Human Type 3 Iodothyronine Selenodeiodinase Is Located in the Plasma Membrane and Undergoes Rapid Internalization to Endosomes

Munira Baqui, Diego Botero, Balazs Gereben, Cyntia Curcio, John W. Harney, Domenico Salvatore, Kenji Sorimachi, P. Reed Larsen, and Antonio C. Bianco

From the Thyroid Section, Division of Endocrinology, Diabetes and Hypertension, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts 02115

The type 3 iodothyronine selenodeiodinase (D3) is an integral membrane protein that inactivates thyroid hormones. By using immunofluorescence cytometry confocal microscopy of live or fixed cells transiently expressing FLAG-tagged human D3 or monkey hepatocarcinoma cells expressing endogenous D3, we identified D3 in the plasma membrane. It co-localizes with Na,K-ATPase α, with the early endosomal marker EEA-1 and clathrin, but not with two endoplasmic reticulum resident proteins. Most of the D3 molecule is extracellular and can be biotinylated with a cell-impermeant probe. There is constant internalization of D3 that is blocked by sucrose or methyl-β-cyclodextrin-containing medium. Exposing cells to a weak base such as primaquine increases the pool of internalized D3, suggesting that D3 is recycled between plasma membrane and early endosomes. Such recycling could account for the much longer half-life of D3 (12 h) than the thyroxine activating members of the selenodeiodinase family, type 1 (D1; 8 h) or type 2 (D2; 2 h) deiodinase. The extracellular location of D3 gives ready access to circulating thyroid hormones, explaining its capacity for rapid inactivation of circulating thyroxine and triiodothyronine in patients with hemangiomas and its blockade of the access of maternal thyroid hormones to the human fetus.

Thyroid tissue is confined to and is present in all vertebrates. Its role is to synthesize and secrete polyyiodinated thyrotone molecules that mediate gene expression in virtually every vertebrate tissue through ligand-dependent transcription factors. Thyroxine (T4) is the primary product of thyroid secretion, a pro-hormone that must be activated by deiodination to 3,5,3′-triiodothyronine (T3) by either type 1 or 2 iodothyronine deiodinases (D1 or D2) to initiate thyroid action. To balance the activation pathway, both T4 and T3 are irreversibly inactivated by monodeiodination of the tyrosyl ring of the iodothyronines, a reaction catalyzed by the type 3 iodothyronine deiodinase (D3). These three enzymes constitute a family of selenocysteine (Sec)-containing integral membrane oxidoreductases (1).

Changes in the activity of D3 modulate both global and local tissue thyroid status. In the global sense, D3 expression is increased by T3 and reduced in hypothyroidism or iodine deficiency, thus accelerating or retarding T3 inactivation to maintain homeostasis (2–4) or to alter plasma T3 concentrations such as occurs during tadpole metamorphosis or during fetal life (5–7). More complex are the alterations in D3 activity in specific tissues dictated by developmental programs that permit precisely timed changes in their differentiation. For example, during metamorphosis in Xenopus laevis tadpoles, the eyes must shift from a lateral to a more rostral and dorso-lateral location to permit overlapping visual fields. Retinal cells follow this shift with an asymmetrical growth, a process that is thyroid hormone-dependent. To develop asymmetrically, however, a subset of dorsal cells must grow at a slower rate. This is achieved by an increase in D3 expression in these cells, thus producing transient local hypothyroidism (8). Whereas D3 thus serves an essential physiological role, its inappropriate overexpression in large hemangiomas has recently been shown to cause a unique clinical syndrome termed consumptive hypothyroidism. This occurs in infants (and rarely in adults) when D3-catalyzed inactivation of T3 and T4 occurs more rapidly than maximum thyroidal production (9–11).

Understanding the cellular biology of D3 can shed light on its function, the potential physiological cofactors, and the mechanisms regulating its degradation. The two T3-activating enzymes, D1 and D2, are also integral membrane proteins. Whereas D1 is located in the plasma membrane (PM), D2 is an endoplasmic reticulum (ER)-resident protein (12). Nothing is known about D3 other than it is an integral membrane protein with a putative transmembrane domain near the NH2 terminus (reviewed in Ref. 1). The present studies demonstrate that D3 is primarily in the PM with most of its molecule in the extracellular space.

