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Expression analysis of the thyrotropin-releasing hormone receptor (TRHR) in the immune system using agonist anti-TRHR monoclonal antibodies

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Abstract Monoclonal anti-rat thyrotropin-releasing hormone (TRH) receptor (TRHR)-specific antibodies (mAb) were generated by immunization with synthetic peptides of rat TRHR partial amino acid sequences; one (TRHR01) was directed against a sequence (84–98) in the extracellular portion of the rat TRHR reported to be constant among different species, including man, and the second (TRHR02) recognizes the C-terminal region sequence 399–412. In lysates from GH_4C_1 cells, a clonal rat pituitary cell line, both mAb recognize the TRHR in Western blot analysis, and TRHR02 immunoprecipitates the TRHR. Incubation of GH_4C_1 cells with the mAb causes a fluorescence shift in fluorescence-activated cell sorting analysis. The cells were stained specifically by both mAb using immunocytochemical techniques. Furthermore, TRHR01 is agonistic in its ability to trigger Ca^{2+} flux, and desensitizes the TRH receptor. We tested for TRHR in several rat organs and found expression in lymphoid tissues. TRHR01 recognizes the human TRHR, and analysis of human peripheral blood lymphocyte and tonsil-derived leukocyte populations showed receptor expression in non-activated and phytohemagglutinin-activated T and B cells.

Key words: Thyrotropin-releasing hormone; Receptor; Monoclonal antibody; Immune system

1. Introduction

Thyrotropin-releasing hormone (TRH) is the first hypophysiotropic hormone whose structure was elucidated and chemical synthesis achieved [1,2]. TRH was discovered in the hypothalamus and characterized for its ability to stimulate pituitary thyrotropin secretion; thereafter, it was identified in several extrahypothalamic brain structures and in other organs and tissues [3]. TRH receptors (TRHR) have been studied extensively (for a review, see [4]). TRH acts via the phosphatidylinositol-calcium-protein kinase C transduction pathway, although the existence of other effectors has been claimed, including adenylyl cyclase [5]. Evidence [6,7] is consistent with the fact that $G_{q/11}$ proteins [8,9] couple the TRHR to a phospholipase C that hydrolyzes phosphatidylinositol 4,5-diphosphate, although some experimental data strongly suggest that other G proteins couple TRHR to different enzymatic systems that may act as TRH transducers [10–13]. cDNA cloning from mouse thyrotropic cells [14] and rat pituitary-derived clonal cell strains GH_4C_1 and GH_3 [15,16] showed that the TRHR belongs to the seven transmembrane-spanning G protein-coupled membrane receptor family. Two receptor isoforms generated by alternative splicing have been identified [17]. Although considerable information has accumulated on TRHR function, and specific residues have been implicated in hormone binding [18–22], for a complete understanding of TRHR biology, improved knowledge is necessary of the molecular mechanisms involved in receptor regulation and in its interaction with transducers.

We generated TRHR-specific monoclonal antibodies (mAb) by immunizing mice with synthetic peptides of amino acid sequences in the first extracellular loop (84–98) and the intracellular C-terminal region (399–412) of the rat TRHR. Here we describe the production and characterization of these mAb, as well as a functional analysis based on their ability to mimic TRH activity. We analyzed receptor expression in rat lymphoid organs by Western blot; taking advantage of the cross-reactivity with the human sequence, we studied TRHR in human peripheral blood mononuclear cells (PBMC) as well as tonsil cells and show its expression in unstimulated and phytohemagglutinin (PHA)-activated T and B cells.

2. Materials and methods

2.1. Immunization and cell fusions

Two peptides of rat TRHR amino acid sequences deduced from the large isoform cDNA [16] were synthesized on an automated peptide synthesizer (AMS422 Abimed, Langefeld, Germany); they correspond to the linear sequences 84–98 (TDSIYGSWVYGYVGC) and 399–412 (CTYGYSLTAKQEKI). The peptides were keyhole limpet hemocyanin (KLH)-conjugated (Pierce, Rockford, IL, USA) and BALB/c mice immunized as described [23].

Spleen and/or lymph node cells from immunized mice were fused with the P3X63-Ag8.653 myeloma cell line (CRL 1580, American Type Culture Collection, Manassas, VA, USA) following standard protocols [24,25]. Supernatants were tested for antibodies in enzyme-linked immunoassays (EIA), and positive hybridomas were cloned.

