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Pharmacology

Activation of erythropoietin receptor in the absence of hormone by a peptide that binds to a domain different from the hormone binding site

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ABSTRACT **Applying a homology search method previously described, we identified a sequence in the extracellular dimerization site of the erythropoietin receptor, distant from the hormone binding site. A peptide identical to that sequence was synthesized. Remarkably, it activated receptor signaling in the absence of erythropoietin. Neither the peptide nor the hormone altered the affinity of the other for the receptor; thus, the peptide does not bind to the hormone binding site. The combined activation of signal transduction by hormone and peptide was strongly synergistic. In mice, the peptide acted like the hormone, protecting against the decrease in hematocrit caused by carboplatin.**

We previously had described a method for identifying peptides that would selectively inhibit internalization of insulin, insulin-like growth factor 1, and other receptors (1–3). We used the same MHC class I protein (MHC-I) sequence D^k-(61–85) as probe in a homology search with the erythropoietin (EPO) receptor (EPO-R) as target. We identified a homologous sequence in the extracellular domain of the receptor, distant from the hormone binding site, in a region suggested to participate in dimerization (4). An EPO-R-derived peptide (ERP) was synthesized identical to that sequence. Now we describe the wholly unexpected finding that EPO-R, which normally is activated by hormone-induced dimerization, can be activated by ERP in the absence of hormone. The peptide does not bind to the hormone binding site; it apparently complexes with its identical sequence in the homology domain. Interestingly, EPO and ERP together produce a dramatic synergy of effect. Finally, we show that in mice ERP has the same effect as EPO.

MATERIALS AND METHODS

Reagents. Recombinant human EPO was purchased from R & D Systems. Antiphosphotyrosine mAb PY-99 and rabbit polyclonal antibodies against JAK2, STAT5, and EPO-R were from Santa Cruz Biotechnology; antiphosphotyrosine antibody 4G10 was from Upstate Biotechnology (Lake Placid, NY); GammaBind G Sepharose beads were from Amersham Pharmacia Biotech; [^{125}I]EPO (specific activity 300 Ci/mmol) was from Amersham Pharmacia; and human fetal liver Marathon-Ready cDNA library was from CLONTECH.

Peptide. ERP peptide was synthesized by American Peptide (Sunnyvale, CA) by standard solid-phase method, purified to >95%, and further characterized by HPLC and mass spectrometry. Storage was at −20°C as a powder or as 1 mM stock solution in water before use.

Cell Lines and Culture Conditions. TF-1 cell line (bone marrow, erythroleukemia, human, ATCC CRL-2003) was used for studies of EPO-R activation and intracellular signaling. Cells were maintained in RPMI medium 1640 supplemented with 5 mM glutamine, 100 units/ml of penicillin, 10 μg/ml of streptomycin, 10% bovine fetal serum (HyClone), and 1 ng/ml of granulocyte/macrophage colony-stimulating factor.

Chinese hamster ovary (CHO) cells overexpressing the human EPO-R (hEPO-R) were used in peptide and hormone binding studies. CHO/EPO-R cell line was obtained by stably transfecting the hEPO-R gene into CHO cells. Briefly, hEPO-R gene was constructed by PCR from human fetal liver cDNA library by using the following 5′ and 3′ primers, respectively: GGGAATTCATGGACCACCTCGGGGCGTCCCTC and GGGAATTCCTAAGAGCAAGCCACATAGCTGGG. *Eco*RI restriction sites were introduced on both ends, and hEPO-R gene was subcloned into mammalian expression vector pCR3.1 (Invitrogen). Expression of EPO-R was confirmed in binding assays using [^{125}I]EPO. For control experiments, CHO cells were transfected with expression vector pCR3.1 that carries hEPO-R gene in inverted sequence, and thus the protein cannot be expressed. Cells were stably transfected by using Lipofectin reagent (GIBCO/BRL), as described (1). Transfected cells were maintained in F12 medium containing 10% calf serum, 2 mM glutamine, 100 units/ml of penicillin, and 10 μg/ml of streptomycin.

Cell Stimulation for *in Vitro* Signaling Assays. Cells were grown in RPMI medium 1640 (supplemented as above) to approximately 1×10^7/ml, centrifuged ($200 \times g$, 5 min), and resuspended in medium with 5% serum and no granulocyte/macrophage colony-stimulating factor (GM-CSF). Cells were starved 12–16 h at 37°C in 5% CO_2, centrifuged, and resuspended to the original density in medium without serum or GM-CSF, and 2 ml was used in each stimulation experiment. For peptide and hormone concentrations and other experimental conditions, see figure and table legends.

