Amino-terminal dimerization of an erythropoietin mimetic peptide results in increased erythropoietic activity

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Background: Erythropoietin (EPO), the hormone involved in red blood cell production, activates its receptor by binding to the receptor's extracellular domain and presumably dimerizing two receptor monomers to initiate signal transduction. EPO-mimetic peptides, such as EMP1, also bind and activate the receptor by dimerization. These mimetic peptides are not as potent as EPO, however. The crystal structure of the EPO receptor (EBP) bound to EMP1 reveals the formation of a complex consisting of two peptides bound to two receptors, so we sought to improve the biological activity of EPO-mimetic peptides by constructing covalent dimers of EMP1 and other peptide mimetics linked by polyethylene glycol (PEG).

Results: The potency of the PEG-dimerized EPO peptide mimetics both *in vitro* and *in vivo* was improved up to 1,000-fold compared to the corresponding peptide monomers. The dimers were constructed using peptide monomers which have only one reactive amine per molecule, allowing us to conclude that the increase in potency can be attributed to a structure in which two peptides are linked through their respective amino termini to the difunctional PEG molecule. In addition, an inactive peptide was converted into a weak agonist by PEG-induced dimerization.

Conclusions: The potency of previously isolated peptides that are modest agonists of the EPO receptor was dramatically increased by PEG-induced dimerization. The EPO receptor is thought to be dimerized during activation, so our results are consistent with the proposed 2:2 receptor : peptide stoichiometry. The conversion of an inactive peptide into an agonist further supports the idea that dimerization can mediate receptor activation.

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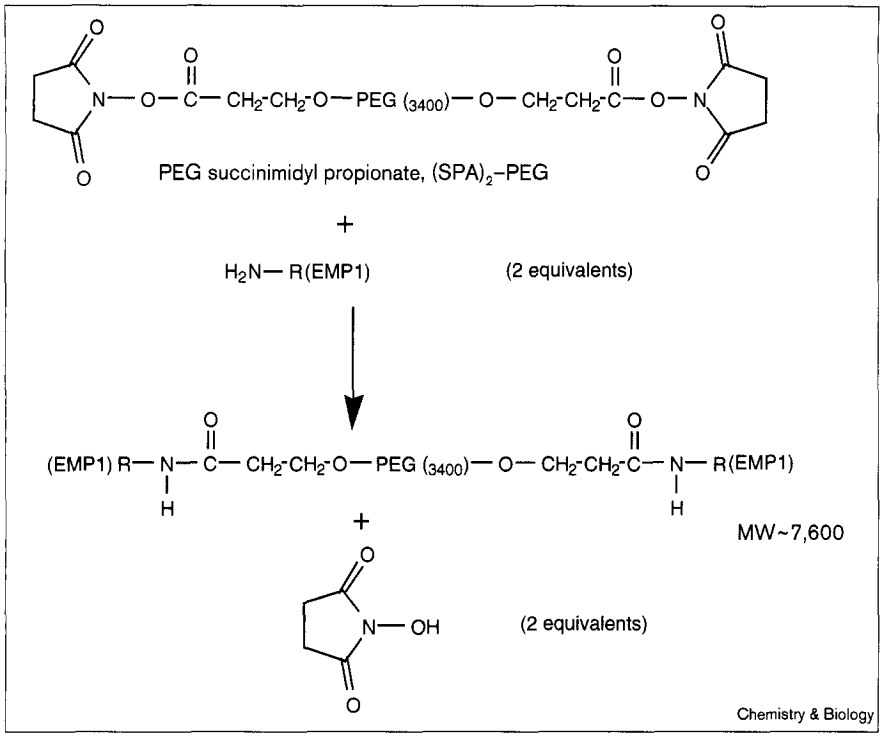
Introduction

The regulation of red blood cell production is under the control of the glycoprotein hormone erythropoietin (EPO) [1,2]. EPO is made in the mammalian kidney in response to hypoxia (low circulating oxygen levels), and released into the circulation to stimulate the maturation and differentiation of erythroid red blood cell precursors. Certain pathological conditions result in low or inadequate production of EPO and recombinant human EPO has been developed as a therapeutic to treat the resultant anemia [2]. The proliferative properties of EPO are mediated through its interaction with, and activation of, a type-I cytokine receptor, a transmembrane protein, found on responsive cells, that is thought to be activated via a dimerization mechanism [3–5].

Recently, a family of EPO-receptor agonist peptides have been discovered [6] and the X-ray crystal structure of an EPO-mimetic peptide bound to the extracellular ligand binding domain (erythropoietin binding protein; EBP) of the EPO receptor was reported [7]. The X-ray structure revealed that two mimetic peptide monomers mediate the dimerization of the EPO receptor in a 2:2 peptide : receptor complex. Solution-phase experiments have demonstrated that an EBP dimer can be effectively stabilized for study using a chemical cross-linking reagent [7]. Other experiments have shown that the EPO receptor can be activated by antibodies that dimerize the receptor [8,9] or by mutations that allow two EPO receptors to become covalently dimerized by the formation of novel disulfide bonds [10–12]. Evidence suggests that EPO itself can promote dimerization of its EBP domain in solution [13]. Further, evidence shows that other type-I cytokine receptors, such as the growth hormone receptor, exist as dimers with their cognate ligands, suggesting that receptor dimerization plays a critical role in activation of this receptor class [14]. The EPO receptor agonists identified by Wrighton *et al.* [6] activate the EPO receptor but display

Figure 1



The general route taken to generate the PEG-coupled peptide dimers used in these studies.

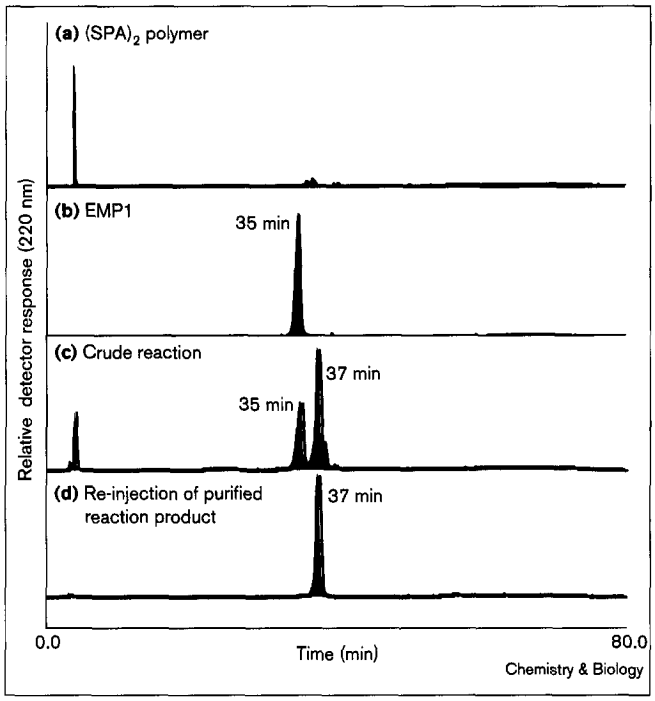
potency significantly less than EPO. We postulated that this was due to the thermodynamically unfavorable four-membered peptide–receptor complex that needs to form. Here, we demonstrate that the biological activity of an EPO mimetic peptide can be significantly improved by covalent dimerization. The overall entropic disadvantage of a 2:2 receptor:ligand interaction is reduced by converting it into a 2:1 receptor:ligand interaction. Furthermore, we show that an inactive mimetic peptide can be converted into a weak agonist by covalent peptide dimerization with a polyethylene glycol (PEG) linker.