2.2. EIA and isotype determination

EIA were performed essentially as described [26], using synthetic peptides (3 μg/ml in PBS, 100 μl/well) adsorbed to 96-well plates

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Abbreviations: EIA, enzyme-linked immunoassay; GAM, goat anti-mouse immunoglobulin antibody; FITC, fluorescein isothiocyanate; KLH, keyhole limpet hemocyanin; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; PHA, phytohemagglutinin; PO, peroxidase; SF, supernatant fluid; TRHR, yhyrotropin-releasing hormone receptor; TBS, Tris-buffered saline

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(Maxisorb, Nunc, Denmark). Antibody isotypes were determined in EIA using peroxidase (PO)-labeled subclass-specific antisera (ICN, Irvine, CA, USA).

2.3. Stable transfection and cell culture

Rat TRHR cDNA (large isoform) subcloned into the pLTR-neoE eukaryotic expression vector was expressed in NIH-3T3 cells (CRL-1658, ATCC). Cells were cultured in Dulbecco's modified Eagle: Ham's F-12 medium (1:1 mixture, Sigma Chemicals, St. Louis, MO, USA) containing phenol red, 0.13% $NaHCO_3$, 10% FBS and penicillin/streptomycin. For transfection experiments, trypsin-treated confluent cells were plated at a 1:15 dilution in 100 mm diameter culture dishes. After 12–16 h, cells were transfected using a standard calcium phosphate coprecipitation protocol [27] at 30 μg of DNA/dish, incubated for 18–24 h, trypsin-treated and replated in selective medium containing 0.8 mg/ml geneticin (G-418, Gibco BRL, Paisley, UK). TRHR expression was confirmed and quantified after 3 weeks of culture in selective medium, following [^{3}H]methyl-histidyl-TRH (Dupont NEN, Boston, MA, USA) binding to transfected cells (data not shown). GH_4C_1 cells (CCL-82.2, ATCC) were grown in monolayer culture in Ham's F10 medium with 10% FBS [28].

2.4. Flow cytometry analysis

GH_4C_1 T cells (2×10^5/100 μl) were stained as described [29] with anti-TRHR mAb or a mouse isotype-matched control mAb, mIgM (1 μg/50 ml/well for 60 min, 4°C). When necessary, cells were permeabilized with 100 μl of 70% ethanol for 10 min at 4°C before staining. The anti-human growth hormone receptor mAb hGHR05 [29] was used as an isotype-matched control.

Human peripherla blood lymphocytes (PBL) or tonsil-derived lymphocytes were purified by Ficoll-Paque (5.7 g/l, Pharmacia) and activated with PHA (0.5%; Difco, Detroit, MI, USA), or ionomycin (1 μM; Sigma) for 5 days at 37°C, 5% CO_2 in the presence of human interleukin-2 (IL-2) (rIL-2; Hoffmann-La Roche, Nutley, NJ). Human PBL or tonsil-derived cells were stained with biotin-labeled anti-TRHR mAb and streptavidin-FITC or -phycoerythrin (PE), simultaneously with PE- or FITC-labeled anti-human leukocyte differentiation markers for B (CD19, Coulter) or T cells (CD4 and CD8, Coulter). Double staining of cells was determined as above.

2.5. Immunoprecipitation, SDS-PAGE and Western blot analysis

Cells (1×10^7) were centrifuged and resuspended in 100 μl of lysis buffer (50 mM Tris-HCl, pH 7.6, 1% Nonidet P-40, 250 mM NaCl, 0.5 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate and a cocktail of protease inhibitors). After incubation (20 min, 4°C), samples were centrifuged ($15\,000\times g$, 15 min, 4°C) and the pellet discarded. Protein extracts (SF) were separated in 12.5% SDS-PAGE [30] and transferred to nitrocellulose membranes for Western blot analysis as described [29].

Immunoprecipitation was performed as previously described [31]. Briefly, SF fractions were precleared by incubation with anti-mouse IgM-agarose (20 μg; Sigma) for 60 min at 4°C and centrifugation ($15\,000\times g$, 1 min), then immunoprecipitated with mAb TRHR02 (5 μg/sample) for 90 min at 4°C, followed by anti-mouse IgM-agarose (20 μg, 60 min, 4°C). Samples were centrifuged ($15\,000\times g$), agarose pellets were washed twice with lysis buffer, three times with 50 mM Tris-HCl, pH 7.6, resuspended in Laemmli buffer and electrophoresed.

