Isolation of a Complementary DNA Clone for Thyroid Microsomal Antigen

Homology with the Gene for Thyroid Peroxidase

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Abstract

The thyroid microsomal antigen (MSA) in autoimmune thyroid disease is a protein of ~ 107 kD. We screened a human thyroid cDNA library constructed in the expression vector lambda gt11 with anti-107-kD monoclonal antibodies. Of five clones obtained, the recombinant β-galactosidase fusion protein from one clone (PM-5) was confirmed to react with the monoclonal antiserum. The complementary DNA (cDNA) insert from PM-5 (0.8 kb) was used as a probe on Northern blot analysis to estimate the size of the mRNA coding for the MSA. The 2.9-kb messenger RNA (mRNA) species observed was the same size as that coding for human thyroid peroxidase (TPO). The probe did not bind to human liver mRNA, indicating the thyroid-specific nature of the PM-5-related mRNA. The nucleotide sequence of PM-5 (842 bp) was determined and consisted of a single open reading frame. Comparison of the nucleotide sequence of PM-5 with that presently available for pig TPO indicates 84% homology. In conclusion, a cDNA clone representing part of the microsomal antigen has been isolated. Sequence homology with porcine TPO, as well as identity in the size of the mRNA species for both the microsomal antigen and TPO, indicate that the microsomal antigen is, at least in part, TPO.

Introduction

For more than two decades, antibodies against an unidentified thyroid microsomal antigen have been observed and studied in patients with autoimmune thyroid disease, in particular Hashimoto's thyroiditis (1, 2). In the past two years, rapid progress has been made in elucidating the nature of this microsomal antigen. By Western blot analysis (3) and by immunoprecipitation (4, 5), the microsomal antigen has been identified as a protein of ~ 107,000 D. Furthermore, there is strong evidence accumulating that the microsomal antigen is thyroid peroxidase (TPO).[1] Thus, using sera from patients with Hashimoto's thyroiditis, a significant correlation was observed between microsomal antibody titers and anti-TPO activity (6, 7). TPO bioactivity can be immunoprecipitated by sera containing microsomal antibodies (6, 8). Finally, immobilized, purified TPO can selectively adsorb antimicrosomal, but not antithyroglobulin, antibodies (9).

Recently, monoclonal antibodies have been produced against the 107-kD human thyroid microsomal antigen (10). In the present report we describe the use of these monoclonal antibodies to obtain a complementary DNA (cDNA) clone representing part of this antigen. Comparison of the nucleotide sequence from part of this clone with the nucleotide sequence of porcine thyroid peroxidase (11, 12) now demonstrates the identity of these two antigens, at least in part.

Methods

Screening of human thyroid cDNA library. The construction of a human thyroid cDNA library in the expression vector lambda gt11 (13) has been previously described (14). This library consists of ~ 10^6 recombinants with an average insert size of 1.1 kb. The expanded library was plated (~ 3×10^4 plaque-forming U/150-mm dish) in *Escherichia coli* Y1090 cells. After 3–3.5 h, nitrocellulose filters previously saturated with isopropyl β-D-thiogalactopyranoside were applied for 3 h. The filters were then exposed to antimicrosomal monoclonal antibodies (1:100 dilution) in ascitic fluid according to the method of Huynh et al. (15) with the exception that bound antibody was detected with anti-mouse IgG (Cappel Laboratories, Cochranville, PA) conjugated to horseradish peroxidase (CooperBiomedical, Inc., Malvern, PA) (1:100 dilution overnight at 4°C). The peroxidase substrate was 0.5 mg/ml 4 chloro-1-naphthol (Sigma Chemical Co., St. Louis, MO), 10 mM imidazole, 0.0125% H_2O_2.

Western blot analysis. Lysogens of selected lambda gt11 clones were made in Y1089 cells for generation of fusion proteins (15). Bacterial lysates were prepared by freeze-thawing and sonication (15). Aliquots (~ 20 μg protein) were applied to 7.5% polyacrylamide gels. The electrophoresed proteins were transferred onto nitrocellulose paper as previously described (14). The filters were then rinsed once with Tris-buffered saline (TBS) buffer (50 mM Tris, pH 8.0, 150 mM NaCl) and then for 20 min in TBS containing 20% fetal calf serum, followed by the same protocol used to screen the library. As a control, mouse ascitic fluid IgG 1 monoclonal antiserum (Cappel Laboratories) was used.

