

## Recombinant expression and purification of the N-terminal extracellular domain of the parathyroid hormone receptor

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Received 13 December 2006, and in revised form 22 February 2007

Available online 6 March 2007

### Abstract

Our goal is to elucidate the nature of the bimolecular interaction of parathyroid hormone (PTH) with its receptor, the parathyroid hormone receptor type-1 (PTHr1). In order to study this interaction, we are aiming to obtain a three-dimensional structure of the PTH–PTHr1 bimolecular complex. Due to the very low expression levels of endogenous PTHr1, a recombinant form is required for structural analysis. However, the extreme hydrophobicity of the transmembrane regions of PTHr1 makes heterologous expression of PTHr1 difficult. Therefore, we sought to express the N-terminal extracellular domain (N-ECD) of PTHr1, a region that plays a pivotal role in ligand interaction. We expressed the N-ECD in both bacterial (*Escherichia coli*) and insect (*Sf9*) cells. The form produced in *E. coli*, a fusion-protein with thioredoxin, is soluble. However, removal of the fusion partner from a partially purified preparation results in dramatic loss of yield of the N-ECD. Expression in *Sf9* cells, however, facilitates purification of a soluble form of the N-ECD. Isothermal calorimetry demonstrates that this N-ECD binds PTH-(1–34), albeit with lower affinity than the full-length receptor. This report describes the expression and purification of milligram quantities of the isolated N-ECD of PTHr1. The receptor fragment retains the ability to bind its cognate peptide ligand, an important pre-requisite for subsequent structural studies.

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**Keywords:** Parathyroid hormone receptor; G protein-coupled receptor; Heterologous expression

Parathyroid hormone (PTH)<sup>1</sup> is the principal regulator of blood Ca<sup>2+</sup> concentrations [1]. In conditions of low blood Ca<sup>2+</sup> levels, PTH is secreted and interacts with its cognate receptor, the parathyroid hormone receptor type-1 (PTHr1), present on target cells in bone and kidney, to mobilize Ca<sup>2+</sup> into the bloodstream. Although PTH generally stimulates bone resorption, when administered intermittently in low doses, it has been shown to have the opposite effect, namely an anabolic action on bone, stimu-

lating bone formation [2]. Therefore, there is growing interest in the therapeutic efficacy of PTH and related peptides for the treatment of osteoporosis. Indeed, a biosynthetic peptide comprising the N-terminal 34 residues of human PTH, hPTH-(1–34), is an approved drug for osteoporosis [3]. Our work focuses on elucidating the nature of the bimolecular interaction of PTH with PTHr1, with the ultimate goal of using this knowledge to design novel and improved compounds for the treatment of osteoporosis and related disorders.

The bimolecular interface of the PTH–PTHr1 complex has been extensively studied using a variety of molecular and biochemical techniques (reviewed in Refs. [4,5]). We and others have used photoreactive PTH analogues to systematically probe regions of interaction with PTHr1 [6–13]. However, the degree of resolution achievable using the technique of photoaffinity scanning is reaching its limit

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<sup>1</sup> Abbreviations used: PTH, parathyroid hormone; PTHr1, parathyroid hormone receptor type-1; N-ECD, N-terminal extracellular domain; GPCRs, G protein-coupled receptors; LIC, ligation-dependent cloning; Gly<sub>3</sub>, 3-residue glycine; tNOE, transferred nuclear Overhauser effect; STD, saturated transfer difference.

[14]. In order to obtain a more refined map of the PTH–PTHr1 interface, we are aiming to elucidate the three-dimensional structure of the PTH–PTHr1 bimolecular complex by X-ray crystallography and/or NMR.

