Studies of 64 kDa antigen(s) and corresponding autoantibodies in patients with thyroid-associated ophthalmopathy

by

Zhiguang Zhang

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements of the degree of
Doctor of Philosophy

Department of Medicine, Division of Experimental Medicine
McGill University
Montreal, Quebec

ISBN 0-612-05818-2

Abstract

The ophthalmopathy associated with Graves' hyperthyroidism (GH) and Hashimoto's thyroiditis is an autoimmune-mediated inflammation of extraocular muscle and orbital connective tissue. The association of thyroid-associated ophthalmopathy (TAO) with autoimmune thyroid disease (ATD) can be explained by the expression on thyroid and eye muscle of shared or cross-reactive Ag(s) targeted by autoimmunity. The best candidate for such Ag(s), at present, are 64 kDa membrane molecules. When incubated with cultured human eye muscle, thyroid, and skeletal muscle cells *in vitro*, autoAbs in TAO patient sera immunoprecipitated a 64 kDa Ag from the first two tissues, but not from skeletal muscle. In dot blot experiments using D1 fusion protein, a fragment of a cloned 64 kDa protein, autoAbs were detected in the sera of 47% of patients with TAO and 57% of those with GH without ophthalmopathy but in only 5% of normal subjects. Short random fragments (100 to 200 bp) of 1D cDNA, a cloned 64 kDa protein, were cloned and expressed in E. coli as fusion proteins using the pUEX1 vector. Four antigenic peptides recognized by TAO sera were cloned and sequenced. When a panel of sera was screened for recognition of each peptides, sera from TAO patients reacted to one or more peptide significantly more frequently than sera from normal controls. A quantitative reverse transcriptase polymerase chain reaction (RT-PCR) methodology was used to investigate the tissue distribution of 1D mRNA and quantitate the levels of expression of transcripts of this specificity from a variety of human tissues. Expression of 1D was highest in thyroid and eye muscle, consistent with a high level of expression of this protein in these tissues. These studies indicate that 64 kDa proteins are expressed in eye muscle and thyroid, and that autoAbs reactive with the Ag(s) are present in the early stages of thyroid eye disease.

Résumé

L'ophtalmopathie associée avec l'hyperthyroïdisme de Graves et l'hypothyroïdisme (maladie d'Hashimoto) est une inflammation autoimmune des muscles oculaires et des tissus conjonctifs de l'oeil. L'association entre l'ophtalmopathie associée avec le thyroïdisme (OAT) et les maladies autoimmunes de la thyroïde peut être expliquée en proposant l'existance d'un ou de plusieurs antigènes similaires trouvés sur la surface des cellules provenant de la thyroïde et des muscles oculaires qui sont ciblés par un processus autoimmun. Présentement, l'antigène qui répond le plus précisément à ce critère est une molécule membranaire de 64 kilodaltons. Les patients atteints d'OAT ont des anticorps sériques capables de vent reconnaitre et de se lier à une molécule de 64 kilodaltons trouvée sur la surface de cellules en culture provenant des muscles oculaires et de la thyroïde mais pas de cellules derivées des muscles squelettiques. Des autoanticorps réagissant avec la protéine D1, un fragment de la protéine de 64 kilodalton, sont détectés par la technique de l'*immunodot blot* dans le sérum de 47% de patients avec OAT, 57% des patients avec hyperthyroïdisme de Graves sans ophtalmopathie et dans le sérum de seulement 5% de personnes normales. Quatre morceaux d'ADN qui codent pour des épitopes antigèniques ont été clonés d'une librairie composée de petit morcaux d'ADN générés au hazard (100 à 200 bases) à partir de l'ADN complémentaire 1D correspondant à la protéine de 64 kilodaltons. Les séra de patients atteints d'AOT ont réagi plus fréquemment avec les peptides dérivées de ces quatres morceaux d'ADN, que les séra d'un groupe témoin. La distribution et la quantité d'ARN messager spécifique pour 1D ont été analysées dans une variété de tissus humains par la technique quantitative de la *reverse polymerase chain reaction*. Les tissus avec la quantité la plus élevée d'ARN messager 1D furent les muscles oculaires et la thyroïde. Ces résultats démontrent que la protéine de 64 kilodaltons est exprimée sur la surface des cellules des muscles

oculaires et de la thyroïde et que les autoanticorps qui reconnaissent cette molécule sont présents durant les premières phases des maladies autoimmunes thyroïdiennes.

Table of Contents

ABBREVIATIONS

2-D	Two-dimensional
Ab	Antibody
AChE	Acetylcholinesterase
ADCC	Antibody-dependent cell-mediated cytotoxicity
Ag	Antigen
APC	Antigen presenting cell
ATD	Autoimmune thyroid disease
BSA	Bovine serum albumin
cDNA	Complementary DNA
CHO	Chinese hamster ovary
CT	Computerized tomography
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPS	Exophthalmos-producing substance
FCS	Fetal calf serum
GAD	Glutamic acid decarboxylase
GH	Graves' hyperthyroidism
HSP	Heat shock protein
HT	Hashimoto's thyroiditis
Ig	Immunoglobulin
Kb	Kilobase
kDa	Kilodalton

LATS	Long-acting thyroid stimulator
LPCA	Leukocyte procoagulant activity assay
MHC	Major histocompatibility complex
MIF	Migration inhibition factor
MMLV	Moloney murine leukemia virus
MW	Molecular weight
mRNA	Messenger RNA
NP40	Nonidet P40
OD	Optical density
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
RT-PCR	Reverse transcriptase-PCR
PEMM	Pig eye muscle membrane
PBMC	Peripheral blood mononuclear cells
RIA	Radioimmunoassay
SDS	Sodium dodecyl sulfate
SDS PAGE	SDS polyacrylamide gel electrophoresis
SD	Standard deviation
T_3	Triiodothyroxine
T_4	Thyroxin
TAO	Thyroid-associated ophthalmopathy
TCR	T cell receptor
Tg	Thyroglobulin
TPO	Thyroid peroxidase
TRAb	TSH Receptor antibody
TRH	Thyrotropin-releasing hormone
TSH	Thyroid-stimulating hormone

List of Figures

Chapter II

Chapter III

Chapter IV

Chapter V

List of Tables

Chapter I

Chapter II

Chapter III

Chapter IV

ACKNOWLEDGMENTS

It is a pleasure for me to acknowledge all of the following people who have contributed toward the completion of this work.

I have been fortunate to have had the opportunity to work with two research supervisors: Dr. Jack R. Wall who provided me with guidance, encouragement, inspiration, support and the opportunity to travel and meet other researchers, which helped to broaden my professional experience; and Dr. Nicole F. Bernard, whose scientific excellence has set an example for me to follow. I thank her for providing me with guidance, encouragement, patience, support and technical expertise.

I would like to express my appreciation to Dr. Joyce Rauch for her constant support, helpful advice and encouragement.

I am most thankful to Dr. Harry L. Goldsmith for his support and encouragement.

I especially want to thank Drs. Erwin Schurr, Malcolm Baines and Jean Deschênes for their constructive criticism, helpful advice and constant encouragement.

I wish to thank Dr. Jaques How for his encouragement, support and helpfulness.

I thank, with great appreciation, Dr. Marian Ludgate for her willingness to collaborate and for providing valuable materials including 1D, D1 cDNA and 1D-CHO cells as well as technical advice and support.

Many thanks to Ms. Famane Chung Lee for her helpfulness, encouragement and friendship.

I feel fortunate to have worked with Dr. Andrée Boucher and Dr. Mario Salvi. Their encouragement, helpful advice and willingness to share information with me has made my work easier.

I am thankful to all of the many members of my laboratory over the years who were not only colleagues but friends with whom I have shared memorable experiences.

I thank Dr. Marianna Newkirk for generously agreeing to let me use her Genetic GCG Program.

I would like to express my appreciation to Dr. Ming Tsao for his encouragement, helpfulness and friendship.

I thank Dr. R. Brown and members of the Department of Surgery, Dr. W. P. Duguid and members of the Department of Pathology, and Drs. B. Arthurs and M. Flanders of the Department of Ophthalmology at the Montreal General Hospital for providing animal and human tissues.

I wish to also acknowledge the support of the staff of the Department of Surgery at the Montreal Children's Hospital for providing human tissues.

PREFACE

In accordance with the Guidelines Concerning Thesis Preparation I have taken the option, according to section (7), of writing the experimental part of the thesis (Chapters II to V inclusive) in the form of original papers already published in learned journals or submitted for publication. This provision reads as follows:

> "The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated text (see below), of an original paper, or papers. In this case the thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. in appendices) to allow a clear and precise judgment to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interests of cohesion.
>
> It is acceptable for theses to include as chapters authentic copies of papers already published, provided these are duplicated clearly on regulation thesis stationery and bound as an integral part of the thesis. Photographs or other materials which do not duplicate well must be included in their original form. In such instances, connecting texts are mandatory and supplementary explanatory material is almost always necessary.
>
> The inclusion of manuscripts co-authored by the candidate and others is acceptable but the candidate is required to make an explicit statement on who contributed to such work and to what extent, and supervisors must attest to the accuracy of the claims, e.g. before the Oral Committee. Since the task of the Examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear. Candidates following this option must inform the Department before it submits the thesis for review."

Thus, each chapter of this thesis bears its own Abstract, Introduction, Methods, Results, Discussion and Bibliography. Also, as required by the Guidelines, there is a common abstract, a general introduction (Chapter I) and a general discussion (Chapter VI) as well as claims to originality and suggestions for further research. A list of abbreviations is placed at the beginning of Chapter I; it serves to define the symbols used therein, and in subsequent chapters of the thesis.

The published and submitted manuscripts are as follows:

Chapter II. Zhang Z-G, Salvi M, Miller A, Bernard NF, Arthurs B, Wall JR. Restricted tissue reactivity of autoantibodies to a 64 kDa eye muscle membrane antigen in thyroid-associated ophthalmopathy. Clin. Immunol. Immunopathy. 62: 183-189, 1992

Chapter III. Zhang Z-G, Dong Q, Rodien P, Alcalde L, Bernard N, Boucher A, Salvi M, Arthurs B, Vassart GM, Ludgate M, Wall JR. Antibodies in the serum of patients with autoimmune thyroid disorders reacted with a recombinant 98 amino acid fragment of a full length 64 kDa eye muscle membrane protein which is also expressed in the thyroid. Autoimmunity. 13: 151-157, 1992

Chapter IV. Zhang Z-G, Wall JR, Bernard NF. Identification of antigenic epitopes of a 64 kDa antigen recognized by the serum of patients with thyroid-associated ophthalmopathy. Clin. Immunol. Immunopathy. (Submitted for publication).

Chapter V. Zhang Z-G, Wall JR, Bernard NF. Tissue distribution and quantitative of gene expression a 64 kDa antigen recognized by the serum of patients with thyroid-associated ophthalmopathy. J. Clin. Endocrino. Metab. (Submitted for publication).

In Chapter II, the candidate was responsible for all of the experiments. However, Ms. A. Miller helped the candidate with the absorption experiments and Drs. M. Salvi and B. Arthurs provided the clinical data used in this study.

In Chapter III, the candidate was responsible for all of the experiments. Drs. M. Salvi, B. Arthurs and A. Boucher provided clinical data used in this study. The D1 fragment was provided by Dr. G. M. Vassart's laboratory (Q. Dong, L. Alcalde, M. Ludgate) at the Libre Universite of Bruxelles.

In Chapter IV, the candidate was responsible for all of the experiments.

In Chapter V, the candidate was responsible for all of the experiments.

CHAPTER I

GENERAL INTRODUCTION

1. INTRODUCTION

The ophthalmopathy associated with Graves' hyperthyroidism and Hashimoto's thyroiditis is an autoimmune-mediated inflammation of extraocular muscle and orbital connective tissue. Although the disorder has many names (Table 1), the most appropriate is "thyroid associated ophthalmopathy (TAO)" which emphasizes that the disorder is not unique to Graves' disease (Wall, 1991; Perros and Kendall-Taylor, 1992). TAO symptoms range from discomfort and lid retraction to disfiguring proptosis (eye protrusion), diplopia (double vision) and blindness (Werner, 1969; Gamblin et al., 1983).

The lack of specific diagnostic signs and corroborating laboratory tests makes it difficult to ascertain the precise prevalence of TAO in patients with autoimmune thyroid disorders (ATD). Of patients with ophthalmopathy, about 85% have associated Graves' hyperthyroidism (GH), 10% have Hashimoto's thyroiditis (HT) and the rest have thyroid immunologic abnormalities in the absence of the classic clinical features of HT or GH. This latter condition is called "euthyroid Graves' disease" (Salvi et al., 1990; Beck, 1989; Jacobson and Gorman, 1984). Therefore most, if not all, patients with TAO have an autoimmune thyroid disorder.

There is also a close temporal relationship between the onset of ATD and TAO. In more than two thirds of patients, hyperthyroidism and ophthalmopathy occur within two years of each other (Gorman, 1983). About 10% of TAO patients have disease manifestation severe enough to consider the use of anti-inflammatory or immunosuppressive drugs. The other 90% have mild to moderate disease, with symptoms that usually disappear once the thyroid problem has been treated (Nordyke et al., 1988; Wall, 1991). Any hypothesis about the etiology and

pathogenesis of TAO must explain the unique relationship of ophthalmopathy with ATD.

There is increasing evidence for the possibility that ATD and TAO share pathogenic mechanisms (Weetman, 1991; Wall et al., 1990). The hypothesis upon which the work for this thesis is based is that the mechanism underlying the association of ATD with TAO is cross-reactivity between a shared thyroid and orbital tissue antigen (Ag): development of autoimmunity directed at a molecule present in one tissue would, therefore, increase the likelihood that the other tissue will also become a target for autoimmune attack. Several thyroid and orbital muscle Ags have been identified as targets for autoantibodies (autoAbs) in patients with TAO. The precise role of these cross-reactive tissue Ags in the pathogenesis of ATD and TAO and in the association between these two autoimmune phenomena is an area of intense investigation.

2. THE THYROID AND THYROID ANTIGENS

2.1 Normal thyroid gland

In the adult, the thyroid gland weighs approximately 10 to 20 g. The normal thyroid gland is a bilobed structure with a connecting isthmus which overlies the second or third cartilaginous rings of the trachea. It has a rich vascular and lymphatic supply (Feind, 1978; Komorowski and Hanso, 1988). The thyroid gland is composed mainly of thyroid follicular cells (thyroid epithelial cells), arranged in follicles bound together by a thin sheath of connective tissue (Dumont et al., 1992). The main component of the thyroid is a clear proteinaceous colloid which is stored inside the thyroid follicles. Normal thyroid function is essential for proper growth

and metabolism. Its absence is especially severe in infants and young children, in whom arrested physical and mental development can occur.

Normal function of the thyroid gland is under the control of the hypothalamic-anterior pituitary-thyroid axis, and is regulated through a negative feedback loop (Brownstein, 1974; Larsen, 1982). Thyrotropin-releasing hormone (TRH), a tripeptide synthesized in the hypothalamus, acts to promote the release and synthesis of Thyrotropin (thyroid-stimulating hormone, TSH) by binding to the TSH secreting cells in the adenohypophysis (Brolin, 1947; Jackson, 1982). TSH binds to its receptor on thyroid epithelial cells which respond in a variety of ways, including secretion of thyroid hormone and thyroid gland growth (Wetzel et al., 1965; Knopp et al., 1970; Lamy et al., 1986). Thyroid hormones in turn inhibit the synthesis and release the TSH from the pituitary. Therefore as the secretion of thyroid hormone decreases, TSH secretion increases, which results in an activation of thyroid function and growth (Aizawa et al., 1978; Caldwell et al., 1985).

2.2 Thyroid antigens

There are three distinct and well characterized thyroid autoAgs to which autoAbs are commonly detected in the serum of patients with ATD: i) thyroglobulin (Tg), ii) thyroid peroxidase (TPO formerly named microsomal antigen) and iii) TSH receptor. AutoAbs to the TSH receptor are believed to be pathogenic in Graves' disease (Volpé, 1990). Abs to TPO and Tg are more closely associated with HT (Salvi et al., 1988). Their role in thyroid disease pathogenesis is not well defined.

2.2.1 Thyroglobulin and anti-thyroglobulin antibodies

Thyroglobulin is the major protein component of the follicular colloid. It is a prohormone from which thyroid hormones (Thyroxine, T4; Triiodothyronine, T3) are formed. Tg is composed of two identical subunits each with a molecular weight (MW) of 330,000 (Kondo et al., 1985). Tg was considered to be a "sequested antigen" until the presence of low levels in normal human serum was demonstrated by radioimmunoassay (RIA) (Van Herle et al., 1973; Roitt and Torrigiani, 1967). Tg is a secretory product of the thyroid gland and produced only by this organ (Ericson, 1983).

Roitt et al. (1956) first demonstrated the presence of Ab to Tg in the serum of patients with HT. Significant titers of anti-Tg Abs are found by hemagglutination in 70% of patients with HT, in 30% of those with Graves' disease and in a small percentage of patients with thyroid carcinoma and other thyroid disorders (Pinchera et al., 1979). There is evidence for molecular similarity between Tg and major histocompatibility complex (MHC) class II associated invariant chain (Koch et al., 1987) and muscle acetylcholinesterase (AChE) (Schumacher et al., 1986; Ludgate et al., 1986). However, anti-Tg antibodies are not likely to play a role in the pathogenesis of TAO, since such autoAbs are not associated with this autoimmune disease (Salvi et al., 1990; Bresler et al., 1990).

2.2.2 Thyroid peroxidase and anti-thyroid peroxidase antibodies

TPO is a membrane bound hemoprotein enzyme (Taurog et al., 1970), active only in the presence of H_2O_2 (Magnusson et al., 1987). TPO plays a key role in thyroid hormone biosynthesis by catalyzing both iodination of tyrosyl residues and coupling of iodotyrosyl residues in Tg to form T3 and T4 (Taurog et al., 1974). Iodination and the initial phase of thyroid hormone secretion occur at or near the

apical microvilli of follicles (Wetzel and Spicer, 1965; Nagasaka et al., 1971; Nakagawa et al., 1985).

Microsomal Ag was described more than three decades ago. Sera from patients with thyroid autoimmunity often reacted to microsomal antigen, an easily denatured protein present on the surface and in the cytoplasm of thyroid cells (Belyavin and Trotter, 1959). Recently, human sera reactive to this Ag were shown to precipitate human TPO prepared from thyroid tissue (Portman et al., 1985) Monoclonal Abs developed to microsomal Ag cross-reacted with TPO (Portman et al., 1988). Comparisons of assays for Ab to microsomal Ag and to TPO have revealed a high correlation between assays for the two molecules (Mariotti et al., 1990). The microsomal Ag is now known to be TPO (Czarnocka et al., 1985). Cloning and sequencing of a cDNA for a full length human TPO mRNA revealed a 3048 bp open reading frame encoding a protein of 933 amino acids with a MW of 103,026 dalton (Kimura et al., 1987; Magnusson et al., 1987).

Kohno et al. demonstrated that autoAbs to TPO can inhibit the enzyme activity of this molecule as measured by iodide uptake assays (Kohno et al., 1986). Genomic DNA studies showed that patients homozygous for a frameshift mutation in the TPO gene presented with congenital goiter (Abramowicz et al., 1992). Some anti-TPO Abs with ability to fix complement can mediate killing of cultured thyroid cell monolayers of cultured thyroid cells (Strakosch et al., 1982). Anti-TPO Abs have been detected in virtually all patients with HT, in most patients with Graves' disease, but much less in frequently in these with other thyroid disorders (Volpe, 1990). There is no evidence that TPO autoAbs are involved in the pathogenesis of TAO (Amino et al., 1976; Salvi et al., 1990).

2.2.3 TSH receptor and TSH receptor antibodies

In 1956, a serum factor with thyroid-stimulating activity, distinct from TSH, was identified in the serum of patients with hyperthyroid Graves' disease. It was named long-acting thyroid stimulator (LATS) (Adams and Purves, 1956). LATS is a thyroid-stimulating autoAb which can mimic most, if not all, the effects of TSH. It can bind to the TSH receptors located on the membrane of follicular cells (Doniach, 1960; Dumont, 1971) and thereby upregulate thyroid function by inducing the release of thyroid hormones T3 and T4 (Hinds et al., 1981). With the cloning of the human thyroid TSH receptor (Libert et al., 1989; Nagayama et al., 1989), the pathogenic role of LATS or TRAb in Graves' disease was characterized (see section on Graves' disease **2.3.2**).

Further study of Graves' disease by Adams and Purves led them to suggest that presence of LATS was more often associated with the eye changes of this disorder than with the hyperthyroidism (Adams and Purves, 1957). Subsequent studies have been unable to demonstrate a correlation between LATS and TAO (Pinchera et al., 1965; McKenzie and McCullagh, 1968; Atta et al., 1990). Although some clinical studies showed a higher prevalence of TRAb positivity in patients with TAO, no correlation between the Ab titer and the severity of the ophthalmopathy was found (Bonnyns et al., 1968). Chang et al. (1990) compared the level of TRAb with orbital computerized tomography (CT) findings in untreated patients with Graves' disease and found no association between TRAb and ophthalmopathy. More work is needed to clarify the relationship between TRAb and TAO.

2.3 Autoimmune thyroid disorders

2.3.1 Hashimoto's thyroiditis (HT)

HT is the name given to a wide spectrum of clinical and pathologic findings, ranging from absence of signs and symptoms to severe hypothyroidism, from an enlarged thyroid (goiter) to an atrophic gland, from scattered clusters of thyroid infiltrating lymphocytes to extensive chronic thyroid inflammation. The diagnosis of HT is generally based on the presence of hypothyroidism or goiter, but it may be based on the detection of thyroid Abs or on the results of histologic studies (Ladenson, 1991). Various circulating anti-thyroid Abs (Woolner et al., 1959; Greenberg, 1970) and other immune phenomena are associated with HT, including *in situ* immune complex deposition and basement membrane changes in the gland (Shamsuddin and Lane, 1981; Pfaltz and Hedinger, 1986), induced expression of MHC class II Ags on the thyroid epithelial cells (Hanafusa, 1983; Aichinger et al., 1985) and presence of circulating anti-TPO Abs and/or Anti-Tg Abs in more than 90% of patients (Ladenson, 1990).

2.3.2 Graves' disease

Graves' disease is defined by hyperthyroidism associated with a diffusely hyperplastic goiter resulting from production of an Ab directed against the TSH receptor. The term hyperthyroidism means sustained thyroid hyperfunction associated with maintenance of increased thyroid hormone biosynthesis and release from the thyroid gland (Braverman and Utiger, 1991). It is now clear that the hyperthyroidism of Grave's disease is caused by TRAb. After binding to the TSH receptor, this Ab acts as a TSH agonist, stimulating adenylate cyclase activity and cAMP generation (McKenzie, 1958; Zakarija et al., 1985; Smith et al., 1988). The symptoms of hyperthyroidism include nervousness, sweating, intolerance to heat, rapid heart beat, fatigue, weight loss with increased appetite, muscle weakness, tremor and diarrhea (Trzepacz et al., 1989). The extra thyroidal features

of this disorder include not only ophthalmopathy but also localized myxedema (pretibial myxedema) and thyroid acropathy (clubbing of the digits and swelling of the end of the long bones) (Nixon and Samols, 1970; Elte and Hensen, 1983).

3. ORBITAL TISSUES AND THYROID-ASSOCIATED OPHTHALMOPATHY

3.1 Normal eye muscle

The human orbit contains seven muscles: the four rectus muscles (the superior, the medial, the inferior and the lateral rectus), the two oblique eye muscles (inferior and superior oblique), and the levator palbebrae superioris muscles. The levator muscle controls the movements of the upper eyelid; the other six govern eye movements. The extraocular muscles are surrounded by a fibrous connective tissue sheath (Spencer and Porter, 1988).

There is clear evidence that extraocular muscles are antigenically and functionally distinct from other skeletal muscles (Kaminski et al., 1990). Extraoculor muscles develop from neuroectoderm while skeletal muscle develops from mesoderm (Gans and Northcutt, 1983; Sevel, 1986). Eye muscles express a unique myosin heavy chain (Wierczorck et al., 1985) and distinct tropomyosin isoforms compared with those of skeletal muscle (Briggs et al., 1988). Eye muscle has a higher innervation ratio and a higher metabolic activity than skeletal muscle and exhibits a variety of ultrastructural characteristics distinct from skeletal muscle (Martinez et al., 1977; Ringel et al., 1978).

3.2 Eye muscle in TAO patients

On gross examination, the extraocular muscles from patients with TAO are 2-8 times larger than normal, dark red and edematous. Histologically, numerous signs of inflammation can be observed in eye muscle from TAO patients including: numerous inflammatory cells (lymphocytes, neutrophils and plasma cells), mucopolysaccharide infiltration, some damage and degeneration of muscle fibers. (Mulvany, 1944; Tengroth, 1964; Havard, 1979; Trokel and Jakobiec, 1981; Wall et al., 1993). These inflammatory changes in the extraocular muscles lead to marked muscle thickening and thus ophthalmopathy.

The expression of Heat shock protein (HSP) in orbital tissue has also been reported. Using cultured fibroblasts from different parts of the body, the 70 kDa HSP was found to be more abundant in retroorbital fibroblasts than in fibroblasts from other sites in patients with TAO (Heufelder et al., 1991). Based on this observation, Heufelder et al. postulated that HSP expression on orbital fibroblasts may be pathogenic in TAO. In eye muscle tissue sections from TAO patients stained for expression of MHC Ags and HSP, the eye muscle fibers and other interstitial cells expressed both class II MHC Ag and HSP-72 while eye muscle from normal subjects do not. (Hiromatsu et al., 1986; Hiromatsu et al., 1993; Hiromatsu et al., 1994). Although the significance of expression of HSP on orbital tissues is not clear (McGregor, 1992), MHC class II Ags play a role in presenting Ag to the immune system and thus can participate in immune and autoimmune responses. The expression of these cell surface glycoproteins is normally restricted to B lymphocytes, macrophages, dendritic cells and other antigen presenting cells (APC). In ATD, thyroid follicular cells express MHC II molecules, while thyroid tissue from normal individuals do not (Hanafusa et al., 1983). This observation led Bottazo et al. (1983) to propose a primary role for MHC class II Ag expression in induction of thyroid autoimmunity in humans. According to this hypothesis, the aberrant expression of MHC class II Ag could permit recognition of autoAgs by

"intolerant" helper T cells, thus triggering autoAb production. More recent evidence from various sources suggests, however, that MHC class II Ag expression is secondary to organ destruction (Iwatani et al., 1986; Khoury, 1987; Voorby et al., 1990; Margolese et al., 1994). Aberrant expression of this molecule can be important in aggravating and maintaining an autoimmune process through the presentation of tissue specific Ags to the immune system. The observation of MHC class II Ag and HSP on the surface of eye muscle cells in TAO patients supports the contention that eye muscle is a target of an autoimmune reaction in this disorder.

About 40-90% of patients with Graves' disease will have some ocular manifestation (Jones et al., 1969; Sridama and DeGroot, 1989; Enzmann et al., 1979; Burch and Wartofsky, 1993), which can be either overt or occult. The ocular manifestations are commonly divided into two types: those characterized by i) protrusion and ii) infiltrative processes (Table 2). CT and ultrasonographic examinations demonstrate marked enlargement of extraocular muscles, particularly the medial rectus, in most patients with GH (Enzmann et al., 1979; Wiersinga et al., 1989; Trokel and Jakobiec, 1981). Table 3 summarize the characteristic features of TAO seen by CT (Char, 1990) .

3.3 History

Many theories have been proposed to explain the development of TAO. The first mention of hyperthyroid patients with associated ophthalmopathy appeared in the medical literature over 200 years ago. Parry (1825) was the first to describe an association between diffuse goiter and eye signs. Graves (1835) was the first to propose the thyroid as having an important role in the etiology of TAO. Von Basedow (1840) was the first to provide a complete description of the clinical ophthalmological features now known as TAO (Werner, 1972). Some early

explanations for the occurrence of the clinical finding of exophthalmos (eye protrusion), characteristic of TAO include: i) orbital muscle laxity with venous congestion (Cooper, 1849) and ii) orbital edema (Thomson, 1924). These explanations have since been shown to be inaccurate (Dobyns, 1950). Based on data obtained from experiments on dogs, Bernard (1852) concluded that cervical autonomic sympathetic stimulation was the cause of thyroid ophthalmopathy. McLean and Norton (1958) have since demonstrated that this is not the case for human thyroid eye disease.

Today, most researchers believe that TAO is an autoimmune disorder - a conclusion based on a great deal of direct and indirect evidence (Perros and Kendall-Taylor, 1992; Gorman and Bahn, 1989; Weetman, 1992; Wall et al., 1993; Boucher et al., 1992; Volpé, 1993). Firstly, most if not all patients with ophthalmopathy have associated ATD (Salvi et al., 1991). Secondly, the orbital tissue of TAO patients is infiltrated by lymphocytes and immunocompetent mononuclear cells (Trokel and Jakobiec, 1981; Campbell, 1984; Weetman et al., 1989). Thirdly, therapeutic interventions known to modulate immune responses such as immunosuppressive agents (Werner, 1966; Guy et al., 1989; Kendall-Taylor et al., 1988; 1992; Weetman et al., 1983; Prummei et al., 1989), plasmapheresis (Glinoer et al., 1986; Dandona et al., 1979; Atabay et al., 1993) and local irradiation (Beierwaltes, 1953; Lloyd and Leone, 1992) have successfully treated patients with TAO without surgical intervention. Fourthly, TAO is often associated with other autoimmune disorders including insulin dependent diabetes mellitus (Metz et al., 1982), rheumatoid arthritis (Wall et al., 1981), pernicious anemia (Furszyfer et al., 1971) and myasthenia gravis (Spoor et al., 1980; Mornex and Orgiazzi, 1980). Fifthly, TAO has the classical markers of an autoimmune process, including circulating autoAbs and sensitized T lymphocytes reactive with orbital Ags (Wall et al., 1993; Boucher et al., 1992).

3.4 Etiology and pathogenesis of TAO

Many theories have been proposed to explain the association of ATD, particularly Graves' hyperthyroidism, with ophthalmopathy.

3.4.1 Pituitary factors in TAO

One of the classical long standing theories proposed to explain the mechanism underlying for the association of TAO with ATD is that TSH, a TSH derivative, or an exophthalmos-producing substance (EPS) of pituitary origin but distinct from TSH, may in combination with "abnormal serum immunoglobulin (Ig)" from patients with TAO, lead to ophthalmopathy (Bolonkin et al., 1975). The evidence for this hypothesis included the observations that i) the TSH molecule is exophthalmogenic in experimental animals (Winand and Kohn, 1970), ii) the TSH molecule can be partially digested with pepsin into a fragment with exophthalmogenic activity but not thyroid-stimulating ability (Kohn and Winand, 1971), iii) this cleavage product, together with TSH, can induce exophthalmos and the biochemical changes characteristic of exophthalmos in a guinea pig model of exophthalmos (Winand and Kohn, 1973) and iv) a gamma-globulin from the sera of exophthalmic patients increased the *in vitro* binding of TSH and the TSH derived exophthalmogic factor to the cell surface of guinea pig retro-orbital tissues (Winand and Kohn, 1972).

Evidence against a role for pituitary derived factor(s) in TAO pathogenesis is now overwhelming. It is well known that, in hyperthyroid TAO patients, TRAb stimulates thyroid function leading to increased circulating levels of T4 and T3. These thyroid hormones in turn suppress TSH secretion resulting in low TSH levels

in blood (Volpé, 1990). The low level of circulating TSH observed in hyperthyroid TAO patients does not support a role for this molecule in the development of ophthalmopathy in humans. In addition, pituitary factors are implicated in causing exophthalmos in the guinea pig animal model by targeting the retrobulbar Harderian gland of these animals. Since humans do not possess such a structure it is unlikely that the experimental observations of Winand and Kohn are applicable to human TAO (Jacobson and Gorman, 1984). Moreover, further studies have failed to support the notion that TSH or pituitary factors play an important role in the pathogenesis of human TAO (Davies et al., 1978; Waring et al., 1986).

3.4.2 Thyroglobulin and TAO

Another theory proposed to explain the association of TAO with ATD was based on the work of Kriss et al.. They postulated that Tg from hyperplastic thyroid acini leaked into regional lymphatics and was transported to cervical lymph nodes. Anti-Tg Abs were then produced which resulted in the formation of immune complexes. According to this hypothesis, Tg in immune complexes adhered to eye muscle and subsequently a series of immunological events were triggered including muscle cell injury, release of cellular protein, histamine release, leukocyte attraction and sensitization of lymphocytes to muscle antigens which could result in a variety of tissue lesions (Kriss, 1970; Konishi et al., 1974; Kriss et al., 1975).

The work of other investigators supports the presence of Tg in normal eye muscle. Mullin et al. (1977) demonstrated that the specific involvement of eye muscles in patients with TAO was due to patients' lymphocytes responding to Tg or a Tg derivative on eye muscle by delayed type hypersensitivity as assessed by quantitation of a particular lymphokine, migration inhibition factor (MIF). Another observation supporting a role for Tg reactivity in TAO is the presence of significant

sequence similarity between portions of Tg and AChE, a muscle protein. Ludgate et al. identified an epitope shared between Tg (between residues 2376 and 2464) and *Torpedo*-AChE (between residues 206 and 294) recognized by sera from patients with ATD (Ludgate et al., 1989). However, levels of Abs to this epitope(s) in the seta of patients with ATD did not correlate with the presence of eye disease.

Little evidence exists at present for a role for Tg as a shared thyroid-eye muscle Ag whose targeting by the immune system underlies the association of ATD with TAO. Evidence against a role for Tg and anti-Tg Abs in TAO pathogenesis include the fact that there is no correlation between the presence of TAO and levels of free Tg, Ab bound Tg or anti-Tg Abs (Brohee et al., 1979; Yamamoto et al., 1983). Furthermore, anti-Tg Abs are found more frequently in patients with HT than in those with GH, but less than 5% of patients with HT have TAO (Salvi et al., 1991). Moreover, the presence of Tg on eye muscle has not been confirmed by some investigators (Kodama et al., 1982).

3.4.3 Thyroid and orbital tissue cross-reactivity

The observed association between ATD and TAO in the same individuals suggests that these two autoimmune disorders share some kind of pathogenic mechanism. While the nature of immunologic links between the two autoimmune disorder is not clear the existence of shared antigenic determinants present on thyroid and eye muscle tissue targeted by an autoimmune response initially limited to one of these tissues could explain the association (Wall et al., 1993).

This putative shared Ag could be an identical protein found in both thyroid and orbital muscle or different molecules bearing an epitope(s) seen as the same by auto-reactive T cells or autoAbs (Schlomchik et al., 1990; Vincent et al., 1987). Antigenic cross-reactivity as the mechanism underlying the association between

two organ specific autoimmune disease is not a new concept. The laboratory of A.L. Notkins produced a number of monoclonal Abs. obtained by fusion of lymphocytes from patients with autoimmune endocrinological diseases and mouse myeloma cells. which reacted with Ags in multiple organs (Haspel et al.. 1983: Satoh et al.. 1983). They suggested that multiple-organ reactive Abs may in part explain the association of organ-specific autoimmune disease in polyendocrinopathies with an autoimmune basis. There is good evidence that the association of insulin dependent diabetes mellitus in patients with stiff man syndrome is due to the presence of autoreactivity to glutamic acid decarboxylase (GAD). an Ag found on islets and in brain neurons (Baekkeskov et al.. 1990). Abs from patients with TAO have been found to recognize a 64 kDa Ag on both thyroid and eye muscle (Salvi et al.. 1988: Dong et al.. 1991).

Evidence for the existence of Ags shared between the eye muscle and the thyroid and of autoimmunity against such shared antigens in patients with TAO has come from several laboratories (Hiromatsu et al.. 1988: Dong et al.. 1991). Hiromatsu et al. reported the presence of cytotoxic Abs in the sera of TAO patients reactive with eye muscle and thyroid cells. This Ab could be absorbed by pre-incubation of patients' sera on monolayers of thyroid cells and eye muscle cells. but not unrelated cells (Hiromatsu et al.. 1988). Miller et al. (1986) showed by ELISA that TAO patients' sera contained Abs reactive with both thyroid and eye muscle membrane Ags. By Western blotting. sera from TAO patients bound a protein of approximately 64 kDa present in several tissues including eye muscle. thyroid. other skeletal muscle. orbital connective tissue and pancreas (Salvi et al.. 1988). Absorption experiments demonstrated that the 64 kDa Ag reacting with Abs in TAO patients' sera. was expressed in eye muscles and thyroid but not in brain. spleen or smooth muscle (Wall et al.. 1990). Another 64 kDa protein. recognized by sera from patients with ATD. called 1D. was cloned by Dong et al. from a

thyroid cDNA expression library (Dong et al., 1991). By Northern blotting 1D appeared to be expressed only in thyroid and eye muscle tissue and not in skeletal muscle. A purified beta-galactosidase fusion protein containing 1D was recognized by Abs from patients with TAO and ATD (Tanaka et al., 1992; Zhang et al., 1992b).