MATERIALS AND METHODS

DNA Constructions and Transfections—the human D3 cysteine (Cys) mutant (CysD3) was generated by replacing Sec144 with Cys using overlap extension PCR. The CysD3 carboxyl FLAG construct (D3C-FLAG) was engineered in a D10 vector fusing the epitope by an EcoRI/XbaI-based strategy as described previously for D1 and D2. These carboxyl FLAG D1 and D2 constructs (D1C-FLAG and D2C-FLAG) encode full-length Cys mutant deiodinases fused to the FLAG peptide at the COOH terminus (12, 13). Human embryonic kidney (HEK-293) or mouse Neuro2A neuroblastoma cells (NB-2A) were transfected along with TKGH to monitor transfection efficiency as described and were treated with 600 μg/ml G418.

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used ~48 h later. Transfection efficiency was estimated by measuring human growth hormone in the media (12).

**Antibodies and Chemicals**—The primary antibodies used for immunofluorescence cytochemistry (IF) include the monoclonal antibody anti-T3-FLAG M2 and its biotinylated derivative (Sigma); rabbit anti-FLAG, monoclonal anti-Na,K-ATPase a, rabbit anti-early endosome antigen-1 (EEA-1) (Affinity Bioreagents, Golden CO); affinity-purified goat anti-Bip antibody, Research Diagnostics, Inc., (Flanders, NJ); and monoclonal anti-clathrin heavy chain TD-1 antibodies, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Secondary antibodies were used at 1.25 μg/ml and included goat anti-rabbit IgG fluorescein isothiocyanate (FITC) and goat anti-mouse IgG Texas Red-X (Molecular Probes, Eugene, OR) or 1 μg/ml FITC-conjugated F(ab’), fragment donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). Peroxidase-conjugated secondary antibodies were visualized with 3,3’-diaminobenzidine (DAB), which was made with 0.3 M sucrose for 15 min to inhibit the clathrin-mediated endocytic pathways.

**Subcellular Fractionation and Western Analysis**—The cytosol and the microsomal fractions of HEK-293 cells transiently expressing D3C-FLAG were prepared after cells were sonicated in lysis buffer-1 (200 mM HEPES buffer, pH 7.5, containing 5 mM sodium pyrophosphate, 5 mM EGTA, 1 mM MgCl2, 1 mM phenylmethylsulfonfluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM iodacetamide, and 1 mM N-ethylmaleimide) and ultracentrifuged at 100,000 × g for 1 h as described (14). Proteins were resolved by SDS-PAGE and processed for Western analysis as described (15).

**IF and Confocal Microscopy**—Studies were conducted in monkey hepatocarcinoma cells (NCL-E6) that endogenously express D3 (16) and in transfected NB-2A or HEK-293 cells grown directly on glass slides (12). Cells were transfected with D3C-FLAG and processed in two different ways. (i) Paraformaldehyde-fixed cells were permeabilized either with digitonin (25–50 μM) or acetone as indicated, followed by incubation with primary antibodies, as indicated, for 1 h at room temperature (RT), rinsed in phosphate-buffered saline (PBS) containing 1% BSA (PBS/BSA), and incubated for 30 min with the appropriate species-appropriate secondary antibodies. (ii) Nonpermeabilized cells were incubated twice with cold PBS and incubated for 1 h at 4 °C with mouse anti-FLAG (1:100) and rabbit anti-D3-18 (1:80) antibodies diluted in cold DMEM containing 10% FBS. Cells were then fixed and blocked with PBS/BSA containing 1% goat serum and incubated with secondary antibodies as above (17). In all studies, the slides were mounted in Vectashield® mounting medium with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and examined by confocal microscopy on a Bio-Rad MRC-1024/2P system interfaced with a Zeiss Axiovert microscope.

