BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **228,** 21–28 (1996)
ARTICLE NO. 1611

Epitope-Tagging of a Functional Thyrotropin Receptor: Detection of the Native Receptor on Intact Cells

Kunihiko Tanaka,* Yuji Nagayama,* Hironori Yamasaki,† Hideki Hayashi,‡ Hiroyuki Namba,§ Shunichi Yamashita,§ and Masami Niwa*

**Department of Pharmacology 1 and †First Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki, ‡Department of Enzyme Genetics, Institute of Enzyme Research, University of Tokushima, Tokushima; and §Department of Cell Physiology, Atomic Disease Institute, Nagasaki University School of Medicine, Nagasaki 852, Japan*

Received September 6, 1996

To facilitate immunological detection of thyrotropin receptor (TSHR), we inserted a *c-myc* epitope within the unique, 50 amino acid segment of the ectodomain (TSHRmyc). When stably expressed in 293 human embryonal kidney (HEK) cells, TSHRmyc demonstrated high affinity TSH binding and the ability to produce cAMP in response to TSH. Binding of the *myc* monoclonal antibody 9E10 to 293-TSHRmyc cells could be detected with [^{125}I] anti-mouse IgG. No competition was observed between TSH and 9E10 binding to 293-TSHRmyc. Immunoprecipitation by 9E10 of TSHRmyc revealed TSHR forms of ~95 and ~100 kDa. Endoglycosidase digestion identified the ~95 kDa species as the single chain precursor with high mannose carbohydrate. The ~100 kDa single chain receptor contained mature, complex carbohydrate. No smaller species of TSHR subunits or proteolytic fragments was observed. Again TSH did not inhibit immunoprecipitation of TSHRmyc by 9E10. These data demonstrate that the normally functioning *c-myc* epitope-tagged TSHR can be detected directly and in native form with a readily available anti-*myc* 9E10 and without the need for prior affinity capture. Lack of competition between 9E10 and TSH suggests that at least part of the 50 amino acid segment in TSHR ectodomain is not a TSH binding site. This epitope-tagged TSHR will be valuable for further studies on the synthesis and trafficking of TSHR.

The structure of TSHR has attracted considerable attention, not only because of its physiological significance in thyroid cell function, but also because of its role in thyroid autoimmunity (1-3). Polyclonal (4,5) and, in particular, monoclonal antibodies (mAb) to TSHR generated by a number of laboratories (6-9) have proven invaluable in their ability to detect and characterize TSHR expressed various expression systems, such as prokaryotic, insect and mammalian cells. Of these, mammalian cells provide the greatest opportunity for studying TSHR under relatively physiological conditions.

Monoclonal antibodies have been used to identify TSHR on the surface of transfected mammalian cells by flow cytometry, as well as in cell homogenates by immunoblotting. However, most progress has been achieved in understanding TSHR synthesis and trafficking by immunoprecipitation with mAb of precursor-labeled transfected L cells (10). Unfortunately, even this excellent system has some inherent difficulties and liabilities. In particular, the mAb cannot adequately characterize precursor-labeled TSHR by *direct* immunoprecipitation. Thus, prior capture of TSHR from a relatively large number of cells is required using a mAb-saturated affinity matrix column (10). Further, the mAb used in these studies preferentially recognize denatured TSHR (6). These drawbacks, as well as the expense of the large amount of mAb required for such studies, makes attractive the development of an alternative approach. One such approach is the epitope-tagging of TSHR so that it can be directly immunoprecipitated by a highly effective, generally available mAb. Over the past six years since the molecular cloning of the human TSHR (11-13), numerous efforts have been made by a number of groups

0006-291X/96 $18.00

to epitope tag TSHR. To date, no effective procedure has been reported. In the present study, we describe the successful epitope tagging of TSHR with human *c-myc* peptide.

