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Binding Properties of Monoclonal Antibody to the Cytoplasmic Domain of Transferrin Receptor

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ABSTRACT. Hybridomas secreting monoclonal antibodies to transferrin receptor (TFR) were isolated. One of these antibodies, U-1, recognized the cytoplasmic domain of TFR and the others, N-2 and W-3, recognized its cell surface domains. Only antibody W-3 competed with transferrin (TF) for binding to TFR. Antibody U-1 bound to purified TFR but not to ^{35}S- or ^{125}I-TFR in cell extracts. ^{125}I-Antibody U-1 bound to TFR alone in cell extracts when TFR was bound to antibody N-2-Sepharose 4B, but even in the presense of cell extracts it did not bind to TFR bound to antibody W-3-Sepharose 4B. Antibody W-3 co-precipitated TFR and a protein of about 30 kDa from cell extracts, and also reacted with the 30 kDa protein in cell extracts in the absence of TFR. Based on these results, the existence of two different states of the cytoplasmic domain of TFR is discussed.

Transferrin (TF), an iron-carrier protein of mammals, is internalized into cells by receptor-mediated endocytosis. The cycle of TF and transferrin receptor (TFR) has been studied extensively (for reviews, see Refs. 8 and 16). Diferric TF binds to TFR on the cell surface and the receptor-ligand complex is internalized into cells via coated pits and vesicles, and then in the acidic compartment of the cell, the iron is released from TF and transported into the cytosol. Finally the complex of TFR and apo-TF recycles to the cell surface and intact apo-TF is released from TFR into the medium, while TFR enters the next cycle. TFR is a transmembranous glycoprotein that is present as a homo-dimer and associates covalently with fatty acid. Recently the complete primary structure of human TFR was deduced from the nucleotide sequence of cDNA (15, 21).

However, in spite of extensive investigations, there are still many problems about the mechanism of the TF and TFR cycle, as there are about the mechanisms of other receptor-ligand systems. Recently introduction of a deletion of 36 amino acids in the cytoplasmic domain of TFR by in vitro mutagenesis of a cDNA expression vector for human TFR was found to result in block of endocytosis of TFR, without affecting its binding to TF, but that modification of phosphorylation sites within this domain did not affect its endocytosis (18). These results suggest that the

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Abbreviations used: TF, transferrin; TFR, transferrin receptor; MEM, minimal essential medium; DMEM, Dulbecco's modified minimal essential medium; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; BBS, borate buffered saline.

cytoplasmic domain is essential for internalization of TFR, but that receptor phosphorylation is not. Moreover, mutations of the LDL receptor in familial hypercholesterolemia that cause truncation of the cytoplasmic domain or result in substitution of an amino acid within this domain were recently shown to impede concentration of the receptor in coated pits and so prevent its internalization, although the receptor has normal ability to bind LDL (6, 12). These findings suggest that the cytoplasmic domain may direct the receptor to be endocytosed with coated pits. The cytoplasmic domain of receptors may also be essential for other steps of the process of the receptor-ligand cycle. So characterization of the cytoplasmic domain seems important for understanding the mechanism of the cycle. Monoclonal antibodies have proved useful for analyses of the structure-function relations of functional proteins, such as the acetylcholine receptor (13, 27), cobra venom toxin (2), diphtheria toxin (9, 30) and various viral proteins, such as influenza virus hemagglutinin (7, 24). Several monoclonal antibodies against TFR, such as B3/25 (26) and OKT9 (25), have been reported, but most of them were obtained by fusion of myeloma cells with spleen cells from mice immunized with a cell line having TFR, not with purified TFR itself. Moreover, no monoclonal antibodies against other domains of TFR than the extracellular domain have yet been reported.

Therefore, in this work we isolated several monoclonal antibodies against TFR that recognized the cytoplasmic domain or the cell surface region of TFR by immunization with purified TFR. These antibodies were further characterized using radioactive TFR in the pure form and in crude cell extracts. The existence of two conformational states of the cytoplasmic domain is discussed.

MATERIALS AND METHODS

Materials. The following materials were used: DEAE-cellulose (DE 52) (Whatman); Na ^{125}I, Bolton-Hunter reagent and ^{35}S-methionine (Amersham); CNBr-activated Sepharose 4B and Protein A-Sepharose 4B (Pharmacia); human transferrin (Midori Jyuji); anti-human transferrin rabbit IgG (Cappel); microtest assay plates (Becton Dickinson); polyethylene glycol 4,000 (Nakarai Chemicals); and succinimidyl 4-(p-maleimido phenyl) butyrate (SMPB) (Pierce). Anti-human TFR rabbit IgG was obtained from Dr. S. Taketani.

Cells and cell culture. HeLa cells and FL cells (human amnion) were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% calf serum. WI-38 human fibroblasts and SP2/0 mouse myeloma cells were cultured in Dulbecco's modified MEM (DMEM) supplemented with 15% fetal calf serum.

Isolation of TFR from human placenta. TFR was isolated from human placenta by affinity chromatography on conjugated anti-transferrin antibody-Sepharose 4B and conjugated transferrin-Sepharose 4B as described previously (23).

