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Localization of Type I Iodothyronine 5'-Deiodinase to the Basolateral Plasma Membrane in Renal Cortical Epithelial Cells*

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Type I iodothyronine 5'-deiodinase is an integral membrane protein catalyzing the phenolic ring deiodination of thyroxine. We recently showed that the substrate binding subunit of this ~50-kDa protein is selectively labeled with *N*-bromoacetyl-L-thyroxine, allowing ready identification of the type I enzyme without the need to maintain catalytic activity. In this study, we used both affinity labeling and catalytic activity to determine the regional distribution of this enzyme in rat kidney and to localize the enzyme to specific plasma membrane domain(s) of renal epithelial cells. The type I enzyme was present exclusively in tubular epithelial cells of the outer renal cortex and co-purified with basolateral plasma membranes; the renal medulla lacked activity. LLC-PK$_1$ cells, derived from the proximal convoluted tubule, have abundant type I 5'-deiodinating activity. We used this homogenous cell line to verify that the type I enzyme was localized to the cytosolic surface of the basolateral membrane. Digitonin permeabilization increased affinity labeling of the enzyme 4-fold, and ~75% of the affinity label was incorporated into the 27-kDa substrate binding subunit. Affinity labeling of the type I enzyme in LLC-PK$_1$ cells mimicked the affinity labeling of the substrate binding subunit of type I 5'-deiodinase in rat kidney (Köhrle, J., Rasmussen, U. B., Ekenbarger, D. M., Alex, S., Rokos, H., Hesch, R. D., and Leonard, J. L. (1990) *J. Biol. Chem.* 265, 6155-6163). Subcellular fractionation of LLC-PK$_1$ cell homogenates showed that both affinity labeled and catalytically active type I enzyme were present on the cytosolic surface of the basolateral region of the renal cell membrane.

Type I iodothyronine 5'-deiodinase (5'D-I)[1] is an integral membrane protein with a M_r of 50,000-60,000 catalyzing the 5'-deiodination of thyroxine (for review see Ref. 1). In mammals, this enzymatic reaction produces >75% of the biologically active thyroid hormone, T_3, found in the circulation (2). Despite extensive examination of the cellular and subcellular distribution of this membrane-bound enzyme, its localization remains controversial.

In the kidney, T_4 to T_3 converting activity has been reported in collagenase-dispersed rat kidney cells (3). However, this study provided no information regarding the distribution of the enzyme among the cells of the proximal or distal convoluted tubule, collecting duct, or glomerular epithelium. Subcellular localization of whole kidney homogenates revealed that 5'D-I and Na^+/K^+-ATPase were similarly enriched in partially purified plasma membranes (4), although only modest recovery of the labile 5'D-I was observed. In contrast, 5'D-I has been localized to both the endoplasmic reticulum (5-7) and the plasma membrane (8) in the liver. Localization to the endoplasmic reticulum was based primarily on co-purification of 5'D-I with glucose-6-phosphatase, a marker for smooth endoplasmic reticulum, and the failure of 5'D-I to co-sediment with liver alkaline phosphatase (5-7), a plasma membrane enzyme localized to the extracellular surface of the canalicular plasma membrane (9). As with all of these early studies, the relatively modest enrichment of 5'D-I achieved by subcellular fractionation, the selection of marker enzymes, and the difficulties in separating serosal and canalicular plasma membranes confound analysis of the subcellular distribution of 5'D-I.

A major obstacle in the analysis of the subcellular location of 5'D-I has been the difficulty in maintaining catalytic activity during fractionation. Enzyme lability, potential problems of latent enzyme pools in vesicle preparations, and the lack of universally accepted assay conditions contribute to the problems encountered. These obstacles have been overcome by the specific affinity labeling of 5'D-I with the alkylating T_4 derivative, $BrAcT_4$. $BrAcT_4$ irreversibly inhibits 5'D-I and specifically labels a protein that has all of the properties of the substrate binding subunit of 5'D-I (5'D-SBS) (10-12).

On denaturing SDS-PAGE gels, this integral membrane protein has a M_r of 27,000. Similar studies by Visser and coworkers (13) identified a 27-kDa 5'D-subunit in liver membranes using $BrAcT_3$ and demonstrated that $BrAcT_3$ irreversibly inactivates the hepatic 5'D-I (14). Under nondenaturing conditions, gel filtration of detergent-soluble 5'D-I showed that deiodinating activity and the $BrAcT_4$-labeled 5'D-SBS co-eluted with an apparent M_r of 50,000-55,000 (11). More recent studies used the affinity labeled 5'D-SBS to follow the distribution of 5'D-I after isopycnic density gradient centrifugation and gel filtration and showed that the $BrAcT_4$-labeled holoenzyme had a Stokes' radius of 3.78 nm, a $s_{20,w}$ of 3.67 and a calculated M_r of 54,700 (12); values in agreement with those reported previously for catalytically active 5'D-I (15).

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[1] The abbreviations used are: 5'D-I, type I iodothyronine 5'-deiodinase; PMSF, phenylmethylenesulfonyl fluoride; PTU, 6-*n*-propyl-2-thiouracil; T_4, thyroxine; T_3, 3,5,3'-triiodothyronine; rT_3, 3,3',5'-triiodothyronine; 3,3'-T_2, 3,3'-diiodothyronine; $BrAcT_4$, *N*-bromoacetyl-L-thyroxine; EMD 21388, 4',6-$(OH)_2$,3',5'-Br_2,3-CH_3-flavonone; SDS, sodium dodecyl sulfate; SBS, substrate binding subunit; PAGE, polyacrylamide gel electrophoresis; tDOC, taurodeoxycholate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

These data suggest that 27-kDa 5'D-SBS is a component of a larger ~50-kDa multimeric protein, possibly a dimer. The identity of the other subunit(s) of 5'D-I remains to be established.

The availability of established renal epithelial cell lines from defined regions of the nephron, and well known markers for both the apical and basolateral plasma membranes (16–20), provides an ideal source of homogeneous cells for the unambiguous determination of the subcellular localization of 5'D-I. In this study, we utilized affinity labeling of 5'D-I in intact renal epithelial cells derived from the proximal convoluted tubule, LLC-PK_1, to identify 5'D-I in subcellular fractions and determined the subcellular distribution of both catalytically activity and affinity-labeled enzyme. 5'D-I was localized to the cytosolic surface of the basolateral cell membrane.