Results and discussion

The mechanism for EPO receptor activation appears to be related to receptor dimerization, and the EPO mimetic peptides appear to act in this way also [7]. To probe this biochemical mechanism further, we sought to utilize simple dimers of synthetic peptides as probes of the receptor activation mechanism. Our approach employed an amine-reactive difunctional PEG molecule, succinimidyl propionate $(SPA)_2$–PEG, to form a linear polymer molecule with EPO mimetic peptides on each end. The difunctional activated PEG used in these experiments has an approximate molecular weight (MW) of 3,400 and has amine-reactive succinimidyl groups at both ends. This reactivity was used to couple two equivalents of EMP1 (MW = 2,092; an EPO receptor agonist peptide) to the polymer with the concomitant liberation of two succinimidyl moieties resulting in a PEG–$(EMP1)_2$ product (MW = ~7,600; Figure 1). EMP1 contains two potentially reactive amines, one at the amino terminus of the peptide and one in the sidechain of the single lysine within the peptide sequence, GGTYSCHFG-PLTWVCKPQGG (single-letter amino acid code), so that a number of different connectivities between the two molecules are possible. A monofunctional PEG5,000 with a single reactive succinimidyl group was used as a control; this species could potentially couple to either or both of the peptide amines. The total estimated mass of the PEG dimer and a monoconjugated PEG peptide would be similar using these strategies.

The difunctional reaction was carried out with EMP1 in excess, and the major reaction product was monitored (Figure 2) and purified by reverse-phase high performance liquid chromatography (HPLC). Under these analytical conditions the $(SPA)_2$ polymer and Tris-inactivated polymer did not appear to bind the column, whereas a major reaction product with a retention time of 37 min was identified (Figure 2c). EMP1 demonstrated a retention time of 35 min, and the excess peptide utilized in the reaction was clearly distinguishable from the nascent reaction product. Analysis of the recovered material after preparative chromatography using analytical reverse-phase HPLC showed a single species with a retention time similar to the major reaction product before purification (Figure 2d for EMP1–PEG dimer; representative data shown for EMP1 only). The recovered product was lyophilized to dryness, resuspended and examined for the ability to compete with

Figure 2



Reverse-phase HPLC analysis of $(SPA)_2$–PEG reaction with EMP1. Experimental conditions are described in the text. **(a)** $(SPA)_2$ polymer does not bind to the column under the analytical conditions employed. **(b)** EMP1 demonstrated a retention time of ~35 min. **(c)** The crude reaction mix after Tris–HCl quench shows a main product peak at 37 min and unreacted EMP1 at 35 min. **(d)** Reinjection of the main product peak after preparative HPLC purification and lyophilization demonstrates a single species at ~37 min.

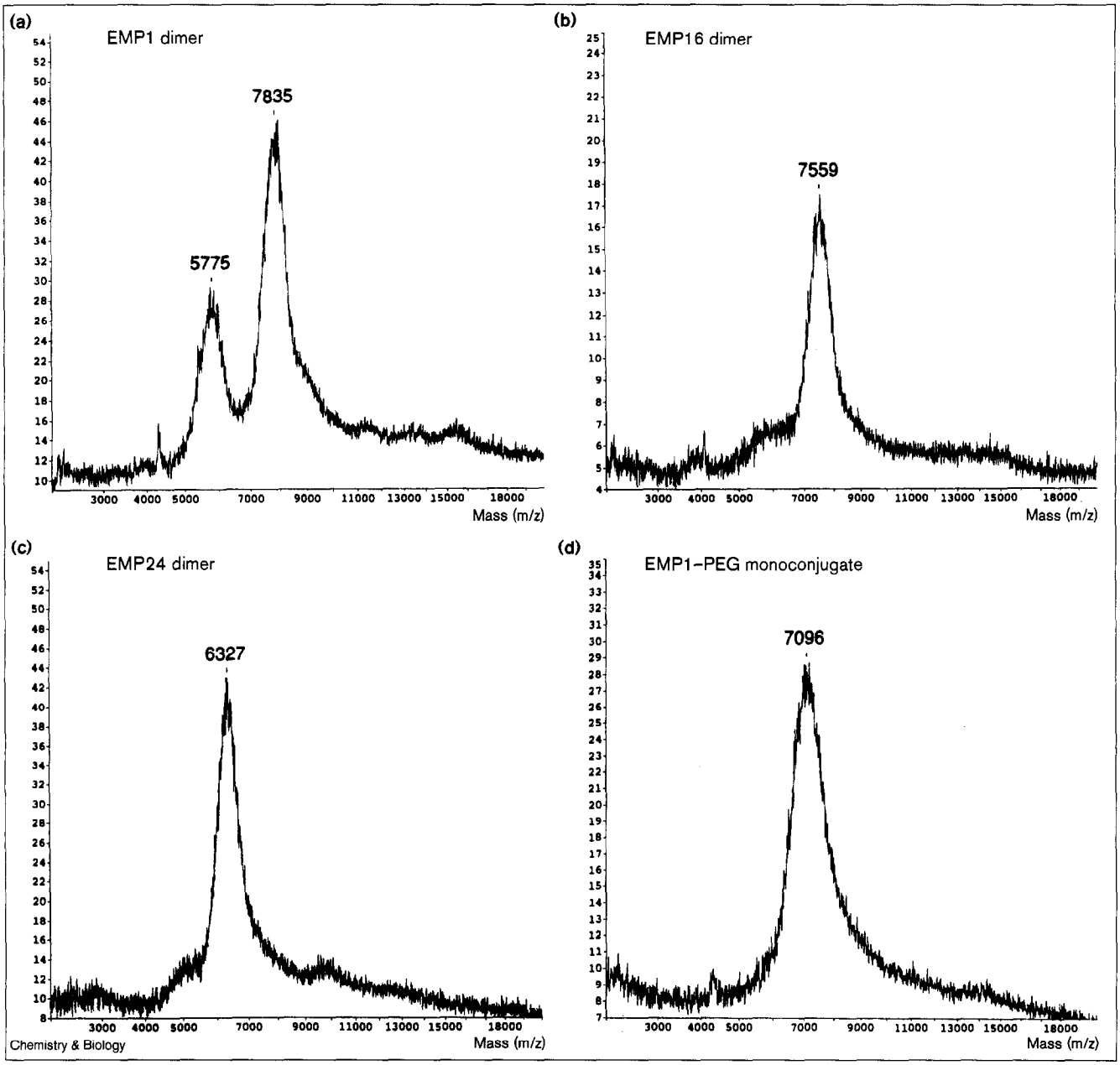
[^{125}I]-labeled EPO for receptor binding, laser desorption mass spectral analysis, and for the ability to support the proliferation of EPO-responsive cells. Similar experimental protocols were used for all PEG–dimer peptides and PEG–monomer peptides reported in this study.

Matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) mass spectral analysis supports the presence of the expected dimeric product of EMP1 (Figure 3a), as indicated by a predominant species with a centroid mass of 7,835 (expected charge to mass ratio [m/z] of about 7,600; see Table 1). Use of this analytical tool may, however, have resulted in the fragmentation of the molecule, because a molecular species corresponding to a peptide–PEG monomer (5,775 m/z) was also observed. The complexity of this spectra was unexpected, and further investigation of the main reaction product by electrospray mass spectrometry resulted in the observation that the purified product could not be ionized using this technique, suggesting that a single non-ionizable species was present in the sample. An alternative explanation is that some amount of PEG–peptide product is present along with the dimer. In either case, the dimer peptide product was easily identifiable. Other peptides in the series demonstrate much simpler spectra: a single species more stable to ionization is indicated in the EMP16–PEG dimer spectrum (Figure 3b; expected m/z of about 7,550, observed 7,559) and in the EMP24–PEG dimer spectrum (Figure 3c; expected m/z of about 6,300, observed 6,327). The dimer reaction products were recovered from conjugation mixtures where the peptide was in excess so as to limit monoconjugation and enhance dimer formation. Monoconjugation and diconjugation of EMP1 were clearly demonstrated through the use of a monovalent PEG 5,000 (see Figure 3d) to afford a control sample of matched molecular weight (expected m/z of about 7,100, observed 7,096) and a diconjugated peptide as well (Table 1). Historically the analysis of PEG-containing biopolymers has proven problematic [15] and there appears to be an observed difference in the behavior of the PEG–peptide conjugate molecules even though a similar chemical linkage is employed in the dimerization of these peptides. Mass spectral and yield data for all peptides are summarized in Table 1.