Structural analyses of proteins require large amounts of sample. Due to the very low expression levels of endogenous PTHr1, a recombinant form is required to achieve sufficient quantities of material for study. We recently described the expression and purification of a recombinant form of full-length PTHr1 expressed in a mammalian system [15]. This partially purified receptor retained the ability to bind hPTH-(1–34), exhibiting an affinity of  $104 \pm 10$  nM in a radioligand–receptor binding assay. NMR studies were attempted using this preparation, but no meaningful data were obtained due to problems arising from the detergent used to solubilize the protein. Aside from solubility issues, the yields of protein obtained from this expression system were low and scale-up produced a preparation that proved difficult to purify. The extreme hydrophobicity of the seven-transmembrane regions of PTHr1 presents a problem for overexpression in heterologous systems. Therefore, we pursued an alternative approach as a starting point for our long-range plans regarding structural analyses of the receptor: expression of the isolated N-terminal extracellular domain (N-ECD) of PTHr1. PTHr1 belongs to the class II family of G protein-coupled receptors (GPCRs), members of which are characterized by long N-ECDs that are known to play pivotal roles in interactions with their respective ligands. Although ligand binding within this family also involves other domains of the receptors, many of the key binding determinants reside within the N-ECD [16]. Indeed, the current model for PTH–PTHr1 interaction proposes at a minimum a two-step binding mechanism in which the C-terminal half of hPTH-(1–34) interacts with the N-ECD, thereby anchoring the hormone to the receptor and presenting the N-terminal “activation” region to the J-domain of PTHr1 [13,17]. We, therefore, reason that elucidating the interaction of hPTH-(1–34) with the N-ECD of PTHr1 will provide important insights pertaining to the overall nature of the bimolecular interface of the hormone with the full-length receptor.

In this study, we report the expression and purification of the isolated N-ECD of PTHr1 using two expression systems. The form of the N-ECD produced in *E. coli* appears to misfold, but the baculoviral expression system facilitated purification of several milligrams of the N-ECD that was shown to bind hPTH-(1–34), an important demonstration of functionality and a pre-requisite for subsequent structural studies.

## Materials and methods

### Cloning of N-ECD for *E. coli* expression system

The coding sequence of full-length PTHr1, previously cloned into pcDNA 3.1 [18], served as the template for amplification of the coding sequence for PTHr1(23–190).

The primer set GACGACGACAAGATGTACGCGCTGGTGGATGC and GAGGAGAAGCCCCGGTTCAAATCATGCCCAGGCGG (the underlined sequences correspond to the ligation-independent cloning (LIC) overhangs to facilitate subsequent ligation into pET32LIC; the double-underlined sequence corresponds to a stop codon) were used in “hot-start” PCR, using 10 ng of template, 0.4  $\mu$ M of each primer (Sigma Genosys, St. Louis, MO), 2.5 U of *Pfu* Ultra DNA polymerase (Stratagene, Cedar Creek, TX) and 0.2 mM of each of dATP, dTTP, dGTP and dCTP (Roche, Indianapolis, IN) in a Hybaid thermocycler (95 °C for 5 min, followed by 5 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, followed by 24 cycles of 95 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min, followed by 1 cycle of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 10 min). The amplified product was inserted into pET32LIC (Novagen, Madison, WI) as per manufacturer’s protocol. NovaBlue GigaSingles competent cells (Novagen) were transformed with the resulting construct and clones harboring the construct of interest were screened by colony-PCR. Cloning was confirmed by DNA sequence analysis (Tufts University Core Facility, Boston, MA). pET32LIC-PTHr1(23–190) served as the template in an inverse PCR to create pET32LIC-His<sub>6</sub>-PTHr1(23–190). Briefly, a forward primer (5′-GCGCACTAGTTACGCGCTGGTGGATGC-3′) and a reverse primer (5′-GCGCACTAGTGTGATGGTGTGATGGTGTGATCATCTTGTCTCGTCATC-3′) were used such that priming occurred in opposite directions from the extreme 3′ end of the enterokinase-cleavage sequence. This resulted in the replication of pET32LIC-PTHr1(23–190) with the addition of sequence encoding a His<sub>6</sub> tag (single-underlined sequence in reverse primer) and a *Spe*I site (double-underlined sequence in reverse primer) at one end, immediately downstream of the enterokinase-cleavage site, and upstream of the PTHr1(23–190) sequence, and a *Spe*I site (double-underlined sequence in forward primer) at the other end of the linear molecule. The PCR conditions and cycle parameters were as above, except an elongation time of 12 min was used. The resulting product was digested with *Spe*I (New England Biolabs, Ipswich, MA) and subsequently ligated using T4 DNA ligase (Promega, Madison, WI) to itself to create pET32LIC-His<sub>6</sub>-PTHr1(23–190).

