Vol. 166, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1990 Pages 50-60

EXPRESSION OF HUMAN PARATHYROID HORMONE IN ESCHERICHIA COLI

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Received June 28, 1989

Human parathyroid hormone (hPTH) is a peptide hormone consisting of 84 amino acids. Using the expression plasmid pKK223-3 with the strong tac-promoter, we have produced a variant of hPTH in E. coli. From the expression plasmid construct the expected product was hPTH with an N-terminal extension of Met-Gly. The peptide was extracted from E. coli cells and purified by high performance liquid chromatography. In two different gel electrophoresis systems including identification by immunoblotting the product behaved exactly as an hPTH standard. N-terminal amino acid sequence analysis of the purified product showed traces of Gly-hPTH. At least 90% of the expressed product was N-terminally blocked, suggesting the presence of N-formyl-methionine. This variant of hPTH did not stimulate adenylate cyclase activity in rat osteosarcoma cell membranes.

Human parathyroid hormone (hPTH) is an 84 amino acid peptide which is secreted from the parathyroid glands. The primary translation product is a 115 amino acid preprohormone, and the prepro part is cleaved off during secretion, yielding the 84 amino acid mature hormone (1).

hPTH is a principal homeostatic regulator of blood calcium and phosphate through its actions on kidney and bone (2,3). Prolonged exposure to low doses of an N-terminal fragment of PTH stimulates bone formation in vivo (4,5), while chronic overproduction (hyperparathyroidism) leads to demineralization.

Abbreviations: PTH, parathyroid hormone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PBS, phosphate buffered saline (0.15 M NaCl, 0.015 M NaH_2PO_4, pH 7.4); IPTG, isopropyl-β-D-thiogalactoside; fMet, N-formyl-methionine.
hormone, we have cloned its cDNA for expression in E. coli.

0006-291X/90 $1.50

The different domains within the PTH molecule appear to be specialized with respect to conveying its biological action in bone and kidney (2).

Therefore in order to learn more about the structure-function relationships of hPTH, and also to be able to do physiological studies with the intact hormone, we have cloned its cDNA for expression in E. coli.

MATERIALS AND METHODS

Strains, media and growth of cells

E. coli strains used were BJ5183 (6) and JM 103 (7). LB-medium contains 5 g Bacto Yeast Extract, 10 g Bacto Tryptone and 10 g NaCl per l, if necessary supplemented with 50 mg/l of ampicillin. For expression of hPTH, JM103 was grown in LB-medium with ampicillin. At an A_{600} of 0.2, IPTG (Pharmacia) was added to a final concentration of 1 mM.

Materials

Restriction enzymes, other DNA-modifying enzymes and [^{125}I]-antirabbit-IgG were from Amersham. The N-terminal-specific anti-PTH antibody was from Chemicon, the preparation of the other antibodies has been described earlier (8). Synthetic hPTH(1-84) was from Sigma or Bacham.

Cloning of hPTH cDNA

Poly(A) selected RNA was isolated from human parathyroid adenomas immediately after surgery. cDNA was prepared and cloned into the PstI site of pBR322 by the GC-tailing method as described by Maniatis et al. (9), and E. coli-strain BJ5183 was transformed by the method of Hanahan (7). The library was screened with synthetic oligonucleotides, based on the published sequence of cDNAs for hPTH (10).

DNA sequencing was done according to the methods of Maxam and Gilbert (11) and Chen and Seeburg (12).

Radioimmunoassay

Radioimmunoassay of hPTH was carried out as described (8) using a monospecific, polyvalent antiserum reactive against epitopes between amino acids 44 and 68 in hPTH.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of SDS was performed as described by Laemmli (13). Gels were silver stained with a BioRad silver staining kit according to the manufacturers recommendations.

For electrophoresis in the presence of acetic acid and urea, the gel was made up with a solution containing 4.5 M urea and 0.9 M HAc. Freeze-dried samples were dissolved in a sample buffer containing 0.9 M HAc, 8 M urea, 2% 2-mercaptoethanol and 0.05% pyronine Y.

