Production and characterization of recombinant insulin-like growth factor-I (IGF-I) and potent analogues of IGF-I, with Gly or Arg substituted for Glu$^3$, following their expression in *Escherichia coli* as fusion proteins

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ABSTRACT

The development of an efficient expression system for insulin-like growth factor-I (IGF-I) in *Escherichia coli* as a fusion protein is described. The fusion protein consists of an N-terminal extension made up of the first 46 amino acids of methionyl porcine GH ([(M$^1$)]-pGH) followed by the dipeptide Val-Asn. The latter two residues provide a unique hydroxylamine-sensitive link between [M$^1$]-pGH(1-46) and the N-terminal Gly of IGF-I. Downstream processing of the fusion proteins involved isolation of inclusion bodies, cleavage at the Asn-Gly bond, refolding of the reduced IGF-I peptide and purification to homogeneity. This expression system was also used to produce two variants of IGF-I in which Glu$^3$ was substituted by either Gly or Arg to give [Gly$^3$]-IGF-I and [Arg$^3$]-IGF-I respectively. Production of milligram quantities of IGF-I peptide was readily achieved. The purity of the IGF-I, [Gly$^3$]-IGF-I and [Arg$^3$]-IGF-I was established by high-performance liquid chromatography and N-terminal sequence analysis. [Gly$^3$]-IGF-I and [Arg$^3$]-IGF-I were more potent than IGF-I in biological assays measuring stimulation of protein synthesis and DNA synthesis or inhibition of protein breakdown in rat L6 myoblasts. Both analogues bound very poorly to bovine IGF-binding protein-2 and slightly less well than IGF-I to the type-1 receptor on rat L6 myoblasts. We conclude that reduced binding to IGF-binding proteins rather than increased receptor binding is the likely explanation for the greater biological potency of the analogues compared with IGF-I.

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INTRODUCTION

Insulin-like growth factor-I (IGF-I) is a potent anabolic growth factor, the actions of which *in vitro* and *in vivo* have been extensively described and recently reviewed by Humbel (1990) and Sara & Hall (1990). The actions of IGF-I on cultured cells are dependent in most cases on it binding to the type-1 IGF receptor on the cell surface. The biological actions of IGF-I *in vitro* and *in vivo* are modified by interaction with a family of insulin-like growth factor-binding proteins (IGFBPs) (Clemmons, 1989; Rutanen & Pekonen, 1990). Previously, we have shown that the binding of IGF-I to purified bovine IGFBP-2 requires the presence of the N-terminal tripeptide of IGF-I, specifically the Glu residue at position 3 (Szabo, Mottershead, Ballard & Wallace, 1988; Bagley, May, Szabo *et al.* 1989). In cell lines that secrete IGFBPs into the culture medium the anabolic effects of IGF-I are enhanced by removal of Glu$^3$ in the IGF-I sequence (Bagley *et al.* 1989). This was established by the addition of purified IGFBPs which inhibit the actions of IGF-I but not the N-terminal truncated variant des(1-3)IGF-I (Ross, Francis, Szabo *et al.* 1989).

Evaluation of the potential applications of potent analogues of IGF-I both *in vitro* and *in vivo* would be facilitated by the ready availability of these...
peptides. Recently, we described an expression system in mammalian cells for the production of secreted recombinant IGF-I and des(1−3)IGF-I (McKinnon, Ross, Wells et al. 1991). However, the yield of peptide from this expression system was low and the cost of production in mammalian expression systems was high. We have previously achieved efficient expression in *Escherichia coli* of a mammalian protein, porcine growth hormone (pGH), by optimizing DNA sequences of a multiple-copy plasmid (Vize & Wells, 1987). In the present report we describe a bacterial expression system for fusion protein constructs containing DNA coding for pGH and IGF-I sequences that are linked by a hydroxylamine-sensitive Asn-Gly bond. This expression system has enabled us to produce large quantities of IGF-I and several N-terminal analogues of IGF-I with enhanced biological potency.

MATERIALS AND METHODS

Materials

Restriction enzymes and deoxynucleotides were purchased from Pharmacia-LKB Pty Ltd, Sydney, New South Wales, Australia. Reference recombinant human IGF-I was kindly provided by Genentech Inc., South San Francisco, CA, U.S.A. Recombinant human IGF-II (hIGF-II) was kindly provided by Eli Lilly, Indianapolis, IN, U.S.A. Bovine IGF-I was purified from bovine colostrum to homogeneity as described previously (Francis, Upton, Ballard et al. 1988). All other molecular biology materials were from Bresatec Ltd, Adelaide, South Australia, Australia.

Plasmid construction and bacterial strain

The expression vector pGIXSC.4, based on the parent plasmid pKT52, provided the basis for further plasmid constructs (Vize & Wells, 1987). This vector contains the *trc* promoter, an optimized ribosome binding site and spacer region together with modified pGH cDNA sequences. When propagated in strains of *E. coli* which overproduce *lac* repressor, addition of isopropyl-β-D-galactoside (IPTG) induces high-level expression of methionyl pGH ([Met*]-pGH) as inclusion bodies (Vize & Wells, 1987).

