Accurate processing and secretion in the baculovirus expression system of an erythroid-cell-stimulating factor consisting of a chimaera of insulin-like growth factor II and an insect insulin-like peptide

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A synthetic gene encoding the signal peptide and the N-terminal sequence of bombyxin, an insect insulin-like peptide, and the 58 amino acids of the C-terminal sequence of human insulin-like growth factor II (IGF II) has been expressed using the baculovirus system. This synthetic chimaera was obtained by amplification of four overlapping oligonucleotides using *Taq* polymerase and cloning into the transfer vector pBluebac. The construct was integrated by homologous recombination into the *Autographa californica* nuclear polyhedrosis genome. *Spodoptera frugiperda* Sf9 insect cells infected with the recombinant baculovirus secreted an accurately processed peptide consisting of the ten N-terminal

amino acids of bombyxin and the 58 C-terminal amino acids of IGF II. The N-terminal glutamine of bombyxin was changed to asparagine to facilitate sequencing of the synthetic peptide. The chimaera was five times more potent than human recombinant IGF II in its capacity to stimulate thymidine incorporation into erythroid cells of fetal bovine liver in a serum-free medium. It stimulated erythroid colony formation in the presence of 2 munits/ml erythropoietin in cells cultured over a monolayer of stromal cells of fetal liver. Artificial chimaeras as described here may prove useful for the production of insulin, IGF I and other peptides as secreted proteins in insect cells.

INTRODUCTION

We found that small peptides (tentatively named erythrotropins) isolated from bovine fetal tissues stimulated thymidine incorporation and haemoglobin synthesis in fetal calf erythroid cells [1]. The isolation and characterization of these peptides indicated that they belonged to the insulin-like growth factor (IGF) family. The most abundant of these peptides in fetal bovine serum was isolated and sequenced and it corresponded to the sequence of bovine IGF II [2,3]. Our results and those of many other investigators indicate that both IGF I and II play an important role in erythropoiesis *in vitro* [4–10] and *in vivo* [11,12]. Therefore IGFs may be useful as therapeutic agents in anaemia and generally as anabolic hormones in diseases where growth hormone may be ineffective or have undesirable side effects [13,14].

IGFs are particularly attractive as targets for site-directed mutagenesis because they are small non-glycosylated peptides which have multiple binding sites with a large number of proteins. For instance, it is possible to decrease the binding activity to serum binding proteins without affecting the binding affinity to IGF receptors [15,16]. Therefore they are exceptional compared with the vast majority of growth factors in the sense that mutagenesis can actually result in a higher biological activity [17]. It would be a great advantage if IGFs could be synthesized with the baculovirus expression system, which is known for being able to produce large amounts of mammalian proteins. Unfortunately, when the IGF II precursor was integrated into the baculovirus, although it was expressed, it resulted in a protein that was not processed or secreted [18]. Alternatively, IGF II has been synthesized as a baculovirus fusion protein [19]. In the present paper we show how a chimaera of IGF II and the N-terminal sequence of the gene encoding the insulin-like peptide bombyxin is correctly processed and secreted in the baculovirus

expression system. The recombinant protein has a higher thymidine-incorporation-stimulating activity in fetal calf erythroid cells than recombinant human IGF II. This strategy could be useful for the synthesis of recombinant insulin-like peptides and probably other secreted mammalian proteins in the baculovirus expression system.

MATERIALS AND METHODS

Materials

All materials for the baculovirus expression system and the TA cloning kit were obtained from Invitrogen (Sorrento Valley, CA, U.S.A.). *Taq* polymerase was from Bio-Can Scientific, Mississauga, Ontario, Canada. Restriction enzymes and the Vent sequencing kit were from New England Biolabs, Mississauga, Ontario, Canada. All culture materials were obtained from Gibco-BRL, Burlington, Ontario, Canada. Oligonucleotides were synthesized by the Sheldon Biotechnology Centre, McGill University, Montreal, Canada.