### Cloning of N-ECD for baculoviral expression system

The primer set GCGGAATTCATGGGGACCGCCCGGATC and GCGGGATCCTCAGTGTGATGGTGTGATGGTGTCCACCTCCAATCATGCCCAGGCGGTC was used for cloning PTHr1(1–190). The bold sequences in the forward and reverse primers correspond to *Eco*R1 and *Bam*H1 recognition sites, respectively, to facilitate subsequent ligation into pVL1392; the underlined sequence in the reverse primer encodes a 3-residue glycine linker; followed by a His<sub>6</sub> epitope (italics). The double-underlined sequence corresponds to a stop codon. The PCR condi-

tions used were as above. The PCR product was purified (QIAquick Spin, Qiagen, Valencia, CA), and treated sequentially with *Eco*R1 and *Bam*H1 (New England Biolabs). The resulting product was ligated with *Eco*R1- and *Bam*H1-treated pVL1392 overnight at 16 °C. NovaBlue GigaSingles competent cells were transformed with this pVL1392-PTHR1(1–190) construct, and clones confirmed as detailed above.

#### *Expression and purification of N-ECD from E. coli*

pET32LIC-PTHR1(23–190) or pET32LIC-His<sub>6</sub>-PTH1(23–190) were used to transform the *E. coli* expression strain Rosetta-gami (DE3) pLysS (Novagen) as per manufacturer's protocol. Resulting colonies were used to inoculate L-broth, supplemented with 100 µg/ml ampicillin, and grown overnight at 37 °C with agitation at 200 rpm. Cultures were diluted 1/100 into 1 L of fresh L-broth supplement with ampicillin and grown to an OD<sub>600</sub> of 0.5–1.0. Protein expression was induced by the addition of 0.25 mM isopropyl-L-β-thiogalactopyranoside and the cultures incubated for an additional 3 h. Cells were harvested by centrifugation at 6000g for 15 min at 4 °C and frozen at –70 °C. For purification of recombinant protein, pellets were thawed, resuspended in MCAC buffer (25 mM sodium phosphate, 500 mM NaCl, pH 7.4) supplemented with Complete Mini protease inhibitor tablets (Roche), and lysed by 4 rounds of freeze/thaw. The lysed cells were clarified by spinning at 35,000g in a JA-25.50 rotor (Beckman, Fullerton, CA) for 30 min at 4 °C. Fusion proteins were purified through nickel–nitrilotriacetic acid agarose (HiTrap chelating column, Amersham Biosciences, Piscataway, NJ) equilibrated with MCAC buffer, and eluted with a step gradient up to 200 mM imidazole. Eluates were concentrated by ultrafiltration through Amicon Ultra 15 concentrators (Millipore, Billerica, MA), and purity was assessed by SDS–PAGE. Protein concentration was determined (Bio-Rad Dc Protein Assay, Hercules, CA) using bovine serum albumin as the standard. Vector-encoded fusion tags were cleaved by treating the partially purified protein preparation with enterokinase (New England Biolabs) for 16 h at room temperature. The resulting mixture was subsequently diluted with MCAC buffer and purified through a nickel–nitrilotriacetic acid agarose column.

#### *Expression and purification of N-ECD from Sf9 cells*

Recombinant baculovirus was created by co-transfecting *Sf9* cells, maintained in TNM-FH medium (BD Biosciences, San Diego, CA), with the pVL1392-PTHR1(1–190) construct and linearized BaculoGold™ DNA (BD Biosciences) as per manufacturer's protocol. After five days, medium was harvested and the virus was subjected to two rounds of amplification by infecting fresh *Sf9* cells. Once sufficient quantities of recombinant virus had been generated, cultures of *Sf9* cells were scaled up to 10 L scale in the GRASP Antibody and Cell Culture Core (Tufts-

New England Medical Center, Boston, MA). Three separate 10 L cultures were initiated and infected with recombinant baculovirus at a multiplicity of infection of 5 and maintained for 72 h before harvesting medium. Medium was processed through a filter with a molecular weight cut-off (MWCO) of 10 kDa and concentrated down to approximately 400 mL. The concentrated filtrate from 10 L of culture was split into two and each batch applied to a nickel–nitrilotriacetic acid agarose column and eluted with a step gradient up to 160 mM imidazole. The eluates were concentrated by ultrafiltration through Amicon Ultra 15 concentrators (10 kDa MWCO), purified through a second nickel–nitrilotriacetic acid agarose column, concentrated, and pooled together. Total protein quantities at various stages of the purification process were assayed as described above, and purity of the N-ECD was estimated by densitometric analysis (ImageJ software, National Institutes of Health) of protein separated by SDS–PAGE.