An M13mp8 clone (MIGF), containing synthetic human IGF-I sequences optimized for expression in *E. coli* (Sproat & Gait, 1985), was a gift from M. Gait. *E. coli* strain JM101 (*lacI*') (Messing, 1979) was used for all cloning and expression studies. Vectors were transformed into JM101 at 37°C on minimal agar containing 100 µg ampicillin/ml (Sigma, St Louis, MO, U.S.A.). Minimal agar contains 60 mmol K₂SO₄/1, 33 mmol KH₂PO₄/1, 1.7 mmol Na₃C₆H₅O₇/1, 7.6 mmol (NH₄)₂SO₄/1, 0.8 mmol MgSO₄/1, 10 mmol glucose/l and 1.5 µmol thiamine/l.

The following nucleotides were used for in-vitro mutagenesis: oligo A, 5'-CAGGGTTTCCGG-GCCGTTGAAAGCCACAGCTCCACGAA-3'; oligo 46, 5'-GCACAGGTTTCTCCGGGCGTT-AACCTGGATGAGTACCTCTGTC-3'; oligo Gly3, 5'-ACCGCAGGGTACCAGGCCCGTGGA-3'; and oligo Arg3, 5'-ACCGCAGGG-TAAGCGGCCCCGTAA-3'. Oligo MG20 (5'-AAGTAGAGCCACGTCACCG) was used as a sequencing primer. All oligonucleotides were synthesized by Bresatec Ltd.

Construction of clones expressing [Met*]-pGH(1−46)-Val-Asn-IGF-I

The procedure employed for the construction of plasmids expressing [Met*]-pGH(1−46)-Val-Asn-IGF-I and its variants, including the deletion of pGH sequences, is outlined in Fig. 1. Sequences coding for methionyl hIGF-I were cloned, in frame, 3' to the pGH cDNA in pGIXSC.4. This allowed expression of a protein consisting of the first 188 amino acids of [Met*]-pGH followed by methionyl IGF-I. In-vitro mutagenesis using oligo A was used to replace the three missing C-terminal amino acids of pGH, to introduce an additional Asn residue and to remove the leading methionine from methionyl hIGF-I. This allows the potential cleavage of the IGF-I peptide from pGH by introducing a hydroxylamine-sensitive Asn-Gly bond. Mutagenesis (Zoller & Smith, 1983; Wood, Gitschier, Lasky & Lawn, 1985) was directed to the EcoRI–HindIII fragment (Fig. 1) after cloning into M13mp8 (Vieira & Messing, 1982). Following verification of the entire DNA sequence by dideoxy sequencing, the fragment was ligated into the plasmid vector to create plasmid [Met*]-pGH-Asn-IGF-I.

Two deletions were constructed to determine expression levels of [Met*]-pGH-IGF-I fusion proteins with various lengths of N-terminal pGH extension. The first, plasmid [Met*]-pGH-Cys-Ala-Asn-IGF-I, was created by restricting plasmid [Met*]-pGH-Asn-IGF-I with Aval and PvuII. This construct directs expression of a fusion protein lacking [Met*]-pGH amino acids 89−188. The second deletion was made by in-vitro mutagenesis of plasmid [Met*]-pGH(1−88)-Cys-Ala-Asn-IGF-I (as described above) using oligo 46. We also introduced a valine codon, GT T, to create a unique Hpal restriction site at the [Met*]-pGH junction with IGF-I, increasing the versatility of the vector (Fig. 1). The resultant plasmid, [Met*]-pGH(1−46)-Val-Asn-IGF-I, directs the expression of IGF-I as a fusion protein with 48
amino acids at the N terminus which can be removed by cleavage with hydroxylamine.

**Construction and expression of [Met]'-pGH(1–46)-Val-Asn-IGF-I analogue clones**

To obtain expression of variant recombinant molecules with Gly or Arg substitutions at position 3 in place of the normal Glu in IGF-I, the plasmid [Met]'-pGH(1–46)-Val-Asn-IGF-I was further modified by in-vitro mutagenesis. Oligo Gly, and oligo Arg were designed to introduce the specific changes to the Glu residue of IGF-I, that is a codon change from GAA to GGT and from GAA to CGT respectively.

Following construction of vectors for expression of the various deleted [Met]'-pGH-IGF-I fusion proteins, transformed cultures were tested for levels of induced protein. Individual colonies were inoculated into 1 ml minimal medium containing 100 μg ampicillin/ml and grown at 37°C until the absorbance at 600 nm was 0.8. Following induction with IPTG for 1 h, the cells were centrifuged and the resulting cell pellet was resuspended in 80 μl 2% (w/v) sodium dodecyl sulphate (SDS) plus 10% (v/v) β-mercaptoethanol and heated at 100°C for 5 min. Samples were analysed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) on homogeneous 15% (w/v) acrylamide gels.

