Expression and Characterization of a Recombinant Human Parathyroid Hormone Secreted by Escherichia coli Employing the Staphylococcal Protein A Promoter and Signal Sequence*


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Human parathyroid hormone (hPTH) is a peptide hormone consisting of 84 amino acids (hPTH(1-84)). Employing the promoter and signal sequence of Staphylococcus aureus-protein A we have expressed hPTH in Escherichia coli. The expressed proteins are secreted to the growth medium, allowing for rapid and easy purification of the desired products. By amino acid sequence analysis and mass spectrometry, we have shown that the major excreted product is correctly processed human identical hPTH(1-84). The purified recombinant hPTH(1-84) stimulates adenylate cyclase activity in rat osteosarcoma cell membranes to exactly the same extent as synthetic parathyroid hormone standards, indicating that the recombinant product has full biological activity.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and other DNA-metabolizing enzymes were obtained from New England Biolabs. Anti-rabbit-IgG was from Amersham Corp., and the NH2-terminal-specific anti-PTH antibody was bought from CHEMICON. The production and characterization of the other antiserum has been described earlier (7). Synthetic hPTH(1-84) and [Ne3,Nle3,Tyr4]hPTH(1-34)amide were from Sigma.

Bacterial Strains, Plasmids, and Recombinant DNA Methods—E. coli strain B5183 (8) was obtained from Dr. F. Lacroute (Centre de Génétique Moléculaire du C.N.R.S., Gil-sur-Yvette, France). The cloning of hPTH cDNA has been described elsewhere.

If not otherwise stated, recombinant DNA methods were performed according to Maniatis et al. (9). DNA sequencing was performed on plasmid DNA with Sequenase (United States Biochemical Corporation) according to the supplier's manual. The oligonucleotides used were synthesized with an automated machine (KabiGen AB, Sweden) as described (10).

Cell Growth and Preparation of Cellular Fractions—For testing of PTH-production, E. coli was grown in 2YT medium (16 g of Bacto Tryptone; 16 g of Bacto Yeast Extract; 10 g of NaCl/liter) containing 0.4% glucose and 0.5 g/liter of ampicillin. Cells were harvested by centrifugation at 10,000 x g for 10 min, and the supernatant was taken as the growth medium fraction. The periplasmic fraction was prepared by the osmotic shock method described by Nossal and Heppel (11). The soluble intracellular fraction was prepared by sonicating the cell pellet remaining after extraction of the periplasmic proteins. The cell pellet was suspended in phosphate-buffered saline, 0.05% Tween 20 and sonicated 5 x 15 s on ice in a model W-10 Sonicator (Ultrasonics). Cell debris was spun down, and the supernatant was used as the soluble intracellular fraction.

Radioimmunoassay—Radioimmunoassay of hPTH was carried out as described (7) using an antiserum reactive against epitopes between amino acids 44 and 68 in hPTH. Polyclonal antibodies against hPTH were produced in rabbits against synthetic peptides corresponding to the amino acid sequences of hPTH(44-68) and hPTH(69-84). The assay was performed at a dilution of 1:5,000 in assay buffer (1 M NaCl; 50 mM Tris-HCl; 0.1% bovine serum albumin; pH 8.6) in a final volume of 50 μl. The incubation period was 48 h.

Detection of hPTH in the cell growth supernatant was performed by radioimmunoassay using antibodies against synthetic peptides corresponding to the amino acid sequences of hPTH(44-68) and hPTH(69-84). The assay was performed at a dilution of 1:5,000 in assay buffer (1 M NaCl; 50 mM Tris-HCl; 0.1% bovine serum albumin; pH 8.6) in a final volume of 50 μl. The incubation period was 48 h.

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The abbreviations used are: human parathyroid hormone, hPTH; PTH, parathyroid hormone; bPTH, bovine PTH; PAGE, polyacrylamide gel electrophoresis; hPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate.

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Expression of Human Parathyroid Hormone in E. coli

a buffer containing 0.1 M Tris-HCl, pH 7.5, 17% glycerol, 4% SDS, 0.05% bromphenol blue, and 2% β-mercaptoethanol and incubated on a boiling water bath for 5 min before being loaded on a 15% gel. Electrophoresis was run for 2-3 h at 600 V constant voltage.

