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Human Thyroid Hormone ß1 Receptor Produced by Recombinant Baculovirus-infected Insect Cells

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SUMMARY: Thyroid hormone receptors are nuclear proteins which regulate transcription in a hormone dependent manner. The baculovirus expression system was used for the overexpression of the ß1 isoform of the human thyroid hormone receptor. The baculovirus produced receptor binds tri-iodothyronine with high affinity, is specifically immunoprecipitated with a ß1 specific antibody, and binds to DNA that contains a known thyroid hormone receptor recognition site. Large scale production and purification of baculovirus produced receptor will be useful for structure-function analyses and studies of transcriptional regulation.

The steroid and thyroid hormone receptor superfamily is composed of a group of structurally related, ligand-activated transcription factors (1). These receptors are characterized by a highly conserved, cysteine-rich DNA binding domain, a carboxyl-terminal ligand-binding domain, and putative domains for transcription activation and/or repression. There are multiple thyroid hormone receptor (TR) isoforms that are derived from two structural genes, α and ß (2). TRs bind to specific DNA elements (TREs) and either positively or negatively regulate transcription in a hormone dependent manner (3).

We are currently investigating the molecular mechanism of TR mediated repression of the thyroid-stimulating hormone α subunit gene (3). For these biological and biochemical studies, it would be useful to have a source for the purification of large quantities of receptor. TR isolation from natural sources has been difficult because of its low levels of expression. In this report, the baculovirus expression

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Abbreviations:
TR, thyroid hormone receptor; hTRß1, human thyroid hormone receptor ß1; TREpal, thyroid hormone response element-palindrome.

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system was used for the abundant production of human TRß1 (hTRß1) isoform in an eukaryotic system (4). Spodoptera frugiperda (Sf9) cells were infected with recombinant baculovirus containing the entire hTRß1 coding sequence. The recombinant virus was engineered by homologous recombination *in vivo* in which the viral polyhedrin gene was replaced with a recombinant polyhedrin gene containing the cDNA of the hTRß1 under the control of the viral polyhedrin promoter (4).

MATERIALS AND METHODS

Construction of hTRß1 expression plasmid: A 1.5 Kb BamHI/XbaI DNA fragment containing the hTRß1 cDNA was inserted into the BamHI/XbaI cleaved baculovirus expression vector pVL1393 to yield plasmid phTRß1.

Baculovirus expression system and preparation of cellular extracts: General baculovirus procedures (insect cell culture, transfection, isolation and purification of recombinant plaques, infection) were performed as described elsewhere (4). Crude cell extracts from Sf9 cells or COS cells were prepared as described previously (5). In brief, virus infected Sf9 cells were harvested 7 days post-infection and the cell pellet was washed twice with PBS and once with a buffer containing 10mM Tris-HCl pH 8.0, 1mM EDTA and 1mM DTT (TED). The cell pellet was resuspended in TED buffer containing 400mM KCl and 20% glycerol (v/v) and flash frozen in liquid nitrogen. The frozen pellet was thawed on ice and centrifuged to obtain the receptor-containing supernatant. Aliquots of the crude extract were stored at -70 C.

SDS-PAGE: SDS-polyacrylamide gel electrophoresis was performed using a buffer system described by Fling & Gregerson (6). 20 ug of each recombinant baculovirus-infected cell suspension, derived from purified recombinant plaques, were analyzed by electrophoresis through a 10 % gel.

Gel shift assays: A synthetic oligonucleotide (TREpal) that contains the palindromic sequence, 5' AGGTCATGACCT, was annealed and filled-in with [^{32}P] dCTP and unlabeled dNTPs using the Klenow fragment of DNA polymerase I, according to manufacturers instructions (New England Biolabs, Beverly, MA). The radioactive probe was incubated with crude cell extracts containing hTRß1 and thyroid hormone, as described elsewhere (5). Protein-DNA complexes were resolved on a 6% native polyacrylamide gel. Gels were dried and exposed for autoradiography.