2.6. Calcium determination

Changes in intracellular calcium concentration were monitored using the fluorescent probe Fluo-3AM (Molecular Probes, Eugene, OR, USA). NIH-3T3 cells transfected with cDNA encoding the rat TRHR and pLTR-neoE-transfected control cells were suspended in RPMI containing 10% FBS and 10 nM HEPES, then incubated with 12 μl/10^6 cells of Fluo-3AM (300 μM in DMSO) for 30 min at 37°C as previously described [31].

2.7. Immunocytochemistry

GH_4C_1 and pLTR-neoE-transfected NIH-3T3 cells were cultured on glass coverslips, then fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4) for 30 min at room temperature. After three washes with PBS, cells were incubated in 0.1% sodium borohydride for 30 min to quench free aldehyde groups, permeabilized with 0.02% Triton X-100 in PBS for 30 min and incubated with 0.2% gelatin in PBS to prevent non-specific antibody binding. Cells were incubated alone or with antibody (TRHR01, TRHR02 or hGHR05, 1:5 supernatant dilution) in PBS for 1 h at room temperature, and immunoperoxidase stained using the avidin-biotin-PO complex (Vectastain Kit, Vector Laboratories, Burlingame, CA, USA). The chromogen (0.06% diaminobenzidine in 0.03% H_2O_2) was incubated for 5–10 min, depending on microscopic evaluation of wet-mount coverslips.

2.8. Animal studies

Normal adult male Wistar rats (200–250 g) were used to study TRHR in lymphoid tissues. Rats were killed by decapitation, and organs (thymus, mesenteric lymph nodes and spleen) were placed in dry ice and maintained at −80°C until processed. They were then placed in lysis buffer as for immunoprecipitation assays and homogenized using a Polytron (Kinematica A). Homogenates were centrifuged ($20\,000\times g$, 20 min, 4°C) and the supernatant maintained at −80°C until electrophoresis and blotting were performed. Protein loading was controlled using a protein detection kit (Pierce). Electrophoresis and Western blots were performed as above.

3. Results

3.1. Characterization of TRHR-specific mAb

Spleen and lymph node cells from two mice immunized with synthetic peptides of rat TRHR amino acid sequences 84–98 and 393–412 (found in the first extracellular loop and the intracellular C-terminal region, respectively) [16] were fused with the P3X63-Ag8.653 myeloma cell line. After culture and screening, one hybrid producing mAb to the first extracellular loop (TRHR01) and another producing mAb to the C-terminal region (TRHR02) were selected for further stabilization and characterization. Isotype determinations showed that both mAb are IgM, κ.

To determine mAb specificity, binding studies were performed using fluorescence-activated cell sorting analysis. A significant fluorescence shift was observed in intact rat pitui-

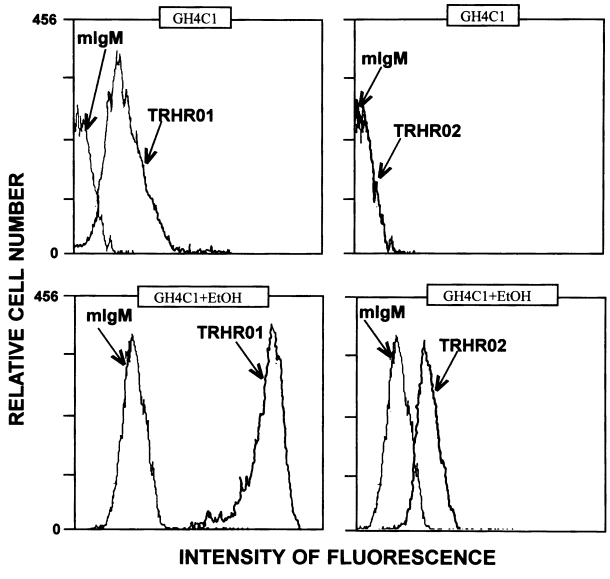

Fig. 1. mAb binding to the GH_4C_1 line. GH_4C_1 cells in exponential growth, intact (GH4C1, upper panels) or permeabilized with ethanol (GH4C1+EtOH, lower panels), were stained with mAb TRHR01, TRHR02 or mIgM (hGRH05) as indicated, followed by GAM-FITC as described in Section 2. Histograms show fluorescence intensity of mAb staining (x axis) versus relative cell number (y axis), compared with that of an isotype-matched control mAb. The figure depicts one representative experiment of five performed.