MATERIALS AND METHODS

Construction and stable transfection of TSHRmyc cDNA. The epitope-tagged TSHR cDNA was constructed by a site-directed mutagenesis system (Mutan-K, Takara). Amino acids 338 to 349 of the TSHR ectodomain was replaced with the human *c-myc* peptide. Single-stranded cDNA template was prepared from the wild-type (wt) human TSHR cDNA (11) in M13mp19 phage vector. Oligonucleotide used was 5′-ATT-GTT-GGG-TAC-GAG-GAA-CAG-AAG-CTT-ATC-TCG-GAG-GAG-GAT-CTC-CTC-GCT-CAT-TAT-TAC-GTC-3′, where the underlined nucleotides encode the human *c-myc* sequence, EEQKLISEEDLL. The mutated and adjacent regions were confirmed by dideoxynucleotide sequencing. The cDNA encoding the epitope-tagged TSHR was excised by EcoR I and Xba I digestion, and ligated into the EcoR I/Xba I-digested eukaryotic expression vector pCR3 (Invitrogen). The resultant plasmid, designated pCR-TSHRmyc, as well as pSV2-NEO-ECE-wtTSHR (11) were stably transfected into 293HEK cells (ATCC,CRL 1573) using Lipofectin (GIBCO-BRL). Cells were grown in DMEM (GIBCO-BRL) supplemented with 10% FBS in the presence of appropriate antibiotics. Stably transfected cells were selected with 800 mg/L G418 (Geneticin, Wako). Several surviving clones were isolated with cloning cylinders.

Cellular cAMP measurements and [^{125}I]TSH ligand binding. For cAMP measurements, the cells, seeded in 24-well culture plates (1×10^5) and cultured overnight, were incubated for 60 min at 37 C in DMEM with 0.5 mmol/L 3-isobutyl-1-methylxanthine and the indicated concentrations of bovine (b) TSH (Sigma). Concentrations of cellular cAMP were determined with a commercial RIA kit (Yamasa). For radioligand binding studies, the cells, seeded in 24-well culture plates (2×10^5) and cultured overnight, were subjected to hormone binding with [^{125}I]bTSH (TRAb kit, Cosmic) as described previously (14). Each experiment involved duplicate wells and was repeated at least twice.

Cell surface anti-c-myc antibody binding. The cells, seeded in 24-well culture plates (2×10^5) and cultured overnight, were washed with PBS and incubated for 1 hr at 4 C in DMEM with 0.1% BSA (Sigma) containing 9E10 (1:1000 dilution). After extensive washing with cold PBS, the cells were incubated for 1 hr at 4 C in DMEM with 0.1% BSA containing [^{125}I] goat anti-mouse IgG (1:200 dilution, ICN). After rinsing with cold PBS, the cells were solubilized with 1% SDS and bound [^{125}I] goat anti-mouse IgG was counted in a γ-counter. Each experiment involved duplicate or triplicate wells and was repeated at least twice. Hybridoma cells for 9E10 were obtained from ATCC (CRL 1729).

Metabolic labeling and immunoprecipitation of wtTSHR. Confluent cells in 6-well culture plates were washed 2$\times$ with cold PBS and incubated in 0.5 mL (per well) methionine- and cysteine-free DMEM (Sigma) for 1 hr at 37 C, and then in the same medium with 100 mCi/L [^{35}S]methionine and [^{35}S]cysteine (Tran^{35}S Label, ICN) for 2-4 hrs at 37 C. In pulse-chase experiments, the cells were further incubated for the indicated periods of time in complete DMEM with 10 % FBS. After extensive washing with cold PBS, the cells were scraped into 1 ml of buffer 1 (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 100 mg/L phenylmethylsulfonyl fluoride (PMSF) and 20 mg/L leupeptin), homogenized with a Teflon homogenizer, and centrifuged for 10 min at 500 x g at 4 C to remove nuclei. The supernatant was recentrifuged for 30 min at 15,000 x g at 4 C. The membrane pellet was solubilized by incubation in 1 ml of solubilizing buffer (buffer 1 with 1% Triton X-100) for 1 hr at 4 C with constant rotation. The lysate was then centrifuged for 30 min at 15,000 x g at 4 C. To remove non-specific immunoprecipitants, the supernatant was precleared by incubating with 2 μl non-immune mouse serum and 50μl Protein A-Sepharose CL4B (a 1:4 suspension in the solubilizing buffer, Pharmacia) for 1 hr at room temperature. The precleared supernatant was then incubated with 2 μl 9E10 and 50 μl 1:4 suspension of protein A-Sepharose CL4B overnight at 4 C with constant rotation. Immune complexes were isolated by centrifugation and washed 5$\times$ with the solubilizing buffer. The pellet was dissolved in Laemmli buffer, boiled for 3 min and analyzed by 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