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. Proteins were dissolved in 300 ml of water containing 1.9 M HAc, 8 M urea, 2% β-mercaptoethanol and 0.05% pyronin Y. Electrophoresis was run in 0.9 M HAc at 180 V till the dye had migrated close to the end of the gel.

Proteins fractionated by SDS-PAGE were transferred electrophoretically to Immobilon polyvinylidene difluoride transfer membranes (Millipore). The filters were incubated for 1 h in a buffer containing Tris buffered saline with 5% non-fat dry milk for 1 h at room temperature. Antibody incubations and washes were performed according to Towbin et al. (13). Cock anti-PTH antiserum that reacts with epitopes positioned after DNA coding for the promoter and signal peptide of S. aureus-protein A. This plasmid should express a fusion protein consisting of the protein A signal peptide and hPTH(1-84). Thus, hPTH(1-84) could be expected to be translocated to the periplasmic space (6), and the signal peptide should be cleaved off during this process.

The construction of the expression plasmid is outlined in Fig. 1. The BglIII-XbaI fragment containing the entire hPTH coding region was excised from the plasmid pSSH'hPTH10' and inserted between the BamHI and XbaI sites in pUC19. This plasmid (designated pUC10PTH) was then cleaved with AvaI and NsiI and inserted between the AvaI and XbaI sites in pKPR3 giving the plasmid pKPR3PTH. To get a protein A identical signal peptide correctly positioned in front of the PTH coding sequence, pKPR3PTH was cleaved with AvaI and NdeI. A synthetic oligonucleotide (see Fig. 1) was then inserted between these sites to give the final expression plasmid pSPTH. The correct sequence of the expression plasmid was confirmed by sequencing of plasmid DNA.

Production of hPTH—Several E. coli strains were transformed with the expression plasmid pSPTH, and different cellular fractions were tested for hPTH production by radioimmunoassay. Of the strains tested B5183 gave the highest overall level of expression, and this strain was therefore chosen for a more detailed study of hPTH expression.

A time course of the PTH production in this strain transformed with pSPTH is shown in Fig. 2. While the overall production of PTH increased in parallel with the OD600 of the culture, the localization of hPTH-immunoreactive material changed as a function of growth time. At early growth stages most of the PTH was located in the periplasmic space, while more than 80% of the total PTH immunoreactivity had accumulated in the growth medium at the stationary phase of growth. It was also apparent that the secretion process must be very efficient in that only a very small fraction of the total hPTH-related material was found in the intracellular fraction.
Expression of Human Parathyroid Hormone in E. coli

**FIG. 1.** Construction of the expression plasmid pSPTH. For details, see text.

**FIG. 2.** hPTH production as a function of culture growth. Cells were grown as described, and the amounts of hPTH immuno-reactive material in the periplasmic (PP), medium (M), and intracellular (IC) fractions were determined by radioimmunoassay. OD<sub>600</sub> of the culture (OD) was determined in dilutions to about OD 0.5, and the total amount of PTH immunoreactivity produced is also shown (tot).

medium and the periplasmic space to SDS-PAGE and immunoblotting.

In Fig. 3 (lane 1) is shown an experiment where proteins from the growth medium have been subjected to immunoblotting using the middle/COOH-terminal antibody. Four major bands can be seen, one 9.5-kDa band comigrating with the PTH standard, two smaller bands with M<sub>r</sub> values of about 6,000 and 5,500, and one larger band with an M<sub>r</sub> of about 13,500. As discussed below some of these peptides have been purified and subjected to NH<sub>2</sub>-terminal amino acid sequence analysis.

As can be seen from Fig. 3 (lane 2), four major hPTH-related peptides could be detected after immunoblotting of the periplasmic proteins. One of these peptides had an M<sub>r</sub> of 9,500 and comigrated exactly with the hPTH standard.

The largest of the immunoreactive peptides from the periplasm has an M<sub>r</sub> of about 14,500 and suggestively represents the signal sequence hPTH fusion protein with an uncleaved signal sequence.