Competitive binding analysis of EMP1 dimers on EBP beads revealed a 50% inhibitory concentration (IC_{50}) of 20 μM for the purified dimer, a value four-fold greater than that for EMP1 monomers in the same assay (Table 2 and Figure 4) indicating some loss of binding avidity upon PEG conjugation. Polymer alone, when inactivated by treatment with Tris–HCl, demonstrated a detectable competition binding signal but this signal was modest (<10%) at the IC_{50} of the PEG–EMP1 dimer. In general, when analyzed by this method, all dimer peptides suffered a modest loss in competitive binding ability, as shown in Table 2. This is not the case for intact EPO receptors on cells, however: the respective EMP1 peptide dimers demonstrate slightly improved binding as compared to the monomer peptide (0.02 μM and 0.07 μM, respectively). The difference in these values is likely to be due to differences in assay formats: the immobilized EBP system would not be expected to allow receptor dimerization, whereas the cell-associated receptors should dimerize freely upon peptide binding.

EPO-responsive cell proliferation studies were performed on cells expressing the murine, truncated human or native human EPO receptors, and the PEG–EMP1 dimer demonstrated 50% effective dose (ED_{50}; the amount of peptide required to stimulate [^{3}H]thymidine incorporation by 50% of the experimental maximum) values of 0.01, 0.0015 and 0.001 μM, respectively (Table 2 and Figure 5). In all three cell lines, the parent peptide, EMP1, demonstrated an ED_{50} of 0.1 μM, indicating a 10-fold increase in potency in the murine receptor line and almost 60- or 100-fold in the cells expressing human receptors. These ED_{50} data clearly demonstrate that the covalent dimers are significantly more active than the parent monomers. Polymer

Figure 3



MALDI–TOF mass spectral analysis. Conditions were as described in the text. **(a)** Spectrum of EMP1 dimer. **(b)** Spectrum of the EMP16 dimer product. **(c)** Spectrum of EMP24 dimer product. **(d)** Spectrum of the EMP1–PEG monoconjugate. All main centroid masses are consistent with the expected dimeric or monomeric peptide–PEG species.

alone, when inactivated by treatment with Tris–HCl, demonstrated no activity in the cell proliferation assay. These values are determined with the concentration reported in terms of micromoles of molecules so that each micromole of peptide dimer contains two equivalents of peptide monomer. Given that these values are at least 10-fold greater than the monomer peptide values, a simple increase in effective peptide concentration cannot account for the difference in activity.

A second EPO mimetic peptide, EMP16, with the sequence GGTYSCHFGPLTWVCKPQ [16], was subjected to a similar PEG-dimerization protocol to that described above for EMP1. This peptide is more active than EMP1 in the cell proliferation assay *in vitro* as well as in the exhypoxic mouse bioassay *in vivo*. The dimer product of PEG–EMP16 was more active on the murine and truncated human cell lines than the unconjugated parent compound, but the gain in potency was much

Table 1

Yield of peptide conjugation reaction and apparent molecular mass of product.

Peptide	Sequence	Mass	Conjugation reagent	Main product mass (centroid m/z)	Yield (% of theoretical)
EMP1	GGTYSCHFGPLTWVCKPQGG	2092	$(SPA)_2$–PEG (MW ~3400)	7835	69
			m-SPA–PEG (MW ~5000)	7096 (peak 1)	–
				12036 (peak 2)	–
EMP16	GGTYSCHFGPLTWVCKPQ	1978	$(SPA)_2$–PEG	7559	54
EMP34	Ac-GGTYSCHFGPLTWVCKPQGG	2133	$(SPA)_2$–PEG	7862	30
EMP35	GGLYACHMGPMTWVCQPLRG	2177	$(SPA)_2$–PEG	7872	37
EMP24	SCHFGPLTWVCK	1375	$(SPA)_2$–PEG	6327	45

See Figure 3 for further details.

more modest (Table 2). Unexpectedly, the EMP16–PEG dimer demonstrated reduced activity for the cell line bearing the native human receptor, about 1.7-fold less than the parent peptide. The reason for this is unclear but appears to reside in the differing ability of the peptide–dimer to mediate receptor activation in the cytoplasmic space upon peptide dimerization, given that essentially no change in the ability to compete for [^{125}I]-labeled EPO binding was observed (Table 2). This is not unreasonable considering the difference in the control regions of these two receptors: the truncated receptor has been manipulated to delete the 40 carboxy-terminal amino acid residues, which have been suggested to contain negative regulatory control elements. In any case, the experimental evidence derived from the murine and truncated human cell lines suggests that dimerization of the peptides with PEG can result in improved potency but that it does not work uniformly on all peptide sequences.

Determination of the exact connectivity of the two peptides with PEG within the EMP1–PEG dimer and the EMP16–PEG dimer was problematic, because each peptide molecule had two potentially reactive amines (a free α-amino terminus and an ε-amine on a lysine sidechain). To explore further the effect of connectivity in these molecules, we PEG-dimerized peptide molecules that contain only an internal lysine group by using an EMP1 analog acetylated at the amino terminus (EMP34) and a sequence analog (EMP35) which has only one reactive amino-terminal amine. *In vitro* proliferation studies with these compounds suggest that potential dimerization through the free amino terminus has the most profound effect on bioactivity giving rise to a species up to 1000-fold more active than the monomeric parent peptide (EMP35–PEG dimer; Table 2). Conjugation through the lysine sidechain had a limited or a negative effect on activity (EMP34–PEG dimer) as did mono- or di-PEG conjugation (Table 3). These data support the conclusion that the

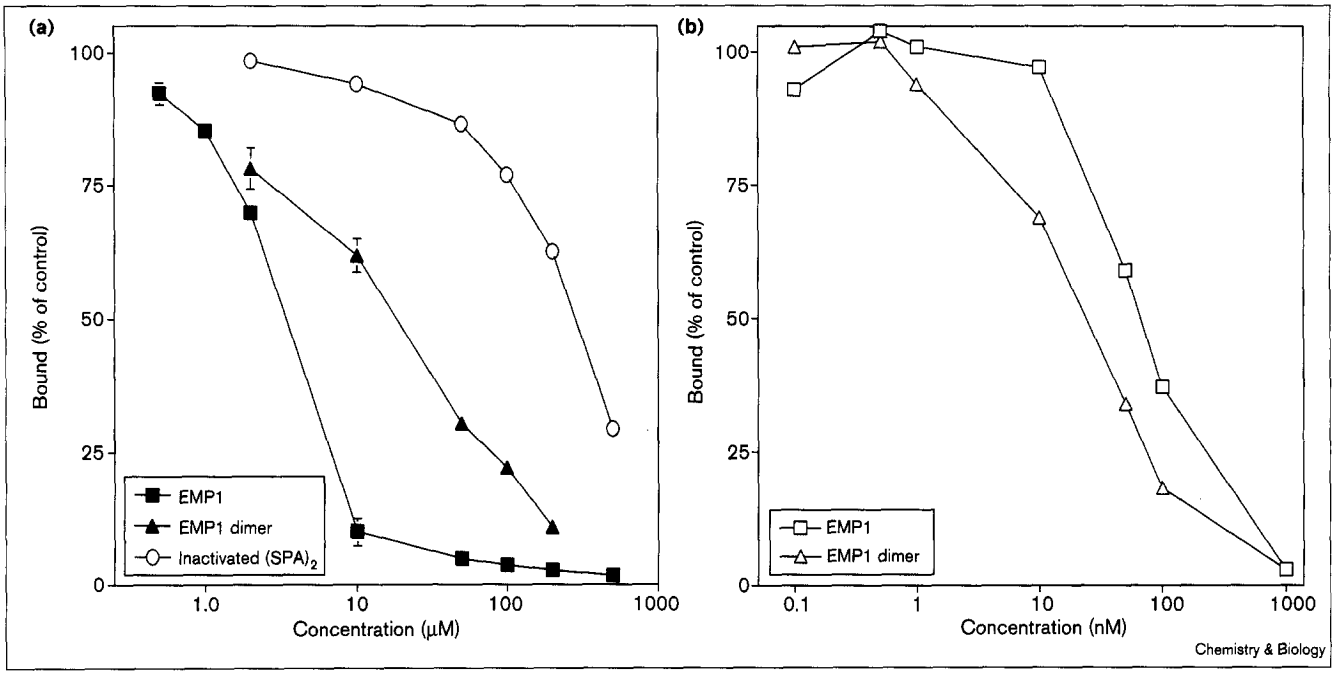
Table 2

EPO receptor binding and cell proliferation studies.