Deglycosylation of TSHRmyc. The receptor protein purified by immunoprecipitation was denatured for 10 min at 95 C in 0.5% SDS and 1% β-mercaptoethanol. The samples were incubated with 100 U N-glycosidase F or with 100 U endoglycosidase H (both from New England Biolabs) in the presence of 100 mg/L PMSF, 20 mg/L leupeptin and 10 μmM phosphoramidon for 2 hrs at 37 C. The enzyme was omitted from control incubation.

RESULTS

Functional properties of TSHRmyc. TSHR was epitope-tagged with a human *c-myc* peptide at amino acids 338–349, a part of the 50 amino acid ''insertion'' (amino acids 317-366), so-called because it is absent from the homologous gonadotropin hormone receptor ectodomains. We characterized the functional properties of two different clonal 293HEK cell lines expressing TSHRmyc (293-TSHRmyc-7 and -11), as well as of a 293HEK cell line expressing wtTSHR. Scatchard analysis of [^{125}I]TSH binding in the presence of increasing concentrations of unlabeled bTSH indicated a TSH binding affinity for 293-TSHRmyc of 3-5 $\times$ 10^{-9} M, somewhat

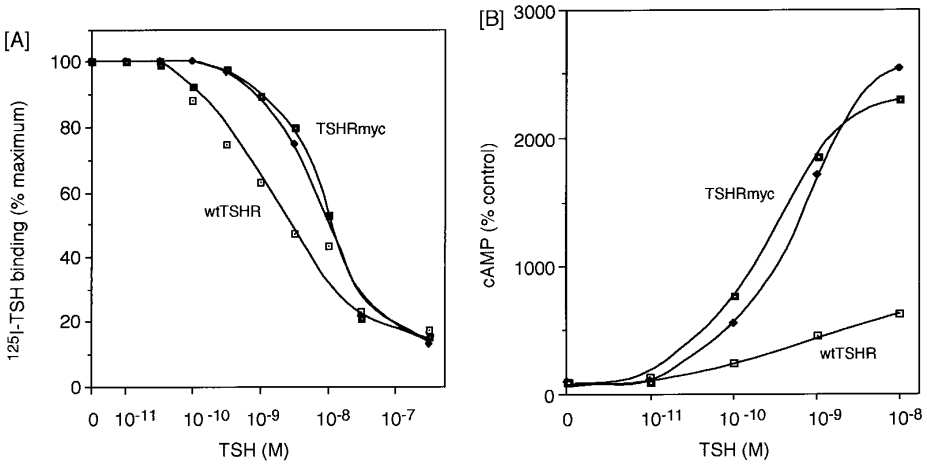

FIG. 1. (A) Competition inhibition by unlabeled bTSH of [^{125}I]bTSH binding to 293HEK cells expressing wtTSHR, TSHRmyc-7 and TSHRmyc-11. (B) cAMP response to bTSH stimulation in 293HEK cells expressing wtTSHR, TSHRmyc-7 and TSHRmyc-11. Conditions for cell culture, TSH binding and the cAMP response assay are described in *Materials and Methods.* Data are representative of two separate experiments; each point represents the mean of duplicate values in duplicate dishes of cells.

lower than for 293-wtTSHR (5×10^{-10} M)(Fig.1A). The number of the receptors expressed per cell for 293-TSHRmyc-7 and 293-TSHRmyc-11 clones (9.43×10^4 and 10.5×10^4, respectively) was apparently greater than for 293-wtTSHR (2.72×10^4). In terms of cAMP response, both 293-TSHRmyc clones responded extremely well to TSH stimulation (Fig.1B). As anticipated from the greater number of TSHR on 293-TSHRmyc clones relative to 293-wtTSHR, the amplitude of cAMP response to TSH was greater in the former cells.