Immunoblotting

Proteins fractionated by PAGE were transferred electrophoretically to Immobilon PVDF Transfer Membranes (Millipore), and staining of the filters and antibody was performed according to the manufacturers recommendations. Cock anti-PTH antiserum that reacts with epitopes within amino acid number 44-68 (8) was used (dilution 1:8000) as a primary antibody and anti-cock-IgG (dilution 1:1000) as the secondary antibody. As a tertiary antibody we used [^{125}I]-antirabbit-IgG from donkey. The N-terminal specific anti-hPTH rabbit antiserum was used (dilution 1:1000) with a secondary [^{125}I]-antirabbit-IgG from donkey.

Extraction of PTH from E. coli cells

Cells from 3 liters of culture were harvested by centrifugation at OD_{600}=1.0, and were washed 2 times in PBS containing 0.1 mM PMSF. The pellet was resuspended in 4 ml of lysozyme solution (10 mg/ml in water) and left at room temperature for 15 min. The wet weight of this suspension was determined, before being frozen at -70 °C. After thawing on ice, the suspension was sonicated 3 x 20 s with a Model W-10 Sonicator (Ultrasonics). PTH was then

extracted essentially as described by Aurbach (14). After ether precipitation and acetone washing (14) the dry powder was extracted with 20% acetone, 0.1% HAc, and the extracted material was used as the starting material for further purification. The yield of immunoreactive material at this stage was 200 µg per l of original culture, corresponding to a yield of about 20%.

HPLC-purification

PTH was further purified by reversed phase HPLC using a Vydac RP C18 protein/peptide column. A 25 cm x 4.6 mm column was used employing LDC constametric pumps model I and III, LDC gradient master, LDC spectromonitor III (LDC, Milton Roy Co, Riviera Beach, FL, USA) and a Vitatron 2 channel recorder. The experimental conditions were as follows: Eluants: A: 0.1% triflouroacetic acid in filtered and distilled H_2O; B: 70% acetonitrile in A. Flow: 1.0 ml/min. Gradient: 35-55% B (linear) in 48 min. Washing with 100% B for 10 min and equilibration with 35% B.

Amino acid sequence analysis

This was done on material separated by SDS-PAGE as described by Matsudaira (15) in a model 477A Protein Sequencer with an on-line 120A phenylthiohydantoin amino acid analyzer from Applied Biosystems (Foster City, CA, USA). All reagents were obtained from Applied Biosystems. Amino acid sequencing was performed by Dr. K. Sletten, Inst. of Biochemistry, University of Oslo.

Biological activity measurements

Recombinant hPTH was purified on HPLC and freeze dried. It was dissolved in distilled water and tested in the adenylate cyclase assay using of UMR 106 rat osteosarcoma cell membranes. Membranes were prepared and the assay carried out as previously described (16,17). The experiments was performed in triplicate determinations which differed by less than 17%.

RESULTS

Cloning and sequencing of cDNA for hPTH

A cDNA library was made as described in Materials and Methods. 66 out of 34 000 tested clones hybridized to the probes, and two of these were chosen for further study. DNA-sequencing showed that both clones contained the entire coding region for preproPTH, and that the sequence of the insert in clone 6 was identical to the sequence published by Hendy et al. (10). In clone 10 however, the sequence diverged from the published sequence in a position just upstream of the start codon (fig.1). This clone contained a double in-frame

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hPTH cDNA (10)          TAT Gtg aag ATG ATA CCT
                                    Met Ile Pro

pSSHPTH-10                    T ATG ATG ATA CCT
                                Met Met Ile Pro

bPTH cDNA (22)                T ATG ATG tct gca
                                Met Met Ser Ala
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Fig.1: The sequence of preproPTH cDNA from pSSHPTH10.
The plasmid pSSHPTH10 was purified by CsCl-centrifugation, and the insert was sequenced by the method of Maxam and Gilbert (11). The sequence round the translation start is compared to the sequences of cDNA for hPTH 10 and bovine PTH 22.