Peptide stock solutions were thawed immediately before use. Cell suspension was treated with peptide or hormone at 37°C in 5% CO_2, then 10 ml of ice-cold cell wash buffer (PBS with 1 mM orthovanadate, 50 mM NaF, 20 mM β-glycerophosphate, and 2 mM sodium pyrophosphate) was added to each Falcon tube and centrifuged (4°C, $200 \times g$). Medium was aspirated, and the wash was repeated twice with buffer. Then, on ice, 0.6 ml of lysis buffer [100 mM Hepes/300 mM NaCl/2% Triton X-100/100 mM NaF/10 mM EDTA/4 mM sodium pyrophosphate containing 0.02 mg/ml aprotinin, 0.02 mg/ml pepstatin A, 0.02 mg/ml leupeptin, 0.02 mg/ml chymostatin, and 0.0002 mg/ml peptidase inhibitor AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride] was added. Cells were suspended by pipetting and transferred to Eppendorf tubes, left on ice about 30 min, then centrifuged ($14{,}000 \times g$, 4°C, 10

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Abbreviations: MHC-I, MHC class I protein; EPO, erythropoietin; EPO-R, EPO receptor; ERP, EPO-R-derived peptide; CHO, Chinese hamster ovary.

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MHC-I,D^k-(61-85)	E R E T Q I A K G N E Q S F R V D L R T L L R Y Y
EPO-R-(194-216)	Q R V E I L E G R T E C V L S N L R G R T R Y

FIG. 1. Amino acid sequence of EPO-R domain with homology to MHC-I peptide D^k-(61–85). To identify the most similar sequence to MHC-I peptide D^k-(61–85) in EPO-R, we used BESTFIT (GCG Software Package version 8.0 with default parameters: rescaled PAM250 matrix, gap creation penalty 3.00, gap extension penalty 0.10) as in our previous paper (1). Output of BESTFIT is shown as the 23-mer aligned underneath the MHC-I peptide. Location of the homologous sequence in EPO-R is indicated at left, numbering according to precursor receptor protein, as per Swissprot data bank. Traditional numbering of D^k-(61–85) is for mature protein; Swissprot numbering is 85–109. Black boxes, identity; gray boxes, similarity. Identity 24%, similarity (including identity) 40%.

min). The supernatant lysate was used for immunoprecipitations.

Immunoprecipitation, SDS/PAGE, and Western Blotting. Antibodies (1–4 μg) were bound to protein-G Sepharose beads before addition of cell lysates (20–40 μl of slurry in 4× diluted lysis buffer) by 2-h incubation at room temperature. Cell lysates were cleared by centrifugation at 4°C, and supernatants were incubated with specific antibodies bound to protein-G Sepharose with end-over-end rotation overnight at 4°C. Beads were washed three times with 2× diluted lysis buffer and once with 0.5 M Tris, pH 6.5. SDS sample buffer (50 μl) was added, and the proteins were separated on 8% polyacrylamide mini-gel. After transfer, poly(vinylidene fluoride) membranes (Millipore) were treated for 1 h in blocking buffer TST (Tris, pH 7.4/0.15 M NaCl/0.075% Tween 20/0.02% NaN_3) with 0.5% dry skim milk powder and incubated with the appropriate primary antibody overnight at 4°C at 1:1,000 dilution. The membrane was washed and incubated with appropriate alkaline phosphatase-conjugated secondary antibody (1:2,000 dilution) at room temperature for 2 h.