**Fermentation and isolation of inclusion bodies**

The bacterial strain carrying the required plasmid, that is plasmid [Met]'-pGH(1–46)-Val-Asn-IGF-I, plasmid [Met]'-pGH(1–46)-Val-Asn-[Gly]-IGF-I or plasmid [Met]'-pGH(1–46)-Val-Asn-[Arg]-IGF-I, was selected as a single colony and used to inoculate a 20ml starter culture. The medium employed contained 0.2 mol glucose/l, 2.3 mmol MgSO₄/1, 30 mmol NH₄Cl/l, 6.9 mmol K₂SO₄/1, 12 mmol KH₂PO₄/1, 18 mmol Na₂HPO₄/1, 0.3 mmol Na₃C₆H₅O₇/1, 30 μmol MnSO₄/1, 30 μmol ZnSO₄/1, 3 μmol CuSO₄/1, 72 μmol FeSO₄/1, 0.12 mmol thiamine/l and 50 μg ampicillin/ml, with the final pH adjusted to 7.2 by addition of NH₄Cl. Following growth at 37°C for 20 h, a 15 litre fermentor (Chemap, Volketswil, Switzerland) was inoculated with the 20ml starter culture. The bacteria were grown in 13 litres of the above medium without ampicillin at 37°C, 55% pH, and pH 7.0 until the absorbance at 600 nm was approximately 20. Regulation of temperature, pH, oxygen tension and glucose carbon supply was under automatic control. Subsequently, the cultures were induced with 0.32 mmol IPTG/l for 5 h. Inclusion body formation was monitored by phase-contrast microscopy. Cells were harvested by centrifugation, suspended at 40% (v/v) in a solution of 20 mmol Tris/l and 50 mmol NaCl/l at pH 8.5 and homogenized at 62 MPa. The homogenate was diluted to 10% (v/v) with water and the inclusion bodies were collected by centrifugation. The inclusion bodies were washed by suspension in a solution containing 20 mmol Tris/l, 5 mmol EDTA/l and 0.02% (w/v) lysozyme at pH 8.0, incubated for 3 h at 20°C, collected by centrifugation and the wet paste stored at -80°C.

**Extraction and cleavage of the N-terminal fusion protein**

For all fusion protein constructs, 25 g inclusion bodies were resuspended at 10% (w/v) in a solution of 8 mol urea/l, 50 mmol glycine/l, 10 mmol EDTA/l and 10 mmol dithioerythritol/l at pH 9.2. The solubilized inclusion bodies were desalted on a BP 113 column (113 mm diameter×150 mm; Pharmacia-LKB Pty Ltd) packed with Sephadex G-25M (Pharmacia Pty Ltd) and equilibrated with a solution containing 8 mol urea/l and 50 mmol glycine/l at pH 9.2. Cleavage of the Asn-Gly bond linking the N-terminal extension peptide to the IGF sequence was carried out essentially as described by Bornstein & Balian (1977) by addition of NH₂OH to the desalted inclusion bodies to a final concentration of 3 mol/l, adjusting the pH to 9.2 with LiOH and incubating the mixture at 45°C for 4 h. The reaction was terminated by chromatography on Sephadex G-25M as described above.

**Reduction, refolding and purification of the IGF peptide**

Prior to refolding, the desalted cleavage reaction products were diluted with desalting buffer to a final protein concentration of 8 mg/ml. The cleaved IGF was then reduced by adding 8 mmol β-mercaptoethanol/l, mixed with three volumes of 0.1 mol Tris–HCl/l (pH 8) and incubated for 5 min at room temperature. Refolding of the reduced peptide was started by the addition of an equal volume of a solution containing 0.1 mol Tris/l, 2 mol urea/l and 0.2 mmol 2-hydroxyethyl disulphide/l (Aldrich, Milwaukee, WI, U.S.A.) and adjustment to pH 8 with 6 mol HCl/l. The refolding reaction was allowed to proceed for 15 h at 25°C and the reaction monitored by analysis on a microbore C4 reverse-phase high-performance liquid chromatography (HPLC) column (2.1 mm diameter×100 mm; Brownlee Laboratories, Santa Clara, CA, U.S.A.) employing a gradient from 20 to 80% (v/v) acetonitrile over 30 min in the presence of 0.1% (v/v) trifluoroacetic acid (TFA) and at a flow rate of 0.2 ml/min. The refolding reaction was terminated by acidification to pH 2.5 with the addition of concentrated HCl.

The cleaved and refolded IGF peptide was purified by reverse-phase HPLC chromatography on a C4
Prep-Pak column (25 mm diameter x 100 mm; 30 nm pore size, 15 μm particle size; Millipore-Waters, Millford, MA, U.S.A.). The acidified refolding products were adjusted to 0.1% TFA and then approximately 500 mg peptide pumped on to the C4 column. Protein, monitored by absorbance at 280 nm, was eluted at a flow rate of 15 ml/min with a gradient of 0–40% (v/v) 2-propanol over 30 min in the presence of 0.1% TFA. Fractions of 1 min were collected and analysed by microbore C4 reverse-phase HPLC as described above. This step was repeated until all the peptide had been processed. Equivalent fractions containing IGF-I peptide from each cycle of chromatography, as determined by comparison with standard IGF-I, were pooled for further processing. The selected IGF-I-containing fractions from each cycle were processed in 8 mg batches by pumping directly onto a Pharmacia Mono S HR 5/5 column (Pharmacia Pty Ltd) equilibrated with 0.01 mol ammonium acetate/1 buffer (pH 4.8), in 10% (v/v) acetonitrile. Protein, monitored by absorbance at 280 nm, was eluted at a flow rate of 1 ml/min with a gradient of 0.01–1 mol ammonium acetate/1 (pH 4.8), in 10% acetonitrile and fractions analysed as described above on microbore C4 reverse-phase HPLC. Fractions from the cation-exchange step with the same elution time as reference recombinant IGF-I on the C4 analytical column were pooled. These IGF pools were then subjected to three sequential reverse-phase HPLC steps on a C18 Prep-Pak column (25 mm diameter x 100 mm; 12.5 nm pore size, 10 μm particle size; Millipore-Waters) as described previously (Francis et al. 1988). The steps involved (i) employing an acetonitrile gradient with heptafluorobutyric acid as the counter ion, (ii) a 1-propanol gradient and heptafluorobutyric acid counter ion, and (iii) an acetonitrile gradient with TFA as the counter ion. At each stage, fractions to be pooled for the next step were selected after analysis on the microbore C4 HPLC column.