Immunoprecipitation: Cell extract (15 µg protein) was incubated with 0.5 nM [^{125}I]-T3 in buffer (50 mM NaCl, 2 mM EDTA, 15 mM Tris pH 7.6, 1 mM DTT, 10% glycerol) for 16 hrs at 4 C. The T3-bound receptors were then incubated for 16 h at 4 C with anti-peptide antibodies (0.5 µl) specific for the hTRß1 or hTRα2 receptors (7). Antibody-receptor complexes were precipitated by adding 30 µl of a 50% slurry of protein A/G agarose beads (Schleicher & Schuell, Keene, NH), incubated with agitation for 1 hr at 4 C and washed three times with ice cold buffer before retained radioactivity was measured.

T3 Binding Affinity: Affinity of T3 binding to receptor was performed using the methods of Schueller et al. (8) and Inoue et al. (9). Extract (0.4 µl) was incubated with 0.01 nM [^{125}I]-T3 (NuClin Diagnostic, Northbrook, IL) at 4 C for 12 hrs in 200 µl of the buffer described above containing increasing concentrations of T3 (0.01nM -1.0 nM) . Receptor affinity constants (K_a) were calculated using Scatchard plot analysis of the bound and free ligand fractions.

RESULTS

Sf9 cells were co-transfected with wildtype baculovirus DNA and phTRß1. Seven days post-infection, cell culture media was used for plaque assays. Ten putative recombinant plaques were isolated and subjected to two additional rounds of plaque purification. Infections with all ten viral isolates were performed and cells were harvested seven days post-infection.

Crude extracts of infected Sf9 cells were analyzed by SDS-PAGE (Fig. 1). Cells infected with wild type baculovirus DNA, expressed a prominent protein (29.5 kDa) representing the polyhedrin protein (Fig. 1, lane 2). Infection with putative recombinant baculovirus produced a prominent band with an estimated mass of 55 kDa and the polyhedrin protein was absent, consistent with the disruption of this gene in the recombinant virus (Fig. 1, lane 1). Nine of the ten putative recombinant isolates expressed the 55kDa protein (data not shown), which is the predicted size of hTRß1. Mock-infected cells (Fig. 1, lane 3) did not show either of the 29.5 or the 55 kDa bands.

To verify that the recombinant viruses expressed hTRß1, an immuno-precipitation of crude cell extracts was performed using anti-hTRß1 antibody (Fig 2a). As a control, anti-hTRα2 antibody was used. Extracts were preincubated with [^{125}I]-T3 to allow concomitant determination of T3-binding by the immunoprecipitated proteins. Cells infected with recombinant baculovirus contained [^{125}I]-T3-labeled proteins that were specifically immunoprecipitated by the ß1 antibody as did a positive control

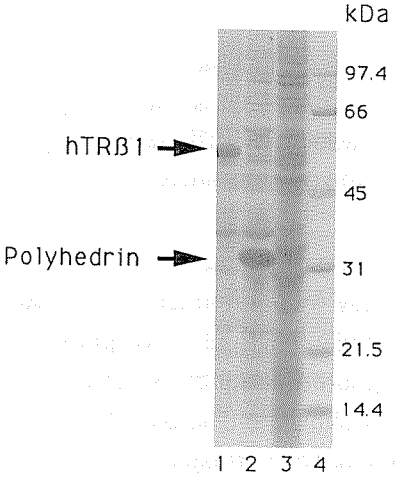

Fig. 1. SDS-PAGE analysis of total cell extracts from uninfected, wild type virus, and phTRß1 infected Sf9 cells. About 20µg of total cell extract was analyzed on a 10 % gel under reducing conditions. Lane 1, phTRß1 infected cells, arrow indicates a major protein of estimated Mr 55 kDa. Lane 2, wild type baculovirus (AcNPV) infected cells, arrow indicates the major protein of estimated Mr 29.5 kDa, corresponding to the polyhedrin protein. Lane 3, uninfected Sf9 cells. Lane 4, molecular weight markers with molecular mass indicated in kDa; phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme.