Relationship of the c-myc epitope to the TSH binding site. The *c-myc* epitope tag in the TSHR 50 amino acid ''insertion'' was of value in examining the role of this region in the TSH binding site. Thus, we tested the ability of TSH and the anti-*myc* 9E10 to compete with each other for binding to 293-TSHRmyc. Addition of 9E10 did not significantly affect maximal [^{125}I]TSH binding (Fig.2A) or the maximal cAMP response to TSH stimulation (Fig.2B) in 293-TSHRmyc. Furthermore, in the experimental system whereby 9E10 binding could be detected using [^{125}I]anti-mouse IgG, addition of 10^{-9} and 10^{-7} M bTSH did not significantly inhibit 9E10 binding to 293-TSHRmyc (Fig.2C). These data suggest no competition between TSH and 9E10 for binding to the TSHRmyc.

Use of the c-myc epitope tag in studying TSHR synthesis. As TSHRmyc-7 and -11 possess identical functional properties, we used one clone (TSHRmyc-7) in the following studies. First, we established the efficacy of 9E10 in immunoprecipitating TSHRmyc. Following [^{35}S]-methionine and [^{35}S]-cysteine precursor labeling of proteins in cells expressing TSHRmyc and wtTSHR, 9E10 immunoprecipitated specific bands of ~100 and ~95 kDa in the former cells (Fig.3). These bands, consistent with single subunit forms of TSHR, were specific for TSHRmyc because no corresponding bands was seen in 293-wtTSHR. We were unable to visualize specific bands corresponding to TSHR subunits. Addition of TSH again did not inhibit 9E10 immunoprecipitation of TSHRmyc (data not shown).

Further experiments were undertaken to characterize these two species of TSHR proteins in a more detail. To examine the trafficking of TSHRmyc, we performed pulse-chase experiments in which 293-TSHRmyc were metabolically labeled for 2 hr (pulse) and subsequently incubated for up to 8 hrs with unlabeled amino acids (chase). As shown in Fig.4, only the ~95 kDa band was detectable at the end of 2 hr pulse. During the chase period, this band