**Cell Biotinylation Assays**—For cell surface biotinylation, HEK-293 cells transiently expressing FLAG-D3C-iodothyronine selenodeiodinase were washed with ice-cold PBS twice and then incubated in 1 mM MCl2 and 0.1 mM CaCl2 (PBS2+) and incubated for two 20-min periods at 4 °C with 10 μg/ml sulfo-NHS-LC-biotin (sulfo-biotin; Pierce) diluted in PBS2+. They were then quenched twice for 20 min (4 °C) with 100 mM glycine in PBS. For intracellular biotinylation, cells were probed for 30 min at room temperature with 1 mg/ml biocytin (Pierce) in PBS and then washed 3 times with PBS2+ containing 2% β-mercaptoethanol. After either method of biotinylation, cells were harvested and lysed for 30 min at 4 °C in lysis buffer-2 (50 mM Tris, pH 8.0, 150 mM NaCl, 1.0% Triton X-100, 1 mM MgCl2) containing 1,000 units of DNase (Promega, Madison, WI), 1 mM phenylmethylsulfonfluoride and 1 × protease inhibitors mixture (Roche Diagnostics). The lysate was centrifuged at 12,000 × g for 10 min at 4 °C. The supernatant incubated for 2 h with streptavidin-agarose beads (Pierce) at 4 °C. The beads were washed twice with lysis buffer-2 containing 500 mM NaCl, twice with the lysis buffer-2, and once with 50 mM Tris-HCl, pH 7.5, and finally the complexes were processed for Western analysis with anti-FLAG M2 antibody (1:3000). In some experiments, as indicated, cells were first treated at 37 °C with DMEM containing 0.3 M sucrose for 15 min to inhibit the clathrin-mediated endocytic pathway (18) or with 10 mM methyl-β-cycloartenol (MC) for 30 min (19), a compound that inhibits clathrin- and caveola-mediated endocytic pathways.

**In Vivo Immunoprecipitation (IP)**—HEK-293 cells transiently expressing D3C-FLAG or D10 vector were metabolically labeled and processed for in vivo IP as described elsewhere (20). Some cells were treated with 10 mM MJCD during the last 30 min of the labeling phase. The cells were then washed with cold buffer A (PBS containing 1% BSA and 10 μg/ml goat anti-mouse IgG) for 20 min followed by incubation on ice with the biotinylated anti-FLAG antibody (1:300) diluted in buffer A for 30 min. Diagonstics were then washed once with buffer A and twice with buffer A containing 5% FBS, harvested, and lysed with lysis buffer. The streptavidin-agarose beads were added to the solubilized fraction, and immune complexes were pelleted by brief centrifugation. Samples were resolved by 4–15% SDS-PAGE and processed for autoradiography.

**Studies of D3 Internalization**—HEK-293 cells transiently expressing D3C-FLAG or D10 were surface biotinylated with SULFO-SS-biotin in PBS2+ for 60 min (4 °C) followed by incubation for 5–10 min at 37 °C with DMEM containing 10% FBS to allow internalization of biotinylated proteins (21). Similarly treated cells were incubated with 0.3 mM primaquine in DMEM with 5 mM HEPES, pH 7.4, to inhibit the recycling limb of the endocytic pathway (22). The cells were then washed with ice-cold PBS2+ and the extracellular biotinylated proteins were freed of biotin by treatment with 50 mM reduced GSH solution and processed for streptavidin-agarose beads pull-down and Western analysis using FLAG M2 antibody (1:3000).

**RESULTS**

**D3 Is Located in the Plasma Membrane**—NCL-E6 monkey hepatocarcinoma cells express high level of endogenous D3 (23). These cells were acetone-treated and processed for IF with primary antibody D3-18, and the images show a pattern in the periphery of these cells suggesting a plasma membrane location (Fig. 1A, a and b). To analyze the cellular expression of this enzyme under controlled conditions using highly specific monoclonal antibody, we prepared a plasmid containing a recombinant human D3 protein tagged with FLAG at its COOH terminus (D3C-FLAG). Because of the inefficient translation of selenoproteins in general, the Sec residue at position 144 was replaced with Cys, and the protein was transiently expressed in various cell types. This D3 retains the capacity to convert T3 to 3,3’-diiodothyronine (T2) in a saturable fashion with a Km of 150 nM (not shown). Western analysis of cytosolic and Na+,K+ ATPase (ph1 11)-washed microsomal fractions of D3C-FLAG expressing HEK-293 cells using anti-FLAG and a primary antibody (D3-18) revealed an identical band of the predicted size (33 kDa) only in the microsomal fraction of the cell lysate (Fig. 1B).