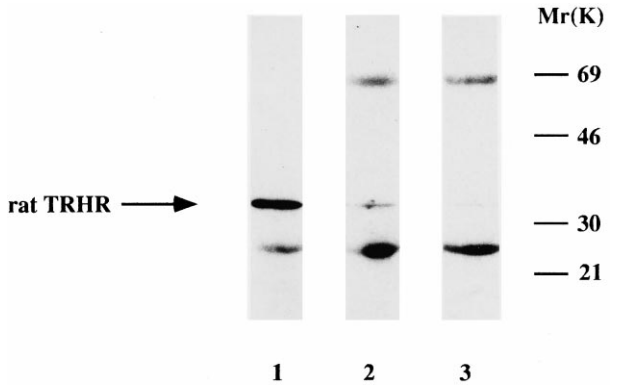

Fig. 2. Immunoprecipitation of GH_4C_1 cell lysates by TRH02. GH_4C_1 cell lysates (1×10^7 cells) were immunoprecipitated using 5 μg/ml of TRHR02. The pellet was immunoblotted using TRHR02 (10 μg/ml; lane 1), TRHR01 (10 μg/ml; lane 2) and isotype-matched mAb (10 μg/ml; lane 3). Other bands correspond to the heavy and light chains of the immunoprecipitating immunoglobulin. A representative autoradiogram is shown. The arrow indicates the position of rat TRHR.

tary GH_4C_1 cells incubated with TRHR01, whereas no binding was detected using TRHR02 or a control mAb (Fig. 1A,B). When cells were ethanol permeabilized, both TRHR01 and TRHR02, but not the control mAb, induced a fluorescence shift (Fig. 1C,D). Both mAb recognize a specific band in Western blot analysis of GH_4C_1 cell lysates (not shown). TRHR02 immunoprecipitates a GH_4C_1 cell lysate protein that is recognized in Western blot analysis by both mAb (Fig. 2).

To ensure the specificity of these anti-rat TRHR mAb, NIH-3T3 cells were transfected with cDNA encoding the rat TRHR subcloned into the pLTR-neoE eukaryotic expression vector. Receptor expression was confirmed and quantified using [^{3}H]methyl-TRH binding analysis, as described in Section 2. Transfected cell lysates were used to confirm mAb reactivity in Western blot analysis; TRHR02 specifically recognized a single band not present in pLTR-neoE-transfected cells (Fig. 3).

To further test specificity, GH_4C_1 cells were immunostained with TRHR01 (Fig. 4A) and TRHR02 (Fig. 4B). No staining was observed in fibroblasts not expressing TRHR, or in GH_4C_1 cells in which control mAb hGHR05 was used (not shown). The immunoperoxidase end product was located at the cell periphery, as a diffuse reaction distributed throughout the cytoplasm and as an intense granular precipitate within the cytoplasm (Fig. 4C,D). No nuclear staining was seen. Distribution of the immunoreactive material was similar using both mAb, but was fainter in TRHR02 staining of non-permeabilized cells, concurring with the intracellular localization of the epitope (data not shown).

3.2. TRHR01 mimics TRH-induced Ca^{2+} mobilization

We analyzed the effect of the mAb on TRH-induced Ca^{2+} mobilization. Fluo-3AM-loaded NIH-3T3 cells transfected with rat TRHR cDNA were preincubated with purified mAb before TRH stimulation, resulting in blockage of TRH-promoted Ca^{2+} mobilization by mAb TRHR01. No effect was seen when cells were preincubated with control or TRHR02 mAb (not shown). We also tested whether the mAb has intrinsic TRH-like activity, as TRH function blockade in Ca^{2+} mobilization assays could be due to either agonist or antagonist activity leading to receptor desensitization. Stimulation with mAb TRHR01 induces a rapid, transient increase in Ca^{2+} concentration and desensitizes the TRHR in response to TRH; TRH stimulation also induces receptor desensitization to the TRHR01 mAb (Fig. 5A–D). As expected, pLTR-neoE-transfected 3T3 cells did not respond to either ligand (TRH of TRHR01) (Fig. 5E,F). Fluo-3AM loading equivalence was determined by Ca^{2+} mobilization in response to the calcium ionophore ionomycin (5 μg/ml). The activity of mAb TRHR01, specific for the first extracellular loop (amino acids 84–98) of the TRHR, thus indicates an important role for this region in ligand-induced biological activity.

3.3. The TRHR is expressed in T and B cells and in lymphoid organs

Recent evidence indicates a possible role for TRH in the development of certain lymphoid cells [32], and indirect data support the presence of the TRHR in thymocytes [33]; we thus tested for TRHR in extracts of rat lymphoid organs. Electrophoresis and Western blot of these extracts with TRHR02 showed a specific protein corresponding to the rat TRHR (Fig. 6) in thymus, mesenteric lymph nodes and spleen extracts, whereas no reaction was observed in control liver extracts.