Construction of the recombinant baculovirus

The synthetic gene shown in Figure 1 was synthesized by the method of Dillon and Rosen [20]. Four sense and antisense strands indicated by arrows in Figure 2 (reaction 1) and containing 84, 84, 84 and 88 nucleotides with overlapping sequences of 18 nucleotides were joined with *Taq* polymerase as illustrated. The gene was amplified with *Taq* polymerase using two oligonucleotide primers which contained the *Nhe*I restriction sites required for the ligation with the pBluebac transfer vector from Invitrogen (Figure 2, reaction 2). The synthetic gene (Figure 1) contained the signal peptide of bombyxin (amino acids –19 to –1) and the first ten amino acids of bombyxin [21] with two

Abbreviations used: IGF, insulin-like growth factor; AcNPV, *Autographa californica* nuclear polyhedrosis virus; BOMIGF, Asn-Gln-Pro-Gln-Met-Val-His-Thr-Tyr-human IGF II-(9–67); Ac[BOMIGF], recombinant baculovirus in which the signal peptide of bombyxin + BOMIGF (Figure 1) has been integrated to the baculovirus polyhedrin promoter; IMDM, Iscove's modified Dulbecco's medium.

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Figure 1 Sequence of the chimera of bombyxin and IGF II

The amino acid sequence and corresponding nucleotide sequence utilized for the synthesis of the synthetic gene are shown. Amino acids -19 to 10 correspond to the signal peptide and the N-terminal section of bombyxin [21] with the exception of amino acids 1 (Asn) and 5 (Met) indicated in boxes. Amino acids 10–68 correspond to amino acids 9–67 of human IGF II [22,23]. A termination codon (TAG) followed the C-terminal amino acid (Glu). Accurate processing [21] should result in the synthesis of BOMIGF, amino acids 1–68.

modifications, namely replacement of amino acids 1 (Gln to Asn) and 5 (Glu to Met). Amino acid 10 (Cys) was common to both bombyxin and human IGF II, and amino acids 10–68 corresponded to residues 9–67 of mature human IGF II [22,23]. A termination codon (TAG) followed immediately after the C-terminal glutamic acid. The product of the amplification reaction was ligated with the TA cloning kit of Invitrogen to pCR1000 (Figure 2, reaction 3), and the entire sequence was confirmed with the Sequenase sequencing kit (US Biochemicals, Cleveland, OH, U.S.A.). The plasmid was digested with *NheI* and ligated to the pBluebac transfer vector (Figure 2, reaction 4), which was originally created by Vialard et al. [24] and contains the *Lac* gene for identification of recombinants by blue colour selection. Positive clones were selected using lifts of bacterial colonies showing hybridization with the bombyxin–IGF II probe by standard techniques [25]. Several clones were sequenced (Circumvent kit, New England Biolabs) to confirm the integrity and correct orientation of the insert after the polyhedrin promoter. Clones that showed 100% identity after sequencing with the Vent polymerase (New England Biolabs) and with the accurate orientation within the polyhedrin promoter were co-transfected (Figure 2, reaction 5) with the wild-type linearized genomic *Autographa californica* nuclear polyhedrosis virus (AcNPV) baculovirus using the calcium phosphate precipitation method [26].

Successful recombination was followed using 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside in the transfection medium as an indicator of the marker enzyme β -galactosidase [27]. Serial dilutions of the transfection supernatants were prepared and used to infect Sf9 cells plated in tissue culture dishes of 6 cm diameter. The infected cells were covered with agarose and the formation of blue plaques was followed for several days [27]. The process of screening for recombinants was as follows: (1)

identification and collection of agarose plugs from blue plaques; (2) expansion of the viral plaques in Sf9 cells plated in 3.5-cm dishes; (3) identification of positive recombinants by dot hybridization using IGF II probes [3]; (4) extraction of DNA from positive recombinants from 750 μ l supernatants to be used for PCR employing as primers 5' and 3' flanking regions of the polyhedrin gene, as indicated in the Invitrogen kit.