Hybridoma. Monoclonal antibody was prepared as described previously (10). That is, purified TFR protein (50 μg) in complete Freund's adjuvant was injected intraperitoneally (i.p.) into five BALB/c mice of 12 weeks old. From one week later, 50 μg of antigen mixed with incomplete Freund's adjuvant was injected i.p. once a week for 3 weeks. One week after the last immunization, 50 μg of the antigen was injected i.p. as a booster. Three days later, spleen cells (2.1×10^8) from the immunized mice were fused with SP2/0 cells (5.28×10^7) using 45% (w/v) polyethylene glycol 4,000 and 15% (v/v) dimethyl sulfoxide. After fusion, the cells were seeded into 96-well tissue culture plates and cultured in HAT medium (1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, 1×10^{-5} M thymidine). Culture supernatants from wells in which hybridomas grew were subjected to radioimmuno-assay. For this a mixture of

^{125}I-labeled TFR (purified), anti-mouse IgG rabbit IgG-conjugated Sepharose 4B, incubation solution [bovine serum albumin at 3 mg/ml, 0.2% Triton X-100, 0.15 M NaCl and 10 mM KPO_4 (pH 7.5)], and the culture supernatant were incubated overnight at 4°C in an Eppendorf tube with gently rotation. The beads were then washed five times with the incubation solution and the radioactivity in each tube was counted in a gamma counter. Cells giving positive results were cloned by the limiting dilution method using macrophages as feeder cells.

Purification of monoclonal antibody. The positive clones were injected into the peritoneum of BALB/c mice. After about 2 weeks, ascites containing antibody was obtained from the mice and treated with 40% ammonium sulfate (pH 8.0). The precipitate was solubilized in 10 mM phosphate buffer (pH 8.0), dialysed against the same buffer and applied to a DE52 column equilibrated with the same buffer. Monoclonal antibodies were eluted with a linear gradient of 0–0.2 M NaCl in 10 mM phosphate buffer (pH 8.0). The immunoglobulin types were determined by the Ouchterlony double immunodiffusion method (17).

Radioiodination of proteins. Anti-mouse IgG rabbit IgG, anti-peptide rabbit serum and purified TFR were labeled with Na^{125}I by the chloramine-T method (19). Monoclonal antibodies and TF were labeled with Na^{125}I using lactoperoxidase (5). The plasma membrane fraction isolated from fresh human placenta was solubilized with 120 mM octylglucoside and subjected to ultra-centrifugation, and the supernatant (4.5 mg protein/ml) was radioiodinated with Bolton-Hunter reagent.

Synthesis of peptide and preparation of anti-peptide rabbit serum. The primary structure of the cytoplasmic domain of human TFR is reported to be as follows: $_2$HN-Met Met Asp Gln Ala Arg Ser *Ala Phe Ser Asn Leu Phe Gly Glu Pro Leu Ser Tyr Thr Arg Phe Ser Leu Ala Arg* Gln Val Asp Gly Asp Asn Ser His Val Glu Met Lys Leu Ala Val Asp Glu Glu Glu Asn Ala Asp Asn Asn Thr Lys Ala Asn Val Thr Lys Pro Lys Arg Cys Ser Gly----(15, 21). The peptides covering positions 8–27 (italics) and 18–27 of the TFR were synthesized by standard procedures (29). The peptide composed of the position 8–27 and additional cysteine residue at C-terminus was also synthesized and conjugated covalently with bovine serum albumin (BSA) using succinimidyl 4-(p-maleimido phenyl) butyrate (SMPB) through the C-terminal cysteine residue of the peptide. About two molecules of the peptide were coupled per molecule of BSA, which was measured by mass-spectrometory. This conjugate was injected subcutaneous into two rabbits (100 μg/rabbit per injection) with complete Freund's adjuvant. Several booster injections were given of the same dose in Freund's incomplete adjuvant, and sera were collected and tested by radioimmunoassay (see below). The serum showing the highest titer was used for experiments.

Binding experiments. Binding of antibodies to peptide was assayed in two ways. First, the synthetic peptide (0.1 mg/ml in 99% methanol) was added to wells of micro-titer plates and dried in the wells. The wells were then washed with borate buffered saline containing 2 mg/ml BSA (BSA-BBS). All subsequent washes were done with the same solution. Then the wells were incubated with BBS containing 2% calf serum and 0.02% azide for one day at 4°C, and then stored. The plates were washed and incubated with various concentrations of antibodies for 4 h at 37°C, washed, and incubated with ^{125}I-labeled anti-mouse IgG for 4 h. Finally the wells were washed again, cut out and counted in a gamma counter. Second, the synthetic peptide was conjugated covalently with CNBr-activated Sepharose 4B (3 mg peptide/ml beads) and 40 μl of this peptide-Sepharose 4B solution was incubated with ^{125}I-labeled and unlabeled antibodies (specific activity: U-1, 1.0×10^7 cpm/μg, N-2, 1.4×10^7 cpm/μg) for 5 h. The Sepharose was then washed 5 times with the incubation solution and its associated radioactivity was counted.

The association constants of the monoclonal antibodies with TFR were determined by a competitive binding assay (9) and Scatchard analysis (20) using ^{125}I-labeled antibodies, unlabeled antibodies and Sepharose 4B conjugated with a solubilized human placenta plasma membrane fraction (3.5 mg protein/ml Sepharose).

Binding of antibody U-1 to the complex of TFR and antibody N-2-Sepharose 4B was assayed as follows. Antibodies were conjugated to CNBr-activated Sepharose 4B (amount of Sepharose: 2 mg antibody/1 ml for antibody N-2, 6 mg/ml for antibody U-1, and 5 mg/ml for antibody W-3). HeLa cells precipitated by centrifugation were dissolved in extraction solution [1.0% Triton X-100, 3 mg/ml BSA, 0.15 M NaCl, 2 mM PMSF, 10 mM phosphate buffer (pH 7.4)], and cell extracts obtained by ultra-centrifugation were used. Mixtures of 60 μl of antibodies-Sepharose 4B, 20 μl of ^{125}I-labeled antibodies (with the specific activities indicated in Fig. 4) and 300 μl of cell extract (prepared from 0.25 ml of the cell pellet) added in various orders were incubated at 4°C. Finally the Sepharose was washed five times with extraction solution and counted.