Northern blot analysis. Poly(A)+ messenger RNA (mRNA) was prepared from Graves' thyroid tissue and from normal human liver tissue by the method of Chirgwin et al. (16), followed by oligo(dT) affinity chromatography. mRNA (7 μg/lane) was applied to 2.2 M formaldehyde agarose gels (1.5%) and transferred by blotting to Nytran paper (Schleicher & Schuell, Inc., Keene, NH). The paper was probed with the indicated cDNA inserts, recovered from pUC18 into which they had been subcloned from lambda gt11. The probes consisted of

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Received for publication 6 February 1987 and in revised form 22 July 1987.

1. *Abbreviation used in this paper:* TPO, thyroid peroxidase.

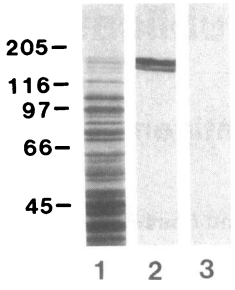

Figure 1. Western blot analysis of the PM-5 fusion protein. The fusion protein of PM-5 was generated in a Y1089 lysogen. Aliquots of bacterial lysate (20 μg protein) were applied to a 7.5% polyacrylamide gel. After electrophoresis and transfer to nitrocellulose paper, the proteins were probed with antimicrosomal antigen monoclonal antibody (see Methods). Lane *1* shows Coomassie Blue staining of the gel. Molecular weight markers were myosin (205 kD), β-galactosidase (116 kD), rabbit muscle phosphorylase B (97 kD), bovine serum albumin (66 kD), and egg albumin (45 kD). Lane *2* indicates binding of specific monoclonal antiserum to the transferred proteins shown in lane *1*. Lane *3* demonstrates lack of interaction of a control monoclonal antibody with the same proteins.

the entire PM-5 cDNA and a 0.6-kb pig TPO cDNA, fragment J (11). The probes were labeled by nick translation (17) to a specific activity of $\sim 2 \times 10^8$ cpm/μg. Hybridization conditions were as previously described (14, 18).

Nucleotide sequencing. cDNA was subcloned into M13mp18 and M13mp19 and sequenced by the dideoxy termination method (19). Comparison of nucleotide sequences was made by the I-FIND program of Bionet.

Results

Screening of the human thyroid cDNA library in lambda gt11 with monoclonal antibodies against the 107-kD human thyroid microsomal antigen yielded five clones. After plaque purification, the phage from each clone was used to make lysogens in Y1089 bacteria (15), and the bacterial lysates from these were used for Western blot analysis. The recombinant β-galactosidase fusion protein in only one clone (PM-5) was confirmed to react with the monoclonal antiserum (Fig. 1). Interestingly, this fusion protein was a doublet. Control monoclonal antiserum of the same subtype did not react with this fusion protein (Fig. 1).

The cDNA insert from PM-5 (~ 0.8 kb) was isolated, subcloned into the Eco RI site of pUC18, and subsequently used as a probe to estimate the size of the mRNA coding for the entire protein. Northern blot analysis indicated hybridization to a 2.9-kb species (Fig. 2). The probe did not bind to human liver mRNA, indicating the thyroid-specific nature of the PM-5-related mRNA. When a 0.6-kb cDNA probe from porcine TPO was hybridized to human thyroid mRNA, a similar 2.9-kb mRNA species was identified (Fig. 2). As with PM-5, the TPO probe did not bind to human liver mRNA.