D3C-FLAG-expressing HEK-293 (Fig. 1A, c and d) and NB-2A (Fig. 1A, e and f) cells were processed for IF using anti-FLAG M2 antibody and analyzed by confocal microscopy. D3 is distributed in the periphery, confirming a PM location. The similar IF staining pattern in the periphery to that in the NCL-E6 cells that constitutively express wild type Sec-containing D3 (Fig. 1A, a and b) validates the use of the FLAG-tagged Cys D3 mutant in these studies.

To determine whether the D3 was also expressed in the ER membrane, we exposed D3C-FLAG-expressing HEK-293 cells to 25 μM digitonin to permeabilize the PM but not the ER (12). D3 staining with anti-FLAG M2 antibody was present only at the cell periphery (Fig. 2A, a and b), different from the pattern in acetone-treated cells. To analyze further for D3 expression in the ER membrane, both HEK-293 and NB-2A cells transiently expressing D3C-FLAG were co-stained with anti-FLAG M2 and anti-Bip, an ER-specific marker. The D3 signal was clearly distinct from that of the ER marker (Fig. 2A, a and d). Similar findings were obtained with another ER-specific marker, calnexin (not shown).

To confirm PM localization, HEK-293 cells transiently expressing D3C-FLAG were co-stained anti-Na,K-ATPase α antibody, a typical PM marker (Fig. 2B, a–d). The confocal analysis indicates that a substantial fraction of transiently expressed D3 co-localizes with the Na,K-ATPase PM staining (Fig. 2B, c–f).
The primary anti-D3 antibody (D3-18) is directed against residues 53–68, just COOH-terminal to the predicted transmembrane domain of D3 (residues 16–41). To confirm co-localization of the COOH-terminal FLAG epitope and this peptide, non-permeabilized HEK-293 cells transiently expressing D3C-FLAG were incubated with anti-FLAG M2 and anti-D3-18 antibodies, fixed, and then stained with secondary antibodies. Both antibodies detected D3 on the surface of the transfected cells, and confocal analysis confirmed their co-localization in the extracellular space (Fig. 3A). By using the same antibodies, acetone-permeabilized D3-expressing cells also showed a similar co-localization (Fig. 3B).

An independent approach was used to demonstrate the presence of D3 in the PM of transfected HEK-293 cells. These were subjected to cell surface biotinylation with the cell-impermeant sulfo-biotin probe (see “Materials and Methods”). Cells were lysed with buffer containing 1% Triton X-100 and the biotinylated proteins isolated with streptavidin-agarose beads. Western analysis with anti-FLAG M2 antibody showed a D3 band, confirming the presence of biotinylated D3C-FLAG (Fig. 3C).

As controls we employed HEK-293 cells transiently expressing D1C-FLAG or D2C-FLAG, as they are located in PM and ER, respectively (12). D1C-FLAG was also biotinylated by the cell-impermeant reagent, whereas D2C-FLAG was not (Fig. 3C). However, D2 could be biotinylated with biocytin, a cell membrane-permeable probe, indicating it was present but not accessible to sulfo-biotin (Fig. 3C).

The chronology of D3 appearance in the PM was determined by combining [35S]Met-Cys metabolic labeling with cell surface biotinylation of D3C-FLAG-expressing HEK-293 cells (see “Materials and Methods”) (24). At various times after cells were chased with unlabeled amino acids and surface-biotinylated,
IgG Texas Red-X (b) and rabbit anti-D3-18 (1:80; phase contrast) were incubated with mouse anti-FLAG M2 (1:100; b cells are HEK-293 transiently expressing D3C-FLAG except where of co-localization are shown in d). Cys metabolically labeled, chased with complete media for 0–20 h. 