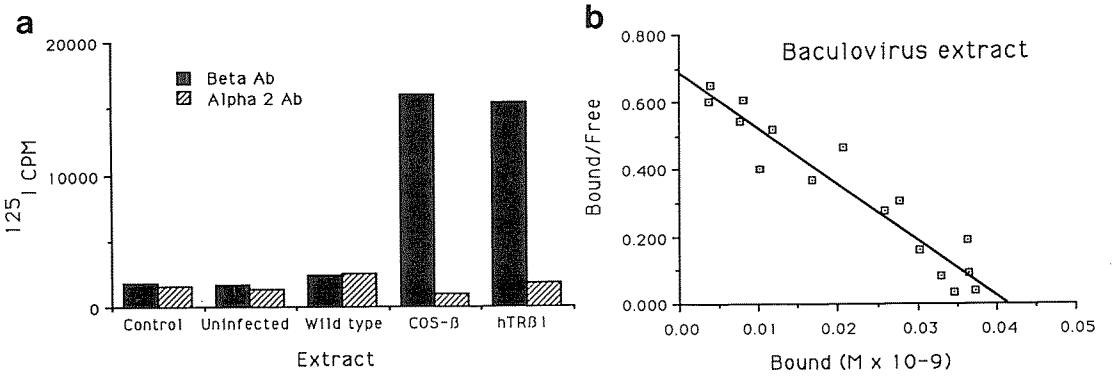

Fig. 2a. [^{125}I]-T3 binding profiles of immunoprecipitated human thyroid hormone receptor ß1 produced in Baculovirus-infected insect cells or in COS cells. Extracts from independent isolates of recombinant baculovirus-infected cells were examined for expression of hTRß1 using immunoprecipitation with either a specific anti-hTRß1 antibody or with an antibody specific for the hTRα2 splicing variant. In this figure, only one of the extracts containing the 55 kDa protein is shown. For comparison, an extract of hTRß1 expressed in COS cells was used as a positive control. The various extracts (7.5 µl) were incubated with 0.5 nM [^{125}I]-T3 and immunoprecipitated with anti-hTRß1 or anti-hTRα2 anti-peptide antibody and protein A/G agarose beads.

Fig. 2b. Scatchard analysis of [^{125}I]-T3 binding to hTRß1 containing Sf9 extracts. phTRß1 infected Sf9 cell extract (0.5 µg) was incubated with 0.01 nM [^{125}I] T3 and various concentrations of T3 as described in *Materials and Methods*.

receptor preparation derived from hTRß1 expressed in COS cells. Background levels of immunoprecipitated protein were seen in cells infected with wild type virus or uninfected cells.

To investigate the thyroid hormone binding characteristics of the hTRß1, diluted aliquots of crude cell extracts were incubated with increasing amounts of [^{125}I]-T3. A representative Scatchard plot analysis is shown in Figure 2b. The calculated K_a from multiple Scatchard analysis was 1.2×10^{10} (M^{-1}), comparable to published values (8) and to the K_a measured for *in vitro* translated hTRß1 and for hTRß1-containing COS extracts (data not shown).

To determine the binding of the baculovirus-expressed hTRß1 to TREpal, a DNA-protein interaction assay was performed using crude cell extracts containing hTRß1 and radioactively labelled TREpal. DNA-protein complexes were resolved by non-denaturing gel electrophoresis (Fig. 3). As a positive control, receptor expressed in COS cells and previously shown to bind both T3 and DNA was studied in parallel (lane 1) (5). Two prominent bands correspond to hTRß1-DNA complexes. A typical recombinant-virus infected Sf9 cell extract (lane 3) also shows two prominent DNA-protein complexes, indicating that baculovirus produced hTRß1 binds TREpal. In parallel gel shift experiments, the extracts were preincubated with [^{125}I]-T3 prior to binding to unlabeled TREpal (data not shown). These experiments confirmed that the protein-DNA complexes contained TR. The reason for the slightly faster mobility of the Sf9 produced hTRß1 is not known but may be due to differential post-translational