	EPO competitive binding IC_{50} (μM)		EPO ED_{50} (μM)		
Peptide	EBP beads	EPO receptor	Murine receptor	Truncated human receptor	Human receptor
EMP1	5	0.07	0.1	0.1	0.1
EMP1–PEG dimer	20	0.02	0.01 (10X)	0.0015 (67X)	0.001 (100X)
EMP16	8	0.07	0.08	0.02	0.0115
EMP16–PEG dimer	15	0.02	0.01 (8X)	0.002 (10X)	0.02 (–1.7X)
EMP34 (*N*-acetyl)	20	ND	0.03	0.06	0.01
EMP34–PEG dimer	60	ND	0.2 (–7X)	0.05	0.2 (–20X)
EMP35 (terminal NH_2)	3	0.2	0.1	0.08	0.1
EMP35–PEG dimer	15	0.0025	0.006 (16X)	0.001 (80X)	0.0001 (1000X)
Tris inactivated polymer	300	ND	IA	IA	IA

ND, not determined. IA, inactive. See Figure 4 and text for further details.

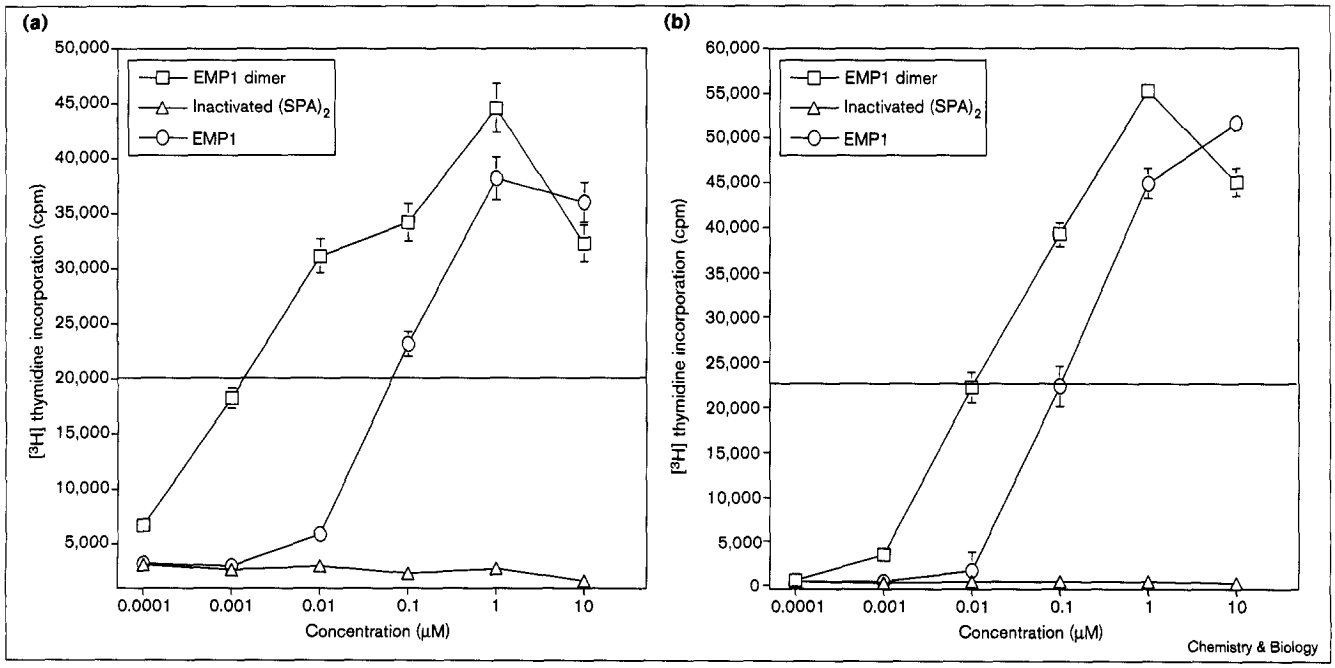
Figure 4



Graphic representation of the analysis of the competitive binding of the EMP1 dimer and EMP1 with [^{125}I]-labeled EPO to **(a)** EBP beads or **(b)** TF-1 cells. These data are summarized numerically in Table 2; see text for further details.

creation of a head-to-head dimer (both peptides attached through the amino terminus) using a PEG linker greatly enhances the potency of EPO-mimetic peptides, and approaches a level almost 1000-fold greater than the free

Figure 5



Cell proliferation studies with EMP1 and the EMP1 dimer on cells bearing truncated **(a)** human or **(b)** murine EPO receptors. The increase in activity at the same concentration of dimeric or monomeric peptides is striking. These data are numerically summarized in Table 2; further details are in the text.

Table 3

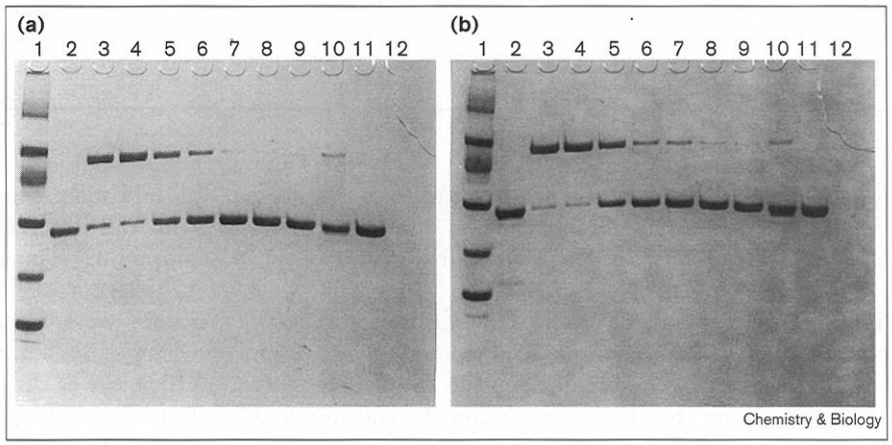
Analysis of nondimer PEG conjugates of EMP1.

Peptide	EPO Competitive Binding IC_{50} (μM)	EP0-ED_{50} (μM)		
		Murine receptor	Truncated human receptor	Human receptor
m-SPA-PEG/EMP1 7096 mass	60	2.0	0.4	0.5
m-SPA-PEG/EMP1, 12036 mass	40	1	0.1	0.6

parent peptide (EMP35 versus EMP35 dimer; Table 2). Furthermore, this effect was not observed upon simple covalent attachment of linear PEG to EMP1 (Table 3), indicating that dimerization is the critical determinant for this increased activity. The more active component in the EMP1–PEG dimer is likely to be the amino-terminal dimer, because conjugation of the amino-terminal acetylated EMP34 occurs at a greatly reduced rate and requires a much longer conjugation time than does EMP1 (see Materials and methods section). The amino- and carboxy-terminal glycine residues of EMP1 were not visualized in the X-ray crystal structure, so the exact role played by the terminal residues is unclear [7,16]. It should be noted that the individual peptide sequence and the placement of the terminal glycines appear to be essential determinants of the level of increased activity or whether any increase is observed at all [16].