The amounts and purity of IGF-I polypeptides produced were determined by comparing their spectrophotometric absorbance at 215 and 280 nm with those of porcine insulin (Sigma) and reference recombinant IGF-I, by reverse-phase chromatography on a Novapak C18 radial compression cartridge as described previously (McKinnon et al. 1991). N-terminal sequencing was performed on a gas-phase sequencer ( Applied Biosystems, Foster City, CA, U.S.A.; model 470A) by employing the methods of Hunkapiller, Hewick, Dreyer & Hood (1983).

IGF-I radioimmunoassay

Measurement of immunoreactivity was carried out as described previously using the Adelaide polyclonal anti-IGF-I serum (Ballard, Johnson, Owens et al. Journal of Molecular Endocrinology (1992) 1990). Reference recombinant IGF-I was used as tracer and standard in the assay.

IGF binding to cell receptors

Binding to the type-1 or type-2 IGF receptors on rat L6 myoblasts was measured using 125I-labelled recombinant IGF-I (Genentech) and hIGF-II respectively (Ross et al. 1989). Briefly, radiolabelled ligand was added in the presence of increasing concentrations of unlabelled peptides in a total volume of 0.5 ml to myoblast monolayers in 24-place multiwells. Following incubation at 2°C for 18 h, the monolayers were washed at 0°C to remove unbound radioligand and the bound radioactivity was measured. Binding was expressed as the percentage of that occurring in the absence of unlabelled IGF peptide.

Effect of IGF peptides on DNA synthesis

Confluent monolayers of L6 rat myoblasts in 24-place multiwell dishes were washed twice over 2 h with Dulbecco’s-modified minimal essential medium (DMEM). Each multiwell dish was subsequently incubated for 24 h in DMEM containing IGF peptides at the indicated concentrations. During the last 4 h of this period, 1 μCi (5 nmol) [3H]thymidine was added per well (Ross et al. 1989). Cells were harvested as described below for protein synthesis measurements. DNA labelling in test wells was expressed as the percentage incorporation relative to a control without added IGF.

Effect of IGF peptides on protein synthesis

Stimulation of protein synthesis in L6 rat myoblasts by growth factors was measured as described previously (Francis, Read, Ballard et al. 1986). Briefly, the lyophilized peptides for assay were dissolved in 0.01 mol HCl/l and diluted into a buffer solution of 0.01 mol potassium phosphate/l (pH 7.4), containing 0.9% (w/v) NaCl and 0.1% (w/v) bovine serum albumin (Sigma; radioimmunoassay grade). Confluent monolayers of L6 rat myoblasts in 24-place multiwell dishes were washed twice over 2 h with DMEM to remove any residual fetal bovine serum. Each multiwell dish was subsequently incubated for 18 h in DMEM containing IGF peptides at the indicated concentrations and 1 μCi [3H]leucine/well. Stimulation of protein synthesis was measured as the increased incorporation of [3H]leucine into total cell protein over an 18-h incubation period above a buffer control.

Effect of IGF peptides on intracellular protein breakdown

Confluent monolayers of rat L6 myoblasts in 24-place multiwells were prelabelled for 18 h in growth medium
with [3H]leucine as the only source of leucine other than 5% (v/v) fetal bovine serum, after which unstable proteins were degraded during a 2-h chase period in the presence of 2 nmol unlabelled leucine/l. Protein degradation was determined as the percentage of labelled protein that is degraded to trichloroacetic acid-soluble radioactivity over a 4-h measurement period in the presence of added peptides (Ballard, Read, Francis et al. 1986). Breakdown rates were expressed as the percentage inhibition relative to the rate in serum-free DMEM plus the sample diluent.

Measurement of binding to bovine IGFBP-2

The relative binding of the various IGF peptides to bovine IGFBP-2 (bIGFBP-2) purified from MDBK cell-conditioned medium was measured as described previously (Szabo et al. 1988). Briefly, 5 ng bIGFBP-2 were incubated with 125I-labelled hIGF-I in the presence or absence of competing unlabelled IGFs at room temperature for 2 h. Free 125I-labelled hIGF-I was separated from IGFBP-bound label by incubating the reaction mixture with activated charcoal at 4°C for 30 min and then centrifuging the mixture. The radioactivity associated with the supernatant was determined and used to calculate the percentage of added radioactivity bound to bIGFBP-2 in the supernatant. Binding was expressed as the percentage of 125I-labelled hIGF-I bound in the absence of added unlabelled IGF peptide.