The production of the recombinant protein was assessed first by PAGE of cell extracts and cell culture supernatants using the Tris/Tricine system [28] followed by electroblotting to poly(vinylidene fluoride) membranes (Immobilon; Millipore, Marlborough, MA, U.S.A.) and Western blotting using an IGF II antibody (UBI Biotechnologies, Lake Placid, NY, U.S.A.). However, this method was not as sensitive and specific as required for an unequivocal identification. The protein bands with the expected molecular mass were faint, and sometimes the interpretation of the results was ambiguous because of the presence of other positive proteins. These problems are probably due to artifacts caused by the SDS denaturation step. Therefore the best method of identification turned out to be measurement of the ability of supernatants of the infected cells to stimulate thymidine incorporation into calf erythroid cells [29]. This method is not necessarily specific for IGF II [4,29] but has the advantage of being able to detect very small amounts of this growth factor. Furthermore, only recombinants producing biologically active peptides are selected. Sf9 cells (2.8×10^6) were plated in 6 cm Lux-contour cell culture dishes [27] and infected with the recombinant viruses in Sf900 serum-free medium (Gibco). After 2–3 days, the supernatants and cells were separated by centrifugation. Supernatants were acidified with trifluoroacetic acid to a final concentration of 1% (v/v) and extracted by reversed-phase h.p.l.c. using C18 cartridges (Sep-Pak; Millipore) as previously described [30]. The cells were lysed in 3 ml of acid-

Figure 2 Techniques utilized for the synthesis of the recombinant baculovirus Ac[BOMIGF]

1, Synthetic gene completed with the *Taq* polymerase from a template consisting of four overlapping oligonucleotides coding for the BOMIGF gene; 2, amplification of the synthetic gene with 5'- and 3'-oligonucleotides including *NheI* restriction sites; 3, TA cloning of the amplified PCR product using the vector pCR1000; 4, clone with the expected sequence was digested with *NheI* and ligated into the pBluebac baculovirus transfer vector; 5, simultaneous transfection of the BOMIGF-vector and a linearized wild-type baculovirus DNA into Sf9 insect cells. The recombinant baculovirus containing BOMIGF was isolated by plaque purification.

extraction mixture [1,30], sonicated for 2 min and extracted as indicated for the supernatants. The extracts were lyophilized (Speedvac centrifuge; Savant, Fisher Scientific, Montreal, Quebec, Canada), suspended in modified, serum-free F12 medium [29] and tested for their capacity to stimulate thymidine incorporation into calf erythroid cells [29]. The modified F12 medium was later replaced with a mixture of F12/Iscoe's modified Dulbecco's medium (IMDM) (3:1, v/v) supplemented with 100 μ M α -thioglycerol, 10 μ g/ml gentamicin and 30 μ g/ml bovine transferrin (ICN, Richmond, CA, U.S.A.).

Production and purification of the recombinant protein

A typical preparation was as follows. Sf9 cells (2×10^8 in 200 ml of suspension culture) were infected in Sf900 serum-free medium with the recombinant baculovirus as a multiplicity of infection of 14 as indicated by Piwnicka-Worms [27]. At 3 days after infection the suspensions were centrifuged at 300 *g* for 10 min. The supernatant was cooled to 4°C, acidified with trifluoroacetic