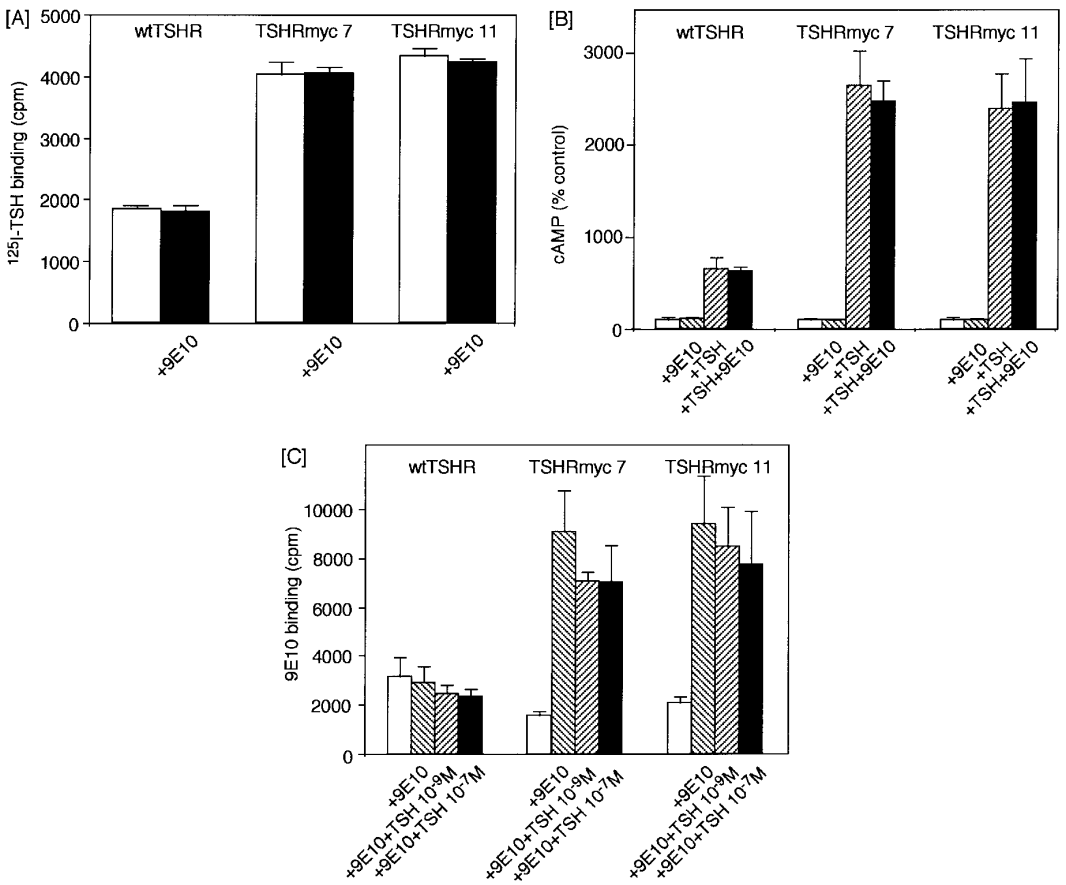

FIG. 2. (A) Competition inhibition by 9E10 of [^{125}I]TSH binding to 293HEK cells expressing wtTSHR, TSHRmyc-7 and TSHRmyc-11. (B) cAMP response to bTSH and 9E10 stimulation in 293HEK cells expressing wtTSHR, TSHRmyc-7 and TSHRmyc-11. (C) Competition inhibition by TSH of 9E10 binding to 293HEK cells expressing wtTSHR, TSHRmyc-7 and TSHRmyc-11. Cell culture and TSH and 9E10 binding assays are described in *Materials and Methods.* Data are representative of two separate experiments; each point represents the mean ± SD of triplicate values determined in triplicate dishes of cells.

progressively diminished and completely disappeared after 6 hr chase. In contrast, the ~100 kDa species became apparent after 2 hr of chase and increased in amount thereafter. Again, no specific smaller TSHR species was observed. Immunopurified TSHRmyc was characterized further by endoglycosidase digestion (Fig.5). Digestion with N-glycosidase F, which catalyses complete deglycosylation, reduced both the ~100 and the ~95 kDa monomeric forms of receptor into the same ~85 kDa species. Only the ~95 kDa species was sensitive to endoglycosidase H, which catalyses the removal of exposed high mannose residues, and again resolved into the same 85 kDa species. These data suggest that the ~95 kDa protein is a high mannose-rich precursor species that is progressively transformed into a ~100 kDa mature protein containing complex carbohydrates.

DISCUSSION

Epitope-tagging of TSHR, when expressed in mammalian cells, has been a long-standing unattained goal. Our rationale for choosing the *c-myc* epitope in conjuction with the site described in the present study was that the hydropathy profile of TSHR suggests that the 50

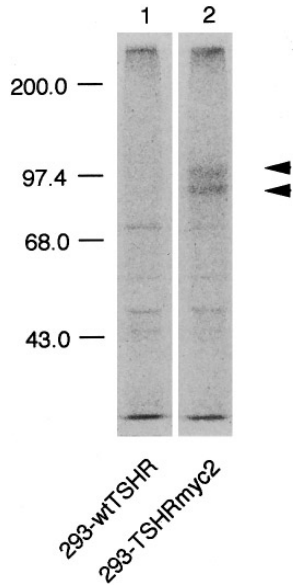

FIG. 3. Immunoprecipitation by 9E10 and SDS-PAGE of TSHRmyc. Cell culture, metabolic labeling, immunoprecipitation and SDS-PAGE are described in *Materials and Methods.* Lane 1, 293HEK cells expressing wtTSHR; lane 2, the cells expressing TSHRmyc. The arrows on the right indicate the ~95 and ~100 kDa TSHR proteins. The sizes of the molecular mass markers are indicated on the left in kDa.

amino acid ''insertion'' (residues 317-366) is very hydrophillic and is, therefore, likely to be on the surface of the molecule (11). Fortunately, this approach was successful. The *c-myc* epitope-tagged TSHR (TSHRmyc) binds TSH with high affinity and responds well to TSH stimulation of cAMP pathway.