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium, antibiotics, Hanks' salt solution, glucose, and trypsin were obtained from GIBCO. Supplemented bovine calf serum (heat-inactivated) was obtained from Hyclone, Inc. EMD 21388 was kindly provided by Dr. K. Irmscher, E. Merck, Darmstadt, Federal Republic of Germany. Culture flasks and plasticware were from Nunc. L-T_4 and L-T_3 were from Sigma; L-rT_3 was from Calbiochem; and L-3,3'-T_2 was from Henning Berlin GmBH. 6-*n*-Propyl-2-thiouracil was obtained from Sigma; sodium iopanoate was from Sterling Winthrop Research Institute. Anti-Na^+/K^+-ATPase antisera (α-subunit, holoenzyme) was a gift from by Dr. Thomas Smith (Brigham and Women's Hospital); and anti-alkaline phosphatase antisera was purchased from U. S. Biochemical Corp. $Na^{125}I$(~17 Ci/mg) was purchased from Du Pont-New England Nuclear. All other reagents were of the highest purity commercially available.

N-$BrAcT_4$ was synthesized as described previously (11) and purified by thin layer chromatography ($CHCl_3$:MeOH:HAc, 90:5:5 (v/v) or ethyl acetate:acetic acid, 90:10 (v/v)) on silica gel plates. The product was >90% pure as judged by reverse phase high performance liquid chromatography on a 0.46 × 25-cm μBondapak 10-μm C18 column (Waters) using 35–90% acetonitrile gradient in 0.1% trifluoroacetic acid at a flow rate 1 ml/min. UV absorption was monitored at 254 nm.

L-[3'-or 5'-^{125}I]rT_3 (~2200 Ci/mmol) and *N*-BrAc-L-[3'- or 5'-^{125}I]T_4 (~2200 Ci/mmol) were prepared by radioiodination of 3,3'-T_2 and *N*-$BrAcT_3$, respectively, using the method of Weeke and Orskov (21), and products were purified as described previously (11). Radioiodinated iodothyronines were >95% pure with $^{125}I^-$ as the major contaminant.

Culture Conditions

LLC-PK_1 cells were obtained from the American Tissue Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% supplemented calf serum, 50 units/ml penicillin, and 90 μg/ml streptomycin, in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cells were fed three times weekly and were subcultured (10,000 cells/cm^2) when they reached confluence (3–5 days). Cells were carried for up to 20 passages (the longest time tested) without altering the expression of 5'D-I activity.

Affinity Labeling of 5'D-I with $BrAcT_4$

Confluent monolayers (25-cm^2 flasks) of LLC-PK_1 cells were washed free of growth medium with 3 × 5 ml of PBS (PBS: 20 mM potassium phosphate buffer (pH 7.4) containing 150 mM NaCl) and incubated at 37 °C for 0–20 min in 1 ml of Hanks' solution containing 1 × 10^6 cpm of BrAc-[^{125}I]T_4 (final concentration, 10 nM) and 0.05% digitonin. Affinity labeling was terminated by washing the cells with 3 × 5-ml washes with ice-cold PBS. Cells were then harvested by scraping and collected by centrifugation at 500 × *g* for 10 min. The labeled cells were resuspended in 100 μl of lysis buffer (20 mM HEPES buffer (pH 7.0), 1 mM EDTA, 0.1 mM PMSF, 1 mM dithiothreitol), sonicated for 10 s at 30 watts, and kept at 4 °C until use. Cell sonicates could be stored at −20 °C for up to 1 month without altering the pattern of affinity labeled proteins. Cell sonicates were then mixed with SDS-PAGE sample buffer to achieve a final concentration of 2 mg/ml protein, 50 mM Tris-HCl (pH 6.8), 2% mercaptoethanol, 1% SDS, 10% glycerol, and 0.002% bromphenol blue. Proteins were denatured by heating at 70 °C for 5 min prior to gel analysis.

Crude LLC-PK_1 cell membranes were prepared from BrAc-[^{125}I]T_4 labeled cells by homogenizing the labeled cells in 500 μl of lysis buffer containing 10% (v/v) calf serum and sedimenting through a 1 M sucrose cushion in lysis buffer at 250,000 × *g* for 45 min. This procedure removes >95% of the unincorporated $BrAcT_4$ without affecting the quantity or distribution of the $BrAcT_4$-labeled proteins.

Kinetics of $BrAcT_4$ Inactivation of 5'D-I in Intact LLC-PK_1 Cells

$BrAcT_4$-mediated enzyme inactivation and BrAc-[^{125}I]T_4 incorporation into LLC-PK_1 cell protein were measured, in parallel, in 25-cm^2 flasks of confluent cells. All flasks were washed free of growth medium with 2 × 5 ml of Hanks' solution and permeabilized with 1 ml of 0.05% digitonin in 100 mM potassium phosphate buffer (pH 7.0), containing 1 mM EDTA. Inactivation reactions were done in triplicate flasks and started by adding $BrAcT_4$ to 1 nM final concentration. Flasks were incubated at 37 °C for 1–10 min. Enzyme inactivation was stopped by adding 10 μM [^{125}I]rT_3 and 20 mM dithiothreitol, and 5'D-I activity was determined in the cells by incubating the flasks for an additional 20 min at 37 °C followed by measuring the amount of radioiodine liberated from [^{125}I]rT_3 in the medium. Data are reported as units/flask, where one unit equals the release of 1 pmol of radioiodine·min^{-1}.

BrAc-[^{125}I]T_4 labeling of LLC-PK_1 cell proteins was started by adding 1 nM BrAc-[^{125}I]T_4 (3800 cpm/fmol) and stopped at 1–10 min by addition of 10 μM rT_3. Cells were harvested and processed as described above. SDS-electrophoresis analysis was done on 50 μg of cell protein and the label incorporation into individual bands determined by densitometry.

Preparation of Subcellular Fractions from Rat Kidney and LLC-PK_1 Cells

Rat Kidney—Renal cortical tubules and glomeruli were separated by mechanical disruption as described by Spiro (22). Contamination of tubules by glomeruli was determined by visual inspection of 50 random fields by phase contrast microscopy. Brush border membranes were prepared by the method of Booth and Kenny (18). Basolateral plasma membranes were prepared as described by Forbush (23).

