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cDNA-directed expression of human thyroid peroxidase

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A human thyroid peroxidase cDNA, hTPO-1 [(1987) Proc. Natl. Acad. Sci. USA 84, 5555–5559], was expressed in human Hep G2 cells using a vaccinia virus cDNA-expression system. When examined by immunoblot analysis, the level of hTPO-1 protein expression reached a maximum approx. 24 h after infection and remained at a similar level up to 72 h post-infection. The expressed protein was enzymatically active as measured by guaiacol oxidation. Monoclonal antibody-assisted immunoaffinity column chromatography was used for partial purification of vaccinia-expressed hTPO-1, resulting in more than 300-fold higher specific activity and a measurable difference spectrum of the hTPO-1 (Fe^{3+})-CN complex.

Peroxidase; cDNA expression; Vaccinia virus; Immunopurification; (Human thyroid)

1. INTRODUCTION

Thyroid peroxidase (TPO; donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) is one of the key enzymes involved in the biosynthesis of thyroid hormones. It catalyzes both iodination and coupling of tyrosine residues in thyroglobulin to produce thyroxine (T_4) and triiodothyronine (T_3) [1,2]. The enzyme is a glycosylated hemoprotein [3] of about 107 kDa as estimated on SDS-polyacrylamide gels [4–8]. The prosthetic group is probably protoporphyrin IX [1,8,9]. Several reports have established that TPO is a major component of the thyroid microsomal antigen, one of the autoimmune antigens against which patients having thyroid autoimmune disorders develop antibodies [4,10–12].

The human TPO cDNA has recently been cloned and its nucleotide and deduced amino acid sequences determined [12–15]. The hTPO-1 cDNA we isolated is 3048 nucleotides in length and codes for a protein with 933 amino acids [13]. Here, we describe, for the first time, expression of the human TPO cDNA (hTPO-1) using a vaccinia-virus expression system. This expressed protein was highly active as measured by guaiacol oxidation after enrichment through an immunoaffinity column.

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Abbreviations: TPO, thyroid peroxidase; TK, thymidine kinase; BudR, 5-bromodeoxyuridine

2. MATERIALS AND METHODS

Human TPO cDNA, hTPO-1, used here was constructed by joining phTPO-2.8 to phTPO-5′ at a *Bgl*II site (435–460 bp in [13]). Human TPO was purified as in [8] and rabbit anti-porcine TPO antibody has been produced [11]. Phosphatase-conjugated goat anti-rabbit IgG antibody was purchased from KPL Laboratories (Gaithersburg, MD). Vaccinia virus (strain WR), recombinant vector pSC11 and human TK^- 143 cells were obtained from Dr Bernard Moss (National Institute of Allergy and Infectious Diseases). CV-1 (ATCC CCL 70) and Hep G2 (ATCC HB 8065) cells were from the American Type Culture Collection.

The vaccinia virus expression system was used to express full-length hTPO-1 cDNA. The insert was first made blunt-ended by DNA polymerase Klenow fragment and ligated to the dephosphorylated *Sma*I site of recombinant plasmid pSC11 [16]. The plasmid containing hTPO-1, or no insert as control, was then allowed to be integrated into vaccinia virus according to [16,17]. Briefly, CV-1 cells were infected with wild-type vac-

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cinia virus prior to transfection with calcium phosphate precipitates of vaccinia virus DNA and recombinant plasmid pSC11 containing cDNA insert or no insert. Virus particles were harvested after 2 days and recombinant virus selection was initiated by infecting human TK^- cells with the harvested viruses in the presence of 5-bromodeoxyuridine (BudR). The basis for this selection is as follows: plasmid pSC11 has portions of the thymidine kinase (TK) sequences that flank vaccinia virus promoters and the *SmaI* cloning site. Recombination takes place between TK sequences in the vector and those in the wild-type virus genome, producing recombinant viruses which are no longer capable of synthesizing native TK protein due to disruption of the TK gene by plasmid insertion. Only viruses without an active TK gene can survive in the presence of BudR. In order to distinguish recombinant viruses from those having spontaneous mutations in their TK gene, expression of the β-galactosidase gene, which occurs in the pSC11 plasmid, was also used for selection. After three successive BudR and β-galactosidase selections, vaccinia virus producing hTPO-1, designated vhTPO-1, was identified by immunoblotting using rabbit anti-porcine TPO antibody.