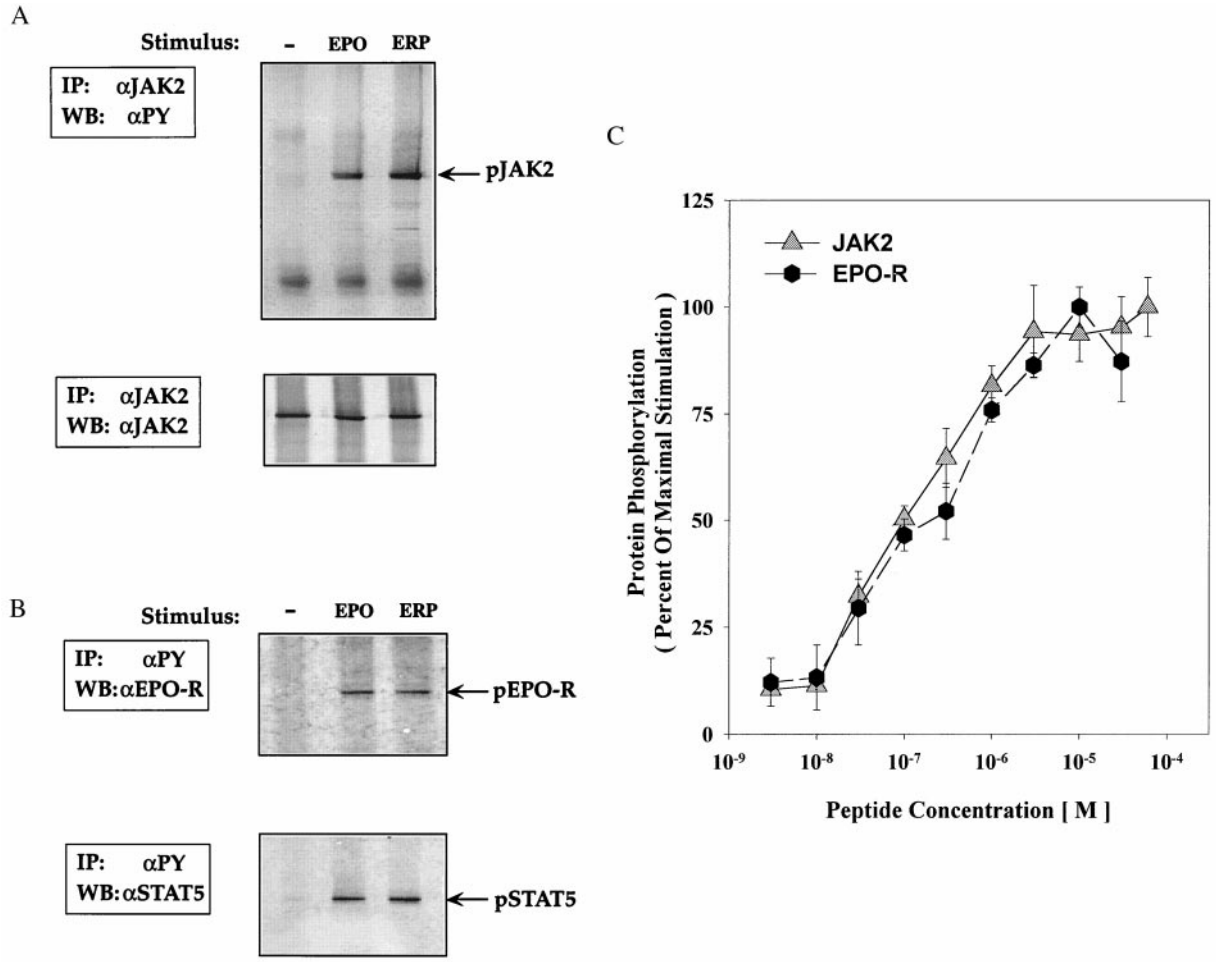

FIG. 2. Activation of EPO-R signaling pathway by peptide derived from the homology domain. (*A*) Phosphorylation of JAK2. TF-1 cells were stimulated in RPMI serum-free medium for 30 min at 37°C with 1 unit/ml EPO or 10 μM ERP. After cell lysis, immunoprecipitation (IP) was done with anti-JAK2 antibody (αJAK2), followed by Western blot analysis (WB) with antiphosphotyrosine antibody, 4G10, (αPY, *Upper*) or anti-JAK2 antibody (αJAK2, *Lower*). Cells were stimulated with hormone or peptide as indicated at top. Panels are digital images of Western blots. Arrow at right indicates position of phosphorylated JAK2 (pJAK2). Lowest band on upper panels represents a protein phosphorylated in the absence of hormone or peptide. (*B*) Phosphorylation of EPO-R and STAT5. Cells were stimulated as for *A*. After lysis and IP with αPY99 antibody, proteins were separated on 8% SDS/PAGE, and WB was performed by using anti-EPO-R antibody (αEPO-R, *Upper*) or anti-STAT5 antibody (αSTAT5, *Lower*). Arrows at right indicate position of phosphorylated EPO-R (pEPO-R) and STAT5 (pSTAT5). Other symbols as in *A*. Cells were unstimulated or incubated with hormone or peptide as indicated. (*C*) Dose-response curve for activation of EPO-R signaling pathway by ERP. TF-1 cells were stimulated for 30 min at 37°C with ERP at concentrations shown. Cell lysis, IP, and quantification of JAK2 phosphorylation as described in *A*. Points are mean ± SEM of densitometry data of three independent experiments, expressed as percent of maximum stimulation in each experiment. EC_{50} of 100nM ERP shown here may be compared with 1.5 units/ml EPO (not shown).

