Identification of the Membrane Receptor Binding Domain of Thyroglobulin

INSIGHTS INTO QUALITY CONTROL OF THYROGLOBULIN BIOSYNTHESIS* (Received for publication, February 18, 1997, and in revised form, June 6, 1997)

Hakim Mezghrani, Hassan Miziat, Joel Courageot, Razika Oughidenni, Paul Bastiani, and Raymond Miquelis

From the Laboratoire de Biochimie, In génierie des Protéines, UMR 6560, Institut Fé dératif Jean Roche, Facul té de Médicine-Nord, Boulevard P. Dramard, 13916 Marseille Cedex 20, France

The last stages of thyroglobulin maturation occur in the thyroid follicular lumen and include thyroid hormone formation and glycan completion. In this compartment, newly secreted thyroglobulins interact with a thyrocyte membrane receptor that prevents their premature lysosomal transfer and degradation. Both GlcNAc moieties and thyroglobulin peptide determinants are involved in receptor interaction. Here we used monoclonal antibodies (mAbs) directed against human thyroglobulin either to inhibit (mAb78) or to enhance (mAb240) the thyroglobulin binding and to identify the region of the thyroglobulin involved in the receptor recognition.

Peptides containing the mAb epitopes were obtained by immunoscreening cyanogen bromide-derived native human thyroglobulin peptides and a cDNA thyroglobulin expression library. Three peptides, localized in the thyroglobulin N-terminal domain, were obtained. Peptides N1 (Ala1148–Gln1285) and N2 (Ser789–Met1009) were recognized by mAb240 and mAb78, respectively. None of them bound the receptor. The third peptide, N3 (Ser789–Met1172), (i) overlapped all or part of the N1 and N2 peptide sequences and was recognized by both mAbs, (ii) carried two complex glycans at Asn797 and Asn928, of which a subset presented accessible GlcNAc residues, and (iii) inhibited the thyroglobulin binding to FRTL5 cell membrane preparations. The N3 peptide includes tyrosine residues that have been reported to be involved in hormone formation. These results suggest that structural modifications closely associated with hormone formation within this domain act as sensors for the receptor interaction and thus for the intrafollicular retention or lysosomal homing of the prohormone.

Thyroglobulin, the major glycoprotein produced by the thyroid gland, is the substrate for the biosynthesis of the two thyroid hormones, tetra-(thyroxine) and triiodothyronine. Mature thyroglobulin is a dimer with a Mr of 660,000, consisting of two apparently identical chains, each containing 67 tyrosyls and 2748 amino acid residues (numbered to exclude a 19-residue leader sequence) in the human (1). The glycoprotein is synthesized and partially matured within thyrocytes (intramonomer disulfide bonding, glycosylation, sulfatation, phosphorylation) and then secreted into the follicular lumen. In this compartment, thyroglobulin is further processed by iodination, hormonogenesis, and completion of some complex type oligosaccharide units (2).

Iodination of thyroglobulin tyrosyls is thought to be mediated by the thyroperoxidase at the thyrocyte apical cell surface. About 10–50 atoms of iodide are associated with each molecule (3). Hormone formation per se requires thyroperoxidase-mediated coupling of two iodotyrosyls. The coupling reaction leaves iodothyronine and dehydroalane at the acceptor and donor sites, respectively (4, 5). Thyroglobulins from various species have been compared. The hormonogenic iodotyrosyl residues (no more than four per monomer) are in both the N- and C-terminal domains of the prohormone, and their positions are well conserved (6–11). The hormonogenic 580 C-terminal residue domain is cysteine-poor, presents no repeated sequence, and structure predictions suggest that it is flexible (1). In contrast, the N-terminal domain (encoded by exon 1 to exon 15, residues 1–1209) consists of tandem repeats rich in cysteine residues, called type 1 repeats, interrupted by unrelated sequences (1, 12). These cysteine-rich repeats are probably involved in disulfide bonds, suggesting that iodotyrosyl coupling results in a highly coordinate three-dimensional structure with a limited flexibility. In vivo kinetic experiments have shown the following: (i) production of mature prohormones requires intralumenal retention, and (ii) mature thyroglobulins preferentially participate in the secretion of hormones in the venous flow, after thyroglobulin internalization and its proteolytic cleavage in the lysosomal system (2, 13, 14). These findings and the observation that thyroglobulin iodination is closely associated with glycan completion (15) led us to investigate and demonstrate a quality control system that prevents lysosomal homing and degradation of iodine-poor immature thyroglobulin. This control depends on the recognition of a subset of molecules by an endogenous receptor and suggests that there is a recycling mechanism by which receptor-bound immature thyroglobulins are passed back to the thyroperoxidase iodination site via the Golgi apparatus (16). Thyroglobulin binding to the thyrocyte receptor is optimal at acidic pH (17, 18), which is consistent with an interaction in prelysosomal compartments. The thyroglobulin receptor interacts with both carbohydrate and peptide determinants of the prohormone (15–17), and we have obtained and characterized anti-thyroglobulin monoclonal antibodies wherein some inhibit and others enhance thyroglobulin binding to its receptor (18).