RESULTS

Analysis of [Met1]-pGH-Val-Asn-IGF-I expression in small-scale cultures

A series of vectors expressing IGF-I as part of a fusion protein with N-terminal [Met1]-pGH was constructed (Fig. 1). The plasmid [Met1]-pGH-Val-Asn-IGF-I propagated in E. coli strain JM101 when induced with IPTG directs efficient expression of a fusion protein of approximately 26 kDa. The level of expression was higher than that observed with [Met1]-pGH alone, when vector pGHXSC.4 is used (data not shown). Since there are solubility problems with recombinant [Met1]-pGH, arising from the tendency of the recombinant protein to self associate and form aggregates (Fridman, Aguilar & Hearn, 1990) and because we wished to minimize the pGh component of the fusion protein, we proceeded to delete the C-terminal coding portions of the pGh component in novel expression cassettes. There was little effect of this deletion on induced protein levels, indicating an increased overall yield of IGF-I (Fig. 2). All the fusion proteins were contained within large inclusion bodies (data not shown). Expression of the two analogues [Met1]-pGH(1-46)-Val-Asn-[Gly3]-IGF-I and [Met1]-pGH(1-46)-Val-Asn-[Arg3]-IGF-I was similar to that described for the IGF-I-containing construct.

Production of folded IGF-I peptide from the [Met1]-pGH(1-46)-Val-Asn-IGF-I fusion protein

The recombinant IGF-I and its analogues [Gly3]-IGF-I and [Arg3]-IGF-I were produced in E. coli as a fusion protein also containing the first 46 N-terminal amino acids of [Met1]-pGH ([Met1]-pGH(1-46)). These two amino acid sequences were linked by the dipeptide Val-Asn, providing a convenient Hpal restriction site for expression vector construction. Furthermore, the inclusion of the Asn residue immediately prior to the N-terminal Gly of IGF-I created the only Asn-Gly bond in the fusion protein, thus allowing specific chemical cleavage with hydroxylamine (Bornstein & Balian, 1977). Cleavage at the sensitive Asn-Gly bond by hydroxylamine generated two new peaks on microbore reverse-phase HPLC with all of the fusion proteins (Fig. 3a and b). Analysis of the reduced cleavage products of [Met1]-pGH(1-46)-Val-Asn-IGF-I revealed a peak eluting at 19 min in the gradient, the same elution time as reduced IGF-I standard. The peak eluting at 22 min was not positively identified but possibly represents the N-terminal [Met1]-pGH(1-46)-Val-Asn component of the fusion protein. As would be expected for this latter peptide, its elution position was not altered by the redox conditions of the refolding reaction. In contrast, the putative reduced IGF-I peptide generates two peaks eluting earlier, with one of these at 15 min, the position expected for IGF-I (Fig. 3c). This material was purified to yield a homogeneous preparation of IGF-I (Fig. 3d).

Typical fermentations yielded 60–90 g wet weight of inclusion bodies/13 litres and each purification cycle started with 25 g wet weight of inclusion bodies. As an example, in one purification cycle for IGF-I, the starting inclusion bodies contained 4.47 g total peptide, as determined by analytical reverse-phase HPLC (Fig. 3a). Hydroxylamine cleavage of this material produced 912 mg peptide in the putative IGF-I peak as described above (Fig. 3b). After the refolding step this yielded 262 mg peptide that co-eluted in the same peak with added IGF-I on analytical C4 reverse-phase HPLC (data not shown). The final yield of homogeneous IGF-I peptide after cation-exchange and preparative reverse-phase HPLC steps was 25 mg. Recoveries were low due to the rigorous removal of side fractions at each step.
Figure 1. Construction of plasmids expressing methionyl porcine GH(1-46)-Val-Asn-insulin-like growth factor-I fusion protein ([Met']-pGH(1-46)-Val-Asn-IGF-I) and its variants. Sequences encoding human IGF-I (hIGF-I) are represented by open boxes. Numbering of amino acids proceeds from the N terminus of [Met']-pGH or IGF-I. The hydroxylamine-labile Asn-Gly bond is indicated by open triangles. Step 1: an M13mp8 clone containing synthetic hIGF-I sequences (mIGF) was restricted with Ncol, the site filled in with Klenow DNA polymerase I and deoxynucleotides and the IGF fragment isolated following digestion with HindIII. Ligation of this fragment to PvuII- and HindIII-cut pGHXSC.4, which also contained the ampicillin resistance gene (amp), gave in-frame cloning of [Met']-pGH and IGF-I sequences. Step 2: sequences deleted by the cloning step 1 plus a codon for Asn to create an Asn-Gly bond were replaced by in-vitro mutagenesis using oligo A. This procedure is described in Materials and Methods. Step 3: plasmid [Met']-pGH-Asn-IGF-I (p[Met']-pGH-Asn-IGF-I) was digested with AvaI and PvuII. Following filling in of the AvaI site, the vector was isolated and religated, resulting in deletion of sequences coding for [Met']-pGH amino acids 89-188. Step 4: deletion mutagenesis in vitro using oligo 46 (see Materials and Methods) was used to remove all but the N-terminal 46 amino acids of [Met']-pGH while creating a unique restriction enzyme site, HpaI, at the junction between [Met']-pGH and IGF-I.
N-terminal sequencing of the purified peptides gave only the expected sequence for IGF-I, [Arg³]-IGF-I or [Gly³]-IGF-I during 20–40 cycles of Edman degradation. The purified recombinant peptides were also characterized in an hIGF-I radioimmunoassay (Fig. 4). Fusion protein-derived IGF-I produced here showed almost identical potency in this assay as reference recombinant hIGF-I that had been used to prepare the radiolabelled ligand. The amounts of peptides required for half-maximal competition in the assay were as follows: reference recombinant hIGF-I, 402 pg; fusion protein-derived IGF-I, 402 pg; bovine IGF-I, 521 pg; [Gly³]-IGF-I, 521 pg and [Arg³]-IGF-I, 771 pg.