The smaller immunoreactive bands probably represent proteolytic degradation products of hPTH. The major smaller bands from the periplasm have M<sub>r</sub> values of about 6,500 and 5,500, and the smaller of these proteins comigrates with a protein also found in the growth medium. The 14.5-kDa band seen in the periplasmic fraction has never been observed in the growth medium fraction, indicating either that this peptide remain in the periplasm, or that it is cleaved during or after excretion to the medium. The smaller bands might correspond to cleavage of the PTH molecule at about position 25 and 35. These regions are known to be susceptible to proteolysis in other systems (17) and might be acted upon by a variety of proteases. In all experiments the predominant band is the band comigrating with the PTH standard (Fig. 3 is a representative example). However, this band generally seem to constitute a greater proportion of the total immunoreactive material in the growth medium than in the periplasm.

**Purification of hPTH Species from the Growth Medium**—PTH was concentrated from the growth medium by chromatography on S-Sepharose, and PTH in the fractions was detected as described under "Experimental Procedures" (data not shown). Fractions containing hPTH(1-84) were then subjected to reverse-phase HPLC. As shown in Fig. 4A, a major peak (fractions 32 and 33) with the same retention time as standard hPTH could be identified. Proteins from this and from the other major peak (fraction 16) were freeze-dried and subjected to SDS-PAGE and immunoblotting. As shown in Fig. 5A (lanes 1 and 2), the peak with the same retention time as hPTH mainly consists of two proteins, a main component with an M<sub>r</sub> identical to an hPTH standard and a minor component with an M<sub>r</sub> of about 13,500. Both of these peptides react with an anti-PTH antibody on immunoblots (Fig. 5B, lanes 5 and 6). The main component (hereafter called recombinant hPTH) was further purified by another round of reverse-phase HPLC as described. As shown in Fig. 4B, this procedure resolved the two components into two peaks. The major peak eluted exactly like an hPTH(1-84) standard (Fig. 4, B and C). Finally, when this peak and standard hPTH(1-84) were cochromatographed one symmetric peak appeared, indicating that the recombinant hPTH behaved exactly as the hPTH standard under these experimental conditions (Fig. 4D). SDS-PAGE of the peak fraction showed one band co-
Expression of Human Parathyroid Hormone in E. coli

Fig. 4. Purification of recombinant hPTH from the growth medium of E. coli BJ5183. Recombinant hPTH was purified as described under "Experimental Procedures." A, chromatogram (A.<.) of the first HPLC purification. Fractions used for SDS-PAGE and second HPLC purification are indicated. B, chromatogram of the second HPLC purification of fractions 32 and 33 from panel A. The peak of recombinant hPTH is indicated in black. C, second HPLC run of 1 μg of standard hPTH(1–84). D, cochromatography of the recombinant PTH peak from panel B and 1 μg of standard hPTH(1–84).

Fig. 5. SDS-PAGE and immunoblotting of peaks from first and second HPLC purification. Proteins from the fractions indicated in Fig. 4 were subjected to SDS-PAGE and immunoblotting as described. Molecular weight standards are indicated. A, Coomassie Brilliant Blue staining of the filter after SDS-PAGE and blotting of fractions from the first HPLC run. B, autoradiogram of an immunoprobed filter (using the middle/COOH-terminal antiserum) of the same samples electrophoresed on a parallel gel (20 times less material was loaded on this gel than on the one shown in panel A). C, Coomassie Brilliant Blue and, D, silver staining of the gel after SDS-PAGE of the proteins in the recombinant hPTH peak indicated in Fig. 4B. Loadings were as follows. 1 and 5, fraction 32; 2 and 6, fraction 33; 3 and 8, molecular weight standards; 4 and 7, fraction 16; 9 and 11, recombinant hPTH peak, 1 μg; 10 and 12, hPTH(1–84) standard, 3 μg.

migrating with the hPTH standard (Fig. 5, C and D), suggesting that the recombinant hPTH was essentially pure and that it behaved exactly like the hPTH standard also in this separation system.