#### *Isothermal calorimetry*

hPTH-(1–34) was synthesized in our laboratory using a Symphony peptide synthesizer (Protein Technologies, Tucson, AZ), purified by HPLC and lyophilized. The peptide was prepared in PBS supplemented with 1 mM acetic acid. N-ECD was dialyzed with this buffer prior to commencing isothermal calorimetry. Isothermal titration calorimetry was performed using a MicroCal VP-ITC titration microcalorimeter (Northampton, MA) at 25 °C. A series of 10 µL injections (for a total of 29 injections) of hPTH-(1–34) (400 µM) were added sequentially into N-ECD (40 µM). The heat of reaction per injection (microcalories per second) was determined by integration of the peak areas using the Origin Version 7.0 software. The heats of dilution were determined in parallel control experiments by injecting 400 µM hPTH-(1–34) solution in the PBS buffer supplemented with acetic acid. These heats of dilution are subtracted from the corresponding N-ECD–hPTH-(1–34) binding experiments before curve-fitting.

#### *SDS–PAGE, Western blot and N-terminal sequencing*

The electrophoresis of purified N-ECD was carried out using 12% Bis–Tris gels (Invitrogen Life Technologies, Carlsbad, CA). Gels were stained with SimplyBlue™ Safe-Stain solution (Invitrogen). For Western blots, electroblotting was performed using PVDF membranes (Invitrogen). Following transfer, membranes were blocked in 5% non-fat milk in TBST buffer (50 mM Tris–Cl, pH 7.5, 0.9% NaCl, and 0.05% Tween 20) for 1 h, then incubated with a 1:1000 dilution of goat anti-hPTH1 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or a 1:500 dilution of HRP-conjugated anti-His<sub>6</sub> monoclonal antibody (Roche) for 1 h at RT. HRP-conjugated swine anti-goat IgG (1:1000 dilution, Roche) antisera was used as the secondary antibody for detecting samples probed with the anti-hPTH1 primary antibody. Membranes were

developed with West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) as per manufacturer’s protocol. For N-terminal sequencing, the N-ECD sample was electro-transferred to the PVDF membrane, stained with Simply-Blue™ SafeStain solution, and excised. Sequencing was performed in the Core Facility, Tufts University, Boston, MA.

Detection of glycosylation

The N-ECD was run through a 12% Bis-Tris gel and the gel was stained with Pro-Q stain (Molecular Probes, Eugene, OR) as per manufacturer’s protocol.

Results and discussion

E. coli expression system

The coding sequence for residues 23–190 of PTHR1 (i.e. the N-ECD lacking the 22-amino acid signal sequence; Fig. 1a) was cloned into the pET32-LIC expression vector

and the resulting construct (Fig. 1b) was used to transform E. coli Rosetta-gami (DE3) pLysS. This strain is optimized for expression of proteins that contain codons rarely used in E. coli and allows disulfide bond formation in the bacterial cytosol. This system facilitated high-level expression of the recombinant N-ECD in a soluble form (Fig. 2). The fusion