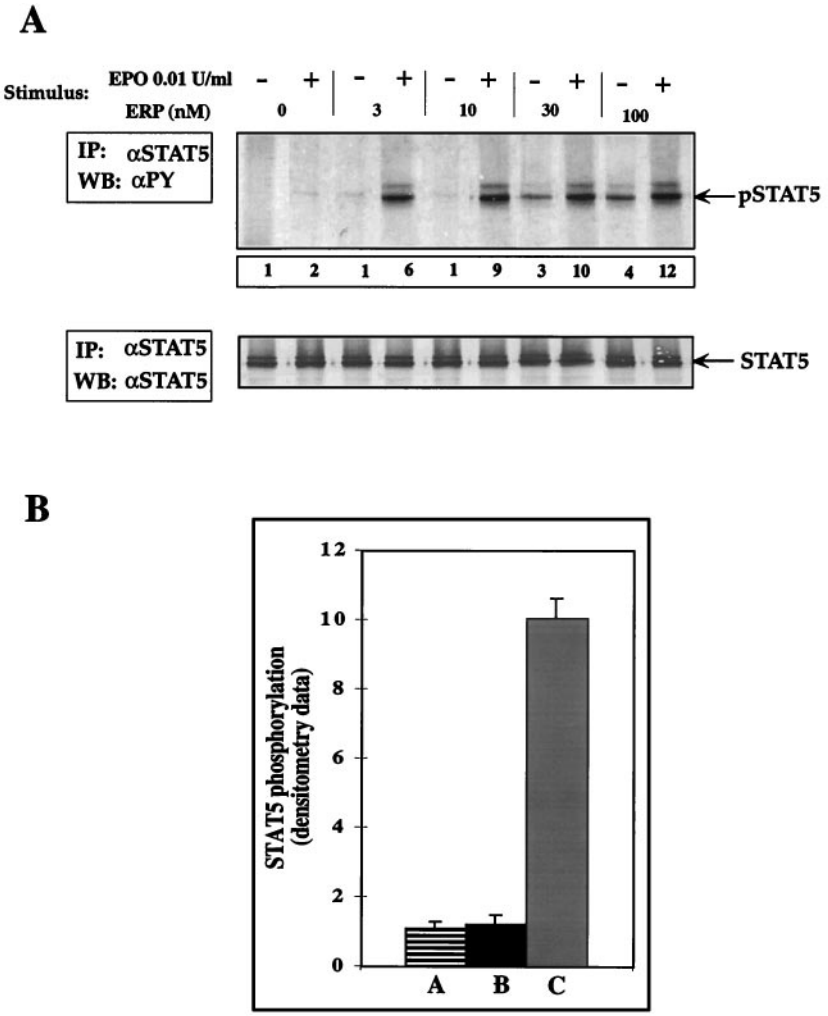

FIG. 3. Synergy between ERP and EPO. (*A*) Cells were stimulated with hormone in the presence and absence of peptide at concentrations indicated. Doses of hormone and peptide were chosen so as to yield weak signals on their own. Incubation conditions, conventions, and symbols were as in Fig. 2. After cell lysis, immunoprecipitation (IP) was done with αSTAT5 antibody, Western blot analysis (WB) with αPY (*Upper*) or αSTAT5 (*Lower*). Densitometry data as multiples of unstimulated readings are presented below upper panel. (*B*) Observed synergistic effects of EPO and ERP. STAT5 phosphorylation at 0.01 units/ml EPO and 10 nM ERP is presented. A = hormone alone, B = peptide alone, C = observed effect of peptide and hormone together. Data are mean ± SEM ($n = 3$). Units on *y*-axis are multiples of baseline data for untreated cells.

Membranes were washed, and color was developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Cappel). The Western blots were scanned, and intensity of the protein bands was quantitated.