Because of the previous evidence that the microsomal antigen is TPO, and because of the identical sizes of mRNA

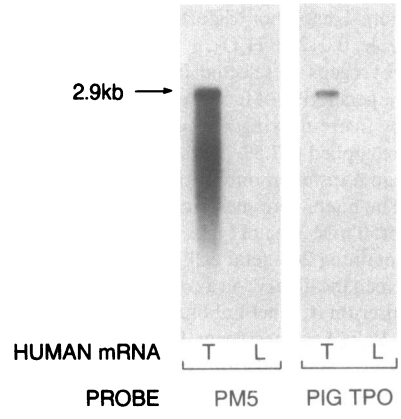

Figure 2. Northern blot analysis of human thyroid and liver mRNA. 7 μg each of human thyroid mRNA (lane *T*) and human liver mRNA (lane *L*) were electrophoresed on a 1.5% agarose gel and were then transferred to Nytran paper as described in Methods. The mRNA was probed with the nick-translated 0.8-kb cDNA insert from clone PM-5 (*left panel*) and with a 0.6-kb porcine TPO cDNA fragment J (*right panel*) (11). Sizing of the 2.9-kb mRNA species was on the basis of DNA and RNA ladders (Bethesda Research Laboratories, Gaithersburg, MD).

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                                                    28                                  55
AAT GAG GTT GTC ACA GAT GAT GAC CGC TAT TCT GAC CTC CTG ATG GCA TGG GGA
Asn Glu Val Val Thr Asp Asp Asp Arg Tyr Ser Asp Leu Leu MET Ala Trp Gly

                                                    82                                  109
CAA TAC ATC GAC CAC GAC ATC GCG TTC ACA CCA CAG AGC ACC AGC AAA GCT GCC
Gln Tyr Ile Asp His Asp Ile Ala Phe Thr Pro Gln Ser Thr Ser Lys Ala Ala

                                                   136                                  163
TTC GGG GGA GGG TCT GAC TGC CAG ATG ACT TGT GAG AAC CAA AAC CCA TGT TTT
Phe Gly Gly Gly Ser Asp Cys Gln MET Thr Cys Glu Asn Gln Asn Pro Cys Phe

                                                   190                                  217
CCC ATA CAA CTC CCG GAG GAG GCC CGG CCG GCC GCG GGC ACC GCC TGT CTG CCC
Pro Ile Gln Leu Pro Glu Glu Ala Arg Pro Ala Ala Gly Thr Ala Cys Leu Pro

                                                   244                                  271
TTC TAC CGC TCT TCG GCC GCC TGC GGC ACC GGG GAC CAA GGC GCG CTC TTT GGG
Phe Tyr Arg Ser Ser Ala Ala Cys Gly Thr Gly Asp Gln Gly Ala Leu Phe Gly

                                                   298                                  325
AAC CTG TCC ACG GCC AAC CCG AGG CAG CAG ATG AAC GGG TTG ACC TCG TTC CTG
Asn Leu Ser Thr Ala Asn Pro Arg Gln Gln MET Asn Gly Leu Thr Ser Phe Leu

                                                   352                                  379
GAC GCG TCC ACC GTG TAT GGC AGC TCC CCG GCC CTA GAG AGG CAG CTG CGG AAC
Asp Ala Ser Thr Val Tyr Gly Ser Ser Pro Ala Leu Glu Arg Gln Leu Arg Asn

                                                   406                                  433
TGG ACC AGT GCC GAA GGG CTG CTC CGC GTC CAC GGC CGC CTC CGG GAC TCC GGC
Trp Thr Ser Ala Glu Gly Leu Leu Arg Val His Gly Arg Leu Arg Asp Ser Gly

                                                   460                                  487
CGC GCC TAC CTG CCC TTC GTG CCG CCA CGC GCG CCT GCG GCC TGT GCG CCC GAG
Arg Ala Tyr Leu Pro Phe Val Pro Pro Arg Ala Pro Ala Ala Cys Ala Pro Glu

                                                   514                                  541
CCC GGC AAC CCC GGA GAG ACC CGC GGG CCC TGC TTC CTG GCC GGA GAC GGC CGC
Pro Gly Asn Pro Gly Glu Thr Arg Gly Pro Cys Phe Leu Ala Gly Asp Gly Arg