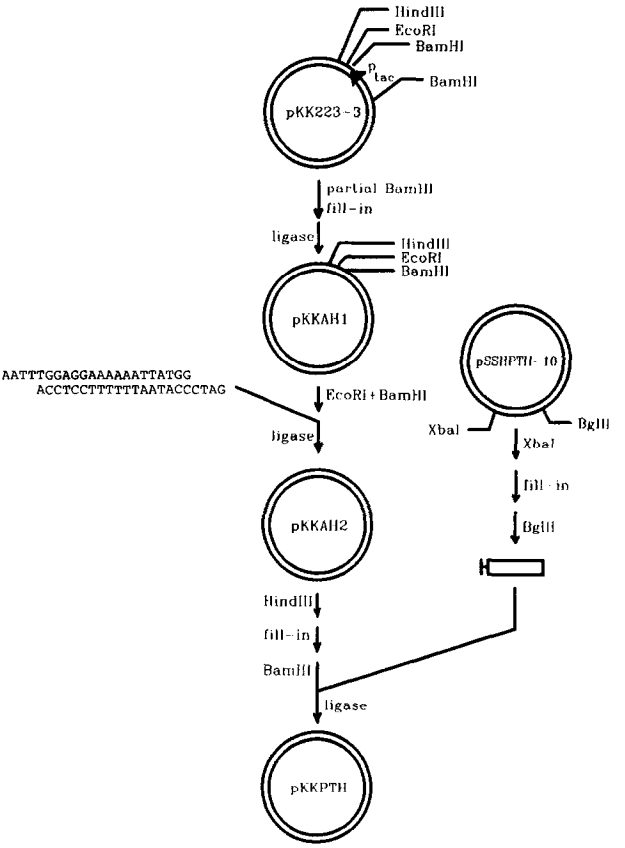

Fig.2: Construction of the hPTH expression plasmid pKKPTH.
For details, see text.

ATG at the beginning of the open reading frame, suggesting the possibility of alternative translation start-points. This sequence probably represented a 4 bases deletion from the sequence published by Hendy et al. (10), and was not found in other clones that were sequenced (data not shown). The plasmid containing this insert was designated pSSHPTH10.

Construction of an hPTH expression plasmid

pSSHPTH10 was used to construct an expression plasmid as outlined in fig.2. To destroy the BamHI-site outside the polylinker, the expression vector pKK223-3 (18) was partly digested with BamHI, and the sticky ends were filled in by the Klenow fragment of DNA polymerase I and religated with T4 DNA ligase. A vector-clone containing a unique BamHI-site in the polylinker was picked (pKKAH1), and a synthetic oligonucleotide containing a Shine-Dalgarno

sequence and an initiation codon was inserted between the EcoRI and BamHI sites to give the plasmid pKKAH2.

The plasmid pSSHPTH10 was digested with XbaI, filled in by Klenow polymerase and cut with BglII. The resulting 282 bp BglII-XbaI fragment was isolated and inserted between the BamHI and a blunt-ended HindIII site in pKKAH2 to give the final construct pKKPTH. In this construct the hPTH cDNA is under the transcriptional control of the IPTG-inducible **tac**-promoter (19) and the rrnb ribosomal RNA transcription terminator (20). This construct should express hPTH with a Met-Gly amino-terminal extension.

Expression of hPTH

For testing the expression level the cells were harvested at late log phase (A_{600}=1.0), disrupted by sonication, and radioimmunoassay was performed on both the soluble and the insoluble fraction after sonication. The expression level usually was about 2 mg PTH/l culture with more than 95% in the soluble fraction, indicating that Met-Gly-PTH does not aggregate in inclusion-bodies, in contrast to many other exogenous peptides produced in E.coli (21). The radioimmunoassay also showed that the PTH-related material was immunologically indistinguishable from hPTH (data not shown).

Analysis of the expression products by immunoblotting

Cell extracts were fractionated by SDS-PAGE and subjected to immunoblotting. As shown in fig.3 (lanes B and D) two PTH-immunoreactive peptides could be demonstrated in this experiment, and one of these co-migrated with the PTH-standard (lane C). This peptide could not be detected in extracts from control cells transformed with the vector alone (data not shown). The other band (approximately 14 kDa) corresponded to a dominant band seen when silver staining a part of the same gel (fig.3, lane A). The 14 kDa silverstained band probably mainly represents an E. coli protein, but as discussed below the antibody reactivity to this band probably is not due to unspecific binding, but reflects co-migration of a higher MW species of PTH made by E. coli.