3.5 Cellular autoimmunity in TAO

While autoAbs of a particular specificity are a hallmark of TAO, the role of cellular autoimmunity in TAO is not well defined. T cell reactivity to orbital Ags in patients with TAO has been studied using several assays and peripheral blood mononuclear cells (PBMC) as a source of T cells (How et al., 1987). One such assay is the MIF test where patients' PBMC are mixed with orbital Ag; if PBMC recognize Ag they release MIF. Munro et al. (1973) demonstrated that lymphocytes from all 15 patients with TAO tested responded positively to a crude human eye muscle Ag(s) preparation. Because the Ag preparation used in this study was not purified, it was not possible to know to which Ag the TAO patients' T cells reacted. When partially purified Ag(s) were substituted in the MIF test, "Tg" was found to stimulate a positive MIF response (Mullin et al., 1977). Another assay measuring T cell reactivity, the leukocyte procoagulant activity assay (LPCA), is based upon the cellular collaboration between lymphocytes and monocytes (Schwartz, 1985; How et al., 1990). Stimulation of T cells by an appropriate Ag results in production of a lymphokine(s) which induces monocytes to elaborate LPCA. Cohen et al. (1992) demonstrated that PBMC from patients with TAO elaborated significantly higher levels of LPCA in response to eye muscle cytosol than PBMC obtained from normal healthy subjects.

Antibody-dependent cell-mediated cytotoxicity (ADCC) directed at eye muscle may be a mechanism for tissue damage in TAO. This form of cell killing is mediated by killer (K) cells (closely related to NK cells) and a specific Ab. In ADCC a target cell Ag is bound by specific Ab and then the K cell lyses the target through interaction of the Fc portion of the Ab (Bogner et al., 1987). A significant positive correlation was noted, in TAO patients, between the specific lysis of cultured eye muscle cells and the degree of eye muscle involvement (Hiromatsu et al., 1987). Sera from TAO patients also supported killing by ADCC of thyroid cells, skeletal muscle cells but not orbital fibroblasts or endothelial cells (Zhang et al., 1989; Hiromatsu et al., 1989).

T cell proliferation has also been used to examine recognition of orbital tissue Ags by T cells from TAO patients (Wall et al., 1978; Tanaka et al., 1992). Approximately 33% of TAO patients, 5% of patients with GH or HT without evident eye disease, and no normal subjects have PBMC which proliferate following stimulation with the D1 fusion protein (Tanaka et al., 1992).

3.6 Humoral autoimmunity of TAO

3.6.1 Antibodies to orbital tissue antigens

Although eye muscle is generally reported as being the most likeliest target tissue for autoimmune attack in TAO, there is also some evidence for autoreactivity with orbital connective tissue Ags and the lacrimal gland. By Western blotting Bahn et al. (1989) identified, in the sera of patients with GH, Abs against a 23 kDa protein on fibroblasts and proposed a possible pathogenic role for these Abs. However, since the reactivity of these Abs against retroorbital fibroblasts was no different from that against fibroblasts from other parts of the body, and because

these Abs were found in GH patients with or without ophthalmopathy, the pathogenic role of Abs of this specificity in TAO is suspect.

Although several eye muscle reactive autoAbs can be detected in the sera of TAO patients by a variety of assays, including ELISA (Kodama et al., 1982; Kendall-Taylor, 1984; Miller et al., 1986), immunofluorescence (Mengistu et al., 1986), protein A binding (Faryna et al., 1985), SDS-PAGE and Western blotting (Salvi et al., 1988), little is known about the molecular structure, subcellular distribution, tissue specificity, or function of these autoAgs.

In 1982, Kodama et al. demonstrated, by ELISA, that an Ab reactive with a partially purified soluble eye muscle Ag was detected in the serum of 74% of patients with active TAO (Kodama et al., 1982). Subsequently the presence of eye muscle Abs was confirmed by other studies (Atkinson et al., 1984; Faryna et al., 1985; Ahmann et al., 1987). Cytotoxic Abs directed against eye muscle cell surface Ag(s), as measured by ADCC, were identified in the sera of 30-40% of patients with early stage ophthalmopathy. One cytotoxic Ab reacted against a thyroid cell surface Ag, different from TPO, and cross-reacted with an eye muscle Ag (Hiromatsu et al., 1988). This cytotoxic Ab could be absorbed by eye muscle and thyroid tissue, but the target Ag to which it reacted remains to be identified. By Western blotting, Abs in the sera of patients with TAO that bound to eye muscle membrane Ags of various sizes, namely 55 kDa, 64 kDa and 95 kDa, were also detected (Zhang et al., 1989; Salvi et al., 1991).

3.6.2 64 kDa thyroid/ eye muscle autoantigens.

The hypothesis upon which this thesis is based is that the mechanism underlying the association of TAO with ATD is the expression on the thyroid and eye muscle of a shared or cross-reactive Ag targeted by autoimmunity. The best

candidate for such an Ag at present is a 64 kDa membrane molecule which is i) expressed on eye muscle and thyroid and ii) recognized by Abs in the sera of a large proportion of TAO patients and iii) recognized only rarely by Abs in the sera of normal individuals. While the role of this molecule in the pathogenesis of TAO is not clear, the presence of Abs to this antigen has been very useful as a diagnostic tool (Miller A et al., 1992; Zhang et al., 1992b; Zhang et al., 1992c).

Salvi et al. were the first to report the presence of Abs to a 64 kDa eye muscle membrane Ag which were detected in 50% of patients with TAO. Abs of this specificity were also detected in 20% of patients with GH without evident eye disease, but not in normal subjects (Salvi et al., 1988). When the presence of Abs to this 64 kDa Ag was correlated with disease stage, sera from 75% of patients with early, severe, TAO contained Abs of this specificity. Anti-64 kDa Abs were detected in 33% of patients with GH with no apparent eye disease and in 17% of patients with HT with no eye disease but were not detected in patients with nonautoimmune thyroid disorders or in sera from the 25 normal subjects included in this survey (Salvi et al., 1991).

Sera from TAO patients contained autoAbs able to immunoprecipitate a 64 kDa molecule from human eye muscle and thyroid but not from skeletal muscle. A proportion of these sera and all sera from patients with ATD without ophthalmopathy also immunoprecipitated a distinct 66 kDa molecule from skeletal muscle (Zhang et al., 1992a). These results were confirmed by Hiromatsu et al. (1993) by quantitative Western blotting. Using rat tissue membrane preparations, Hiromatsu et al. showed that TAO sera reacted strongly with a 64 kDa protein in eye muscle, and weakly with a 66 kDa protein in skeletal muscle.

The 64 kDa Ag expressed by human eye muscle, thyroid and skeletal muscle was further characterized by two-dimensional (2-D) gel electrophoresis and Western blotting (Boucher et al., 1994). Pretreatment of eluted 60-70 kDa

solubilized membrane Ag from eye muscle, skeletal muscle and thyroid with N-glycosidase F, to eliminate charge heterogeneity resulting from glycosylation differences, changed the pI and MW of the molecules from each of these tissues recognized by TAO sera. The thyroid/eye muscle 64 kDa molecules recognized by TAO sera were shown to be glycoproteins and to be different in eye muscle and thyroid based on their distinct pI. The glycoprotein from skeletal muscle recognized by TAO sera appeared to have a higher MW (66 kDa) and a pI different from the Ag in eye muscle and thyroid recognized by the same sera. Trypsin digestion of size separated (60-70 kDa), eluted material from these three tissues prior to isoelectric focusing and Western blotting generated a fragment of about 30 kDa in the three tissues which was recognized by Abs in TAO sera (Boucher et al., 1991; Boucher et al., 1992).

Dong et al. (1991) studied a 64 kDa thyroid/eye muscle Ag. They cloned a 64 kDa Ag from a thyroid cDNA expression library by screening with a pool of sera from HT patients. The full length 572 amino acid protein, corresponding to a MW of 63-64 kDa, was called 1D and a 98 amino acid fragment of this protein was named D1. Using D1 as a probe in Northern blotting of poly(A)+ RNA from various tissues revealed 3.9kb transcripts in normal human thyroid and extraocular muscle, but not skeletal muscle. D1 affinity-purified autoAbs bound to a molecule of 64 kDa by Western blotting of human thyroid tissue (Dong et al., 1991).

Based on computer analysis 1D is predicted to consist of a short transmembrane segment, an intracellular domain where D1 is situated, and a longer extracellular domain (Ludgate, personal communication; Zhang et al., 1992d). Bernard et al. (1994) screened a panel of sera from patients with and without ophthalmopathy for the presence of Abs reactive with the full-length 1D molecule. Chinese hamster ovary (CHO) cells transformed with 1D and untransformed CHO cells were stained with the serum panel by immunofluorescence. Results obtained

by immunofluorescence were compared with those obtained by immunoblotting with 1D transformed or untransformed CHO solubilized cell membrane. Approximately 40% of patients with TAO had detectable serum antibodies to 1D, in both tests. The greatest prevalence of Ab reactivity was for patients with GH without evident eye disease where 70% reacted with 1D transformed CHO cells by immunofluorescence. This suggests that antibodies to the 1D protein may be an early marker of ophthalmopathy in patients with GH. Although 1D and the 64 kDa Ag have a similar size, tissue distribution and are recognized by autoAbs in TAO patients, their identity remains to be confirmed by amino acid sequencing of purified human 64 kDa eye muscle or thyroid antigen and comparison with that deduced for 1D.

3.6.3 Antigenicity of 1D antigen

The availability of a full length cDNA clone for 1D permits an examination of its antigenic make up by epitope mapping technology. In order to understand the molecular and cellular basis for the pathogenesis of TAO, it is important to identify the antigenic segments on 1D recognized by the auto-Abs in TAO patients. The precise identification of the antigenic epitope(s) of molecules recognized by the immune system has the potential to aid in the diagnosis and prognosis of disease, and identification of targets for immunomodulation (Horsfall et al., 1991; Momburg et al., 1993).

The immune response to external and internal Ags involves specific receptors on T and B cells (Bona, 1991). Antigenic determinants, or epitopes, are the portions of antigenic molecules that are recognized by Abs or T cell receptors (TCR). The determinants which interacts with Ab or TCR Ag binding sites can consist of either amino acids which are continuous (linear) in the primary sequence

of protein, or of amino acids which are discontinuous (assembled) in the primary sequence, but are brought together as a results of the folding of the protein to its native, functional, shape (Benjamin et al., 1984; Jemmerson, 1987).

Recognition of Ag by T cells usually requires processing of antigen into short linear peptides for presentation. The consensus of opinion is that peptides of less than 12 (about 8-9) amino acids can bind to TCR when presented in the context of the appropriate MHC molecule (Jardetzky et al., 1991; Hunt et al., 1992; Stern et al., 1994; Jardetzky et al., 1994). Information on the nature of the protein epitopes recognized by B cells is more complex. Based upon molecular modeling of Abs reacting with Ags, the majority of B-cell epitopes are thought to be conformational (Blundell et al., 1987). Various approaches have been used to locate Ab binding sites (epitopes) in proteins, including studies of binding to protein fragments or synthetic peptides (Kazim and Atassi, 1980; Geysen et al., 1987), binding to variant or mutated forms of antigen (Urbanski and Margoliash, 1977; Peterson and Seed, 1987) and proteolytic cleavage (Sheshberadaran and Payne, 1988). A library of peptides expressed in an expression vector would provide an unlimited supply of peptide fragments. By this method, the elucidation of both linear and conformational epitopes are possible, although at present this technique is mainly used for B-cell epitopes (Mehra et al., 1986; Prigent et al., 1990; Ceriani et al., 1992).

If a specific cDNA is inserted into an expression vector, the corresponding peptide is directly synthesized by the infected cell as a fusion protein (Stanley and Luzio, 1984). This fusion protein consists of a bacterial polypeptide linked to a eukaryotic polypeptide. The fusion protein is detected with Ab directed to the protein expressed by the inserted sequences. beta-galactosidase is a commonly used carrier protein (Mole and Lane, 1989). Analysis of polyclonal Abs using libraries of random DNA fragments expressed in E. coli provides a rapid method for

mapping their binding sites. Such a system has been successfully used in the mapping of important protein Ags (Scandella et al., 1988; Henry et al., 1990; Prigent et al., 1990; Libert et al., 1991). In essence the method is the same as using overlapping synthetic peptides, except that the size of the peptide can be made larger, allowing a more native fold of the protein (Laver et al., 1990). Synthesis as a fusion protein with E. coli beta-galactosidase may have an additional stabilizing effect (Stanley et al., 1987).

Table 1. Synonyms used to describe thyroid-associated ophthalmopathy*

Graves' ophthalmopathy	Thyroid exophthalmos
Von Basedow's ophthalmopathy	Exophthalmi ophthalmopathy
Dysthyroid ophthalmopathy	Exophthalmic ophthalmoplegia
Endocrine ophthalmopathy	Progressive exophalmos
Thyroid ophthalmopathy	Endocrine orbitopathy
Thyroid myopathy	Dysthyroid orbitopathy
Endocrine exophthalmos	Ophthalmic Grave' disease
Endocrine eye lesion	Malignant exophalmos

*Perros C and Kendall-Taylor P (1992)

Table 2. Abridged classification of ocular changes of Graves' disease*

Class	Definition
0	No signs or symptoms
1	Only signs (upper eyelid retraction and stare with or without eyelid lag or proptosis): no symptoms
2	Soft-tissue involvement (symptoms and signs)
3	Proptosis
4	Extraocular muscle involvement
5	Corneal involvement
6	Sight loss (optic nerve involvement)

*Char DH (1990)

Table 3. Computerized tomography findings in Thyroid-associated ophthalmopathy*

Enlarged extraocular muscle: tendonous insertion often spared
Normal orbital fat
Occasional slight bowing of medial wall
Occasional inferior rectus enlargement on axial scan: simulates an orbital tumor
Occasional optical compression of the optic nerve by enlarged extraocular muscles
Rare lacrimal gland enlargement
Absence of
Orbital masses
Vascular engorgement
Sinus involvement

*Char DH (1990)

CHAPTER II

Restricted tissue reactivity of autoantibodies to a 64 kDa eye muscle membrane antigen in thyroid-associated ophthalmopathy

PREFACE

The association observed between autoimmune thyroid disease (ATD) and thyroid associated ophthalmopathy (TAO) suggests that these two autoimmune disorders share a pathogenic mechanism.

AutoAbs to a 64 kDa human eye muscle and thyroid membrane protein had previously been demonstrated in the sera from patients with TAO by Western blotting. This Chapter demonstrates that several tissues could neutralize the ability of sera from TAO patients to detect a 64 kDa Ag from eye muscle in Western blots. In a separate set of experiments sera from TAO patients were used to immunoprecipitate labeled cell membrane molecules expressed on primary cell cultures established from several tissues. This approach revealed that sera recognized a 64 kDa molecule on thyroid and eye muscle membrane and a 66 kDa Ag on skeletal muscle membrane.

ABSTRACT

We studied the tissue specificity of eye muscle (EM) membrane-reactive autoantibodies detected in the serum of patients with thyroid-associated ophthalmopathy (TAO). In preliminary studies, such antibodies were shown to react, in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting, with human thyroid (THY) and other human skeletal muscle (HSM) membrane antigens. We carried out absorption with human EM (HEM), THY, and HSM membranes of sera from patients with TAO and autoimmune thyroid disease without ophthalmopathy which reacted with one or more of 55-, 64-, and 95-kDa antigens in pig eye muscle (PEM) membrane in immunoblotting, the majority of which were also cytotoxic to HEM cells in an antibody-dependent cell-mediated cytotoxicity assay. In Western blotting, serum antibodies reactive with PEM membrane antigens of 55, 64, and 95 kDa were cross-absorbed by HEM, THY, and HSM but not by spleen or brain membranes and showed some species specificity, being absorbed by pig and human, but not bovine, EM membranes. When incubated with cultured HEM, THY, and HSM cells *in vitro*, autoantibodies in TAO sera immunoprecipitated a 64-kDa antigen from the first two tissues, but not from HSM, suggesting a specific binding to autoantigenic epitopes in HEM and THY. Sera from patients with TAO as well as those from patients with thyroid autoimmunity without ophthalmopathy immunoprecipitated a ~66-kDa protein, shown to be distinct from the 64-kDa antigen. The restricted immunological cross-reactivity of antibodies to a THY and HEM 64-kDa membrane antigen is discussed in the context of the association of ophthalmopathy with thyroid autoimmunity. Further experiments are needed to show whether autoantibodies to the 64-kDa eye muscle and thyroid shared antigen are cytotoxic, and thus likely to play a major role in the pathogenesis of the eye disease, or just markers of the

orbital autoimmune process.

INTRODUCTION

The extraocular muscle is considered to be the main target of the autoimmune reactions of thyroid-associated ophthalmopathy (TAO) (1-3), although there is also evidence for immunological reactions against orbital connective tissue antigens (4, 5) and the lacrimal gland (6). The identity and significance of the principal eye muscle antigens are, at the present time, unknown.

Eye muscle (EM) antibodies were first detected in the serum of patients with TAO using an enzyme linked immunosorbent assay (ELISA) incorporating a monoclonal antibody-purified soluble EM antigen (7). Human EM (HEM) membrane-specific antibodies could not be identified by ELISA since nonspecific binding to HEM was found in the serum of normal subjects (8, 9). HEM cell surface reactive cytotoxic antibodies have been detected in the serum of TAO patients by an antibody-dependent cell cytotoxicity (ADCC) assay (10) which, by absorption on various tissue monolayers, were shown to cross-react with human thyroid (THY) and skeletal muscle (HSM) cell surface antigens (11).

Recently, we identified, by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, autoantibodies to a HEM membrane antigen of 64 kDa (12) and to pig EM (PEM) membrane antigens of 55, 64, and 95 kDa in approximately 50% of patients with TAO (13). Antibodies reactive with the 64-kDa antigen most closely correlated to the principal clinical features of ophthalmopathy (13). Serum from TAO patients was also shown to react in immunoblotting with a protein of 64 kDa in THY (12), and subsequent reports of absorption experiments by ELISA and immunoblotting have confirmed

that HEM-reactive antibodies cross-react with THY membranes (14, 15).

Until now, serological studies from various laboratories, including our own, (8, 13, 16), have invariably shown cross-reactivity of EM antibodies with HSM membrane antigens arguing against the existence of EM-specific autoantigens and the corresponding antibodies in the serum of patients with TAO (17, 18). In the present study we performed cross-absorption experiments, by Western blotting, to investigate the extent of tissue cross-reactivity of EM autoantibodies in TAO. We have also carried out immunoprecipitation of HEM, THY, and HSM cell membrane antigens with sera from TAO patients which contain (i) antibodies to the 64 kDa EM antigen and (ii) antibodies which are cytotoxic to HEM cells in ADCC. We confirm the existence of a 64-kDa membrane autoantigen which is a target for HEM and THY cross-reactive antibodies in TAO and, by immunoprecipitation, we demonstrate its specific expression on HEM but not other HSM membranes.

MATERIALS AND METHODS

Sera from the following groups of patients were studied: (i) TAO: nine patients, seven women and two men, aged 32-63 (mean age 50 years) of whom eight had associated Graves' hyperthyroidism and one had no apparent thyroid disease ("euthyroid Graves' disease"). The ophthalmopathy was active in seven cases and was "burnt out" in two cases, and its duration ranged from 6 months to 20 years. The clinical appearance of the eye disease was arbitrarily graded as mild (two cases), moderate (three cases), and severe (three cases). Since this work was not designed to study the prevalence of immunological parameters in TAO, previously reported by our group (13), TAO patient sera were selected on the basis of the detection of antibodies reactive with one or more recognized EM antigens in

immunoblotting, or of cytotoxic antibodies to EM cells in an ADCC assay, or both. (ii) Autoimmune thyroid disease without evident eye disease: six patients, five women and one man, aged 25-78 (mean age 46 years), of whom five had Hashimoto's thyroiditis and one had Graves' hyperthyroidism. Five normal subjects, three women and two men, aged 23-62 (mean age 40 years), in whom serum EM antibodies were not detected, were studied as controls. A summary of the clinical details of the patients, and of their serum reactivities in Western blotting and ADCC, is given in Table 1.

Antigen preparation

Normal HEM, spleen, and brain were obtained at autopsy less than 6 hr after death. Normal HSM and THY tissues were obtained, fresh, at operation. Pig and bovine EM were obtained, fresh, from animals used for surgical research. All tissues were kept frozen at - 70°C until processing. Batches of pooled autopsy and surgical specimens were prepared in order to minimize variations between experiments. Tissues were thawed in phosphate-buffered saline (PBS) containing a cocktail of protease inhibitors [aprotinin, phenylmethylsulfonyl fluoride (PMSF), benzamidine, ∈-aminocaproic acid, orthophenanthroline] rinsed of blood, cleaned of adipose tissue then minced with scissors, and homogenized in a Polytron mechanical blender. For the preparation of membrane fractions homogenates were first centrifuged at 400g for 15 min in order to remove whole cells, debris, and nuclei, then the supernatant was centrifuged at 100,000g for 30 min. The final pellet was retained as the "membrane fraction" and stored until used.

SDS-PAGE and Western Blotting

SDS-PAGE was performed according to the method of Laemmli (19) using an 8% separating gel and a 4% stacking gel in a mini-gel apparatus (Bio-Rad Laboratories, Richmond, CA). PEM membrane was applied at a concentration of 100 μg protein in 30-μl aliquots per well and run under reducing conditions. In each experiment molecular weight (MW) standards (Bio-Rad Laboratories) were included. The gel was run at 100 V for 60 min then transferred onto nitrocellulose paper at 100 V for 60 min in transfer buffer (0.02 mol Tris, 0.2 mol glycine, 20% methanol; pH 8.3). The strip with protein MW standards was stained with amido black and destained with 5% methanol and 7% acetic acid. Filter strips containing PEM membrane were blocked in 3% gelatin in Tris-buffered saline (0.2 mol Tris-HCl, 0.09 mol NaCl; pH 7.4) (TBS) at 37°C for 60 min. Strips were washed for 30 min in Tween-TBS (TBS, 0.5% Tween 20) and then incubated with absorbed or nonabsorbed serum, diluted 1:25 in 1% gelatin in TBS, for 2 hr at 37°C. TBS instead of serum was used in control strips. The paper was then washed with Tween-TBS and incubated with an alkaline phosphatase (AP)-conjugated anti-human IgG (γ chain specific) antiserum diluted 1:1500 in 1% gelatin TBS for 2 hr at 37°C. Strips were again washed with Tween-TBS and developed with 5-bromo-4-chloro-3-indolyl phosphatetoluidine and p-nitro blue tetrazolium chloride (Bio Rad Laboratories) for 15 min. Finally, strips were washed in distilled water for 5 min to remove excess substrate. In order to evaluate the accuracy of MW assignment to bands we performed several experiments where biotin-conjugated MW standards were electrophoresed adjacent to the antigen. These filters were stained with appropriate sera and then incubated with a biotin $F(ab')_2$ fragment of a goat anti-human IgG + IgM antiserum, diluted 1:1000. After washing the filters were incubated with AP-conjugated streptavidin diluted 1:700 and developed as described above.

Absorption

Liquid phase absorption of serum antibody activity was carried out by incubating serum samples in small test tubes with increasing concentrations (0-6 mg/ml) of PEM, THY, HSM and other membranes. After overnight incubation at 4°C, supernatants were collected and tested, in quadruplicate and in the same assay, for antibody activity against PEM membrane in immunoblotting. Results were expressed as persistance or loss of reactivity (identified as bands) to EM antigens of 55, 64, and 95 kDa.

Cell culture and immunoprecipitation

HEM, THY, and HSM cell cultures, obtained from several donors, were prepared as described previously (11). Briefly, 8.5-10 x 10^5 cells were incubated at 4°C for 30 min with 1 ml freshly prepared 1 mmol N-hydroxy-succinimidobiotin (NHS-d-Biotin, Sigma, St. Louis, MO). After extensive washing with PBS, 1 ml of Nonidet-P40 (NP-40) buffer (1% NP-40, 1 mmol EDTA, 50 mmol Tris-HCl, 150 mmol NaCl, pH 8.0) and 10 µl protease inhibitor [2 mg/ml PMSF in dimethylformamide (DMF)] were added to the culture dish and incubated at 4°C for 1 hr. The lysed cells were centrifuged at 200g at 4°C for 10 min and the supernatant was collected and further centrifuged in a microfuge at 13,000g, with the resulting supernatant (NP-40 lysate) retained for immunoprecipitation. Each 0.5- to 1.0-ml NP-40 lysate was preabsorbed with normal human serum to block nonspecific binding and was then incubated with patient or normal serum at 4°C for 16 hr. The mixture was then transferred to a tube containing 25 µl 50% protein A-Sepharose and centrifuged for 15 sec. After washing the pellets were run in a standard SDS-PAGE. Proteins were then transferred onto nitrocellulose paper and

after blocking with gelatin, identified by either horseradish peroxidase- or AP-linked streptavidin conjugates.

RESULTS

Absorption experiments in immunoblotting

Sera were absorbed with the following two panels of tissue membranes: (i) PEM and membranes prepared from THY, HSM, human brain, and spleen and (ii) EM of human, porcine, and bovine origin, then tested for reactivity with PEM membrane antigens in Western blotting. Absorption experiments were performed many times with the same, and different, TAO sera with essentially the same results. Data presented here are representative of these experiments. Control experiments were performed many times using antibody-negative sera from both patients with TAO and normals (results not shown). Immunoblotting of TAO sera on PEM membranes most often showed a strong reactivity at 64 kDa, sometimes accompanied by weaker reactivities at 55 and 95 kDa. Reactivity with a 64 kDa PEM antigen was, in all experiments, absorbed out by HEM, THY, and HSM membranes, but not by spleen or brain membranes, used as control tissues. Figure 1 shows one such representative experiment carried out with the serum from a patient with TAO (Patient No. 8, Table 1), which reacts strongly with the 64 kDa protein and more weakly with the 55 kDa protein. HEM, PEM, and THY membranes almost completely absorbed the antibody activity and HEM absorbed out the antibodies at lower protein concentrations than PEM, suggesting a denser distribution of the autoreactive antigenic epitopes on the membrane of human cells. In this experiment spleen membrane was used as a control tissue and did not absorb out PEM antibody activity. PEM autoantibody reactivity at 64 kDa was

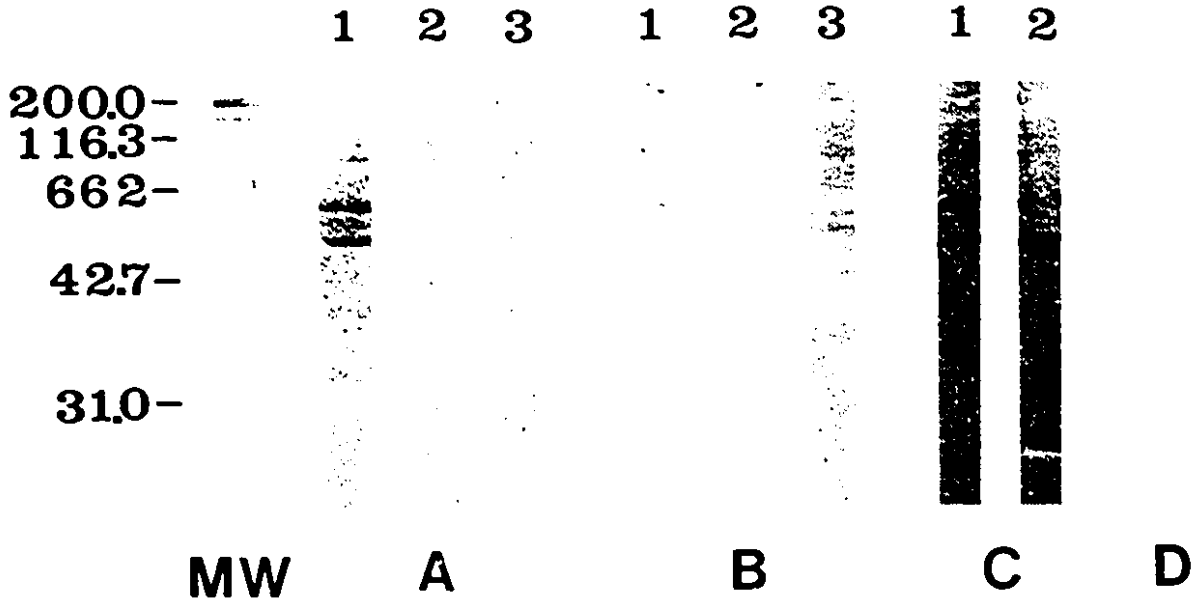
TABLE 1

Clinical and Immunological Findings of Patients with Thyroid Autoimmunity with or without Ophthalmopathy, Whose Sera Were Tested in Absorption and Immunoprecipitation Studies

Group	Patient/ subject no.	Sex/age	Thyroid disease	Eye disease		EM[a] ADCC	PEM blot (kDa)[b]
				Activity	Severity		
TAO[d]	1	F/49	GH[c]	"Burnt out"	Mild	+	55, 64, 95
	2	F/48	GH	Yes	Moderate	+ +	64
	3	M/42	GH	Yes	Mild	–	55, 64
	4	F/57	GH	"Burnt out"	Severe	+	64
	5	F/51	GH	Yes	Mild	+	55, 64
	6	M/32	GH	Yes	Severe	+ +	NT[e]
	7	F/43	GH	Yes	Severe	NT	64
	8	F/63	GH	Yes	Mild	NT	55, 64
	9	F/62	GH	"Burnt out"	Severe	—	55
ATD[f]	10	M/37	GH	—	—	—	64
	11	F/25	HT[g]	—	—	NT	—
	12	F/62	HT	—	—	+ + +	55
	13	F/52	HT	—	—	+	—
	14	F/29	HT	—	—	—	NT
	15	F/78	HT	—	—	NT	—
Normals	16	M/23	—	—	—	NT	—
	17	F/56	—	—	—	—	—
	18	F/62	—	—	—	NT	—
	19	F/23	—	—	—	—	—
	20	M/36	—	—	—	—	NT

[a] Antibody-dependent cell-mediated cytotoxicity (ADCC) against eye muscle (EM) cells.
[b] Western blot analysis on SDS-electrophoresed pig eye muscle (PEM) membranes.
[c] Graves' hyperthyroidism.
[d] Thyroid-associated ophthalmopathy.
[e] Autoimmune thyroid disorders without ophthalmopathy.
[f] Hashimoto's thyroiditis.
[g] Not tested.

Figure 1. Immunoblot analysis of 64 kDa antigen recognized by serum from patients with TAO. SDS-polyacrylamide gel electrophoresis of pig eye muscle membranes and Western blotting with serum from a patient with thyroid-associated ophthalmopathy (Patient No. 8, Table 1) following liquid phase absorption with membranes prepared from human eye muscle (lane 1, 0 mg/ml; lane 2, 5 mg/ml) (A); thyroid (lane 1, 0 mg/ml; lane 2, 3 mg/ml; lane 3, 6 mg/ml) (B); skeletal muscle (lane 1, 0 mg/ml; lane 2, 3 mg/ml; lane 3, 6 mg/ml) (C); spleen (lane 1, 0 mg/ml; lane 2, 3 mg/ml; lane 3, 6 mg/ml) (D); or pig eye muscle (lane 1, 0 mg/ml; lane 2, 2 mg/ml; lane 3, 5 mg/ml; lane 4, 7 mg/ml (E). MW, molecular weight standards. Serum dilution was 1/50. Pig eye muscle membranes (100 μg/well) were run in all lanes of A, B, C, D, and E. Strip (F) was run with PBS.


1 2 3 1 2 3 1 2
200.0-
116.3-
66.2-
42.7-
31.0-
MW
A
B
C
D

also invariably absorbed out by HSM membranes (Fig. 1) and by cardiac muscle and other striated muscle as well (data not shown).

We then tested the same sera for EM species specificity. Sera were incubated with membranes prepared from pig, human, and bovine EM and then tested with PEM membranes in immunoblotting as above. Experiments were carried out on the same serum shown in Fig. 1. The results are summarized in Fig. 2 which shows that the 64, 95, and 55 kDa reactive antibodies had some species selectivity being absorbed out by human and pig EM, but not by bovine EM.

Immunoprecipitation

The cross-reactivity of EM and striated muscle antigens suggested from absorption experiments might have been the result of poor specificity of the immunoblotting technique used to test for PEM membrane-reactive autoantibodies. Since HSM membranes might have absorbed 64 kDa-reactive antibodies as a result of nonspecific antigen recognition, we decided to immunoprecipitate antigenic membrane proteins from HEM, THY and HSM, using appropriately selected sera, prior to separation in SDS-electrophoresis. A representative experiment of immunoprecipitation of HEM membrane antigens is shown in Fig. 3, in which a 64 kDa protein is precipitated by the serum from three TAO patients (Nos. 1, 2, and 3, Table 1) but not that from a normal subject (No. 20, Table 1). Two of these patient sera also had cytotoxic antibodies to HEM cells in ADCC. While other less reactive proteins of various molecular weights were precipitated from HEM by these and other TAO sera, their specificity and significance are not known. Two of the six patients with autoimmune thyroid disease without evident ophthalmopathy (Nos. 10 and 11, Table 1) also immunoprecipitated a 64 kDa HEM membrane

Figure 2. Immunoblot analysis of species specificity of 64 kDa antigen recognized by serum from patients with TAO. SDS-polyacrylamide gel electrophoresis of pig eye muscle membranes and Western blotting with serum from a patient with thyroid-associated ophthalmopathy (Patient No. 8, Table 1), following liquid phase absorption with eye muscle membranes prepared from various species, namely pig (lane 1, 0 mg/ml; lane 2, 3 mg/ml; lane 3, 6 mg/ml) (A); human (lane 1, 0 mg/ml; lane 2, 3 mg/ml; lane 3, 6 mg/ml) (B); or bovine (lane 1, 0 mg/ml; lane 2, 3 mg/ml; lane 3, 6 mg/ml) (C). Pig eye muscle membranes (100 μg/well) were run in all lanes of A, B, and C. Strip (D) was run with PBS. MW, molecular weight standards.