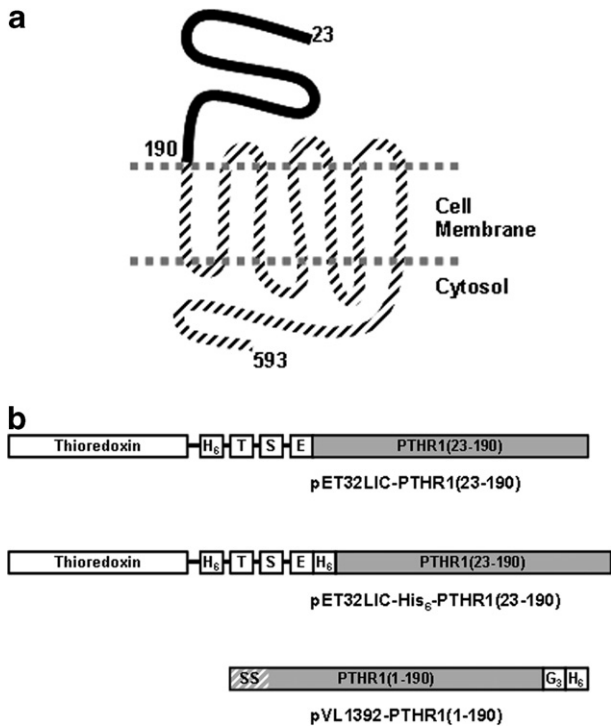


Fig. 1. Overview of cloning strategy. (a) Topological representation of PTHR1. The N-ECD, lacking the 22-residue signal sequence due to processing, is highlighted in black. (b) Schematic structure of the constructs used in this study. The bacterial expression vector pET32-LIC-PTH1(23–190) directed the expression of the N-ECD, without the 22-residue signal sequence of PTHR1, fused to an N-terminal construct encoding thioredoxin, His<sub>6</sub> (H<sub>6</sub>), S-tag (S) and cleavage sites for thrombin (T) and enterokinase (E). pET32LIC-His<sub>6</sub>- PTHR1(23–190) incorporated an additional His<sub>6</sub> epitope between the enterokinase cleavage site and the N-ECD coding sequence. pVL1392-PTH1(1–190) was used to generate recombinant baculovirus directing the expression of the N-ECD, incorporating the native signal sequence of PTHR1 (SS) and a C-terminal His<sub>6</sub> epitope separated from the N-ECD sequence by a 3-residue glycine linker (G<sub>3</sub>).

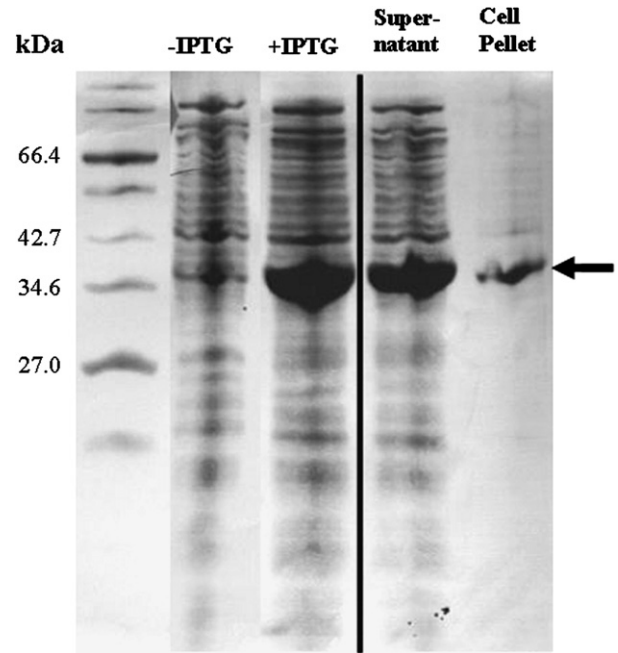


Fig. 2. Expression of a soluble form of the N-ECD of PTHR1 in E. coli. Coomassie-stained SDS-PAGE gel showing the expression of the N-ECD fusion protein in E. coli upon induction with IPTG. When cells were lysed, the fusion protein was recovered in the soluble fraction (supernatant), with very little evident in the cell pellet. The position of the fusion protein is indicated by the arrow.

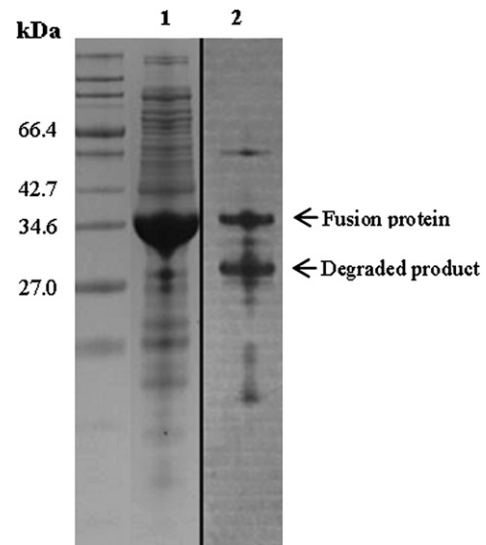


Fig. 3. Partial purification of the N-ECD of PTHR1. Coomassie-stained SDS-PAGE gel showing whole cell lysate (lane 1) and the eluate obtained from metal-chelate affinity chromatography (lane 2).