The specificity of these mAb and the cross-reactivity of TRHR01 with human TRHR, as the 84–98 sequence is also found in the human receptor, led us to test for TRHR expression in various leukocyte populations. Resting and PHA- or ionophore-activated PBMC were analyzed in flow cytometry by double-color staining using biotin-labeled TRHR01 in conjunction with specific B and T cell markers. Based on these assays, TRHR is expressed in PBMC-derived B (30% of $CD19^+$ cells) and T cells (40% $CD4^+$ and 18% $CD8^+$). Tonsil-derived B (70% of $CD19^+$ cells) and T cells (30% $CD4^+$ and 8% $CD8^+$ cells) also express the TRHR (Fig. 7). TRHR expression in B and T cells is unaltered following PHA activation, indicating constitutive TRHR expression in these cells (Fig. 7).

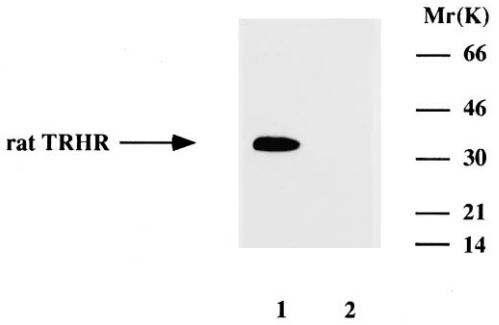

Fig. 3. Western blot analysis of NIH-3T3 cells transfected with cDNA encoding the rat TRHR. NIH-3T3 cells transfected with the cDNA encoding the rat TRHR (lane 1) or pLT-neoE-transfected NIH-3T3 cells (lane 2) (1×10^7 cells) were lysed and immunoblotted as described in Section 2 using TRHR02 (10 μg/ml). Protein loading was controlled using a protein detection kit (Pierce). Arrow shows the position of rat TRHR.

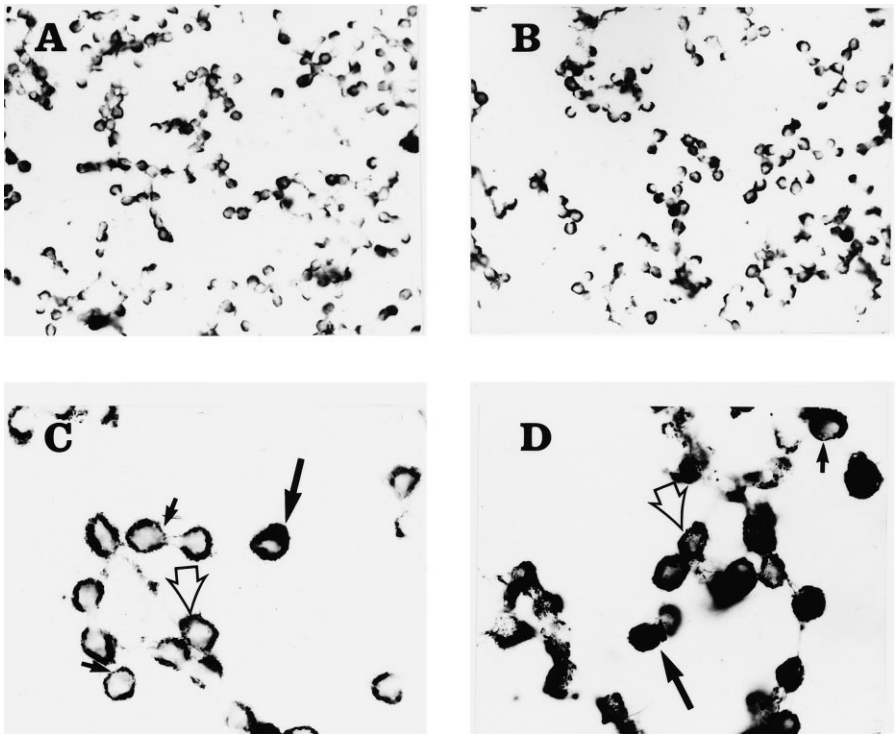

Fig. 4. Immunocytochemistry of GH_4C_1 cells using TRHR01 and TRHR02. Immunostaining of GH_4C_1 cells using TRHR01 or TRHR02 is shown in panels A and B, respectively. Note that there is a significant number of intensely stained cells, whereas others are weakly labeled. High-power light micrographs show the differential distribution of immunoreactive material in GH_4C_1 cells stained with TRHR01 (C) or TRHR02 (D). The reaction is restricted to the cell periphery (small arrows), intense granular staining within the cytoplasm (open arrows) or diffuse cytoplasmic immunoprecipitate (long arrows). A and B: magnification, 340×; C and D, 850×.