LLC-PK_1 Cells—Confluent cell monolayers from ten 75-cm^2 flasks were washed free of culture medium with 3 × 10 ml of ice-cold PBS and scraped from the flasks into a total of 15 ml of ice-cold PBS. Cells were collected by centrifugation at 500 × *g* for 10 min and the supernatant aspirated. All subsequent isolation steps were done at 4 °C, unless otherwise indicated. Cells were then lysed by one freeze-thaw cycle, resuspended in 2 ml of lysis buffer, and homogenized in a Teflon-glass homogenizer.

Subcellular fractionation of LLC-PK_1 cells was done on Percoll gradients at 4 °C as described (24). In brief, 500 μl of cell homogenate was layered onto 11 ml of 16% Percoll in 250 mM sucrose, 20 mM HEPES buffer (pH 7.0), 1 mM EDTA, 0.1 mM PMSF, 1 mM dithiothreitol and centrifuged in a Sorvall T865.1 rotor at 23,000 rpm for 25 min. 500-μl fractions were collected from the top and kept at 4 °C until use. Subcellular organelles were identified by marker enzymes as follows: apical plasma membrane–alkaline phosphatase (EC 3.1.3.1) (25) and γ-glutamyl transpeptidase (EC 2.3.2.2) (19); basolateral plasma membrane–Na^+/K^+-ATPase (EC 3.6.1.3) (26); mitochondria–succinic dehydrogenase (EC 1.3.99.1) (26); endoplasmic reticulum–glucose-6-phosphatase (EC 3.1.3.9) (25); lysosomes–acid phosphatase (EC 3.1.3.2) (27); and cytosol–lactate dehydrogenase (EC 1.1.1.27) (28). Specific density was determined with density markers beads (Pharmacia LKB Biotechnology Inc.).

Preparation of Apical and Basolateral Membranes from LLC-PK_1 Cells

Apical plasma membranes were prepared as described by Booth and Kenny (18), and basolateral plasma membranes were prepared by the methods of Forbush (23) and Simons and Virta (29). For the isolation of apical membranes or basolateral membranes, affinity-labeled cells from five flasks were scraped from the dish, suspended in a total of 1 ml of 10 mM mannitol, 2 mM Tris-HCl (pH 7.1) (apical membranes) or 1 ml of 250 mM sucrose, 20 mM HEPES buffer (pH 7.0), 1 mM EDTA, 0.1 mM PMSF, and 1 mM dithiothreitol (basolateral membranes). Cells were then lysed by one freeze-thaw cycle and homogenized with 10 strokes of a Teflon-glass homogenizer and kept at 4 °C.

Alternatively, cells were grown to confluence on 10-cm dishes and 5'D-I was affinity-labeled with BrAc-[^{125}I]T_4 as described above. Following the removal of the labeling medium, the cell monolayer was washed with 3 × 5 ml of PBS, 1 mM $MgCl_2$, and 1 mM $CaCl_2$. Apical plasma membranes were removed by overlayering the cell monolayer with a nitrocellulose membrane (0.45 μm pore) equilibrated with the wash buffer. The filters were dried for 4 min under a stream of N_2, and the apical membranes adhering to the filter were removed by lifting. Cell remnants containing the basolateral membranes were left on the culture dish and were washed 2 × 5 min with PBS, scraped from the dish, and collected by centrifugation at 1,000 × *g* for 10 min. The pellet was resuspended in 100 μl of lysis buffer and 20 μl of 5 × SDS-electrophoresis sample buffer added. Proteins were denatured by heating at 70 °C for 5 min prior to gel analysis.

The purity of the basolateral membrane preparations were determined by Western blot analysis (30) using Na^+/K^+-ATPase and alkaline phosphatase as specific markers of the basolateral and apical plasma membrane, respectively. Increasing amounts of cell homogenate (1, 2, and 5 μg of protein) and purified membrane (0.5, 1, and 2 of μg protein) were applied to nitrocellulose under vacuum, using a 72-well slot blot chamber (Schleicher & Schuell). Na^+/K^+-ATPase and alkaline phosphatase were identified using anti-Na^+/K^+-ATPase antisera (α-subunit, holoenzyme) and anti-alkaline phosphatase antisera, respectively. Immune complexes were developed using Protein G-gold conjugate with silver enhancement according to the manufacturer's instructions (Bio-Rad) or the Vectastain ABC kit with peroxidase detection system according to manufacturer's instructions (Vector Labs). Both detection systems yielded equivalent results. Immune complexes were quantitated using an LKB Ultroscan XL scanning laser densitometer.

Iodothyronine 5'Deiodinase Assay

5'-Deiodinating activity was determined as described previously (31) at 10 μM [^{125}I]rT_3 (100 cpm/pmol) and 20 mM dithiothreitol. Reactions were done in triplicate for 10 min at 37 °C and product formation quantitated as described previously (31). Enzyme activity is expressed as units, where 1 unit equals the release of 1 pmol radioiodine·min^{-1}.

SDS-Electrophoresis Analysis

Affinity radiolabeled proteins (25–50 μg) were separated on 12.5% SDS-polyacrylamide slab gels as described by Laemmli (32), with 4 mM thioglycolic acid present in the upper tank buffer (11). Gels were then fixed in a solution composed of 10% HAc (v/v) and 25% isopropyl alcohol (v/v) and dried. Dried gels were exposed to XAR-5 film (Kodak) at −70 °C with intensifying screens for 1–5 days. Radioautographs were analyzed by scanning densitometry.

Two-dimensional Gel Analysis

Two-dimensional gel analysis was done as described by O'Farrell (33) without urea. In brief, BrAc-[^{125}I]T_4-labeled, tDOC-solubilized membrane protein (40 μg) from cells labeled in the absence (~300,000 cpm) or presence (~50,000 cpm) of the 5'D-I inhibitor, 10 μM iopanoic acid, were suspended in 20 μl of lysis buffer containing 5 mM tDOC, 2% ampholines (Pharmalytes 3-10 and 5-8; 2:3 ratio), and 2% Nonidet P-40 and focused on 1.5 × 110-mm gel tubes for 16 h at 500 V. Isoelectric focusing gels were then treated for 5 min in 50 mM Tris-HCl (pH 7.8), 2% mercaptoethanol (v/v), 1% SDS (w/v), 10% glycerol (v/v), layered onto a 12.5% SDS-polyacrylamide slab gel and fixed in place with 1% agarose. Electrophoresis in the second dimension was done under reducing conditions as described above.