acid to a final concentration of 1% (v/v) and extracted by reversed-phase h.p.l.c. as indicated above using one cartridge per 25 ml of medium. The extract was concentrated and applied to two Waters I-125 columns attached in series [30]. Several identical separations were required to process the complete extract. Fractions corresponding to molecular masses of 5–14 kDa were pooled, concentrated and applied to a Vydac C4 semipreparative column (1 cm $\times$ 25 cm). They were eluted at a speed of 2 ml/min using a gradient of solution A [water/acetonitrile/trifluoroacetic acid (99:1:0.1 by vol.)] to solution B [water/acetonitrile/trifluoroacetic acid (20:80:0.1, by vol.)] as follows: 10 min from 0 to 25% B, 30 min from 25 to 45% B and 20 min from 45 to 80% B. Portions of volume 15 μ l were taken, evaporated and tested for biological activity using the bioassay of calf liver erythroid cells as indicated above. Fractions with thymidine-incorporation-stimulating activity were pooled and applied to four Waters I-125 gel-filtration columns attached in series and eluted at a speed of 0.6 ml/min. Fractions with thymidine-incorporation-stimulating activity were pooled and applied to a Brownlee Aquapore column of 10 cm long preceded by a similar precolumn 3 cm long. The peptides were eluted at a speed of 0.3 ml/min using a gradient between solutions A and B as follows: 10 min from 0 to 22% B, 10 min from 22 to 38% B and 10 min from 38 to 55% B. A single peak of absorbance eluted at 24 min contained the fractions with biological activity, as indicated in the thymidine-incorporation assay. Samples were used for amino acid analysis (Beckman 6300 amino acid analyser), N-terminal sequencing (Sheldon Biotechnology Centre, McGill University) and electrophoresis in precast gels (high-density SDS/PAGE; Phastsystem, Pharmacia, Baie d'Urfe, Quebec, Canada).

Erythroid colony formation

A brief description of the methods for the clonogenic assay follows (full details are available from L.F.C. on request). In summary, haematopoietic progenitor cells were obtained from frozen bovine fetal liver cells thawed with warm IMDM and treated with NH_4Cl to destroy the mature erythroid cells selectively. The cells were separated by Ficoll/Paque. Mononuclear cells at the interface were collected, washed once, suspended in IMDM and plated at a cell density of 10^4 cells/ml over a monolayer of stromal cells previously seeded in 12-well plates (Corning). The stromal cells were originally prepared from fetal liver endothelial-like cells and had undergone on average ten passages. The medium was supplemented with 0.8% (w/v) methylcellulose, 2% (v/v) fetal bovine serum, 60 μ g/ml transferrin, 8 μ g/ml cholesterol, 5.6 μ g/ml oleic acid, 8 μ g/ml L- α -phosphatidylcholine, 0.3 μ g/ml vitamin A, 0.33 μ g/ml vitamin E, 130 μ g/ml haemin, 30% F12 medium, 100 nM α -thioglycerol and 2 munits/ml recombinant erythropoietin. IGF II or the recombinant BOMIGF was added at a final concentration of 500 ng/ml. After 72 h, the number of erythroid colonies was counted in a phase-contrast microscope.

RESULTS AND DISCUSSION

Construction of a recombinant baculovirus encoding a bombyxin–human IGF II chimaera

The baculovirus expression system has been successfully used for the synthesis of a large variety of mammalian proteins. Insect cells have the possibility of recognizing many mammalian signals for the processing and intracellular localization of proteins driven by the polyhedrin promoter of the virus [31,32]. The polyhedrin protein normally accounts for 30% of the total proteins synthe-

Figure 3 Stimulation of thymidine incorporation into erythroid cells of fetal bovine liver by extracts of infected Sf9 insect cells

Erythroid cells (2.8×10^6) were infected with Ac[BOMIGF] (multiplicity of infection = 10). After the time periods indicated on the abscissa, cell lysates and culture supernatants were subjected to reversed-phase chromatography as indicated in the Materials and methods section, and samples of these extracts were tested for their capacity to stimulate [^3H]thymidine incorporation. ●, effect of extracts from cell culture supernatants; ○, extracts from cell lysates.