The purpose of the present study was to use anti-thyroglobulin antibodies to identify the receptor binding domain of the

* This work was supported by institutional funding from the Centre National de la Recherche Scientifique and grants from l’Assistance Publique de Marseille and the Program Hospitalier de Recherche Clinique 1994 (to R. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 33 04 91 96 20 69; Fax: 33 04 91 65 75 95.
human prohormone. We thereby mapped the receptor binding domain to the N-terminal thyroglobulin region encoded by exons 10 to 14. The domain presents incompletely processed oligosaccharide units bearing accessible GlcNAc residues and includes tyrosyl residues reported to be either iodinated or involved in hormonogenesis.

MATERIALS AND METHODS

Reagents

The antithyroglobulin monoclonal antibodies (mAbs,\(^1\) Immunotech, Marseille, France) were used those previously shown to block (mAb 78) or to enhance (mAb 240) the binding of thyroglobulin to its receptor (18). Plasmid cDNA pEX human thyroglobulin libraries (19) were kindly provided by Y. Malthiery (Faculté de Medecine, Angers, France). Endoglycosidase F/N-glycosidase F (N-glycanase) from Flavobacterium meningosepticum was from Boehringer Mannheim, and Clostridium perfringens neuraminidase was from Sigma.

Preparation and Analysis of Human Thyroglobulin Peptides

CNBr Peptides Purification—Peptide fragments from iodine-poor human thyroglobulin (gift from B. Mallet and P-J Lejeune, Faculté de Medecine, Marseille, France) were aliquot fractions of previously described preparations (20). Briefly, thyroglobulin (2.8 atoms of iodine/mol, traces of T, 10 mmol T4/mol) was from a patient with a single colloid goiter. Purified thyroglobulin was treated with cyanogen bromide (CNBr), and the resulting digest was eluted by chromatography on a Sephadex G-200 column in I3 propionic acid. Five fractions (I-V) were collected (void volume to gel-included fractions), dialyzed, and lyophilized.

Preparative Electrophoresis—CNBr peptide fragments of fraction II were further separated using a model 491 Prep Cell preparative electrophoresis apparatus (Bio-Rad). Peptides (20 mg) were solubilized in buffer A (60 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 5% bromphenol blue). Samples were heated (5 min at 90 °C) and loaded onto a 12% acrylamide running gel on small Prep Cell gel column (28 mm inner diameter). Electrophoresis was carried out (200–250 V/40 mA constant current) for 12 h. After elution of the bromphenol blue, 2.5 ml fractions were collected at a flow rate of 1 ml/min.

Prestained standards were used to estimate molecular masses. Peptide fractions called fraction II (26 kDa, corresponding to collected fractions 22–30), II2 (28 kDa, fractions 40–46), II3 (40 kDa, fractions 65–82), II4 (44 kDa, fractions 182–171), and II5 (50 kDa, fractions 191–206) were pooled, dialyzed at 4 °C for 24 h against ammonium acetate, and lyophilized. The purified hormonogenic CNBr N-terminal domain (Asn-Met)\(^2\); see Refs. 20–22) was a gift of B. Mallet and P.-J. Lejeune.

SDS-Polyacrylamide Gel and Immunoblotting—Fractions I to II5 were further characterized by SDS-PAGE (12% acrylamide) and immunoblotting using mAb78 or mAb240 (1–3 µg/ml) for 1 h and 2.5 µl of a Gal elution buffer (50 mM M-α-D-glucosidase) in a gradient of trifluoroacetic acid/acetonitrile (peptide N2) or 10% SDS-polyacrylamide gels, excision of the major band, and electroelution (peptide N3).

Peptide Sequencing—Purified N2 and N3 peptides (500 pmol) were concentrated and washed onto a Problott polyvinylidene difluoride membrane (Protein Sample Preparation Cartridge, Applied Biosystems) and sequenced. Automated Edman degradations were performed with a 476A protein sequencer (Applied Biosystems) with an on-line 140A analyzer for identification of the phenylthiohydantoin-amino acid derivatives separated on a phenylthiohydantoin C18 reverse-phase HPLC column in a gradient of trifluoroacetic acid/acetonitrile (peptide N2) or 10% SDS-polyacrylamide gels, excision of the major band, and electroelution (peptide N3).