Hep G2 cells infected by vhTPO-1 were harvested in phosphate-buffered saline (pH 7.2) at various times after infection as indicated, and sonicated for 5 s. Cell lysates thus obtained were then directly subjected to SDS-polyacrylamide gel electrophoresis (100 μg protein/well) [18]. Blotting was performed using a semi-dry blotter (Integrated Separation Systems, Hyde Park, MA) via the method of Kyhse-Andersen [19]. After treatment with antibodies, final color development of the alkaline phosphatase reaction was carried out using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates (KPL Laboratories). Protein concentration was determined using BCA protein assay reagent (Pierce, Rockford, IL).

Immunopurification of the expressed hTPO-1 protein was carried out by means of immunoaffinity column chromatography using monoclonal antibody 38E to human TPO as described [8]. Briefly, Hep G2 cells, harvested after approx. 50 h post-infection, were homogenized in 20 mM Tris buffer, pH 7.4, containing 0.2% sodium cholate, 0.1 M KCl, and 0.1 mM KI. After sedimentation of cell debris, the supernatant was directly applied onto a column of Sepharose 4B coupled with monoclonal antibody 38E [8]. The column was washed with 50 mM borate buffer, pH 9.0, containing 0.5% sodium cholate, 1 M KCl and 1 mM KI. The expressed hTPO-1 was eluted with 0.2 M NH_4OH containing 0.5% sodium cholate, 1 M KCl, and 1 mM KI [8]. Peak fractions were combined, then concentrated and desalted using Centricon 30 (Amicon, Danvers, MA) in 20 mM Tris buffer (pH 7.4), containing 0.1 mM KI.

TPO activity was determined in 0.1 M Tris (pH 7.4) at 20°C by using guaiacol oxidation on an Aminco DW-2000 spectrophotometer. Guaiacol oxidation was measured by following the absorbance change at 470 nm in the presence of 30 mM guaiacol and 0.5 mM H_2O_2.

3. RESULTS AND DISCUSSION

The recombinant virus, vhTPO-1, obtained as outlined in section 2, was used to infect Hep G2 cells. The human hepatoma Hep G2 cell line was chosen to express hTPO-1 in this study, since this liver-derived cell line is known to possess ample intracellular membranes and to express a variety of heme proteins, therefore containing sufficient heme. Cells were harvested at various times after infection as indicated, subjected to immunoblotting, and found to possess hTPO-1 protein (fig.1). The size of the expressed protein is the same as that of the purified protein (about 107 kDa) [4,8,11]. Expressed hTPO-1 appeared as early as 6 h after infection (not shown) and the level of synthesis reached a maximum approx. 24 h after infection (fig.1). The level of expressed protein remained elevated up to 72 h post-infection, suggesting that the hTPO-1 expressed protein is not particularly labile. Based on the immunoblot band intensity and comparisons with purified TPO, the amount of hTPO-1 expressed protein was estimated to be about 0.3–0.6 μg/100 μg cell lysate.

