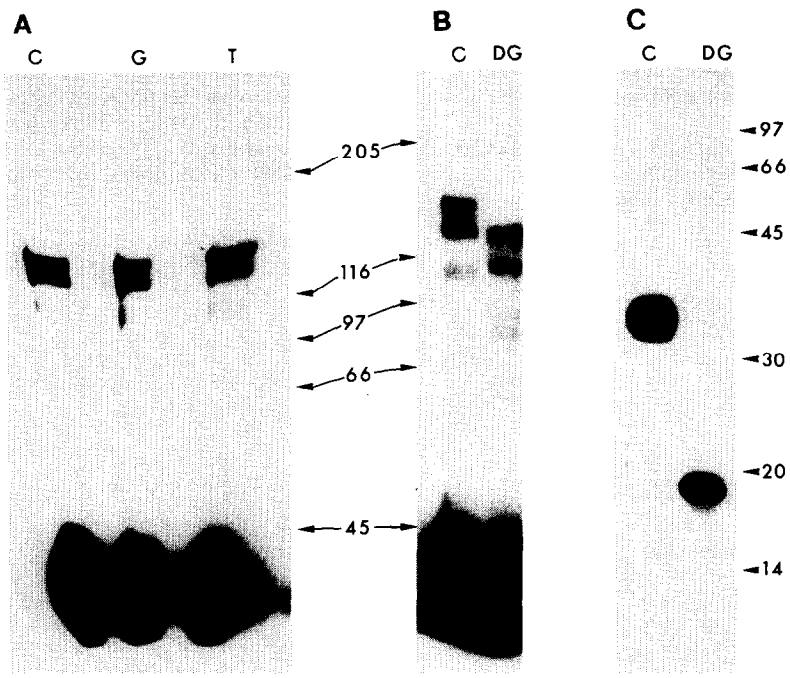

Fig. 3. Contribution of carbohydrates to the molecular mass of the proteins cross-linked with Epo. (A) Control cells (C) and cells cultured 48 h with glucosamine (G) or tunicamycin (T) were labeled with ^{125}I-Epo and cross-linked with disuccinimide suberate before analysis by SDS-PAGE. (B, C) Cross-linked ^{125}I-Epo-receptor complexes from untreated cells were immunoprecipitated with anti-Epo antiserum, incubated without (C) or with (DG) glycohydrolases as described in section 2 and analysed by SDS-PAGE using 7.5% (B) or 12.5% (C) polyacrylamide gel. Numbers indicate the molecular masses in kDa of marker proteins.

plexes were lowered after deglycosylation decreasing from 144 kDa to 121 kDa for the larger size complex and from 124 kDa to 102 kDa for the smaller size complex. Epo being a highly glycosylated protein with both O- and N-linked carbohydrates, the efficiency of the deglycosylation reactions was assessed by examining Epo deglycosylation. Small aliquots of the reaction mixture were analysed on 12.5% polyacrylamide gel to verify the efficiency of the carbohydrate removal reactions (note that, as always reported, the efficiency of the cross-linking reaction was low). Fig. 3C shows that Epo molecular mass decreased from 34 kDa to 19 kDa after glycohydrolase action. Since the reported molecular mass of Epo protein moiety is 18399 Da [11], we could estimate that deglycosylation was complete. Thus nearly all the molecular mass lowering of the cross-linked complexes could be attributed to Epo deglycosylation. After allowing for the contribution of removing carbohydrate from Epo, the molecular masses of the Epo cross-linked proteins changed only very little after deglycosylation: in 5 experiments we calculated values of 104 ± 5 kDa and 100 ± 6 kDa for the larger size protein and 86 ± 4 kDa and 82 ± 4 kDa for the smaller size protein before and after deglycosylation respectively.

3.3. *Conclusions*

Our results show that glycosylation of the receptor is not required for Epo binding. We have also demonstrated that the 100 and 85 kDa proteins cross-linked with Epo are very slightly if at all glycosylated. Consequently these proteins cannot be derived by glycosylation of the 55 kDa protein whose cDNA was cloned recently [8]. The relationship between these proteins remains to be determined. Other covalent modifications can increase the size of a protein: ubiquitin fixation for example was reported for some membrane receptors (see [12] for review). Alternatively the 85 and 100 kDa proteins may not be related to the 55 kDa protein. Authors have reported results suggesting that the Epo receptor may be a complex of disulfide-linked proteins [1,6,13]. It is possible that Epo could be cross-linked to proteins of the complex (the 85 and 100 kDa proteins) different from the true Epo binding moiety (the 55 kDa protein). We are currently testing this hypothesis.

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