protein was partially purified by metal-chelate affinity chromatography (Fig. 3) and subjected to enterokinase cleavage to remove the vector-encoded tags (Fig. 4). However, only the vector-encoded fusion tags were recovered from this process. Various other chromatography regimes were attempted (combinations of metal-chelate, ion-exchange, S protein-affinity and size-exclusion chromatographies, before and/or after enterokinase treatment), but none proved successful in isolating the N-ECD free from its fusion partner (data not shown). Use of thrombin to remove the fusion tags similarly failed to yield the isolated N-ECD (data not shown). In an attempt to prevent the cleaved N-ECD from being proteolytically degraded following its release from the fusion tags, a second His<sub>6</sub> site was engineered into the pET32LIC-PTH1(23–190) construct immediately 3' to the enterokinase site and 5' to the N-ECD coding sequence (Fig. 1b). Similar to pET32LIC-PTH1(23–190), this construct directed high-level expression of a soluble fusion protein, but once again, enterokinase treatment of the partially purified protein only yielded the vector-encoded fusion tags (data not shown). The failure to isolate the N-ECD free from its fusion partners suggests that the N-ECD is degraded, possibly due to misfolding. Although the fusion protein is soluble, it is thought likely that the thioredoxin component of the vector-encoded tag masks the insolubility of the misfolded N-ECD.

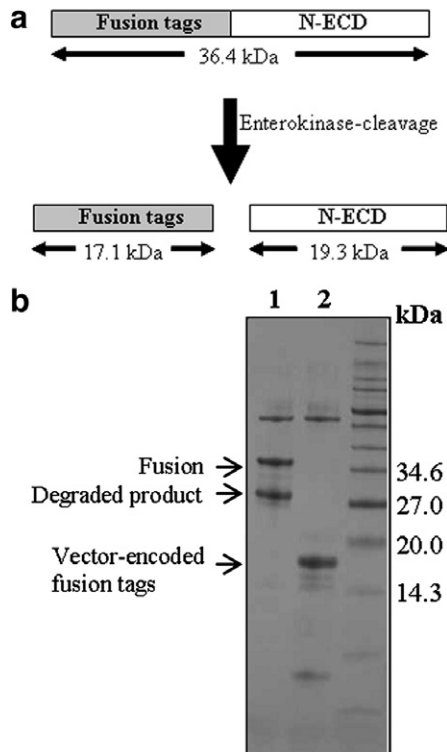


Fig. 4. Removal of fusion tags by enterokinase. (a) Schematic showing products expected upon treatment of the fusion protein with enterokinase. (b) Coomassie-stained SDS-PAGE gel showing partially purified fusion protein before (lane 1) and after (lane 2) treatment with enterokinase. N-terminal sequencing of the ~17 kDa band obtained showed this to correspond to the vector-encoded fusion tag.

### Baculoviral expression system

Due to anticipated problems of producing a human protein in a prokaryotic expression system, we used a baculovirus expression system in parallel to the bacterial expression system in order to produce a recombinant form of the N-ECD. The coding sequence for the entire N-ECD of PTHR1 (i.e. residues 1–190, including the native signal sequence) was amplified by PCR, incorporating a 3-residue glycine (Gly<sub>3</sub>) linker at the C-terminus followed by a His<sub>6</sub>-tag. This construct was cloned into the pVL1392 transfer vector (Fig. 1b), and together with linearized baculoviral DNA, was used to co-transfect *Sf9* insect cells. This approach allowed us to produce a recombinant baculovirus incorporating the N-ECD coding sequence, and this virus successfully directed the expression of a soluble form of the N-ECD that was secreted into the culture medium (Fig. 5). N-terminal sequencing of this protein confirmed that the signal sequence was processed (data not shown). Large-scale suspension culture of *Sf9* cells infected with this recombinant baculovirus facilitated the purification of the N-ECD by metal-chelate affinity chromatography (Fig. 6a) with a yield of approximately 0.6 mg pure protein per liter of culture. A typical purification is summarized in Table 1. The N-ECD of PTHR1 contains four glycosylation sites [6]; the purified preparation was shown to be glycosylated (Fig. 6b). It should be noted, however, that the glycosylation pattern of recombinant proteins expressed in insect cells is different from those expressed in mammalian systems [19]. However, it was previously shown that