\(^1\) The abbreviations used are: mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; CNBr, cyanogen bromide; HPLC, high performance liquid chromatography; Tg, thyroglobulin; WGA, wheat germ agglutinin.

medium. After amplification and regrowth, fusion protein synthesis was induced by a 2-h incubation at 42 °C. Nitrocellulose disks were treated with chloroform vapor for 15–30 min, incubated in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl\(_2\), 3% bovine serum albumin, 1 µg/ml DNase I, 40 µg/ml lysosome) for 4 h at room temperature and washed in Tris-buffered saline. For immunoblotting disks were incubated for 2 h in the presence of mAb78 or mAb240 (1–5 µg/ml). Selected immunoreactive clones were grown in Luria Bertani medium containing 100 µg of ampicillin per ml at 37 °C to obtain an optical density at 550 nm of 0.4. Protein expression was induced to obtain large quantities of hybrid protein (23). Bacterial extract (5 µl) was dissolved in SDS-PAGE sample buffer, separated by 8% SDS-PAGE, and transferred to nitrocellulose. Immunodetection was performed as described above.

The sequence of the cDNA insert of the positive clone was determined by the dyeoxy-mediated chain termination method with T7 DNA polymerase (Sequenase, U. S. Biochemical Corp.).

Iodination—Human thyroglobulin (10 µg) was iodinated withIODOGEN (50 µg) and Na\(^125\)I (1 mCi) in 50 µl of 50 mM Tris-HCl, pH 8.5, for 15 min at 4 °C. The reaction was stopped by dilution with 1 ml of PBS, and Tg was isolated by gel filtration on a Sephadex S-200 column. The specific activity was 95 µCi/µg. The N3 peptide (4 µg, specific activity 10 µCi/µg) was iodinated using the same procedure.

Glycosidase Treatments—Endoglycosidase H treatment was carried out overnight digesting the reduced N3 peptide (1.5 µg) in 20 µl of 2 milliliters of Endo-H in 100 mM sodium phosphate buffer, pH 6.0, containing 0.1% Triton X-100 and 0.03% SDS. N-Glycanase treatment was also performed on the reduced N3 peptide (1.5 µg) by incubation at 37 °C for 20 h with 1.5 milliliters of N-glycanase in 50 µl of 50 mM acetate buffer, pH 5.0, 10 mM EDTA, 0.3% Triton X-100, 0.1% SDS, 1% β-mercaptoethanol. The reaction was stopped by heating for 3 min at 100 °C and subjecting the sample to SDS-PAGE under denaturing conditions. The proteins were blotted onto nitrocellulose which was then incubated with mAb78 (diluted 1/100), added for 2 h, and alkaline phosphatase-conjugated anti-mouse antibodies for 2 h.

In an additional experiment to assess the extent of deglycosylation at various concentrations of N-glycanase, aliquot fractions of radiolabeled \(^125\)I-N3 peptide (10 ng, 120,000 cpm) were treated in the same medium with N-glycanase at each 25, 2.5, 0.25, 0.025, 0.0025, and 0.00025 milliliters. The samples were resolved by SDS-PAGE under reducing conditions, and the gel was scanned and analyzed with a PhosphorImager (Bio-Rad, Les Ulis, France).

Neuraminidase treatment was performed on aliquots (30 ng) of the radiolabeled N3 peptide by reaction with 25 milliliters of C. perfringens neuraminidase buffer, pH 5.0, for 20 h at 37 °C. The reaction was stopped by heating for 3 min at 80 °C, and the sample was then extensively dialyzed against phosphate buffer.

Lectin Affinity Chromatography—Incompletely processed complex type oligosaccharides bearing accessible GlcNAc residues were detected using wheat germ agglutinin (WGA) and Bandeiraea simplicifolia II BSS II-conjugated agarose columns. A minor modification of established method (18, 24) for WGA affinity chromatography was used. Briefly, 20 µl of radiolabeled \(^125\)I-N3 peptide (before (250,000 cpm) or after neuraminidase treatment (330,000 cpm)) was applied to WGA columns (150 µl) and allow to stand for 1 h. The columns were washed with 15 ml of 10 mM phosphate buffer, pH 7.4, 0.15 M NaCl, 0.2% Nonidet P-40 (wash buffer), and then with 5 ml of a Gal elution buffer (10 mM α-D-galactose in wash buffer) and then with 5 ml of a GlcNAc elution buffer (10 mM α-D-glucosamine in wash buffer), 0.5-ml fractions were collected and counted in a gamma counter. BSS II affinity chromatography was performed as described previously for the native thyroglobulin (15, 18). The column (300 µl) was equilibrated with loading buffer consisting of 50 mM phosphate buffer, pH 7.4, 0.15 M NaCl, 0.1% bovine serum albumin, and 0.1 mM CaCl\(_2\), MnCl\(_2\), and MgCl\(_2\). After equilibration, 40 µl of \(^125\)I-N3 peptide (500,000 cpm) was loaded onto the column and allowed to interact for 1 h. The unbound fraction was recovered by adding 30 ml of loading buffer. The bound fraction was recovered by addition of a buffer containing 0.5 M GlcNAc. 0.5-ml fractions were collected.