EPO-R Competition Binding Assay. CHO/EPO-R cells were maintained in F12 media as described above. Cells were centrifuged and washed with binding buffer (F12/0.2% BSA/0.02% NaN_3), resuspended, and counted by using trypan blue as indicator of viability. Each reaction contained approximately 1×10^6 cells, [^{125}I]EPO (300 pM, specific activity 300 Ci/mmol) and unlabeled EPO (0.3–61 nM). ERP at final concentration 25 μM was present or absent in a final reaction volume of 100 μl. Alternatively, competitive binding of [^{125}I]ERP in the presence or absence of 500 pM EPO was performed. Reaction mixtures were the same except that 50,000 cpm of [^{125}I]ERP (specific activity 10 Ci/mmol), radiolabeled as described (2), was added. Competition was with 0.03–250 μM unlabeled ERP. After overnight incubation in triplicate at 4°C, 80 μl of each reaction was layered onto 200 μl of oil (70% dibutyl phthalate, 30% phthalic acid) and centrifuged (10,000 × *g*, 1 min, room temperature). The centrifuge tubes were immersed in an ethanol bath containing dry ice, the bottoms of the tubes were snipped off, and the cell pellets were transferred to a gamma counter.

Mice. C57BL/6J male mice were obtained from the Jackson Laboratories, marked, distributed randomly into cages, and allowed to acclimatize for 2 weeks. At 8 weeks of age (day 0), PBS or carboplatin (120 mg/kg) was administered i.p. Carboplatin-treated animals were given daily i.p. injections as follows: PBS alone, 1.5 μg/kg EPO or 0.03 mg/kg ERP. On days 0, 4, and 8, small tail-blood samples were taken for hematocrit, and the animals were weighed.

RESULTS

Identification of Biologically Active Peptide. We showed previously (1) that the insulin receptor contained a sequence homologous to the MHC-I peptide D^k-(61–85) and that a synthetic peptide identical to that sequence bound to the receptor in the region from which the peptide was derived. Moreover, binding of the peptide inhibited receptor internalization. Here we applied the same search procedure to a dimerizing receptor, human EPO-R (P19235), with the result shown in Fig. 1. The homology domain is in the extracellular portion of the receptor, only 35 residues distant from the membrane, in a region assumed to participate in hormone-induced dimerization. No comparable matches were found with inverted probe sequence or randomly scrambled probe or

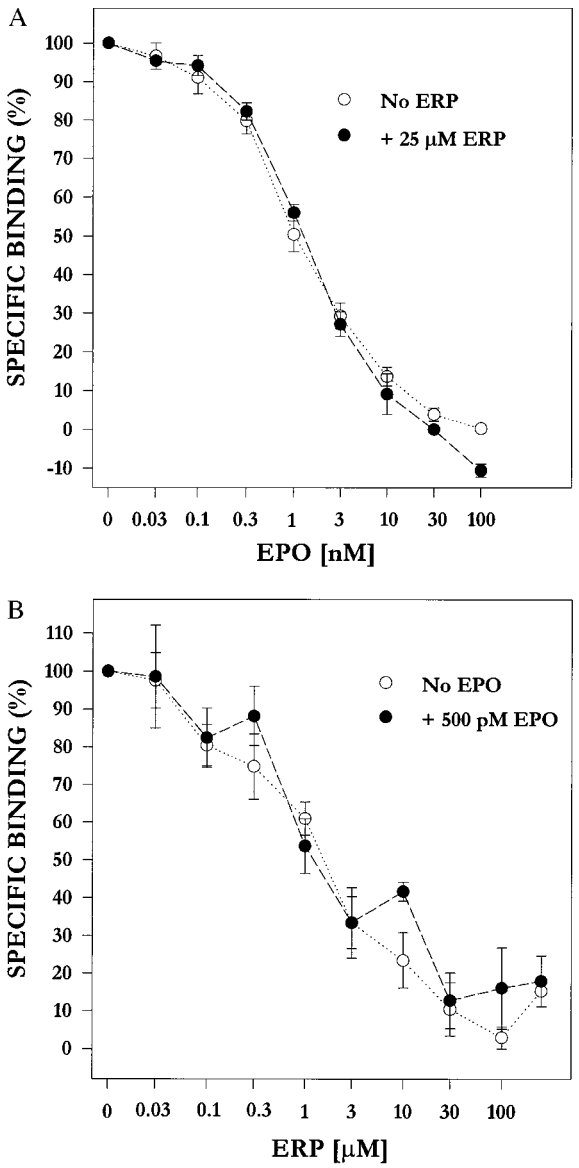

FIG. 4. Binding of EPO and ERP to EPO-R. (*A*) Competitive binding of [^{125}I]EPO in the presence (closed symbols) and absence (open symbols) of 25 μM ERP. (*B*) Competitive binding of [^{125}I]ERP in the presence (closed symbols) and absence (open symbols) of 500 pM EPO. See *Materials and Methods* for details. Data represent percent of maximum specific binding. The cell line shown expresses about 10^5 receptors per cell, as measured by binding of [^{125}I]EPO or [^{125}I]ERP. In contrast, the control cell line (see *Materials and Methods*) bound nearly undetectable amounts of [^{125}I]EPO or [^{125}I]ERP.

target sequence. A peptide (ERP) identical to the homologous sequence shown was synthesized.

