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

(Received for publication, December 12, 1990)

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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-PK1 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-PK1 cells mimicked the affinity labeling of the substrate binding subunit of type I 5'-deiodinase in rat kidney (Köhrlle, J., Rasmussen, U. B., Ekenbarger, D. M., Alex, S., Rotok, H., Hesch, R. D., and Leonad, J. L. (1990) J. Biol. Chem. 265, 6155-6163). Subcellular fractionation of LLC-PK1 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)† is an integral membrane protein with a M, 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, T3, 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, T4 to T3 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 label 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 copurification 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 by the arylation T4 derivative, BrAcT3. BrAcT3 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, of 27,000. Similar studies by Visser and co-workers (13) identified a 27-kDa 5'D-Sbsubunit in liver membranes using BrAcT3 and demonstrated that BrAcT3 irreversibly inactivates the hepatic 5'D-I (14). Under non-denaturing conditions, gel filtration of detergent-soluble 5'D-I showed that deiodinating activity and the BrAcT3-labeled 5'D-SBS co-eluted with an apparent M, 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 BrAcT3-labeled holoenzyme had a Stokes' radius of 3.78 nm, a spherical of 3.67, and a calculated M, of 54,700 (12); values in agreement with those reported previously for catalytically active 5'D-I (15).
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-PK1, 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, Hank's salt solution, glucose, and trypsin were obtained from GIBCO. Supplemented bovine serum (heat-inactivated) was obtained from HyClone, Inc. EMD 21588 was kindly provided by Dr. K. Irmscher, E. Merck, Darmstadt, Federal Republic of Germany. Culture flasks and plasticware were from Nunc. L-T, and L-T were from Sigma; L-rT, was from Calbiochem; and L-3',5'-T, was from Henning Berlin GmbH. N-BrAcT, was obtained from Swant, and radioactive iodine was obtained from Mallinckrodt Whinthrop Research Institute. Anti-Na'/K'-ATPase antisera (α-subunit, holozyme) was a gift from Dr. Thomas Smith (Brigham and Women's Hospital); and anti-alkaline phosphatase antisera was purchased from U. S. Biochemical Corp. Na'11263 (17 Ci/mg) was purchased from Du Pont-New England Nuclear. All other reagents were of the highest purity commercially available.

N-BrAcT, was synthesized as described previously (11) and purified by thin layer chromatography (CHCl3: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 Bondapak 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 264 nm.

5'-BrAcT, was incubated with 3,3'T2 (2200 Ci/mmol) 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-PK1, cells was done on Percoll gradients as described (24). In brief, 500 μl of cell homogenate was layered onto 11 ml of 15% 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 T805.1 rotor at 25,000 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) (30); mitochondrial succinic dehydrogenase (EC 1.3.99.1) (28); endoplasmic reticulum–glucose-6-phosphatase (EC 3.1.3.2) (25); lactic acid dehydrogenase (EC 1.1.1.27) (28). Specific density was determined with density markers (Pharmacia LKB Biotechnology Inc.).

Affinity Labeling of 5'D-I with BrAcT, Platelet antibody (pH 7.0), 1 mM EDTA, 0.1 mM PMSF, 1 mM dithiothreitol, and 5'D-I activity was determined in the cells by incubating the cells for an additional 20 min at 37°C according to the method of BracT,-mediated enzyme inactivation and BrAcT-stable incorporation into LLC-PK; cell proteins were measured, in parallel, in 25-cm2 flasks of confluent LLC-PK; cells. 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), and 5'D-I activity was determined as described above.

Preparation of Subcellular Fractions from Rat Kidney and LLC-PK; 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 randomly selected phase contrast microscopically. Basolateral plasma membranes were prepared by the method of Booth and Kenny (18). Basolateral plasma membranes were prepared as described for Forbus (23). LLC-PK; Cells—Confluent cell monolayers from 10-75 cm2 flasks were washed free of culture medium with 3 × 10 ml of ice-cold PBS and scraped from the flask 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-PK1, 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 15% 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 T805.1 rotor at 25,000 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) (30); mitochondrial succinic dehydrogenase (EC 1.3.99.1) (28); endoplasmic reticulum–glucose-6-phosphatase (EC 3.1.3.2) (25); lactic acid dehydrogenase (EC 1.1.1.27) (28). Specific density was determined with density markers (Pharmacia LKB Biotechnology Inc.).

Preparation of Apical and Basolateral Membranes from LLC-PK; Cells

Apical plasma membranes were prepared as described by Booth and Kenny (18), and basolateral plasma membranes were prepared by the methods of Forbus (23) and Simon and Virta (29). For the isolation of apical membranes or basolateral membranes, affinity-labeled cells from five flasks were scraped from the dishes, 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.