Miscellaneous Methods

Protein was determined by the method of Bradford (34) using human immunoglobulin G as the standard.

RESULTS

Early work on the subcellular localization of 5'D-I in rat kidney (4) ignored the diversity of cell types present in the kidney and the regional specialization of the nephron. In preliminary experiments, >85% of the 5'D-I activity was found in the outer 3 mm of the renal cortex with an abrupt loss of enzyme activity occurring at the cortical-medullary junction. The renal medulla had undetectable levels of 5'D-I activity. Separation of the renal cortex into glomeruli and cortical tubules showed that the isolated tubules had twice the 5'-deiodinating activity of that in glomeruli (Table I). When the glomeruli were corrected for cortical tubule cell contamination little or no 5'D-I activity remained, suggesting that the 5'D-I present in glomeruli was due to contaminating tubular elements.

Since the crude membrane preparations used for 5'D-I assays are composed of sealed vesicles with both right-side-out and inside-out orientations, we examined whether orientation of the catalytic site toward the vesicle lumen resulted in enzyme latency. Crude plasma membrane vesicles were prepared from renal cortex tubules by differential centrifugation (800,000 × g_{min} pellet) and then separated on 5–20% linear Ficoll gradients. Shown in Fig. 1 is the distribution of 5'D-I determined in the native and tDOC-solubilized membranes. Detergent-solubilized vesicles showed a ~2-fold increase in 5'D-I activity, suggesting that up to half of the enzyme activity in these preparations was latent. In all subsequent experiments, 5'D-I activity was determined in the

TABLE I

Distribution of 5'-D-I along the major elements of the rat renal cortex

The renal cortical tubules and glomeruli were prepared as described under "Materials and Methods." The identity of the isolated fractions was confirmed by microscopy and the contaminating elements counted in 50 separate fields. 5'-D-I activity was determined as described under "Materials and Methods." Data are representative of six separate preparations.

Morphology	5'-Deiodinase activity	Contamination (contaminating structure)
	units/mg protein	%
Whole cortex	50	
Intact tubules	62	15 (glomeruli)
Glomeruli	33	30 (tubules)

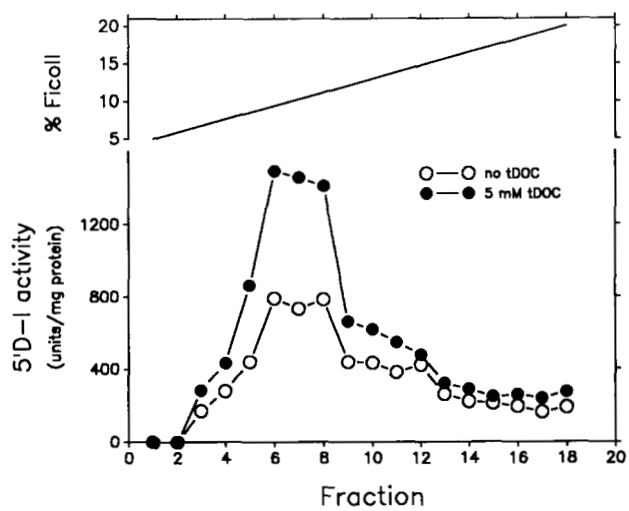

FIG. 1. **Effects of detergent solubilization of 5'D-I activity in renal membrane vesicles.** Kidney tubules were prepared as described in the legend to Table I and homogenized in 5 volumes (w/v) of 250 mM sucrose, 20 mM HEPES (pH 7.0), 1 mM EDTA, and 1 mM dithiothreitol. Homogenates were centrifuged as 500 × *g* for 10 min to remove cell debris, and crude plasma membranes were collected by centrifugation at 1,600 × *g* for 10 min. Membranes were resuspended in homogenizing buffer and layered onto a 10-ml 5–20% linear Ficoll gradient in homogenizing buffer and centrifuged at 35,000 rpm for 3 h. 500-μl fractions were collected and 5'D-I activity determined in the absence or presence of 5 mM tDOC as described under "Materials and Methods." Data are the means of closely agreeing (±10%) triplicate determinations.

presence of 5 mM tDOC to ensure maximal recovery of 5′D-I activity.

The distribution of 5′D-I among specific membrane fractions of renal cortical tubule epithelium is shown in Table II. Both 5′D-I and Na^+/K^+-ATPase were enriched 13–15-fold in basolateral plasma membranes with 30–40% recovery of enzymatic activity. In contrast, 5′D-I was absent from the brush border plasma membranes and did not co-purify with either alkaline phosphatase or γ-glutamyl transpeptidase, two enzyme markers of the apical cell surface. By comparison, only 2–5-fold enrichments of 5′D-I, Na^+/K^+-ATPase, and γ-glutamyl transpeptidase were observed in the heavy microsomal fraction composed of a mixture of membranes from endoplasmic reticulum, the apical and basolateral plasma membranes.

Identification of 5′D-I in Intact LLC-PK$_1$ Cells—Since 5′D-I was limited in the outer renal cortex, and we previously showed that cells derived from the proximal convoluted tubule of pig kidney (LLC-PK$_1$) had abundant 5′D-I activity (35), we used this homogeneous cell line to confirm the distribution of affinity labeled 5′D-I among the specialized domains of the plasma membrane. Shown in Fig. 2 is the pattern of $BrAcT_4$-labeled proteins obtained by incubating intact LLC-PK$_1$ cells with the affinity label. Prominently labeled bands were observed at 30, 27, and 25 kDa, with minor bands occasionally observed at 90 and 55 kDa. To ensure access of the affinity label to all cellular compartments, the effect of selected permeabilizing agents on $BrAcT_4$ labeling of intact LLC-PK$_1$ cell proteins was examined. Cells permeabilized with 0.05% (w/v) digitonin showed a 3–4-fold increase in $BrAcT_4$ labeling with a preferential accumulation in the 27-kDa band, as judged by scanning densitometry. Little or no 27-kDa protein was released from the cells into the medium by permeabilization and ~75% of the affinity label was associated with this protein. $BrAcT_4$ labeling of other bands was marginally affected. Cells treated with either 0.1% (w/v) saponin or up to 0.1 M dimethyl sulfoxide showed variable increases in affinity labeling.