Immunoprecipitation. Subconfluent FL or WI-38 cells in 100 mm dishes were incubated with [^{35}S]-methionine (0.15 mCi/ml in methionine-free MEM) for 6 h at 37°C, and then washed and dissolved in 0.75 ml of extraction solution and centrifuged. Volumes of 250 μl of the resulting supernatant were mixed with 60 μl of each antibody-Sepharose 4B and incubated overnight at 4°C. The Sepharose was washed with extraction solution at least six times, and then immune-complexes were dissociated by boiling with 2% SDS and subjected to SDS-polyacrylamide gel electrophoresis (10% gel) (11). After electrophoresis, the gel was fixed and examined by fluorography as described by Bonner and Laskey (1). The gel was then dried and exposed to Kodak X-ray film at −80°C.

For applications of ^{125}I-placenta membrane fraction and purified TFR, 50 μl (about 30 μg of proteins, 4.4×10^7 cpm) and 40 μl (about 0.1 μg, 4×10^5 cpm), respectively were used for each lane.

For indirect immunoprecipitation, Protein A-Sepharose 4B was used.

For application of TFR precipitated from FL cells, after incubation of the ^{35}S-methionine labeled FL cell extract with antibody N-2-Sepharose 4B, the beads were washed and the antigen bound to the antibody was dissociated by incubation with 0.1 M glycine (pH 2.35), BSA at 3 mg/ml and 0.2% Triton X-100 for 6 h at 4°C. The detached antigen was separated from the beads by filtration and dialysed against 10 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl. This fraction was then immunoprecipitated with antibody U-1-Sepharose 4B.

Immunoblotting. TFR was analyzed by immunoblotting as described elsewhere (14).

RESULTS

Properties of monoclonal antibodies against TFR. Monoclonal antibodies against purified TFR were produced and purified as described under MATERIALS AND METHODS. Three antibodies, named N-2, U-1 and W-3, were characterized further. All of them were identified as IgG_1 by Ouchterlony diffusion analysis.

Various concentrations of ^{125}I-labeled antibody N-2, U-1 or W-3 were incubated overnight at 4°C with human cells. Then the cells were washed and cell-associated radioactivity was counted. ^{125}I-Antibodies N-2 and W-3 bound to human cells in a saturable manner, but ^{125}I-antibody U-1 showed no significant binding (Fig. 1). There results suggested that antibodies N-2 and W-3 bound to surface regions of TFR, whereas antibody U-1 bound to a site within the cytoplasmic or near the transmembranous region. Consistent with these ideas, when FL cells were labeled metabolically with ^{3}H-palmitate and treated with trypsin, to cut off extracellular

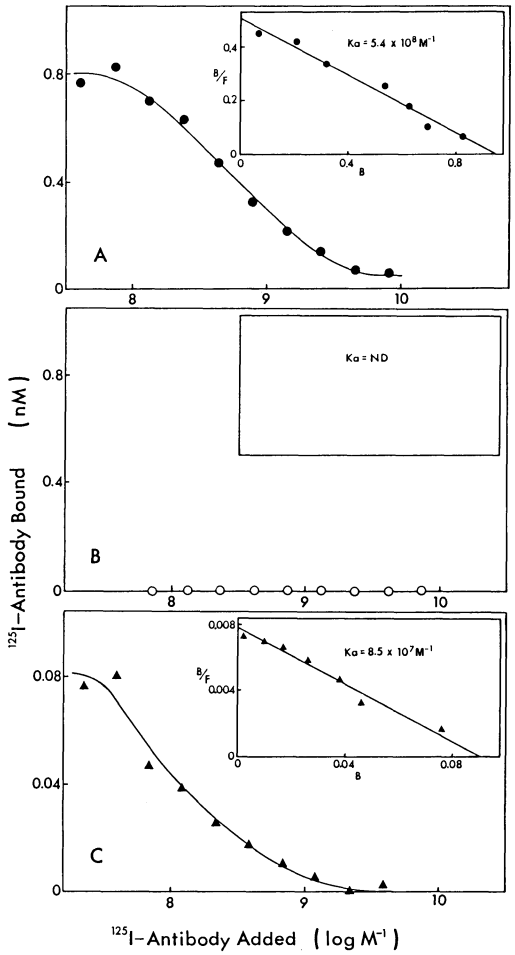

Fig. 1. Bindings of ^{125}I-antibodies N-2, U-1 and W-3 to cultured cells. FL cells (A and B) or HeLa cells (C) were seeded into 24-well tissue culture trays and cultured for 2 days at 37°C in MEM containing 10% calf serum to semi-confluency. Various concentrations of ^{125}I-labeled and unlabeled (A) antibody N-2 (final specific activity, 5.4×10^5 cpm/μg protein), (B) U-1 (1.2×10^6 cpm/μg protein) or (C) W-3 (4.9×10^6 cpm/μg protein) were added to wells and incubated for 16 h at 4°C. For panel C, before addition of antibody, the cells were washed with MEM containing 1% BSA and incubated in the same medium for 1 h at 37°C and then the cycle was repeated once more. The cells were then washed three times with phosphate buffered saline containing 3 mg/ml BSA, solubilized in 0.1 N NaOH, transferred to test tubes and counted. Data are corrected for nonspecific binding assayed in the presence of excess unlabeled antibodies (1.2 μM). Insets show Scatchard plots (20) of data and the calculated association constant (Ka). ND: not determinable.