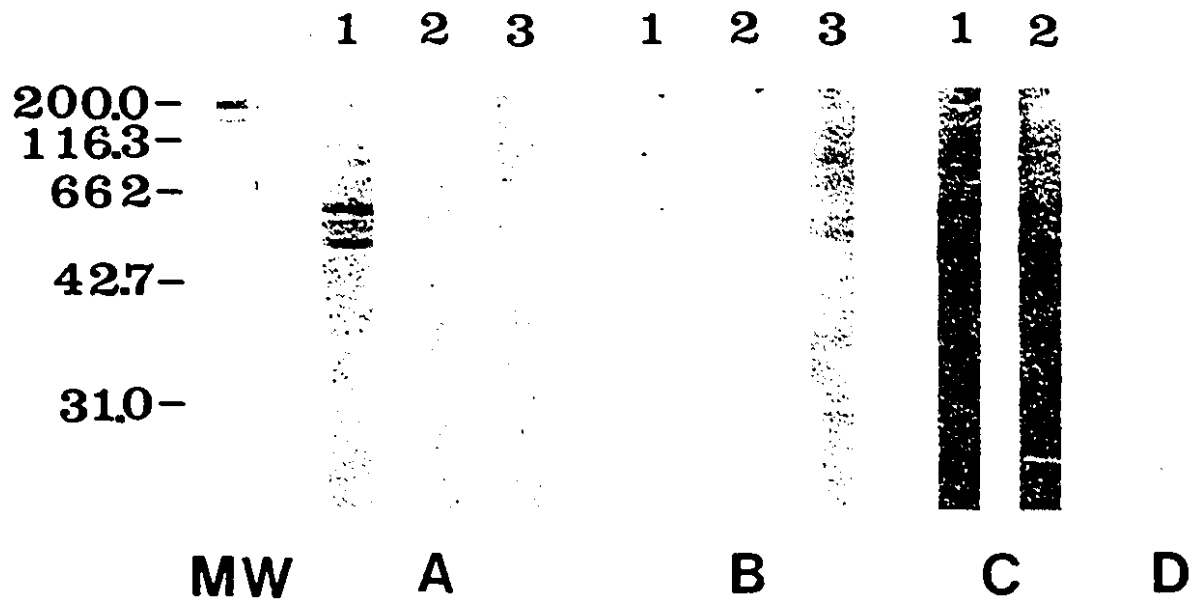
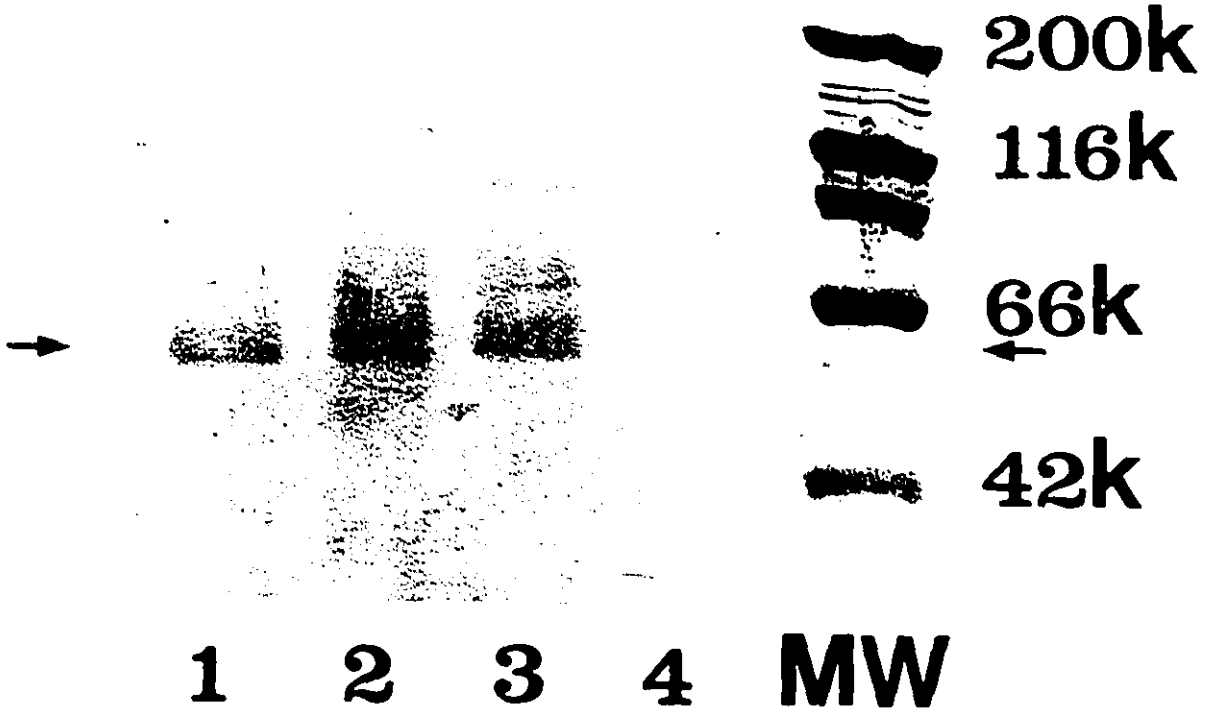

1 2 3 1 2 3 1 2
200.0–
116.3–
66.2–
42.7–
31.0–
MW
A
B
C
D

Figure 3. Surface biotinylation and immunoprecipitation of 64 kDa antigen from human eye muscle cells. SDS electrophoresis and Western blotting of biotin-labeled human eye muscle membrane proteins immunoprecipitated by sera from patients with thyroid-associated ophthalmopathy (Patient Nos. 1, 2, and 3, Table 1) (lanes 1-3) and from a normal subject (Subject No. 20, Table 1) (lane 4). MW, molecular weight standards. The arrows indicate bands at 64 kDa in lanes 1-3.


200k
116k
66k
42k
1
2
3
4
MW

antigen (not shown).

Figure 4 shows a representative example of immunoprecipitation of THY membrane antigens. A 64 kDa protein was precipitated by the serum from two TAO patients (Nos. 1 and 7, Table 1), from one patient with Graves' hyperthyroidism (No. 10, Table 1), and from two with Hashimoto's thyroiditis without ophthalmopathy (Nos. 14 and 15), but not by the serum from a normal subject (No. 19, Table 1). Thyroid peroxidase (110 kDa) was not precipitated by either of the Hashimoto patients' sera, despite the fact that both had significant (≥1:400) titers of antimicrosomal antibodies as measured in passive hemagglutination. Interestingly, the two TAO sera used in this experiment (Nos. 1 and 7, Table 1) also immunoprecipitated a 64 kDa protein from HEM cell membranes which, in the earlier absorption studies by immunoblotting, were shown to react with a 64 kDa PEM and THY shared membrane antigen (results not shown). Immunoprecipitation of membrane proteins from HSM cells by the same panel of sera from patients with thyroid autoimmunity with and without ophthalmopathy was also carried out and is summarized in Fig. 5. Sera from TAO patients (Nos. 1, 4, and 5, Table 1) as well as those from patients with autoimmune thyroid disease without eye disease (Nos. 12 and 13, Table 1) immunoprecipitated a 66kDa protein, while normal sera did not (Nos. 16,17 and 18, Table 1). When immunoprecipitated HEM and HSM membrane proteins were electrophoresed in the same gel it is clearly seen that the 64 kDa HEM protein migrated distinctively from a HSM protein of 66 kDa (Fig. 5).

DISCUSSION

Studies of the immunological mechanisms leading to the orbital inflammatory changes of TAO have provided evidence for the existence of shared

Figure 4. Surface biotinylation and immunoprecipitation of 64 kDa antigen from human thyroid cells. SDS electrophoresis and Western blotting of biotin-labeled human thyroid membrane proteins immunoprecipitated by sera from patients with thyroid-associated ophthalmopathy (Patient Nos. 1 and 7, Table 1) (lanes 5 and 6), a patient with Graves' hyperthyroidism without evident ophthalmopathy (Patient No. 10, Table 1) (lane 2), two patients with Hashimoto's thyroiditis without ophthalmopathy (Patient Nos. 14 and 15, Table 1) (lanes 3 and 4), and a normal subject (Subject No. 19, Table 1) (lane 1). MW, molecular weight standards. The arrow indicates bands at 64 kDa in lanes 2, 3, 5, and 6.

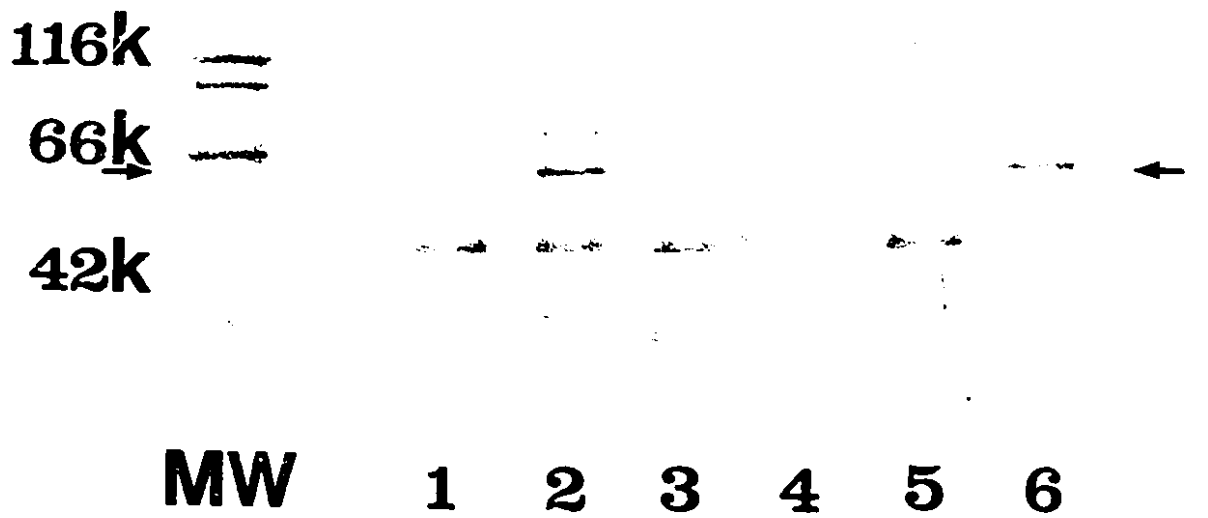
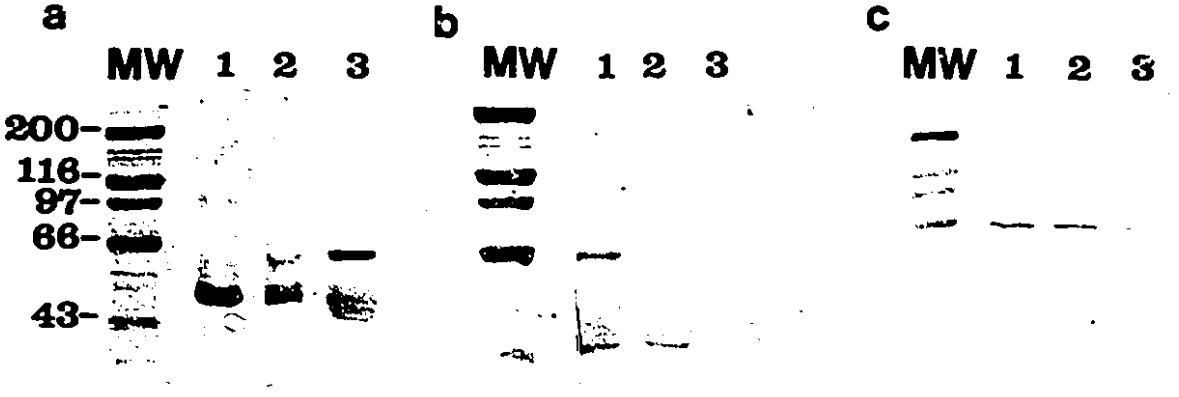

116k
66k
42k
MW
1
2
3
4
5
6

Figure 5. Surface biotinylation and immunoprecipitation of antigens from human eye and skeletal muscle cells. SDS electrophoresis and Western blotting of human membrane proteins immunoprecipitated from skeletal muscle, by serum from a patient with Graves' hyperthyroidism without ophthalmopathy (Patient No. 12, Table 1) (lane 1) and from a normal subject (Subject No. 16, Table 1) (lane 2) and eye muscle, by serum from a patient with thyroid-associated ophthalmopathy (Patient No. 1, Table 1) (lane 3) (a); skeletal muscle, by serum from a patient with thyroid-associated ophthalmopathy (Patient No. 4, Table 1) (lane 1) and from two normal subjects (Subject Nos. 17 and 18, Table 1) (lanes 2 and 3) (b); and skeletal muscle, by serum from two patients with Graves' hyperthyroidism without evident ophthalmopathy (Patient Nos. 12 and 13, Table 1) (lanes 1 and 2) and from a patient with thyroid-associated ophthalmopathy (Patient No. 5) (lane 3) (c).


a
MW 1 2 3
200-
116-
97-
66-
43-
b
MW 1 2 3
c
MW 1 2 3

thyroid and orbital tissue antigens. These include (i) an epitope shared by orbital connective tissue membrane and thyroglobulin (20), (ii) a region of molecular sequence homology shared by thyroglobulin and acetylcholinesterase (21), (iii) the possible existence of TSH receptors in orbital connective tissue (5), and (iv) a cell surface thyroid and eye muscle shared antigen, different from thyroid peroxidase (now recognized as the thyroid microsomal antigen), which is the target of cytotoxic antibodies in ADCC (11).

From immunoblotting studies we reported serum antibodies reactive with a 64 kDa HEM membrane antigen in 50% of patients with TAO which also reacted with orbital connective tissue and THY in a smaller proportion of cases (12). These data raised the possibility that there may be an antigen(s) common to the various tissues tested which could be a target(s) for cross-reactive autoantibodies in the serum of patients with TAO. Cross-absorption experiments in the present study provide clear evidence that the 64 kDa EM antigen, as well as antigens of 55 and 95 kDa, share epitopes with antigens found in THY membranes, as we also described in a previous preliminary report (15).

Cross-reactivity of EM-reactive autoantibodies with other striated muscle antigens has also been reported in studies from our laboratory (12, 13) and by other investigators (8, 16). We have also performed studies with mouse monoclonal antibodies which were shown to react, in ELISA, with EM antigens as well as with antigens expressed on cardiac muscle and skeletal muscle membranes (18). These findings are consistent with recent data reported by Weetman *et al.* (17), who demonstrated sensitization of T and B cells to EM and skeletal muscle antigens in patients with autoimmune thyroid disorders. The present absorption data confirm that, as determined by standard electrophoretic separation of proteins in SDS-polyacrylamide gels followed by Western blotting, autoantibodies in the

serum of patients with TAO react with EM and skeletal muscle membrane antigens of 55, 64, and 95 k.

In order to analyze in more detail the molecular basis of striated muscle antibody reactivity in TAO, we immunoprecipitated HEM and HSM membrane proteins. The rationale for this was based on (i) the knowledge that a greater specificity of antibody/antigen binding is obtained by selective *in vitro* labeling of cell membrane proteins (22) and (ii) the fact that since SDS may alter or denature cell membrane proteins, including target autoantigens, standard immunoblotting may only detect a subset of antibodies present in the serum of patients with autoimmune disorders, such as TAO. Our inability, in earlier studies (13), to demonstrate a close correlation between the presence of cytotoxic antibodies, as determined in ADCC assays, and reactivity to a 64 kDa EM antigen in immunoblotting, is consistent with both observations. By labeling cultured HEM cells *in vitro* and incubating with serum from TAO patients, we were able to confirm that the principal antigen targeted by serum autoantibodies is a 64 kDa membrane protein. In these studies most of the sera which bound to intact HEM cells *in vitro* were also cytotoxic to the same HEM cell targets in an ADCC assay. Moreover, since a 64 kDa protein was also immunoprecipitated from THY cell membranes, the possibility of a membrane antigen shared between HEM and THY, which is the target for cytotoxic autoantibodies in TAO, was again suggested (15). For example, two of the cytotoxic sera which immunoprecipitated a 64 kDa protein from both HEM and THY cell membranes were shown by absorption and immunoblotting to cross-react with this protein in the two tissues. On the other hand, in the present study, we were not able to immunoprecipitate HSM cell membrane proteins of 64, 55, or 95 kDa by sera reactive with this antigen(s) in EM membrane. Indeed, the 66 kDa protein precipitated from HSM cell membrane by sera from patients with autoimmune thyroid disorders is

unrelated to the presence of the eye disease. We have no direct molecular evidence as to why, in absorption experiments carried out in immunoblotting, this HSM membrane protein absorbed out the activity of antibodies to a 64 kDa PEM membrane antigen, although this may have been on the basis of alteration in the expression of native autoepitopes on the membrane fractions by SDS treatment. The fact that a few of the sera from patients with autoimmune thyroid disease without ophthalmopathy precipitated similar proteins from HEM membranes may indicate that these patients do, indeed, have an early or minimal eye muscle involvement which was not evident at clinical examination.

The present work shows that the immunoprecipitation technique is more specific than immunoblotting in identifying proteins expressed on the membrane of EM cells which are the specific targets for autoantibodies in the serum of patients with TAO. The 64 kDa EM (and perhaps other) membrane antigen may also be the target of those autoantibodies that are cytotoxic to HEM cells in ADCC (11), although this is yet to be proven. The study also confirms the existence of EM and THY antigen cross-reactivity, which may be the immunological basis for the association of ophthalmopathy with autoimmune thyroid disease (23). Recent evidence from the laboratory of Vassart and colleagues (24), of a 64 kDa protein encoded for by a gene expressed in EM and THY membranes, but not HSM membranes, confirms the present findings and substantiates our notion of restricted immunological cross-reactivity as the basis for this association. Indeed, preliminary collaborative studies (M. Salvi, J. R. Wall, and M. Ludgate unpublished observation) have shown that the recombinant 64 kDa protein is bound in a dot blot assay by EM and thyroid cross-reactive sera. In conclusion, the reactivity of 64 kDa EM autoantibodies in TAO is not organ-specific, as generally accepted for potentially pathogenic antibodies in autoimmune disorders (25), but restricted to a THY cross-reactive epitope(s) of the same MW (23). Finally, cross-

reactivity of HEM and PEM autoantigens confirms the suitability of PEM membranes for routine use in immunoblotting to detect serum autoantibodies which are markers of TAO.

REFERENCES

1. Weetman, A. P., Cohen, S., Gatter, K. G., Fells, P. and Shine, B. (1989) Immunohistochemical analysis of the retrobulbar tissues in Graves' ophthalmopathy. Clin. Exp. Immunol. 75: 222-227.
2. Feldon, S. E. and Weiner, J. M. (1982) Clinical significance of extraocular muscle volumes in Graves' ophthalmopathy. Arch. Ophthalmol. 100: 1266-1271.
3. Wall, J. R., How, J, Salvi, M. and Hiromatsu, Y. (1987) Can endocrine exophthalmos now be viewed as separate from thyroid autoimmunity? Bailliere's Clin. Immunol. 1: 141-163.
4. Bahn, R. S., Gorman, C. A., Johnson, C. M. and Smith, T. J.(1989) Presence of antibodies in the sera of patients with Graves' disease recognizing a 23 kDa fibroblast protein. J. Clin. Endocrinol. Metab. 69: 622-628.
5. Rotella, C. M., Zonefrati, R., Toccafondi, R., Valente, W. and Kohn, L., (1986) Ability of monoclonal antibodies to the thyrotropin receptor to increase collagen synthesis in human fibroblasts: An assay which appears to measure exophthalmogenic immunoglobulins in Graves' sera. J. Clin. Endocrinol. Metab. 62: 357-364.
6. Wall, J. R., Trewin, A., Fang, S. Z., Ingbar, S. H. and Braverman, L. E. (1978) Studies of immunoreactivity to human lacrimal gland fractions in patients with ophthalmic Graves' disease. J. Endocrinol. Invest. 1: 253-258.

7. Kodama, K., Sikorska, H., Bandy-Dafoe, P., Bayly, R. and Wall, J. R. (1982) Demonstration of a circulating autoantibody against a soluble eye muscle antigen in Graves' ophthalmopathy. Lancet 2: 1353-1356.
8. Kadlubowski, M., Irvine, W. J. and Rowland, A. C. (1986) The lack of specificity of ophthalmic immunoglobulins in Graves' disease. J. Clin. Endocrinol. Metab. 63: 990-995.
9. Miller, A., Sikorska, H., Salvi, M. and Wall, J. R. (1986) Evaluation of an enzyme-linked immunosorbent assay for the measurement of autoantibodies against eye muscle membrane antigens in Graves' ophthalmopathy. Acta Endocrinol. (Copenhagen) 113: 514-522.
10. Wang, P. W., Hiromatsu, Y., Laryea, E. A., How, J. and Wall, J. R. (1986) Immunologically mediated cytotoxicity against human eye muscle cells in Graves' ophthalmopathy. J. Clin. Endocrinol. Metab. 63: 316-320.
11. Hiromatsu, Y., Fukazawa, H., Guinard, F., Salvi, M., How, J. and Wall, J. R. (1988) A thyroid cytotoxic antibody that cross-reacts with an eye muscle cell surface antigen may be the cause of thyroid-associated ophthalmopathy. J. Clin. Endocrinol. Metab. 21 67: 565-570.
12. Salvi, M., Miller, A. and Wall, J. R. (1988) Human orbital tissue and thyroid membranes express a 64 kDa protein which is recognized by autoantibodies in the serum of patients with thyroid-associated ophthalmopathy. FEBS Lett. 232: 135-139.
13. Salvi, M., Bernard, N., Miller, A., Zhang, Z.-G., Gardini, E. and Wall, J. R. (1991) Prevalence of antibodies reactive with a 64 kDa eye muscle membrane antigen in thyroid-associated ophthalmopathy. Thyroid 1: 207-213.
14. Weightman, D., and Kendall-Taylor, P. (1988) Cross-reaction of eye muscle antibodies with thyroid tissue in thyroid-associated ophthalmopathy. J. Endocrinol. 122: 201-206.

15. Wall, J. R., Salvi, M. and Bernard, N. (1990) Eye disease and autoimmune thyroid disorders: Mechanisms for the association. In "Graves' Ophthalmopathy" (J. R. Wall and J. How, Eds.), pp. 63-77, Blackwell, Boston.
16. Ahmann, A., Baker, J. R., Weetman, A. P., Wartofski, L., Nutman, T. B. and Burman, K. D. (1987) Antibodies to porcine eye muscle in patients with Graves' ophthalmopathy: Identification of serum immunoglobulins directed against unique determinants by immunoblotting and enzyme-linked immunosorbent assay. J. Clin. Endocrinol. Metab. 64: 454-460.
17. Weetman, A. P., Fells, P. and Shine, B. (1989) T and B cell reactivity to extraocular and skeletal muscle in Graves' ophthalmopathy. Br. J. Ophthalmol. 73: 323-327.
18. Teboul, B., Triller, H., Chung, F., Bernard, N., Zhang, Z.-G., Wall, J. R. and Salvi, M. (1991) Muscle and species reactivity of mouse monoclonal antibodies to human eye muscle membrane antigens. Clin. Immunol. Immunopathol. 59: 104-116.
19. Laemmli, U. K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227: 680-684.
20. Kuroki, T., Ruf, J., Whelan, L., Miller, A. and Wall, J. R. (1985) Antithyroglobulin monoclonal and autoantibodies cross-react with an orbital connective tissue membrane antigen: A possible mechanism for the association of ophthalmopathy with autoimmune thyroid disorders. Clin. Exp. Immunol. 62: 361-370.
21. Ludgate, M., Dong, Q., Dreyfus, P. A., Zakut, H., Taylor, P., Vassart, G. and Soreq, H. (1989) Definition at the molecular level of a thyroglobulin-acetylcholinesterase shared epitope: Study of its pathophysiological significance in patients with Graves' ophthalmopathy. Autoimmunity 3: 167-176.

22. Hussain, R., Kaushal, N. A. and Ottesen, E. A. (1985) Comparison of immunoblot and immunoprecipitation methods for analyzing cross-reactive antibodies to filarial antigens. J. Immunol. Methods 84: 291-301.

23. Wall, J. R., Salvi, M., Bernard, N., Boucher, A. and Haegert, D. (1991) Thyroid-associated ophthalmopathy: A model for the association of organ-specific autoimmune disorders. Immunol. Today 12: 150-153.

24. Dong, Q., Ludgate, M. and Vassart, G. (1991) Cloning and sequencing of a 64 kDa autoantibode expressed in the thyroid and the extraocular muscle. J. Clin. Endocrinol. Metab. 72: 1375-1381.

25. Winter, W. E. and MacLaren, N. K. (1987) To what extent is "polyendocrine" serology related to the clinical expression of disease? Bailliere's Clin. Immunol. 1: 109-123.

CHAPTER III

Antibodies in the serum of patients with autoimmune thyroid disorders react with a recombinant 98 amino acid fragment of a full length 64 kDa eye muscle membrane protein which is also expressed in the thyroid

PREFACE

A 64 kDa eye muscle/thyroid membrane Ag could be detected by Abs in TAO sera by several methods: Western blotting, immunoprecipitation and 2-D gel electrophoresis. At the early stage of a research project aimed at cloning the 64 kDa molecule recognized by sera from TAO patients from human thyroid and eye muscle cDNA libraries, a report appeared from the laboratory of Dr. G. Vassart and colleagues at the IRIBHN, ULB, Brussels of the isolation of a cDNA encoding a 64 kDa Ag cloned from a human thyroid cDNA expression library by screening with a pool of sera from patients with ATD. The full length 572 amino acid protein, corresponding to a MW of 63-64 kDa, was called 1D and a 98 amino acid fragment of this protein was named D1. Using D1 as a probe in Northern blotting of mRNA from various tissues revealed 3.9 kb transcripts in normal thyroid and eye muscle, but not skeletal muscle. D1-affinity purified autoAbs from TAO patients bound to a molecule of 64 kDa human thyroid Ag by Western blotting.

The similarities between the 64 kDa molecule identified by Western blotting, immunoprecipitation and 2-D gel electrophoresis by Dr. Jack Wall's research group and the molecule encoded by 1D cDNA by Dr. G. Vassart's research group were compelling enough at the time to encourage the development

of collaborative projects aimed at elucidating the relationship between the two molecules and characterizing 1D further. The availability of a unique and well characterized panel of sera from patients at various stages of ATD and TAO, at the Thyroid studies Center of the Montreal General Hospital, would be essential for correlating the presence of Abs specific for 1D with clinical findings. The availability of a cDNA clone for 1D permitted expansion, expression, manipulation and a level of characterization that could not be achieved with a molecule for which a cDNA clone was unavailable.

ABSTRACT

We have tested sera from patients with autoimmune thyroid disorders with or without ophthalmopathy for immunoreactivity, in a dot blot assay, against a recombinant 98 amino acid fragment of a cloned 64 kDa protein, Dl, which is expressed in human eye muscle and thyroid, in the form of a Lac Z fusion protein. Tests were positive in 19 out of 40 patients with established thyroid-associated ophthalmopathy (TAO), in 12 out of 21 patients with Graves' hyperthyroidism (GH) without clinically evident ophthalmopathy, in 5 out of 10 patients with thyroid autoimmunity and lid retraction but no other signs of ophthalmopathy, in 4 out of 23 patients with Hashimoto's thyroiditis (HT) without evident ophthalmopathy and in 2 out of 18 patients with benign adenoma or multinodular goitre, but in only 2 out of 37 normal subjects tested. SDS-polyacrylamide gel electrophoresis and Western blotting for an antibody reactive with a 64 kDa antigen in pig eye muscle membranes was also carried out on sera from patients with TAO and GH. While immunoblotting for antibodies reactive with a 64 kDa protein was more often positive in patients with TAO, in whom 58% had serum antibodies which reacted with a 64 kDa protein, this was not the case in patients with GH without eye signs in whom the prevalence of positive immunoblot tests was 35%. Overall there was a fairly close correlation between the two tests although there were many exceptions. While the apparent discrepancy between antibodies recognizing the human recombinant 98 amino acid peptide in dot blot and those binding to a 64 kDa protein in pig eye muscle membranes in immunoblot could be explained by the existence of more than one epitope on the native protein, it is possible that the peptide D1, and the 64 kDa molecule are different. Although the relationship of these antibodies to the ophthalmopathy is uncertain, the high prevalence of antibodies to the D1 fusion protein in patients

with GH without evident ophthalmopathy (but who may have subclinical disease) and in patients with thyroid autoimmunity with lid retraction, which many believe to be an early stage of ophthalmopathy, raises the possibility that these antibodies may be an early marker of the eye disease in patients with thyroid autoimmunity. Further molecular studies will show whether D1, and the 64 kDa protein are the same or different molecules while prospective clinical studies of these patient groups will be necessary to determine the significance of the corresponding antibodies.

INTRODUCTION

While it is generally accepted that thyroid-associated ophthalmopathy (TAO) is an autoimmune disorder (1, 2) the identity and nature of the target antigen(s) is still poorly understood. Although many workers (1-5) favor the extraocular muscle as being the principal site of the autoimmune reaction others (6, 7) believe that the periorbital connective tissue is the more important target. Various eye muscle-derived autoantibodies have been detected in the serum of patients with TAO (5, 8-11) including those that are cytotoxic to eye muscle cells in antibody-dependent cell-mediated cytotoxicity (ADCC) (12-16). Recent interest in a 64 kDa eye muscle membrane antigen, which is the target of antibodies in 50% of unselected patients with TAO and in 74% of those with more recent disease (17), has been reinforced by the isolation, from a normal human thyroid cDNA expression library, of a cDNA clone (D1) encoding a 98 amino acid peptide which was also expressed in eye muscle but not other skeletal muscle (18). This peptide was shown to be a fragment of a full length 572 amino acid protein corresponding to a mol wt of 64 kDa (19). Although it has not be proven that the cloned protein and that targeted by TAO sera in Western blotting are the same,

preliminary results from our laboratories suggests that this may be the case (Rodien, Wall, Ludgate unpublished observations). In the present study we have tested sera from patients with TAO and those with autoimmune thyroid disorders without evident ophthalmopathy for reactivity against Dl, in the form of a Lac Z fusion protein, in a dot blot immunoassay. We demonstrated high prevalences of antibodies to the recombinant peptide in patients with (TAO), Graves' hyperthyroidism (GH) without clinically evident ophthalmopathy and in those with thyroid autoimmunity associated with lid retraction .

MATERIALS AND METHODS

Clinical subjects

The following groups of patients and subjects were studied: Group I: Thyroid-associated ophthalmopathy. Nine males and 31 females aged 20-69 (mean age 41 yr.), with established eye disease, 21 of whom had eye disease of less than one year's duration and 19 of more than one year in whom the inflammatory changes were "burnt-out" in all cases. All patients were euthyroid, having been treated with radioactive iodine in 35 cases or by thyroidectomy in 5 cases. The ophthalmopathy was associated with Graves' hyperthyroidism in 35 patients and Hashimoto's thyroiditis in 5 patients all of whom were euthyroid on thyroxine replacement therapy.

Group II: Graves hyperthyroidism. Six males and 15 females age 20-74 (mean age 38 yr.) in whom the diagnosis was based on hyperthyroidism, diffuse goitre and detectable TSH receptor antibodies. All were euthyroid having been treated with radioactive iodine in all cases and had no evident ophthalmopathy. CT scans were not carried out in these patients.

Group III: Lid retraction associated with thyroid autoimmunity. One man and 9 women, aged 21-63 (mean age 39 yr.) with severe lid lag and retraction but no other evidence for established ophthalmopathy. Six of these patients, who had Graves' hyperthyroidism in the past, were euthyroid on thyroxine replacement treatment. In 4 patients the lid signs were associated with Hashimoto's thyroiditis, diagnosed from the typical firm, irregular goitre, significant (≥1/400) titers of thyroid microsomal antibodies and aspiration needle biopsy evidence for lymphocytic infiltration and Hurtle cells.

Ophthalmological examination was carried out on these patients at the thyroid eye clinic by one of us (BA). Apart from variable degrees of lid retraction, minor lid swelling and lag in all, there were no inflammatory changes or proptosis, intraocular pressure was normal and eye muscle function full. CT scan, carried out on 8 of these patients, was abnormal in 4 showing increased thickness of the levator palpebrae/superior rectus muscle mass.

Group IV: Hashimoto's thyroiditis. Four men and 19 women aged 25-74 (mean age 45 yr.) with no evidence for established ophthalmopathy, all of whom were euthyroid on thyroxine replacement therapy.

Group V: Non autoimmune thyroid disorders. Eighteen women aged 32-65 (mean age 46 yr.) with a single benign adenoma (5 patients), colloid nodular goiter (7 patients), or multinodular goiter (6 patients), with no evidence of thyroid autommunity or ophthalmopathy.

Group VI: Normal subjects. Eight males and 32 females aged 25-60 (mean age 40 yr.) with no evidence of goitre or thyroid autoimmunity, no past history of other autoimmune disorders and no family history of autoimmune thyroid disease or other autoimmunity.

Antigens

Pig eye muscle membranes (PEMM) were prepared as described in previous publications from this laboratory (10, 17). Briefly, fresh pig muscle was obtained at sacrifice of normal pigs used for surgical research. Following homogenization, cell debris and nuclei were removed by centrifugation at 3,000 g. The supernatant from this step was centrifuged at 108,000g and the supernatant discarded. The obtained pellet was washed three times in PBS, the protein concentration determined and aliquots stored at -70°C until use in SDS-PAGE.

Dl protein, the recombinant protein used as antigen in dot blot assay, was a 98 amino acid peptide expressed as a Lac Z fusion protein by a cDNA clone (Dl) obtained from screening a normal human thyroid cDNA expression library in lambda gt 11 with a pool of Hashimoto's thyroiditis patients sera, as described in Refs 18 and 19. The expressed peptide, which corresponded to 294 base pairs of DNA, was a novel peptide sharing no significant homologies with any other protein entered into the EMBL DNA Databank release 22 or the Swiss-PROT Protein Databank release 13 data bases. Clone affinity purified antibodies (Ig) bound to a 64 kDa human thyroid membrane protein in Western blotting. In Northern blotting Dl hybridized to a 3.9 kb transcript in poly (A+) mRNA prepared from normal human thyroid and eye muscle but not other skeletal muscle. When Dl cDNA was used to probe the same thyroid library a cDNA encoding a full length 572 amino acid protein, corresponding to a MW of 63-64 kDa, was obtained (19). Recombinant Dl peptide was prepared for dot blot assay as lysis plaques (20,000-30,000 per filter) blotted onto the surface of 8x8 cm nitrocellulose filters soaked in IPTG.

Control antigen

The control antigen was a Lac Z fusion protein expressed by a cDNA clone encoding a fragment of the human beta pregnancy protein 1, isolated from a human placental library.

Dot blot

Nitrocellulose discs onto which had been blotted DI, or control, lysis plaques were cut into small wedges. A single wedge was incubated with an individual serum sample. Filters were incubated, in small covered petri dishes at room temperature for 1 hr, with serum diluted 1:40, in Tris HCl 10mM pH 8; NaCl 150mM; NP40 0.05%; powdered milk 0.5% buffer. Following this step the filters were washed three times in buffer without milk then incubated with an anti-human IgG-alkaline phosphate conjugate diluted 1:2000 in Tris NaCl; 0.1% milk buffer, at 37°C for 1 hr 15 min. After washing, substrates, 5-bromo-4chloro-3-inodolyl phosphate toluidine and p-nitro blue tetrazolium chloride (BCIP/NBT) (Bio-Rad, Richmond, CA) in 0.1 M NaCO3; 1.0 mM MgCl2 pH 9.8 (carbonate buffer), was added and the reaction stopped with water. A positive reaction with the DI protein was identified as a dark blue/black coloration of the plaques. In negative tests the plaques stained a much lighter bluish color. Tests were read, blind, by two observers and the final result expressed as positive (+, ++), borderline positive (±) or negative (-). Only reactivity of + or ++ was considered significant. Equal numbers of randomized patient and normal sera were tested in each assay and each serum was tested against both the DI protein and human beta pregnancy protein 1, as negative control.

SDS-PAGE and Western blotting

SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to the standard method of Laemmli (20) using an 8% separating and 4% stacking gel in a mini gel apparatus (Bio-Rad). PEMM was applied at a concentration of 100 µg protein in 30 µl aliquots per well and run under reducing conditions with mercaptoethanol. In each experiment molecular weight (MW) standards (Bio-Rad) were included. The gel was run at 100 V for 60 min then transferred to nitrocellulose paper at 100 V for 60 min in transfer buffer (0.02 mol/l Tris; 0.2 mol/l glycine; 20% methanol, pH 8.3). Filter strips containing PEMM were blocked with 3% gelatin in Tris-buffered saline (0.2 mol/l Tris-HCl; 0.09 mol/l NaCl, pH 7.4) (TBS), at 37°C for 60 min. Strips were washed for 30 min in Tween-TBS (TBS, 0.5% Tween 20), then incubated with serum diluted 1:25 in 1% gelatin TBS, for 2 hr at 37°C. TBS instead of serum was used in control strips. The paper strips were then washed with Tween-TBS and incubated with an alkaline phosphatase-conjugated anti-human IgG (γ chain specific) anti-serum diluted 1:1500 in 1% gelatin TBS, for 2 hr at 37°C. Strips were again washed with Tween-TBS and developed with BCIP/NBT (Bio-Rad) for 15 min. Finally, strips were washed in distilled water for 5 min to remove excess substrate.

Other methods

Serum levels of T4 and T3 were measured using commercial radioimmunoassay kits. Antithyroid microsomal (thyroid peroxidase) and TSH receptor antibodies were measured using commercial hemagglutination ("Thymune M", Burroughs Welcome Ltd., Beckenham, U.K.) and radio receptor assay ("TRAK", Henning Berlin Gmbh) kits, respectively.

Statistical analyses

Prevalances of antibodies to the Dl protein or a 64 kDa eye muscle membrane protein in immunoblotting in patient and normal groups were analyzed statistically using X^2 tests (2x2 contingency tables with Yates correction), or the Fisher's exact test.

RESULTS

A typical dot blot assay for immunoreactivity with the Dl fusion protein is depicted in Figure 1. The results of assays carried out on sera from patients with autoimmune thyroid disorders, with or without ophthalmopathy, patients with non-immunological thyroid disorders and normal subjects are summarized in Table 1. Dot blots were positive in 19 out of 40 (47%) patients with established TAO, 12 out of 21 (57%) patients with GH without evident ophthalmopathy, 5 out of 10 patients with GH or HT and lid retraction but no other manifestations of ophthalmopathy, 4 out of 23 (17%) patients without ophthalmopathy, 2 out of 18 (11%) patients with benign adenoma or multinodular goiter and 2 out of 37 (5%) normal subjects. The prevalances of positive tests were significantly greater than that for normals for patients with TAO, GH and thyroid autoimmunity with lid retraction (Fisher's exact test at least $P<0.05$), but not for the other two groups (P=NS) (Table 1).