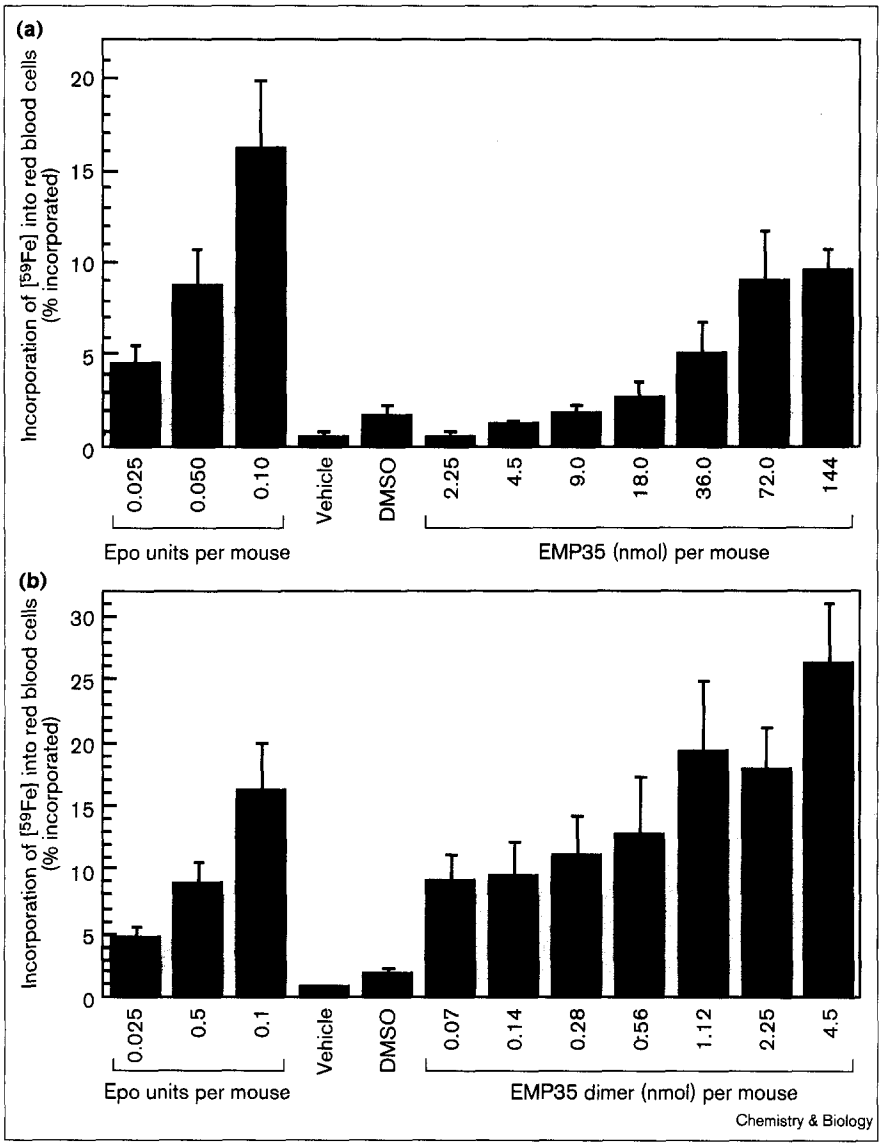
Obvious from the data presented above is the conclusion that the attachment of the PEG polymer to the amino terminus of the two peptide monomers does not result in any steric hinderance which would prevent the interaction of the peptide dimer with the two receptors. Examination of the crystal structure [7] suggests that the peptide termini point out into space above the two receptor subunits, so that attachment of the polymer would not be expected to compromise the ability of the peptide to interact with the receptor monomers. Indeed, covalent EMP1 dimers linked via the carboxyl terminus of EMP1 also appear to have improved receptor activation potential [17]. To confirm that the peptide dimers retained the ability to dimerize EBP in solution, however, a bifunctional sulphydryl reactive cross-linker (DPDPB) was used to capture and stabilize a mimetic-dependent EBP dimeric structure [7]. As shown in Figure 6, a dimeric EBP product is formed upon co-incubation of the peptide, EMP1 (or EMP1 dimer), DPDPB and EBP. The receptor dimer product is identical in the two different peptide preparations because a covalent bond joins two EBP monomers and the peptides are apparently released during electrophoretic separation in the presence of sodium dodecyl sulfate (SDS). These data support the idea that EMP1 and the EMP1 dimer mediate

Figure 6



SDS–PAGE analysis of DPDPB cross-linking of EBP in the presence of EMP1 or the EMP1 dimer. Peptides and EBP (22 μM) were incubated in the presence of the heterobifunctional sulfhydryl-reactive cross-linking reagent DPDPB (1.1 mM) in matched reagent mixtures which differ only by the concentration of **(a)** EMP1 or **(b)** the EMP1 dimer. Lane 1 contains molecular weight markers of 14.4, 20.1, 30, 43, 63 and 94 kDa; lane 2: EBP (5 μg); lane 3: EBP, DPDPB, EMP1 (40 μM); lane 4: EBP, DPDPB, EMP1 (20 μM); lane 5: EBP, DPDPB, EMP1 (10 μM); lane 6: EBP, DPDPB, EMP1 (5 μM); lane 7: EBP, DPDPB, EMP1 (1 μM); lane 8: EBP, DPDPB, EMP1 (0.5 μM); lane 9: EBP, DPDPB, EMP1 (0.25 μM); lane 10: EBP, no DPDPB, EMP1 (40 μM); lane 11: EBP, DPDPB, no EMP1; Lane 12: no EBP, DPDPB, EMP1 (40 μM). (b) The same lanes but with the EMP1 dimer in place of EMP1. Both EMP1 and the EMP1 dimer promote the concentration-dependent formation of receptor dimer which is captured by DPDPB. The EMP1 dimer appears to produce slightly more dimer product at each concentration. In the absence of cross-linker (lane 10) both EMP1 and the dimer promote the formation of a small amount of receptor dimer that can be reversed with reducing agents (data not shown) presumably acting through the lone free sulphydryl on EBP.

Figure 7



Exhypoxic mouse bioassay. **(a)** Stimulation of [^{59}Fe] incorporation into nascent red blood cells by EPO and EMP35. **(b)** Stimulation of [^{59}Fe] incorporation into nascent red blood cells by EPO and the EMP35 dimer.

formation of a soluble receptor dimer, and from this it can be concluded that EBP dimerization is a property of the EPO mimetics, EMP1 and the EMP1 dimer.