regions of TFR, and then solubilized with a non-ionic detergent and subjected to immunoprecipitation, antibody U-1 precipitated a ^{3}H-labeled fragment with a MW of about 25 K, which was detected by autoradiography, whereas antibody N-2 did not give any specific band (data not shown). Fatty acid is known to be associated covalently with the region of TFR that is in close proximity to the plasma membrane, so the fragment precipitated by antibody U-1 may be the portion of TFR

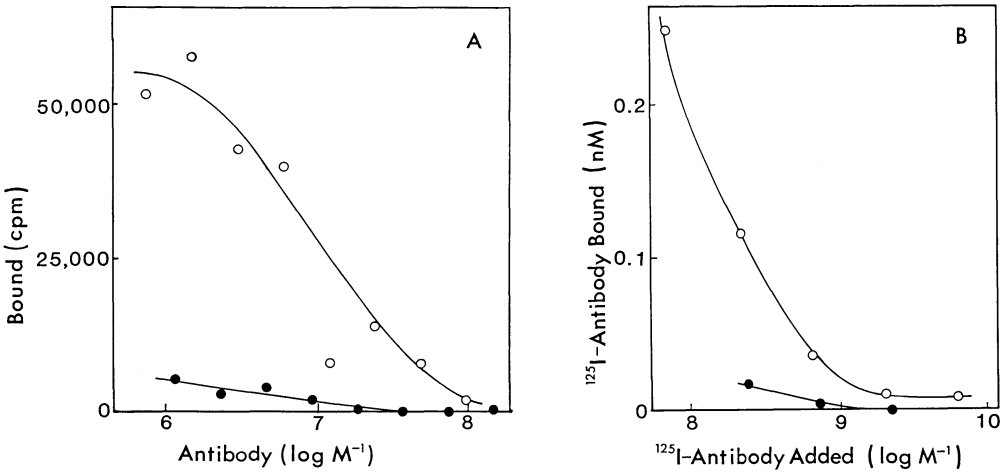

Fig. 2. Binding of antibodies N-2, U-1 and W-3 to synthetic peptide of the N-terminal region of TFR (peptide 8–27) A. Bindings of antibodies W-3 (●) and U-1 (○) to peptide 8–27 measured by solid-phase RIA as described under MATERIALS AND METHODS. Values are corrected for nonspecific binding measured in micro-titer plates not coated with the peptide. B. Bindings of ^{125}I-antibodies N-2 (●) and U-1 (○) to conjugated peptide 8–27-Sepharose 4B measured as described under MATERIALS AND METHODS. Values are corrected for nonspecific binding assayed in the presence of excess unlabeled antibodies (8.2 μM).

not exposed on the cell surface. Thus the results suggested that antibody U-1 binds to a cytoplasmic or transmembranous domain of TFR.

The complete primary structure of human TFR deduced from the nucleotide sequence of cDNA has been reported (15, 21). Therefore, we next tried to determine the sequence recognized by antibody U-1 using synthetic peptides. We synthesized a peptide composed of 20 amino acids corresponding to Ala_8-Arg_{27} of the N-terminal portion of TFR, which is located in the center of the cytoplasmic domain of the molecule (see MATERIALS AND METHODS) and overlaps the deleted region (Arg_6-Ala_{41}) of the endocytosis-defective mutant TFR made by L.C. Kuhn and coworkers (18). The abilities of the monoclonal antibodies to bind to this peptide were examined by two methods (Fig. 2A and B).

Figure 2A shows the result of solid phase radio-immunoabsorbent assay using micro titer plates coated with the synthetic peptide. Significant binding of U-1 to the peptide was observed. On assay of direct binding of ^{125}I-labeled monoclonal antibodies to the synthetic peptide conjugated with Sepharose 4B, ^{125}I-antibody U-1 also bound to the peptide-Sepharose 4B (Fig. 2B), but antibodies N-2 and W-3 did not (Fig. 2A, B). These results confirm that the epitope recognized by U-1 antibody is located in the cytoplasmic domain of Ala_8-Arg_{27} of TFR. Antibodies U-1, N-2 and W-3 did not bind to another synthetic peptide, Leu_{18}-Arg_{27} of TFR, which is the C-terminal half of the peptide Ala_8-Arg_{27}. We also obtained polyclonal antibody against the synthetic peptide Ala_8-Arg_{27}+Cys by immunizing rabbits with a conjugate of this peptide and bovine serum albumin. This polyclonal antibody bound to both synthetic peptide and purified TFR (data not shown).

Inability of monoclonal antibody U-1 to bind to TFR in cell extracts. Cell

extracts were prepared from FL or WI-38 cells labeled with ^{35}S-methionine by lysis with a non-ionic detergent, and the abilities of antibodies N-2 and U-1 to precipitate ^{35}S-methionine labeled TFR in the extracts were examined. In extracts of both strains, antibody N-2 precipitated TFR, but antibody U-1 did not (Fig. 3, lanes 1–5). This result was confirmed using iodinated detergent-solubilized placental membranes (lanes 6 and 7). But ^{125}I-labeled TFR purified from placenta (which was used for immunization and assay in isolation of hybridoma) was precipitated by antibody N-2 or U-1 (lanes 8 and 10). When constant amounts of purified ^{125}I-TFR and various amounts of monoclonal antibodies were incubated and then immunoprecipitated with protein A-Sepharose 4B pre-coated with rabbit anti-mouse IgG antibodies, antibodies N-2 and U-1 showed the same kinetics of binding to the purified TFR (data not shown). The affinity constants of antibodies N-2 and U-1 to