The relationship between dot blot reactivity to Dl and parameters of ophthalmopathy in patients with established TAO was assessed. The prevalences of positive dot blot tests In patients with clinical evidence for eye muscle involvement [11 out of 23 (48%)] and in those without eye muscle disease clinically [8 out of 17 (47%)] were similar and the difference was not significant (X^2 test. P=NS). While tests were slightly more often positive in patients with mild

Figure 1. Representative dot blot immunoassay for autoantibodies reactive with a recombinant 64 kDa antigen fragment. Dot blot immunoassay for autoantibodies reactive with a recombinant 98 amino acid fragment of a 64 kDa eye muscle membrane protein expressed in the form of a Lac z fusion protein. A=strongly positive (++) (TAO serum): B=positive (+) (TAO serum): C=negative (-) (normal serum): D=control phage (human beta pregnancy protein 1) tested with TAO serum.

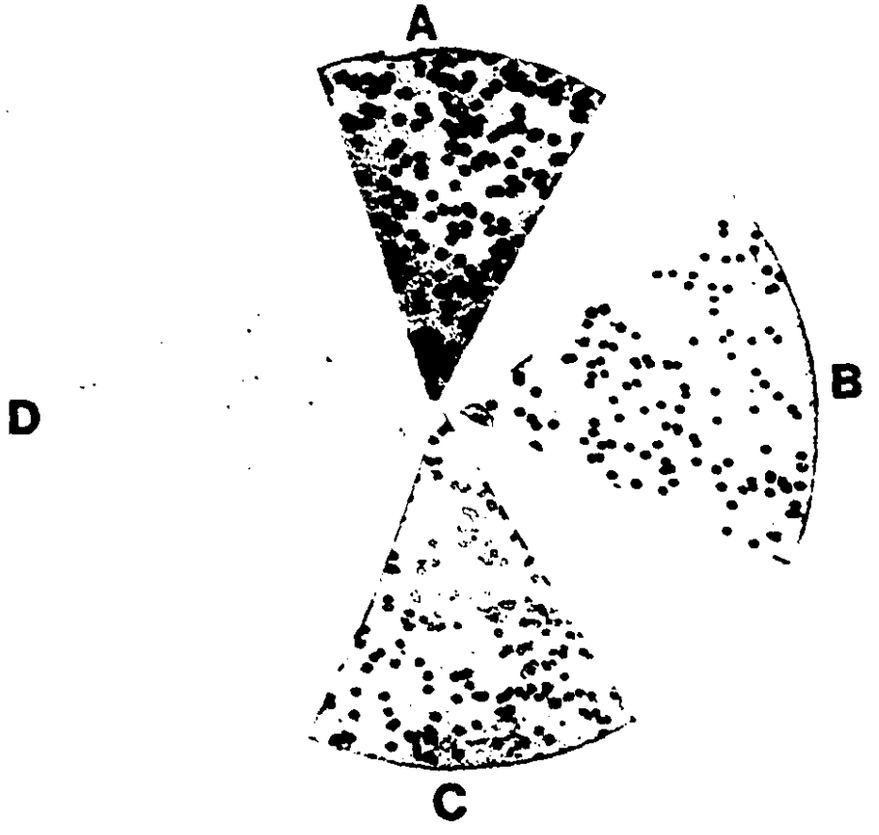

A
B
C
D

Table 1. Immunoreactivity with a recombinant 98 amino acid fragment of a 64 kDa protein in dot blot assay of sera from different groups of patients and normal subjects

Group	Patients number	Positive Dot blot*	
TAO	40	19(47%)	p≤0.001
GH	21	12(57%)	P≤0.001
Thyroid autoimmunity +lid retraction	10	5(50%)	p≤0.05
HT	23	4(17%)	NS**
Goitre/single nodule	18	2(11%)	NS
Normals	37	2(5%)	

*Reactivity against D1, a recombinant 98 amino acid fragment of a 64 kDa eye muscle protein, assessed as a color change of lytic plaques blotted onto nitrocellulose paper.
**NS=Not significant

eye disease [9 out of 16 (56%)] compared to those with severe disease [10 out of 24 (42%)], this difference was not significant (P=NS). Finally, tests were most often positive in patients with shorter duration eye disease [10 out of 14 (71%)] than in those whose serum had been tested more than one year following diagnosis [9 out of 26 (35%)], but the difference just failed to reach statistical significance (P=NS). (Results not shown).

Western blotting for reactivity with a 64 kDa antigen in crude PEMM was also carried out on 60 of the sera from patients with GH or TAO. Those sera giving the strongest reactions in dot blot usually also gave the strongest bands on Western blotting. The correlation between the two tests is summarized in Table 2. Overall the association between positive reactivity in dot blot and Western blot was significant (Fisher's exact test, $P<0.05$), although there were many exceptions. Thus while sera from 17 out of 24 patients with GH or TAO giving positive dot blot tests gave positive Western blots with a 64 kDa PEMM antigen, immunoblotting for reactivity with a 64 kDa antigen was also positive in 12 out of 36 patients giving negative dot blots. In patients with TAO immunoblot was more often positive (58%) than the dot blot assay while in patients with GH but no evident eye disease the prevalence of positive reactivity to a 64 kDa antigen in immunoblotting was lower (35%), although differences were not significant (Fisher exact test. P=NS (Table 3).

DISCUSSION

To summarize the main results we have demonstrated immunoreactivity against a recombinant 98 amino acid fragment of a cloned human 64 kDa eye muscle protein which is also expressed in the thyroid (18), in the sera of 47% of patients with established TAO in 50% of a small group of patients with thyroid

Table 2. Correlation between dot blot assay and Western Blotting in all patients with thyroid-associated ophthalmopathy or Graves' hyperthyroidism.

Dot blot assay*	Western blotting** Positive (n=29)	Western blotting Negative (n=31)
Positive (n=24)	17	7
		P≤0.01#
Negative (n=36)	12	24

*Dot blot immunoreactivity against a 98 amino acid fragment of a 64 kDa protein.
**SDS-PAGE and Western blotting for reactivity against a 64 kDa protein in PEMM.
#Statistical analysis refers to difference between sera positive and negative groups.

Table 3. Correlation between dot blot assay and Western blotting in patients with thyroid-associated ophthalmopathy and Graves' hyperthyroid.

Group	Positive Western blotting*	Positive Dot blot**
TAO (n=40)	23 (58%)	19 (47%)
GH (n=21)	8/20 (38%)	p=NS# 12 (57%)

*Band at 64 kDa in SDS-PAGE and Western blotting with PEMM.
**Immunoreactivity against D1 in an dot blot assay.
#Difference between TAO and GH assessed using Fisher's Exact test.

autoimmunity and with lid retraction but no signs of progressive ophthalmopathy and in 57% of those with GH without evident ophthalmopathy. In patients with TAO, positive dot blot tended to be associated with mild eye disease of recent onset although the correlations were not statistically significant. While there was a fairly good overall correlation between the results of dot blot assays and immunoblotting for reactivity with a 64 kDa protein in PEMM, there were many exceptions: sera from a few patients with negative dot blot assays gave positive reactivity in immunoblotting while, conversely, some patients with negative immunoblots reacted with the 98 amino acid peptide fragment in dot blot. Since the 98 amino acid peptide comprises less than one fifth the number of amino acids of the full length 64 kDa molecule it is likely to contain less of the reactive epitopes than the parent molecule and targeted by a lower proportion of sera. Reactivity of the peptide fragment in a dot blot but negative immunoblot could be explained by the existence of a family of antibodies reactive with the 64 kDa protein some of which bind to an epitope(s) in the full length protein which is destroyed or denatured during SDS-PAGE.

Although it is not proven that the Dl protein and the 64 kDa eye muscle membrane protein which is targeted by TAO sera in immunoblotting (10, 17) are the same molecule we believe this to be the case for the following reasons: (i) the two proteins correspond to the same MW; (ii) autoantibodies from patients with thyroid autoimmunity, affinity purified by Dl, bind to thyroid membranes at the same position, at 64 kDa, as unpurified antibodies (19), (iii) the pattern of reactivity of whole serum and Dl-affinity purified antibodies with a 64 kDa protein in THY and eye muscle isolated by two dimensional (isoelectric focusing/SDS-PAGE) gel electrophoresis are identical for patients with Graves' hyperthyroidism with or without ophthalmopathy (Boucher *et al.*, submitted); and (iv) while there are certainly discrepancies between immunoblot and dot blot results those sera

giving the strongest reactivity with Dl generally gave the strongest bands in Western blot in the present study. In order to prove this one must microsequence the 64 kDa protein obtained as single spot from two dimensional gel electrophoresis and compare with the sequences of Dl. This work is in progress in our laboratory.

A key finding from this study was the high prevalence of positive dot blot tests in patients with GH without evident ophthalmopathy and in a small group of those with GH or HT associated with lid retraction. Similar findings in these groups were reported for antibodies to a 64 kDa protein in Western blotting (17-21). Since CT scanning was not carried out in patients with GH we cannot exclude the possibility that these patients had ophthalmopathy at the time of study, although eye muscle function was normal in all patients and they did not have proptosis or increased intraocular pressure. The lid lag and retraction of our patients were not due to "sympathetic hypersensitivity" since all were euthyroid at the time of study. The relationship between lid retraction and progressive ophthalmopathy is not clear. Levator palpebrae superiors muscle inflammation, manifested as lid retraction and lag may be: (i) a precursor of extraocular muscle inflammation and therefore an early sign of TAO as suggested by others (22): (ii) an isolated auto-immune-mediated myositis which could occur alone or in association with TAO; or (iii) a feature of TAO resulting from synkinetic activity of the superior rectus muscle in overcoming inferior rectus restriction, or due to enlargement and fibrosis of the levator muscle itself (23). Since the greatest prevalences of antibodies to D1, were in patients with GH without eye disease and in those with lid signs only, it will be necessary to carry out prospective studies of these patients before a role of the antibodies in the pathogenesis of TAO can be claimed. Despite uncertainty about their significance these findings do raise the possibility that antibodies to D1, and those reactive with a 64 kDa eye muscle

membrane protein may appear early in the course of TAO and be good markers for the development of ophthalmopathy in patients with autoimmune thyroid disorders, in particular Graves' hyperthyroidism .

Dong *et al.* (19) showed, in Northern blotting, that the RNA message for the D1 peptide was expressed in eye muscle and thyroid but not other skeletal muscle. We demonstrated earlier from extensive absorption experiments in immunoblotting that a 64 kDa protein was expressed in the eye muscle and thyroid (24). In that study we were able to immunoprecipitate a 64 kDa antigen from intact thyroid and eye muscle cells, but not from systemic skeletal muscle cells with sera from patients with TAO suggesting that absorption with crude membrane fractions may be less specific than Northern blotting and immunoprecipitation. Finally, in recent studies, we have identified a single molecule corresponding to a MW of 64 kDa and isoelectric point of ≈6.9 in two dimensional gel electrophoresis of PEMM and human eye muscle and thyroid membranes, but not human skeletal muscle membranes (25). Cross-reactivity of autoantibodies with a 64 kDa protein antigen expressed in eye muscle and thyroid could explain the very common association of ophthalmopathy with autoimmune thyroid disorders (26).

REFERENCES

1. Wall JR. (1984) Autoimmunity and Graves ophthalmopathy. In Gorman C, Waller R, Dyer J. eds. The eye and orbit in Thyroid disease. N.Y., Raven Press, p. 103-111.
2. Wall JR, Salvi M, Bernard N. (1989) Eye disease and autoimmune thyroid disorders: mechanisms for the association. In Wall JR, How J. eds. Pathogenesis and Management of Graves' ophthalmopathy. Contemporary issues in Endocrinol. and Metab., Blackwell Scientific Pubs. Inc. 1: 63-77.

3. Weetman AP, Cohen S, Gatter K, Fells P, Shine B. (1989) Immunohistochemical analysis of the retrobulbar tissues in Graves' ophthalmopathy. Clin Exp. Immuno. 75: 222-227.
4. Ahmann A, Baker JR, Weetman AP, Wartolski L, Nauman TB, Burman KD. (1987) Antibodies to porcine eye muscle in patients with Graves' ophthalmopathy. Identification of serum immunoglobulins directed against unique determinants by immunoblotting and enzyme-linked immunosorbent assay. J Clin Endocrinol Metab. 64: 454-460.
5. Faryna M, Nauman T, Gardas A. (1985) Measurement of autoantibodies against human eye muscle plasma membranes in Graves' ophthalmopathy. Br Med J. 290: 454-460.
6. Bahn RS, Gorman CA, Woloschak GE, David CS, Johnson PM, Johnson CM. (1987) Human fibroblasts *in vitro*: A model for the study of Graves' ophthalmopathy. J Clin Endocrinol Metal. 65: 665-670.
7. Jacobson DH, Gorman CA. (1984) Endocrine ophthalmopathy: current ideas concerning etiology, pathogenesis and treatment. Endocr Rev. 5: 200-220.
8. Kodama K, Sikorska H, Bandy-Dafoe P, Bayly R, Wall JR. (1982) Demonstration of circulating autoantibody against a soluble eye muscle antigen in Graves' ophthalmopathy. Lancet. 2: 1353-1356.
9. Kendall-Taylor P, Atkinson S, Halcombe M. (1989) A specific IgG in Graves ophthalmopathy and its relation to retro-orbital and thyroid autoimmunity. Brit Med J. 288: 1183-1186.
10. Salvi M, Miller A, Wall JR. (1989) Human orbital tissue and thyroid membranes express a 64 kDa protein which is recognized by autoantibodies in serum of patients with thyroid-associated ophthalmopathy. FEBS Lett. 232: 135-139.

11. Mengistu M, Laryea EA, Miller A, Wall JR. (1986) Clinical significance of a new autoantibody against a human eye muscle soluble antigen detected by immunofluorescence. Clin Exp Immunol. 65: 19-27.
12. Wang PW, Hiromatsu Y, Laryea EA, How JR. (1986) Immunologically mediated cytotoxicity against human eye muscle cells in Graves' ophthalmopathy J Clin Endocrinol Metab. 63: 316-320.
13. Hiromatsu Y, Fukazawa H, How J, Wall JR. (1987) Antibody-dependent cell-mediated cytotoxicity against human eye muscle cells and orbital fibroblasts in Graves' ophthalmopathy-roles of Class II MHC antigen expression and interferon-γ action on effector and target cells. Clin Exp. Immunol. 70: 593-603.
14. Hiromatsu Y, How J, Fukazawa H, Guinard F, Salvi M, How J, Wall JR. (1988) A thyroid cytotoxic antibody that cross-reacts with an eye muscle cell surface antigen may be the cause of thyroid associated ophthalmopathy. J Clin Endocrinol Metab. 67: 565-570.
15. Hiromastu Y, Cadarso L, Salvi M, Wall JR. (1990) Significance of cytotoxic eye muscle antibodies in patients with thyroid-associated ophthalmopathy. Autoimmunity. 5: 205-213.
16. Hiromatsu Y, Wang PW, Wosu L, How J, Wall JR. (1987) Mechanisms of immune damage in Graves' ophthalmopathy. Hor Res. 26: 198-207.
17. Salvi M, Bernard N, Miller A, Zhang Z-G, Gardini A, Wall JR. (1991) Prevalence of antibodies reactive with a 64 kDa eye muscle membrane antigen in thyroid-associated ophthalmopathy. Thyroid. 1: 207-213.
18. Elisei R, Dong Q, Weightman D, Ludgate M. (1991) Cloning and sequencing of candidate eye muscle antigen in Graves' ophthalmopathy. In Proc 10th International Thyroid Congress. The Hague, Netherlands, p. 166. Feb. Abstract

19. Dong Q, Ludgate M, Vassart G. (1991) Novel 64 kDa autoantigen recognized by patients with autoimmune thyroid disease. J Clin Endocrinol Metab. 72: 1375-1381.

20. Laemmli UK. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature. 227: 680-684.

21. Miller A, Arthurs B, Boucher A, Liberman A, Bernard N, Salvi M, Wall JR. Significance of antibodies reactive with a 64 kDa eye muscle membrane antigen in patients with thyroid-associated ophthalmopathy. Thyroid (in press)

22. McLean JM, Norton EWD. (1959) Unilateral lid retraction without exophthalmos. Arch Ophthalmol. 61: 681-686.

23. Feldon SE, Levin L. (1990) Graves' ophthalmopathy: V. Etiology of upper eyelid retraction in Graves' ophthalmopathy. Brit Ophthal. 74: 484-485.

24. Zhang Z-G, Salvi M, Miller A, Bernard N, Arthurs B, Wall JR. Restricted tissue reactivity of autoantibodies to a 64 kDa eye muscle membrane antigen in thyroid-associated ophthalmopathy. Clin Immunol Immunopath (in press)

25. Boucher A, Zhang Z-G, Bernard N, Salvi M, Wall JR. (1991) Immuno precipitation of eye muscle cell membrane antigens and two dimension gel electrophoresis confirm the importance of a 64 kDa eye muscle antigen in Thyroid-Associated Ophthalmopathy. In Proc 10th International Thyroid Congress. The Hague, Netherlands, p. 17, Feb. Abstract

26. Wall JR, Salvi M, Bernard NF, Boucher A, Haegert D. (1991) Thyroid-associated ophthalmopathy-a model for the association of organ-specific autoimmune disorders. Immunology Today. 12: 150-153.

CHAPTER IV

Identification of antigenic epitopes of a 64 kDa antigen recognized by the serum of patients with thyroid-associated ophthalmopathy

PREFACE

In Chapter III we demonstrated immunoreactivity against the D1 fusion protein in sera of 47% of patients with established TAO and in 57 % of those with GH, a group of patients which may have early stage subclinical ophthalmopathy at the time of study. There was a good overall correlation between reactivity of patients sera by dot blot assay and by Western blotting for reactivity with a 64 kDa protein in PEMM.

To further dissect the antigenicity of the 1D molecule, short random fragments (100 to 200 bp) of 1D cDNA were cloned and expressed in E. coli as fusion proteins using the pUEX1 vector. The 1D fragment cDNA library was screened with a pool of sera from patients with TAO in order to identify antigenic peptides recognized by TAO autoAbs.

ABSTRACT

Recombinant DNA techniques were used to identify antigenic epitopes on a 64 kDa autoantigen expressed on human thyroid and eye muscle and recognized by sera from patients with thyroid-associated ophthalmopathy (TAO). Random fragments of full length 1D cDNA, which encodes the 64 kDa Ag, were cloned and expressed in E. coli as fusion proteins. Antigenic peptides were detected by colony blotting with sera from TAO patients and identified by sequencing the corresponding cDNA inserts. Four antigenic peptides were characterized and positioned with respect to the 1D amino acid sequence. One antigenic peptide (G-8) was found to be close to the C terminal end of 1D and three others (G-2, G-4, and G-5) were clustered at sites between amino acids 76-205. The immunoreactivities of sera from 15 patients with TAO and 9 patients with Graves' hyperthyroidism (GH) and 6 patients with Hashimoto's thyroiditis (HT), both without clinical evidence of ophthalmopathy, were tested against the four peptides and full-length 1D protein by Western blotting. Compared to normal subjects, sera from TAO patients were found to react more often with at least one of the four peptides or with full-length 1D protein [8 of 15 (53%)]. Sera from patients with TAO also reacted more often with G-4 and/or G-5 peptides than did those from normal subjects [7 of 15 (47%) TAO sera versus 1 of 12 (8%) sera from normal subjects]. Based on peptide amino acid sequences and the reaction pattern of the serum panel 6 immunogenic epitopes were identified on the 1D molecule.

INTRODUCTION

Thyroid-associated ophthalmopathy (TAO) is an autoimmune disorder characterized by infiltration of the extraocular muscles and surrounding connective tissue with lymphocytes and other mononuclear cells (1, 2). Although the mechanism for the association of ophthalmopathy with autoimmune thyroid disease (ATD) is not fully understood, one possibility is the existence of shared or cross-reactive antigen (Ag) expressed by thyroid and eye muscle such that reactivity elicited to an Ag present on one tissue will be recognized on the other tissue (3, 4). Although several eye muscle reactive antibodies (Abs) can be detected in the sera of patients with TAO (5-8), at present, the best candidate for such an Ag is a 64 kDa membrane molecule, which is expressed on both eye muscle and thyroid. The 64 kDa molecule is recognized by Abs in the sera of a large proportion of TAO patients and only rarely by Abs in the sera of individuals without TAO or ATD (9).

A cDNA, called 1D, which encodes a 64 kDa protein, has recently been cloned by Dong et al. (10) from a thyroid cDNA expression library screened with a pool of sera from Hashimoto's thyroiditis (HT) patients. The initial clone, called D1 which encoded a 98 amino acid fragment of the full length protein, was used to rescreen the library from which the full-length 1D cDNA was obtained (10). By Northern blotting analysis 1D mRNA was shown to be present in human eye muscle and thyroid, but not skeletal muscle. Quantitative RT-PCR has been used to identify and compare 1D mRNA expression in human tissues. 1D expression was greatest in thyroid and muscle tissues, and found to be more abundant in eye muscle than skeletal muscle (11). Using the D1 fusion protein as Ag in dot blot experiments, Zhang et al. (12) demonstrated that 19 of 40 (48%) sera from TAO patients, 4 of 23 (17%) sera from HT patients without evident ophthalmopathy, and only 2 of 37 (5%) sera from normal subjects reacted with expressed D1 fusion

protein. These results support the importance of 1D as an Ag targeted by the immune system in TAO patients.

We report here the identification and localization of peptides of 1D recognized by sera from TAO patients. This was achieved by construction of a sublibrary consisting of randomly cut 100-200 bp fragments of the 1D gene ligated into the expression vector pUEX (13). Colonies resulting from the transformation of Escherichia (E). coli with this ligation mixture were screened with a pool of sera from patients with TAO. Immunoreactive peptides were identified by sequencing the corresponding DNA inserts and positioning them within the 1D protein structure. The recognition of these antigenic determinants by auto-Abs from a panel of patients with TAO and ATD was assessed.

MATERIAL AND METHODS

Human subjects

Human subjects were classified into several groups based on their disease status. Group 1 included 2 males and 13 females aged 30-63 yrs (mean age 42 yrs) with TAO and established eye disease; the ophthalmopathy was associated with GH in 13 patients and HT in 2 patients. Group 2 included one male and 8 females aged 21-59 yrs (mean age 38 yrs) with GH without evident ophthalmopathy; diagnosis was based on hyperthyroidism, diffuse goiter, and detectable TSH receptor Abs. Group 3 included one male and 5 females aged 26-61 yrs (mean age 39 yrs) with HT but with no clinical evidence of ophthalmopathy. Group 4 included 3 males and 9 females aged 23-62 yrs (mean age 41 yrs) with no evidence of goiter or thyroid autoimmunity as normal controls.

1D cDNA fragment library construction

A 3.8 kb fragment containing full-length 1D cDNA was released from λgt11-1D by digestion with EcoRi (10). The 1D cDNA was purified by agarose gel electrophoresis and electroelution. The 1D cDNA was fragmented by digestion with DNase I under conditions precalibrated to produce fragments of approximately 100-200 bp (14). A fraction of the digestion mixture was run on a 2.0% agarose gel to verify the size of the randomly generated fragments.

Since it is essential that the resulting DNA fragments have blunt ends for efficient adapter ligation, the 1D cDNA DNase I digestion fragments were filled with ^{32}p-dCTP using the Klenow fragment of E. coli DNA polymerase (15). Three pmol of DNA fragments and 250 pmol of each adapter (adapter A: 5' GATCCG-GCAACGAAGGTACCATGG; adapter B: 5'CCATGGTACCTTCGTTGCCG) were linked by T4 DNA ligase prior to fractionation on a 2.0% low-melting point agarose gel (Haymerie et al., 1986). The portion of the gel containing fragments of 100-200 bp was excised and then ligated into pUEX vector. Vector was prepared by digesting 5 mg of pUEX (Amersham, Buckinghamshire, UK) with BamHI followed by ligation with 40 pmol of adapters A and B by T4 DNA ligase. E. coli DH5α competent cells were then transformed with pUEX vector with or without inserts. The size of the resulting cDNA inserts was confirmed by the polymerase chain reaction (PCR) using 5' CCTGGAGCCCGTCAGTATCGGC and 3' CTAGAGCCGGATCGATCCGGTC primers, which bind to DNA immediately flanking the BamHI cut site of pUEX (16).

Immunological screening of the 1D fragment library

DH5α cells, transformed with the 1D cDNA fragment library, were plated onto Luria Broth (LB; 10g Bacto-tryptonl, 5g Bacto-yeast extract, 5g NaCl/L) agar and grown at 30°C. Confluent bacterial colonies were transferred to a nitrocellulose filter (Millipore, Bedford, MA) and incubated for another 2 hr at 42°C. Each filter was put onto two sheets of Whatman 3MM paper previously soaked in 5.0% SDS and heated for 15 min at 95°C. Filters were washed with buffer (10mM Tris-HCl, pH 8.0; 150 mM NaCl; and 0.05% Tween 20, TNT) and blocked with 3% skim milk power in TNT for 1 hr. The pool of TAO patients' sera used for screening the fragment library was first preabsorbed to a filter grown on DH5α cells transformed with wild type pUEX containing no insert DNA. After preabsorption, sera diluted 1:1000 were incubated with filters for 2 hr at 37°C. Filters were washed 5 times in TNT buffer at 25°C for 15 min and incubated for 1hr at 25°C with alkaline phosphatase (AP)-conjugated anti-human IgG diluted 1: 3000. The filters were developed with 5-bromo-4-chloro-3-indolyl phospha-tetoluidine (BCIP) and nitro blue tetrazolium chloride (NBT) substrates. Positive clones were selected, subcloned and rescreened in the same way.

Positive clones were grown overnight in 5 ml of LB medium at 275 rpm at 37°C overnight. 1.5 ml of the each culture was centrifuged for 15 sec at 13,000 rpm. Cells were resuspend in ice cold lysis buffer (glucose 50 mM; EDTA 10 mM; 25 nM Tris-HCl, pH 8.0; and lysozyme 4mg/ml). After ethanol precipitation, the nucleic acids were extracted with an equal volume of phenol-chloroform-isoamyl alcohol (24: 24: 1). The supernatant was reprecipitated with 2 volumes of ethanol and centrifuged. The pellet was resuspended in Tris-EDTA pH 7.6 (TE) buffer and further purified by DNA-resin (CloTech, Palo Alto, CA).

Nucleotide sequencing of selected positive clones containing cDNA inserts was performed by the dideoxynucleotide chain termination method (17), using the Sequenase sequencing kit (US Biological Cor., Cleveland, OH) with

oligonucleotide primers (5' GGGGATTGGTGGCGACGACTCCTGG 3' CTAGAGCCGGATCGATCCGGTC), which bind to DNA flanking the BamHI cut site of the pUEX vector. Purified double-stranded plasmid DNA was denatured with fresh NaOH (18) to generate a single-stranded template. Reaction products were separated by electrophoresis on a 6% acrylamide urea gel.

Polyacrylamide gel electrophoresis (PAGE) and Western blotting analysis of positive clones

Bacterial colonies containing pUEX vector with inserts of interest were prepared for SDS-PAGE. Positive clones were grown in LB medium at 30°C to an OD_{600} of 0.4. The incubation temperature was then increased to 40°C for another 2 hr. The cells were harvested by centrifugation at 12,000g for 1 min. Pellets were sonicated and resuspended in 1 mM phenylmethylsulfonylfluoride (PMSF). The bacterial extract was dissolved in electrophoresis sample buffer (50 mM Tris-HCl, pH 6.8; 100 mM dithiothreitol; 2.0% SDS; 0.1% bromophenol blue; and 10% glycerol). The samples were heated at 100°C for 3 min and centrifuged at 12,000g for 1 min at room temperature.

SDS-PAGE was performed according to the method of Laemmli (19) using an 7.5% separating and 4% stacking gel. Bacterial extracts containing fusion protein were applied at a concentration of 20-50 mg of protein in a 30 µl volume per track. High molecular weight (MW) standards (Bio-Rad, Richmond, CA) were run simultaneously in each experiment. The gel was electrophoresed at 100V for 60-90 min and transferred onto nitrocellulose paper in transfer buffer (20 mM Tris-HCl; 20 mM glycine; 20% methanol, pH 8.3). Filter strips containing fusion protein were blocked with 3% milk power in Tris-buffered saline (200 mM Tris-HCl; 90 mM NaCl, pH 7.4) (TBS) at 4°C overnight. Strips were washed for 30

min in Tween-TBS (TBS; 0.5% Tween 20), and incubated with sample sera previously absorbed with wild type pUEX fusion protein containing no insert (13). The paper strips were then washed with Tween-TBS and incubated with an AP-conjugated anti-human IgG (1: 3000) in TBS for 1 hr at 37°C. Strips were again washed with Tween-TBS and developed with BCIP/NBT.

Expression of full-length 1D in pUEX

A 1780 bp fragment, containing full-length 1D cDNA, was released from a Bluescript SK+ vector by digestion with Xho 1 and Bam H1. After purification, the 1780 bp 1D cDNA was ligated with Xho 1 and Bam H1 digested pUEX vector. Western blotting using 1D fusion protein reactive with sample sera was performed as described above.

Statistics

The prevalences of positive immunoreactivity for sera from patients with TAO, ATD and normal subjects to each antigenic peptide were compared using a X^2 test.

RESULTS

Analysis of antigenic determinants in 1D

The strategy developed by Mehra et al. (20) was used to define antigenic determinants recognized by anti-64 kDa Abs in patients with TAO. This approach involved the preparation of a library of DNA fragments coding for short peptides

by digesting 1D cDNA with DNaseI in order to generate fragments in the 100 to 200 bp range. The resulting DNA fragments were first linked to adapters, fractionated by size and inserted into the expression vector pUEX. Inserts of 100-200 bp are expected to code for peptides of approximately 30 to 60 amino acids. The library consisted of approximately 8 x 10^5 clones, all of which were screened.

In order to verify that DNase I digestion of 1D generated fragments of the expected size, 36 clones were randomly selected and subjected to PCR. Twenty five clones were found to contain inserts in the 100 to 200 bp range and no inserts were detected in other clones (not shown). The first round of screening was carried out with a pool of sera from four patients with TAO (Table 1), two of the whom had Abs reactive with a 64 kDa human eye muscle membrane protein by Western blotting, but did not react with the D1 fusion protein by ELISA (21) or dot blotting. The other two sera reacted by ELISA and dot blotting with the D1 fusion protein, but did not react with a 64 kDa human eye muscle membrane Ag by Western blotting.

Four positive clones were isolated, sequenced and positioned on the 1D nucleotide map by matching to the 1D cDNA sequence using Genetics GCG program (Genetics Computer Group, Madison, WL). The 4 deduced immunoreactive peptides were 36-52 amino acids long (Table 2). The corresponding amino acid sequences were deduced from the 1D nucleotide sequence. Immunoreactive peptides G-2 (residues 76-112), G-4 (residues 127-179) and G-5 (residues 156-205) were all positioned to hydrophilic domains of the 1D amino acid sequence. Immunoreactive peptide G-8 (residues 412-450) partially overlaps with the D1 98 amino acid fragment (residues 426-524) and is located near the C-terminus of the 1D molecule (Fig. 1).

Figure 2 shows the deduced antigenicity profile of the 1D 64 kDa protein. The antigenicity profile is based on calculations of hydrophilicity, surface

Table 1. Reactivity of TAO sera used to screen a 1D fragment library

Patient No.	D1-fusion protein#	64 kDa protein
1	+*	–**
2	+	–
3	–	+
4	–	+

#D1 is a 98 amino acid fragment of 1D protein.
*Reactivity assessed by ELISA and immunoblot using D1 fusion protein as antigen.
**Reactivity assessed by Western blotting using solubilized eye muscle membrane as antigen.

Table 2. Characteristics of 4 antigenic peptides of 1D thyroid/eye muscle antigen

Antigenic peptides	Number of amino acids	Position in 1D amino acid sequence
G-2	36	76-112
G-4	52	127-179
G-5	49	156-205
G-8	38	412-450

Figure 1. Location and characteristics of 4 antigenic peptides in the nucleotide sequence of 1D. The nucleotide sequence analysis of positive clones were determined by dideoxynucleotide chain termination method. A is a schematic representation of the nucleotide sequence of 1D and the corresponding location of antigenic peptides G2, G4, G5, G8 and D1. B is a hydrophilicity plot of 1D and C shows the surface probability of 1D deduced by Genetic GCG program (Genetic Computer Group) based on nucleotide sequence of 1D.

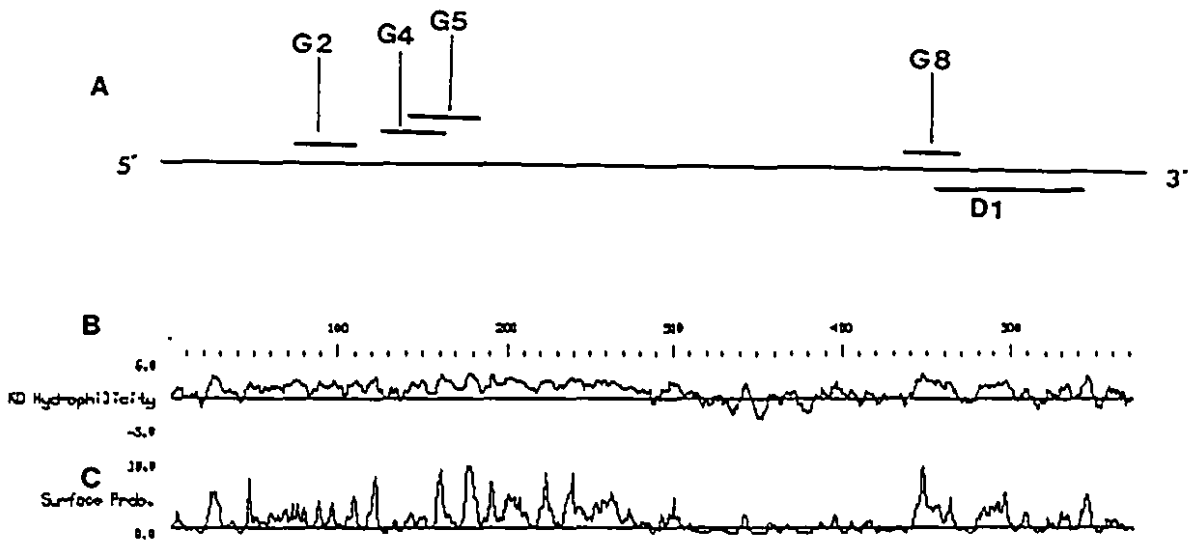
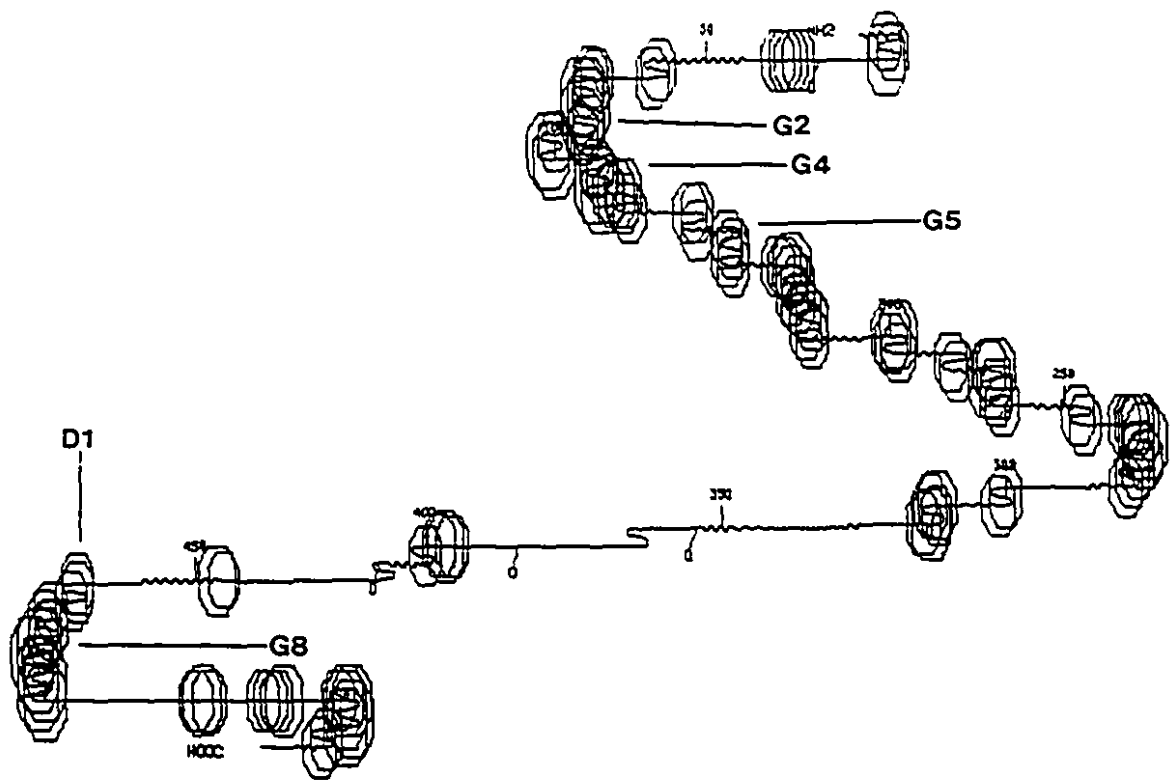

A
G2
G4
G5
G8
5'
3'
D1
B
C

Figure 2. Predicted antigenicity profile for 1D, a 64 kDa protein. The antigenicity profile is based on calculation of hydrophilicity, surface probability and flexibility to predict the position of Ab binding sites by assigning an antigenic index to amino acid regions. The regions predicted to have a high antigenic index (≥1.2) are marked by elongated octagons. The location of identified antigenic peptides are indicated: G2, G4, G5 and G8.