**Materials and Methods**

NaCl, 0.5% SDS to release the immunocomplexes, diluted to 1 ml with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% SDS to release the immunocomplexes, diluted to 1 ml with 50 mM Tris-HCl, pH 7.5, and the pull-down with streptavidin-agarose beads loaded in the same gel. E–G, HEK-293 cells transiently expressing D3C-FLAG (E), D1C-FLAG (F), or D2C-FLAG (G) were [35S]Met/Cys metabolically labeled, chased with complete media for 0–20 h, 

[35S]Met/-Cys-labeled D3C-FLAG was immunoprecipitated with anti-FLAG M2 antibody and the PM D3 pool isolated on streptavidin-agarose beads (Fig. 3D). It is notable that at all time points the biotinylated (PM) D3 signal is at least 10-fold less intense than that of the total lysate D3, indicating the presence of a substantial pool of intracellular D3. However, the PM D3 signal was most intense at 4–6 h relative to earlier or later times (Fig. 3D), indicating that previously synthesized D3 continues to enter and exit the PM and that D3 is a relatively stable protein. The D3 half-life was ~12 h by pulse-chase analysis (Fig. 3E) as compared with the shorter half-lives of D1C-FLAG and D2-C-FLAG proteins (~7 and ~2 h, respectively) (Fig. 3, F and G).

**D3 in the Plasma Membrane Is Internalized Predominantly by a Clathrin-dependent Mechanism**—Most proteins in the PM undergo internalization through the endocytic pathway. To determine whether this is the case for D3, we exposed HEK-293 cells transiently expressing D3C-FLAG to either hypertonic media (0.3 M sucrose), a procedure that inhibits receptor-mediated endocytosis by preventing the formation of clathrin-coated pits (18), or to 10 mM MβCD, which extracts cholesterol from the PM and inhibits both caveolin- and clathrin-dependent endocytosis (25–28). After incubation, cells were exposed to sulfo-biotin and analyzed by Western blotting using anti-FLAG M2 antibody. Both treatments markedly and specifically increased the PM D3 while not affecting cell permeability, as demonstrated by the absence of D2 biotinylation in similarly treated cells (Fig. 4, A and B) and trypan blue staining (not shown).

The D3 internalization was also investigated by immunoperoxidase analysis of PM D3 in intact [35S]Met-Cys pulse-labeled cells with or without MβCD (20). These were incubated with biotinylated anti-FLAG antibody, lysed, isolated on streptavidin agarose beads, and resolved by SDS-PAGE. Consistent with the direct biotinylation experiments, MβCD-sensitive [35S]Met/-Cys-labeled D3C-FLAG was clearly present in much higher quantity on the surface of MβCD-exposed than in control cells (Fig. 4C). The D3 endocytic pathway is likely to include internalization via a clathrin-dependent mechanism because in addition to the sensitivity to hypertonic medium (Fig. 4A–C), confocal analyses indicate core-localization of D3C-FLAG with clathrin (Fig. 4D, c and d).

Evidence of D3 recycling was obtained using an assay that utilizes cell surface biotinylation with a glutathione-sensitive (cleavable) biotinylation reagent (see “Materials and Methods”) (21). After biotinylation of D3C-FLAG-expressing cells at 4 °C, cells were re-warmed at 37 °C to allow internalization of biotin-tagged cell surface proteins. At the indicated times, the HS-biotin moiety was cleaved from non-internalized cell surface D3 by exposure to 50 mM GSH. The cell lysates were subjected to streptavidin pull-down and Western analysis.