4. Discussion

Study of the biochemical characteristics of TRHR has been hampered by the lack of appropriate reagents. Detergent-based analysis failed mainly because detergents disrupt receptor interaction with the labeled ligand, and as the ligand could not be covalently cross-linked to its receptor (data not shown). Straub et al. [10] nevertheless established the structure of the murine TRHR based on its cDNA sequence; this structure, corresponding to that of a G protein-coupled receptor (GPCR), has been confirmed by various groups and reported for several species. Monoclonal antibodies have been widely employed for the characterization and biochemical analysis of several receptors, including those of the GPCR superfamily [34]. Here we describe the production and characterization of two mAb that specifically bind TRHR in GH_4C_1 pituitary cells, and analyze their function based on their ability to mimic TRH activity.

Synthetic peptides of rat TRHR sequences were chosen as immunogens as they permit preselection of receptor regions of interest. Several sequences were chosen in a first approach, based on various criteria: first, on the amino acid sequence analysis, which allows selection of receptor regions that are species-specific or are present in other species, enabling generation of specific or potentially cross-reactive mAb. The second criterion was for sequences that include amino acids implicated in ligand binding, which would be useful in generating mAb that might affect TRH activity, and third, sequence-based structural requirements that might give rise to peptides that resemble receptor conformations. These criteria do not ensure the desired immune response, however, as seen for immunogens including sequences from the TRHR second and third extracellular loops, which failed to elicit antibody responses in our hands. The amino acids in these two loops are important in the initial interaction of TRH with its receptor [35]. Production of mAb with possible agonist activity should nonetheless not be restricted to sequences known to be involved in the ligand binding site. It has been shown that mAb against epitopes not involved in the ligand binding site of the growth hormone receptor can mimic growth hormone (GH) activity by stabilizing an active conformation of this receptor [29]. In vitro, TRHR01 recognizes a peptide corresponding to a sequential epitope in the first extracellular loop (amino acid sequence 84–98), a sequence also present in the human TRHR. TRHR02 binds an epitope in the intracellular C-terminal region of the rat TRHR (amino acids 399–412).

In several assays, including flow cytometry, Western blot, immunoprecipitation and immunohistochemistry, TRHR01 and TRHR02 bind specifically to the rat GH_4C_1 cell line, which expresses a large number of TRHR copies. Consistent with the intracellular location of the epitope recognized by TRHR02, cell permeabilization is necessary to allow TRHR02 interaction with the receptor in flow cytometry assays. TRHR01 binding increases after permeabilization; this treatment may improve exposure of the sequential epitope recognized, or make accessible receptor molecules located in other intracellular compartments.

Both TRHR01 and TRHR02 recognize a specific band in Western blot analysis of GH_4C_1 cell lysates; this band is also detected by polyclonal antibodies from animals immunized with other rat TRHR sequences (not shown). TRHR02 im-