G2
G4
G5
D1
G8

probability and flexibility to predict the position of Ab binding sites by assigning an antigenic index to amino acids regions. There is a good correlation between the positions of the antigenic peptides defined through screening and a high antigenic index as predicted from the antigenicity profile.

Immunocharacterization of TAO sera

The immunoreactivity of sera from group 1 (n=15), group 2 (n=9), and group 3 (n=6) patients and group 4 normal subjects (n=12) to the 4 1D peptides was assessed. Following preabsorption to remove nonspecific and b-galactosidase reactive Abs, each patient's serum was incubated with nitrocellulose strips containing each of the 4 size-separated 1D-β-galactosidase fusion proteins. The Western blotting results are summarized in Tables 3 and 4.

Of the sera from group 1 patients (TAO), 3 of 15 (20%) reacted with G-2, 4 of 15 (27%) with G-4, 5 of 15 (33%) with G-5, and 2 of 15 (13%) with G-8 (Table 3). Of 12 normal subjects (group 4), none reacted with G-5 and only one reacted with G-2, G-4, and G-8 fusion proteins by Western blotting (data not shown). Forty-seven % (7/15) of sera from group 1 patients reacted with G-4 and/or G-5 peptides compared with 8% (1/12) sera from group 4 normal subjects ($p < 0.05$). Sera from group 2 and group 3 patients tended to react with one or more of these antigenic peptides more frequently than sera from normal subjects [4 of 9 (44%) from group 2, 2 of 6 (33%) from group 3, and 1 of 12 (8%) from normal subjects: p = not significant (N.S.)].

Western blotting of full-length 1D fusion protein was also carried out using sera from the same panel of patients with TAO or ATD. Sera from these patients had a tendency to be more reactive with 1D than those from normal subjects [6 of 15 (40%) group 1 patients, 4 of 9 (44%) group 2 patients, 2 of 6 (33%) group 3

Table 3. Reactivity of sera from group 1 patients with full length and 1D and 4 1D-derived antigenic peptides

Patient ID	Group	Diagnosis	1D (1-572)*	G-2 (76-112)	G-4 (127-179)	G-5 (156-205)	G-8 (412-450)
1	1	TAO	+**	+	+	-	-
2	1	TAO	-	-	-	+	-
3	1	TAO	-	-	-	-	-
4	1	TAO	-	-	-	-	-
5	1	TAO	+	-	-	-	-
6	1	TAO	+	-	-	+	-
7	1	TAO	+	+	+	-	-
8	1	TAO	+ +	+	+ +	+ +	+ +
9	1	TAO	-	-	+	+	-
10	1	TAO	-	-	-	-	-
11	1	TAO	-	-	-	-	-
12	1	TAO	+ +	-	+	+ +	+ +
13	1	TAO	-	-	-	-	-
14	1	TAO	-	-	-	-	-
15	1	TAO	-	-	-	-	-

*The corresponding 1D sequence position of each peptide is indicated in parentheses.
**-, + and + + correspond to negative, positive and highly positive reaction by Western blotting, respectively.

Table 4. Reactivity of sera from group 2 and 3 patients with full length 1D and 4 1D-derived antigenic peptides

Patient ID	Group	Diagnosis	1D (1-572)*	G-2 (76-112)	G-4 (127-179)	G-5 (156-205)	G-8 (412-450)
16	2	GH	-**	-	-	-	-
17	2	GH	+	+	-	+	+
18	2	GH	++	-	++	++	++
19	2	GH	-	-	-	-	-
20	2	GH	+	+	+	-	-
21	2	GH	-	-	-	+	-
22	2	GH	+	-	-	-	-
23	2	GH	-	-	-	-	-
24	2	GH	-	-	-	+	-
25	3	HT	-	-	-	-	-
26	3	HT	-	-	-	-	-
27	3	HT	+	-	+	+	+
28	3	HT	-	-	-	-	-
29	3	HT	-	-	-	-	-
30	3	HT	++	+	-	-	-

*The corresponding 1D sequence position of each peptide is indicated in parentheses.
**-, + and ++ correspond to negative, positive and highly positive reaction by Western blotting, respectively.

patients, compared to one of 12 (8%) normal subjects; p=N.S.]. Overall 8 of 15 (53%) group 1 patients, 4 of 9 (44%) group 2 patients, and 2 of 6 (33%) group 3 patients reacted with either peptides or full-length 1D protein. Sera from group 1 patients reacted significantly more frequently with peptides and/or full-length 1D protein than did those from normal subjects [group 1, 53%; group 4 8%; p <0.025].

Several distinct patterns of reactivity were observed in sera from patients with TAO and ATD. The majority of sera recognized more than one immunoreactive peptide and also reacted with the full-length 1D protein. On the other hand, some sera (Table 3 #5 and Table 4 #22) reacted with full-length 1D protein but no peptides, while others (Table 3 #2 and #9) reacted with peptides, but not with full-length 1D protein.

The reactivity patterns of TAO and ATD patients' sera with the 4 1D peptides reveal that each peptide contains at least one immunogenic epitope. Antigenic peptides G-4 (residues 127-179) and G-5 (residues 156-205) partially overlap (Fig.1). Some sera reacted with both G-4 and G-5, while other sera reacted with either G-4 or G-5 only (Tables 3 and 4). These results suggest that at least 3 epitopes are localized within the amino acid sequence 127-205: one between amino acid 127 and 156 [recognized by sera from patients 1, 7, 20 and 27 (Tables 3 and 4)]; one between amino acid 156 and 179 (recognized by sera from patients 8, 9, 12 and 18); and one between amino acid 179 and 205 (recognized by sera from patients 2, 6, 17, 21, 22 and 24).

Antigenic peptide G-8, a 38 amino acid peptide, partially overlaps D1, the 98 amino acid antigenic peptide (Fig.1) described by Dong et al. (1991). However the serum panel did not react identically with G-8 and D1 (Table 5). All of the sera reacting with G-8 also reacted with D1, while only a subset of 1D reactive sera recognized G-8. Therefore, at least two epitopes appear to be located in the amino

Table 5. Reactivity of some sera from group 1, 2 and 3 with G-8 and D1 antigenic peptides

Patient ID	Group	Diagnosis	G-8 (412-450)*	D1 (426-524)
1	1	TAO	-**	+
2	1	TAO	-	+
3	1	TAO	++	+
4	1	TAO	++	+
5	2	GH	+	+
6	2	GH	++	+
7	2	GH	-	+
8	3	HT	+	+
9	3	HT	-	+

*The corresponding 1D sequence position of each peptide is indicated in parentheses.
**-, + and ++ correspond to negative, positive and highly positive reaction respectively of by ELISA to D1 and by Western blotting to G-8.

acid 412-524 region, one between amino acid 450 and 524 and one between amino acid 426 and 450 (Table 5).

DISCUSSION

The results presented here describe the identification of some of the immunogenic peptides of 1D, a 64 kDa Ag expressed by thyroid and eye muscle that is recognized by sera from patients with TAO. Short random fragments (100-200 bp) of 1D cDNA were cloned and expressed in E coli. cells as fusion proteins using the pUEX vector. Four immunoreactive peptides recognized by TAO patient sera were cloned and sequenced. Based on a model predicting antigenicity, all four antigenic peptides are localized to areas of 1D that exhibit a high antigenic index. When a panel of sera were screened for recognition of each peptide, sera from GH (47%) and HT (33%) patients reacted to one or more peptides more frequently than sera from normal controls (8%). Sera from patients with TAO reacted significantly more often with peptides G-4 and G-5 than did those from normal subjects.

To precisely identify the antigenic determinants, it is necessary to prepare a large number of well-characterized peptide fragments or chemical derivatives from the original protein Ag and to then test these derivatives for immunological activity (22, 23). The use of recombinant cDNA methodology to deduce amino acid sequences that comprise specific antigenic determinants in a protein was originally developed by Mehra et al. (20). In essence, the method is the same as using overlapping synthetic peptide probes (24), with the exception that the size of the peptide probe can be made longer, possibly allowing for a more native folding of the resulting peptide.

The pUEX plasmid is an expression vector that produces a b-galactosidase hybrid protein (b-galactosidase plus foreign peptides). The presence of a polylinker in the reading frame greatly simplifies and increases the flexibility of cloning, as compared with expression in lgt 11, which has only one restriction enzyme cut site option. Since screening is carried out with specific Abs, only 1D inserts in the correct reading frame are selected. The b-galactosidase is not enzymatically active but is important because it confers insolubility on the hybrid protein. Recombinants with cDNA fragments of less than 400 bp express stable hybrid proteins at levels comparable with those produced by the vector alone (25%-40% of the total SDS-extractable protein). The insolubility of these hybrid proteins protects them against proteolysis and facilitates their purification (25).

The goal of this study was to characterize the small antigenic peptides recognized by autoAbs from patients with TAO. Such antigenic peptides could then be used as specific Ag in diagnostic tests (e.g. ELISA) for the detection of antibodies to clinically relevant epitopes of the 64 kDa molecule. In this study we characterized four such antigenic peptides recognized by sera from TAO and ATD patients. In most cases, sera from patients with TAO recognized more than one antigenic peptide, demonstrating that patients with TAO have a polyclonal reactivity to the 1D protein. Some sera recognized only the full-length 1D fusion protein, suggesting that some autoAbs require more conformational or discontinuous antigenic determinants for recognition, react with parts of 1D that were poorly expressed in the fragment library, or react with parts of 1D that were not isolated from the fragment library.

One interesting finding in our study is that sera from patients with TAO reacted significantly more often with G-4 and G-5 antigenic peptides (47%) than did those from normal subjects (8%). These results show that Abs to the G4/G5 antigenic 1D peptides are associated with TAO. It is interesting to note that the

prevalence of Abs of this specificity in TAO is very similar to that of Abs reactive with a 64 kDa eye muscle membrane protein by Western blotting (9, 26).

Natural history studies reveal that most patients with HT will have no clinical ophthalmopathy at any stage of their disease. This observation is in contrast to the hypothesized role for Abs reactive to 1D in TAO. Possible explanation for this discrepancy include 1) the small sample size of the present study and 2) the possibility that Abs to 1D are markers of a thyroid autoimmune process rather than of ophthalmopathy. The role of these Abs in disease pathogenesis should be resolved by a large prospective study of newly diagnosed patients with GH and HT.

Sera from patients with GH without evident eye disease also had a high prevalence (44%) of immunoreactivity to antigenic peptides of 1D. This observation is in accord with a recent study which demonstrated that reactivity to 1D expressed by CHO cells is greatest (73%) in patients with GH without evident eye disease (27). These results are consistent with the notion that such Abs may be an early marker of ophthalmopathy in GH patients. It has been shown that approximately 80-90% of patients with GH have ophthalmopathy, as determined by the sensitive method of CT scanning of the extraocular muscle, at the time of diagnosis of hyperthyroidism, even though clinical signs ophthalmopathy may be minimal (28, 29). Since CT scanning was not carried out in these patients with GH, we cannot exclude the possibility that these subjects had early stage ophthalmopathy at the time of study, although none exhibited proptosis or abnormal eye muscle function.

Two of the TAO sera (Table 3, #7 and #12) used to screen the 1D sublibrary reacted with the D1 fusion protein but not with a 64 kDa eye muscle membrane protein by Western blotting. These sera did, however, react with full-length 1D fusion protein. Serum #7 reacted with peptides G-2 and G-4, and serum

#12 reacted with peptides G-4, G-5 and G-8. One of the sera (Table 3, #1) used to screen the 1D sublibrary reacted with the 64 kDa protein of eye muscle membrane, but did not react with D1 fusion protein. This serum was shown to recognize peptides G-2, G-4 and full-length 1D fusion protein. Even though Abs against the 1D molecule and the 64 kDa eye muscle membrane protein are often present in the sera of patients with TAO, it is not known whether the two molecules identified by different methods are the same. In fact, the pI of the 64 kDa protein was found to be about 6.0-7.0 (30, 31) from 2-D gel electrophoresis and Western blotting of human eye muscle and thyroid membrane while the pI of the 1D molecule, deduced from Genetic GCG program (Genetic Computer Group), was 10.3 (Zhang et al. unpublished observation). These observations suggest that they may be different molecules. Microsequencing of the purified 64 kDa protein from eye muscle membrane will resolve this issue.

REFERENCES

1. Wall JR, Bernard N, Boucher A, Salvi M, Zhang Z-G, Kennerdell J, Tyutyunikov A and Genovese C (1993) Pathogenesis of thyroid-associated ophthalmopathy: an autoimmune disorder of the eye muscle associated with Graves' Hyperthyroidism and Hashimoto's thyroiditis. Clin Immunol. Immunopathol. 68: 1-8.
2. Weetman AP, Cohen S, Gatter KC, Fells P and Shine B (1989) Immunohistochemical analysis of the retrobulbar tissues in Graves' ophthalmopathy. Clin. Exp. Immunol. 75: 222-227.
3. Hiromatsu Y, Fukazawa H, Guinard F, Salvi M, How J and Wall JR (1988) A thyroid cytotoxic antibody that cross-react with an eye muscle cell surface

antigen may be the cause of thyroid associated ophthalmopathy. J. Clin. Endocrinol. Metab. 67: 565-570.

4. Zhang Z-G, Salvi M, Miller A, Bernard N, Arthurs B and Wall JR (1992) Restricted tissue reactivity of autoantibodies to a 64 kDa eye muscle membrane antigen in thyroid-associated ophthalmopathy. Clin. Immunol. Immunopathol. 62: 182-189.
5. Kodama K, Bandy-Dafoe P, Sikorska H, Bayly R and Wall JR (1982) Demonstration a circulating autoantibody against a soluble eye muscle antigen in Graves' ophthalmopathy. Lancet. 8312: 1353-1356.
6. Kendall-Taylor P, Atkinson S and Holcombe M (1984) A specific IgG in Graces' ophthalmopathy and its relation to retro-orbital and thyroid autoimmunity. Br. Med. J. 288: 1183-1186.
7. Miller A, Sikorska H, Salvi M and Wall JR (1986) Evaluation of an enzyme-linked immunosorbent assay for the measurement of autoantibodies against eye muscle membrane antigens in Graves' ophthalmopathy. Acta Endocrinol. (Copenh) 113: 514-522.
8. Salvi M, Miller A and Wall JR (1988) Human orbital tissue and thyroid membrane express a 64 kDa protein which recognized by autoantibodies in the serum of patients with thyroid-associated ophthalmopathy. FEBS Lett. 232: 135-139.
9. Miller A, Arthurs B, Boucher A, Liberman A, Bernard N, Salvi M and Wall JR (1992) Significance of antibodies reactive with a 64 kDa eye muscle membrane antigen in patients with thyroid autoimmunity. Thyroid 2: 197-202.
10. Dong Q, Ludgate M and Vassart G (1991) Cloning and sequencing of a novel 64 kDa autoantigen recognized by patients with autoimmune thyroid disease. J. Clin. Endocrinol. Metab. 72: 1375-1381.

11. Zhang Z-G, Wall JR and Bernard N. (1994) Tissue distribution and quantitation of a gene expressing a 64 kDa antigen associated with thyroid-associated ophthalmopathy. Submitted.

12. Zhang Z-G, Dong Q, Rodien P, Alcade L, Bernard N, Boucher A, Vassart G, Ludgate M and Wall JR (1992b) Autoantibodies in the serum of patients with ATD react with a recombinant 98 amino acid fragment of a 64 kDa eye muscle recombinant protein which is also expressed in the thyroid. Autoimmunity. 13: 151-157.

13. Prigent SA, Stanley KK and Siddle K (1990) Identification of epitopes on the human insulin receptor reacting with rabbit polyclonal antisera and mouse monoclonal antibodies. J. Biol. Chem. 265: 9970-9977.

14. Libert F, Ludgate M, Dinsart C and Vassart G (1991) Thyroperoxidase, but not the thyrotropin receptor contains sequential epitopes recognized by autoantibodies in recombinant peptides expressed in the pUEX vector. J. Clin. Endocrinol. Metab. 73: 857-860.

15. Haymerie H, Herz J Bressan GM, Frank R and Stanley KK (1986) Efficient construction cDNA libraries in plasmid expression vectors using an adaptor strategy. Necleic Acids Res. 14: 8615-8624.

16. Higuchi R (1989) Simple and rapid preparation of samples for PCR. In: Erlich HA (ed) PCR technology. Stockton Press. pp. 31-38.

17. Sanger F, Nicklen S and Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. 74: 5463-5467.

18. Messing J and Vieira J (1982) A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. Gene 19: 269-276.

19. Laemmli UK (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature. 227: 680-684.

20. Mehra V, Sweetser D and Young RA (1986) Efficient mapping of protein antigen antigenic determinants. Proc. Natl. Acad. Sci. 83: 7013-7017.

21. Zhang Z-G, Bernard N, Ludgate M and Wall JR (1992d) Epitope mapping of a human 64 kDa membrane autoantigen associated with thyroid autoimmunity and ophthalmopathy. Proceedings 66th Annual Meeting of the American Thyroid Association, Rochester, MN, September. Thyroid 2 (Suppl. 1): 74

22. Horsfall AC, Hay FC, Soltys AJ and Jones MG (1991) Epitope mapping. Immunology Today. 12: 211-213.

23. Momburg f, Fuchs S, Drexler J, Busch R, Post M, Hammerling GJ and Adorini L (1993) Epitope-specific enhancement of antigen presentation by invariant chain. J. Exp. Med. 178: 1453-1458.

24. Atassi MZ (1984) Antigenic structures of proteins. Eur. J. Biochem. 145: 1-20.

25. Stanley KK and Luzio JP (1984) Construction of a new family of high efficiency bacterial expression vectors: identification of cDNA clones coding for human liver proteins. EMBO. J 3: 1429-1434.

26. Salvi M, Bernard N, Miller A, Zhang Z-G, Gardinie E and Wall J. (1991) Prevalence of antibodies reactive with a 64 kDa eye muscle membrane antigen in thyroid-associated ophthalmopathy. Thyroid 1: 204-213.

27. Bernard N, Nygen TN, Tyutyunikov A, Stolarski C, Scalise D, Genovese C, Hayes MB, Ludgate M and Wall JR (1994) Antibodies against 1D, a recombinant 64-kDa membrane protein, are associated with ophthalmopathy in patients with thyroid autoimmunity. Clin. Immunol. Immunopathol. 70: 225-233.

28. Enzmann DR, Donaldson S and Kriss JP (1979) Appearance of Graves' disease on orbital computed tomograph. J. Comput. Assist. Tomogr. 3: 815-819.

29. Werner SC (1974) Ultrasonographic evidence of a consistent orbital involvement in Graves' disease. N. Engl. J. Med. 290: 1447-1450.

30. Boucher A, Bernard N, Miller A, Rodien P, Salvi M and Wall JR (1994) Two dimensional gel electrophoresis identifies minor differences in immunologically cross-reactive 64 kDa autoantigens in the thyroid and eye muscle. J. Endocrinol. Invest. 17: 7-13.

31. Wall JR, Hayes M, Scalise D, Stolarski C, Nebes V, Kiljanski M, Salvi M and Sato M (1994) Native gel electrophoresis and isoelectric focusing of a 64 kDa eye muscle protein shows that it is an important target for serum autoantibodies in patients with thyroid-associated ophthalmopathy and not expressed in other skeletal muscle. Submitted.

CHAPTER V

Tissue distribution and quantitative of gene expression a 64 kDa antigen recognized by the serum of patients with thyroid-associated ophthalmopathy

PREFACE

Dong et al. used D1, a 298 bp 1D cDNA fragment, as a probe to study 1D mRNA expression in different human tissues by Northern blotting. They demonstrated a 3.9 kb transcript in thyroid and eye muscle, but not in skeletal muscle. Ross et al. and our research team studied the tissue distribution of 1D mRNA by the more sensitive RT-PCR. Both laboratories demonstrated that 1D mRNA could be amplified from several other tissues. Because amplification of target DNA sequences by PCR is exquisitely sensitive and because this method has the potential of amplifying a few copies of template into a detectable signal, no conclusion could be drawn from these experiments regarding the comparative abundance of specific 1D mRNA between tissues. These studies were repeated with appropriate controls to exclude the possibility of amplification of non-specific template in a manner which permitted quantitation of the levels of 1D mRNA expression from various human tissues. This Chapter describes the use of a quantitative RT-PCR to further investigate the tissue distribution of 1D mRNA and quantitate the levels of expression of transcripts of this specificity from a variety of human tissues.

ABSTRACT

We have developed a competitive reverse transcriptase-polymerase chain reaction (RT-PCR) assay for the quantitation of 1D (a cloned 64 kDa protein) mRNA levels in preparations of total RNA from variety of human tissues. This competitive RT-PCR assay is based on coamplification of a competing 1D cDNA internal template (pGEM-1D') and target cDNA. 1D specific mRNA was quantitated in ten human tissues. The level of 1D transcript expression in these tissues decreases in the following order: thyroid (5 pg/μg total RNA) > eye muscle (3.2 pg/μg) > skeletal muscle (2.4 pg/μg) > ovary (2 pg/μg) > cerebellum (0.4 pg/μg), kidney (0.33 pg/μg), pancreas (0.27 pg/μg), spleen (0.22 pg/μg) and thymus (0.19 pg/μg) > retina (0.016 pg/μg). The greater expression of the 1D molecule in thyroid and eye muscle supports the notion that this molecule may be involved as a target of autoimmune attack in thyroid-associated ophthalmopathy (TAO).

INTRODUCTION

Progressive inflammation of the extraocular muscles and the surrounding orbital connective tissue occurs in most patients with Graves' hyperthyroidism (GH) and in a small proportion of those with Hashimoto's thyroiditis (HT). This condition is named Thyroid-Associated Ophthalmopathy (TAO) (1). Of all patients with TAO 85% have GH, 10% have HT and 5% have other thyroid immunological abnormalities (2). A variety of eye muscle reactive autoantibodies have been detected in the sera of patients with TAO (3, 4).

It has been hypothesized that the association of TAO with autoimmune thyroid disease (ATD) is due to the expression of a shared or cross-reactive

antigen (Ag) by thyroid and eye muscle. A 64 kDa membrane molecule has emerged as the best candidate for an Ag underlying the association of TAO and ATD, as it is expressed on both eye muscle and thyroid and is recognized by Abs in the sera of a large proportion of TAO patients and only rarely by Abs in the sera of individuals without TAO or ATD (5).

A 64 kDa Ag expressed in the human thyroid and extraocular muscle has been cloned by Dong et al (6). from a thyroid complementary deoxyribonucleic acid (cDNA) expression library. The full length cDNA, named 1D, encodes a 572 amino acid protein, corresponding to a MW of 63-64 kDa. A 98 amino acid fragment of this protein was named D1. The use of D1 as a probe in Northern blotting revealed 3.9 kb transcripts of poly(A)+ ribonucleic acid (RNA) from normal human thyroid and eye muscle, but not skeletal muscle. The more sensitive reverse transcriptase polymerase chain reaction (RT-PCR) methodology has been used by Ross et al. (7) and by us (8) to investigate the tissue distribution of 1D messenger RNA (mRNA). 1D amplification products were observed when eye muscle, skeletal muscle, thyroid, and cardiac muscle RNA were used as the PCR template.

This report describes the use of quantitative RT-PCR to further investigate the tissue distribution of 1D mRNA and to quantitate the levels of expression in a variety of human tissues. Our results demonstrate that 1D mRNA is expressed in human thyroid, eye muscle, skeletal muscle, ovary, thymus, spleen, kidney, cerebellum, pancreas and retina. The level of 1D transcript expression in these tissues decreases in the following order: Thyroid (5 pg/μg total RNA) > eye muscle (3.2 pg/μg) > skeletal muscle (2.4 pg/μg) > ovary (2 pg/μg) > cerebellum (0.4 pg/μg), kidney (0.33 pg/μg), pancreas (0.27 pg/μg), spleen (0.22 pg/μg) and thymus (0.19 pg/μg) > retina (0.016 pg/μg).

MATERIALS AND METHODS

RNA isolation

Surgical specimens, placed in liquid nitrogen immediately after excision, were the source of most of the human tissues used in this study. Tissues were stored at -70°C until RNA extraction. Total RNA was isolated from human tissues by the guanidine isothiocyanate method (9, 10), with the exception of pancreas, thymus and retina RNA, which were obtained from ClonTech (Palo Alto, CA). Briefly, frozen human tissues were homogenized using a Polytron in a denaturing solution (4.0 M guanidine isothiocyanate; 0.2 mM b-2-mercaptoethanol; 42 mM sodium citrate; 0.83% N-lauryl sarcosine). Nucleic acids were extracted with an equal volume of a 24:24:1 (vol:vol:vol) phenol: chloroform: isoamyl alcohol mixture. Following centrifugation at 10,000 x g for 20 min the top aqueous phase, containing RNA, was harvested and precipitated with an equal volume of isopropanol. The resulting pellet was washed with 70% ethanol and dried. The RNA pellet was then resuspended in diethylpyrocarbonate (DEPC)-treated water. The purity and yield of total RNA were determined spectrophotometrically by measuring absorbance at 260 and 280 nm. The integrity of the resulting RNA was verified by formaldehyde agarose gel electrophoresis and visualized under ultraviolet (u.v.) light by staining of ribosomal RNA (rRNA) bands with ethidium bromide (11). RNA was considered intact and therefore appropriate for use as a template for cDNA synthesis and PCR if the 28S band of rRNA appeared to be twice as intense as the 18S band (12).

CHO cells expressing 1D

CHO.5 is a clonal population of Chinese hamster ovary (CHO) cells stably expressing 1D. CHO.5 cells were cultured in F-12 nutrient medium supplemented with 10% fetal calf serum (FCS), 100 units (U)/ml penicillin, 100 ng/ml streptomycin, 2.5 μg/ml amphotericin B, 200 μg/ml gentamycin, 2 mM L-glutamine and 300 μg/ml neomycin. RNA extraction from CHO.5 cells was performed as described above.

First strand cDNA synthesis

Reverse transcription was carried out using 4.0 μg of total RNA in a volume of 30 μl containing 1.0 μg random hexamer primers (Promega, Madison, WI), 1 mM deoxyribonucleoside triphosphates (dNTPs) (Promega), 20 U of RNasin and 400 U of Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega). RNA was denatured at 70°C for 10 min and chilled on ice. After incubation for 60 min at 37°C, the temperature was raised to 95°C for 5 min to inactivate the reverse transcriptase and then cooled to 4°C (13, 14).

To test the yield and efficiency of the reverse transcriptase reaction, 4.0 μg CHO.5 total RNA was reverse transcribed as above, in the presence of 2-5 μCi ^{32}P-dCTP. The reaction mixture was sampled prior to reverse transcription to determine the total counts per minute (c.p.m) and trichloroacetic acid (TCA)-precipitable c.p.m (background). After reverse transcription, the reaction mixture was sampled for measurement of incorporated ^{32}P-dCTP (15). The amount of cDNA synthesized was calculated by multiplying the fraction of total dCTP incorporated into TCA-precipitatable c.p.m by the number of nM of each dNTP in the reaction and the average weight of the 4 dNTPs. The formula used to calculate

the efficiency of reverse transcription is: amount of cDNA synthesized (μg)/amount of mRNA (μg) present at the start of the reaction x 100%.

Creation of competitive templates

Quantitative RT-PCR is based on the comparison of the amount of template-specific PCR products generated following the simultaneous amplification of cDNA derived from a particular tissue source and a known quantity of competitive DNA template added to the same reaction tube. Competitive template DNA for these experiments was prepared by digesting 20 mg of λgt11-1D with 80 U EcoRI for 2 hr. Digestion products were separated by electrophoresis on a 1.2% low-melting point agarose gel. The band containing the 1.78 kb 1D fragment was excised from the gel, extracted and precipitated. EcoRI digested 1D was then ligated into EcoRI digested dephosphorylated pGEM-7Zf (Promega). Recombinant plasmids were transformed into competent Escherichia (E) coli DH5a cells (BRL, Gaithersburg, MD) according to the protocol provided by the manufacturer. Two to 4 ng of ligation mixture was incubated with 100 ml of competent cells on ice for 30 min followed by a 45 sec heat shock at 42°C. S.O.C. medium (2% bactotryptone; 0.5% yeast extract; 10 mM NaCl; 2.5 mM KCl; 20 mM $MgCl_2$-$MgSO_4$; 20 mM glucose) was added to the transformed DH5a cells and the cells were grown for 1 hr in a 37°C shaking water bath (225 rpm). One hundred μl of transformed cells were then plated onto petri dishes of LB agarose containing 100 mg/ml ampicillin and incubated for 16 hr at 37°C.

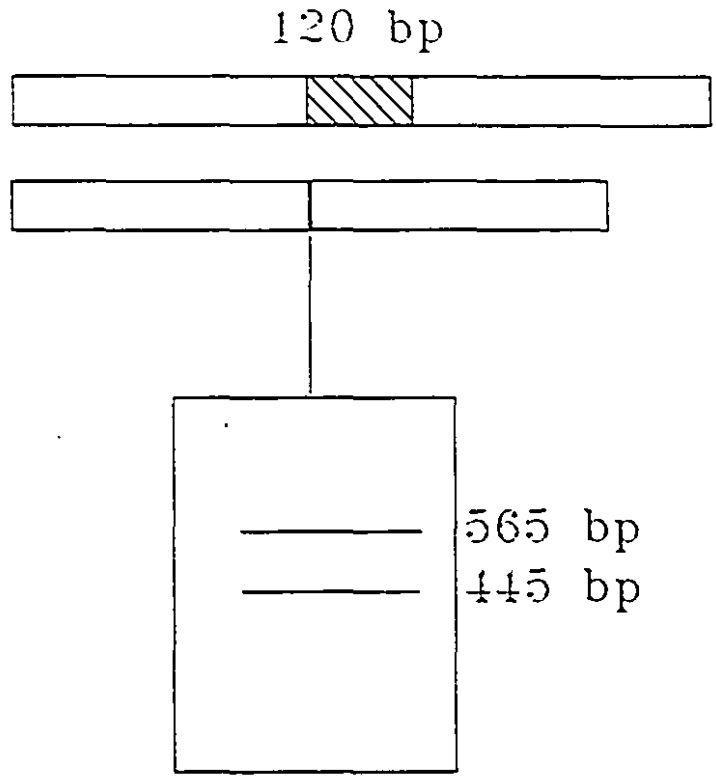
Resulting colonies were selected for mini-preparation of plasmid DNA. The presence of 1D inserts in plasmid DNA prepared from these clones was screened by PCR using the 5' (nucleotide 1174-1195) and 3' (1598-1619) forward and reverse primers (Sheldon Biotechnology Center, Montreal, Quebec) deduced from

digested pGEM-1D was then ligated with a gel purified 120 bp HaeIII treated pBR322 DNA fragment. The ligation mixture was transformed into competent DH5a cells as described above. The resulting colonies were screened by PCR using the same 5' and 3' primers described above. A pGEM-1D subclone (pGEM-1D') containing an insert of expected size (445+120 bp) was selected for use as the competitive internal control for quantitative PCR assays (Fig. 1). pGEM-1D' was grown for 16 hr at 37°C and DNA was extracted and purified by a plasmid DNA isolation kit (ClonTech). The DNA yield was determined by measuring the absorbance at 260 nm and the size of the insert DNA was verified by agarose gel electrophoresis.

PCR amplification

The PCR method is based on the repetitive cycling of three reactions (denaturation, primer binding, and DNA synthesis), which vary only in incubation temperature (16). The reagents required to amplify a target DNA include two single-stranded oligonucleotide primers, designed to be complementary to known sequences of the target DNA, the 4 dNTP, and the heat-stable enzyme Taq DNA polymerase (17). The first step in the PCR procedure is heat denaturation of double-stranded DNA. As the temperature is reduced, the two DNA primers anneal to complementary sequences on opposite strands of the target DNA. The next step is the actual synthesis of new complementary second strands of DNA, which occurs through the extension of each annealed primer and is catalyzed by Taq polymerase in the presence of excess dNTPs. After extension of the primers, the cycle is repeated 25-35 times (18). The PCR products are analyzed by agarose gel electrophoresis.

Figure 1. Design of an internal control for quantitating 1D expression. The internal control (pGEM-1D') was made by inserting a 120 bp fragment into the internal EspI restriction enzyme cut site of the pGEM-1D insert.


120 bp
565 bp
445 bp

DNA amplification was carried out in PCR buffer (10 mM Tris-HCl, pH 8.4, containing 50 mM KCl, 0.1 mg/ml gelatin and 2 mM $MgCl_2$) supplemented with 50 mM dNTPs, 0.25 μM each of the 5' and 3' specific primers, and 2.0 U of Taq polymerase in a final volume of 50 μl. The mixture was overlaid with 50 μl of mineral oil and amplified in sequential cycles of 94°C for 45 sec, 60°C for 45 sec and 72°C for 2 min. All PCR components used in the reaction were tested for possible contamination of DNA in a simultaneous PCR reaction. PCR of genomic DNA derived from the tissues was negative, presumably because the primers span one or more introns.

Analysis of amplified products

Two μl of each PCR sample was analyzed in parallel with pBR322 DNA size markers (Boehringer Mannheim, Mannheim, FRG) by electrophoresis on a 1.8% agarose or 6 M urea-8% polyacryamide gel (PAGE). DNA bands were visualized by staining with ethidium bromide followed by exposure to u.v. light. Quantitation of the DNA bands was performed at a Bogen TC-1 Biology Workstation, using *SigmaPlot* custom software (Sigma, St. Louis, MO). A high-speed video camera was used to acquire a video image of the gel, with output directly to a computer-based system for data analysis and storage. Ethidium fluorescence associated with DNA bands in the gel was proportional to the amount of DNA present. Bands of interest were selected on the video screen for analysis and the integrated fluorescence intensity associated with each band determined by computer. To compare the inter-assay results, the reaction yields (densitometric units) were evaluated by comparing the intensity of the PCR product band with that of the 500 bp pBR322 size marker used as a standard.

RESULTS

Efficiency of reverse transcription

pGEM-1D' DNA, used as the internal control in these experiments was already double-stranded and therefore was not subject to any of the factors that affect efficiency of reverse transcription of mRNA into cDNA prior to amplification. The efficiency and reproducibility of this step was evaluated in separate experiments using CHO.5 total RNA as template and conditions identical to those used to reverse transcribe tissue specific total RNA. Results from 6 experiments revealed that cDNA first strand synthesis had an efficiency of 46.2 ± 6.9%. This value was subsequently used as a correction factor for calculating concentrations of 1D specific mRNA from human tissues from RT-PCR reaction yields.

In order to evaluate the reproducibility of the competitive PCR step, CHO.5 cDNA samples prepared as described above by reverse transcription, were subjected to amplification at the same time and under the same condition used to carry out competitive RT-PCR of human tissue specific total RNA. In 6 experiments, the concentration of 1D specific mRNA from CHO.5 was found to be 0.6 ± 0.058 pg/mg total RNA (range 0.53 to 0.66 pg/mg total RNA).