Peroxidase activity was determined by guaiacol oxidation using a cell lysate expressing hTPO-1 proteins (table 1). Cells harvested 48 h after infection with either wild-type vaccinia virus or recombinant virus containing plasmid pSC11 only displayed no activity. These results indicate that measurable peroxidase activity was indeed derived from the cDNA-expressed hTPO-1. The activity was detected in 24 h post-infection cells and reach-

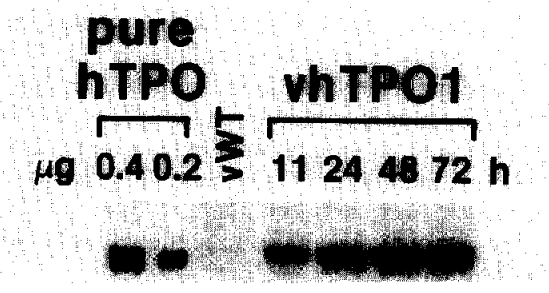

Fig.1. Immunoblot analysis of vaccinia virus-expressed hTPO-1. vhTPO-1 infected Hep G2 cells were harvested at various post-infection times as indicated (11, 24, 48, 72 h). Wild-type vaccinia infected cells (vWT) were harvested 48 h after infection. Cells were processed as described in section 2 and 100 μg protein of the cell lysate was loaded per lane. The same results as those for vWT were obtained by using vaccinia virus carrying plasmid pSC11. A dilution series of purified human TPO (0.2, 0.4 μg) was electrophoresed on the same gel to estimate the amount of expressed hTPO-1 protein.

Table 1

Guaiacol oxidation activity of vaccinia virus-expressed hTPO-1 protein

Post-infection time (h)	Guaiacol oxidation activity
vWT (48)	ND
vhTPO-1 (11)	ND
(24)	0.032 ± 0.001
(48)	0.181 ± 0.006
(72)	0.256 ± 0.004
hTPO-1 after immunoaffinity column	80.8 ± 2.3

Guaiacol oxidation activity was measured as described in the text. Values represent the specific activity expressed in ΔA_{470}/min per mg protein. ND, no detectable activity. Activity determination was performed at least three times per sample and values are means ± SD

ed maximal level at 72 h (table 1). No measurable activity was observed in the 11 h sample. These data are interesting because the level of immunodetectable hTPO-1 protein in 11 h post-infection cells was at least 1/4 that of maximally expressed proteins obtained in the 24–72 h post-infection cells (fig.1). These results indicate the existence of a lag time (~24 h) between expressed protein level and activity expression (fig.1 and table 1). This may be due to post-translational modification of the protein, which is a necessary requirement for activity. Potential post-translational modifications might be glycosylation or signal peptide cleavage. The presence of signal peptide at the N-terminus of TPO has been predicted based on the cDNA-deduced amino acid sequence [12,13,15]. It might be expected that changes in glycosylation or signal peptide cleavage would be detectable from differences in mobility on SDS-polyacrylamide gels, however, no obvious differences are seen in the inactive protein present at 11 h post-infection and the most active protein present at 72 h (fig.1). The pertinent post-translational modification might be too subtle for detection on gels. Another possibility that could account for the lag time in activity is that heme incorporation does not occur during or soon after protein synthesis.

The Hep G2 cell lysate after solubilization with sodium cholate [8] was further subjected to partial purification of hTPO-1 protein using monoclonal antibody to human TPO [8] as described in section

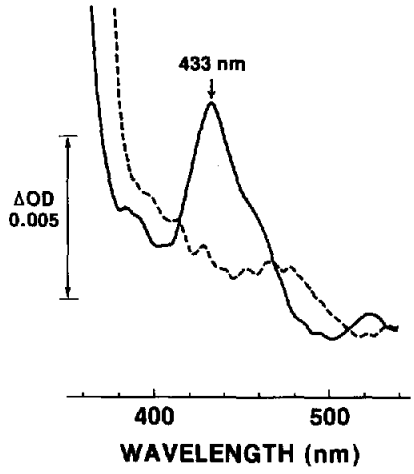

Fig.2. Difference spectrum of expressed hTPO-1(Fe^{3+})-cyanide complex. Solutions containing enriched hTPO-1 (——) or control protein (– – –) were divided into two cuvettes. Non-specific proteins that were eluted from an immunoaffinity column with the same buffer as for eluting hTPO-1 were prepared using Hep G2 cells infected with wild-type vaccinia virus and were used as control protein. A crystal of KCN was then added to a sample cuvette and the difference spectrum recorded on an Aminco DW-2000 spectrophotometer.