Affinity label incorporation into permeabilized LLC-PK$_1$ cells increased for up to 20 min and then remained constant for up to 40 min of incubation. Loss of cellular protein from the permeabilized cells was not apparent for the first 15 min of incubation and then progressively increased so that only 30% of the total cell protein remained after 40 min of incubation. Despite the loss of total cell protein, little or no 27-kDa protein was lost from the cells. Based on these findings, 20 min was chosen as the optimal time for affinity labeling.

TABLE II

Subcellular distribution of 5′-D-I in isolated membrane fractions from renal tubular cells

Renal cortical tubules were prepared as described under "Materials and Methods." Cortical tubule heavy microsomes and basolateral plasma membranes prepared as described under "Materials and Methods." Brush border membranes (apical cell membranes) were prepared separately. Data are presented as relative specific activity defined as the activity in the individual fraction/starting enzyme activity. The starting enzyme activities in renal tubule homogenates were as follows: alkaline phosphatase, 860 units/mg protein; γ-glutamyl transpeptidase, 640 units/mg protein; Na^+/K^+-ATPase, 16 units/mg protein; 5′-D-I, 0.04 units/mg protein, where 1 unit = 1 nmol product/min.

Enzyme	Relative specific activity		
	Heavy microsomes	Brush border plasma membrane	Basolateral plasma membrane
Alkaline phosphatase	0.9	4.2	<0.1
γ-Glutamyl transpeptidase	2.0	6.0	<0.1
Na^+/K^+-ATPase	3.2	2.1	14.6
5′-D-I	5.2	1.2	13.0

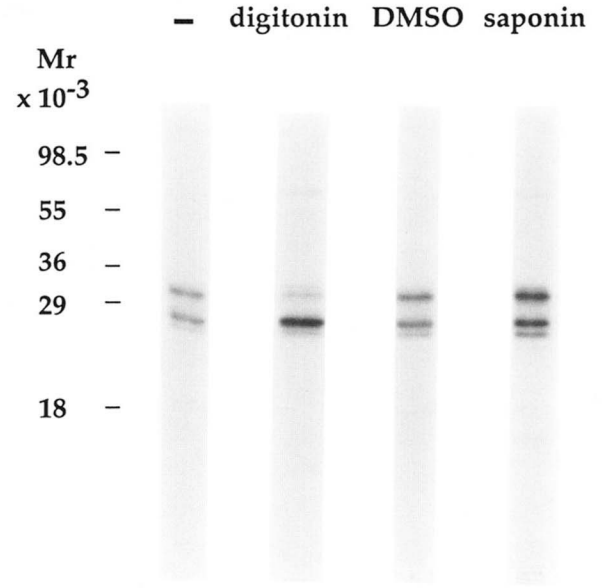

FIG. 2. **$BrAcT_4$ labeling of proteins in intact LLC-PK$_1$ cell.** Cells were preincubated for 5 min in the absence or presence of 0.05% digitonin, 100 mM dimethyl sulfoxide, or 0.1% saponin in Hanks' salts solution followed by affinity labeling with 10 nM $BrAc[^{125}I]T_4$ for 20 min at 37 °C. Proteins in cell sonicates were separated on 12.5% SDS-PAGE gels as described under "Materials and Methods." Radioautograms were developed at −70 °C for 24 h.

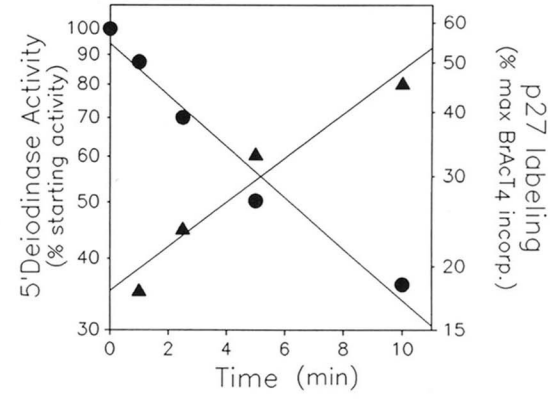

FIG. 3. **Kinetics of $BrAcT_4$ inactivation of 5′D-I and 5′D-SBS labeling in permeabilized LLC-PK$_1$ cells.** Triplicate flasks (25 cm^2) of confluent cells were preincubated with 0.05% digitonin in Hank's salts solution for 5 min, followed by affinity labeling with 1 nM $BrAc[^{125}I]T_4$ at 37 °C for the times indicated. Parallel culture flasks were incubated with 1 nM $BrAcT_4$ at 37 °C for the times indicated, followed by determination of 5′D-I activity as described under "Materials and Methods." Cells were harvested by scraping, sonicated, and 50 μg of $BrAc[^{125}I]T_4$-labeled protein separated on 12.5% SDS-PAGE gels. The percentage of maximal affinity label incorporation was calculated using the quantity of $BrAc[^{125}I]T_4$ incorporated into the 27-kDa protein after 40 min of incubation as maximal labeling. Data are reported as the means of closely agreeing (±10%) triplicate flasks. 5′D-I activity in untreated controls was 20 units/flask. ●, 5′D-I activity; ▲, p27 labeling.

To establish the relationship between the affinity-labeled 27-kDa protein and 5′D-I in LLC-PK$_1$ cells, we determined the kinetics of $BrAcT_4$ inactivation of 5′D-I and compared them with the kinetics of affinity labeling of the 27-kDa protein. As shown in Fig. 3, the time-dependent inactivation of 5′D-I by $BrAcT_4$ followed pseudo-first order kinetics and was directly related to the accumulation of the affinity label by the 27-kDa protein in intact cells, with equivalent rate

constants (k) of 0.04 min^{-1}. In contrast, label incorporation into the 30- and 25-kDa bands was near maximal after 2.5 min of incubation and was not blocked by a 10,000-fold molar excess of the 5'D-I substrate, rT_3 (data not shown).

To confirm the identity of the $BrAcT_4$-labeled 27-kDa substrate binding subunit of 5'D-I (5'D-SBS) in LLC-PK_1 cells, the effects of reversibly-binding 5'-deiodinase inhibitors and selected substrates on $BrAcT_4$ labeling were studied. Competitive 5'D-I inhibitors such as iopanoate (a radiocontrast agent) and EMD 21388 (a synthetic flavone) selectively blocked labeling of the 27-kDa protein by >90%. Similarly, the enzyme substrates rT_3 and T_4 and the product T_3 blocked $BrAcT_4$ labeling of the 27-kDa protein by >95%. $BrAcT_4$ labeling of all other bands were unaffected by any of these compounds.