The recombinant hPTH from the HPLC purification was then subjected to NH2-terminal amino acid sequencing as described, and the result is shown in Table I. We were able to determine unambiguously 45 amino acids from the NH2-terminal, and the determined sequence was identical to the known sequence of hPTH (18, 19). The sequence analysis also indicated that the recombinant hPTH was more than 90% pure after the three purification steps employed. The repetitive yield in this analysis was 94.5% for Leu in positions 7, 11, 15, 24, 28, and 41, and 94.5% for Val in positions 2, 21, 31, and 35.

To further substantiate the conclusion that the 9.5-kDa protein is intact hPTH(1–84), we performed acetic acid-urea polyacrylamide gel electrophoresis and immunoblotting on proteins from various stages of the purification. In this separation system proteins are separated according to a combination of charge and size. The results of such an experiment are shown in Fig. 6. It can be seen that the main immunoreactive protein in the fraction containing recombinant hPTH comigrates with the PTH standard also in this separation system (lanes 2 and 5).

To show that the purified recombinant hPTH represented the intact hormone, we performed mass spectrometry as described under "Experimental Procedures." The plasma desorption mass spectrum obtained from the recombinant hPTH adsorbed on the sample foil covered with nitrocellulose is shown in Fig. 7. A molecular mass of 9426 ± 9 daltons could be calculated from the single-charged and the double-charged molecular ions present in the spectrum. The theoretical molecular mass of hPTH calculated from the amino acid composition is 9425 daltons, thus corresponding nicely to the value determined for the recombinant hPTH by mass spectrometry.

<p>| NH2-terminal amino acid sequences of PTH species purified from the growth medium |
|------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
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<tr>
<td>PTH immunoreactive peptides</td>
<td>A: SVSEIQLMHNLGKHLNSMERWLRKKLQ</td>
<td>B: SVSEIQ</td>
<td>C: EWLRRKLQ</td>
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TABLE I

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Expression of Human Parathyroid Hormone in E. coli

We have also sequenced the corresponding 9.5-kDa protein isolated from the periplasm, and we found that also this protein had an NH$_2$-terminal amino acid sequence identical to hPTH (data not shown).

The immunoblot analysis of the HPLC-peaks also revealed two other peptides that reacted with the anti-PTH antibody, namely a 6.0-kDa peptide from fraction 16 (Fig. 4A, and Fig. 5, lanes 4 and 7) and the 13.5-kDa minor constituent of the peak containing recombinant hPTH (Fig. 5, lanes 2 and 6). These peptides probably are the same as the three largest peptides detected in immunoblots of unfractionated material from freeze-dried medium shown in Fig. 3 (lane 1). The 6.0-kDa peptide from fraction 16 was cut out from the filter shown in Fig. 5A (lane 4) and subjected to NH$_2$-terminal amino acid sequence analysis. The amino acid sequence showed that this peptide was a fragment of hPTH, starting at amino acid 22 and probably extending all the way through to the COOH-terminal end of hPTH (1–84) (Table I).

The NH$_2$-terminal sequence of the 13.5-kDa peptide (Table I) was determined by cutting out the Coomassie-stained band after SDS-PAGE and blotting of proteins from the minor peak from the second HPLC run (Fig. 4B).

As shown in Table I, this peptide has an NH$_2$-terminal amino acid sequence that is identical to that of hPTH, despite being about 4 kDa larger than hPTH(1–84). At present the molecular identity of this 13.5-kDa peptide is unclear. It is interesting to note, however, that an hPTH-immunoreactive peptide with the same $M_r$ is observed also when hPTH is expressed as an intracellular peptide in E. coli. Thus, the production of the 13.5-kDa peptide does not seem to be a consequence of the expression of hPTH as a secreted peptide but might possibly be due to a readthrough of the PTH stop codons or to some kind of covalent modification of hPTH introduced inside the E. coli cell.

**Biological Activity**
The purified recombinant hPTH was tested for biological activity in the adenylate cyclase assay as described, and it can be seen that the recombinant hPTH stimulated adenylate cyclase to the same extent as the hPTH(1–84) and [Nle°,Nle°°,Tyr°°°]hPTH(1–34)amide standard, indicating that the recombinant hPTH has a specific biological activity that in this assay is indistinguishable from that of the standards (Fig. 8).