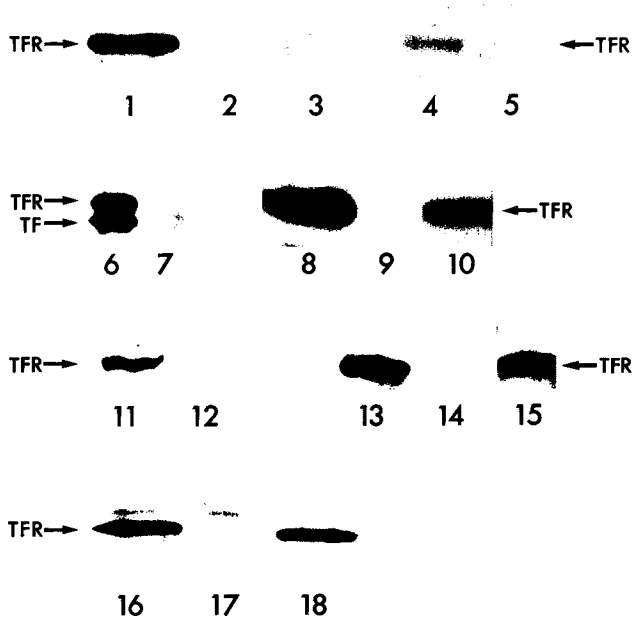

Fig. 3. Immunoprecipitation of TFR by antibodies N-2, U-1 and polyclonal antibody against peptide 8−27+Cys. Lanes 1, 2 and 3, ^{35}S-methionine-labeled FL cell extracts were directly immunoprecipitated. Lanes 4 and 5, ^{35}S-methionine-labeled WI-38 cell extracts. Lanes 6 and 7, ^{125}I-labeled placental membrane extract. Lanes 8, 9 and 10, ^{125}I-labeled TFR (purified). Lanes 11 and 12, TFR obtained from ^{35}S-methionine-labeled FL cell extracts with antibody N-2-Sepharose 4B. Lanes 13, 14 and 15, the same as lanes 11 and 12 except for addition of unlabeled FL cell extract that had been adsorbed with antibody N-2-Sepharose 4B for lanes 14 and 15. For immunoprecipitation, antibody N-2-Sepharose 4B (lanes 1, 4, 6 and 8), antibody U-1-Sepharose 4B (lanes 2, 5, 7, 10, 11, 13 and 14), antibody W-3-Sepharose 4B (lane 15), or, unrelated monoclonal antibody-Sepharose 4B as a control (lanes 3, 9 and 12) was used. Exposures of the autoradiograph were 1.5 h for lanes 1–5, 8–15 and 48 h for lanes 6 and 7. An intensifying screen was used for lanes 8–10 to enhance autoradiography. Lanes 16, 17 and 18: ^{35}S-methionine-labeled FL cell extracts were indirectly immunoprecipitated with antibody N-2 (lane 16), antibody U-1 (lane 17) or polyclonal antibody against peptide 8−27+Cys (lane 18). Exposures of the autoradiograph was 4.5 h for lanes 16–18. The locations of TFR and TF are indicated by arrows. For lane 6, ^{125}I-labeled TF was co-precipitated with TFR, because iodination of the placental membrane extract resulted in labeling of the ligand-receptor complex.

TFR, determined as described under MATERIALS AND METHODS, were $3.4 \times 10^8\ M^{-1}$ and $3.3 \times 10^8\ M^{-1}$, respectively. Thus it is unlikely that the inability of antibody U-1 to immunoprecipitate TFR in cell extracts was due to its low binding ability. Furthermore, antibody U-1 could precipitate TFR recovered from the immunoprecipitate by antibody N-2 of a ^{35}S-methionine-labeled FL cell extract (lane 11). So the possibility that the cytoplasmic domain of TFR was degraded in the cell extracts could be excluded. On the contrary, antibody U-1 could not precipitate purified TFR that had been re-mixed with a cell extract adsorbed by antibody N-2 (lane 14), whereas antibody W-3 could (lane 15). Unlike antibody U-1, polyclonal antibody against the synthetic peptide Ala_8-Arg_{27}+Cys could precipitate TFR in ^{35}S-methionine-labeled FL cell extracts (lane 18). This polyclonal antibody may contain antibodies that recognize different epitopes of the Ala_8-Arg_{27} peptide from that recognized by antibody U-1 and thus can precipitate TFR even in extracts. This result also showed that the cytoplasmic region of TFR in cell extracts was intact. These findings suggest several possibilities: i) The cytoplasmic domain of TFR that binds to antibody U-1 may have a reversibly different conformation in purified TFR. ii) The molecules that associate with cytoplasmic domains of TFR may mask the binding site of antibody U-1, and iii) other molecules that react with antibody U-1 may be present in cell extracts, and these may cause competitive inhibition of immunoprecipitation of TFR by antibody U-1.

The binding site of antibody U-1 is exposed by binding of antibody N-2 to TFR of cells extracts. We next examined the above possibilities. Mixtures of ^{125}I-labeled antibody U-1, HeLa cell extract and conjugated antibody N-2-Sepharose 4B added in various orders were incubated and then the radioactivities associated with the Sepharose were counted. The results in Fig. 4 show that ^{125}I-antibody U-1 bound to a mixture of antibody N-2-Sepharose 4B and the extract (column 1) but not to antibody N-2-Sepharose 4B alone (column 3). Moreover ^{125}I-antibody U-1 bound as well to Sepharose after incubation of antibody N-2-Sepharose 4B with the cell extract with (column 1) and without (column 2) washing before its addition. These results, and the fact that no specific molecule other than TFR was immunoprecipitated by antibody U-1, exclude possibility iii), because if there had been any cross-reacting material, omission of pre-washing (column 2) would have competitively inhibited the association of radioactivity. If there is no cross-reaction, the equal levels of radioactivity with and without washing indicate that antibody N-2-Sepharose 4B trapped all the TFR in the extract or that although excess TFR was present in the solution without washing, it did not compete with TFR trapped on the Sepharose for binding of antibody U-1. So next, the order of mixing of antibodies was reversed, that is, the cell extracts were incubated first with ^{125}I-antibody U-1, and then antibody N-2-Sepharose 4B was added, and the first incubation was prolonged and the second one was shortened for enhancing differences. In this case, the radioactivity associated with N-2-Sepharose was much less than on first incubation of the extract and N-2-Sepharose (columns 4 and 5). When purified TFR was used instead of the cell extracts, appreciable ^{125}I-antibody U-1 bound to TFR, irrespective of the order of mixing (columns 8 and 9). These data suggest that antibody U-1 cannot bind to its binding site on TFR in cell extracts, but that it can bind to TFR bound to antibody N-2-Sepharose. The associated radioactivity after first incubation with ^{125}I-antibody U-1 (column 5) was low like that observed when the extract was first incubated alone and then the iodinated antibody and the Sepharose were added (column 6). Moreover when the iodinated antibody, Sepharose and the