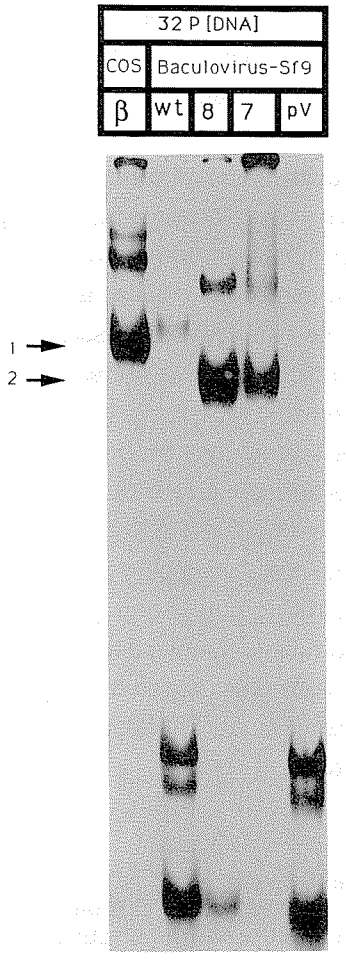

Fig. 3. Gel shift analysis using hTRß1 expressing Sf9 extracts. 15µl of of Sf9 cell extract (lanes 2, 3, and 4) or hTRß1 containing COS extract (lane 1) was incubated with 50 femtomoles of [^{32}P] labelled TREpal. Lane 2, wildtype virus infected extract; lane 3, Sf9 cells infected with recombinant-virus from one hTRß1 plaque; lane 4, pVL 1393 (vector) infected extract.

modification or associated proteins in COS cells. Extracts from cells infected with wildtype baculovirus (lane 4) or pVL1393 vector (lane 2) were used as negative controls for non-receptor proteins that might interact with TREpal.

DISCUSSION

In this study, we have demonstrated the production of recombinant hTRß1 in the baculovirus expression system. Several lines of evidence suggest production of authentic hTRß1. First, the expressed protein binds thyroid hormone hormone with high affinity that is characteristic of the T3 receptor. Second, the receptor is specifically immunoprecipitated by an anti-peptide antibody to hTRß1. Third, it binds to a known T3 receptor DNA binding site (TREpal). SDS-polyacrylamide gel electrophoresis

indicates that hTRß1 may account for as much as 5-10% of total cellular protein in infected Sf9 cells. By hormone binding criterion only, the ratio of functional hTRß1 compared to total hTRß1 protein present appears to be fairly high, although precise quantification must await further purification.

The baculovirus expression system utilizes an eukaryotic (insect cells) vehicle to produce the foreign protein. It has become increasingly evident that eukaryotic proteins often require one or more post-translational modification(s) in order to render them biologically active. The use of Sf9 cells as an eukaryotic system has found widespread acceptance for the high level expression of foreign genes to produce biochemical quantities of biologically active proteins (10). The baculovirus-mediated production of human glucocorticoid receptor (11) as well as the current report of TR expression indicates that the system may be well suited for the production of the steroid/thyroid superfamily of ligand-induced transcription factors. A T7 expression system has been used to express hTRß1 in E. coli, which was shown to bind hormone and DNA (12). It will be of interest to compare the properties of receptors prepared from these two sources. Following purification to homogeneity, detailed biochemical studies may be performed to define in molecular terms the properties of the receptor involved in dimerization, DNA binding, ligand binding, trans-activation and trans-repression. Moreover, sufficient amounts of pure receptor may ultimately facilitate structural analysis of receptor.

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