2. The partially purified hTPO-1 exhibited a difference spectrum, when complexed with cyanide, with λ_{max} at 433 nm (fig.2). This is similar to that reported in [20] and clearly indicates that the expressed hTPO-1 is a heme-protein. After immunoenrichment, the hTPO-1 exhibited greater than 300-fold higher specific activity as measured by guaiacol oxidation (table 1). This is the first report describing the production of an enzymatically active cDNA-expressed mammalian peroxidase. Further studies using site-directed mutagenesis should help in gaining insights into the structure-function relationships of these peroxidases.

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REFERENCES

[1] DeGroot, L.J. and Niepomniszcze, H. (1977) Metabolism 26, 665–718.
[2] Nunez, J. (1980) in: The Thyroid Gland (De Visscher, M. ed.) pp.39–59, Raven, New York.
[3] Rawitch, A.B., Taurog, A., Chernoff, S.B. and Dorris, M.L. (1979) Arch. Biochem. Biophys. 194, 244–257.

[4] Czarnocka, B., Ruf, J., Ferrand, M., Carayon, P. and Lissitzky, S. (1985) FEBS Lett. 190, 147–152.
[5] Kajita, Y., Morgan, D., Parks, A.B. and Smith, B.R. (1985) FEBS Lett. 187, 334–338.
[6] Hamada, N., Grimm, C., Mori, H. and DeGroot, L.J. (1985) J. Clin. Endocrinol. Metab. 61, 120–128.
[7] Hamada, N., Portmann, L. and DeGroot, L.J. (1987) J. Clin. Invest. 79, 819–825.
[8] Ohtaki, S., Kotani, T. and Nakamura, Y. (1986) J. Clin. Endocrinol. Metab. 63, 570–576.
[9] Ohtaki, S., Nakagawa, H., Nakamura, S., Nakamura, M. and Yamazaki, I. (1985) J. Biol. Chem. 260, 441–448.
[10] Portmann, L., Hamada, N., Heinrich, G. and DeGroot, L.J. (1985) J. Clin. Endocrinol. Metab. 62, 1001–1003.
[11] Kotani, T., Umeki, K., Matsunaga, S., Kato, E. and Ohtaki, S. (1986) J. Clin. Endocrinol. Metab. 62, 928–933.
[12] Libert, F., Ruel, J., Ludgate, M., Swillens, S., Alexander, N., Vassart, G. and Dinsart, C. (1987) EMBO J. 6, 4193–4196.
[13] Kimura, S., Kotani, T., McBride, O.W., Umeki, K., Hirai, K., Nakayama, T. and Ohtaki, S. (1987) Proc. Natl. Acad. Sci. USA 84, 5555–5559.
[14] Libert, F., Ruel, J., Ludgate, M., Swillens, S., Alexander, N., Vassart, G. and Dinsart, C. (1987) Nucleic Acids Res. 15, 6735.
[15] Magnusson, R.P., Chazenbalk, G.D., Gestautas, J., Seto, P., Filetti, S., DeGroot, L.J. and Rapoport, B. (1987) Mol. Endocrinol. 1, 856–861.
[16] Chakrabarti, S., Brechling, K. and Moss, B. (1984) Mol. Cell. Biol. 5, 3403–3409.
[17] Mackett, M., Smith, G.L. and Moss, B. (1984) J. Virol. 49, 857–864.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Kyhse-Andersen, J. (1984) J. Biophys. Biochem. Methods 10, 203–209.
[20] Hosoya, T. and Morrison, M. (1967) J. Biol. Chem. 242, 2828–2836.