Of interest, much higher receptor numbers and cAMP responses to TSH stimulation were observed in 293-TSHRmyc than in 293-wtTSHR. Among the possible explanations for this phenomenon are:- (i) the CMV promoter used to express TSHRmyc is generally stronger than

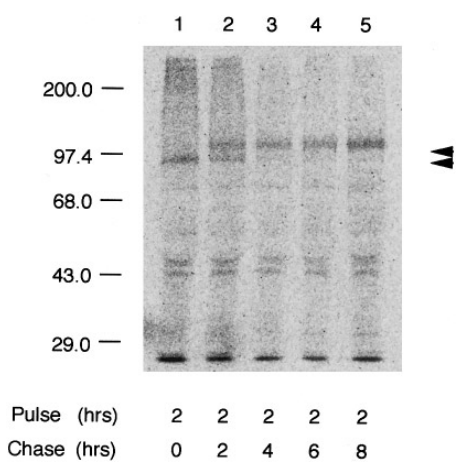

FIG. 4. Pulse–chase of TSHRmyc expressed in 293HEK cells. Pulse was for 2 hrs, and chases were 0 to 8 hrs. The arrows on the right indicate the ~95 and ~100 kDa TSHR proteins. The sizes of the molecular mass markers are indicated on the left in kDa.

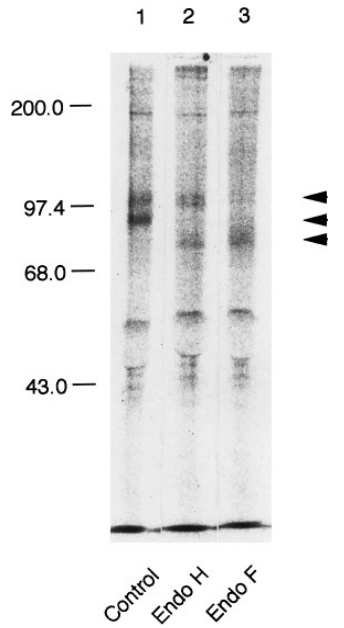

FIG. 5. Endoglycosidase digestion of TSHRmyc. The immunopurified TSHR proteins were digested with *N*-glycosidase F (Endo F, lane 2) and endoglycosidase H (Endo H, lane 3) as described in *Materials and Methods.* The arrows on the right indicate the ~85 kDa, ~95 and ~100 kDa TSHR proteins. The sizes of the molecular mass markers are indicated on the left in kDa.

the SV40 promoter used in the vector containing wtTSHR (15) and (ii) the ~2.7 kb TSHRmyc cDNA lacks ~1.3 kb of 3′-noncoding region of the receptor cDNA recently been reported to suppress steady state levels of TSHR mRNA (16). In contrast, the cDNA for wtTSHR (4 kb) contains the entire 3′-noncoding region (11).

The slightly lower affinity for TSH of 293-TSHRmyc relative to 293-wtTSHR may reflect the four-fold greater number of receptors on the former cells. Thus, the higher number of receptors could introduce an artifact into Scatchard analysis consequent to non-saturation of binding sites. Alternatively, there may be negative cooperativity among overexpressed receptors (17). Finally, we cannot exclude the possibility that introduction of the *c-myc* epitope directly, or indirectly, alters the TSH binding site.