***In Vitro* Activity of Peptide.** Fig. 2 demonstrates the activation of the EPO-R signaling pathway by EPO and also by peptide in the absence of hormone. The lanes show unstimulated control, activation by hormone, and activation by peptide alone. Fig. 2*A* demonstrates the phosphorylation of JAK2, and Fig. 2*B* shows the phosphorylation of the receptor itself and also of STAT5.

Fig. 2*C* presents the densitometry data as a log dose-response curve. Here, we show both EPO-R and JAK2 phosphorylation; a similar EC_{50} value (about 100 nM) was found for STAT5 phosphorylation. These curves reach a maximum, and at the highest concentrations that could be used, there was no indication of the typical reduction in efficacy seen at high concentrations of the natural hormone (bell-shaped curve) (5, 6).

Table 1. ERP inhibits internalization of EPO-R

Peptide	Time, min	Surface receptors, (% of total) Mean	(SEM)
None	5	65.3	(3.7)
	10	53.3	(6.2)
	20	55.3	(2.1)
	40	53.8	(0.6)
ERP, 15 μM	5	78.3	(1.4)
	10	78.5	(3.0)
	20	73.0	(2.0)
	40	81.8	(6.1)

Internalization of EPO was measured in TF-1 cells as described (1). Surface receptors were determined by the difference between total specific binding of labeled EPO and residual binding after treatment at acid pH. Data are mean ± SEM of triplicates from three experiments.

Synergy Between Hormone and Peptide. When hormone and peptide were present together, each at a concentration insufficient to initiate detectable signaling, they were strongly active in combination. This dramatic synergy of effect between peptide and hormone is illustrated in Fig. 3*A*, with phosphorylation of STAT5 as criterion of receptor activation. Fig. 3*B* summarizes the degree of synergy. Individually tested at low concentrations, both compounds gave only background level stimulation, but when combined, the effect was at least 10 times greater.

To determine whether the synergy was caused by increased affinity of EPO or ERP for EPO-R, we established a cell line that overexpresses EPO-R (CHO/EPO-R) and a control CHO cell line transfected with vector containing inverted EPO-R gene. Competition binding experiments were carried out with labeled and unlabeled hormone and with labeled and unlabeled peptide. Fig. 4*A* shows that the peptide, even at 25 μM, had no effect on the affinity of EPO. Fig. 4*B* illustrates the reciprocal experiment, showing that EPO had no effect on the affinity of ERP. Binding of radiolabeled EPO or ERP to the control cell line was very low and was not competed by EPO or ERP. Thus, these results indicate that the hormone and peptide bind to different sites on the receptor, with K_d approximately 0.7 nM and 1 μM, respectively. Clearly, the explanation for the synergy must be sought at some point in the activation process distal to the binding sites.

We reported previously for insulin receptor (IR) and IGF-1R that peptides derived from these two receptors and identified by homology to MHC-I D^k- (61–85) region selectively inhibit receptor internalization of IR and IGF-1R, respectively (1). Here, if inhibition of internalization produced a very large increment of cell surface receptors, the synergy between peptide and hormone might possibly be explained. As shown in Table 1, however, although ERP did inhibit internalization, it caused only about a 50% increase in the number of cell surface receptors, far short of accounting for the 10-fold or greater observed degree of synergy.

Fig. 5*A* demonstrates that whereas increasing concentration of EPO beyond that producing maximum effect resulted in decreased signaling (bell-shaped dose-response curve, suggested previously for proliferation assays; ref. 5), this did not occur with ERP. Moreover, as shown in Fig. 5*B*, the peptide prevented the decrease of signaling and produced full receptor activation, even when a high concentration of EPO was present.