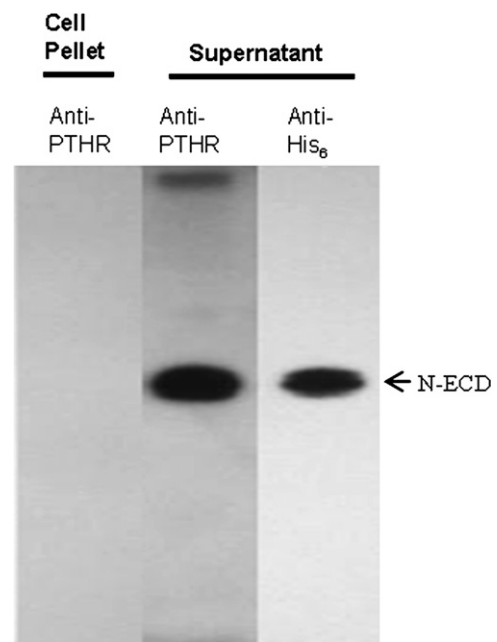


Fig. 5. Western blot to detect expression of a soluble form of the N-ECD of PTHR1 in *Sf9* cells. *Sf9* cells infected with recombinant baculovirus secreted the recombinant N-ECD into the medium. The cell pellet and supernatant fractions were probed with antibodies recognizing either PTHR1 or the His<sub>6</sub> epitope.

glycosylation is not necessary for ligand-binding by PTHR1 [20]. Isothermal calorimetry was used to assess the ability of this recombinant protein to interact with hPTH-(1–34). This analysis demonstrated that the N-ECD can bind hPTH-(1–34), exhibiting a dissociation constant,  $K_D$ , of 6.2  $\mu\text{M}$  (Fig. 7). This is considerably weaker binding than occurs to the full-length receptor (approximately 100 nM [15]), but it is within the anticipated range.

Our central goal is to elucidate the structure of the PTH–PTHR1 bimolecular complex with the ultimate hope of using this knowledge to obtain insights into the fundamental basis of molecular recognition in hormone–receptor interactions in general, and more specifically, in the design of new compounds for the treatment of osteoporosis and related disorders. We have succeeded in generating a functional form of the N-ECD of PTHR1 in quantities sufficient to allow us to initiate structural studies. Currently,

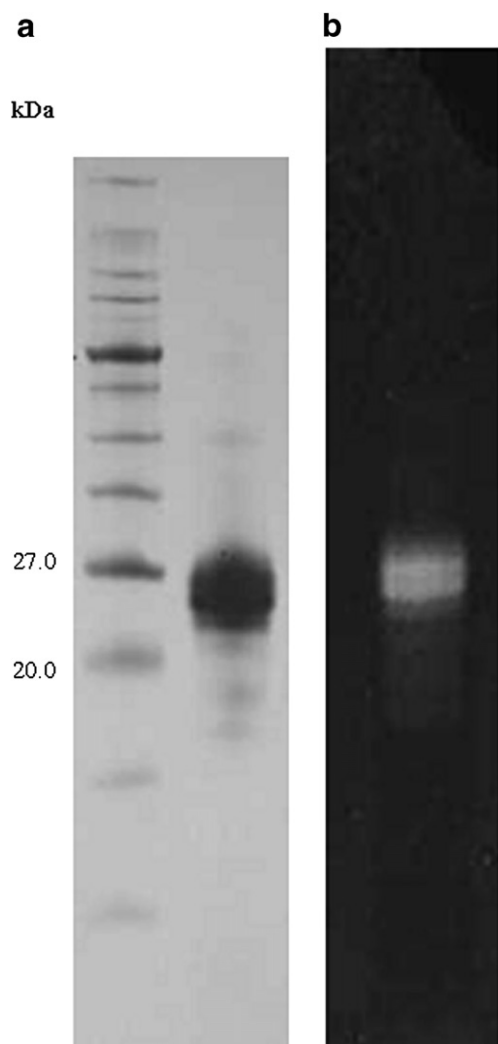


Fig. 6. Purification of the N-ECD of PTHR1. (a) Coomassie-stained SDS-PAGE gel showing eluate obtained from metal-chelate affinity chromatography. The predicted molecular weight of this protein is 20.3 kDa, but the observed MW appears closer to 25 kDa, as judged by SDS-PAGE. (b) Use of a fluorescent stain for carbohydrate moieties show this protein to be glycosylated.