The ability of the anti-*myc* 9E10 to bind TSHRmyc expressed on the surface of intact cells indicates that the mAb, although elicited against a synthetic peptide (18), can bind to the native receptor. This result also suggests that the site where the *c-myc* peptide was introduced (amino acids 338-349) must be exposed on TSHR exterior, as predicted (see above). Our data are consistent with those of Harfst *et al.* that a polyclonal antibody against TSHR amino acids 313-330 (primarily within the 50 amino acid region) can recognize TSHR on flow cytometry with intact cells (5). An interesting difference between the two studies is that 9E10 does not compete with TSH for binding to transfected 293HEK cells, whereas competition between TSH and the antiserum to residues 313-330 was observed (5). One possible explanation for the discrepancy between these studies is that residues 313-330 contain one facet of the multiple TSH contact points (19) and that this site is closely upstream of a non-contact segment (338-349). The fact that entire 50 amino acid segment (residues 317-366) can be deleted without loss of high affinity binding (20,21) could reflect the redundancy in the contact points necessary for high affinity TSH binding or function (19). Alternatively, competition between TSH and the antiserum to 313-330 could occur via steric or allosteric effects.

For a number of reasons, the expression of TSHRmyc in 293 HEK cells represents a powerful tool for the study of the metabolic turnover and trafficking of TSHR in mammalian cells. First, the sensitivity of the detection system makes unnecessary prior enrichment of TSHR on an affinity matrix, a procedure that could possibly introduce quantitative and qualitative artifacts. Second, TSHRmyc can be detected under native conditions. This phenomenon may be useful in a number of future projects, such as use of 9E10 for confocal microscopic analysis of intracellular trafficking of native TSHR. Further, affinity-purified native TSHR may be of value for the detection of TSHR autoantibodies in human disease.

Single subunit TSHR have been observed previously in cultured FRTL5 rat thyroid cells (22), transfected CHO cells (23-25) and are particularly abundant in transfected L cells (6,10). Our precursor labeling studies of 293-TSHRmyc confirm the observations of Loosfelt *et al.* in L cells that the single subunit, high mannose form of TSHR is a precursor of the larger single subunit TSHR with mature, complex carbohydrate (10). Moreover, it is of interest that TSHRmyc in 293HEK cells appears to be essentially entirely in the single chain, uncleaved form. It has been suggested that transfected L cells, unlike thyroid cells, have a reduced proficiency for TSHR maturation, thereby leading to accumulation of single subunit forms of TSHR (10). 293HEK cells could display this phenomenon in an even more exaggerated manner. The possibility that the *c-myc* epitope at residues 338-349 may prevent cleavage of TSHR into two subunits seems very unlikely in view of the fact that mutant TSHR that lacks the entire 50 amino acid region (317-366) in which the *c-myc* epitope resides still cleave into two subunits (23). In future studies, transfection of TSHRmyc into different cells types could provide a useful tool to directly compare, without cellular disruption or protein denaturation, TSHR trafficking and processing in these cells.

In summary, we have succeeded in epitope-tagging TSHR in stably-transfected mammalian cells. This modified, but normally functioning receptor can be detected directly and in native form with a readily available mAb and without the need for prior TSHR affinity capture. Lack of competition between the mAb and TSH suggests that at least part of the unique, 50 amino acid segment in TSHR ectodomain is not a TSH binding site. This epitope-tagged TSHR will be valuable for further studies on the metabolic processes involving TSHR.

ACKNOWLEDGMENTS

We thank Professor Basil Rapoport, Thyroid Molecular Biology Unit, Veterans' Administration Medical Center, and the University of California, San Francisco, California for critical reading of the manuscript. This research was partly supported by Uehara Memorial Foundation, Tokyo, Japan.