Quantitative PCR coamplification of native 1D cDNA and pGEM-1D'

In order to define the relationship between the amount of template DNA present at the start of the reaction and the quantity of amplified product present at the end of the reaction, amplification of pGEM-1D' DNA was performed with between 0.001 and 50 pg starting material for 28 cycles. The final quantity of

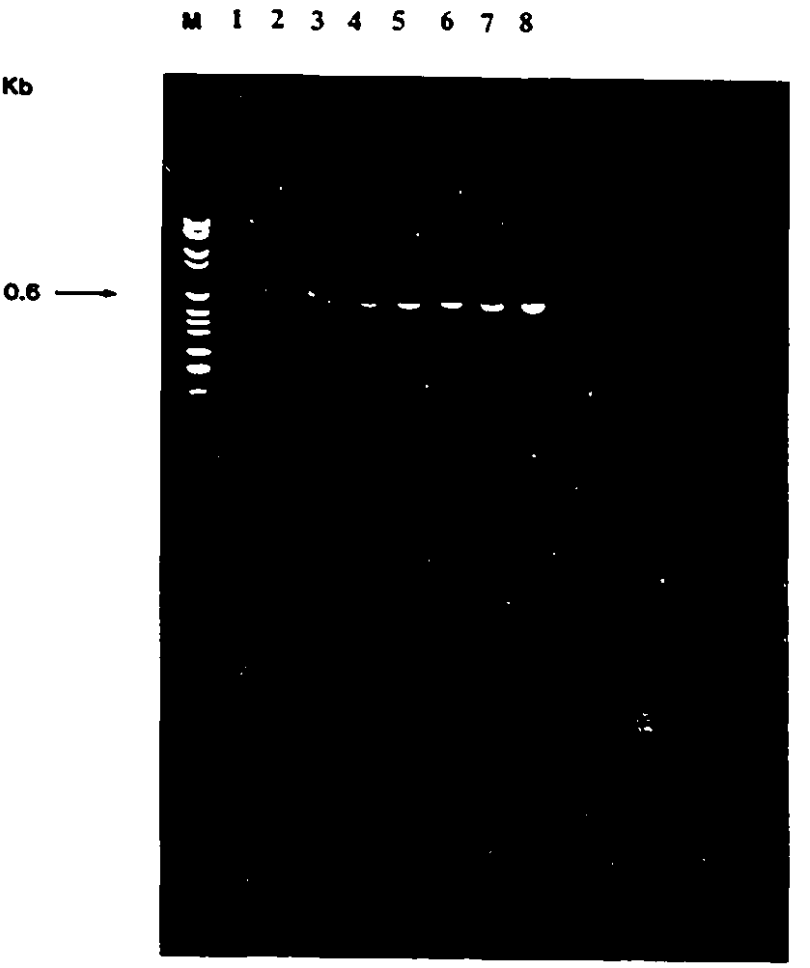
pGEM-1D' specific product (565 bp) varied logarithmically in a dose dependent manner over a range of 3 orders of magnitude with the amount of template introduced at the start of the reaction (Fig. 2).

To determine the kinetics of coamplification of pGEM-1D' and prototypic 1D, 1.0 pg of pGEM-1D' and 0.4 μg of reverse transcribed CHO.5 RNA were coamplified for up to 36 PCR cycles. The 2 bands of expected size (445 bp, 565 bp) increased in intensity with the number of amplification cycles (Figure 3). In addition, the quantity of native 1D PCR product increased linearly up to cycle 28 and reached a plateau around cycle 30. Amplification proceeded, with the same efficiency for both templates, through the exponential and nonexponential phases to the plateau phase. This observation confirmed that despite a difference of 120 bp in size between the two templates, the product ratio of pGEM-1D'/native 1D cDNA remained constant throughout the 36 cycles. Since the amplification efficency was identical for both targets throughout the amplification process, the amount of 1D-specific cDNA in cDNA from a defined amount of RNA from a particular tissue can be determined by comparison with the amount of pGEM-1D' product generated at any cycle number in the range examined. We chose to amplify all subsequent reactions for 28 cycle, because amplification for this number of cycles was in the logarithmic phase and highly sensitive to the quantity of template available at the start of the reaction.

The effect of varying the ratio of prototypic 1D-specific CHO.5 cDNA and pGEM-1D' cDNA present at initiation of amplification is shown in Figure 4. A constant amount of cDNA (from 0.4 μg CHO.5 RNA) mixed with variable amounts of pGEM-1D' (0.001 pg to 5 ng) were amplified for 28 cycles. The results in Figure 4 demonstrate that as the concentration of the pGEM-1D' present at initiation increased the concentration of the corresponding PCR product (565 bp) increased, while that of the product from prototypic 1D mRNA from CHO.5

Figure 2. Quantity of pGEM-1D' specific PCR amplification products varies depending on the amount of template present at initiation of reaction. PCR was carried out for 28 cycles. (a) Amplification products were electrophoresed on a 1.8% agarose gel and stained by ethidium bromide. Lane M, size markers (HaeIII digested pBR322); lane 1, control reaction without template; pGEM-1D' template concentration at start of reaction: lane 2, 0.001 pg; lane 3, 0.01 pg; lane 4, 0.1 pg; lane 5, 0.5 pg; lane 6, 1.0 pg; lane 7, 5.0 pg; lane 8, 50 pg. (b) Plot of quantitative densitometry reading obtained by computer-based video image analysis of the gel shown in (a), using procedures described in Materials and Methods. The mean ± SD valves of 4 separate experiments.

a



b

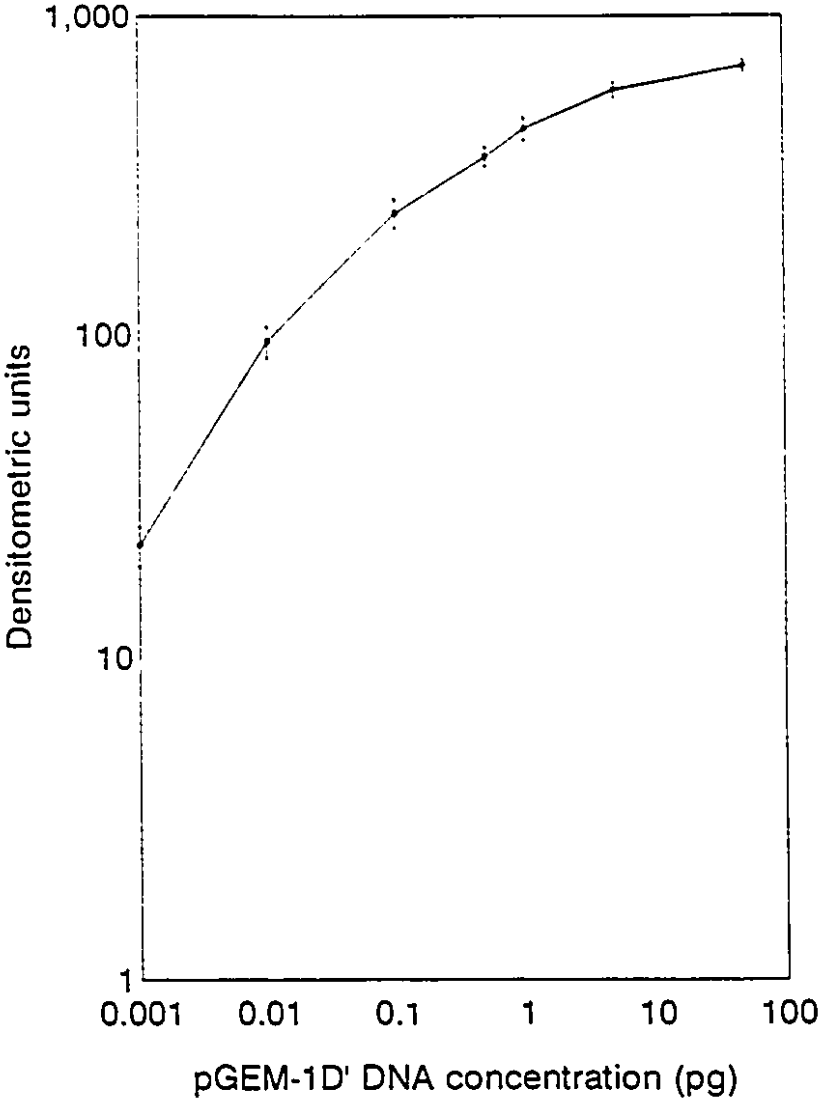
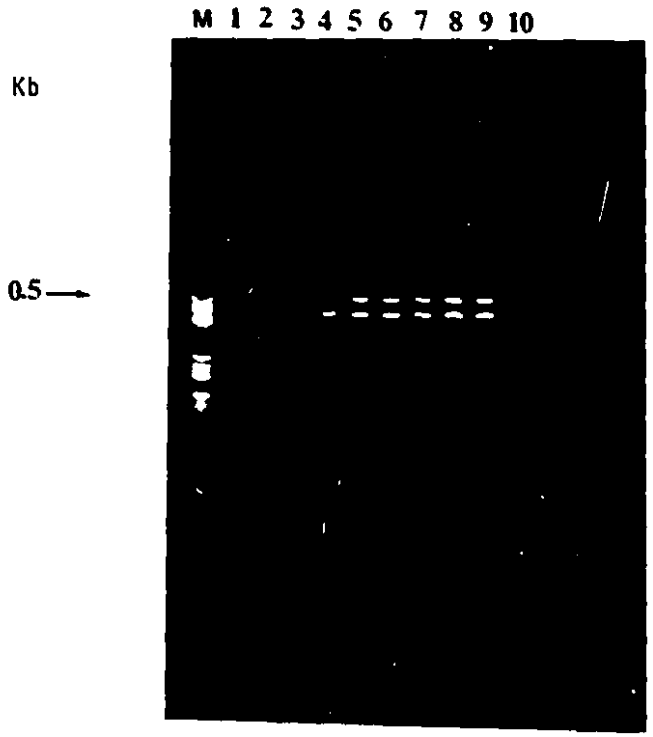

1,000
100
10
1
Densitometric units
0.001
0.01
0.1
1
10
100
pGEM-1D' DNA concentration (pg)

Figure 3. Analysis of coamplification of CHO.5 cDNA and pGEM-1D' DNA for varying cycle numbers. (a) CHO.5 cDNA (synthesized from 0.4 μg total RNA) and 0.1 pg pGEM-1D' were coamplified for 20 cycles. lane 1: 22 cycles. lane 2: 24 cycles. lane 3: 26 cycles. lane 4: 28 cycles. lane 5: 30 cycles. lane 6: 32 cycles. lane 7: 34 cycles. lane 8: 36 cycles. lane 9. Two μl of each reaction was electrophoresed on a 1.8% agarose gel. DNA was stained by ethidium bromide. Lane M contains size markers (HaeIII digested pBR322): lane 10 a simultaneous reaction containing all the reagents presents in reactions amplified in lanes 1-9 but without DNA template. (b) Plot of quantitative densitometry reading obtained by computer-based video image analysis of the gel shown in (a). as described in Materials and Methods. The mean ± SD valves of 4 separate experiments.

a



b

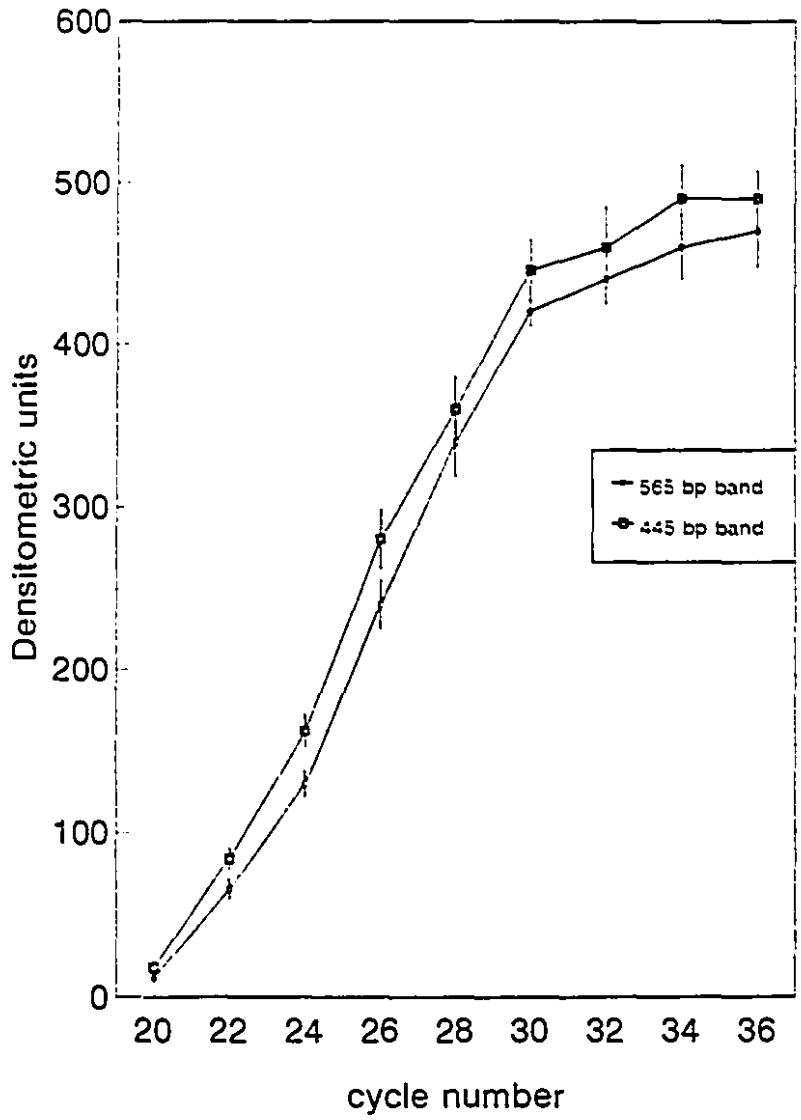
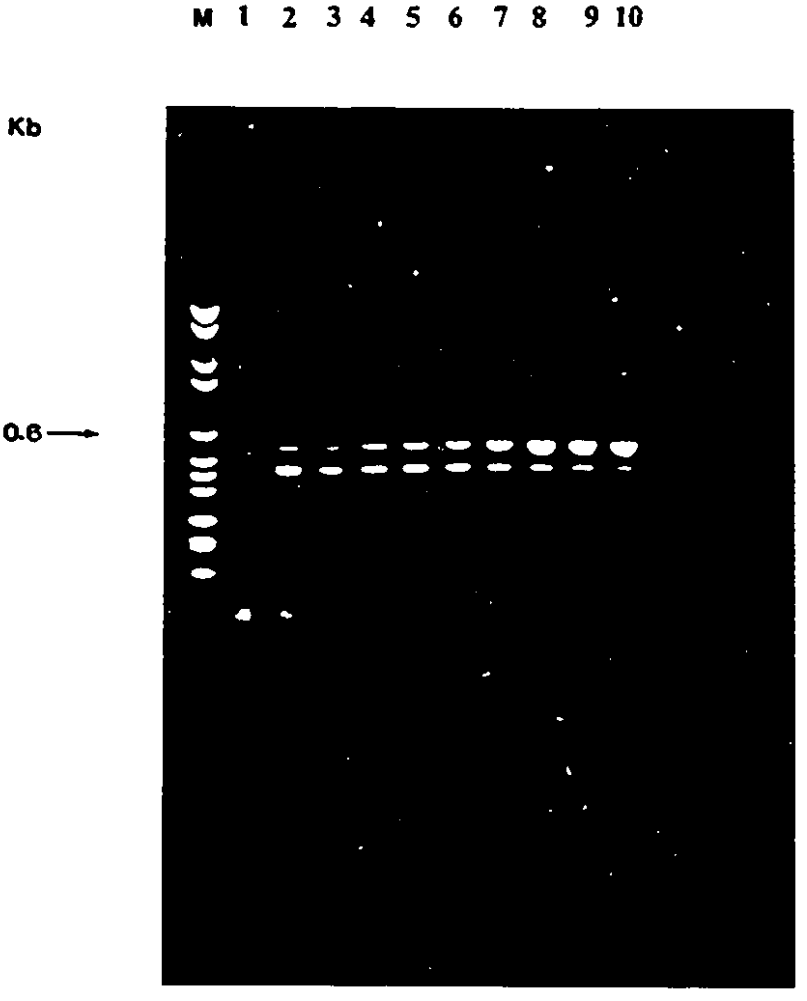
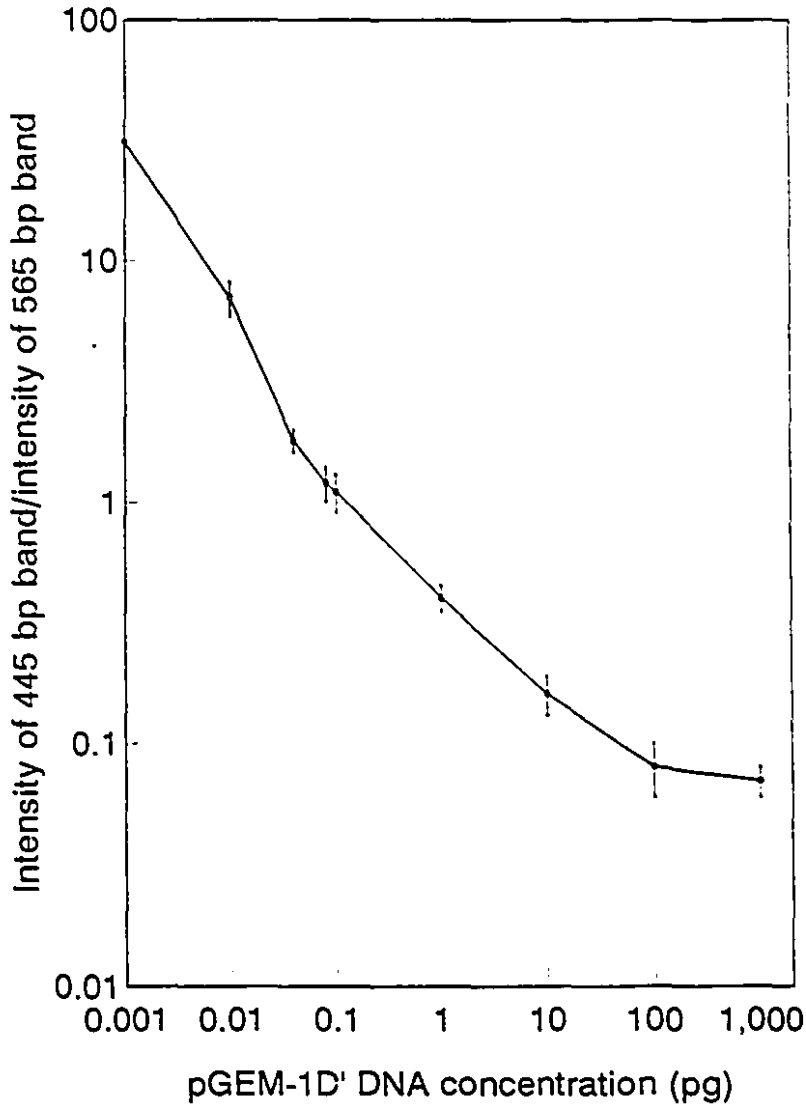

600
500
400
300
200
100
0
Densitometric units
20
22
24
26
28
30
32
34
36
cycle number
565 bp band
445 bp band

Figure 4. Analysis of coamplification of varying ratios of CHO.5 cDNA to pGEM-1D' DNA. Varying quantities of pGEM-1D' DNA were coamplified with a constant amount of CHO.5 cDNA (synthesized from 0.4 μg total RNA). Two μl of the reaction was electrophoresed on a 1.8% agarose gel. DNA was stained by ethidium bromide. (a) Lane M, size markers (HaeIII digested pBR322); lane 1, control reaction without template; amount of internal control added to reaction: lane 2, 0.001 pg; lane 3, 0.01 pg; lane 4, 0.04 pg; lane 5, 0.08 pg; lane 6, 0.1 pg; lane 7, 1.0 pg; lane 8, $5x10^{1}$ pg; lane 9, $5x10^{2}$ pg; lane 10, $5x10^{3}$ pg. (b) Plot of quantitative densitometry readings obtained by computer-based video image analysis of the gel shown in (a), using procedures described in Materials and Methods. The mean ± SD valves of 4 separate experiments.

a



b


100
10
1
0.1
0.01
Intensity of 445 bp band/intensity of 565 bp band
0.001
0.01
0.1
1
10
100
1,000
pGEM-1D' DNA concentration (pg)

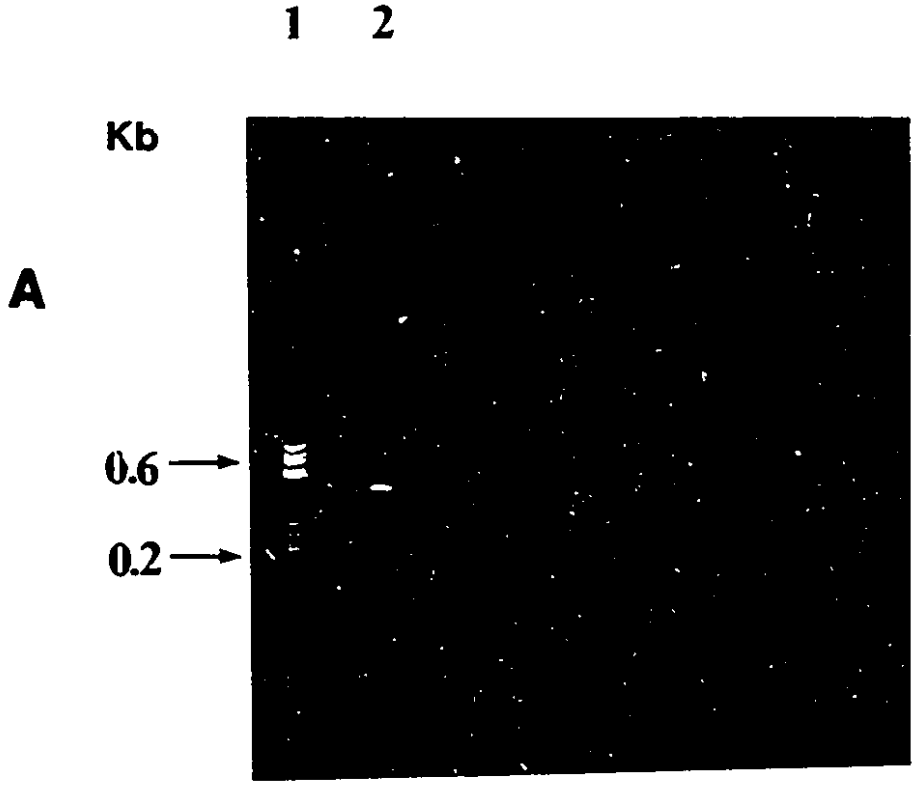
cells (445 bp) decreased. Because both templates were amplified with similar kinetics and efficency (see above), the concentration of pGEM-1D' yielding a PCR product band of equivalent intensity to that from CHO.5 cDNA represents a reaction where the initial quantity of 1D specific cDNA was equal to that of competing pGEM-1D' levels.

Analysis of 1D mRNA expression in various human tissues

Using this methodology mRNA specific for 1D was detectable in a variety of human tissues. All PCR products were found to be the predicted size (445 bp) on agarose gels. The specificity of these 445 bp amplification products was verified by restriction enzyme digestion with EspI, which resulted in fragments of the predicted size of 93 and 352 bp (Fig. 5), and Southern blotting with a 1D specific probe (8).

First, 40 ng of reverse transcribed RNA from each tissue was subjected to PCR in the presence of between 0.01 and 100 pg of pGEM-1D' DNA. This permitted an estimation of the quantity of pGEM-1D' DNA required to achieve equivalence following coamplification with 0.2 to 0.4 μg of reverse transcribed RNA from each tissue. Once this was determined, 0.2-0.4 μg of reverse transcribed RNA from each tissue was coamplified with serial 2-fold dilutions of pGEM-1D' DNA, which included the concentration yielding equivalence with that tissue specific cDNA in the previous set of experiments. Figure 6 shows thyroid cDNA reverse transcribed from 0.4 μg total RNA coamplified with serial 2-fold dilutions of pGEM-1D' ranging from 1.25-40 pg by PCR. As the amount of pGEM-1D' increased, amplification of thyroid 1D-cDNA diminished. From this analysis and taking into account an efficiency factor of 0.46 for the reverse transcription step required to produce cDNA from thyroid mRNA, the amount of

Figure 5. EspI digestion of 1D mRNA amplified by RT-PCR. PCR was performed using procedures described in Materials and Methods and 10 μl PCR products was digested with Esp I. Digestion fragments were analyzed on (a) 1.8% agarose gel and (b) 6 M urea 8% PAGE. Following electrophoresis, DNA bands were stained with ethidium bromide. (a) Lane 1, size markers (HaeIII digested pBR322); lane 2, EspI digested PCR products fragments. (b) Lanes 1, size markers (Bgl I and Hinf I digested pBR328); lane 2, size markers (HaeIII digested pBR322); lane 3, EspI digested PCR product fragments.


1
2
Kb
A
0.6
0.2

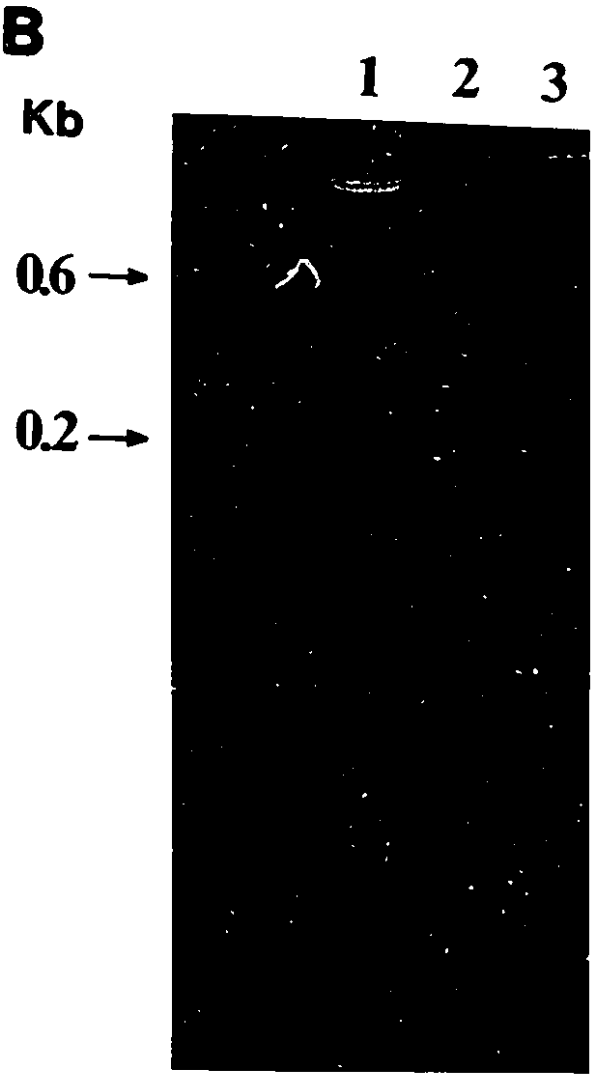
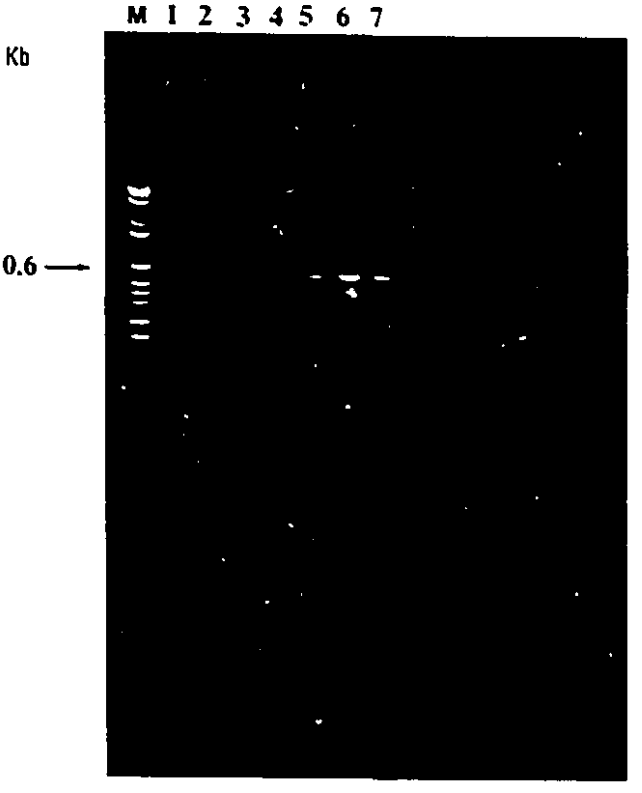
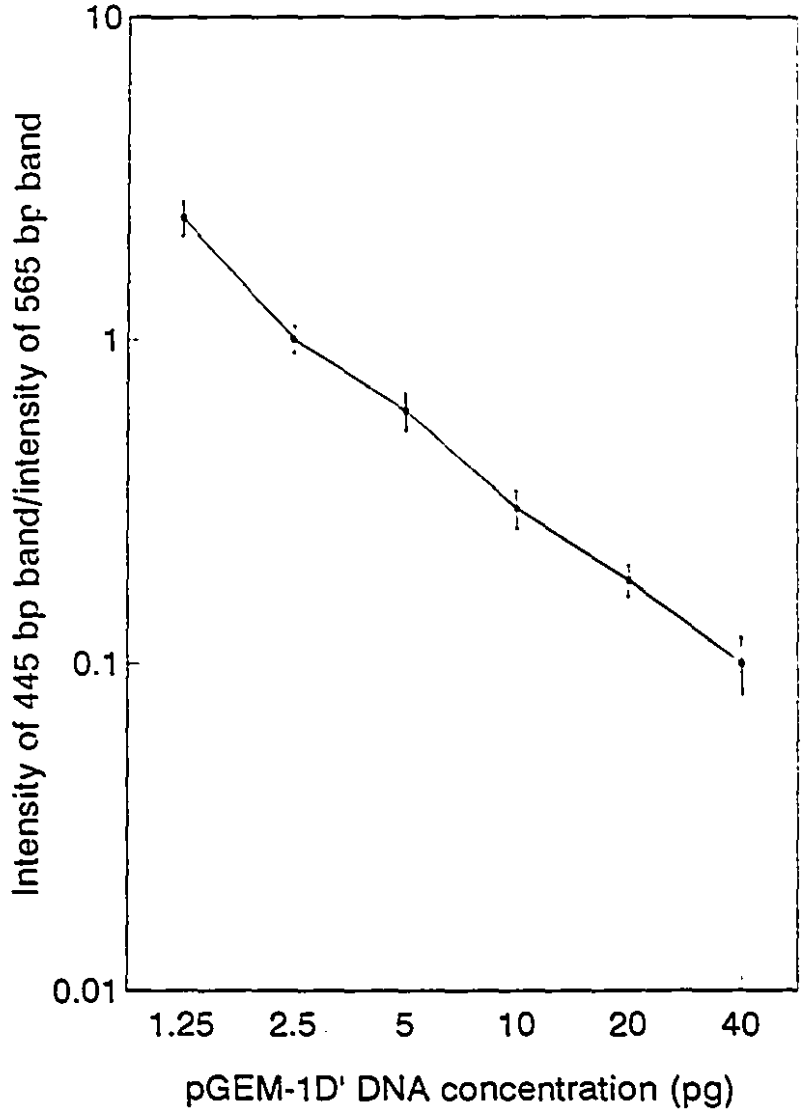

B
1
2
3
Kb
0.6
0.2

Figure 6. Quantitation of thyroid 1D mRNA by competitive RT-PCR. Thyroid cDNA (synthesized from 0.4 ug total RNA) and 2-fold serial dilutions of pGEM-1D' were coamplified by PCR (see Materials and Methods). (a) Two μl of each reaction mixture was electrophoresesed on a 1.8% agarose gel. DNA bands were stained with ethidium bromide. Lane M, size markers (HaeIII digested pBR322); lane 1, control reaction without template; amount of pGEM-1D' DNA added to each reaction: lane 2, 1.25 pg; lane 3, 2.5 pg; lane 4, 5.0 pg; lane 5, 10 pg; lane 6, 20 pg; lane 7, 40 pg. (b) Plot of quantitative densitometry readings obtained by computer-based video image analysis of the gel shown in (a), using procedures described in Materials and Methods. The mean ± SD valves of 3 separate experiments.

a



b


10
1
0.1
0.01
Intensity of 445 bp band/intensity of 565 bp band
1.25
2.5
5
10
20
40
pGEM-1D' DNA concentration (pg)

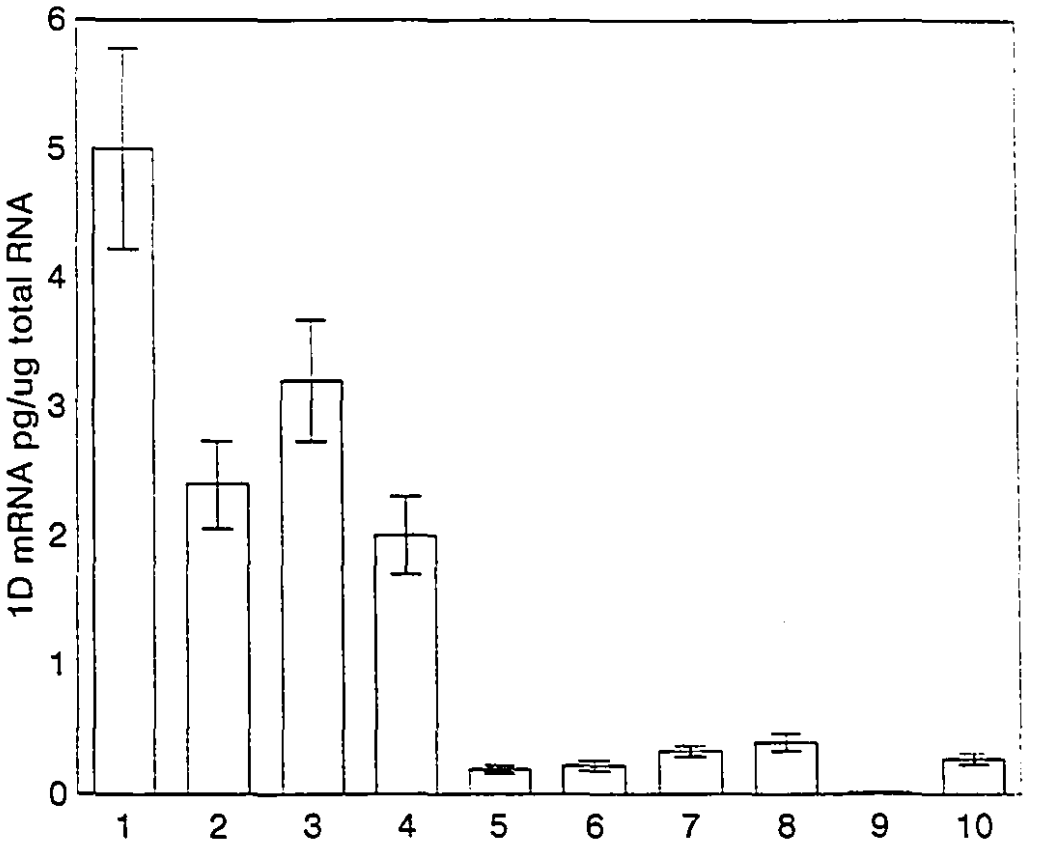
1D specific mRNA in thyroid was deduced to be 5.0 ± 0.28 pg/μg total RNA. This analysis was extended to eye muscle where the concentration of 1D specific RNA was 3.2 ± 0.47 pg/μg total RNA, skeletal muscle (2.4 ± 0.33 pg/μg total RNA), ovary (2.0 ± 0.31 pg/μg total RNA), cerebellum (0.4 ± 0.07 pg/μg total RNA), kidney (0.33 ± 0.04 pg/μg total RNA), pancreas (0.27 ± 0.05 pg/μμg total RNA) spleen (0.22 ± 0.04 pg/μg total RNA), thymus (0.19 ± 0.03 pg/μg total RNA), and retina tissue (0.016 ± 0.003 pg/μg total RNA) (Fig. 7).

The concentration of 1D specific mRNA from total RNA for each of 10 human tissues was determined from 4 separate RT-PCR experiments. All of the experiments included control reactions performed under identical conditions, using CHO.5 RNA as template in order to evaluate reproducibility of first strand synthesis and the PCR steps from experiments to experiments. As noted above, the average concentration of 1D specific mRNA in CHO.5 cells was found to be 0.6 ± 0.058 pg/μg total RNA.