***In Vivo* Activity.** Table 2 shows that the peptide was effective *in vivo*, presumably by activating EPO-R. Mice treated with carboplatin suffer a severe decrement in hematocrit. ERP as well as EPO itself protected significantly against the toxic

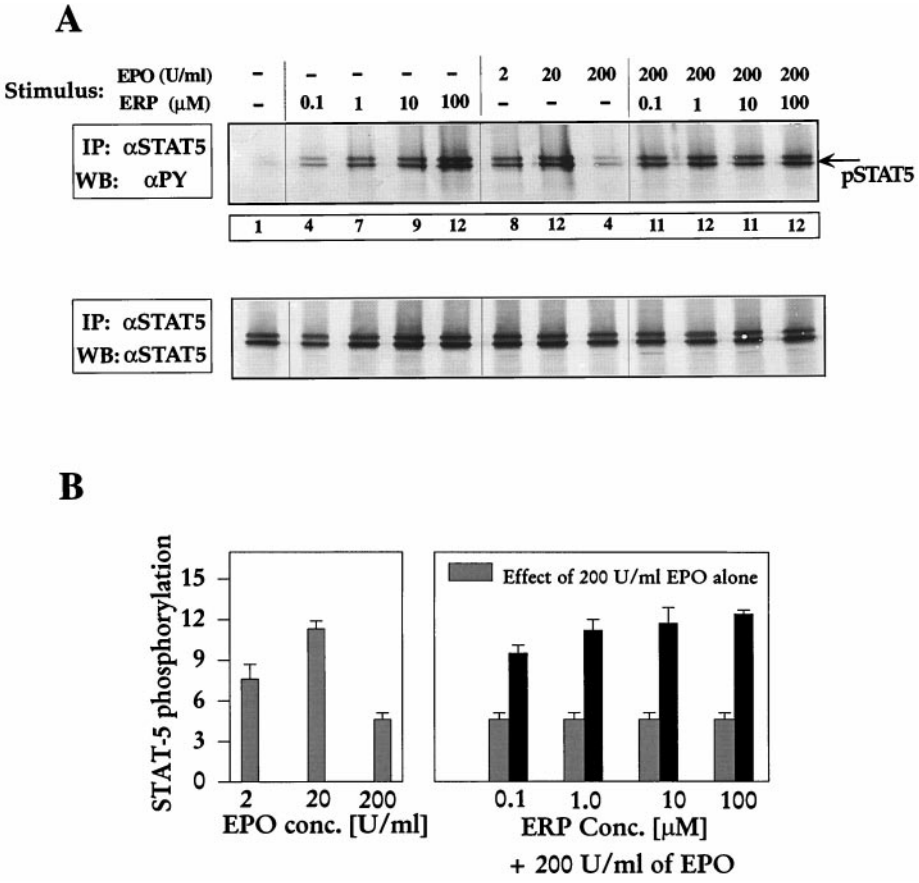

FIG. 5. Effect of ERP on STAT5 phosphorylation at high concentrations of EPO (bell-shaped curve). (*A*) TF-1 cells were stimulated for 30 min at 37°C by incubation with various concentrations of EPO or ERP or both, as indicated. Conventions and symbols are as in previous figures. After cell lysis, immunoprecipitation (IP) was done with αSTAT5 antibody followed by αPY and αSTAT5 Western blot analysis (WB). Densitometry data are shown below upper panel. (*B*) ERP effects on STAT5 phosphorylation at high concentration (200 units/ml) of EPO. (*Left*) EPO bell-shaped dose-response curve with inhibition at 200 units/ml. (*Right*) Comparison between EPO alone at 200 units/ml (gray bars) and in the presence of different ERP concentrations (black bars). Data are mean ± SEM of three independent experiments.

effect of carboplatin on erythropoiesis. The potency of EPO appears about 4-fold higher than ERP.

DISCUSSION

We have shown here and previously (1) that sequences homologous to the MHC-I peptide D^k-(61–85), which are found in the extracellular domain of various receptors, are blueprints for the synthesis of peptides with unexpected properties such as inhibition of receptor endocytosis. The remarkable finding in this paper is that a peptide discovered by such a homology search can activate receptor signal transduction in the complete absence of the natural ligand, and that the peptide interacts outside the hormone binding site. Here we demonstrate the phenomenon for EPO-R and the synthetic peptide ERP, derived from the homology domain. But the phenomenon is by no means limited to EPO; we have found that other dimerizing receptors can be activated by corresponding peptides in the same manner. And homology searches have thus far identified possible receptor activation sites in 33 other receptors that typically form either homodimers or heterodimers (unpublished work).

