Evidence for the Location of the Receptor-Binding Site of Human Erythropoietin at the Carboxyl-Terminal Domain

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Five different peptides (P1: 84-95; P2: 152-166; P3: 52-63; P4: 7-23; P5: 110-123) homologous to relatively hydrophilic regions of human erythropoietin (huEpo) have been synthesized to identify biologically active domains of the hormone. All peptides were able to induce high titers of peptidespecific antibodies in rabbits. Antisera from rabbits induced by recombinant huEpo (rhuEpo) contained a relatively high amount of antibodies preferentially directed against three peptides (P2, P4, and P5), of which P4 comprised the amino-terminal region, P2 the carboxyl-terminus, and P5 an interior region previously described as the receptor-binding site. The same three peptides were able to induce rhuEpo-specific antibodies, whereas P1 and P3 lacked this activity. Only peptide-P2–induced antisera inhibited the biologic activity of rhuEpo in a cell proliferation assay, indicating that the carboxyl-terminal region of the molecule is essentially involved in the biologic function of rhuEpo.

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acid analysis (10% to 15% of total weight of the final complex). The conjugates were dissolved in phosphate-buffered saline (PBS) and mixed with complete Freund's adjuvant (CFA). Preimmune sera were taken before the first subcutaneous (sc) immunization of 0.4 mg antigen in CFA. Two weeks later, a second sc immunization of 0.8 mg antigen in the same adjuvant was performed, which was followed 14 days later by a 5-day intravenous (IV) immunization of 0.1 mg of the antigen with aerosol as adjuvant.13 Five days later the sera were collected. The same immunization protocol was used for the production of rhuEpo-specific antisera, using the hormone coupled to KLH, respectively.

**Microtiter plates were coated with rhuEpo protein (20 μg/mL) or peptide P1-P5 (20 μg/mL) in carbonate buffer (60 mmol/L Na₂CO₃, pH 9.9). Diluted samples of antisera (0.5% bovine serum albumin [BSA] in PBS) were incubated with the immobilized antigen for 2 hours at room temperature and then washed three times with 0.05% Tween 20 in PBS. The plates were then incubated with goat antirabbit Ig F(ab')₂, fragments coupled to alkaline phosphatase (Sigma, Munich, Germany) in 0.5% BSA-PBS. After another three washing steps with 0.05% Tween-PBS and with 0.2 mol/L Tris-HCl, pH 9.5, the plates were developed by use of p-nitrophenyl phosphate in 1 mol/L Tris-HCl, pH 9.5, and bound antibodies were quantified by measuring the amount of p-nitrophenol at 405 nm.

**Gel electrophoresis and immunoblotting.** Samples of purified rhuEpo were separated on Pharmacia 10% to 15% gradient gels using the Phast Gel separation system (Pharmacia). After separation the protein was transferred to nitrocellulose filters by a dry blotting procedure as follows. A dry filter was put on top of the gel and blots were developed with 5-bromo-4-chloro indoxyl phosphate (Sigma) was prepared additionally containing 1 μCi of methyl-3H-thymidine were added to each well. The cells were labeled for 3 hours, and 'H-thymidine incorporation was determined in a TRI-CARB 6660 Liquid Scintillation Counter (Inotech, Wohlen, Switzerland). For inhibition of biologic activity, rhuEpo was preincubated with dilutions of antisera or preimmune serum. To prove the specificity of the neutralizing activity, the sera were administered to the cultures after preadsorption to the specific peptide coupled to sepharose according to Axen et al. 20 The coupling reaction was performed with 30 μg of peptide P2 and 10 μg of BrCN-activated sepharose (Pharmacia-LKB). For preadsorption peptide P2-Sepharose (300 μL) was incubated for 30 minutes with 1 mL of antisera 448. During that time the sepharose beads sedimented. To complete sedimentation the samples were centrifuged for 3 minutes at 1,000 rpm. The supernatant was removed repeatedly after incubation with different portions of peptide-P2-sepharose.

**RESULTS**

Mapping of specific epitopes of rhuEpo. Immunogenic domains of proteins usually are hydrophilic surface structures. Because surface regions of proteins are involved in biologic activities of many proteins there is a likelihood that immunogenic regions and sites of interaction with other components (eg, active centers or binding sites) may be coincident. We used computer analysis to search for huEpo-specific peptides comprising optimal traits according to these criteria.