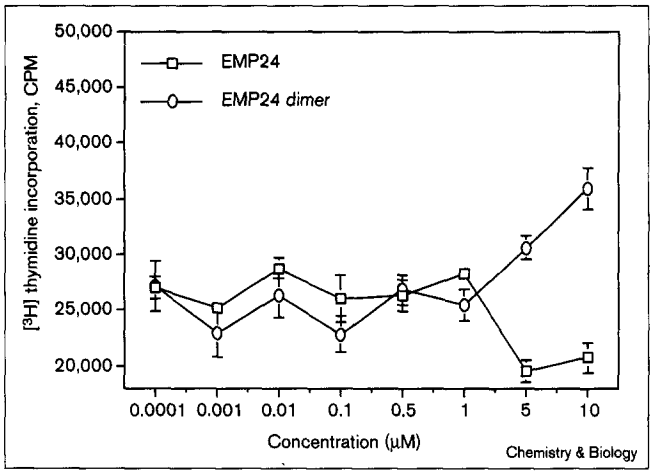
To investigate the ability of EMP–PEG dimers to retain bioactivity *in vivo*, and to examine any potential increase in bioactivity, they were studied in the exhypoxic polycythemic mouse bioassay (see Materials and methods section; Figure 7 and Table 4). This assay is frequently used to estimate the ability of an exogenously administered compound to induce new red blood cell synthesis or to function as EPO or an EPO mimetic. The numbers reported here were generated from sets of 10 animals for each experimental dose, and the data suggest that, on a mole-equivalent basis, the EMP1–PEG dimer is about 10-fold more active than EMP. This value is consistent with the differential effect of the dimer peptide on the murine as compared to truncated or full length human EPO receptor cell lines *in vitro*, in which increased potency values were seen, of 10- and 60–100-fold, respectively. Clearly, the dimers do not activate the murine receptors as efficiently as they do the human receptors and this is confirmed with the results *in vivo*. The EMP16 dimer does not demonstrate significantly increased potency over the monomer peptide *in vitro*, and examination *in vivo* confirmed that the dimer does not have increased potency. In contrast, the EMP35 dimer demonstrated an *in vivo* improvement of 257-fold over the monomer parent (equivalency values of 18 and 0.07 for the monomer and dimer respectively; Table 4).

Table 4

Approximate equivalency of EPO and mimetic peptides *in vivo*.

Peptide	Amount of peptide equivalent to 0.025 units of EPO (nmol)
EMP1	3.8
EMP1 dimer	0.28
EMP16	0.5
EMP16 dimer	0.45
EMP35	18
EMP35 dimer	0.07
EPO	0.0000058

Figure 8



Effect of PEG dimerization on EMP24 activity in EPO-responsive cell-proliferation studies in FDC-P1-derived cell lines containing a human EPO receptor. The peptide alone does not induce proliferation of the EPO-responsive cells, but after PEG dimerization a weak but significant agonist effect is seen. Approximately twice as many counts per minute (cpm) were incorporated by cells incubated with 10^{-5} M peptide dimer compared to cells incubated with 10^{-5} M peptide monomer. The replicate error bars represent the standard deviation of four assay points per concentration of peptide or peptide dimer.

Given that the 257-fold improvement in equivalence for the EMP35 dimer was significantly greater than the 16-fold increase seen in the murine receptor proliferation studies, it is reasonable to suggest that some other factor is responsible for this level of potency. Given that PEG conjugation of polypeptides often translates into increased serum half-life and reduced immunogenicity [15], PEG modification of the EMP35 dimer may have significantly increased its potency *in vivo* as a result of this combination of effects along with improved proliferative properties. From these data it can be reasonably speculated that the full increase in potency on human EPO receptor-bearing cells might be exhibited *in vivo* in nonhuman primates or in humans; confirmation of this conjecture awaits study in these species. Even with the improved potency of the PEG dimers, however, it remains clear that much effort will be required to achieve the level of potency of the EPO molecule itself, which is many times that of the EMP35 dimer. This may be due to the symmetrical fashion in which the two peptides interact with the EPO receptor, whereas the interaction of EPO itself with the receptor would be expected to be asymmetric [7]; this could lead to differences in the receptor signaling assembly which would preclude full activation with the symmetrical mimetic peptide even when it is dimerized.

Furthermore, we have shown that EMP 24, an inactive truncation analog of EMP1 (SCHFGPLTWVCK; which lacks the tyrosine critical for receptor dimerization) [16] can be converted to a weak agonist for the human EPO receptor cell line by PEG dimerization (Figure 8). A 10^{-5} M concentration of the parent peptide has no activity above background whereas the dimeric peptide at the same concentration results in a level of proliferation that incorporates twice as many counts per minute (cpm) of [^{3}H]thymidine over background levels of DNA synthesis. This inactive to active conversion appears to be limited to the human EPO receptor cell line, because the EMP24 dimer did not act as an agonist on the murine EPO receptor cell line (data not shown). It has previously been speculated that such an antagonist to agonist conversion may be possible for ligands that bind, but do not activate, receptors that use dimerization activation mechanisms [18,19]. Although we have provided minimal evidence for such a conversion with EMP24, several other inactive EMP1 derivatives [16], including EMP6 (Tyr4 → Ala) and EMP7 (Tyr4 → Thr), were not converted to agonists using this method (data not shown). It is possible in these cases that PEG-dimerization resulted in a structure that did not allow productive receptor dimerization. This observation makes general application of this technique problematic and suggests that other unknown criteria must be satisfied in order to change an inactive peptide into an agonist.

Significance

The glycoprotein hormone erythropoietin (EPO) is responsible for regulating red blood cell production. Low EPO levels or inadequate production of EPO exists in a number of pathological conditions, and recombinant EPO can be used to treat the resulting anemia. EPO binds to the extracellular portion of its receptor and activation is thought to occur by ligand-dependent dimerization of two receptors which initiates EPO signal transduction. A family of EPO receptor agonist peptides was recently discovered and may provide the basis for future therapeutic agents. The agonist peptides bind to the EPO receptor but do not activate the receptor as effectively as EPO does. We have improved the potency of these mimetic peptides by dimerizing them with a polyethylene glycol (PEG)

linker. The PEG–peptide dimers activate the receptor up to 1000-fold more efficiently *in vitro* (EMP35) and are more effective in an assay *in vivo*. Upon PEG-dimerization the additional molecular bulk of the agents with improved potency retain the ability to dimerize the extracellular domain of the EPO receptor (EPO binding protein, EBP). Not all peptides, such as EMP16, however, respond in the same manner and individual experimentation is required to investigate the possibility for improved potency. The observation that at least one inactive peptide can be converted into a weak agonist demonstrates the potential of the method described here and suggests that the possibility of activating receptors by dimerization of compounds with simple binding capacity exists, provided that they do not induce an inactive receptor conformation. This methodology might be extended to other peptidic and nonpeptidic pharmacophores (the part of a molecule causing the specific physiological effects or binding interactions) which bind to other receptors. The potency of peptide ligands for other receptors thought to have simple dimerization activation mechanisms, such as the thrombopoietin receptor, have been shown to benefit from dimerization [20]. The optimal size of the PEG molecules used in this study has not yet been fully explored and it is reasonable to expect that activation of different receptors will require the use of different PEGs. It is also possible that a difunctional PEG with two different chemical reactivities could be used to form heterodimers of two different peptides or compounds in order to activate receptors whose active form is ultimately composed of two or more heterologous proteins (receptor heterodimers or receptor oligomers). Receptor and protein dimerization activation mechanisms have emerged as an important fundamental concept, and the design of small molecules which can effectively mimic these functions is an area of intensive investigation.

Materials and methods

Reagents

SDS–PAGE gels (10–20% gradient SDS–PAGE plates, 84 x 70 x 1.0 mm, Integrated Separation Systems, Natick, MA) were stained with Coomassie Brilliant Blue R-250 (BioRad). A commercial preparation of activated difunctional polyethylene glycol (PEG-succinimidyl-propionate, $(SPA)_2$–PEG, mean MW ~3,400) was purchased from Shearwater Polymers, (Huntsville, AL) as was the monofunctional reagent, methoxy-PEG-succinimidyl-proprionate (m-SPA–PEG), MW ~5,000. EMP1 and all other peptides were obtained from the Peptide Synthesis Facility RWJ–PRI, (La Jolla, CA) and have been previously described [16]. These peptides were cyclized via oxidation of their intramolecular cysteines, amidated at the carboxyl terminus and mass confirmed by FAB–MS. All were Ellman-reaction negative. Tris base was obtained from BioRad, (Hercules, CA), and (1,4-Di-[3'-(2'-pyridyldithio)propionamido]butane (DPDPB) and trifluoroacetic acid (HPLC grade) were obtained from Pierce Chemical Co (Rockford, IL).