Biological activity of fusion protein-derived IGF-I, [Gly³]-IGF-I and [Arg³]-IGF-I in L6 myoblasts

The anabolic activities of recombinant IGF-I and the analogues [Gly³]-IGF-I and [Arg³]-IGF-I were determined in L6 rat myoblasts. In the protein synthesis assay, all the IGF peptides stimulated the myoblasts to give a maximum response, equivalent to that observed with the addition of 10% (v/v) fetal bovine serum where protein synthesis was stimulated 370% above control cultures (Fig. 5a). The fusion protein-derived IGF-I was equipotent to the bacterial reference recombinant IGF-I supplied by Genentech. In this assay, native bovine IGF-I showed similar activity to both these peptides. Half-maximal responses of that obtained with 10% (v/v) fetal bovine serum occurred at 2.9 ng [Arg³]-IGF-I/ml, 7.9 ng [Gly³]-IGF-I/ml, 15.8 ng fusion protein-derived IGF-I/ml, 15.8 ng reference recombinant IGF-I/ml and 17.1 ng bovine IGF-I/ml. A reproducible difference in the shape of the dose–response curves between the two analogues and IGF-I was also observed. This resulted in greater potency differences between the peptides at 25% of the maximal response level, where the amount of peptide required was 0.76 ng [Arg³]-IGF-I/ml, 1.4 ng [Gly³]-IGF-I/ml and 5.6 ng fusion protein-derived IGF-I/ml.

Protein breakdown of prelabelled-cell protein in L6 myoblasts was inhibited to the same maximal effect by all peptides, approximately 35% over the 4-h period of measurement compared with 22% inhibition by 10% fetal bovine serum (Fig. 5b). The order of potency, with the concentrations required for a half-maximal response given in parentheses, was [Arg³]-IGF-I (0.3 ng/ml) > [Gly³]-IGF-I (0.7 ng/ml) > fusion protein-derived, reference recombinant and bovine IGF-I (1.3 ng/ml).

The ability of the peptides to stimulate DNA synthesis was determined and, at the highest levels tested, achieved 50% of the maximal response achieved with 10% (v/v) fetal bovine serum (Fig. 5c). In the presence of 10% (v/v) fetal bovine serum the incorporation of [³H]thymidine into L6 myoblasts was stimulated 500% compared with control cultures. The order of potency, with the concentrations required to achieve 25% of the maximal response with 10% (v/v) fetal bovine serum given in parentheses, was [Arg³]-IGF-I (7.7 ng/ml) > [Gly³]-IGF-I (24 ng/ml) > fusion protein-derived, reference recombinant and bovine IGF-I (49 ng/ml).
Binding of IGF-I analogues to cell receptors and IGFBPs

To investigate the basis for increased potency of [Gly³]-IGF-I and [Arg³]-IGF-I in the anabolic assays, their relative affinity for the type-1 IGF receptor on L6 myoblasts was determined (Fig. 6a). Competition for binding of [¹²⁵I]-labelled IGF-I increased in the following order, with the concentrations giving half-maximal competition shown in parentheses: [Gly³]-IGF-I (10.0 ng/ml), [Arg³]-IGF-I (8.2 ng/ml), fusion protein-derived IGF-I and bovine IGF-I (5.2 ng/ml) and reference recombinant IGF-I (4.2 ng/ml).

Competition for binding of [¹²⁵I]-labelled IGF-I to purified bIGFBP-2 was similar for all three preparations of IGF-I, with half-maximal displacement at approximately 5 ng peptide/0.25 ml added to the assay (Fig. 6b). However, [Gly³]-IGF-I and [Arg³]-IGF-I competed poorly and, even when more than 250 ng peptide were added in the assay, the analogues displaced less than 50% of the radiolabelled ligand. Thus 25% displacement of radiolabelled IGF-I was achieved with 390 ng [Arg³]-IGF-I, 100 ng [Gly³]-IGF-I but only 1.7 ng fusion protein-derived IGF-I.