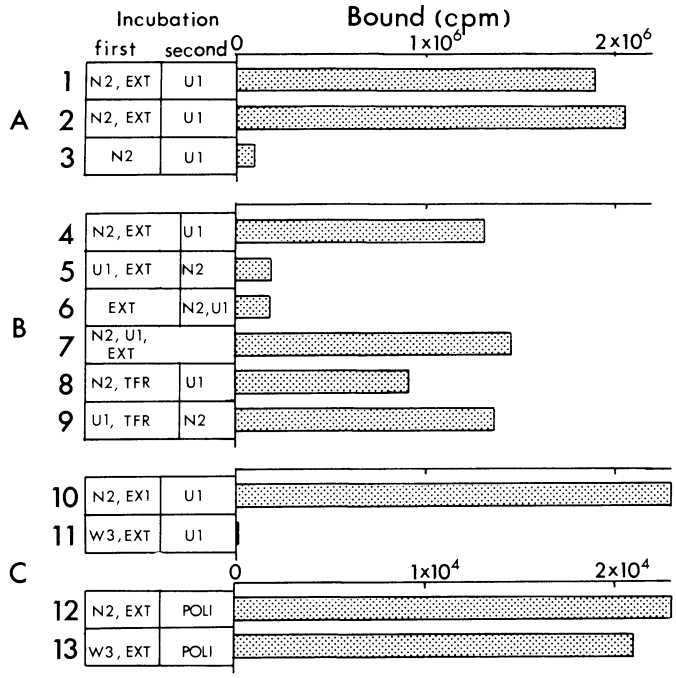

Fig. 4. Binding of ^{125}I-antibody U-1 to a complex of TFR and antibody N-2-Sepharose 4B. ^{125}I-Antibody U-1, HeLa cell extract (or purified TFR) and antibody-Sepharose 4B added in various orders, were incubated and then the binding of U-1 to Sepharose was measured as described under MATERIALS AND METHODS. The order of mixing is indicated on the left of columns. N2, antibody N-2-Sepharose 4B; EXT, HeLa cell extract; U-1, ^{125}I-antibody U-1 (specific activity 4.0–9.0 × 10^6 cpm/μg protein); TFR, purified TFR; W-3, antibody W-3-Sepharose 4B; POLI, ^{125}I-polyclonal antibody against peptide 8−27+Cys (1.52 × 10^7 cpm/μg protein of IgG fraction). For columns 1, 2, 3, 10, 11, 12 and 13 first and second incubations were done overnight, while for columns 4–9 first incubation was for 48 h and second incubation for 3 h. For column 1 only, a washing step was carried out between the first and second incubation periods. The values for columns 4–13 are corrected for nonspecific binding assayed without HeLa cell extracts (extraction solution instead) as shown in column 3. Use of unrelated monoclonal antibody-Sepharose 4B resulted in the same count as that for nonspecific binding.

extract were mixed simultaneously and incubated for the same period, the associated radioactivity was the same as that after first incubation with antibody N-2-Sepharose 4B (column 7).

These results suggest that the binding site of antibody U-1 to TFR in the cell extracts is not exposed conformationally or by other molecules associating with TFR and that the binding of antibody N-2 to TFR causes conformational change and/or release of the covering molecule so that antibody U-1 can approach the binding site.

Monoclonal antibody W-3 did not expose the U-1 binding site and no "Masking Protein" was found. The binding of antibody W-3 to ^{125}I-labeled TFR (purified) was inhibited by TF, and the binding of ^{125}I-labeled TF to cell surface TFR was inhibited by antibody W-3, whereas the bindings of the other antibodies, N-2 and U-1, did not have significant effects on the binding of TF to TFR (data not shown). Antibody W-3 did not bind to TF itself (data not shown). Therefore, the binding

site of antibody W-3 to TFR may be the same as, or near that of TF, unlike that of antibody N-2. Antibody W-3 also inhibited cell uptake of ^{59}Fe through TF and TFR.