Binding Assay—Solid phase assays of thyroglobulin binding to FRTL 5 membranes were performed as follows. Membranes, prepared as described previously (15, 18), were added (20 µg) to 50 µl of PBS buffer) and were incubated with 20 µl of PBS containing 2% bovine serum albumin. Thyroglobulin (5 × 10\(^4\) to 10\(^6\) cpm in 100 µl of a binding buffer containing 25 mM acetate buffer, pH 5.0, 150 mM NaCl, 5 mM CaCl\(_2\), and
FIG. 1. Isolation of a pEX clone encoding a human Tg fragment recognized by mAb240. A pEX human thyroglobulin expression library was plated and screened with mAbs that interfere with the binding of Tg with its receptor. A positive clone was identified (A, left) and isolated (A, right). After amplification and regrowth, fusion protein synthesis was induced (B, lane 1; compare with the noninduced clone used as a control in B, lane 2). The isolated fusion protein Cro-LacZ + fTg was further analyzed by immunoblotting (C) using both mAb240 and mAb78 antibodies. See “Materials and Methods” for details.

RESULTS

Isolation of a Human Thyroglobulin Fragment-Fusion Protein Recognized by mAb240—pEX human thyroglobulin expression libraries were prepared from PstI fragments of thyroglobulin cDNA inserted into the three forms of the pEX plasmid, corresponding to the three reading frames. Between 200 and 700 transformed E. coli cells were plated, corresponding to about seven copies of each fragment in every reading frame. Nitrocellulose culture dish replicas were tested for the presence of peptides encoding the receptor thyroglobulin binding domain by using mAb78 and mAb240 antibodies. One immunoreactive clone recognized by mAb240 was isolated in the initial screening, and only in pEX1 (Fig. 1A). The hybrid protein produced by this positive clone was further characterized. As expected, the length of the Cro-LacZ fusion protein obtained after induction (23) (Fig. 1B, lane 1) was greater than β-galactosidase (M, of the fusion protein about 165,000). Moreover, this fusion protein was recognized by mAb240 only (Fig. 1C). The length of the PstI cDNA fragment in this positive clone was 444 base pairs, and the deduced peptide sequence, we named peptide N1, corresponded to Ala1148-Gln1295 on the human thyroglobulin monomeric molecule (Fig. 4).

No other positive clone was detected with either mAb240 or mAb78 antibodies.

Isolation of CNBr Human Thyroglobulin Peptides Recognized by mAb78 and mAb240—The failure to obtain a thyroglobulin fragment-fusion protein encoding the mAb78 epitope prompted us to search for such immunoreactive peptides from human native thyroglobulin. Five fractions previously obtained after Sephadex G-200 filtration of the CNBr digest from iodine-poor thyroglobulin were used as a source of peptide fragments (Ref. 20 and Fig. 2A). After SDS-PAGE and electroblotting, immunoreactive peptides were found only in fraction I (M, 42,000 with mAb78, ranging from 50,000 to 64,000 with mAb240) and fraction II (M, 38,000, and ranging from 50,000 to 80,000 with both mAbs) (Fig. 2B). Because they included the smallest fragment (about 39 kDa) recognized by both mAbs, peptides of fraction II were further separated by preparative electrophoresis. Immunoblot analysis of five fractions, designated I1 to I5 and ranging from 25 to 55 kDa (Fig. 2A), led to the identification of two peptides fractions. One (I2, about 28 kDa) was recognized by mAb78 and the second (I3, about 40 kDa) by both mAb78 and mAb240 (Fig. 2B). The same results were obtained by immunoblot using single peptides N2 and N3, prepared from fractions I2 and I3, respectively, and devoid of minor contaminants after further purification on reverse-phase HPLC column and/or by SDS-PAGE electrophoresis and electroelution (data not shown).

Table I presents the results of N-terminal sequencing of the purified N2 and N3 peptides and their deduced position in the human thyroglobulin polypeptide chain (1). After 8 Edman degradation cycles of both peptides, we obtained the same sequence, SYREAASG, corresponding to Ser789-Gly796 on the thyroglobulin polypeptide chain, indicating that both peptides began at Ser797. Four additional Edman degradation cycles on peptide N3 failed to identify the residue at position 797. As an Asn residue was expected at this position, but in a context of a potential glycosylation site (NXS), we inferred that Asn797 was glycosylated. Finally, taking into account both their apparent molecular weights and the possible CNBr cleavage sites within the thyroglobulin corresponding domain (see Fig. 4, lower part), we deduced that the C-terminal residues were Met1172 for peptides N2 and N3, respectively.