DISCUSSION

We have reported the production of recombinant...
IGF-I and the biologically potent variant des(1-3)IGF-I following the expression of gene constructs in Chinese hamster ovary cells (McKinnon et al. 1991). In this transformed mammalian cell system, both IGF peptides were secreted into the extracellular medium as biologically active molecules. However, the yield of purified peptide was low, approximately 25 ng/l of conditioned medium processed. On the other hand the yields reported here are much higher, approximately 5 mg/l of fermentation broth. We have achieved this higher yield by the adaptation of an efficient expression system for [Met]-pGH to enable the production of IGF-I and several analogues as fusion proteins linked to residues 1–46 of [Met]-pGH. These fusion proteins are even more efficiently expressed than [Met]-pGH alone, where translational efficiency had been optimized (Vize & Wells, 1987). This conclusion is also supported by visual inspection of the protein gels in Fig. 2, where reducing the length of the pGH N-terminal extension does not appear to reduce the level of fusion protein expression. The improved efficiency may be

**FIGURE 4.** Radioimmunoassay of insulin-like growth factors (IGFs) using a polyclonal antiserum raised against bovine IGF-I and recombinant human IGF-I as radioligand. The peptides tested were fusion protein-derived IGF-I (○), [Gly]-IGF-I (●), [Arg]-IGF-I (▼), reference recombinant IGF-I (▼) and bovine IGF-I (□). Values are means of triplicate determinations; S.E.M. values are indicated where they are larger than the symbol size. In this assay 31% of the added radioactivity was bound in the absence of competing peptides. Non-specific binding in the absence of antibody (1.1% of added radioactivity) has been subtracted.

**FIGURE 5.** Biological effects of insulin-like growth factors (IGFs) on (a) protein synthesis, (b) protein breakdown and (c) DNA synthesis in rat L6 myoblasts. The peptides tested were fusion protein-derived IGF-I (○), [Gly]-IGF-I (●), [Arg]-IGF-I (▼), reference recombinant IGF-I (▼) and bovine IGF-I (□). Values are means of triplicate determinations on three cultures at each peptide concentration; S.E.M. values are indicated where they are larger than the symbol size.
to 1 g fusion protein/l of fermentation broth. The method reported here could be readily scaled up and yields optimized at each step to produce the large quantities of IGF-I or variants required for testing their efficacy as anabolic agents in vitro.

The production of recombinant proteins in E. coli as fusion proteins has been widely reported (Bruggmann, Chaundhary, Gottesman & Pastan, 1991). Furthermore, this approach has also been used for the production of hIGF-I (Forsberg, Palm, Ekebacke et al. 1990), truncated hIGF-I (Forsberg, Baastrup, Brobjer et al. 1989) and hIGF-II (Furman, Epp, Hsiung et al. 1987). In the present report the recombinant protein was successfully produced from inclusion bodies formed in E. coli. This involved first unfolding the polypeptide chain in a denaturating buffer under reducing conditions and then removing the unwanted N-terminal [Met¹]-pGH(1-46)-Val-Asn extension peptide by cleaving the hydroxyamine-sensitive Asn-Gly bond linking it to the IGF-I sequence. The cleavage reaction proceeds with a high yield and a single major product was indicated by analytical reverse-phase HPLC (Fig. 3b). However, the generation of undesirable modified forms of recombinant proteins in various heterologous expression systems has been noted on many occasions (Freedman, 1989; Forsberg et al. 1990). In this context we identified several different forms of recombinant IGF-I and des(1-3)IGF-I produced by transformed Chinese hamster ovary cells (McKinnon et al. 1991). Although we did not establish the basis for their different chromatographic properties, these forms appeared to possess identical biological properties, stimulating protein synthesis and binding to the type-I IGF receptor with equivalent potency.

During refolding of the cleaved IGF-I peptide, two prominent peaks of refolded peptide were produced; the later peak eluting at 16 min was identified as correctly folded IGF-I and subsequently purified (Fig. 3c). We believe that the peak eluting before the major refolded IGF-I peak at 15 min is probably a misfolded isomer, equivalent to that observed previously by ourselves (Bagley et al. 1989) and others (Raschdorf, Dahinden, Maerki et al. 1988). Clearly, the production of recombinant proteins in heterologous expression systems provides ample opportunity for modification of the coded genetic recombinant sequence at any stage from fermentation to downstream processing. Structural analysis of the biologically active products produced in heterologous recombinant DNA expression systems requires the application of extremely sophisticated and time-consuming techniques, such as mass spectrometry and amino acid sequencing (Pramanik, Tsarbopoulos, Labdon et al. 1991).

**FIGURE 6.** Competition by growth factors for the binding of ¹²⁵I-labelled insulin-like growth factor-I (IGF-I) to (a) rat L6 myoblasts and (b) bovine IGF-binding protein-2. The peptides tested were fusion protein-derived IGF-I (○), [Gly³]-IGF-I (●), [Arg³]-IGF-I (▲), reference recombinant IGF-I (▼) and bovine IGF-I (□). Values are means of triplicate determinations at each peptide concentration; s.e.m. values are indicated where they are larger than the symbol size.

Due to an increased rate of translation or to post-translational events such as enhanced inclusion body formation and lowered cell toxicity with the fusion protein. Our attempts at direct expression of methionyl IGF-I yielded no detectable protein in spite of translation in vitro, presumably due to inefficient translation or instability of the peptide in E. coli (data not shown). However, the use of a [Met¹]-pGH-based leader peptide of only 48 amino acids circumvents such problems, resulting in yields of up
were to hand, recombinant IGF-I, been natural blue-staining electrophoresis by ing phase amino acid sequencing and homogeneity on reverse-phase HPLC employing very shallow gradients to establish the purity of our end products. The requirement for very high purity was largely responsible for lowering the yields of final product. Gradient SDS-PAGE was a much less discriminating indicator of the microheterogeneity in the processed recombinant proteins. For example, relatively heterogeneous peptide mixtures, as indicated by reverse-phase HPLC, migrated on denaturing electrophoresis gels as a single band of Coomassie blue-staining peptide (results not shown).