We identified five major hydrophilic regions of the huEpo molecule. 21, 22 The selected peptides display high hydrophilicity values, as well as high surface probability and antigenic index values (Fig 1). Four of them, P1, P3, P4, and P5, also have a high probability of forming α-helical structures, and the carboxyterminal region P2 is predicted to contain β-sheet and turn structures.

To identify rhuEpo-specific antigenic regions on the selected five peptides, several rabbit anti-rhuEpo antisera were examined for binding to the peptides. For this purpose, microtiter plates were coated with the single peptides, and individual antisera were tested in a direct binding assay. The results are shown in Fig 2. All sera tested contained antibody fractions recognizing each of the peptides. However, depending on the peptide, the titers of the peptide-specific fractions strongly diverged. The data show that regions of the huEpo molecule covered by peptides P2, P4, or P5 provide highly immunogenic epitopes for antibody induction in rabbits (Fig 2D, F, and G). Comparison of the binding capacity of these sera to the peptides (Fig 2C
bent assay (ELISA) and immunoblot experiments, we
whereas rhuEpo-specific titers were low in anti-P1 and
Although P1 and P3 induced high peptide specific titers
tutions. 0 50 100 150
antibody titers (915,918, Table 2), whereas the peptide-P1-
tiserum 915 had been reactive in enzyme-linked immunosor-
immunoblots (Fig 4a and c).
structures of the native huEpo molecule covered by the
induced by peptides P2, P4, and P5 (Fig 3B, D, and E),
antisera against P2, P4, and P5 react
characterized for peptide synthesis and further investiga-
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through G) with that to rhuEpo (Fig 2B) shows a difference
Characterization of antisera induced by huEpo peptides.
Partial peptide sequences of proteins per se are suitable as
epitopes, which mimic the antigenic property of the natural
protein to induce antibodies that can recognize the
whole protein molecule.5 Therefore, rhuEpo-derived syn-
thetic peptides P1, P2, P3, P4, and P5 were coupled to KLH
and used for immunization of rabbits. Peptide-specific
antibodies could be induced with all peptides in several
individual animals, and antisera with high titers of antibod-
ies were obtained (Table 1). Antigen specificity was con-
firmed by blocking studies, using free peptides as inhibitors
(data not shown). Antibodies against P1, P2, P3, P4, and P5
also detected native rhuEpo bound to microtiter plates
(Table 1, lane 2; Fig 3). Highest antibody response was
induced by peptides P2, P4, and P5 (Fig 3B, D, and E),
whereas rhuEpo-specific titers were low in anti-P1 and
anti-P3 antisera (Fig 3A and C). These data suggest that the
structures of the native huEpo molecule covered by the
peptides P2, P4, and P5 are exposed on the surface (Table
1; Fig 3). In contrast, peptides P1 and P3 probably do not
represent surface epitopes of rhuEpo. Immunoblot analysis
confirmed that antisera against P2, P4, and P5 react
specifically with denatured rhuEpo (Fig 4b, d, and e).
Although P1 and P3 induced high peptide specific titers
(see Table 1), these antisera did not react with huEpo in
immunoblots (Fig 4a and c).
We further analyzed the capacity of the antisera to bind
to native radiolabeled rhuEpo in solution using an immuno-
precipitation method. Only anti-P2 and anti-P4 antisera
contained relatively high antibody titers specific for the
native rhuEpo molecule (Table 2). Peptide-P3- and -P5-
specific antisera contained only very low rhuEpo-specific
antibody titers (915, 918, Table 2), whereas the peptide-P1-
specific antisera had only background levels of binding
effectivity to rhuEpo. However, because anti–peptide-P5 an-
tiserum 915 had been reactive in enzyme-linked immunosor-
ent assay (ELISA) and immunoblot experiments, we
analyzed antibody fractions of this serum affinity purified
on P5 sepharose. In these antibody fractions antibodies
specifically binding to native rhuEpo clearly could be
identified (915a, Table 2). However, the binding capacity
was very low.
The carboxyl-terminus of Epo is essential for biologic
function. Antibodies with specificity to distinct protein
regions have been described to perturb protein function.12,13
Therefore, antibodies binding to rhuEpo were examined
for inhibitory effects on the biologic activity of the native
hormone in an in vitro proliferation assay.14 As shown in Fig
5A, anti-rhuEpo serum 348 was able to block the function
of rhuEpo totally after preincubation with the hormone for
1 hour before stimulation of the tissue culture. Preimmune
serum had no inhibitory effect on rhuEpo-induced cell
proliferation. To identify functionally important regions of
the rhuEpo molecule, antibodies specific for peptides P2,
P4, and P5 were studied in the same way for their effects on
rhuEpo-induced proliferation. As shown in Fig 5, only
antibodies specific for the carboxyl-terminal peptide P2
(serum 448) were able to block the proliferation of eryth-
roid precursor cells. Antibodies induced by peptide P4
(serum 926) and peptide P5 (serum 915) did not block the
biologic activity of EPO (Fig 5A). However, because the
region (AA 99-129) that is partially covered by peptide
P5 had been shown to be involved in biologic activity,16 we
used P5-specific antibody fractions of serum 915 for neutralizing
the biologic activity of rhuEpo in the proliferation assay.
However, even this affinity-purified antibody fraction lacked
neutralizing activity (data not shown). To prove that the
inhibition of rhuEpo-induced cell proliferation by anti-P5
sera was specific, antiserum 448 was preadsorbed to peptide
P2-Sepharose. As shown in Fig 5B, the neutralizing activity
of serum 448 could be completely removed by a fivefold
preadsorption of serum 448 to P2-sepharose.