Fig. 4 indicates the complete peptide sequence of the immunoreactive peptides N2 (Ser789-Met1008), N3 (Ser789-Met1172), and N1 fusion peptides (Ala1148-Gln1295) (Fig. 4, lower part).
and their positions on the human thyroglobulin polypeptide chain (Fig. 4, upper part). We have observed that the epitope recognized by mAb240 was present on both peptide N3 and the fusion protein N1 (Fig. 3 and Fig. 1, respectively), and the mAb240 epitope is presumably within the sequence Ala1148–Gln1295. We have observed that the epitope recognized by mAb240 was present on both peptide N3 and the fusion protein N1 (Fig. 3 and Fig. 1, respectively), and the mAb240 epitope is presumably within the sequence Ala1148–Gln1295. We have observed that the epitope recognized by mAb240 was present on both peptide N3 and the fusion protein N1 (Fig. 3 and Fig. 1, respectively), and the mAb240 epitope is presumably within the sequence Ala1148–Gln1295.

**Table I**

| Peptide | N-terminal sequence | Apparent Mr | Calculated Mr | Thyroglobulin position |
|---------|---------------------|-------------|---------------|------------------------|
| N2      | SYREAAAG            | 28,000      | 24,987        | Ser^{787–790}–Met^{1083} |
| N3      | SYREAAAGSTGFS       | 39,300      | 42,527        | Ser^{787–790}–Met^{1172} |

FIG. 3. Preparative electrophoresis and immunoblot analysis of human Tg CNBr peptide subfractions. CNBr peptide fragments of fraction II (20 mg) were separated under reducing conditions using a preparative electrophoresis apparatus. After elution of the bromphenol blue, 2.5-ml fractions were collected as described under "Materials and Methods." Aliquots were analyzed by SDS-PAGE electrophoresis on mini-gels (12% resolving gel), and tubes 22–30, 40–46, 65–82, 162–171, and 191–206, corresponding to fractions II1, II2, II3, II4, and II5, respectively, were pooled and further analyzed by repeated SDS-PAGE. A, Coomassie Blue-stained fractions. B, immunoblots of purified fractions probed with mAb78 and mAb240.

FIG. 4. Positions in thyroglobulin and amino acid sequences of isolated peptides. Upper part, diagrammatic representation of human thyroglobulin. The various kinds of repeated motifs and regions of thyroglobulin are represented as boxes. Spaces have been introduced for clarity between contiguous motifs and bear no relationship to gene introns. Open boxes represent the unrelated sequences interrupting some repeats or separating different kinds of repeats (1). Shaded boxes represent the type 1 cysteine-rich repeats (1, 25). The type 3 internal homology (1) is shown in grey boxes, and the acetylcholinesterase-like region (1) is shown as a hatched box. Exon-introns junctions (27) are marked by small arrows or dashed stalks (human) and continuous stalks (other species). Middle part, tyrosine residues potentially involved in hormone formation are represented as lollipops. A, positions of acceptor tyrosine residues (shaded lollipops, positions 5, 1291, 2555, 2569, and 2748) or donor tyrosine residues (open lollipops, positions 5, 130, 986, 1008, 2469, and 2522) in bovine or rabbit thyroglobulin (11). B, positions of acceptor residues (5, 685, 1290, 2572, 2618, and 2765) or donor residues (130, 239) in human thyroglobulin (10). Lower part, the sequences of N2 (Ser^{787–790}–Met^{1083}), N3 (Ser^{787–790}–Met^{1172}), and the fusion protein N1 (Ala^{1148}–Gln^{1295}) are indicated with horizontal arrows N1 (dotted line) and the glycosylation sites of N3 (solid lines) are underlined. The italic letters indicate the amino acid residues common to the N3 and N1 peptides.

Experiments using these peptides as competitors were performed. N3, but not N2, completely abolished the binding (Fig. 6). To check whether conserved amino acid residues of type 1 repeats were involved in binding, we used the N-terminal CNBr homologenic fragment (Asn^{1–Met^{171}}) as competitor. This N-terminal fragment is mainly composed of two type 1 cysteine-rich repeats (about 72% peptide sequence, see Fig. 4 and Refs. 1 and 25). Importantly, neither the N-terminal peptide (Fig. 8), nor the CNBr fragments depleted of the N3 peptide (not shown) inhibited the binding. These data provided evidence that these repeats alone are not directly involved in the thyroglobulin/receptor interaction.

Glycanese Treatment and Lectin Affinity Chromatography of the N3 Peptide—The peptide N3 presents two potential glycosylation sites, i.e. Asn^{797} which is probably glycosylated (see above, Table I) and Asn^{928} (Fig. 4 and Fig. 5). GlcNAc-bearing complex type glycans have been implicated in thyroglobulin-
specific binding (17, 18), and we therefore determined if such glycans and GlcNAc motifs are present on the N3 peptide that carries the receptor binding domain.