PEG peptide dimer and polymer preparations

Tris inactivated polymer. Tris-inactivated polymer was formed by incubation of 5 mM $(SPA)_2$ polymer dissolved in phosphate buffered saline (PBS; Gibco, Gaithersburg, MD) with 50 mM Tris-HCl, pH 7.5 added, and was used without further purification.

Mono-PEG conjugation of EMP1. Mono-PEG conjugates of EMP1 peptide were made using the monofunctional amine reactive polymer analog m-SPA–PEG. The conjugation reaction was carried out with polymer in excess (~three-fold) by suspending 142.5 mg (0.0286 mmol, MW ~5,000) of polymer in 4 ml PBS, pH 7.5, and adding 20 mg EMP1 (0.0095 mmol, MW 2,092) dissolved in 1 ml of 0.1% trifluoroacetic acid (TFA) and the mixture was incubated on ice for 20 h. At that time, the reaction was adjusted to a final concentration of 50 mM Tris by addition of 1 M Tris–HCl, pH 7.5 and incubated on ice for 1 h. Analytical HPLC suggested that there were two main reaction products of essentially equivalent magnitude which were not baseline resolved. Preparative HPLC (using the flatter gradient system described below) and conservative cuts resulted in collection of two product peaks that eluted at ~44 and ~47 min. After lyophilization, 24.8 mg and 16.5 mg of the two species were recovered, respectively. Mass spectral analysis of these two species demonstrated centroid masses of 7,096 (peak 1) and 12,036 (peak 2), indicating the coupling of one or two PEG molecules, respectively, to the peptide.

PEG dimerization of EMP1. The dimerization of EMP1 was carried out by suspending 25 mg (0.0071 mmol) of $(SPA)_2$ polymer [21] in 4 ml PBS, pH 7.5, and adding a three-fold molar excess of EMP1 (0.0213 mmol, 44.5 mg, MW 2092) dissolved in 1 ml of 0.1% TFA. The mixture was incubated on ice for 3 h, then an additional 7.5 mg (0.0036 mmol) of lyophilized EMP1 was added. The final ratio was 3.5 moles of EMP1 to 1 mole of $(SPA)_2$–PEG and the mixture was incubated for an additional 17 h on ice. At that time, the reaction was adjusted to a final concentration of 50 mM Tris by addition of 1 M Tris–HCl, pH 7.5 and incubated on ice for 1 h. After preparative HPLC and lyophilization 38 mg of PEG dimer was recovered, which corresponds to a 69% yield: the theoretical yield was 55 mg, based on a calculated mass of 7,600 mg/mmol.

PEG dimerization of EMP16: The modification of EMP16 was carried out by suspending 11.2 mg (0.0033 mmol) of $(SPA)_2$ polymer in 2.5 ml PBS, pH 7.5, and adding an approximately three-fold molar excess of EMP16 (0.010 mmol, 20 mg, MW 1,978) dissolved in 0.25 ml of 0.1% TFA. This mixture was incubated on ice for 20 h. At that time, 0.25 ml of 1 M Tris-HCl, pH 7.5 was added and the reaction mix was incubated at 4°C for 1 h. The sample was subjected to analytical and preparative HPLC as below, with the main preparative reaction product peak eluting at ~43 min. After preparative HPLC and lyophilization 13.3 mg of PEG dimer was recovered, which corresponds to a 54% yield: the theoretical yield for this experiment was 24.42 mg, based on a calculated mass of 7,400 mg/mmol.

PEG dimerization of EMP34: The dimerization of EMP34 was carried out by suspending 10.5 mg (0.0031 mmol) of $(SPA)_2$ polymer in 2.5 ml PBS, pH 7.5, and adding an approximately three-fold molar excess of EMP34 (0.0094 mmol, 20 mg, MW 2,133) dissolved in 0.25 ml of 0.1% TFA. This mixture was incubated at 4°C for 28 h. At that time, the reaction as monitored by HPLC was estimated to be approximately 30% complete, and the temperature was shifted to ambient. An additional 27 h incubation provided no net increase in product. Because of possible hydrolysis of the reactive polymer an additional 5 mg of polymer was added, and the incubation continued for an additional 16 h. At that time, 0.25 ml of 1 M Tris–HCl, pH 7.5 was added and the reaction mix was incubated at 4°C for 1 h. The long reaction time of this conjugation was probably due to the structure of the peptide which was acetlyated at the amino terminus, leaving the internal lysine as the sole reactive amine. The sample was subjected to analytical and preparative HPLC (using the flatter gradient system described below) with the main preparative reaction product peak eluting at ~48 min. After preparative HPLC and lyophilization, 10.4 mg of PEG dimer was recovered, corresponding to a 30% yield: the theoretical yield for this experiment was 34.4 mg based on a calculated mass of 7,650 mg/mmol.

PEG dimerization of EMP35: The modification of EMP35 was carried out by suspending 2.6 mg (0.00076 mmol) of $(SPA)_2$ polymer in

3.0 ml PBS, pH 7.5, and adding a ~3-fold molar excess of EMP35 (0.00229 mmol, 5 mg, MW 2,177) dissolved in 0.1 ml of 0.1% TFA. This mixture was incubated on ice for 26 h. At that time, 0.25 ml of 1 M Tris–HCl, pH 7.5 was added and the reaction mix was incubated at 4°C for 1 h. The sample was subjected to analytical and preparative HPLC (using the flatter gradient system described below) with the main preparative reaction product peak eluting at ~46 min. After preparative HPLC and lyophilization 2.2 mg of PEG dimer was recovered, corresponding to a 37% yield: the theoretical yield for this experiment was 24.42 mg based on a calculated mass of 7,400 mg/mmol.

PEG dimerization of EMP24: The modification of EMP24 was carried out by suspending 1.2 mg (0.00036 mmol) of $(SPA)_2$ polymer in 0.5 ml PBS, pH 7.5, and an approximately three-fold molar excess of EMP24 (0.0011 mmol, 1.5 mg, MW 2,177) dissolved in 0.05 ml 0.1% TFA was added and the mixture was incubated on ice for 20 h. At that time, 0.1 ml of 1 M Tris–HCl, pH 7.5 was added and the reaction mix was incubated at 4°C for 1 h. The sample was subjected to purification using the analytical HPLC system described below with the main reaction product peak eluting at ~38 min. After preparative HPLC and lyophilization 1 mg of PEG dimer was recovered, corresponding to a 45% yield: the theoretical yield for this experiment was 2.2 mg based on a calculated mass of 6,150 mg/mmol.

Analytical and preparative HPLC

Over the course of the above reactions, the accumulation of product was monitored using analytical reverse-phase HPLC. The analysis was carried out using a Vydac C-18 Protein–Peptide column (0.46 × 25 cm, part no. 218TP54) and a Rainin Gradient HPLC system fitted with a Dynamax dual wavelength detector. At injection, the column was equilibrated in 0.1% TFA in dH_2O and was developed with a 45 min linear gradient (0–100%) of acetonitrile (ACN) containing 0.1% TFA beginning 10 min after injection. The flow rate was held constant at 1 ml/min.

The main reaction product was purified by preparative reverse phase HPLC on the same chromatographic system using a Vydac C-18 Protein–Peptide column (2.2 × 25 cm, no. 218TP15022). The reaction mix (6 ml) was injected after the column was equilibrated at 80:20, H_2O:ACN (both containing 0.1% TFA), at a constant flow rate of 8 ml/min. After a 20 min wash, bound species were eluted by applying a linear gradient of 100% ACN/0.1% TFA over 60 min. The major product peak eluting at 48 min, was collected and lyophilized to obtain EMP1–PEG dimer. These elution conditions were subsequently modified to improve the resolution of some of the conjugation products (*m*PEG–EMP1, EMP34 and EMP35) from reaction by-products. This was accomplished by applying a flatter linear gradient of 20–80% acetonitrile over 60 min. The variation in retention time due to different peptides and elution conditions is described as part of each synthesis, above. The materials recovered after lyophilization of the main product peak from each reaction were analyzed by analytical reverse-phase HPLC, MALDI–TOF mass spectrometry, EPO competitive binding potential and for bioactivity *in vitro*.