At zero time all the biotinylated D3 is sensitive to GSH and is therefore on the cell surface (Fig. 4E). After 5 min of re-warming, biotinylated D3 is present inside the cells as indicated by its resistance to GSH treatment. Longer incubation times did not result in greater biotinylated D3 accumulation inside the cells, which could be due to recycling of the internalized, biotinylated D3 to the cell surface. Consistent with this explanation, when cells were re-warmed in the presence of 0.3 mM primaquine, a weak base that reduces the rate of return of endosomal proteins to the cell surface (22), the pool of internalized (GSH-resistant) biotinylated D3 was increased (Fig. processed for IP with anti-FLAG M2, and resolved by 10% SDS-PAGE; bands were quantified, corrected by growth hormone levels in the media, and plotted as mean ± S.D. of two plates.
HEK-293 transiently expressing D3C-FLAG were [35S]Met/-Cys metabolically labeled, treated with 10 mM MG132 during the last 30 min of labeling, and processed for in vivo IP (see “Materials and Methods”); the arrow indicates the D3 protein. D, cells were fixed and processed for IF (see “Materials and Methods”) with rabbit anti-FLAG (1:600; a) and mouse anti-EEA-1 (1:100; b) antibodies; secondary antibodies were anti-rabbit IgG FITC (a) and anti-mouse IgG Texas Red-X (b); c, co-localization of the two proteins; d, higher magnification of the boxed area in c, showing details of the co-localization area; bar = 10 μm. E, HEK-293 cells transiently expressing D3C-FLAG were cell surface-biotinylated with the GSH-sensitive (cleavable) reagent sulfo-NHS-SS-E, HEK-293 cells transiently expressing D3C-FLAG were cell surface-biotinylated with the GSH-sensitive (cleavable) reagent sulfo-NHS-SS-E.

4E). Exposure of cells to the D3 substrate, T₃, had no effect on the ratio of biotinylated PM D3 to the total Triton X-100-soluble cell D3.

The fate of the internalized D3 was determined by specific confocal IF analysis in cells stained with antibodies to early endosomes antigen-1 (EEA-1) (Fig. 5c). The merged images show the extensive co-localization of D3 and EEA-1 (Fig. 5d). This indicates that most D3 does not follow the endocytic pathway to late endosomes and lysosomes but accumulates in the early endosomes where a short-loop recycling can occur.

**DISCUSSION**

The present studies are the first to describe the cellular biology of D3, a critical component of the thyroid hormone inactivation pathway. Both endogenous D3 in NCLP-6E cells and D3 transiently expressed in embryonic kidney or neuroblastoma cells are found at the cell periphery (Fig. 1). Curiously, however, only a fraction of D3 is in the PM at any point in time, the remainder is localized just internal to this (Figs. 2, A and B, and 5b). This is illustrated clearly in the comparison between the staining of Na,K-ATPase α and D3 (Fig. 2B) which show co-localization of only a portion of the D3 within the membrane marker. D3 is external to the ER compartment, and much of it co-localizes with clathrin or EEA-1, an early endosomal marker (Figs. 4D and 5d). Positive staining of intact cells with antibodies directed against residues 53–68 or the COOH-terminal FLAG epitope and biotinylation of the protein by an impermeant probe confirm the presence of both epitopes in the extracellular space (Figs. 3C and 4, A–C). Thus, circulating iodothyronines are exposed to D3 but not to D2, which is present only in the ER, as is re-confirmed in the present investigation (Fig. 3C) (12).

Whereas both D1 and D3 are present in the PM, previous topological studies of D1 indicated it had a type 1 membrane protein orientation, with the COOH terminus and the catalytic center of the protein remaining in the cytosol as D1 is incorporated into the PM (29). Like D1, D3 contains a single predicted highly conserved transmembrane domain between residues 16 and 41. All five different algorithms used predict that the COOH-terminal portion of newly synthesized D3 is in the ER compartment, although the COOH-terminal portion of newly synthesized D1 is in the cytosol (29). If there is only a single transmembrane domain and the catalytic center is extracellular, it would give ready access of plasma T4 and T3 to this inactivating enzyme. This is consistent with the role of D3 in the placenta, uterus, and fetal liver to block entry of maternal thyroid hormone to the fetus (reviewed in Ref. 1) and also the capacity of the overexpressed enzyme in infantile hemangioendothelioma cells to increase thyroid hormone inactivation rates up to 9-fold (9).