Figure 4 Reversed-phase h.p.l.c. of a partially purified extract of culture supernatants of cells infected with Ac[BOMIGF]

Reversed-phase extracts of cell culture supernatants of Sf9 cells infected with Ac[BOMIGF] were first partially purified on gel-filtration columns as described in the Materials and methods section. Fractions with apparent molecular masses between 5 and 14 kDa were pooled and applied to a Vydac C4 column and eluted with a gradient of acetonitrile in the presence of trifluoroacetic acid as indicated in the Materials and methods section. ●, [^3H]thymidine incorporation observed in fetal bovine erythroid cells cultured with 15 μl portions of the h.p.l.c. fractions

sized at late stages of infection. Unfortunately, insect cells cannot recognize some mammalian signal peptides and are unable to process some mammalian proteins accurately. IGF II has been synthesized using the baculovirus expression system but only as a fusion protein [19]. This method allows the synthesis of large amounts of the peptide. Nevertheless, it is necessary to recover the peptide by CNBr treatment. Furthermore, a biologically active peptide can be obtained only after a complex renaturation

process followed by chromatographic separation of the fraction with the right conformation. The advantages of the baculovirus system (high yield, efficient expression) are then limited by the complexity of the recovery of biologically active peptides. In the discussion of the experiments describing the expression of human IGF II in 3T3 fibroblasts, Burgisser *et al.* [18] indicated that this peptide, although synthesized in insect cells after integration in the baculovirus expression system, was not processed or secreted. Obviously insect cells cannot recognize the natural signal peptide of mammalian IGFs. Tessier *et al.* [33] have developed a new baculovirus vector in which the recombinant sequence of the protein to be synthesized is integrated in the polyhedrin promoter together with the signal peptide of melittin, a secreted insect toxin which is an important component of bee venom. They succeeded in obtaining secretion of a chimaera of the melittin signal peptide and papain, a plant protease. We changed the *Hind*III and *Eco*RI restriction sites of a bovine IGF II cDNA cloned in Bluescript [3] to *Bam*HI and *Pst*I by PCR and ligated it to the multiple cloning site of Tessier's vector containing the melittin signal peptide. Although the correctness of the ligation was confirmed by sequencing and the transfection and recombination in the baculovirus were successful, we were not able to detect any biologically active peptides in supernatants or cell lysates of cells infected with numerous recombinants (L. F. Congote, unpublished work). It is difficult to assess the causes of this negative result. We assumed that the simple addition of an insect signal peptide may not always result in a properly folded and secreted protein and tried therefore to construct another vector with a new signal sequence.

The insulin family of peptides represents one of the most conserved protein structures during evolution. There are insulin-related peptides in *Locusta migratoria* [34] and in the neurosecretory cells of the silkworm [21]. It is therefore reasonable to assume that the signal sequences of these insect insulin-like peptides may be used for the synthesis of mammalian insulins or IGFs. We were particularly interested in bombyxins, the insulin-like peptides of the silkworm, because Adachi *et al.* [21] have found that the precursor of these peptides closely resembles mammalian proinsulins. Therefore we synthesized a chimaera between the signal peptide of bombyxin and the human IGF II gene (Figure 1), using the method of Dillon and Rosen [20]. We were particularly interested in recognizing the accurate processing of the synthetic chimaera by protein sequencing. Therefore we changed the N-terminal glutamine of natural bombyxin to asparagine. This modification prevents the pyroglutamic acid formation observed in bombyxin and therefore the subsequent blockage of the N-terminal synthetic IGF. It is possible that the processing enzymes in insect cells may require the presence of certain amino acids within the N-terminal section of the mature proteins. Therefore we decided to keep the first 10 amino acids of bombyxin in the synthetic gene. It is reasonable to assume that replacement of the N-terminal IGF II amino acid sequence with that of bombyxin should not alter the biological activity of this growth factor, because it is known that this section of the molecule is not essential for receptor binding [17,35]. On the contrary, elimination of potential binding sites in this region of IGFs with plasma IGF-binding proteins eventually increases the biological activity of the synthetic peptide, as has been demonstrated with recombinant IGF I analogues obtained by site-directed mutagenesis [17]. We introduced an additional mutation into the N-terminal region of the synthetic peptide, namely a methionine at position 5. Therefore if Sf9 cells infected with the recombinant virus could not eliminate the signal peptide, this unique methionine within the N-terminal region of the mature IGF could eventually be used for elimination of the signal

Figure 5 Gel-filtration h.p.l.c. of the fractions with thymidine-incorporation-stimulating activity purified on C_4 columns

The bioactive fraction indicated in Figure 4 was applied to four Waters I-125 gel-filtration columns attached in series and eluted as indicated in the Materials and methods section. ●, [3 H]Thymidine incorporation observed in fetal bovine erythroid cells cultured with 8 μ l portions of the h.p.l.c. fractions.