Table 1  
Purification of N-ECD from culture medium of *Sf9* cells

	Total protein (mg)	Purity of N-ECD (%)
Culture supernatant	820	N/A
Flow through	758	N/A
Eluate (round 1)	5.1	85
Eluate (round 2)	4.1	97

The supernatant from a 10 L-scale *Sf9* culture was divided into two batches, and each batch was treated separately, as described in materials and methods. The above data is from a typical purification. N/A, not assayed.

we are using transferred nuclear Overhauser effect (tNOE) and saturation transfer difference (STD) spectroscopies to gain structural and topological information about the hormone [hPTH-(1–34)] while bound to the N-ECD. Also, we now are positioned to initiate crystallography trials in a bid to gain an X-ray structure of both the N-ECD alone and the N-ECD–hPTH-(1–34) complex. In parallel to crystallography trials, we plan to produce isotopically labeled N-ECD suitable for NMR analysis in order to obtain solution structures of both the N-ECD alone and the N-ECD–PTH(1–34) complex. It is envisioned that such three-dimensional structural analyses will provide

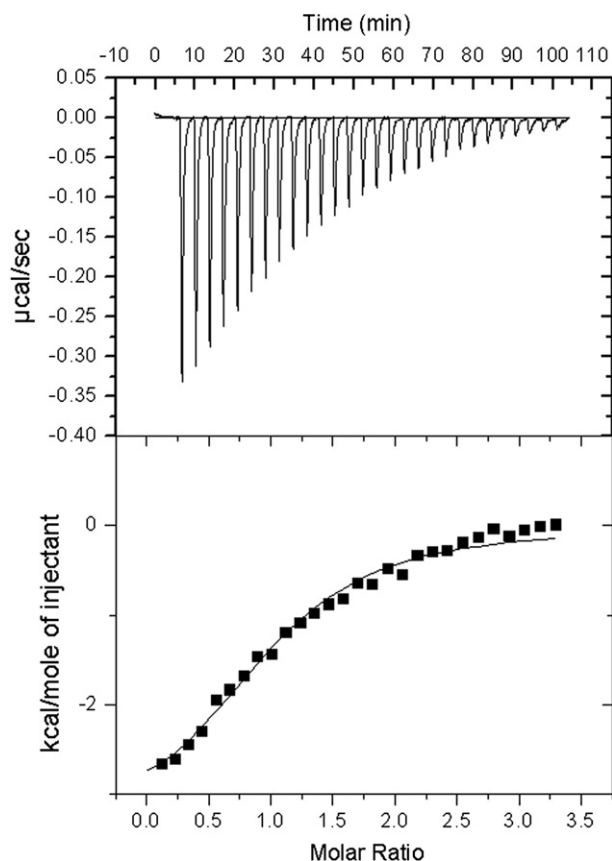


Fig. 7. Functional analysis of recombinant N-ECD produced in *Sf9* cells. Isothermal calorimetry was used to assess the ability of the N-ECD to bind hPTH-(1–34). A representative binding isotherm is shown. Top: baseline-subtracted raw data. Bottom: peak-integrated and concentration-normalized enthalpy change vs PTH/N-ECD ratio.

important insights useful not only for the design of drugs targeting the PTH receptor, but also for elucidating general principles of hormone–receptor interactions that operate for type-II G protein-coupled receptors.

### Acknowledgments

This work was supported by grant DK-47940 (to M.R.) from the National Institutes of Health. We gratefully acknowledge Dr. Douglas Jefferson, Des Lee and Dr. Ann Kane of the GRASP facility at Tufts–New England Medical Center for assistance in large-scale growth of *Sf9* cells and subsequent processing of the medium. The GRASP facility is supported by Grant P30 DK-34928 from the National Institutes of Health.

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