**DISCUSSION**

To our knowledge this work represents the first report on the successful expression and secretion of intact hPTH from E. coli. Other investigators have expressed hPTH as an intracellular peptides in E. coli (20, 21, 36) or have tried to achieve secretion of hPTH from E. coli using hPTH’s own signal sequence (22). In the last report three forms of PTH were produced. One of these, hpreproPTH, became associated with the outside of the inner membrane, while the two other forms, hPTH(3–84) and hPTH(8–84), were intracellular peptides. The authors concluded that the PTH signal sequence is able to confer translocation of hPTH through the inner membrane but that this signal peptide is not cleaved off by the signal peptidase, causing hpreproPTH to remain in the inner membrane.

In contrast, the protein A signal sequence used in the present work seems to be very effective in translocating hPTH through the inner membrane, indicated by the observation that only a few percent of the total hPTH immunoreactivity was located in the intracellular fraction. It also seems that the signal sequence is efficiently cleaved off during secretion. Although molecules probably corresponding to the uncleaved fusion protein could be detected in the periplasmic fraction by immunoblotting, these molecules constituted only a small amount of the total immunoreactive peptides.

A very unexpected finding was that a large amount of the immunoreactive material was excreted to the growth medium. The reason for this is at the moment unclear. It is not, however, due to cell lysis or a general leakage phenomenon because the ratio of hPTH to total protein is much higher (about 15 times, data not shown) in the medium than in the periplasmic fraction. Also the fact that the hPTH species in the medium are partly different from those seen in the periplasmic fraction (see below) argues against a generally unspe-
Expression of Human Parathyroid Hormone in *E. coli*

Wingender et al. (36) recently reported on high yields when hPTH was expressed as an intracellular fusion protein. These authors, however, were unable to produce human identical hPTH(1–84), their main product being Pro-hPTH.

Expression of hPTH as a secreted protein also has the advantage of avoiding the problem with the NH₂-terminal formyl-methionine residue necessary for initiation of translation. Although this formyl-methionine residue can be removed from intracellular proteins by a deformylase and a methionine-amino-peptidase, this often is an inefficient process for heterologous proteins especially when these are highly expressed (35). In the case of hPTH, it has been shown that the removal of this residue is incomplete, even at low expression levels (21). When hPTH is expressed as a secreted protein the NH₂-terminal part of the fusion protein is cleaved off by the signal peptidase, and as long as this cleavage occurs at the right position, the NH₂-terminal of the heterologous protein should be correct. A correct NH₂-terminal is utterly important for hPTH because the biological activity of this hormone is critically dependent on the amino-terminal sequence. For example will the addition or deletion of only 1 amino acid residue usually destroy most of the biological activity (2, 3), although it very recently has been shown that Pro-hPTH has full biological activity (36)? In our expression system the hPTH fusion protein is at least partly correctly processed to hPTH(1–84) during secretion to the growth medium. In addition to hPTH(1–84), however, several hPTH fragments are produced, indicating a certain level of degradation during the secretion process.

Another obvious advantage of our expression system is the ease by which the recombinant hPTH can be purified. As can be seen from Figs. 4 and 5, the recombinant hPTH can be judged to be more than 90% pure after just two purification steps, and the sequence analysis indicates that it is more than 90% pure after the third purification step. This is in contrast to the lengthy purification procedure employed by Rabbani et al. (21) to purify recombinant hPTH expressed as an intracellular peptide in *E. coli*.

In conclusion we have succeeded in expressing and purifying recombinant hPTH in *E. coli*. Since the purified 9.5-kDa protein comigrates with an hPTH(1–84) standard in three different separation systems, has the right NH₂-terminal amino acid sequence, and has a correct molecular mass as determined by mass spectrometry, it seems reasonable to conclude that this protein represents intact hPTH(1–84) produced and excreted in *E. coli*. Furthermore, the purified product has full biological activity as determined in the adenylate cyclase assay. In comparison to other published systems for the expression of hPTH in microorganisms, our system gives higher production and easier purification, since the product is excreted to the growth medium. By using this expression system combined with high density fermentation methods, it should now be possible to produce pure hPTH(1–84) in such quantities that physiological and clinical studies using the intact hormone could be performed on a larger scale.

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Expression of Human Parathyroid Hormone in E. coli


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