DISCUSSION

The use of reverse transcription followed by PCR (RT-PCR) enables the amplification of individual RNA molecules. The high sensitivity of RT-PCR makes it possible to detect mRNA of extremely low abundance (19, 20). Several PCR-based approaches have been developed for quantitating DNA and RNA. One of the original approaches used external heterologous standards, such as mRNA species with a relatively similar level of expression from tissue to tissue (i.e. b-actin or b_2-microglobulin). These standards were amplified in separate reactions and used different primers (21, 22). Such an approach to RNA/DNA quantitation is semi-quantitative, at best, because kinetics for primer hybridization can vary,

Figure 7. 1D specific mRNA levels in various human tissues. The concentration of 1D specific mRNA from total RNA of 10 human tissues. RT-PCR was performed as described in Materials and Methods. The bars represent the mean ± SD valves of 4 separate experiments and are numbered as follows: 1. thyroid: 2. skeletal muscle: 3. eye muscle: 4. ovary: 5. thymus: 6. spleen: 7. kidney: 8. cerebellum: 9. retina: 10. pancreas.


1D mRNA pg/ug total RNA
0
1
2
3
4
5
6
1
2
3
4
5
6
7
8
9
10

thereby influencing amplification efficiency. Moreover high copy number templates, such as b-actin and others, amplify in a completely different manner than low copy number templates and small pipetting differences between tubes can dramatically affect PCR signals.

A more accurate method for quantitation of RNA/DNA by PCR involves the addition of known amounts of an internal control to the amplification mixture (23, 24). The use of an internal control that includes the same primer template sequences as the target template makes it possible to determine, under identical conditions, the absolute amount of target cDNA by allowing known amounts of a competitive internal control to compete with the target for primer binding during the amplification reaction (25).

This report describes the use of this method of competitive PCR for quantitation of 1D specific mRNA in assessing the tissue distribution and quantity of 1D mRNA in a panel of human tissues. The competitive PCR is based on simultaneous amplification of two templates: 1D cDNA and a competing internal control (pGEM-1D'). Because native 1D and pGEM-1D' cDNA templates are present in the same reaction and bind the same primer set, any factors affecting amplification have the same effect on both templates. As a consequence, the ratio of PCR products reflects the ratio between the amount of pGEM-1D' and 1D cDNA present at the start of the reaction, thus allowing the quantitation of native 1D cDNA in a given amount of RNA from the known amount of pGEM-1D'.

Dong et al. (6) used D1, a 298 bp 1D cDNA fragment, as a probe to study 1D mRNA expression in different human tissues by Northern blotting. They demonstrated an abundant 3.9 kb transcript in 5 mg poly $(A)^+$ thyroid RNA and a weaker signal of the same size in an equivalent amount of eye muscle mRNA. No 1D specific mRNA was detected in skeletal muscle. Ross et al. (7) studied tissue distribution of 1D mRNA by the more sensitive RT-PCR. They demonstrated a

weak signal for 1D mRNA expression in all of the tissues tested (eye muscle, eye connective tissue, thyroid, kidney, parathyroid, liver, lymphocytes, and uterus). Amplification of target DNA sequences by PCR is exquisitely sensitive and this method has the potential of amplifying a few copies of template into a detectable signal. Therefore under the conditions used by Ross et al. (7) and in earlier studies from our laboratory (8), it was only possible to obtain qualitative results as to the presence or absence of a particular nucleic acid species.

In this report, quantitative RT-PCR has been used to confirm and extend these observations. 1D mRNA expression was detected in all human tissues tested (thyroid, skeletal muscle, eye muscle, ovary, thymus, spleen, kidney, pancreas, cerebellum, and retina). Quantitation of 1D specific mRNA in these tissues was performed on 4 separate occasions; the inter assay variability was 5-20%. 1D expression was greatest in thyroid and muscle tissues. This transcript was more abundant in eye muscle than skeletal muscle. This may explain why Dong et al. (6) were able to detect 1D in thyroid and eye muscle but not in skeletal muscle by Northern blotting. Approximately 10-fold less 1D specific mRNA was seen in thymus, spleen, pancreas, kidney, and cerebellum. If levels of 1D mRNA reflect levels of 1D-specific protein expression, the tissues with the greatest expression of this protein are thyroid and eye muscle, the two tissues targeted by autoimmunity in TAO.

The abundance of 1D transcripts in thyroid and eye muscle compared with other tissues not targeted in TAO, together with the presence of autoantibodies specific for 1D in 47% patients with TAO, 50% of ATD patients with lid retraction, 17% of patients with HT, and 5% normal subjects, are consistent with the concept that 1D plays a role in the pathogenesis of TAO (26).

The expression of 1D mRNA in skeletal muscle is in agreement with results obtained by immunoprecipitation of solubilized human skeletal muscle membranes

with sera from TAO patients (27). Two-dimensional gel electrophoresis and Western blotting also revealed glycoprotein molecules of 64 kDa in thyroid, eye and skeletal muscle. These antigenically related molecules are all recognized by sera from TAO patients but had different isoeletric points (28). The different isoelectric points of 64 kDa antigen from these 3 human tissue sources could be due to distinctive tissue specific glycosylation patterns or small differences in amino acid sequence. The primer set used to amplify 1D specific cDNA from human tissues all yielded products of the same size and were therefore not sensitive to putative tissue specific differences in 1D sequence accounting for differing isoelectric points.

In summary, we developed an RT-PCR assay for the quantitation of 1D mRNA expression in human tissues. Because this competitive PCR enables quantitative determination of low abundance mRNAs, low level expression of 1D mRNA in tissue such as retina can be quantitated. 1D specific mRNA can be quantitated in all 10 human tissues tested and differs significantly from tissue to tissue. The level of 1D specific mRNA is highest in thyroid (5 pg/μg total RNA) and lowest in retina (0.016 pg/μg total RNA). The higher expression of 1D molecule in thyroid and eye muscle is consistent with a high level of protein expression in these tissues. The elevated levels of this autoantigen in thyroid and eye muscle may in turn account for why both these tissues are targeted in TAO.

REFERENCES

1. Wall JR, Salvi M, Bernard N, Boucher A and Haegert D (1991) Thyroid-associated ophthalmopathy-A model for the association of organ-specific autoimmune disorder. Immunology Today 12: 150-153.

2. Salvi M. Zhang Z-G. Haegert D. Woo M. Liberman A. Cadarso L and wall JR (1990) Patients with endocrine ophthalmopathy not associated with overt thyroid disease have multiple thyroid immunological abnormalities. J. Clin. Endocrinol. Metab. 70: 89-94.
3. Kodama K. Bandy-Dafoe P. Sikorska H. Bayly R and Wall JR (1982) Demonstration a circulating autoantibody against a soluble eye muscle antigen in Graves' ophthalmopathy. Lancet. 8312: 1353-1356.
4. Van der Gaag R. Vernimmen R. Fiebelkorn N. van Dierendonck MC and Kijlstra A (1990) Graves' ophthalmopathy: what is the evidence for extraocular muscle specific antibodies? Int Ophthalmol. 14: 25-30.
5. Miller A. Arthurs B. Boucher A. Liberman A. Bernard N. Salvi M and Wall JR (1992) Significance of antibodies reactive with a 64 kDa eye muscle membrane antigen in patients with thyroid autoimmunity. Thyroid 2: 197-202.
6. Dong Q. Ludgate M and Vassart G (1991) Cloning and sequencing of a novel 64 kDa autoantigen recognized by patients with autoimmune thyroid disease. J. Clin. Endocrinol. Metab. 72: 1375-1381.
7. Ross P. Koenig RJ. Arscott P. Ludgate M. Waiter M. Nelson CC. Kaplan MM and Baker JR (1993) Tissue specificity and serologic reactivity of an autoantigen associated with autoimmune thyroid disease. J. Clin. Endocrinol. Metab. 77: 433-438.
8. Zhang Z-G. Ludgate M. Bernard N and Wall JR (1992) Tissue distribution of a recombinant 64 kDa membrane antigen (1D) determined using the polymerase chain reaction. Clin Res. 40: 2 (Abstract).
9. Chomczynski P and Sacchi N (1987) single-step method of RNA isolation by acid guanidine isothiocyanate-pheno-chloroform extraction. Anal. Biochem. 162: 156-159.

10. Louveau I. Chaudhuri S and Etherton TD (1991) An improved method for isolating RNA from porcine adipose tissue. Anal. Biochem. 196: 308-310.
11. Lehrach H. Diamond D. Wozney JM and Boedtker H (1977) RNA molecular weight determinations by gel electrophoresis under denaturing conditions. a critical reexamination. Biochemistry. 16: 4743-4748.
12. Sambrook J. Fritsch EF and Maniatis T (1989) Molecular cloning: A Laboratory Manual. Cold Spring Harbor Laboratory. Cold Spring Harbor. NY.
13. Murphy LD. Herzog CE. Rudick JB. Fojo AT and Bates SE (1990) Use of the polymerase chain reaction in the quantitation of mdr-1 gene expression. Biochemistry. 29: 10351-10356.
14. Kinoshita T. Imamura J. Nagai H and Shimotohno K (1992) Quantification of gene expression over a wide range by the polymerase chain reaction. Anal. Biochem. 206: 231-235.
15. Siebert PD (1991) RT-PCR: Methods and application. Book 1. ClonTech Laboratories. Inc. Palo Alto. CA. pp. 51
16. Saiki RK. Scharf S. Faloona F. Mullis KB. Horn G. Erlich HA and Amheim N. (1985) Enzymatic amplification of beta-globulin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science. 230: 1350-1354.
17. Saiki RK. Gelfand DH. Stoffel S. Scharf SJ. Higuchi R. Jorn GT. Mullis KB and Erlich HA. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science. 239: 487-491.
18. Erlich HA. Gelfand D and Sninsky JJ (1991) Recent advances in the polymerase chain reaction. Science. 252: 1643-1651.
19. Chelly J. Kaplan JC. Gautron S and Kahn A (1988) Transcription of the dystrophin gene in human muscle and non-muscle tissues. Nature. 333: 858-860.

20. Sarkar G and Sommer SS (1989) Access to a messenger RNA sequence or its protein product is not limited by tissue or species specificity. Science. 244: 331-334.

21. Siebert PD and Fukuda M (1985) Induction of cytoskeletal vimin and actin gene expression by a tumor-promoting phorbol ester in the human leukemic cell line. J. Biol. Chem. 260: 3868-3874.

22. Schmidt EE and Merrill GF (1991) Changes in dihydrofolate reductase (DHFR) mRNA levels can account fully for changes in DHFR synthesis rates during terminal differentiation in a highly amplified myogenic cell line. J. Mol. Cell. Biol. 11: 3726-3734.

23. Wang AM, Doyle MV and Mark DF (1989) Quantification of mRNA by the polymerase chain reaction. Proc. Natl. Acad. Sci. 86: 9717-9721

24. Gilliland G, Perrin S, Blanchard K and Bunn F (1990) Analysis of cytokine mRNA and DNA: Detection and quantitation by competitive polymerase chain reaction. Proc. Natl. Acad. Sci. 87: 2725-2729.

25. Siebert PD and Larrick JW (1992) Competitive PCR. Nature. 359: 557-558.

26. Zhang Z-G, Dong Q, Rodien P, Alcade L, Bernard N, Boucher A, Vassart G, Ludgate M and Wall JR (1992a) Autoantibodies in the serum of patients with ATD react with a recombinant 98 amino acid fragment of a 64 kDa eye muscle recombinant protein which is also expressed in the thyroid. Autoimmunity. 13: 151-157.

27. Zhang Z-G, Salvi M, Miller A, Bernard N, Arthurs B and Wall JR (1992b) Restricted tissue reactivity of autoantibodies to a 64 kDa eye muscle membrane antigen in thyroid-associated ophthalmopathy. Clin. Immunol. Immunopathol. 62: 182-189.

28. Boucher A, Bernard N, Miller A, Rodien P, Salvi M and Wall JR (1994) Two dimensional gel electrophoresis identifies minor differences in

immunologically cross-reactive 64 kDa autoantigens in the thyroid and eye muscle. J. Endocrinol. Invest. 17: 7-13.

CHAPTER VI

GENERAL DISCUSSION

Although the pathogenesis of TAO is unclear, recent studies have contributed greatly to our understanding. At present cross-reactivity against thyroid and eye muscle shared Ag(s) is the best explanation for the association of TAO and ATD.

This thesis has studied the 64 kDa thyroid/eye muscle Ag(s) recognized by Abs in the sera of patients with TAO. Abs specific for cross-reactive 64 kDa eye muscle and thyroid molecules are associated with ophthalmopathy in patients with thyroid autoimmunity. Two 64 kDa Ags have been identified by separate approaches. One 64 kDa Ag was identified by Western blotting of thyroid/eye muscle membrane Ags with autoAbs in the sera of patients with TAO (Salvi et al. 1988). Another 64 kDa Ag, 1D, was cloned by Dong et al. (1991) from a thyroid cDNA expression library by screening with a pool of sera from patients with ATD. **The overall objective of this thesis was to characterize the 64 kDa Ag(s), and to define the prevalence of autoAbs directed at these molecules in patients with TAO.**

This objective was achieved by: 1) Identifying eye muscle/thyroid membrane 64 kDa Ag(s) by Western blotting and immunoprecipitation with sera from TAO patients; 2) Assessing the ability of Ags expressed in different tissues to adsorb Abs reactive with a 64 kDa thyroid/eye muscle membrane Ag from TAO patients' sera; 3) Analyzing the reactivity of sera from TAO and ATD patients to a recombinant 98 amino acid fragment (D1) of 1D, a 64 kDa thyroid/eye muscle Ag, by immuno-dot blot; 4) Comparing the reactivity of sera from TAO and ATD patients to D1 fusion protein and solubilized 64 kDa eye muscle membrane Ag by Western blotting; 5) Assessing and quantitating the tissue distribution of 1D by quantitative RT-PCR; 6) Identifying antigenic peptides of 1D; 7) Cataloguing reactivity of sera from patients with TAO and ATD to each of these 1D antigenic peptides.

The experimental observations upon which the work for this thesis is based is the identification, by SDS-PAGE and Western blotting, of a 64 kDa Ag present in human eye muscle and thyroid membranes which is recognized by Abs in the sera of approximately 50% of patients with TAO (Salvi et al., 1988; Salvi et al., 1991). In Chapter II, we absorbed sera of TAO patients displaying this reactivity with a panel of tissues prior to using the sera in Western blotting to investigate which tissues expressed cross-reactive Ag(s). In Western blotting experiments, TAO patients' serum Abs reactive with a 64 kDa pig eye muscle membrane (PEMM) Ag were absorbed by human eye muscle, thyroid, and skeletal muscle but not by spleen or brain tissue. These observations demonstrated that human eye muscle and thyroid expressed Ag(s) that shared immunological cross-reactivity with each other and with a 64 kDa PEMM Ag recognized by sera from patients with TAO.

AutoAbs in sera from TAO patients were incubated with biotin labeled primary cultures of human eye muscle, thyroid and skeletal muscle cells prior to solubilization and immunoprecipitation. A molecule of 64 kDa was immunoprecipitated from eye muscle and thyroid, but not skeletal muscle. These experiments demonstrated that the 64 kDa Ag was present in the membrane of living eye muscle and thyroid cells and was recognized by sera from TAO patients in its integral cell membrane and native conformation.

In 1991 a cDNA encoding a 64 kDa Ag was cloned by Dong et al. (1991) by screening a thyroid expression library with a pool of sera from patients with ATD. The full-length 572 amino acid protein was named 1D and a 98 amino acid fragment of this molecule was named D1. By Northern blotting the mRNA for 1D was expressed in both thyroid and eye muscle, but not in skeletal muscle. D1 affinity-purified Abs from the sera of TAO patients bound to a 64 kDa protein in human thyroid tissue by Western blotting. Although the identity of molecule

encoded by this cDNA and the 64 kDa molecule recognized by sera of TAO patients by Western blotting was not confirmed, the similarity between the two molecules was compelling enough to obtain the 1D/D1 cDNA clones and to initiate a research program based on its characterization. A panel of sera from patients with TAO and ATD, followed in the Thyroid Studies Center of the Montreal General Hospital, was available to screen for reactivity against D1 fusion protein in a dot blot immunoassay. Immunoactivity against D1 was found in the sera of 47% of patients with TAO and in 57% of patients with GH without evident eye disease. In previous work, reactivity to the thyroid/eye muscle 64 kDa Ag described by Wall et al. (1991a) was found in 50% of patients with TAO and in 33% patients with GH (Salvi et al. 1991).

In order to explore the relationship between the 64 kDa thyroid/eye muscle membrane molecule described by Wall et al. (1991a) and the 64 kDa thyroid/eye muscle Ag encoded by the cDNA cloned by Dong et al. (1991) a panel of sera from TAO patients was screened both by Western blotting of PEMM and by dot blot of D1 fusion protein. There was a good overall correlation between the results obtained by dot blot assay and those obtained by Western blotting for activity with a 64 kDa protein in PEMM. Sera from 17 patients with TAO or GH reacted with 64 kDa Ag(s) by both assays. However, sera from 12 TAO or GH patients with negative dot blot assays reacted positively by Western blotting while 7 patients' sera negative by Western blotting reacted with the D1 fragments by dot blot. Concordance in serum reactivity to these 2 molecules could be due to 1) shared immunological cross-reactivity (or identity) between the 2 Ags or 2) the presence of 2 populations of Abs in many sera from TAO/ATD patients, one of which reacts with the 1D molecule the other reacts with the 64 kDa molecule described by Wall et al. (1991a). The ability of at least some Abs in the sera of TAO/ATD patients to bind a 64 kDa thyroid membrane molecule by Western blotting

following affinity purification on D1 fusion protein suggests some immunological cross-reactivity between the 2 Ags. Discordance in serum reactivity could be due to several reasons: 1) The 98 amino acid D1 peptide comprises less than one fifth of the total amino acid sequence of the full-length 1D molecule and therefore may express only a subset of the potentially immunogenic epitopes of the parent molecule. This may explain why some sera reactive with the full-length 64 kDa molecule did not react with D1. 2) Since PEMM rather than human eye muscle membrane (HEMM) was used as the source of Ag in Western blotting, sera reacting with D1, but not PEMM, may be recognizing a human species specific epitope not expressed in PEMM.

To further dissect the antigenicity of the 1D molecule, short random fragments (100 to 200 bp) of 1D cDNA were cloned and expressed in E. coli as fusion proteins using the pUEX1 vector. Four antigenic peptides were characterized and localized on the 1D molecule. Immunoreactive peptides G-2 (residues 76-112), G-4 (residues 127-179) and G-5 (residues 156-205) were positioned to hydrophilic domains. Immonoreactive peptide G-8 (residues 412-450) partially overlaps with the D1 fragment (residues 426-524) and is located near the C terminal end of the full-length 1D molecule.

The immunoreactivities of a panel of sera from patients with TAO and ATD were tested against these four peptides and the full-length 1D protein by Western blotting. Several distinct reactivity patterns were observed in sera from patients with TAO and ATD. The majority of sera recognized more than one immunoreactive peptide and also reacted with full-length 1D protein. This demonstrated that patients with TAO have a polyclonal reactivity to the 1D protein. Two sera recognized only full-length 1D fusion protein suggesting either that some sera contained auto-Abs which recognized more conformational or

discontinuous antigenic determinants. or reacted with parts of 1D that were not selected from the fragment library.

Six epitopes were localized on the 1D molecule based on peptide amino acid sequences and the reaction pattern of the serum panel against each peptide (#1. residues 76-112; #2. residues 127-156; #3. residues 156-179; #4. residues 179-205; #5. residues 426-450; #6. residues 450-524). Sera from patients with TAO reacted significantly more frequently with G-4 and G-5 peptides (47%) than did those from normal subjects (8%).

An important observation from this study was the high prevalence of positivity to the D1 molecule in sera from GH patients. It has been shown that approximately 80-90% of patients with GH have ophthalmopathy. as determined by CT scanning of the extraocular muscles. at the time of diagnosis of the hyperthyroidism. even though ophthalmopathy may not be clinically evident. CT scanning was not carried out in the group of GH patients whose sera were used in this study. Therefore, although none exhibited proptosis or abnormal eye muscle function they may. according to the findings of Werner et al. (1974) and Enzmann et al. (1979). have subclinical ophthalmopathy. 57% of patients with GH have Abs that recognize D1 and 47% these patients have Abs to G4/G5. If most of these GH patients actually have subclinical/early stage ophthalmopathy Abs to D1 and G4/G5 may be early markers for the development of ophthalmopathy. Antigenic peptides such as G4 or G5 could be used as Ag in simple screening tests such as ELISA to detect Abs to clinically relevant epitopes of the 64 kDa molecule and to monitor patients with GH to determine the relationship between the presence of such Abs and the development of ophthalmopathy.

The tissue expression of 1D was first studied by Northern blotting using D1 as a probe. Dong et al. (1991) demonstrated that 1D was expressed in human eye muscle and thyroid but not skeletal muscle. More recent experiments carried out

by Ross et al. (1993) and by us (1992) using the RT-PCR to investigate the tissue distribution of 1D mRNA demonstrated 1D amplification products when eye muscle, thyroid, skeletal muscle and cardiac muscle RNA were used as PCR template. Because of the extreme sensitivity of the PCR reaction and the methods used, the possibility of amplification of non-specific template could not be excluded.

In order to address this criticism and quantitate the level of 1D mRNA expression in various human tissues, a quantitative RT-PCR assay was developed. A competitive internal control for 1D mRNA amplification was designed and used in these assays. Of the ten human tissues tested, 1D expression was highest in thyroid and muscle tissues. This transcript was more abundant in eye muscle than skeletal muscle. This may explain why Dong et al. was able to detect 1D in thyroid and eye muscle but not in skeletal muscle. Approximately 10-fold less 1D specific mRNA was seen in thymus, spleen, pancreas, kidney and cerebellum. If levels of 1D mRNA reflect levels of 1D-specific protein expression, tissues with the highest expression of this protein are thyroid and eye muscle, the two tissues targeted by autoimmunity in TAO. From mRNA expression experiments 1D expression dose not appear limited to eye muscle and thyroid. Since post transcription events may influence the levels of protein expression from a given amount of mRNA, the actual amount of 1D protein expression on various tissues awaits availability of 1D specific Abs.

The mRNA was extracted from eye muscle tissue (which contains eye muscle cells, fibroblasts and other cells). A single retro-ocular target tissue may not explain all of the eye manifestations of TAO. Both eye muscle and fibroblasts may be targets of autoimmune attack. It is possible that Abs to orbital Ags may be secondary phenomena, while Ag specific T cell may mediate the primary damage. Lessons learned from studying insulin dependent diabetes mellitus may be

applicable to autoimmunity in TAO. In insulin dependent diabetes mellitus. T cells are involved in b-cell killing and. Abs against islet cells were positive in 50-70% new patients and were not play a role in the pathogenesis (Baekkeskov et al. 1990).

It is not known as yet if 1D protein and the 64 kDa eye muscle membrane protein identified by Western blotting are the same molecule. The following points favor the possibility that the two molecules are identical. 1) The molecular weight of both molecules is indistinguishable. 2) Both are expressed in thyroid and eye muscle. 3) Both molecules were recognized by sera from about half of patients with TAO and GH. by a smaller proportion of patients from HT. but rarely by sera from normal subjects. 4) There is an overall tendency for concordance between reactivity to the 64 kDa Ag by Western blotting and that to D1 fusion protein by immuno-dot blotting by individual TAO sera. 5) AutoAbs from patients with TAO affinity-purified by D1 bind to a thyroid membrane molecule of 64 kDa by Western blotting. Several points have emerged supporting the non identity of these molecules: 1) Discordance in serum reactivity patterns to these molecules (species source of Ag and fragment length of Ag must be taken into consideration in interpreting these results) 2) Two-D gel electrophoresis and Western blotting of human eye muscle and thyroid membrane reveal that the pI of the 64 kDa protein was about 6.0-7.0 (Boucher et al. 1994; Wall et al. 1994) while the pI of 1D molecule deduced from Genetic GCG program is 10.3. A definitive conclusion as to the relatedness of these molecules must await microsequencing of the 64 kDa molecule detected by Western blotting in order to directly compare its amino acid sequence with that deduced for 1D.

CHAPTER VII

BIBLIOGRAPHY

Abramowicz MJ. Targovnik HM. Varenaa V. Cochaux P. Krawiec L. Pisarev MA. Propato FVE. Juvenal G. Chester H and Vassart G (1992) Identification of a mutation in the coding sequence of the human thyroid peroxidase gene causing congenital goiter. J. Clin. Invest. 90: 1200-1204.

Adams DD and Purves HD (1956) Abnormal responses in the assay of Thyrotropin. Univ. Otago. Med. School Proc. 34: 11-12.

Adams DD and Purves HD (1957) The role of Thyrotropin in hyperthyroidism and exophthalmos. Metabolism 6: 26-35.

Ahmann A. Baker JR. Weetman AP. Wartofsky L. Kretman TB and Burman KD (1987) Antibodies to porcine eye muscle in patients with Graves' ophthalmopathy: identification of serum immunoglobulins directed against unique determinants by immunoblotting and enzyme-linked immunosorbent assay. J. Clin. Endocrinol. Metab. 64: 454-460.

Aichinger G. Fill H and wick G (1985) In situ immune complexes. lymphocyte subpopulation. and HLA-DR-positive epithelial cells in Hasnimoto' thyroiditis. Lab. Invest. 52: 132-140.

Aizawa T. Koizumi Y. Yamada T. Tawata M. Nagata H. Izumiyamo T and Yoshizaw K (1978) Difference in pituitary-thyroid feedback regulation in hypothyroid patients. depending on the severity of hypothyrpodism. J. Clin. Endocrinol. Metab. 47: 560-565.

Amino N, Hagan SR, Yamada N and Refetoff HS (1976) Measurement of circulating thyroid microsomal antibodies by the tanned red cell haemagglutination technique: its usefulness in the diagnosis of autoimmune thyroid disease. Clin. Endocrinol. (Oxf) 5: 115-125.

Amino N, Yuasa T, Yabu Y, Miyai K and Kumahara Y (1980) Exophthalmos in autoimmune thyroid disease. J. Clin. Endocrinol. Metab. 51: 1232-1234.

Arid RB. (1940). Experimental exophthalmos and the associated myopathy induced by the thyrotropic hormone. Arch Ophthal. 24: 1167-1178.

Atabay C, Schrooyen M, Zhang Z-G, Salvi M, Glinnoer D and Wall JR. (1993) Use of eye muscle antibody measurements to monitor response to plasmapheresis in patients with thyroid-associated ophthalmopathy. J. Endocrinol. Invest. 16: 669-674.

Atta HR, McCreath G, McKillop JH, Forrester JV, Thomson JA and Gray WHW (1990) Ophthalmopathy in early thyrotoxicosis-relationship to thyroid receptor antibodies and effects of treatment. Scot. Med. J. 35: 41-44.

Baekkeskov S, Aanstoot H-J, Christgau S, Reetz A, Solimena M, Cascalho M, Folli F, Richter-Olesen H and Camilli P-D (1990) Identification of the 64 k autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 347: 151-156.

Beck K. (1989) Thyroid antibodies in endocrine ophthalmopathy. Acta Endocrinol. (Copenh) (Suppl 2): 117-122.

Beierwates WH. (1953) X-ray treatment of malignant exophthalmos: a report on 28 patients. J. Clin. Endocrinol. Metab. 13: 1090-1100.

Belyavin G and Trotter WR (1959) Investigations of thyroid antigens reacting with Hashimoto' sera. Evidence for an antigen other than thyroglobulin. Lancet 1: 648-652.

Benjamin DC, Berzofsky JA, East IJ et al. (1984) The antigenic structure of proteins: a reappraisal. Ann. Rev. Immunol. 2: 67-101.

Bernard N, Nygen TN, Tyutyunikov A, Stolarski C, Scalise D, Genovese C, Hayes MB, Ludgate M and Wall JR (1994) Antibodies against 1D, a recombinant 64-kDa membrane protein, are associated with ophthalmopathy in patients with thyroid autoimmunity. Clin. Immunol. Immunopathol. 70: 225-233.

Blackman M, Kappler JW and Marrack P (1988) T cell specificity and repertoire. Immunol. Rev. 101: 5-19.

Bogner U, Wall JR and Schleusener H (1987) Cellular and antibody mediated cytotoxicity in autoimmune disease. Acta. Endocrinol. (Copenh) Suppl 281: 133-138.

Bolonkin D, Tate R, Luber JH and Kohn LD. (1975) Experimental exophthalmos-binding of Thyrotropin and an exophthalmogenic factor derived from Thyrotropin to retro-orbital tissue plasma membranes. J. Biol. Chem. 250: 6516-6521.

Bona CA (1991) Postu'ates defining pathogenic autoantibodies and T cells. Autoimmunity 10: 169-172.

Bonnyns M, Demeester-Mirkine N, Calay R and Bastenie PA (1968) Evaluation of the relationship between long-acting thyroid stimulator, clinical and biological thyrotoxicosis, and exophthalmos. Acta Endocrinol. (Copenh) 58: 581-592.

Bottazzo GF, Pujol-Borrell R and Hanafusa T (1983) Role of aberrant HLA-DR expression and antigen presentation in induction of endocrine autoimmunity. Lancet 2: 1115-1118.

Boucher A, Bernard N, Miller A, Salvi M and Wall JR (1991) Characterization of a thyroid and eye muscle shared antigen by two-dimensional gel electrophoresis and trypsin digestion. The Endocrine Society Abstracts Book: 22.

Boucher A, Bernard N, Miller A, Rodien P and Wall JR (1992) Physicochemical characterization of 64 kDa antigens in TAO. Proceedings 66th Annual Meeting of the American Thyroid Association, Rochester, MN, September. Thyroid 2 (Suppl. 1): 54

Boucher A, Bernard N, Zhang Z-G, Salvi M, Rodien P, Triller H and Wall JR (1992) Nature and significance of orbital autoantigens and their corresponding Autoantibodies in thyroid-associated ophthalmopathy. Autoimmunity 13: 89-93.

Boucher A, Bernard N, Miller A, Rodien P, Salvi M and Wall JR (1994) Two dimensional gel electrophoresis identifies minor differences in immunologically

cross-reactive 64 kDa autoantigens in the thyroid and eye muscle. J. Endocrinol. Invest. 17: 7-13.

Braverman LE and Utigor RD (1991) Introduction to thyrotoxicosis. In: Braverman LE and Utiger RD (eds) Werner and Ingbar's The Thyroid. JB Lippincott. Phildelphia. pp. 645-647.

Bresler HS. Burek CL. Hoffman WH and Rose NR (1990) Autogenic determinants on human thyroglobulin. II. Determinants recognized by autoantibodies from patients with chronic autoimmune thyroiditis compared to autoantibodies from health subjects. Clin. Immunol. Immunopathol. 54: 76-86.

Briggs MM. Jakoby J. Davidowitz J and Schachat FH (1988) Expression of a novel combination of fast and slow troponin C isoforms in rabbit extraocular muscles. J. Muscle. Res. Cell. Motil. 9: 241-247.

Brohee D. Delespesse G. Debisschop MJ and Bonnyns M (1979) Circulating immune complexes in various thyroid disease. Clin. Exp. Immunol. 36: 379-383.

Burch HB and Wartofsky L (1993) Graves' ophthalmopathy: current concepts regarding pathogenesis and management. Endocr. Rev. 14: 747-793.

Campbell RJ (1984) Pathology of Graves' ophthalmopathy. In: Gorman CA. Cambell RJ and Dyer JA (eds). The Eye and Orbital in Thyroid Disease. New York: Raven Press. pp. 25-31.

Caldwell G, Keller HA, Gow SM, Beckett GT, Sweeting VM, Seth J and Toft AD (1985) A new strategy for thyroid function testing. Lancet 1: 1117-1118.

Ceriani RL, Larocca D, Peterson JA, Enloe S, Amiya R, Walkup G and Blank EW (1992) A novel serum assay for breast epithelial antigen sing a fusion protein. Anal. Biochem. 201: 178-184.

Chang TC, Huang KM, Chang TJ and Lin SL (1990) Correlation of orbital computed tomography and antibodies in patients with hyperthyroid Graves' disease. Clin. Endocrinol. (Oxf) 32: 551-558.

Char DH (1990) Eye signs and diagnosis of thyroid ophthalmopathy. In: Char DH (ed). Thyroid Eye Disease. Churchill Livingstone Inc, New York, pp. 35-86.

Cohen M, Salvi M, Miller A, Bernard B and Wall JR (1992) Cell-mediated immunity to orbital tissue antigens in TAO determined using the leukocyte procogulant activity assay. Autoimmunity 11: 225-231.

Cooper W (1849) On protrusions of eyes in connection with anemia, palpitation and goiter. Lancet 1: 55-58.

Czarnocka B, Ruf J, Ferrant M, Carayon P and Lissitzky S (1985) Purification of the human thyroid peroxidase and its identification as the microsomal antigen involved in autoimmune thyroid disease. FEBS Lett. 190: 147-152.

Dandona P, Marshall N, Bidey S, Nathan AW and Havard CWH (1979) Successful treatment of exophthalmos and pretibial myxedema with plasmapheresis. Br. Med. J. 2: 667-668.

Davies TF, Teng CS, McLachlan SM, Smith RB and Hall R (1978) Thyrotropin receptors in adipose tissue, retroorbital tissue and lymphocytes. Mol. Cell. Endocrinol. 9: 303-310.

De Grandi PB, Kraeherbuhl JP and Campiche MA (1971) Ultrastructural localization of calcitonin in the parafolicular cells of pig thyroid gland with cytochrome c-labeled antibody fragments. J. Cell. Biol. 50: 446-456.

Dobyns BM. (1950) Present concepts of the pathologic physiology of exophthalmos. J. Clin. Endocrinol. Metab. 10: 1202-1230.

Dong Q, Ludgate M and Vassart G (1991) Cloning and sequencing of a novel 64 kDa autoantigen recognized by patients with autoimmune thyroid disease. J. Clin. Endocrinol. Metab. 72: 1375-1381.

Doniach I (1960) Types of thyroid growth. Br. Med. Bull. 16: 99-104.

Drescher EP and Benedict WL (1950) Asymmetric exophthalmos. Arch. Ophthalmol. 44: 109-128.

Dumont JE (1971) The action of thyrotropin on thyroid metabolism. Vitam. Horm. 29: 287-343.

Dumont JE, Lamy F, Roger P and Maenhaut C (1992) Physiological and pathological regulation of thyroid cell proliferation and differentiation by thyrotropin and other factors. Physiological Reviews 72: 667-697.

Elte DW and Hensen EJ (1983) Pretibial myxedema: report of 17 cases and review of the literature. Neth. J. Med. 26: 220-223.

Enzmann DR, Donaldson S and Kriss JP (1979) Appearance of Graves' disease on orbital computed tomograph. J. Comput. Assist. Tomogr. 3: 815-819.

Ericson LE (1983) Ultrastructural aspects of iodination and hormone secretion in the thyroid gland. J. Endocrinol. Invest. 6: 311-318.

Faryna M, Nauman J and Gardas A (1985) Measurement of autoantibodies against human eye muscle membranes in Graves' ophthalmopathy. Br. Med. J. 290: 191-193.

Feind CR (1978) Surgical anatomy of the thyroid. In: Thyroid. A fundamental and clinical Text. forth edition. Werner SC and Ingbar SH (eds). Harpes and Row, New York. pp. 426-436.

Fink DJ (1988) Stop and go in the thymus. Immunol Today 9: 377-340.

Furszyter J, McConahey W, Kurland LT and Maldonado JE (1971) On the increased association of Graves' disease with pernicious anemia. Mayo. Clin. Proc. 46: 37- 42.

Gamblin G, Harper DG, Galentine P, Buck DR, Chernow B and Eil C (1983) Prevalence of increased intraocular pressure in Graves' disease: evidence of frequent subclinical ophthalmopathy. N. Engl. J. Med. 308: 420-424.

Gans C and Northcutt RG (1983) Neural crest and the origin of vertebrates: a new head. Science 220: 268-274.

Geysen HM, Tainer JA, Stuart JR, Mason TJ, Alexander H, Getzoff ED and Lerner RA (1987) Chemistry of antibody binding to a protein. Science 235: 1184-1190.

Glinoer D, Etienne-Deserve J, Schrooyen M, Sand G, Hoyoux P, Mahieu P and Winard R (1986) Beneficial effects of intensive plasma exchange followed by immunosuppressive therapy in severe Graves' ophthalmopathy. Acta Endocrinol. (Copenh) 111: 30-38.

Gorman CA (1983) Temporal relationship between onset of Graves' ophthalmopathy and diagnosis of thyrotoxicosis. Mayo. Clin. Proc. 58: 1515-1519.