The presence of D3 in the PM can explain another aspect of the inactivation pathway. In some species, such as amphibians, D1 is not expressed, and in fact, it is also not expressed in human cerebral cortex despite its presence in human liver and kidney (30). Thus, the balance between T₃ activation by D2 and inactivation by D3 is what determines the concentration of T₃ in the nuclei of such tissues. It is not yet certain whether D2 and D3 are expressed in the same or different cells in the brain (1). If both are in the same cell, the differential subcellular localization would still allow for preferential access of D2-
generated T₃ to the cell nucleus. The D3 in D2-expressing cells would then act to limit the availability of the pro-hormone T₄, deiodinating it to reverse T₃, rather than inactivating T₄ produced at the ER membrane.

The rapid accumulation of biotinylated D3 in the PM in the presence of endocytosis inhibitors (Fig. 4, A–C) argues that D3-containing PM regions are normally internalized, becoming part of an endosomal vesicle. These vesicles seem to be predominantly clathrin-coated as there is extensive co-localization of D3 and clathrin (Fig. 4D) and minimal co-localization of D3 and caveolin-1 (not shown). This suggests that during endocytosis, internalized D3 is selected for recycling back to the cell surface. This is supported by the data in the internalization assay in which biotinylated D3 was retained within the cells exposed to primaquine, a potent inhibitor of membrane transport from endosomes to the plasma membrane (Fig. 4E) (22).

This is the first demonstration that a deiodinase undergoes endocytosis, a phenomenon that could have important biological and physiological consequences for D3 activity. For example, the presence of D3 in early endosomes (Fig. 5) suggests that only a minute fraction of internalized D3 progresses to late endosomes and to lysosomal proteolysis, explaining the half-life. In fact, the D3 half-life is ~12 h, a figure that contrasts with the short half-life of D2 (~2 h), the critical ER-resident T₃-producing deiodinase (Fig. 3, E–G). This suggests that the short term adaptation of thyroid status to reduced T₄ production during iodine deficiency or hypothyroidism is due primarily to post-translational up-regulation of D2 and not to a rapid decrease in global D3-mediated thyroid hormone degradation, characteristic of chronic iodine deficiency (1). On the other hand, the substantial quantity of potentially recyclable D3 in the early endosomal pool raises the possibility that an appropriate signal such as starvation or illness could lead to its rapid relocation to the cell surface with a consequent acute inactivation of circulating T₄ and T₃ (1).

During the deiodination reaction the selenol in the active center of the enzyme participates in the nucleophilic attack during which it is oxidized, resulting in transient enzyme inactivation until it is reduced by an as yet unidentified co-factor. The extracellular compartment is known to be an oxidizing environment, making it difficult to reduce the active center after catalysis. Because it has been suggested that a reducing environment is present in the prelysosomal compartment (endosomes) (31), D3 recycling could constitute a mechanism for D3 re-activation. Our preliminary studies do not show any acute effect of T₃ on D3 partitioning within the Triton X-100 soluble pool. However, other stimuli have not been explored.

In conclusion, the present studies show that newly synthesized D3 migrates to the plasma membrane and rapidly undergoes endocytosis to the early endosomal pool, possibly recycling back to the plasma membrane. The signals controlling D3 partition between these two pools are not known, although this process may facilitate regeneration of the selenium-containing active center of the enzyme. The topological studies suggest that the catalytic portion of D3 is extracellular in agreement with the theoretical predictions from the primary amino acid sequence. An active center in the extracellular space would allow D3 ready access to inactivate both the active thyroid hormone T₃ and the prohormone T₄ in both physiological and pathophysiological conditions. Recent IF studies have localized D3 in the endothelial cells of human hemangiomas (10), placenta, umbilical cord, and uterus (11). In addition D3 in the human fetus is expressed in all the epithelial surfaces in contact with amniotic fluid including the skin, bronchial and intestinal epithelia, and amnion syncytiotrophoblast of the placenta. Its position on the PM of these cells would facilitate its putative role to block uncontrolled access of maternal thyroid hormone to the fetus.

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