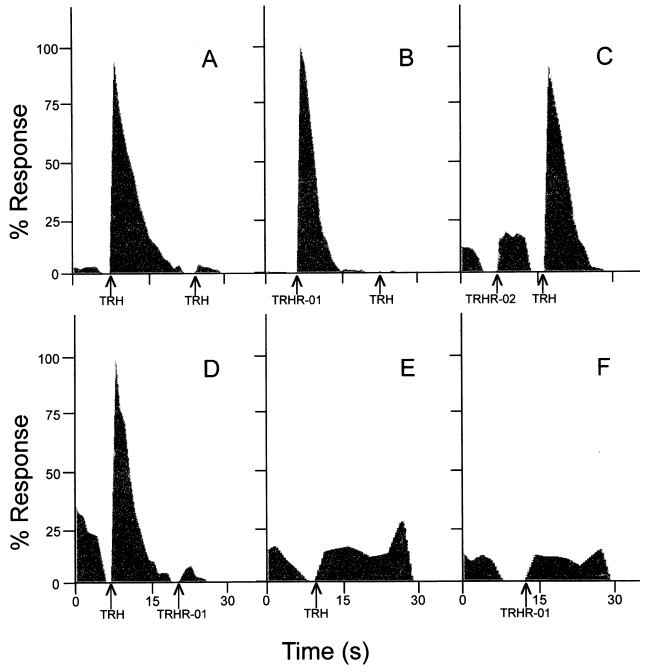

Fig. 5. TRHR01 mAb promotes Ca^{2+} mobilization and desensitizes the TRH-induced response. NIH-3T3 cells transfected with cDNA encoding the rat TRHR were loaded with Fluo-3AM and processed as indicated in Section 2. Ca^{2+} mobilization was induced with TRH (1 μg/ml), TRHR01 or TRHR02 (20 μg/ml). TRH promotes Ca^{2+} mobilization, and cells were desensitized to a second stimulation (A). TRHR01 induces the same activity and desensitizes the cells to a TRH challenge (B). TRHR02 has no effect and does not modify the TRH response (C). TRH also desensitizes the cell to a challenge with TRHR01 (D). As a control, pLTR-neo-transfected NIH-3T3 cells were stimulated by TRH (E) or TRHR01 (F). Results are expressed as a percentage of the TRH-induced calcium response. The figure depicts the results of one experiment representative of five performed. Arrows depict the time of stimulus addition.

munoprecipitated the same protein from GH_4C_1 cell lysates. To demonstrate clearly the specificity of these mAb, NIH-3T3 cells were transfected with the cDNA encoding the rat TRHR. The presence of functional membrane receptors was shown by their ability to bind TRH, and by the response to TRH-promoted Ca^{2+} mobilization. TRHR01 stained the transfected cells and TRHR02 recognized the same band in Western blot analysis of cell lysates, whereas no recognition was observed in pLTR-neoE-transfected 3T3 cells.

In ligand binding and receptor mRNA expression in oocytes, Gautvik et al. [36,37] presented evidence for two TRHR types in rat GH_4C_1 cells; in Southern blot hybridization analysis of GH_4C_1 and GH_3 cells, however, others found a pattern consistent with a single gene copy [15,17]. Ligand binding and Western blot analysis of GH_4C_1 cell lysates using our mAb support the second hypothesis, corroborating the existence of only one rat TRHR type.

The cloning and characterization of a novel subtype of rat TRH receptor, rTRHR2, was recently described [38,39]; this receptor appears to be restricted to the central nervous system, in particular to the sensory neurons of the spinothalamic tract and the spinal cord dorsal horn. This new TRHR lacks 44 C-terminal amino acids of the classic TRHR (rTRHR1), which include the epitope recognized by TRHR02. The TRHR01 mAb recognizes the amino acid sequence TRHR1 (84–98), which has a high degree of identity with the corresponding sequence in rTRHR2, indicating that this mAb may recognize both subtypes.

The 19 amino acid C-terminal extension of the rat TRHR is probably not critical for ligand binding or G protein coupling [15,16]; TRHR02 may thus be of use in determining whether this sequence has a role in receptor regulation. As this mAb immunoprecipitates the receptor, it may also serve to study possible signal transduction molecule association to the TRHR.

Specific immunostaining was observed in GH_4C_1 cells incubated with TRHR01 and with TRHR02. The difference in the expression level between permeabilized and non-permeabilized cells may indicate improved epitope exposure in the ethanol-treated cells. Both antibodies recognize the TRHR specifically, as only cells expressing this receptor were reactive. The location of staining at the cell periphery and plasma membrane conforms with the location expected for a membrane receptor. Intracytoplasmic staining may indicate intracellular receptor pools in the processes of synthesis, endocytosis and trafficking [40–42]. Electron microscopy studies will be necessary to elucidate the precise location of these TRHR pools.

More important is the evidence that TRHR01 has agonistic activity, mimicking TRH-promoted Ca^{2+} mobilization and evoking desensitization of the response to the natural ligand. The same effect occurs in the inverse experiment, in which TRH desensitizes the cells in response to TRHR01. The effect is clearly specific, since the isotype-matched control mAb TRHR02, which recognizes an intracellular epitope, promotes no effect. Neither was any effect observed for TRH or for TRHR01 when Ca^{2+} mobilization assays were performed in pLTR-neoE-transfected NIH-3T3 cells, although they were equally loaded with Fluo-3AM.