Endoglycosidase H treatment using 2 milliunits of enzyme failed to deglycosylate 1.5 $\mu$g of peptide N3 (Fig. 7A). By contrast, three forms of the peptide appeared after treatment with 1.5 milliunits of N-glycanase as follows: the fully glycosylated N3 (about 39 kDa), N3 with one glycan chain (about 36 kDa), and the totally deglycosylated N3 (about 34.5 kDa) (Fig. 7B).

Even when evaluated in reducing conditions, we failed to obtain completely deglycosylated preparations. As shown in Fig. 7B, deglycosylation of 10 ng of $^{125}$I-labeled N3 peptide, using $25 \times 10^{-5}$ to 25 milliunits of glycanase, resulted in the disappearance of only the native 39-kDa peptide and accumulation of both 36- and 34.5-kDa forms. Thus we concluded that N3 was glycosylated at both Asn797 and Asn928 and that the monoglycosylated 36-kDa form was partially resistant to deglycosylation, even by up to 25 milliunits of glycanase (Fig. 7B, forms a–c).

The limited available amount of N3 peptide led us to search for the presence of GlcNAc-bearing glycans by lectin affinity chromatography (Fig. 8) (18, 23). Wheat germ agglutinin binds GlcNAc and, to a lower extent, sialic acid moieties. Fig. 8A shows that a subset of radiolabeled N3 peptide (about 10%) was retained on the column and was selectively released using an elution buffer containing GlcNAc. The same elution pattern was obtained in a control experiment using a neuraminidase-treated N3 peptide (Fig. 8A). This indicates that only GlcNAc moieties were involved in wheat germ agglutinin recognition. These observations were reinforced by the finding that a subset of N3 (about 8.5%) was also selectively retained on a B. simplicifolia II affinity column, another GlcNAc-specific lectin (Fig. 8B). In both cases, no additional binding was observed when unbound fractions were analyzed on fresh columns.

**DISCUSSION**

We identified the thyroglobulin domain involved in receptor binding. It is localized within a stretch of 383 amino acid residues in the N-terminal region, i.e. within the sequence Ser789-Met1172. The antigenic domain recognized by mAb78 was located in the region Ser789-Met1172 (peptide N2), a region encoded by the 3' end of exon 10 and exon 11 (Fig. 5). mAb78 antagonizes the binding of thyroglobulin to its receptor (18) and recognizes the deglycosylated peptides (present data). Peptide determinants involved in receptor interaction are presumably located in this region. Nevertheless, the observation that complete inhibition of thyroglobulin binding was only obtained...
The Receptor Binding Domain of the Thyroglobulin

FIG. 8. Lectin affinity chromatography of the N3 peptide. A, native (○) or neuraminidase-treated (●) 125I-N3 peptides were applied onto WGA columns (150 μl), washed with 15 ml of 10 mM phosphate buffer, pH 7.4, 0.15 M NaCl, 0.2% Nonidet P-40, and then eluted with Gal-elution buffer (1 M D(+)-galactose in wash buffer) and GlcNAc-elution buffer (1 M D(+)-N-acetylglucosamine in wash buffer as described under “Materials and Methods”). B, 125I-N3 peptide was applied onto a BSSII affinity column (300 μl), washed with 50 mM phosphate buffer, pH 7.4, 0.15 M NaCl, 0.1% bovine serum albumin, 0.1 mM CaCl2, MnCl2, and MgCl2 and eluted with 0.5 M GlcNAc in washing buffer.

with the N3 peptide (Ser789-Met1172), but not N2, indicates either the involvement in binding of determinants in the Pro1009-Met1172 sequence, or, alternatively, that this sequence portion is needed for the formation of the three-dimensional structure appropriate for binding. The C-terminal part of N3, namely the Ala1148-Met1172 sequence, is recognized by mAb240, an antibody able to modify the binding domain as judged by its enhancing effect on the thyroglobulin binding (18). This supports the idea that this region is conformationally important.

The thyroglobulin receptor recognizes the Ser789-Met1172 domain consistent with the overall structure and specific determinants of immature molecules being appropriate for intrafollicular retention. These features may then be modified or disappear during maturation to allow the homing of mature prohormone to lysosomes. The Ser789-Met1172 domain fulfills the criteria expected for such a “sensor” domain.