The immunoreactivity of the fusion protein-derived IGF-I in a polyclonal IGF-I radioimmunoassay was comparable to that observed for the reference recombinant hIGF-I and natural bovine IGF-I which has an identical sequence (Fig. 4). This result provides further support for our conclusion that the IGF-I produced from the fusion protein was not significantly altered in any way from natural IGF-I. Previously, we suggested that this polyclonal antisem is sensitive to amino acid sequence changes near the N terminus of IGF-I, so that any significant change in structure might have been expected to have some effect on the epitopes recognized (Bagley et al. 1989). However, substitution of Glu at position 3 with Gly only slightly reduced the immunoreactivity compared with recombinant IGF-I. [Arg^3]-IGF-I, on the other hand, was only about 50% as potent as the fusion protein-derived IGF-I. These results suggest that there was no dramatic alteration in the structure of the analogues. Biological assays of the recombinant peptides in rat L6 myoblasts revealed potencies in the order [Arg^3]-IGF-I > [Gly^3]-IGF-I > IGF-I. All the peptides were capable of stimulating protein synthesis maximally, but the dose–response curves of the variants were different. At the half-maximal dose–response level, [Arg^3]-IGF-I and [Gly^3]-IGF-I were respectively 5.4- and 2.0-fold more potent than reference recombinant or natural IGF-I (Fig. 5a). Similar increases in potency were shown by the variants in protein breakdown and DNA synthesis bioassays (Fig. 5b and c).

Examination of receptor binding by the analogues to the type-I receptor on L6 myoblasts did not offer an explanation for their increased potency. In fact, [Gly^3]-IGF-I and [Arg^3]-IGF-I competed 1.9- and 1.6-fold less effectively than IGF-I respectively, for binding of radiolabelled IGF-I to the myoblasts (Fig. 6a). Thus [Arg^3]-IGF-I and [Gly^3]-IGF-I were more potent than IGF-I in stimulating protein and DNA synthesis as well as inhibiting protein breakdown, even though they showed reduced binding in the IGF-I radioreceptor assay. IGF-I is known to exert its anabolic effects on protein metabolism in L6 myoblasts through the type-I IGF receptor (Ewton, Falen, & Florini, 1987; Ballard, Ross, Upton, & Francis, 1988). Not surprisingly, we found that, like IGF-I, the potent analogues did not compete for binding of radiolabelled IGF-II to these cells (data not shown).

We have shown previously that the increased potency of the truncated variant des(1–3)IGF-I, compared with IGF-I, results from reduced binding to IGFBPs (Ross et al. 1989). Cascieri, Hayes & Bayne (1989) also characterized two analogues of IGF-I with N-terminal alterations that have increased biological potency in Balb/C 3T3 cells and concomitant reduced affinity for the 28kDa IGFBP secreted by these cells. Here we report two analogues of IGF-I with substitutions at position 3 that show poor binding to a purified binding protein, bovine IGFBP-2 (Fig. 6b). [Arg^3]-IGF-I and [Gly^3]-IGF-I were 230- and 59-fold less potent than IGF-I respectively in competing for 12I-I-labelled IGF-I binding to bovine IGFBP-2. This reinforces our earlier work indicating the importance of the Glu^3 residue in binding to this and other IGFBPs (Bagley et al. 1989). It is well established that L6 myoblasts secrete IGFBPs into the extracellular medium (Bagley et al. 1989; McCusker, Camacho-Hubner, & Cleemmons, 1989). Recently, we reported that a number of N-terminal analogues of IGF-I prepared by chemical synthesis, including [Arg^3]-IGF-I and [Gly^3]-IGF-I, displayed very low binding to the IGFBPs present in L6 myoblast-conditioned medium (Wallace, Bagley, May et al. 1989). Consequently, a likely explanation for the increased anabolic activity of [Gly^3]-IGF-I and [Arg^3]-IGF-I, compared with IGF-I, is their reduced binding to the IGFBPs secreted by L6 myoblasts.

A reproducible difference in the shape of the dose–response curves for the analogues compared with IGF-I was observed in both the protein and DNA synthesis assays (Fig. 5a and c). We propose that endogenous IGFBPs secreted by the cells bind small amounts of added IGF-I and effectively inhibit its action, and that a biological response is not observed until the IGFBP is titrated. Because the analogues have much reduced affinity for IGFBPs, and hence would be less affected by this phenomenon, even small amounts of the analogues remain free and are biologically active through binding to the type-I IGF receptor. This interpretation is supported by our observations on the inhibitory effect of added IGFBPs on the actions of IGF-I but not des(1–3)IGF-I in chicken embryo fibroblasts, a cell line that does not secrete IGFBPs into the extracellular medium (Ross et al. 1989).

In this report we have described an efficient
expression system in E. coli for IGF-I as a fusion protein with an N-terminal extension that includes the first 46 amino acids of methionyl pGH. This system has been used to produce significant quantities of IGF-I and two variants, [Gly³]-IGF-I and [Arg²]-IGF-I, which show enhanced biological potency compared with IGF-I.

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