A similar experiment was performed fractionating the proteins by HAc/urea PAGE. In this system the peptides are separated according to both charge and molecular weight. The major 14 kDa silverstained band seen after

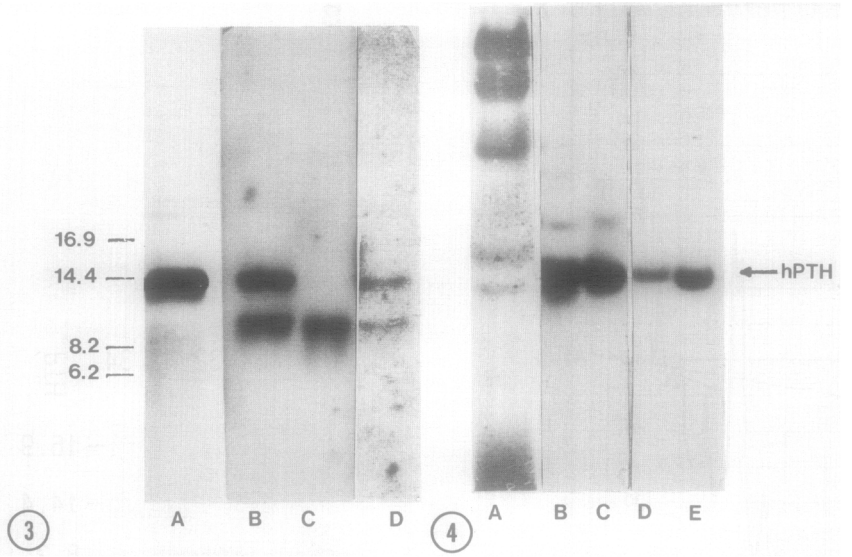

Fig.3: Analysis of expression products by SDS-PAGE and immunoblotting.
PTH expression in E.coli containing the plasmid pKKPTH was induced by IPTG. The cells were disrupted by sonication, and a cell extract was made (14), and subjected to SDS-PAGE (15% gel) under reducing conditions. Lanes were silver stained (A), or electroblotted onto nitrocellulose filters and reacted with antiserum against the mid-region part (B and C) or the N-terminal part (D) of hPTH. Antibody binding was detected by [^{125}I]-antirabbit-IgG. Lane C was loaded with 40 ng of hPTH (Sigma) as a standard, lanes A,B and D were loaded with E. coli extracts containing about 50 ng of hPTH-related material as determined by radioimmunoassay.

Fig.4: Analysis of expression products by HAc-urea polyacrylamide electrophoresis and immunoblotting.
Cell extracts (same as in fig.3) were subjected to HAc/urea PAGE (15% gel). Lanes were silver stained (A) or electroblotted onto nitrocellulose filters and reacted with antiserum against the mid-region part (B,C) or the N-terminal part (D,E) of hPTH. Antibody binding was detected by [^{125}I]-anti-rabbit-IgG. Lanes C and E were loaded with 40 ng of hPTH (Sigma) as a standard, lanes A,B and D were loaded with E. coli extracts containing about 500 ng (A) and 50 ng (B,D) of hPTH-related material as determined by radioimmunoassay.

SDS-PAGE (fig.3, lane A) apparently has split into several bands upon HAc-urea PAGE (fig.4, lane A), but the major immunoreactive band still co-migrated with the PTH-standard (fig.4, lanes B and D).

We also performed an experiment where the proteins were separated by two-dimensional PAGE (data not shown). After running HAc/urea PAGE in the first dimension the band co-migrating with the PTH-standard was run on SDS-PAGE, showing that the major immunoreactive peptide co-migrated with the PTH-standard in both dimensions. It therefore probably represents the expected expression product, Met-Gly-hPTH or a closely related molecular species.