Table 2. Protective effects of EPO and ERP on mice exposed to carboplatin

Treatment	Carboplatin treated	Hematocrit (% change from basal) Mean	(SEM)
None (PBS control)	No	−6.8	(2.2)
PBS	Yes	−42.5	(7.3)
EPO	Yes	−27.9*	(4.2)
ERP	Yes	−22.3*	(4.1)

Data are hematocrit values on day 8 as percent change from basal. n = 7–12 animals per group. * = $P < 0.05$, compared with carboplatin-treated animals receiving only phosphate buffer injections. See *Materials and Methods* for details. Weight measurements indicated normal growth rate during this experiment.

EPO-R belongs to the family of receptors that are typically activated by dimerization, in which the hormone ligand brings together two monomers, as has been shown in crystal structure studies of hormone-receptor complexes (7, 8). D^k-(61–85) is an example of a sequence with the property of self-association, as we demonstrated in previous work (1, 9). It is not surprising, therefore, that a homologous sequence in a receptor also should self-associate, in this case by binding ERP. Interaction between two EPO-R monomer chains is also, in effect, a self-association, which underlies the phenomenon of dimerization and accompanies activation. In other words, the homology domain of monomer 1 interacts with a similar sequence of monomer 2 when brought into proximity by EPO binding. Here we show interaction and activation with peptide ERP. It is tempting to speculate that ERP may be mimicking monomer 2 in its binding to monomer 1, thus shifting a conformational equilibrium of monomer 1 to the active state without need for dimerization of two complete receptor chains.

Indirect evidence presented here is relevant. Increasing concentration of hormone is known to yield a bell-shaped

dose-response curve (Fig. 5), which generally is interpreted to mean that at high concentration the hormone binds to so many monomers that less dimerization (hence less activation) can occur. ERP, in the absence of hormone, causes maximum activation without any decrease in effect at the highest practical concentrations; and most significant, it overcomes the decrease produced by high hormone concentrations. Thus, as suggested above, although the complex of EPO with a single EPO-R chain is inactive (5), the complex of ERP with an EPO-R monomer may be active.

An alternative interpretation, which cannot be excluded, might involve the possibility that ERP facilitates, directly or indirectly, some unspecified process of molecular interaction of EPO-R with some adapter protein, resulting in activation of the EPO-R signaling pathway. Another possibility is that ERP binds to EPO-R without activating it, but also binds to and activates some other receptor, resulting in JAK2, STAT5, and EPO-R phosphorylation, as observed.

The peptide described in this paper differs significantly from previously described peptides that mimic EPO (10, 11). These mimetic peptides bind to a hormone binding site, whereas ERP binds to receptor at a region close to the membrane and far from the hormone binding site, a region that is thought to participate in hormone-induced dimerization. Recently, a nonpeptide small molecule that is capable of activating the signal transduction pathway of murine granulocyte colony-stimulating factor receptor has been reported. This molecule, although binding to a receptor domain other than the hormone binding site, was suggested to act by dimerizing receptor chains (12).

Synergy between peptide and hormone is of obvious practical importance and potential therapeutic benefit. For example, the effect of a suboptimal level of a natural hormone could be amplified, as would be desirable for EPO in kidney disease.

The degree of synergy (Fig. 3*B*) cannot be accounted for by recruiting of more receptors to the cell surface; the maximum possible effect of inhibiting internalization would be to double the signal transduction, inasmuch as more than half of EPO-R is already on the surface at the initial steady state.

The mechanism of synergy is unclear, except that it does not depend on an increased affinity of the hormone or peptide for their respective binding sites; in other words, peptide shifts the hormone EC_{50} to the left without altering K_d and without increasing the maximum effect, as measured by phosphorylation of signaling molecules JAK2, STAT5, and EPO-R itself. Possible explanation of this costimulatory amplification include different conformations of ERP- and EPO-occupied active receptors, resulting in a different pattern of kinase phosphorylation and/or recruitment (as by raft migration) (13, 14).

Results *in vivo* in mice were consistent with those expected from activation of EPO-R. In the absence of added hormone, in mice treated with carboplatin, ERP alone (as well as EPO) protected hematocrit levels from the toxic effects of carboplatin.

In conclusion, we have shown that a homology search based on a MHC-I sequence (1) identifies an extracellular domain in the erythropoietin receptor with the following properties: (*a*) A synthetic peptide identical to that domain activates receptor signal transduction. (*b*) Activation by peptide does not require the presence of hormone. (*c*) Peptide binds to receptor elsewhere than at the hormone binding site. (*d*) Peptide and hormone together produce strongly synergistic activation. (*e*) In mice, peptide acts like the hormone in protecting against carboplatin toxicity.

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