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Alternatively, cells were grown to confluence on 10-cm dishes and 5'-D-I was affinity-labeled with BrAc-[\(^{125}\)I]T\(_3\), as described above. Following the removal of the labeling medium, the cell monolayer was washed with 3 x 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 \(\mu\)m 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 x 5 min with PBS, scraped from the dish, and collected by centrifugation at 1,000 \(\times\) 6 for 10 min. The pellet was resuspended in 100 \(\mu\)l of lysis buffer and 20 \(\mu\)l of 5 x 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 membranes, respectively. Increasing amounts of cell homogenate (1, 2, and 5 \(\mu\)g of protein) and purified membrane (0.5, 1, and 2 \(\mu\)g 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 (a-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.

**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 \(\times\) \(g\)min pellet) and then separated on a 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 absence or presence of 5 mM tDOC as described under "Materials and Methods." Data are representative of six separate preparations.

**Table I**

| Morphology          | 5'-Deiodinase activity (units/mg protein) | Contamination (contaminating structure) |
|---------------------|-----------------------------------------|----------------------------------------|
| Whole cortex        | 50                                      |                                        |
| Intact tubules      | 62                                      | 15 (glomeruli)                         |
| Glomeruli           | 93                                      |                                        |

**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 at 600 \(\times\) \(g\) for 10 min to remove cell debris, and crude plasma membranes were collected by centrifugation at 1,600 \(\times\) \(g\) 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 \(\mu\)l 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.
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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-PK1 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-PK1) 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 BrAcT4-labeled proteins obtained by incubating intact LLC-PK1 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 BrAcT4 labeling of intact LLC-PK1 cell proteins was examined. Cells permeabilized with 0.05% (w/v) digitonin showed a 3-4-fold increase in BrAcT4 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 with the 27-kDa labeling.

Affinity label incorporation into permeabilized LLC-PK1 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.

| 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                       | 13.9                        |

Fig. 2. BrAcT4 labeling of proteins in intact LLC-PK1 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's salts solution followed by affinity labeling with 10 nM BrAc[T4]T4, 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 BrAcT4 inactivation of 5'D-I and 5'D-SBS labeling in permeabilized LLC-PK1 cells. Triplicate flasks (25 cm2) of confluent cells were permeabilized with 0.05% digitonin in Hanks's salts solution for 5 min, followed by affinity labeling with 1 nM BrAc[T4]T4, at 37 °C for the times indicated. Parallel culture flasks were incubated with 1 nM BrAcT4 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 90 µg of BrAc[T4]T4 labeled protein separated on 12.5% SDS-PAGE gels. The percentage of maximal affinity label incorporation was calculated using the quantity of BrAc[T4]T4 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.
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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-1 substrate, T₂ (data not shown).

To confirm the identity of the BrAcT₄-labeled 27-kDa substrate binding subunit of 5’D-I (5’D-SBS) in LLC-PK₁ cells, the effects of reversibly-binding 5’-deiodinase inhibitors and selected substrates on BrAcT₄ 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 rT₃ and T₄ and the product Ta blocked labeling of the 27-kDa protein by >95%. BrAcT₄ 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 BrAcT₄ labeling of the 27-kDa protein, cells were preincubated with 10 μM PTU for 5–20 min, followed by affinity labeling with BrAcT₄. 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 BrAcT₄-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 BrAcT₄-labeled 27-kDa protein in LLC-PK₁ cells is the 5’D-SBS.

Subcellular Distribution of 5’D-I and 5’D-SBS in LLC-PK₁ 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-PK₁ cells 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-PK₁ cells with BrAcT₄ 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 BrAcT₄-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
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Fig. 5. Two-dimensional gel analysis of BrAcT₄-labeled 5'D-SBS done in the absence (A) and presence (B) of 10 μM iopanoate. Confluent monolayers of LLC-PK₁ cells were affinity-labeled with 10 nM BrAc[¹²⁵I]T₄ 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-PK₁ cells (5–10 flasks (80 cm²)) were used as the starting material for subcellular fractionation. BrAc[¹²⁵I]T₄-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 BrAcT₄-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.

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-PK₁ 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 BrAcT₄, as a specific affinity label for 5'D-I (10–12), we found that BrAcT₄ 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-PK₁ cells was blocked by 5'D-I substrates, inhibitors,
and the time-dependent formation of a PTU-enzyme mixed disulfide. In addition, this affinity-labeled protein in LLC-PK1 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-PK1 cells was confirmed by the observation that the rate of BrAcT,-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-PK1 cells is similar, if not identical, to the 5′-D-SBS in liver and kidney membranes (10, 11, 13, 14).

Since LLC-PK1 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-PK1 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-PK1 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, T3, 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 thyroid hormone 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-PK1, and in rat kidney cortical tubular cells. The ability to affinity label this enzyme with BrAcT,- and the simple pattern of nonenzymel-related proteins in LLC-PK1 cells makes these cells an ideal source of the enzyme catalyzing the bioactivation of thyroid hormone.

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