REFERENCES

1. Nagayama, Y., and Rapoport, B. (1992) *Mol. Endocrinol.* **6,** 145–156.
2. Vassart, G., and Dumont, J. E. (1992) *Endocr. Rev.* **13,** 596–611.
3. Nagayama, Y., and Nagataki, S. (1994) *Thyroid Today* **17,** 1–9.
4. Ban, T., Kosugi, S., and Kohn, L. D. (1992) *Endocrinology* **131,** 815–829.
5. Harfst, E., Ross, M. S., Nussey, S. S., and Johnstone, A. P. (1994) *Mol. Cell. Endocrinol.* **102,** 77–84.
6. Loosfelt, H., Pichon, C., Jolivet, A., Misrahi, M., Caillou, B., Jamous, M., Vannier, B., and Milgrom, E. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 3765–3769.
7. Johnstone, A. P., Cridland, J. C., DaCosta, C. R., Harfst, E., and Shepherd, P. S. (1994) *Mol. Cell. Endocrinol.* **105,** R1–R9.
8. Seetharamaiah, G. S., Wagle, N. M., Morris, J. C., and Prabhakar, B. S. (1995) *Endocrinology* **136,** 2817–2824.
9. Nicholson, L. B., Vlase, H., Graves, P., Nilsson, M., Molne, J., Huang, G. C., Morgenthaler, N. G., Davies, T. F., McGregor, A. M., and Banga, J. P. (1996) *J. Mol. Endocrinol.* **16,** 159–170.
10. Misrahi, M., Ghinea, N., Sar, S., Saunier, B., Jolivet, A., Loosfelt, H., Cerutti, M., Devauchelle, G., and Milgrom, E. (1994) *Eur. J. Biochem.* **222,** 711–719.
11. Nagayama, Y., Kaufman, K. D., Seto, P., and Rapoport, B. (1989) *Biochem. Biophys. Res. Comm.* **165,** 1184–1190.

12. Libert, F., Lefort, A., Gerard, C., Parmentier, M., Perret, J., Ludgate, M., Dumont, J. E., and Vassart, G. (1989) *Biochem. Biophys. Res. Comm.* **165,** 1250–1255.
13. Misrahi, M., Loosfelt, H., Atger, M., Sar, S., Guiochon-Mantel, A., and Milgrom, E. (1990) *Biochem. Biophys. Res. Comm.* **166,** 394–403.
14. Chazenbalk, G. D., Nagayama, Y., Kaufman, K. D., and Rapoport, B. (1990) *Endocrinology* **127,** 1240–1244.
15. Boshart, M., Weber, F., Jahn, G., Dorsch-Hasler, K., Fleckenstein, B., and Schaffner, W. (1985) *Cell* **41,** 521–53.
16. Kakinuma, A., Chazenbalk, G., Filetti, S., McLachlan, S., and Rapoport, B. (1996) *Endocrinology* **137,** 2664–2669.
17. Chazenbalk, G. D., Kakinuma, A., Jaume, J. C., McLachlan, S. M., and Rapoport, B. (1996) *Endocrinology* (in press).
18. Evans, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985) *Mol. Cell. Biol.* **5,** 3610–3616.
19. Nagayama, Y., Wadsworth, H. L., Chazenbalk, G. D., Russo, D., Seto, P., and Rapoport, B. (1991) *Proc. Natl. Acad. Sci. USA.* **88,** 902–905.
20. Wadsworth, H. L., Chazenbalk, G. D., Nagayama, Y., Russo, D., and Rapoport, B. (1990) *Science* **249,** 1423–1425.
21. Kosugi, S., Akamizu, T., Takai, O., Prabhakar, B. S., and Kohn, L. D. (1991) *Thyroid* **1,** 321–330.
22. Furmaniak, J., Hashim, F. A., Buckland, P. R., Petersen, V. B., Beever, K., Howells, R. D., and Rees Smith, B. (1987) *FEBS Letters* **215,** 316–322.
23. Russo, D., Chazenbalk, G. D., Nagayama, Y., Wadsworth, H. L., Seto, P., and Rapoport, B. (1991) *Mol. Endocrinol.* **5,** 1607–1612.
24. Russo, D., Nagayama, Y., Chazenbalk, G. D., Wadsworth, H. L., and Rapoport, B. (1992) *Endocrinology* **130,** 2135–2138.
25. Endo, T., Ikeda, M., Ohmori, M., Anzai, E., Haraguchi, K., and Onaya, T. (1992) *Biochem. Biophys. Res. Comm.* **187,** 887–893.