                                                   568                                  595
GCC AGC GAG GTC CCC TCC CTG ACG GCA CTG CAC ACG CTG TGG CTG CGC GAG CAC
Ala Ser Glu Val Pro Ser Leu Thr Ala Leu His Thr Leu Trp Leu Arg Glu His

                                                   622                                  649
AAC CGC CTG GCC GCG GCG CTC AAG GCC CTC AAT GCG CAC TGG AGC GCG GAC GCC
Asn Arg Leu Ala Ala Ala Leu Lys Ala Leu Asn Ala His Trp Ser Ala Asp Ala

                                                   676                                  703
GTG TAC CAG GAG GCG CGC AAG GTC GTG GGC GCT CTG CAC CAG ATC ATC ACC CTG
Val Tyr Gln Glu Ala Arg Lys Val Val Gly Ala Leu His Gln Ile Ile Thr Leu

                                                   730                                  757
AGG GAT TAC ATC CCC AGG ATC CTG GGA CCC GAG GCC TTC CAG CAG TAC GTG GGT
Arg Asp Tyr Ile Pro Arg Ile Leu Gly Pro Glu Ala Phe Gln Gln Tyr Val Gly

                                                   784                                  811
CCC TAT GAA GGC TAT GAC TCC ACC GCC AAC CCC ACT GTG TCC AAC GTG TTC TCC
Pro Tyr Glu Gly Tyr Asp Ser Thr Ala Asn Pro Thr Val Ser Asn Val Phe Ser

                                                   838
ACA GCC GCC TTC CGC TTC GGC CAT GCC ACG A
Thr Ala Ala Phe Arg Phe Gly His Ala Thr
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Figure 3. Nucleotide and amino acid sequence of clone PM-5. The translational reading frame begins at basepair No. 2 in the PM-2 sequence.

species for the human microsomal antigen, human TPO (Fig. 2) and pig TPO (11), we determined the entire nucleotide sequence of PM-5. The human thyroid microsomal antigen cDNA clone (PM-5) consisted of a tract of 842 bp, all within a single reading frame (Fig. 3). Remarkably, comparison of the nucleotide sequence of PM-5 with that of pig TPO revealed an homology of 84% (Fig. 4). This region of homology is located between 2.2 kb and 1.4 kb upstream from the 3'-end of the pig TPO cDNA gene (12).