Figure 6 Final reversed-phase h.p.l.c. of BOMIGF

The fractions with thymidine-incorporation-stimulating activity shown in Figure 5 were applied to a Brownlee Aquapore column and eluted as indicated in the Materials and methods section. ●, [3 H]Thymidine incorporation observed in fetal bovine erythroid cells cultured with 10 μ l portions of the h.p.l.c. fractions.

peptide by CNBr treatment. This unique methionine would also be useful if the insect sequences of the chimaera were found to induce an immune response *in vivo*, because the five N-terminal amino acid residues could be eliminated by CNBr treatment.

The synthetic chimaera of Figure 1 was ligated into the TA cloning vector pCR1000. This allowed us to confirm the sequence and to eliminate clones with mismatches known to occur using *Taq* polymerase. At the same time, it facilitated digestion with the restriction enzyme *Nhe*I, because amplified DNA with restriction sites at the 3' and 5' ends are poorly digested [36]. After integration of correctly amplified genes into the pBluebac vector of Vialard et al. [24], the sequence and correct orientation of the inserted DNA were further confirmed using the Vent polymerase sequencing kit as indicated in the Materials and methods section. Recombinant viruses were made and selected as described above and the recombinant viral stocks amplified. The recombinant baculovirus selected here from a pure plaque was

Figure 7 SDS/PAGE of the purified BOMIGF

The fractions eluted at 24 min during the reversed-phase h.p.l.c. shown in Figure 6 were applied to a high-density gel (Pharmacia) and separated by electrophoresis using the Phastsystem. The arrows and numbers indicate the positions of the molecular-mass markers in kDa (Rainbow low-molecular-mass markers; Amersham). The gel was stained with silver.

named Ac[BOMIGF]. Figure 3 shows the production of a factor with thymidine-incorporation-stimulating activity in the supernatants of Sf9 insect cell cultures after infection with Ac[BOMIGF]. Note that all the activity is present in the supernatant, and practically no activity could be observed in cell extracts. Supernatants of mock-infected cells or cells infected with the wild-type baculovirus AcNPV did not have any thymidine-incorporation-stimulating activity (results not shown). This very sensitive bioassay was used for the purification and selection of positive recombinants.

Purification of the bombyxin–IGF II chimaera (BOMIGF)

We have purified the recombinant protein on a semipreparative scale from batches of 150–200 ml of serum-free medium obtained from suspension cultures of cells infected by the baculovirus. The initial purification was a reversed-phase chromatography followed by gel filtration as indicated in the Materials and methods section. The most efficient purification step was the reversed-phase chromatography using Vydac columns (Figure 4), which eliminated most of the contaminating proteins. Further purification steps, such as gel-filtration h.p.l.c. (Figure 5) and reversed-phase h.p.l.c. using Aquapore columns (Figure 6), resulted in a single peptide as analysed by SDS/PAGE (Figure 7). The apparent molecular mass was between 6 kDa (SDS/PAGE) and

Figure 8 Erythropoietic activity of the recombinant chimaera

(a) Comparison of the effects of BOMIGF (●), human recombinant IGF II (○) and human recombinant erythropoietin (□) on $[^3\text{H}]$ thymidine incorporation into erythroid cells from fetal bovine liver. (b) Effects of 500 ng/ml IGF II and BOMIGF on erythroid colony formation (CFU, colony-forming units) of mononuclear cells of fetal bovine liver cultured over a monolayer of fetal stromal cells in the presence of 2 units/ml erythropoietin. Results are means $\pm$ S.E.M. of three determinations.