The binding activity of the receptor is partially dependent on the presence of disulfide bonds in thyroglobulin; the reduced prohormone has a higher affinity than the native prohormone for the receptor (18). Flexibility of the binding domain, as for example that observed following binding of the mAb240 antibody to thyroglobulin, facilitates the thyroglobulin-receptor interaction (18). Note that the binding domain is in the N terminus of thyroglobulin. Unlike the C-terminal hormonogenic domain, which is cysteine-poor and probably highly flexible, the N-terminal domain contains a sequence repeated 10 times between position 10 and 1177 (in human thyroglobulin), in which the positions of Cys, Pro, and Gly residues are highly conserved (1, 25). The presence of these Cys-rich repeats, predicted to form a rigid three-dimensional structure, probably involved in disulfide bonds (1, 25, 28), may confer a limited and constrained flexibility to this thyroglobulin region. Formation of some disulfide bonds, which leads to the synthesis of dimeric (19 S) or tetrameric (27 S) forms of thyroglobulin, occurs after the release of thyroglobulin into the follicular lumen (29). Formation of such intermonomer disulfide bonds (and also intramonomer bonds) would be facilitated by the H2O2-generating system involved in the oxidation of iodide and the oxidative coupling of tyrosyls into iodothyronines at the apical cell surface (2). Thus, disulfide bonds may form within (or in the vicinity of) the binding domain during intraluminal thyroglobulin maturation. This process may progressively yield folding units with a rigid three-dimensional structure unfavorable for receptor recognition.

Complex-type glycans containing accessible GlcNAc are found in the receptor binding domain; the N3 peptide is glycosylated at Asn797 and Asn928. Although Asn797 was not predicted as a putative glycosylation site because it is part of a hydrophobic domain (Ref. 1 and Fig. 5), it is flanked by amino acids residues favoring N-glycan grafting (Ser2, Gly1, and Leu127(30)). The location of a glycan unit in such a hydrophobic pocket may have consequences on the ability of the glycanase to interact with and cleave the glycan. Deglycosylation of N3 resulted in 2.5- and 4-kDa shifts of the initial molecular mass, corresponding well with the removal of two glycans of 2.5 and 1.5 kDa each. Some glycans were not complete; using lectin affinity columns, we found accessible GlcNAc residues on N3. These GlcNAc residues usually found on immature thyroglobulin (15) may well be those involved in the thyroglobulin-receptor interaction.

Tyrosyl residues may be essential determinants for binding (31). A survey of the literature to identify which tyrosyl residues in the binding domain may be involved in the receptor interaction indicated the following: 1) in human thyroglobulin, Tyr347, Tyr364, and Tyr372 are iodinated in vitro by lactoperoxidase, and 2) Tyr364 is converted to diiodotyrosyl in vivo, whereas Tyr347 has been considered to be an “attractive candidate for the donor of outer iodothyronyl ring” (2, 10). In bovine thyroglobulin, which presents 85% identity with the human thyroglobulin binding domain (Ser789-Met1172), the search for potential dehydroalanine residues indicated that Tyr386 or Tyr1007, which correspond to Tyr385 and Tyr1007, respectively, in the human sequence, are available for hormonogenesis as donor sites (11). Thus, at least five tyrosyl residues, from Ser789 to Met1172, are solvent-exposed. As these residues are modified by both iodination or hormone formation, they may act as sensors in the regulation of the thyroglobulin/receptor binding activity.

A recent reinvestigation of the thyroglobulin type 1 repeat by Molina et al. (25, 32) showed that it is different than other known cysteine-rich modules. It is found in 32 proteins and is very similar to a cysteine protease inhibitor (33). It is thought to control proteolytic events in some of these proteins. Type 1 modules “could function as binders and reversible inhibitors of the protease involved in the proteolytic processing of thyroglobulin” (32). Binding of thyroglobulin type 1 motifs to proteases in endosomes may thus orient and limit the proteolytic cleavage to hormone-containing domains (32). Note that, in our experimental conditions, thyroglobulin peptides containing the type 1...
repeats other than the peptide N3 were unable to bind to the receptor. Therefore, although interaction of type 1 repeats with proteases is by no means excluded, our observations suggest that the binding domain involved in follicular retention requires a domain specifically involved in the prohormone function (iodination or hormone formation) rather than a type 1 repeat module.

In conclusion, we showed that the thyroglobulin domain involved in receptor recognition is localized within a stretch of 383 amino acid residues in the N-terminal region of the prohormone. This domain carries two complex-type oligosaccharide units, up to five tyrosyl residues involved in iodination or hormone synthesis, and could be disulfide-bonded so as to form bridges between internal Cys-rich repeats. These results provide insight into a domain in which coordinate modifications (glycan completion, iodination, hormone synthesis and disulfide bonding) may regulate the receptor/thyroglobulin binding activity and thus the retention of thyroglobulin within the follicular lumen.

Acknowledgments—We are grateful to Drs. B. Mallet and P.-J. Lejeune, Faculté de Médecine, Marseille, France, for supplying human thyroglobulin and human thyroglobulin CNBr peptides. We thank Prof. Y. Malthiéry, Faculté de Médecine, Angers, France, for providing plasminogen activator antibodies mAb78 and mAb240, and Prof. H. Rochat for the facilities for HPLC peptide purification and sequencing. Thanks are also due to Drs. F. Molina and C. Granier, Faculté de Pharmacie, Montpellier, France, for sharing results prior to publication.