DISCUSSION
To further define the biologically active domain of
rhuEpo, rabbit antibodies were raised against five major
hydrophilic peptide regions of the molecule. Three of them,
peptides P2, P4, and P5, were able to provoke the produc-

Fig 1. Computer analysis of the aminoacid se-
quence of huEpo. For the analysis the software of the
University of Wisconsin Genetics Computer Group
(UWGCG) was used. Plots of hydrophilicity (HP),
surface probability (LSP), flexibility (F), and antigenic
index (AI) are displayed in the upper part of the
figure. In the lower part, predicted regions of turn
structures (T), a-helices (H), and b-sheets according
to Chou and Fasman (CF)14 and Garnier, Olguthorpe,
and Robson (GOR)15 are shown. According to the
data, five peptides (P1, P2, P3, P4, and P5) were
selected for peptide synthesis and further investiga-
tions.
tion of anti-rhuEpo antibodies, suggesting that they comprised immunologically relevant structures. In contrast, antibodies against the peptides P1 and P3 were not cross-reactive with rhuEpo.

Sytkowski and Donahue used a similar immunologic approach to map the active site of rhuEpo. Although they were able to raise antibodies against the carboxyl-terminal 20 residue peptide (residues 147-166), their antisera did not cross-react with highly purified huEpo. We show here that antibodies raised against synthetic peptide P2 comprising carboxyl-terminal residues 152-166 are able to recognize the rhuEpo molecule in its native and biologically active as well as in its partially denatured form using radioimmunoprecipitation, ELISA, or immunoblotting, respectively.

We further demonstrate that the carboxy-terminal is at least part of the biologically active site of the rhuEpo molecule. Antisera against the carboxyl-terminal peptide (P2) of rhuEpo were able to block the biologic activity of the hormone completely, whereas antibodies specific for regions other than the carboxyl-terminal sequences failed to do so. The inhibition of biologic activity could be a result of direct blocking of the receptor-binding site or of confor-

Fig 2. Direct binding of rhuEpo-specific antisera to rhuEpo and to selected peptides. rhuEpo-specific antisera from individual rabbits were tested for antigen specificities. Microtiter plates (96 wells) were coated overnight with 0.5% BSA (A), 20 μg/mL of rhuEpo (B), or 20 μg/mL rhuEpo-derived peptides (P1, P2, P3, P4, and P5). Then the plates were saturated with BSA and incubated with various dilutions of rhuEpo-specific antisera. Bound antibody was detected using goat antirabbit F(ab')2 fragment coupled to alkaline phosphatase and quantified by photometric determination of p-Nitrophenol at 405 nm. Values correspond to the average of double determinations.
Table 1. Specific Antibody Titers of Rabbit Anti-rhuEpo Antisera Against the Inducing Antigen

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Antibody Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-rhuEpo</td>
<td>$1.3 \times 10^{-6}$ (rhuEpo)</td>
</tr>
<tr>
<td>Anti-P1</td>
<td>$1.5 \times 10^{-8}$</td>
</tr>
<tr>
<td>Anti-P2</td>
<td>$1.5 \times 10^{-8}$</td>
</tr>
<tr>
<td>Anti-P3</td>
<td>$3.2 \times 10^{-8}$</td>
</tr>
<tr>
<td>Anti-P4</td>
<td>$3.2 \times 10^{-8}$</td>
</tr>
<tr>
<td>Anti-P5</td>
<td>$3.2 \times 10^{-8}$</td>
</tr>
</tbody>
</table>