7.4 kDa (h.p.l.c.), which corresponds to the expected molecular mass of 7.2 kDa. N-terminal-sequence analysis of this peptide agreed exactly with the expected cleavage site of the bombyxin precursor and gave the sequence Asn-Gln-Pro-Gln-Met-Val, including the two new amino acids (Asn, Met) introduced into the synthetic chimaera. Amino acid analysis after an overnight hydrolysis was also in agreement with the expected composition. The amounts of cysteine are underestimated because of partial oxidation. The values were (expected numbers in parentheses): Ala, 3.8 (4); Asx, 5.9 (4); Arg, 5.4 (5); Cys, 3.1 (6); Gly, 5.1 (5); Glx, 8.3 (8); His, 1.1 (1); Ileu, 0.5 (1); Leu, 4.5 (5); Lys, 1.4 (1); Met, 1.1 (1); Phe, 2.1 (3); Pro, 2.4 (3); Ser, 6.3 (6); Thr, 3.7 (4); Tyr, 3.4 (3); Val, 4.6 (5). We were particularly interested in the identification of the recombinant protein. Therefore the h.p.l.c. purification procedure illustrated in Figures 4–6 was designed for a fast, rather than an efficient, purification with high recoveries. Therefore we obtained 20 μg from 200 ml of supernatant instead of 100–200 μg as would be expected from the initial activities of the crude extracts. Preliminary experiments indicate that insect cell lines such as *Trichoplusia ni* egg cells (Invitrogen) produce eight times more recombinant peptide than Sf9 cells (L. F. Congote, unpublished work).

Erythropoietic activity of BOMIGF

The erythropoietic activity of the recombinant protein was evaluated by two methods. The first involved measurement of the protein's capacity to stimulate thymidine incorporation into erythroid cells of fetal calf liver (Figure 8a). Incubation conditions were as indicated in the original bioassay [29], but total incubation volume was reduced to 100 μl and the number of cells was only 3×10^5 . The median effective dose of the recombinant peptide was on average 12 ± 4 ng/ml (mean $\pm$ S.E.M., four experiments), about 5.6 times more potent than the recombinant human IGF II ($P < 0.05$, paired t test). BOMIGF and IGF II were less potent than human recombinant erythropoietin. The plateaus of the dose-response curves of both IGFs (approx. 2400 c.p.m.) seem to be lower than that observed with erythropoietin

(3000 c.p.m.). However, this difference is eliminated if a small amount of erythropoietin is added together with BOMIGF. In fact, incorporation of $[^3\text{H}]$ thymidine in cells incubated with 0.008 ng/ml erythropoietin and 138 ng/ml BOMIGF was 3680 ± 160 c.p.m. in the same experiment. The median effective doses of both IGF II and BOMIGF on thymidine incorporation are higher than those observed with erythropoietin (Figure 8a). Nevertheless, they are about ten times lower than the physiological concentrations of IGF II in cord blood [37]. BOMIGF and IGF II had similar effects in the stimulation of erythroid colony formation in the presence of erythropoietin and monolayers of fetal liver cells (Figure 8b), as required for the action of IGFs in bovine fetal liver. However, this culture system is difficult to evaluate quantitatively, because it is not completely serum-free and requires the presence of erythropoietin and stromal cells (Q. Li and L. F. Congote, unpublished work). We are at present modifying the BOMIGF-pBluebac vector by site-directed mutagenesis with the hope of obtaining new chimaeras which could result in a higher erythropoietic activity.

We think that the introduction of the bombyxin signal peptide as a tool for the synthesis of insulin and insulin-like peptides using the very efficient baculovirus system will eventually decrease the cost of production and facilitate the modification of these useful peptides by site-directed mutagenesis. We are also exploring the possible use of bombyxin chimaeras with peptides that do not belong to the insulin superfamily.

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