PTU inhibits catalysis by forming a mixed disulfide with an oxidized sulfhydryl of the enzyme generated during the catalytic cycle (1). To determine the relationship between the time-dependent formation of a PTU-enzyme mixed disulfide and blockade of $BrAcT_4$ labeling of the 27-kDa protein, cells were preincubated with 10 μM PTU for 5–20 min, followed by affinity labeling with $BrAcT_4$. As shown in Fig. 4, PTU pretreatment progressively blocked the subsequent affinity labeling. Compared with untreated controls, affinity labeling of the 27-kDa protein decreased by 20, 40, and 74% after 5, 10, and 20 min of pretreatment with PTU, respectively, as judged by scanning densitometry.

Shown in Fig. 5 is the distribution of $BrAcT_4$-labeled proteins after two-dimensional gel electrophoresis. The affinity-labeled 27-kDa protein showed a single spot with a pI of 6.1, and this spot was markedly reduced when cells were affinity-labeled in the presence of the 5'D-I inhibitor, iopanoate. As observed previously (11), an increase in the labeling of a 55-kDa protein (pI 4.1) and an 18-kDa protein (pI ~4) were observed when labeling of the 5'D-I was blocked by iopanoate. Taken together, the data indicate that the $BrAcT_4$-labeled 27-kDa protein in LLC-PK_1 cells is the 5'D-SBS.

Subcellular Distribution of 5'D-I and 5'D-SBS in LLC-PK_1

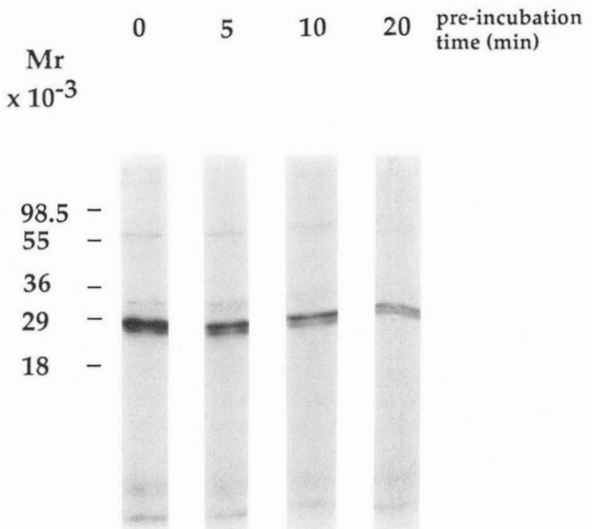

FIG. 4. **Effects of propylthiouracil pretreatment on $BrAcT_4$ labeling of p27 in LLC-PK_1 cells.** Triplicate flasks (25 cm^2) of cells were preincubated for 5–20 min with 10 μM propylthiouracil and the cell monolayers washed free of inhibitor by 2 × 5 ml of Hanks' solution. Cells were then permeabilized for 5 min with 0.05% digitonin in Hanks salts solution, followed by a 20-min labeling period with of 10 nM BrAc[^{125}I]T_4. Cell proteins were separated on 12.5% SDS-PAGE gels as described under "Materials and Methods," and radioautograms were developed at −70 °C for 2 days.

Cells—Shown in Fig. 6 are the profiles of organelle marker enzymes, 5'D-I activity and the 5'D-SBS after fractionation of LLC-PK_1 cell homogenates on self-forming Percoll gradients. Both the 5'D-I activity and the affinity-labeled 5'D-SBS showed sharp co-incident peaks that sedimented with plasma membrane markers and were well separated from the smooth and rough endoplasmic reticulum. Little or no 5'D-I activity or affinity-labeled 5'D-SBS was observed to co-sediment with mitochondria (fractions 18–20) or lysosomes (fractions 16–18), and nuclei were devoid of 5'D-I and 5'D-SBS. As expected, treatment of LLC-PK_1 cells with $BrAcT_4$ inhibited >95% of the 5'D-I activity observed in the gradient.

Since 16% Percoll gradients failed to resolve apical from basolateral membranes, two approaches were used to determine the distribution of affinity-labeled 5'D-I in these two membrane locations. Basolateral membranes from $BrAcT_4$-labeled cells isolated by the method of Simons and Virta (26) showed that both 5'D-SBS and the basolateral membrane marker, Na^+/K^+-ATPase, were enriched 25- and 45-fold, respectively, in these membranes (Table III). In contrast, the apical membranes marker, alkaline phosphatase, was marginally enriched, and little or no affinity-labeled 5'D-SBS was present in the apical membranes adhering to the nitrocellulose used to remove the apical cell border (data not shown).

Using traditional methods (18, 23) for the preparation of these two membrane compartments showed that both the 5'D-SBS and immunoreactive Na^+/K^+-ATPase were enriched 14- and 10–12-fold, respectively, in basolateral membranes with >60% recovery of these two proteins in these isolated preparations (Table III). Alkaline phosphatase, a marker of the apical brush border, was marginally enriched (~2-fold) in basolateral membranes. In contrast, purified apical membranes showed a 10-fold enrichment of alkaline phosphatase, with only 2–3-fold enrichments of either Na^+/K^+-ATPase or the affinity-labeled 5'D-SBS and <3% recovery of the 5'D-SBS or Na^+/K^+-ATPase in these purified membranes (Table III).

To determine whether 5'D-I faced the cytosol or the extracellular space in the basolateral plasma membrane, the vectorial orientation of 5'D-I was examined using the impermanent probe, trypsin. Trypsinization of permeabilized cells decreased the affinity-labeled 5'D-SBS content by 75% (Table IV), as judged by scanning densitometry. Similarly, trypsinization resulted in the loss of >95% of the 5'D-I activity in permeabilized cells. In contrast, treatment of unpermeabilized cells with the impermanent probe had no effect on either the affinity-labeled 5'D-SBS content or 5'D-I activity. These data demonstrate that 5'D-I is orientated toward the intracellular compartment in the basolateral membrane of the cell.