Antibody W-3 was found to differ from antibody N-2 in activity to permit antibody U-1 to bind to crude TFR in cell extracts, but antibody W-3 could bind to this TFR. When conjugated antibody W-3-Sepharose 4B was used instead of antibody N-2-Sepharose 4B, ^{125}I-labeled antibody U-1 did not bind to Sepharose incubated first with cell extracts for a longer time (Fig. 4, column 11), with or without the washing step. The possibility that antibody W-3-Sepharose 4B cannot bind to TFR in cell extracts can be excluded, because ^{125}I-labeled rabbit polyclonal antibody against the peptide Ala_8-Arg_{27}+Cys bound to similar extents to antibody N-2- and W-3-Sepharose 4B pre-incubated with the cell extracts (columns 12 and 13) and antibody W-3-Sepharose 4B could precipitate crude TFR in ^{35}S-methionine labeled cell extracts as well as antibody N-2-Sepharose 4B (see Fig. 5). When W-3-

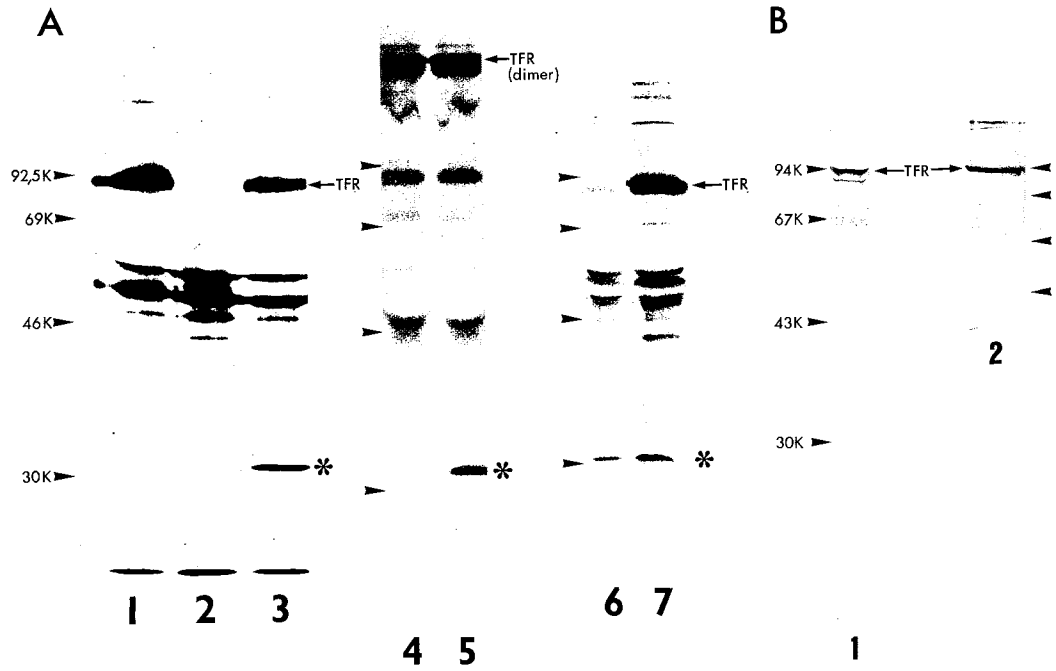

Fig. 5. Co-precipitation of another protein besides TFR by antibody W-3. A. ^{35}S-Methionine-labeled FL cell extracts were immunoprecipitated directly with antibody N-2-Sepharose 4B (lanes 1 and 4), with unrelated monoclonal antibody-Sepharose 4B (lane 2) and with antibody W-3-Sepharose 4B (lanes 3 and 5). For lanes 4 and 5, the samples were electrophoresed in non-reduced conditions; reduced conditions were used for other lanes. Lanes 6 and 7 are the same as lane 3 except for adsorption of the cell extracts by antibody N-2-Sepharose to pre-clear TFR (lane 6) or by BSA-Sepharose as a control (lane 7) before immunoprecipitation. The positions of molecular weight markers are indicated by arrowheads, that of TFR by arrows, and that of protein of about 30 kDa by asterisks. Exposures of the autoradiograph were 5 h for lanes 1–3 and 18 h for lanes 4–7. B. Hela cell extracts were immunoblotted with polyclonal antibody against peptide 8−27+Cys (lane 1) or against to whole molecule of TFR (lane 2) as described in MATERIALS AND METHODS.

Sepharose 4B was first incubated with purified TFR instead of the cell extracts, ^{125}I-labeled antibody U-1 bound to the Sepharose as well as when N-2-Sepharose 4B was used (data not shown). Thus unlike antibody N-2, antibody W-3 seemed not to cause a conformational change of cytoplasmic domains of TFR or release of the molecule covering the U-1 binding site, which was required for binding of antibody U-1. If the latter possibility is true, antibody W-3-Sepharose 4B might precipitate a complex of such a molecule and TFR from cell extracts, and in fact a protein of about 30 kDa was seen as a major band besides TFR and non-specific bands in an autoradiograph on SDS-PAGE analysis of an immunoprecipitate in an ^{35}S-methionine labeled FL cell extract with antibody W-3-Separose 4B (Fig. 5A, lane 3). Since this protein did not react with antibody N-2- or unrelated monoclonal antibody-Sepharose 4B (lanes 1 and 2) or with antibody U-1-Sepharose 4B (data not shown), it must be specifically precipitated with TFR only by antibody W-3. So we investigated the possibility that this protein associated with TFR and covered the binding site of U-1. The 30 kDa protein was precipitated by antibody W-3 to the same extent irrespective of whether reduced or non-reduced condition in SDS-PAGE of the immunoprecipitate. (Fig. 5A, lane 3 and 5). Thus another possibility that antibody W-3 precipitated "heterodimer" consisting of one 90 kDa (intact) and one 30 kDa (cleaved by endogenous cellular proteases) TFR chain was excluded. Furthermore, immuno-blotting of the cell extracts with rabbit antibodies against the peptide Ala_8-ARg_{27}+Cys or against the whole molecule of TFR gave only a single band identical to TFR and no band near 30 kDa (Fig. 5B), strongly supporting the idea that the 30 kDa protein is not a degradative product of TFR. Finally to obtain information about whether coprecipitation of the 30 kDa protein is due to the formation of a complex with TFR or an immunological cross-reactive substance of cell, before immunoprecipitation with antibody W-3 the ^{35}S-methionine labeled cell extract was extensively immunoprecipitated with antibody N-2 to remove TFR (Fig. 5A lane 6). Results showed that TFR was exhaustively precleared, but that nevertheless the 30 kDa protein was precipitated with antibody W-3. Thus the 30 kDa protein is probably not associated with TFR but shares an epitope(s) with TFR. The existence of two different conformational states of the cytoplasmic domain is most probable cause of the difference in binding of antibody U-1.