Mass spectrometry

The approximate molecular mass of the PEG–peptide dimers was determined by MALDI–TOF mass spectrometry on a Finnigan MAT mass analyzer equipped with a 337 nm pulsed nitrogen laser and operated in the positive ion mode with 20 kV acceleration potential. The samples were prepared in an α-cyano-4-hydroxycinnamic acid matrix and the instrument was calibrated with an unrelated peptide or with bovine insulin. Alternatively, 4-nitroaniline was used as the matrix and TFA omitted from the solvent for ionization under non-acidic conditions.

EBP-based competition binding assay

The extracellular domain of the human EPO receptor, EBP, was produced and characterized as described [22]. EBP produced by this method contains one free sulfhydryl group at amino-acid position 181. In order to immobilize EBP to serve as the basis for a competition binding assay, this site was used for covalently attaching EBP to agarose beads [23,24]. Briefly, individual peptides were dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution of 1 mM. All reaction tubes contained 50 µl of EBP beads, 0.5 nM [^{125}I]-labeled EPO and 0–500 µM peptide in a total volume of 500 µL binding buffer (0.2% BSA in PBS). The final concentration of DMSO was adjusted to 2.5% in all peptide assay tubes. Examination of the sensitivity of the assay to DMSO demonstrated that concentrations of up to 25% DMSO (v/v) had no deleterious effect on binding. Nonspecific binding was measured in each assay by inclusion of tubes containing a large excess of unlabelled EPO (1000 nM). Initial assay points with no added peptide were included in each assay to determine total binding. Binding mixtures were incubated overnight at room temperature with gentle rocking. The beads were then collected using Micro-columns (Isolab, Inc.) and washed with 3 ml of wash buffer (5% BSA in PBS). The columns containing the washed beads were placed in 12 × 75 mm glass tubes and bound radioactivity levels were determined in a gamma counter. The amount of bound [^{125}I]-labeled EPO was expressed as a percentage of the control binding (total = 100%) and plotted against the peptide concentration after correction for nonspecific binding. The IC_{50} was defined as the concentration of the analyte which reduced the binding of [^{125}I]-labeled EPO to the EBP beads by 50%.

EPO receptor competition binding analysis

TF-1 cells [25] were maintained in RPMI 1640, 10% fetal calf serum, 1% L-glutamine, 1% penicillin, 0.1% streptomycin and 1 ng/ml of granulocyte–macrophage colony stimulating factor (GM–CSF). [^{125}I]-labeled EPO was obtained from NEN Research Products. Cells were centrifuged and washed once with binding buffer (RPMI 1640, 5% BSA, 25 mM Hepes, pH 7.5, 0.02% sodium azide) resuspended in binding buffer, and counted using trypan blue as an indicator of viability. Each reaction contained approximately 5×10^5 cells, [^{125}I]-EPO (0.5 nM), and either no competitor, peptide or dimer preparation in a final volume of 200 µl. The binding reactions (in duplicate) were incubated overnight at 4°C. Following binding, the tubes were centrifuged at 12,000 rpm for 1 min at 4°C. The supernatant was removed, the cell pellet was resuspended in 100 µl binding buffer, and layered onto 0.7 ml of bovine calf serum. The tubes were centrifuged at 12,000 rpm for 5 min at 4°C, the supernatant was removed, the bottom of the tubes snipped off, and the cell pellets counted in a Micromedic ME plus gamma counter. Nonspecific binding was determined by incubating cells with [^{125}I]-labeled EPO and a 100-fold excess of nonradioactive EPO.

EPO-dependent cell proliferation assays

Cell line FDC-P1/ER, an EPO-dependent line that expresses the murine EPO receptor, was grown and maintained as described previously [16,26]. The hypersensitive cell line FDC-P1/trER that expresses a functional truncated human EPO receptor (missing the carboxy-terminal 40 amino acids) and a cell line expressing a full length human EPO receptor, FDC-P1/HER were also used. All three cell lines exhibit EPO-dependent cellular proliferation and their use has been described [16]. Briefly, cells were maintained in RPMI 1640 media (Gibco/BRL) containing 10% heat-inactivated fetal calf serum and 10 units/ml of recombinant human EPO. For the proliferation assay, cells were grown to stationary phase, centrifuged, washed with RPMI 1640 media (no EPO), and grown for an additional 24 h without EPO. The cells were then counted, resuspended at 800,000 cells/ml and dispensed at 40,000 cells per well. Stock solutions of the peptide dimers (5 mM in PBS) and peptide (10 mM in DMSO) were prepared and dispensed in triplicate to final concentrations of 1×10^{-10} M to 1×10^{-5} M and adjusted to a final volume of 0.2 ml. Final DMSO concentrations of 0.1% (v/v, maximal) or less were shown to have no cellular toxicity or stimulatory effects (data not shown). After a 42 h incubation at 37°C, 1 µCi per well of [^{3}H]thymidine was added and the incubation continued for 6 h, at which time the cells were harvested and counted to assess [^{3}H]thymidine incorporation as a measure of cell proliferation. Results are expressed as the amount of peptide or dimer peptide necessary to yield one half of the maximal activity obtained with recombinant EPO in each assay set. A standard EPO dose-response curve was generated within each assay.

Dimerization of EBP by EPO mimetic peptides

EBP [22] was used to study the ability of EPO mimetic peptides to mediate dimerization of the ligand binding domain of the EPO receptor. Peptide-mediated dimerization was stabilized for study using the non-water soluble homobifunctional sulfhydryl reactive cross-linking reagent, DPDPB [7,16]. Briefly, EBP (22 μM) was incubated in the presence or absence of DPDPB (1.1 mM) and variable concentrations of EMP1 or the EMP1–PEG dimer in 75 μl of PBS, pH 7.5 with all reactions and controls containing a final concentration of 4.4% DMSO and 0.007% TFA. These samples were incubated for 4 h at room temperature and stored at 4°C for 12 h before analysis on reducing and nonreducing SDS–PAGE.

Polycythemic exhypoxic mouse bioassay

Peptides were assayed for activity *in vivo* in a polycythemic mouse bioassay [27]. BDF1 mice were allowed to acclimatize to ambient conditions for 7–10 days. Body weight was determined for each animal and low-weight animals (<15 g) were excluded. Mice were introduced to hypobaric chambers and were kept in a conditioning cycle of 18 h of 0.40 +/- 0.02 atm, and 6 h at ambient pressure for a total of 14 days. Following the 14 day period, mice were removed to ambient pressure for 72 h prior to sample administration. Test samples or recombinant human EPO standards were diluted in assay vehicle, 0.1% BSA in PBS. Peptide sample stock solutions were first solubilized in DMSO. Peptide dimers were solubilized in PBS. Control groups included one group of vehicle alone, and one group of (DMSO) at a final concentration of 1%.

Each dose group contained 10 mice. Mice were injected subcutaneously (scruff of neck) with 0.5 ml of the appropriate sample. Then, 48 hours following the sample injection, the mice were administered an intraperitoneal injection of 0.2 ml of [^{59}Fe] (approx. 18.0 μCi/mg, Dupont, NEN), 0.40 μCi per mouse. Mouse body weight was determined 24 h following [^{59}Fe] administration and the mice were sacrificed 48 h after the [^{59}Fe] injection. Blood was collected from each animal by cardiac puncture and hematocrits determined (heparin was used as the anti-coagulant). Each blood sample (0.2 ml) was analyzed for [^{59}Fe] incorporation in a Packard gamma counter. Mice that had hematocrit values less than 53% were eliminated.

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