In the absence of other data mapping the TRHR residues implicated in TRH binding, our data support a critical role for amino acids 84–98 in TRHR activity. The effect may be due to direct interaction with the ligand binding epitope or to the stabilization of an active receptor state. The latter case has been shown in other receptors such as the human growth hormone receptor (hGHR), for which a mAb that does not compete for GH binding can stabilize an active hGHR conformation [29]. Our data suggest that regions other than those previously implicated in TRH binding may also be important

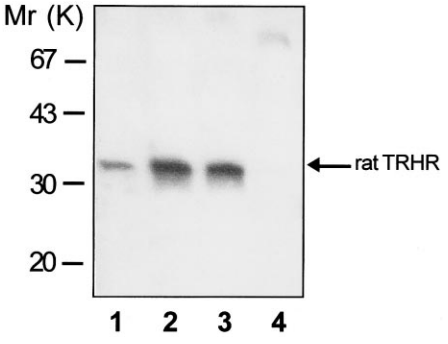

Fig. 6. TRHR is expressed in rat lymphoid organs. Thymus (lane 1), lymph nodes (lane 2), spleen (lane 3) and liver (lane 4) as a control, were homogenized and immunoblotted as described in Section 2 using TRHR02 (10 μg/ml). Protein loading was controlled using a protein detection kit (Pierce). The arrow shows the position of rat TRHR.

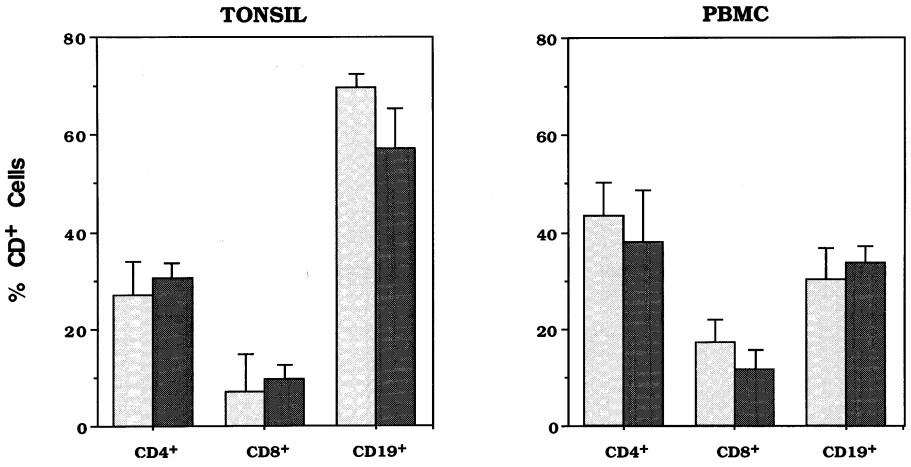

Fig. 7. TRHR expression on human lymphocytes. TRHR expression in resting (white bars) or activated (black bars) tonsil- or PBMC-derived T or B cells, using double color staining with TRHR01 and anti-T (CD4, CD8) or anti-B cell (CD19) antibodies, as indicated. The figure shows the percentage of cells expressing the TRHR, as defined by TRHR01 binding in flow cytometry in various lymphocyte populations defined by the indicated CD markers. Data represent the mean of triplicate determinations, with the S.D. indicated.

for ligand binding, for receptor activation, or for both. A dichotomy between binding and activating regions has been shown for other GPCR, as is the case for chemokine receptors. Assays using chimeric chemokine receptors and mAb suggest a two-step mechanism, indicating dissociation between regions involved in ligand binding and activation [34,43,44].

The finding of TRHR in lymphoid tissues and cells is also interesting, and suggests an unknown role for this hypothalamic hormone in these cells. Studies in athymic mice indicate that TRH and TSH significantly influence the development of lymphoid cells associated with intestinal intraepithelial lymphocytes [45]. Data also support the presence of the TRHR in thymocytes, as it has been shown that TRH promotes thymic reconstitution in mice with anterior hypothalamic area lesions [32]. Northern blot analyses have recently identified TRHR mRNA in immune cells [33]; the use of TRHR01 and TRHR02 extends this to the presence of the protein in lymphoid organs, as well as in unstimulated and PHA-activated PBMC and tonsil-derived B and T cells. These findings indicate constitutive TRHR expression in these cells and confirm previous data showing that activation does not affect TRHR mRNA levels in PBMC [33].

The mAb staining pattern described confirms an active role for TRH in the immune system [46] and opens new perspectives. Their specificity renders these mAb of considerable value for receptor localization analysis in tissues and cell lines, as well as for studies of cell trafficking, hormone binding-related motifs, proteins associated following binding, and residues implicated in receptor regulation. In view of the recent description of the novel TRHR subtype, new experiments are in progress to determine whether these mAb will aid in dissecting the responses triggered by one or the other TRHR type.

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