Gorman CA and Bahn RS (1989) Pathogenesis of Graves' ophthalmopathy. Dev. Ophthalmol. 20: 1-7.

Graves RT (1835) Clinical Lectures. London. Med. Sug. J. 7: 516-517.

Greenberg AH, Czernichow P, Hung W, Shelley W, Winship T and Blizzard RM (1970) Juvenile chronic lymphocytic thyroiditis: clinical, laboratory and histological correlations. J. Clin. Endocrinol. Metab. 30: 293-301.

Guy JR, Fagien S, Donovan JP and Rubin ML (1989) Methylprednisolone pulse therapy in severe dysthyroid optic neuropathy. Ophthalmology 96: 1048-1053.

Hanafura T, Borrell RP, Chiovato L, Russell RCG, Doniach D and Bottazzo GF (1983) Aberrant expression of HLA-DR antigen on thyrocytes in Graves' disease: relevance for autoimmunity. Lancet 2: 1111-1115.

Haspel MV, Onodera T, Prabhakar BS, McClintock PR, Essani K, Ray UR, Yagihash S and Notkins AL (1983) Multiple organ-reactive monoclonal antibodies. Nature 304: 73-76.

Havard CWH (1979) Progress in endocrine exophthalmos. Br. Med. J. 1: 1001-1004.

Henry M, Malthiery Y, Zanelli E and Charvet B (1990) Epitope mapping of human thyroglobulin. J. Immunol. 145: 3692-3698.

Hiromatsu Y, Wosu L, Dornan J and Wall JR (1986) Role of class II antigen expression on normal human eye muscle cells and orbital fibroblasts in Graves' ophthalmopathy. In: The thyroid and autoimmunity. Drexhage HA and Wiersinga WM, eds. Elsevier Science Publishers, pp.272-274.

Hiromatsu Y, Fukazawa H and Wall JR (1987) Cytotoxic mechanisms in autoimmune thyroid disorders and thyroid-associated ophthalmopathy. Endocrinol. Metab. Clin. North. Am. 16: 269-284.

Hiromatsu Y. Fukazawa H. Guinard F. Salvi M. How J and Wall JR (1988) A thyroid cytotoxic antibody that cross-react with an eye muscle cell surface Ag may be the cause of thyroid associated ophthalmopathy. J. Clin. Endocrinol. Metab. 67: 565-570.

Hiromatsu Y. Sato M. Tanaka K. Nonaka K. Chinami M and Fukazawa H(1993) Significance of anti-eye muscle antibody in patients with thyroid-associated ophthalmopathy. Autoimmunity 14: 1-8.

Hiromatsu Y. Tanaka K. Ishisaka N. Kamachi J. Kuroki T Hoshino T. Inoue Y. Wall JR and Nonaka K (1994) Human histocompatibility leukocyte antigen-Dr and heat shork protein-70 expression in eye muscle tissue in thyroid-associated ophthalmopathy. J. Clin. Endocrinol. Metab. (In press).

Hinds WE. Takai. Rapoport B. Filetti S and Clark OH (1981) Thyroid-stimulating immunoglobulin bioassay using cultured human thyroid cells. J. Clin. Endocrinol. Metab. 52: 1204-1210.

Horsfall AC. Hay FC. Soltys AJ and Jones MG (1991) Epitope mapping. Immunology Today. 12: 211-213.

How J. Hiromatsu Y. Wang P. Salvi M and Wall JR (1987) Cellular mechanisms for autoimmune damage in thyroid-associated ophthalmopathy. In: Pinchera A. Ingbar SH and McKenzie JM. eds. Thyroid Autoimmunity. New York: Plenum, pp. 125-133.

How J, Bernard N and Wall JR (1990) Cell-mediated immunity in Graves' ophthalmopathy. In: Wall JR, How J (eds) Graves' Ophthalmopathy. Blackwell Scientific Publications, Boston, pp. 37-46.

Hunt DF, Henderson RA, Shabanowitz J, Sakaguchi K, Michel H, Sevilir N, Cox AL, Appella E and Engelhard VH (1992) Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. Science 255: 1261-1263.

Ivy HK (1972) Medical approach to ophthalmopathy of Graves' disease. Mayo. Clin Proc. 47: 980-985.

Iwatani Y, Gerstein HC, Iitaka M, Row VV and Volpe R (1986) Thyrocyte HLA-DR expression and interferon gamma production in autoimmune thyroid disease. J. Clin. Endocrinol. Metab. 63: 695-708.

Jacobsen DH and Gorman CA (1984) Endocrine ophthalmopathy: current ideas concerning etiology, pathogenesis and treatment. Endocrinol. Rev. 5: 200-220.

Jackson IMD (1982) Thyrotropin-releasing hormone. N. Engl. J. Med. 306: 145-155.

Jardetzky TS, Lane WS, Robinson PA, Madden DR and Wiley DC (1991) Identification of self peptides bound to purified HLA-B27. Nature 353: 326-329.

Jardetzky TS, Brown JH, Gorge JC, Siren LJ, Urban RG, Chi Y, Stauffacher C, Strominger HL and Wiley DC (1994) Three-dimensional structure of a human

class II histocompatibility molecule complexed with superantigen. Nature 368: 711-718.

Jemmerson R (1987) Antigenicity and native structure of globular proteins: low frequency of peptide reactive antibodies. Proc. Natl. Acad. Sci. 84: 9180-9184.

Jones DIR, Munro DS and Wilson GM (1969) Observation on the course of exophthalmos after I-131 therapy. Proc. R. Soc. Med. 62: 15-18.

Kaminski H, Maas E, Spiegel P and Ruff RL (1990) Why are eye muscles frequently involved in myasthenia gravis? Neurology 40: 1663-1669.

Kazim AL and Atassi MZ (1980) A novel and comprehensive synthetic approach for the elucidation of the full antigenic structures, determination of the full antigenic profile of the α-chain of human hemoglobin. Biochem. J. 191: 261-264.

Kendall-Taylor P, Atkinson S and Holcombe M (1984) A specific IgG in Graces' ophthalmopathy and its relation to retro-orbital and thyroid autoimmunity. Br. Med. J. 288: 1183-1186.

Kendall-Taylor P, Crombie AL Stephenson Am, Hardwick M and Hall K (1988) Intravenous Methylprednisolone in the treatment of Graves' ophthalmopathy. Br. Med. J. 297: 1574-1578.

Kendall-Taylor P, Crombie AL and Perros P (1992) High-dose intravenous Methylprednisolone pulse therapy in severe thyroid-associated ophthalmopathy. Thyroid 2 (Supple. 1): 29.

Khoury EL (1987) Aberrant expression of class II HLA antigens by the target cells: Cause or consequence of the autoimmune aggression? Acta Endocrinol. (Copenh) 11: (Suppl 281): 35-41.

Kimura S. Kotani T. McBride OW. Umeki K. Hirai K. Nakayama T and Ohtaki S (1987) Human thyroid peroxidase: complete cDNA and protein-sequence. chromosome mapping and identification of two alternately-spliced mRNAs. Proc. Natl. Acad. Sci. 84: 5555-5559.

Koch N. Lauer W. Habicht J and Dobberstein B (1987) Primary structure of the gene for the murine Ia antigen associated invariant chains (Ii). An alternatively spiced exon encodes a cystein rich domain highly homologous to a repetitive sequence of thyroglobulin. EMBO J. 6: 1677-1683.

Kodama K. Bandy-Dafoe P. Sikorska H. Bayly R and Wall JR (1982) Demonstration a circulating autoantibody against a soluble eye muscle antigen in Graves' ophthalmopathy. Lancet 8312: 1353-1356.

Kodama K. Sikorska H. Bay R. Bandy-Dafoe P and Wall JR (1984) Use of monoclonal antibodies to investigate a possible role thyroglobulin in the pathogenesis of Graves' ophthalmopathy. J. Clin. Endocrinol. Metab.59: 67-73.

Kohn LD and Winard RJ (1971) Relationship of thyrotropin to exophthalmos-producing substance. Formation of an exophthalmos-producing substance by pepsin-digestion of pituitary glycoproteins containing both thyrotropic and exophthalmogenic activity. J. Biol. Chem. 246: 6570-6575.

Kohno Y, Hiyama Y, Shimojo N, Nakajima and Hosaya T (1986) Autoantibodies to thyroid peroxidase in patients with chronic thyroiditis: effect of antibody binding on enzyme activities. Clin. Exp. Immunol. 65: 534-541.

Knopp J, Stolc V and Tong W (1970) Evidence for the induction of iodide transport in bovine thyroid cells treated with thyroid stimulating hormone or dibutyryl cyclic adenosine 3' 5'monophosphate. J. Biol. Chem. 245: 4403-4408.

Komorowski RA and Hanson GA (1988) Occult thyroid pathology in the young adult: an autopsy study of 138 patients without clinical thyroid disease. Hum. Pathol. 19: 689-696.

Kondo Y, Inoue K, Kondo E and Ui N (1985) Electron microscopy of monoclonal antibody-linked chains of thyroglobulin molecules. Mol. Cell. Endoc. 41: 223-227.

Konishi J, Herman MM and Kriss JP (1974) Binding of thyroglobulin and antithyroglobulin immune complex to extraocular muscle membrane. Endocrinology 95: 434-446.

Kriss JR (1970) Radioisotope thyroid lymphography in patients with Graves' disease. J. Clin. Endocrinol. Metab. 31: 315-323.

Kriss JR, Konishi J and Herman M (1975) Studies on the pathogenesis of Graves' ophthalmopathy (with some related observations regarding therapy). Recent. Prog. Horm. Res. 31: 533-566.

Ladenson PW (1991) Diagnosis of hypothyroidism. In: Braverman LE and Utiger RD (eds) Werner and Ingbar's The Thyroid. JB Lippincott, Phildelphia, pp. 1092-1098.

Lamy F, Roger PP, Lecocq RLE and Dumont JE (1986) Differential protein synthesis in the induction of thyroid cell proliferation by thyrotropin, epidermal growth factor or serum. Eur. J. Biochem. 155: 265-272.

Larsen PR (1982) Thyroid-pituitary interaction: feedback regulation of thyrotropin secretion by thyroid hormone. N. Engl. J. Med. 306: 23-32.

Laver WG, Air GM, Webster RG and Smith-Gill SJ (1990) Epitopes on protein antigens: misconceptions and realities. Cell 61: 553-556.

Leiri T, Cochaux P, Targovnik HM, Suzuki M, Shimoda SI, Perret J and Vassart G (1991) A 3' splice site mutation in the thyroglobulin gene responsible for congenital goiter with hypothyroidism. J. Clin. Invest. 88: 1901-1905.

Libert F, Lefort A, Gerard C, Parmentier M, Merret J, Ludgate M Dumont JE and Vassart G (1989) Sequencing and expression of the human thyrotropin (TSH) receptor: evidence for binding of autoantibodies. Biochem. Biophys. Res. Comm. 165: 1250-1255.

Libert F, Ludgate M, Dinsart C and Vassart G (1991) Thyroperoxidase, but not the thyrotropin receptor contains sequential epitopes recognized by autoantibodies in recombinant peptides expressed in the pUEX vector. J. Clin. Endocrinol. Metab. 73: 857-860.

Lloyd WC and Leone CR (1992) Supervoltage orbital radiotherapy in 36 cases of Graves' ophthalmopathy. Am. J. Ophthalmol. 113: 374-380.

Ludgate M. Swillens S. Mercken L and Vassart G (1986) Homology between thyroglobulin and acetylcholinesterase: an explanation for pathogenesis of Graves' ophthalmopathy ? Lancet 2: 219-220.

Ludgate M. Dong Q. Soreq H. Mariotti S and Vassart G (1989) The pathothysiological significance of a thyroglobulin-acetylcholinesterase shared epitope in patients with Graves' ophthalmopathy. Acta Endocrinol. (Copenh) 121: (Suppl. 2): 38-45.

Magnusson RP. Gestautas J. Taurog A and Rapoport B (1987a) Molecular cloning of the structural gene for porcine thyroid peroxidase. J. Biol. Chem. 262: 13885-13888.

Magnusson RP. Chazenbalk GD. Gestautas J. Seto P. Filetti S. DeGroot JL and Rapoport B (1987b) Molecular cloning of the complementary deoxyribonucleic acid for human thyroid peroxidase. Mol. Endocrinol. 1: 856-861.

Margolese H. O'Keefe C. Chung F. Ertug F. Wall JR and Bernard N (1994) Expression of major histocompatibility complex class II antigen in NOD mouse thyroid. Autoimmunity 17: 1-17.

Marines D (1938) Studies on pathological physiology of exophthalmos of Graves' disease. Ann. Intern. Med. 12: 443-453.

Mariotti S. Caturegli P. Piccolo P. Barbesino G and Pinchera A (1990) Antithyroid peroxidase autoantibodies in thyroid disease. J. Clin. Endocrinol. Metab. 71: 661-669.

Martinez AJ. McNeer KW. Hay SH and Watson A (1977) Extraocular muscles: morphogenetic study in humans. light microscopy and ultrastructural features. Acta Neuropathol. 38: 87-93.

Mauri L. Meienberg O. Roth E and Konig MP (1984) Evaluation of endocrine ophthalmopathy with saccadic eye movement. J. Neurol. 231: 182-187.

McGregor AM (1992) Heat shock protein and autoimmune thyroid disease: Too hot to handle? J. Clin. Endocrinol. Metab. 70: 720-723.

McKenzie JM (1958) Delayed thyroid response to serum from thyrotoxic patients. Endocrinology 62: 865-868.

McKenzie JM and McCullagh EP (1968) Observations against a causal relationship between the long-acting thyroid stimulator and ophthalmopathy in Graves' disease. J. Clin. Endocrinol. Metab. 28: 1177-1182.

Mclean JM and Norton EWD. (1958) Unilateral lid retraction without exophthal. Arch. Ophthalmol. 61: 681-686.

Mehra V. Sweetser D and Young RA (1986) Efficient mapping of protein antigen antigenic determinants. Proc. Natl. Acad. Sci. 83: 7013-7017.

Mengistu M, Laryea E, Miller A and Wall JR (1986) Clinical significance of a new autoantibody against a human eye muscle soluble antigen, detected by immunofluorescence. Clin. Exp. Immunol. 65: 19-27.

Metz HS, Woolf PD and Patton ML (1982) Endocrine ophthalmopathy in adolescence. J. Pediatre. Ophthalmol. Strabismus. 19: 58-60.

Miller A, Sikorska H, Salvi M and Wall JR (1986) Evaluation of an enzyme-linked immunosorbent assay for the measurement of autoantibodies against eye muscle membrane antigens in Graves' ophthalmopathy. Acta Endocrinol. (Copenh) 113: 514-522.

Miller A, Arthurs B, Boucher A, Liberman A, Bernard N, Salvi M and Wall JR (1992) Significance of antibodies reactive with a 64 kDa eye muscle membrane antigen in patients with thyroid autoimmunity. Thyroid 2: 197-202.

Mole SE and Lane DP (1989): A practical approach. Vol. 3 Glover DM ed. IRL. Press. Oxford pp. 113-126.

Momburg f, Fuchs S, Drexler J, Busch R, Post M, Hammerling GJ and Adorini L (1993) Epitope-specific enhancement of antigen presentation by invariant chain. J. Exp. Med. 178: 1453-1458.

Mornex R and Orgiazzi JJ (1980) Hyperthyroidism . In: De Visscher M (ed) The Thyroid Gland. Raven Press. New York, pp. 279-362.

Mullin BR, Levinson RE, Friedman A, Henson DE, Winand RJ and Kohn LD (1977) Delayed hypersensitivity in Graves' disease and exophthalmos-identification of thyroglobulin in human orbital muscle. Endocrinology 100: 351-366.

Mulvany JH (1944) The exophthalmos of hyperthyroidism. Am. J. Oppathalmol. 27: 589-612.

Munro E, Lamki L Row VV and Volpe R (1973) Cell mediated immunity in the exophthalmos of Graves' disease as demonstrated by the migration inhibition factor test. J. Clin. Endocrinol. Metab. 37: 286-292.

Nagasaka A, DeGroot LJ, Hati R and Liu C (1971) Studies on the biosynthesis of thyroid hormone: reconstruction of a define in vitro iodinating system. Endocrinology 88: 486-490.

Nagayama Y, Kaufman KD, Seto P and Rapoport B (1989) Molecular cloning, sequence and functional expression of the cDNA for the human thyrotropin receptor. Biochem. Biophys. Res. Comm. 165: 1184-1190.

Nakagawa H and Ohtaki S (1985) Thyroxine (T4) release from thyroglobulin and its T4-containing peptide by thyroid thiol proteases. Endocrinology 115: 33-40.

Nixon DW and Samols E (1970) Acral changes associated with thyroid disease. JAMA. 212: 1175-1181.

Nordyke RA, Gilbert FL Jr and Harada ASM (1988) Graves' disease. Influence of age on clinical findings. Arch. Intern. Med. 148: 626-631.

Parry CH (1825) Collections for the unpublished medical writings of the late Caleb Hillier Parry. Vol. 2. London. Underwoods. pp.111-128.

Perros C and Kendall-Taylar P (1992) Pathogenic mechanisms in thyroid-associated ophthalmopathy. J. Internal. Med. 231: 205-211.

Peterson A and Seed B (1987) Monoclonal antibody and ligand binding sites of the T cell erythrocyte receptor (CD2). Nature 329: 842-846.

Pfaltz M and Hedinger CE (1986) Abnormal basement membrane structures in autoimmune thyroid disease. Lab. Invest. 55: 531-539.

Pinchera A, Fenzi GF, Bartalena L et al. (1979) Thyroid antigens involved in autoimmunity thyroid disorders. In: Autoimmunity in Thyroid disease. Klein. E. and Horster FA. (eds). Schattauer. Stuttgart. pp. 49-67.

Portman L, Hamada N, Heinrich G and DeGroot LJ (1985) Anti-thyroid peroxidase antibody in patients with autoimmune thyroid disease. J. Clin. Endocrinol. Metab. 61: 1001-1003.

Prigent SA, Stanley KK and Siddle K (1990) Identification of epitopes on the human insulin receptor reacting with rabbit polyclonal antisera and mouse monoclonal antibodies. J. Biol. Chem. 265: 9970-9977.

Prummel MF, Mourits MP, Berghout A, et al.(1989) Prednisone and cyclosporine in the treatment of Graves' ophthalmopathy. N. Engl. J. Med. 321: 1353-1359.

Ringel SP, Wilson WB, Barden M and Kaiser M (1978) Histochemistry of human extraocular muscle. Arch Ophthalmol. 96: 1067-1072.

Roitt IM, Doniach D, Campbell RN and Hudson RV (1956) Autoantibodies in Hashimoto's thyroiditis (lymphadenoid goitre). Lancet 2: 820-821.

Roitt IM and Torrigiani G (1967) Identification and estimation of undergraded hyroglobulin in human serum. Endocrinology 81: 421- 429.

Rotella CM, Zonefrati R Toccafondi R Valente WA and Kohn LD (1989) Ability of monoclonal antibodies to the thyrotropin receptor to increase collagen synthesis in human fibroblasts: an assay which appears to measure exophthalmogenic immunoglobulins in Graves' disease. J. Clin. Endocrinol. Metab. 62: 357-367.

Salvi M, Fukazawa H, Bernard N, Hiromatsu Y, How J and Wall JR (1988) Role of autoantibodies in the pathogenesis and association of endocrine autoimmune disorders. Endocr. Rev. 9: 450-466.

Salvi M, Miller A and Wall JR (1988) Human orbital tissue and thyroid membrane express a 64 kDa protein which recognized by autoantibodies in the serum of patients with thyroid-associated ophthalmopathy. FEBS Lett. 232: 135-139.

Salvi M. Bernard N. Miller A. Zhang Z-G. Gardinie F and Wall J. (1991) Prevalence of antibodies reactive with a 64 kDa eye muscle membrane antigen in thyroid-associated ophthalmopathy. Thyroid 1: 204-213.

Salvi M. Zhang Z-G. Haegert D. Woo M. Liberman A. Cadarso L and wall JR (1990) Patients with endocrine ophthalmopathy not associated with overt thyroid disease have multiple thyroid immunological abnormalities. J. Clin. Endocrinol. Metab. 70: 89-94.

Satch J. Probhakar BS. Haspel MV. Ginsberg-Fellner F and Notkins AL (1983) Human monoclonal autoantibodies that react with multiple endocrine organs. N. Engl. J. Med. 309: 217-220.

Scandella D. Mahoney S. Mattingly M. Roeder D. Timmons L and Fulcher CA (1988) Epitope mapping of human factor VIII inhibitor antibodies by deletion analysis of factor VIII fragments expressed in Escherichia coli. Proc. Natl. Acad. Sci. 85: 6152-6156.

Schumacher M. Camp S. Maulet Y. Newton M. MacPhee-Quigley K. Taylor SS. Freidmann T and Taylor P (1986) Primary structure of Torpedo California acetylcholinesterase deduced from its cDNA sequence. Nature 319: 407-409.

Schwartz BS (1985) Antigen-induced monocyte procoagulant activity: requirement for antigen presentation and histocompatibility leukocyte antigen-DR molecules. J. Clin. Invest. 76: 970-977.

Sevel D (1986) The origins and insertions of the extraocular muscles: development, histological features, and clinical significance. Trans. Am. Ophthalmol. Soc. 84: 488-526.

Shamsuddin AKM and Lane RA (1981) Ultrastructural pathology in Hashimoto's thyroiditis. Hum. Pathol. 12: 561-573.

Sheshberadaran H and Payne LG (1988) Protein antigen-monoclonal antibody contact sites investigated by limited proteolysis of monoclonal antibody-bound antigen: protein "footprinting". Proc. Natl. Acad. Sci. 85: 1-5.

Smith BR, McLachlan SM and Furmaniak J (1988) Autoantibodies to the thyrotropin receptor. Endocr. Rev. 9: 106-121.

Spencer RF and Porter JD (1988) Structural organization of the extraocular muscles. In: Butter-Ennever J. ed. Neuroanatomy of the oculamotor system. Amsterdam: Elsevier. pp. 33-79.

Spoor TC, Martines AJ, Kennerdell JS and Mark LE (1986) Dysthyroid and myopathy of the medical rectus: A clinical pathologic report. Neurology 30: 939-944.

Sridama V and DeGroot LJ (1989) Treatment of Graves' disease and the cause of ophthalmopathy. Am. J. Med. 87: 70-73.

Stanley KK and Luzio JP (1984) Construction of a new family of high efficiency bacterial expression vectors: identification of cDNA clones coding for human liver proteins. EMBO. J 3: 1429-1434.

Stanley KK, Herz J and Dickson J (1987) Topological mapping of complement component C9 by recombinant DNA techniques suggests a novel mechanism for its insertion into target membranes. EMBO. J 6: 1951-1957.

Stern LJ, Brown JH, Jardetzky TS, Gorge, Urban RG, Strominger JL and Wiley DC. (1994) Crystal structure of the human class II MHC-DR1 complexed with an influenza virus peptide. Nature 368: 215-221.

Strakosch CR, Wenzel BE, Row VV and Volpe R (1982) Immunology of autoimmune thyroid disease. N. Engl. J. Med. 307: 1488-1507.

Tanaka K, Hiromatsu Y, Ishisaka N, Nonaka K, Ludgate M and Wall JR. (1992) T cell reactivity to a recombinant fragment (D1) of a novel 64 kDa protein in thyroid-associated ophthalmopathy. In: Proceedings, International Symposium on Hashimoto's Thyroiditis, Fukuoka, December. (Abstract).

Taurog A (1970) Thyroid peroxidase-catalyzed iodination of thyroglobulin: inhibition by excess iodide. Arch. Biochem. Biophys. 139: 212-229.

Taurog A, Dorris ML and Lamas L (1974) Comparison of lactoperoxidase- and thyroid peroxidase-catalyzed iodination and coupling. Endocrinology 94: 1286-1294.

Tengroth B (1964) Histological studies of orbital tissues in a case of endocrine exophthalmos before and after remission. Acta Ophthalmol (Copenh) 42: 588-591.

Thomson ES (1924) Orbital edema in exophthalmic goitre. Am. J. Ophthalmol. 7: 27-55.

Trokel SL and Jakobiec FA (1981) Correlation of CT scanning and pathologic features of ophthalmic Graves' disease. Ophthalmology 88: 553-564.

Trzepacz PT, Klien I, Robert M, Greenhouse J and Levey GS (1989) Graves' disease: an analysis of thyroid hormone levels and hyperthyroid signs and symptoms. Am. J. Med. 87: 558-561.

Tunbridge WMG, Evered DC, Hall R, Appleton D, Brewis M, Clark F, Grimley Evans J, Young E, Bird T and Smith PA (1977) The spectrum of thyroid disease in a community: the Wickham survey. Clin Endocrinol. (Oxf) 7: 481-493.

Urbanski GJ and Margoliash E (1977) Topographic determinants on cytochrome c. J. Immunol. 118: 1170-1180.

Van Herle AJ, Uller RPI, Matthews NL and Brown J (1973) Radioimmunoassay for measurement of Thyroglobulin in human serum. J. Clin. Invest. 52: 1320-1327.

Vincent A, Whiting PJ, Schluep M, Heidenreich F, Lang B, Roberts A, Willcox N and Newsom-Davis J (1987) Antibody heterogeneity and specificity in Myasthenia Gravis. Ann. N. Y. Acad. Sci. 505: 106-120.

Volpé R (1990) Immunology of human thyroid disease. In: Volpé R, ed. Autoimmunity in endocrine disease. Boca Raton, Fl: CRC Press, pp.73.

Volpé R. (1993) Graves' hyperthyroidism and endocrine ophthalmopathy-one or two closely related disease. Dev. Ophthalmol. 25: 101-111.

Von Basedow (1840) Exophthalmos durch hypertrophie des Zellgewebes in der Augenhohle. Wochenschr Heilk 6: 197-209.

Voorby HAM, Kabel PJ, de Haan M, Jeucken PHA, Vander Gaag RD, De Baets MH and Drexhabe HA (1990) Dendritic cells and class II MHC expression on thyrocytes during the autoimmune thyroid disease of the BB rat. 55: 9-22.

Wall JR, Henderson J, Strakosch CR and Joyner DM (1981) Graves' ophthalmopathy. Can. Med. Assoc. J. 124: 855-866.

Wall JR, Trewin A, Fang SL, Ingbar SH and Braverman LE (1978) Studies of immunoreactivity to human lacrimal gland fractions in patients with ophthalmic Graves: disease. J. Endocrinol. Invest 1: 253-258.

Wall JR (1986) Role of cellular mechanisms in the orbital autoimmunity of Graves' ophthalmopathy. J. Endocrinol. Invest. 9(Suppl 3): 71.

Wall JR, Salvi M and Bernard N (1990) Eye disease and autoimmune thyroid disorders: mechanism of the association. In: Wall JR, How J (eds) Graves' Ophthalmopathy. Blackwell Scientific Publications, Boston, pp. 63-77.

Wall JR, Salvi M, Bernard N, Boucher A and Haegert D (1991a) Thyroid-associated ophthalmopathy-A model for the association of organ-specific autoimmune disorder. Immunology Today 12: 150-153.

Wall JR (1991b) Pathogenesis and management of thyroid associated ophthalmopathy: an update. Thyroid Today 14: 1-9.

Wall JR, Bernard N, Boucher A, Salvi M, Zhang Z-G, Kennerdell J, Tyutyunikov A and Genovese C (1993) Pathogenesis of thyroid-associated ophthalmopathy: an autoimmune disorder of the eye muscle associated with Graves' Hyperthyroidism and Hashimoto's thyroiditis. Clin. Immunol. Immunopathol. 68: 1-8.

Wall JR, Hayes M, Scalise D, Stolarski C, Nebes V, Kiljanski M, Salvi M and Sato M (1994) Native gel electrophoresis and isoelectric focusing of a 64 kDa eye muscle protein shows that it is an important target for serum autoantibodies in patients with thyroid-associated ophthalmopathy and not expressed in other skeletal muscle. Submitted.

Waring GS, Kodama K, Sikorska H and Wall JR (1986) TSH and orbital antibodies. Lancet 2: 224-225.

Weetman AP, Ludgate M, Mills PV, McGregor AM, Beck L, Lazarus JH and Hall R (1983) Cyclosporin improves Graves' ophthalmopathy. Lancet 2: 486-489.

Weetman AP, Cohen S, Gatter KC, Fells P and Shine B (1989) Immunohistochemical analysis of the retrobulbar tissues in Graves' Ophthalmopathy. Clin. Exp. Immunol. 75: 222-227.

Weetman AP (1991) Thyroid associated eye disease: pathophysiology. Lancet 338:25-28.

Weetman AP (1992) Thyroid-associated ophthalmopathy. Autoimmunity 12: 215-222.

Weightman and Kendall-Taylar (1989) Cross reaction of eye muscle antibodies with thyroid tissue in thyroid-associated ophthalmopathy. J. Endocrinol. 122: 201-206.

Werner SC (1966) Prednisone in emergency treatment of malignant exophthalmos. Lancet 1: 1004-1007.

Werner SC (1969) Classification of the eye changes of Graves' disease. J. Clin. Endocrinol. Metab. 29: 982-984

Werner SC (1972) The eye changes of Graves' disease. Mayo. Clin. Proc. 47: 969-974.

Werner SC (1974) Ultrasonographic evidence of a consistent orbital involvement in Graves' disease. N. Engl. J. Med. 290: 1447-1450.

Wetzel BK. Spicer SS and Wollman SH (1965) Changes in the fine structure and acid phosphatase localization in rat thyroid cells following thyrotropin administration. J. Cell. Biol. 25: 593-618.

Wierczorek DF, Periasamy M, Butler-Browne GS, Whalen RG and Nadal-Ginard B (1985) Coexpression of multiple myosin heavy chain genes in addition to tissue specific ones in extraocular musculature. J. Cell. Biol. 101: 618-629.

Wiersinga WM, Smith T, van der Gaag R, Mourlts M and Koornneet L (1989) Clinical presentation of Graves' ophthalmopathy. Ophthalmic. Res. 21: 73-82.

Wiersinga WM, Smith T, van der Gaag R and Koornneet L (1988) Temporal relationship between onset of Graves' ophthalmopathy and onset of thyroid Graves' disease. J. Endocrinol. Invest. 11: 615-619.

Winand and Kohn LD (1970) Relationship of thyrotropin to exophthalmic producing substance. Purification of homogeneous glycoproteins containing both activities from (3H) labeled pituitary extracts. J. Biol. Chem. 245: 967-975.

Winand RJ and Kohn LD (1972) The binding of (3H) thyrotropin and 3H-labeled exophthalmogenic factor by plasma membranes of retro-orbital tissue. Proc. Natl. Acad. Sci. 69: 1711-1715.

Winand RJ and Kohn LD (1973) Retrobulbar modifications in experimental exophthalmos: the effect of thyrotropin and exophthalmos-producing substance derived from thyrotropin on the 35SO4 incorporation and glycosaminoglycan content of harderian glands. Endocrinology 93: 670-680.

Woods AC (1946) The ocular changes of primary diffuse toxic goitre. Medicine (Baltimore) 25: 113-153.

Woolner LB, McConahey WM and Beahrs OH (1959) Struma lymphomatos (Hashimoto's thyroiditis) and related thyroid disorders. J. Clin. Endocrinol. Metab. 19: 53-83.

Wybar KC (1957) The nature of endocrine exophthalmos. Bibl. Ophthalmol. 49: 119-220.

Yamamoto K, Saito K, Takai T and Yoshida S (1983) Diagnosis of exophthalmos using orbital ultrasonography and treatment of malignant exophthalmos with steroid therapy, orbital radiation therapy and plasmapheresis. Progr. Clin. Biol. Res. 116: 189-205.

Zakarija M, Jin S and McKenzie JM (1988) Evidence supporting the identity in Graves' disease of thyroid-stimulating antibody and thyroid growth-promoting immunoglobulin G as assayed in FRTL5 cells. J. Clin. Invest. 81: 879-884.

Zhang Z-G, Meideros-Neto g, Iacona A, Hiromatsu Y, Salvi M, Triller H, Bernard N and Wall JR (1989) Studies of cytotoxic antibodies against eye muscle antigens in patients with thyroid associated ophthalmopathy. Acta. Endocrinol. (Copenh) 12 (Supple 2): 23-30.

Zhang Z-G, Salvi M, Miller A, Bernard N, Arthurs B and Wall JR (1992a) Restricted tissue reactivity of autoantibodies to a 64 kDa eye muscle membrane antigen in thyroid-associated ophthalmopathy. Clin. Immunol. Immunopathol. 62: 182-189.

Zhang Z-G. Dong Q. Rodien P. Alcade L. Bernard N. Boucher A. Vassart G. Ludgate M and Wall JR (1992b) Autoantibodies in the serum of patients with ATD react with a recombinant 98 amino acid fragment of a 64 kDa eye muscle recombinant protein which is also expressed in the thyroid. Autoimmunity 13: 151-157.

Zhang Z-G. Bernard N. Boucher A. Ludgate M. Tyutyunikov A. Genovese C and Wall JR (1992c) Molecular characterization of 64 kDa eye muscle autoantigen and significance of the corresponding autoantibodies. Proceedings 66th Annual Meeting of the American Thyroid Association. Rochester. MN. September. Thyroid 2 (Suppl. 1): 3.

Zhang Z-G. Bernard N. Ludgate M and Wall JR (1992d) Epitope mapping of a human 64 kDa membrane autoantigen associated with thyroid autoimmunity and ophthalmopathy. Proceedings 66th Annual Meeting of the American Thyroid Association. Rochester. MN. September. Thyroid 2 (Suppl. 1): 74.

CHAPTER VIII

STATEMENT OF ORIGINALITY
SUGGESTIONS FOR FURTHER WORK

1. Statement of originality

The following novel findings and observations have been reported in this thesis:

1). AutoAbs in sera from TAO patients immunoprecipitated a 64 kDa membrane Ag from cultured human eye muscle and thyroid cells demonstrating that sera recognized antigenic epitope(s) present on these tissues in a conformation that was known to be biologically relevant. These observations were the first direct evidence that eye muscle and thyroid may be the common targets of autoimmune reactivity in TAO (Chapter II).

2). Results present in this thesis demonstrated for the first time that sera from nearly half of patients with established TAO and in more than half of those with GH (the majority of patients of this group may have subclinical ophthalmopathy) reacted with recombinant antigenic peptide of a cloned human 64 kDa eye muscle/thyroid Ag. These results suggested that auto-Abs against the cloned 64 kDa Ag may be an early marker of the eye disease in patients with thyroid autoimmunity (Chapter III).

3). A cDNA internal control "pGEM-1D" was created and a modified quantitative RT-PCR technique was used to assess the tissue distribution and abundance of 1D transcripts in various tissues. The abundance of 1D transcripts present in thyroid and eye muscle compared with that in other tissues may explain why these two tissues are targeted by autoimmunity in TAO.

4). Recombinant cDNA techniques were used to identify antigenic epitopes on 1D. Random fragments of the full-length 1D cDNA were cloned and expressed in E.

coli as fusion proteins. Four antigenic peptides which contain epitopes recognized by auto-Abs from patients with TAO were identified. When a panel of sera was screened for recognition of each peptide, sera from TAO patients reacted to one or more peptides significantly more frequently than those from normal controls (53% sera from TAO patients versus 8% of sera from normal controls; $p<0.025$). Forty four percent of GH patients and 33% of HT patients also possessed Abs which reacted with one or more 1D peptides.

2. Suggestions for further work

1). The human eye muscle and thyroid tissue 64 kDa protein identified by Dr. J. Wall's research group by Western blotting should be purified and microsequenced. Its sequence should be compared with that deduced for the 1D molecule in order to provide direct evidence for identity, relatedness or absence of relatedness of these molecules. This work is presently being carried out in Dr. Wall's laboratory.

2). 1D antigenic peptides or full-length 1D fusion protein could be used as Ag in tests such as ELISA for detection of Abs to 1D. Prospective longitudinal study of sera from patients with TAO and ATD using this methodology together with clinical findings at follow up visits would further define the clinical significance of autoAbs to 1D.

3). Monoclonal Abs specific for 1D and the 64 kDa protein identified by Western blotting should be made and their reactivity compared on histological sections of human eye muscle, thyroid and somatic skeletal muscles in order to determine their pattern of reactivity in such tissues specimens.

4). 1D mRNA sense or anti-sense probe should be made. Such probes could be used in *in situ* hybridization of human eye muscle, thyroid and somatic skeletal tissues. This will confirm and more precisely define the expression and cellular distribution of 1D in these tissues.