DISCUSSION

The results in this work were as follows: (a) We showed that the recognition site of monoclonal antibody U-1 was located within the NH_2-terminal region of TFR, Ala_8-Arg_{27}, which is a cytoplasmic domain, while monoclonal antibodies N-2 and W-3 bound to cell surface domains of TFR and antibody W-3 competed with TF for binding to TFR. (b) Antibody U-1 could not bind to TFR in detergent extracts from cells or tissue, but could bind to purified TFR. But it could bind to TFR in the extract when the TFR formed a complex with antibody N-2-Sepharose 4B. (c) On the contrary TFR as a complex with antibody W-3-Sepharose 4B did not bind antibody U-1. Although antibody W-3 co-precipitated a protein of about 30 kDa with TFR, this protein was unlikely to be associated with TFR and so to prevent antibody U-1 from binding to it.

From these results we suggest that the cytoplasmic domain of TFR has two conformational states which are interchangeable. One is that when TFR is purified or formes a complex with antibody N-2 and permits antibody U-1 to approach its

binding site. The other is that in cell extracts or a complex with antibody W-3 that does not permit binding of antibody U-1. Possibly, the binding of antibody N-2 changes the conformation of TFR, resulting in exposure of the binding site of antibody U-1 within the cytoplasmic domain.

In certain receptor systems, such as those of EGF or insulin, binding of the ligand to the extracellular domain of its receptor triggers a function of its cytoplasmic domain such as tyrosine kinase activity, perhaps through conformational change of the receptor molecule. Furthermore binding of some monoclonal antibodies to the receptors mimics this "signal transduction" (4, 22). This is likely, but is not consistent with our observations that antibody W-3, whose binding is like that of TF, did not changed the conformation of TFR, whereas antibody N-2, whose binding is unlike that of TF, had this effect. The fates of ^{125}I-labeled antibody N-2 and W-3 bound to FL cells at 37°C were different: In the steady state, 65% of cell-associated antibody N-2 was internalized into cells (pronase-insensitive), whereas percentage of internalized W-3 was half that of N-2. Moreover during incubation, pronase-insensitive antibody N-2 bound to the cells was not degraded (unpublished data). Unlike antibody N-2, OKT9, a monoclonal antibody against a cell surface domain of TFR, was internalized and degraded with TFR (28). These differences may reflect the different effects of these antibodies on the conformation of TFR, which directs the pathway of TFR migration. Exposure of the binding site of antibody U-1, owing to this conformational change, may also occur when TFR is trypsinized or during its conjugation to CNBr-Sepharose 4B, which involves treatments at high and low pH values, because antibody U-1 could bind to the cytoplasmic fragment of TFR obtained by trypsin treatment of the cell surface or to extracted TFR directly conjugated to CNBr-Sepharose 4B, as described in the results. The effect of TF itself on binding of antibody U-1 requires investigation. Polyclonal antibody against the synthetic peptide recognized the same region as antibody U-1 did, but, unlike antibody U-1, it bound to TFR irrespective of the conformational states of the cytoplasmic domain. Antibody U-1 must recognize a specific region within Ala_8-Arg_{27} and putative conformational change must affect this region. Studies with polyclonal antibodies against each domain of TFR would probably not have demonstrated this change.

Details at the molecular level of how the binding site of antibody U-1 is exposed are unknown. The simplest hypothesis is that in cell extracts, the cytoplasmic domain of TFR has a "folded" conformation and the binding site of antibody U-1 is hidden in the inside of the molecule, but when conformational change is induced by, for example, antibody N-2, this domain "extends" and the binding site is exposed on the surface of the molecule. But the possibility of association of molecules with the cytoplasmic domain of TFR, causing masking of the binding site of antibody U-1, is not completely eliminated (possibility ii described in the RESULTS), although the 30 kDa protein co-precipitated with TFR is unlikely to be associated with TFR and no other proteins co-precipitated by antibody W-3 were found. Conceivably the "masking" molecule is some material other than protein, such as lipid, or a protein not containing methionine. The factor necessary for either "folding" or "masking" must be present in the cell extract, because purified TFR reverted to the states in which the binding site of U-1 was hidden when the cell extract was added (Fig. 3, lane 14). In any case, when antibody N-2 binds to TFR, the conformation of the cytoplasmic domain must change to "extend" or to release the masking molecules.

In the last 10 years, indirect evidence has been obtained for interactions of cell surface receptors with the cytoplasmic cytoskeletal network, such as that cap formation by a specific receptor is associated with a similar distribution of intracellular membrane-associated actin or myosin (3). Although there are no reports of functional linkage between a certain receptor and the actomyosin system, the cytoskelton may be attached to receptors and pull them down or move them. Furthermore, receptors may interact either directly or indirectly with clathrin forming the coat of coated pits and vesicles. The process of migration of receptors mediating endocytosis is complicated and highly systematic. Cytosolic or membranous molecules other than receptors themselves may be necessary for driving and controlling the system and for joining receptors to the cytoskeleton or clathrin. If the conformational change of the cytoplasmic domain of TFR observed here participates in association of the domain of the receptor with the cytoskelton system, its characterization may provide a clue to the mechanism of the system.

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