DISCUSSION

The subcellular localization of the membrane-bound 5'D-I has been a subject of continuing confusion. Since the kidney is a diverse organ composed of highly polarized epithelium and there are well known markers for both the apical and basolateral plasma membrane, we examined the inter-organ and subcellular distribution of 5'D-I in this organ. 5'D-I was limited to the tubular epithelium of the renal cortex. Subcellular fractionation of the cortical tubules showed that 5'D-I co-purified with the basolateral plasma membrane marker Na^+/K^+-ATPase, and like the liver, the 5'-deiodinase failed to co-purify with either apical membrane enzyme marker. Since alkaline phosphatase is restricted to the canalicular surface of the hepatocyte (9), a region analogous to the apical brush border of the renal epithelial cell, the current findings

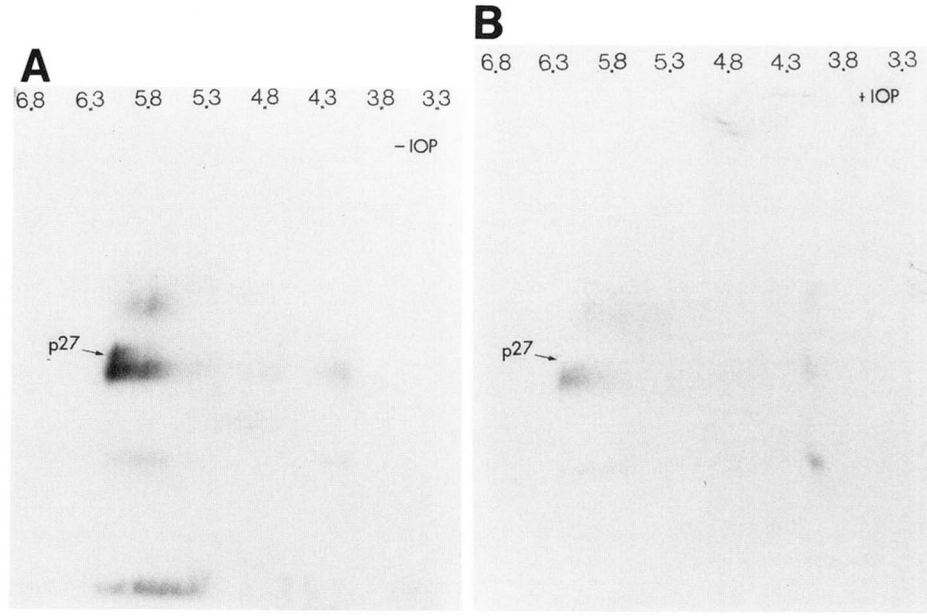

FIG. 5. **Two-dimensional gel analysis of $BrAcT_4$-labeled 5D-SBS done in the absence (*A*) and presence (*B*) of 10 μM iopanoate.** Confluent monolayers of LLC-PK_1 cells were affinity-labeled with 10 nM $BrAc[^{125}I]T_4$ in the absence (*A*) and presence (*B*) of 10 μM iopanoate. 40 μg of radiolabeled cell membranes (~300,000 cpm for *A* and ~50,000 cpm for *B*) were then run on two-dimensional gels as described under "Materials and Methods." Radioautograms were developed at −70 °C for 2 days.

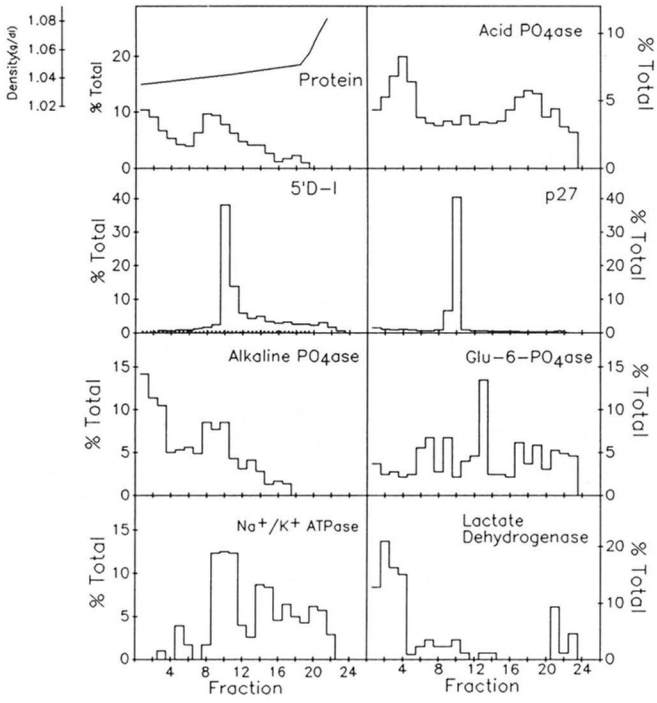

FIG. 6. **Distribution of marker enzymes, 5′D-I and 5′D-SBS, after fractionation on 16% Percoll gradients.** Confluent monolayers of LLC-PK_1 cells (5–10 flasks (80 cm^2)) were used as the starting material for subcellular fractionation. $BrAc[^{125}I]T_4$-labeled cells were prepared as described under "Materials and Methods." 5′D-I activity was determined in parallel, in the presence of 5 mM tDOC, in cell homogenates prepared from untreated (——) and $BrAcT_4$-treated (· · · ·) cells. Subcellular fractionation was done on 16% Percoll gradients. The distribution of alkaline phosphatase and Na^+/K^+-ATPase were determined by Western blot analysis as described under "Materials and Methods." Data are representative of three separate experiments.

TABLE III

Characterization of apical and basolateral plasma membranes from LLC-PK_1 cells

Basolateral plasma membranes were prepared as described by Simons and Virta (26) (Method I). Apical and basolateral membranes were also prepared by the method of Booth and Kenny (18) and Forbush (23), respectively (Method II). Na^+/K^+-ATPase (α-subunit, Method I; holoenzyme, Method II), alkaline phosphatase, and 5′-D-SBS were determined as described under "Materials and Methods" and quantitated by scanning densitometry. Data are reported as means of three closely agreeing (±10%) separate preparations. Numbers in parentheses indicate the recovery of the individual protein in the fraction. For Method I, starting immunoreactive protein levels were: alkaline phosphatase, 0.6; Na^+/K^+-ATPase, 1.5; and 5′-D-SBS, 1.1 AU/mg protein, respectively. For Method II, starting immunoreactive protein levels were: alkaline phosphatase, 13; Na^+/K^+-ATPase, 15; and 5′-D-SBS, 7 AU/mg protein, respectively. AU, arbitrary absorbance units.