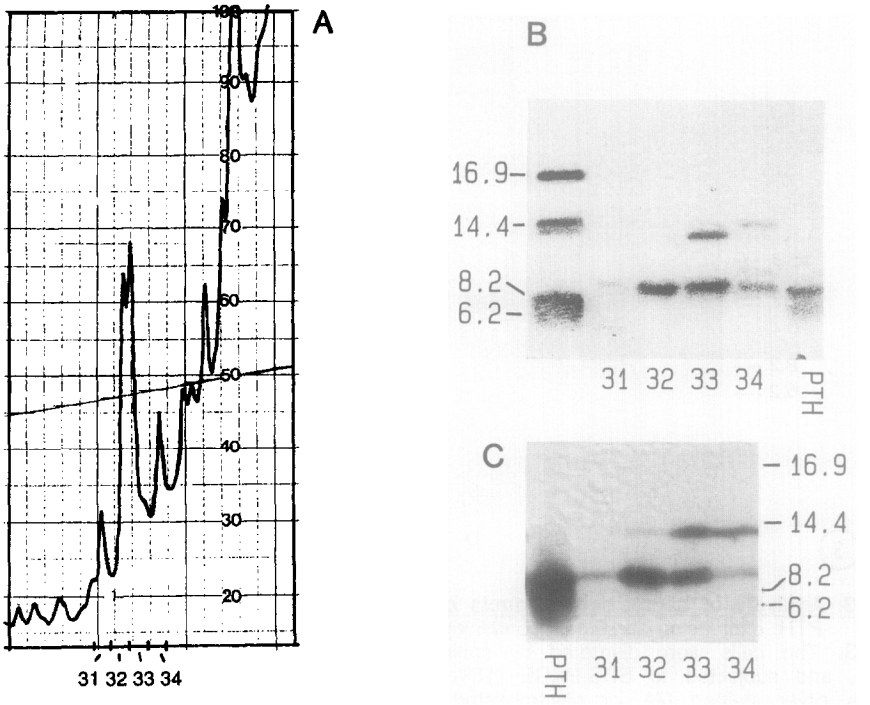

Fig.5: Purification of recombinant hPTH.
Met-Gly-PTH was purified from E. coli extracts by HPLC as described. PTH immunoreactive material in the fractions was detected by immunoblotting. (**A**) Elution profile from the HPLC purification, the fractions containing hPTH immunoreactivity are indicated. (**B**) SDS-PAGE, blotting and Coomassie-staining of the fractions indicated in (A). The fraction numbers are indicated under each lane. (**C**) Immunoblotting of the fractions from panel B using the mid-region antibody (except for the hPTH standard 10 times less material was loaded on the gel in panel C compared to panel B). Molecular weight standards are indicated, 1.5 µg hPTH(1-84) (Sigma) was loaded as a standard.

Purification and amino acid sequencing

The PTH-enriched cell extract was subjected to reverse-phase HPLC as described, and PTH in the fractions was detected by immunoblotting using the middle-region specific antibody. As shown in fig.5 (A and C) PTH-immuno-reactive material could be localized to a peak eluting with a retention time of 32-33 min. On SDS-PAGE and Coomassie-staining this peak was shown to consist of to major components (fig. 5B, fractions 32 and 33). One of these had an MW of about 9.5 kDa and co-migrated with the PTH-standard, the other component had an MW of about 14 kDa. Both of these proteins reacted with the anti-PTH antibody on immunoblotting, while the major 14 kDa polypeptide seen on SDS-PAGE (fig.3, lane A) eluted in the large peak appearing after the elution of PTH (fig.5 A) and did not react with the antibody (data not shown).

This indicates that the antibody reactivity to the 14 kDa protein (see also fig.3, lanes B and D) is due to this protein being PTH-related, and is not caused by unspecific binding to the major 14 kDa protein seen in fig.3 (lane A).

We then performed N-terminal amino acid sequence analysis on the peptide from fraction 32 (fig.5) that co-migrated with the PTH-standard. The sequence analysis gave a very low yield (much less than 10%), indicating that a large fraction of the sample was N-terminally blocked. A probable explanation for this is that fMet-hPTH could be a major constituent of this preparation. However a fraction of the preparation seemed to start with the amino acids X-Ser-Val-Ser-X-Ile which corresponds to the sequence expected if the N-terminal f-methionine residue has been removed to yield Gly-PTH.