Binding of anti-rhuEpo and anti-rhuEpo peptide-specific antisera to the inducing antigens in a solid phase ELISA. Antibody titers were determined by titration of twofold dilutions of different antisera in microtiter plates (96 wells) coated with 20 μg/mL of homologous peptide or 20 μg/mL of rhuEpo. Bound antibody fractions were detected with goat antirabbit F(ab'), fragments coupled to alkaline phosphatase. The procedures and evaluations were performed as described in Fig 2. The values correspond to double determinations of the half maximal optical density at 405 nm.

mational changes through antibody binding to the hormone. However, because of the completeness of the inhibition, we would favor the first assumption. Antibodies raised against carboxyl-terminal peptide 147-166 by Sytkowski and Donahue did neither detect the native Epo molecule nor exhibit a neutralizing activity. However, Sytkowski and Donahue proposed another region as a biologically active domain of rhuEpo, located between residues 99 and 129. In their study, antisera specific for two overlapping peptides from amino acid 99 to 118 and 111 to 129 could block biologic activity by about 75%. Antibodies against one of our peptides from the same region (residues 110 to 123, peptide P5) did not inhibit the biologic activity, although rhuEpo was recognized by these antibodies in ELISA and immunoblot experiments. However, when we tested the binding capacity of P5-specific antiserum 915 to native radiolabeled Epo, we realized that the antiserum was of low rhuEpo-specific antibody titer. Furthermore, antibodies of that serum, which were affinity purified with P5-sepharose, could not neutralize the biologic activity. This finding indicated that the peptide P5 had induced another set of antibody species than the peptides covering the same region of rhuEpo used by Sytkowski and Donahue.

The N-terminal region of huEpo, represented by peptide P4, carried a third relatively immunogenic structure of the peptides analyzed. In the native huEpo this region is
Fig 4. Western blot analysis of rhuEpo peptide-specific antisera. Purified rhuEpo was separated on a 10% to 15% polyacrylamide gradient gel and plotted to nitrocellulose (NC). The NC filter was cut into strips and each strip was incubated with an individual rabbit antiserum, e.g., anti-P1 (a), anti-P2 (b), anti-P3 (c), anti-P4 (d), anti-P5 (e), anti-Epo (f), and rabbit preimmune serum (g). The strips were then incubated with goat antirabbit F(ab'), fragments coupled to alkaline phosphatase and developed with phosphatase substrate (see Materials and Methods).

The synthetic peptides used by us and others cover the total rhuEpo sequence. Using a Kyte-Doolittle plot and a more extended computer analysis (see Materials and Methods) nearly identical peptide sequences were selected for antibody induction. Two peptides with relatively hydrophobic domains could not be analyzed, because their synthesis resulted in insoluble products (aminoacids 20-40 and 60-80). All other peptides (aminoacids 1-26[22]; 7-23 [P4]; 40-59[23]; 52-63 [P3]; 80-99[24]; 84-95 [P1]; 99-118[25]; 110-123 [P5]; 111-129, 131-150, 147-166[26]; and 152-166 [P2]) were recognized as antigen in rabbits. Eight of the peptide-specific antisera and a monoclonal antibody (specificities for aminoacids 1-26[27]; 7-23 [P4]; 40-59, 80-99, 99-118[28];

coupled to the carboxyterminal domain via disulfide bond. Using the immunoprecipitation method, a high amount of rhuEpo-binding activity was detected in P4-specific antisera that could only partially be competed by cold rhuEpo (Table 2). Despite the high binding activity of this serum to native rhuEpo, no inhibition of biologic activity was observed in the huEpo-specific proliferation assay. This finding is in agreement with studies describing activity of anti-huEpo antibodies against the N-terminal region. None of these antisera or a monoclonal antibody specific for this region were able to block biologic activity. Thus, the suggestion that this region is not involved in the receptor-binding domain of rhuEpo is confirmed by our data.