Tissue fraction	Protein	Alkaline phosphatase	Na^+/K^+-ATPase	5′-D-SBS
	% recovery		*-fold enrichment*	
Method 1				
Cell homogenate	100	1 (100%)	1 (100%)	1 (100%)
Membrane basolateral	1.7	5 (8%)	45 (75%)	25 (42%)
Method II				
Cell homogenate	100	1 (100%)	1 (100%)	1 (100%)
Membrane basolateral	5.6	1.9 (10%)	11 (52%)	14 (72%)
Apical	0.7	12 (9%)	2 (2%)	3 (3%)

suggest that the kidney and liver 5′D-I distribution may not differ. In addition, the observation that up to half of the 5′D-I activity is latent in vesicular membrane preparations renders earlier subcellular distribution studies difficult to interpret. This latency probably reflects restricted access of the substrates and cofactors to the enzyme active site and/or the limited diffusion of products generated in the vesicle lumen. This latter possibility may be especially important when determining catalytic activity by measuring the release of radioiodine.

The ability of several renal cortical cell lines to catalyze 5′-deiodination has been examined previously (35). LLC-PK_1 cells retain the properties of the proximal convoluted tubule and have abundant 5′D-I activity making them an ideal source of 5′D-I in a homogeneous cell population.

Using $BrAcT_4$ as a specific affinity label for 5′D-I (10–12), we found that $BrAcT_4$ labeled a 27-kDa protein and that affinity label incorporation was directly related to irreversible enzyme inactivation. Affinity labeling of the 27-kDa protein in LLC-PK_1 cells was blocked by 5′D-I substrates, inhibitors,

TABLE IV

Determination of the vectoral orientation of 5'-D-I in the plasma membrane of LLC-PK$_1$ cells

Triplicate flasks (25 cm^2) of BrAc[^{125}I]T$_4$-labeled and unlabeled cells were permeabilized with 0.05% digitonin for 5 min at 37 °C as indicated. Trypsin (0.1 mg/ml) was added as indicated and the cells then incubated at 37 °C for 5 min. Proteolysis was terminated by the addition of an equal volume of ice-cold Hanks' solution containing 10% calf serum, and the cells were scraped from the dish and collected by centrifugation at 70 × *g* for 5 min. Cell pellets were washed three times with 5 ml of ice-cold PBS and the final cell pellets sonicated in 100 μl of lysis buffer. 5'-D-I activity and BrAcT$_4$-labeled 5'-D-SBS were determined as described under "Materials and Methods." 5'-D-I activity reported as means ± S.E.; 5'-D-SBS reported as the mean of closely agreeing (±10%) triplicate flasks. AU, arbitrary absorbance units.

	Trypsin	5'-D-I	
		Activity	5'-D-SBS
		units/flask	*AU/flask*
Unpermeabilized	−	5.8 ± 1	2.7
	+	5.3 ± 0.9	2.6
Permeabilized	−	7.9 ± 2.4	2.4
	+	<0.1	0.3

and the time-dependent formation of a PTU-enzyme mixed disulfide. In addition, this affinity-labeled protein in LLC-PK$_1$ cells had a pI that was identical to that reported for the 5'D-SBS in rat kidney (11). The relationship between 27-kDa protein and 5'D-SBS in the LLC-PK$_1$ cells was confirmed by the observation that the rate of BrAcT$_4$ inactivation of enzyme activity equaled the rate of affinity labeling of the 27-kDa protein. Recently, Berry *et al.* (36) cloned a cDNA from a rat liver cDNA library that encodes a ~27-kDa protein with all of the properties of 5'D-I. These data indicate that the 27-kDa protein present in LLC-PK$_1$ cells is similar, if not identical, to the 5'D-SBS in liver and kidney membranes (10, 11, 13, 14).

Since LLC-PK$_1$ cells maintain polarity in culture and form tight junctions that limit access to the basolateral portion of the cell, we examined whether this functional barrier might restrict access of the affinity label to the 5'D-SBS. Permeabilized LLC-PK$_1$ cells showed a 3-4-fold increase in the labeling of the 5'D-SBS, suggesting that entry of the affinity label to the intracellular compartment or access to basal portion of the cell was partially restricted.

The identification of the affinity labeled 5'D-I in LLC-PK$_1$ cells eliminates the problems inherent in the determination of catalytic activity and allows the subcellular distribution of 5'D-I to be unambiguously determined. These data show that the enzyme was localized to the cytosolic surface of the basolateral cell membrane. Since the source of this enzyme's substrate is the circulation, it was not unexpected to find the 5'D-I located in close proximity to the vascular bed. The finding that the active site of the enzyme faces the intracellular compartment indicates that the substrate and the product, T$_3$, must cross the cell membrane to enter and/or exit the cell. Recent work by several laboratories (37-39) have suggested that cellular uptake of iodothyronines is, in part, a facilitated process. Others have proposed that endocytosis mediates the entry of thyroid hormones into cells, either by specific interactions with surface iodothyronine receptors (40, 41) or co-internalized in association with several classes of lipoproteins (42, 43). Clearly, the latter mechanism would have little or no selective advantage for hormone metabolizing tissues, since the iodothyronines must cross the bilipid layer to gain access to the cytosolic-facing catalytic site of 5'D-I.

Recent studies have proposed that 5'D-I and protein disulfide isomerase, an enzyme localized to the endoplasmic reticulum, are similar, if not identical, proteins (44, 45). However, the current findings that the enzyme is located in the basolateral plasma membrane of renal epithelial cells and previous work demonstrating that 5'D-I is composed of at least two subunits (10–13) indicate that 5'D-I and protein disulfide isomerase are distinctly different proteins.

In summary, we have identified and characterized the subcellular distribution of the substrate binding subunit of 5'D-I and demonstrated that the enzyme resides in the basolateral portion of the cell membrane in the renal epithelial cell line, LLC-PK$_1$, and in rat kidney cortical tubular cells. The ability to affinity label this enzyme with BrAcT$_4$ and the simple pattern of nonenzyme-related proteins in LLC-PK$_1$ cells makes these cells an ideal source of the enzyme catalyzing the bioactivation of thyroid hormone.

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