REFERENCES
1. Malthiéry, Y., and Lissitzky, S. (1987) Eur. J. Biochem. 165, 491
2. Bjorkman, U., and Ekholm, R. (1990) in Biochemistry of Thyroid Hormone Formation and Secretion (Greer, M. A., ed) pp. 83–125, Raven Press, Ltd., New York
3. Ekholm, R., and Wollman, S. (1975) Endocrinology 97, 1432–1454
4. Gavaret, J.-M., Nunez, J., and Cahnmann, H. J. (1980) J. Biol. Chem. 255, 5281–5285
5. Gavaret, J.-M., Cahnman, H. J., and Nunez, J. (1981) J. Biol. Chem. 256, 9167–9173
6. Rawitch, A. B., Cheroff, S. B., Litwer, M. R., Rouse, J. B., and Hamilton, J. W. (1983) J. Biol. Chem. 258, 2079–2082
7. Dunn, J. T., Anderson, P. C., Fox, J. W., Fassler, C. A., Dunn, A. D., Hite, L. A., and Moore, R. C. (1987) J. Biol. Chem. 262, 16948–16952
8. Fassler, C. A., Dunn, J. T., Anderson, P. C., Fox, J. W., Dunn, A. D., Hite, L. A., Moore, R. C., and Kim, P. S. (1988) J. Biol. Chem. 263, 17366–17371
9. Roe, M. T., Anderson, P. C., Dunn, A. D., and Dunn, J. T. (1989) Endocrinology 124, 1327–1332
10. Lamas, L., Anderson, P. C., Fox, J. W., and Dunn, J. T. (1989) J. Biol. Chem. 264, 13543–13545
11. Ohmiya, Y., Hayashi, H., Kondo, T., and Kondo, Y. (1990) J. Biol. Chem. 265, 9066–9071
12. Merken, L., Simon, M. J., De Martynoff, G., Swillens, S., and Vassart, G. (1985) Eur. J. Biochem. 147, 59–64
13. Bastiani, P., and Simon, C. (1977) Acta Endocrinol. 85, 357–363
14. Miquelis, R., and Simon, C. (1980) Acta Endocrinol. 95, 489–494
15. Bastiani, P., Papandreou, M. J., Blanck, O., Fenouillet, E., Thibault, V., and Miquelis, R. (1995) Endocrinology 136, 4204–4209
16. Miquelis, R., Courageot, J., Jaeg, A., Blanck, O., Perrin, C., and Bastiani, P. (1993) J. Cell Biol. 123, 1685–1706
17. Miquelis, R., Alquier, C., and Monaygu, M. (1987) J. Biol. Chem. 262, 15291–15298
18. Miziaut, H., Bastiani, P., Balivet, T., Papandreou, M. J., Fort, V., Erregragui, K., Blanck, O., and Miquelis, R. (1996) Endocrinology 137, 1570–1577
19. Henry, M., Malthiéry, Y., Zanelli, E., and Charvet, B. (1999) J. Immunol. 164, 3692–3698
20. Marrig, C., Lejeune, P. J., Venet, N., Rolland, M., and Lissitzsky, S. (1986) FEBS Lett. 207, 302–306
21. Mallet, B., Lejeune, P.-J., Baudry, N., Nicoli, P., Carayon, P., and Franc, J.-L. (1995) J. Biol. Chem. 270, 29881–29888
22. Xiao, S., Pulloch, H. G., Tawro, A., and Rawitch, A. (1995) Arch. Biochem. Biophys. 320, 96–105
23. Stanley, K. K., and Lazio, J. P. (1984) EMBO J. 3, 1429–1443
24. Witheheart, S. W., Passatani, A., Reicher, J. S., Holt, G. D., Haltiwanger, R. S., and Hart, G. W. (1989) Methods Enzymol. 179, 82–95
25. Molina, F., Bouanani, M., Pau, B., and Granier, C. (1996) Eur. J. Biochem. 240, 125–133
26. Kyle, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132
27. Parma, J., Christophe, D., Pohl, V., and Vassart, G. (1987) J. Mol. Biol. 196, 769–779
28. Gentile, F., and Salvatore, G. (1993) Eur. J. Biochem. 218, 603–621
29. Edelhoch, H. (1985) in Thyroglobulin, the Prothyroid Hormone (Eggo, M. C., and Burrow, G. N., eds) pp. 1–11, Raven Press, Ltd., New York
30. Imberty, A., and Perez, S. (1995) J. Mol. Biol. 251, 3692–3698
31. Strack, H., and Kohn, D. (1988) J. Biol. Chem. 263, 1327–1332
32. Molina, F., Papandreou, M. J., Blanck, O., Carbonetti, F., and Miquelis, R. (1996) FEBS Lett. 391, 229–231
33. Yamashita, M., and Konagaya, S. (1996) J. Biol. Chem. 271, 1282–1284