Table 2. Detection of Native rhuEpo by Peptide-Specific Antibodies

<table>
<thead>
<tr>
<th>Antiserum No.</th>
<th>Specificity</th>
<th>Working Dilution of Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:1000</td>
</tr>
<tr>
<td>444 P1</td>
<td></td>
<td>0.038 ± 0.001</td>
</tr>
<tr>
<td>+</td>
<td>0.026 ± 0.001</td>
<td>0.019 ± 0.008</td>
</tr>
<tr>
<td>448 P2</td>
<td></td>
<td>5.97 ± 0.03</td>
</tr>
<tr>
<td>+</td>
<td>0.295 ± 0.009</td>
<td>0.203 ± 0.008</td>
</tr>
<tr>
<td>918 P3</td>
<td></td>
<td>0.186 ± 0.019</td>
</tr>
<tr>
<td>+</td>
<td>0.153 ± 0.011</td>
<td>0.103 ± 0.007</td>
</tr>
<tr>
<td>926 P4</td>
<td></td>
<td>1.162 ± 0.013</td>
</tr>
<tr>
<td>+</td>
<td>0.989 ± 0.018</td>
<td>1.092 ± 0.015</td>
</tr>
<tr>
<td>915 P5</td>
<td></td>
<td>0.145 ± 0.010</td>
</tr>
<tr>
<td>+</td>
<td>0.091 ± 0.003</td>
<td>0.065 ± 0.003</td>
</tr>
<tr>
<td>915a* P5</td>
<td></td>
<td>0.754 ± 0.013</td>
</tr>
<tr>
<td>+</td>
<td>0.256 ± 0.001</td>
<td>0.166 ± 0.017</td>
</tr>
<tr>
<td>Control (Epo)</td>
<td></td>
<td>77.3 ± 0.8</td>
</tr>
<tr>
<td>+</td>
<td>11.4 ± 0.1</td>
<td>5.61 ± 0.03</td>
</tr>
</tbody>
</table>

Binding of anti-Epo peptide antibodies in a competitive radioimmunoassay. Binding of radiolabeled whole Epo is given in percent of input radioactivity. Mean values and standard deviations were calculated from triplicates. For the evaluation of binding specificity, cold Epo was added: (−) indicates binding without, and (+) indicates binding with 1.0 μg of cold native Epo as a competitor present.

*Antibody 915a was an affinity-purified antibody fraction eluted from peptide-P5-sepharose. Working dilutions of this antibody were 1:200, 1:400, and 1:800 after adjustment to a protein concentration of 8.4 mg/mL.
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Fig 5. (A) Inhibition of rhuEpo-dependent in vitro proliferation by rhuEpo-specific antisera. Spleen cells of anemic mice were induced to proliferate by purified rhuEpo (0.05 pmol Epo/mL). To analyze the effect of antisera on the biologic activity of rhuEpo, the hormone was preincubated with dilutions of anti-rhuEpo and anti-rhuEpo peptide antisera before incubation of the cell culture. Proliferation rates were determined by \(^{3}H\) thymidine incorporation. rhuEpo was added to tissue culture together with different amounts (0.25 \(\mu\)L/mL, 2.5 \(\mu\)L/mL, and 25 \(\mu\)L/mL) of rabbit preimmune serum; rabbit anti-whole Epo molecule serum 348; rabbit anti-P2 serum 448; rabbit anti-P4 serum 926; and rabbit anti-P5 serum 915. Control samples contained rhuEpo without serum or medium (DMEM, 10% FCS) without rhuEpo. (B) Reversion of neutralization by preadsorption of antisera. To prove that neutralization is caused by specific interaction of the anti-P2 antibody fraction with rhuEpo (0.05 pmol/mL), serum 448 was two times and five times preadsorbed on P2 sepharose and then used in the neutralization assay. The samples analyzed contained: Epo plus nonadsorbed antiserum 448 (+448), Epo plus antiserum 448 preadsorbed twice (+448 2x), and Epo plus antiserum 448 preadsorbed five times (+448 5x). Control samples contained culture medium (MED) without Epo.

110-123 [P5]; 111-129, 131-150\(^a\); and 152-166 [P2]) cross-reacted with the rhuEpo molecule. Only two regions, the carboxyl-terminal region (P2) (aminoacids 152-166) and an interior region (aminoacids 99-129), have been shown to be involved in the function of Epo. However, it is still possible that other parts of the molecule, including the glycosyl residues, also contribute to its biologic function. Further analyses using an extended panel of epitope-specific monoclonal antibodies, site-directed mutagenesis, and the resolution of the three-dimensional structure of rhuEpo and its receptor are warranted to show whether the carboxyl-terminus and the interior region act together perhaps with other regions of the molecule for receptor binding and signal transduction in erythroid progenitor cells.

ACKNOWLEDGMENT

We are grateful to C. Lau, A. Wagner, B. Honisch, and H. Schneider for excellent technical assistance. We thank J.A. Smith and W. Oster for critical reading and A. Mankel and E. Bieker for typing the manuscript.
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Evidence for the location of the receptor-binding site of human erythropoietin at the carboxyl-terminal domain

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