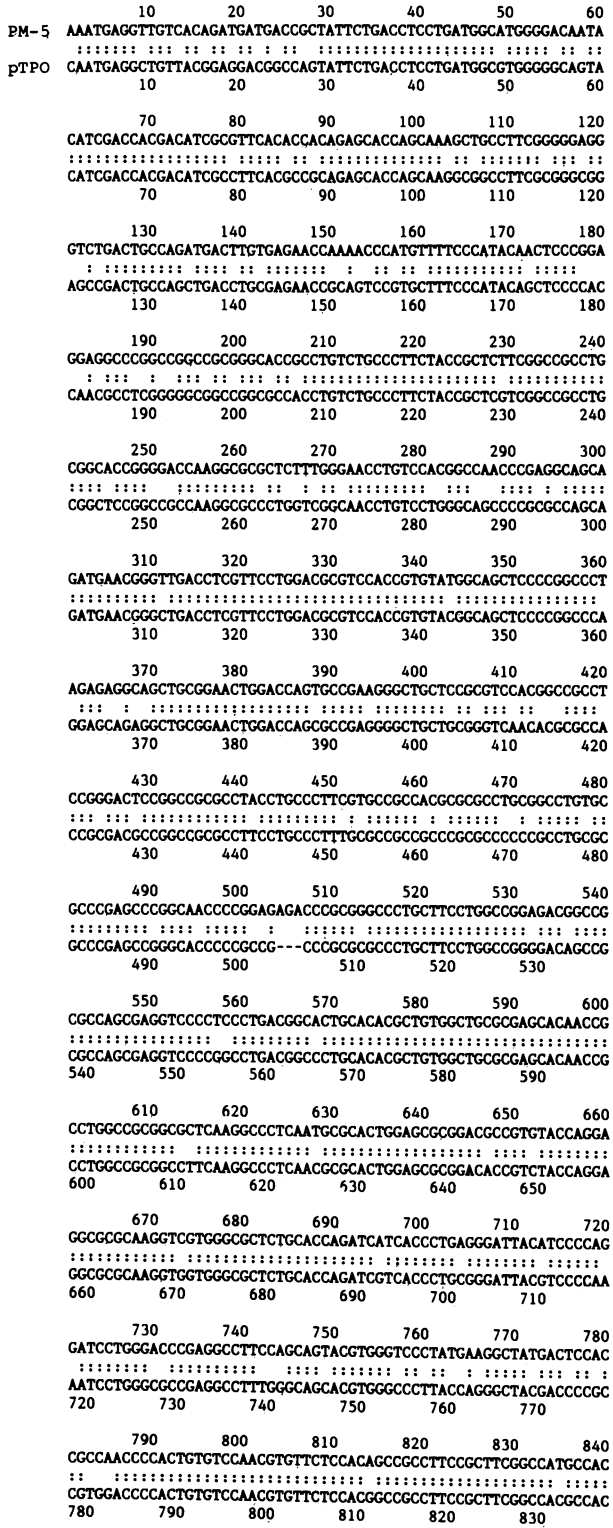

Figure 4. Homology of the nucleotide sequences of clone PM-5 (human) and pig TPO (*pTPO*). Colons indicate nucleotide identity. The numbering of the pig TPO nucleotide sequence is arbitrary because we have not as yet determined the exact length of the gene extending up to the 5' end. However this region of pig TPO spans from 2.2 kb to 1.4 kb from the 3' end (12).

Discussion

Pig TPO was recently cloned in our laboratory using synthetic oligonucleotide probes based on the amino acid sequence of tryptic fragments of highly purified porcine TPO (11, 12). In the present study we screened a human cDNA lambda gt11 library by another method, namely using monoclonal antibodies to detect an expressed protein. Clones with regions of homologous sequence were obtained by each procedure. The < 100% homology between the regions of the two clones is to be expected because they are from two different species, namely pig and human. Despite the fact that the entire gene has not been characterized, the degree of homology noted, as well as the identity in the sizes of mRNA for both the microsomal antigen and TPO, indicate that the microsomal antigen is, at least in part, TPO.

It is interesting that the fusion protein generated by clone PM-5 is a doublet. Because PM-5 is clonal it follows that the fusion protein generated by this clone is derived from one gene, and therefore the doublet is likely to be the result of posttranslational modification of the fusion protein. However we cannot exclude the possibility of multiple transcriptional initiation sites, although this is highly unlikely in view of the fact that the cDNA insert is within the β-galactosidase gene, and transcriptional initiation will begin in the β-galactosidase gene itself.

The present data indicating identity of the thyroid microsomal antigen and TPO genes open the way for the complete characterization of these antigens. This in turn will be very useful in further studies on the pathogenesis of autoimmune thyroid disease. For example, it will permit the development of (*a*) more sensitive and specific assays for autoantibodies, (*b*) the generation of antibodies against specific epitopes on the antigen that can be used for in vivo and in vitro studies on the pathogenesis of autoimmune thyroid disease, (*c*) potential therapy for Hashimoto's thyroiditis using antiidiotypic antibodies, and (*d*) there is now the opportunity for examining the mechanism of regulation of microsomal antigen expression in autoimmune thyroid disease, as well as the relationship between the expression of this gene and other genes such as for the class II histocompatibility antigens.

Acknowledgments

The expert secretarial assistance of Jan Alfstad is gratefully acknowledged.

This work was supported by National Institutes of Health (NIH) grants AM-36182 and AM-19289, as well as by the Veterans Administration. Computer resources used to carry out our studies were provided by the NIH-sponsored Bionet National Computer Resource for Molecular Biology, grant 441 RR-01685-03.

Note added in proof. Since submitting the present paper we have determined the complete nucleotide sequence of both pig and human TPO cDNA. They are both 3.2 kb in length. Irrespective of whether a

pig or human TPO cDNA probe was used, the same size mRNA species was detected (as shown in Fig. 2).

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