Biological activity

PTH-related peptides from fraction 32 (fig.5A) were purified by HPLC as described, and biological activity was tested in the adenylate cyclase assay (16,17). This PTH preparation had no biological activity in concentrations that gave maximal stimulation of the enzyme activity with standard PTH (fig.6).

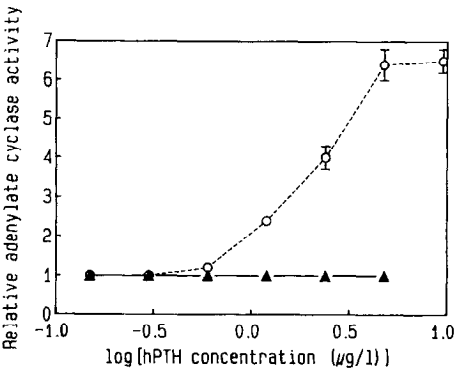

Fig.6: Biological activity of the purified recombinant hPTH. Material from fraction 32 (fig.5) was tested in the adenylate cyclase assay as described in Materials and methods. The concentrations of hPTH was determined by radioimmunoassay. (○ ○) hPTH(1-84) standard, (▲ ▲) recombinant PTH. Standard deviations are indicated. The results are given as the mean of 3 determinations ± standard deviation.

DISCUSSION

The hPTH cDNA-clone used in this study had a sequence different from that previously published by Hendy et al. (10). In our clone 10 there were two consecutive methionine codons at the start of preproPTH instead of one as reported by Hendy et al. (10) (fig.1). This opens the possibility for an alternative translation start of preproPTH, in that the synthesis of Met-preproPTH might occur in addition to the synthesis of preproPTH. It is interesting to note that bovine preproPTH starts with two consecutive methionine residues (22). The significance of the observed sequence difference is unclear, and is currently under investigation in our laboratory.

This report describes the successful expression of slightly modified forms of human parathyroid hormone in E.coli. In immunoblotting experiments the PTH reactive material migrated as a sharp band, and no low molecular weight degradation products could be detected. Hence in our system hPTH apparently was quite stable, in contrast to what was reported by Breyel et al. (23) and Rabbani et al. (24). Compared to these reports we achieved about ten times higher production of hPTH, despite using the same promoter as Breyel et al. (23). A possible explanation for this might be that fMet-Gly-PTH is more stable in E. coli than fMet-PTH which was the major product expected from the constructs of these authors. Very recently Wingender et al. (25) reported high expression of a cro-ß-galactosidase-hPTH fusion protein which after acidic cleavage results in amounts of Pro-hPTH equivalent with ours.

Rabbani et al. (24) reported that the N-terminal methionine was partly cleaved off, yielding a small amount of correctly processed hPTH(1-84). Our data indicate that also when PTH is expressed as Met-Gly-PTH, E. coli is unable to deformylate effectively the initiator fMet. However the N-terminal methionine is apparently rapidly removed from deformylated molecules.

Born et al. (26) showed that the expression of preproPTH in E. coli directed the protein to the surface of the bacterial inner membrane, but that it was not properly processed. The major processed forms were PTH(3-84) and PTH(8-84). In our system we, however, find no indications of production of

hPTH(3-84) or PTH(8-84)as reported by Rabbani et al. (24) and Born et al. (26).

The hPTH-species produced in this work did not stimulate adenylate cyclase in our biological assay system, and hence apparently had no PTH-agonistic activity. The reason for this probably is that the amino terminal end is very important for the biological activity of PTH (2). Few studies have been done with amino terminal extensions of PTH, but addition of tyrosine at position -1, or Tyr-Gly-Gly at positions -3 through -1 caused a marked decline in biological activity (27). Moreover fMet-hPTH has only 10% of the activity of hPTH(1-84) (24). This calls for methods to produce high yields of hPTH with the correct N-terminal sequence. Such studies are in progress in our laboratory.

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