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Extranuclear Localization of Endogenous 11β-Hydroxysteroid Dehydrogenase-2 in Aldosterone Target Cells*

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ABSTRACT

Type 2 11β-hydroxysteroid dehydrogenase (11βHSD2) plays a key role in conferring aldosterone selectivity on the mineralocorticoid receptor (MR) by inactivating intracellular glucocorticoids before they can occupy the MR. 11βHSD2 is a microsomal enzyme expressed in aldosterone target cells, although its subcellular distribution is still unclear. The goal of this study was to determine the subcellular localization of the endogenous 11βHSD2 in renal aldosterone target cells. We generated an antibody against rabbit 11βHSD2 and used it in combination with a nuclear marker and confocal laser scanning microscopy. On Western blots the antibody recognized a single band of approximately 41 kDa in the renal cortical collecting duct, outer medullary collecting duct, submandibular gland and adrenal cortex, whereas the colon, liver, renal medulla, and heart were negative. Immunohistochemistry showed specific reaction in the known aldosterone target cells of the kidney (connecting tubule, cortical collecting duct, and outer medullary collecting duct) with no signals over glomeruli, proximal nephron segments, and blood vessels. Staining for 11βHSD2 was very weak in rabbit colon, and no immunoreactivity could be detected in the heart and brain. Confocal microscopy of kidney sections costained with the 11βHSD2 antibody and the nuclear marker propidium iodide demonstrated that 11βHSD2 is in the cytoplasmic compartment with no evidence for nuclear localization.

Subcellular localization of 11βHSD2 to a cytoplasmic compartment seems ideal for fulfilling its biological function, *i.e.* the efficient inactivation of intracellular glucocorticoids before they occupy MRs, which are predominantly cytoplasmic in the absence of hormone. (*Endocrinology* **139:** 2955–2959, 1998)

THE TYPE 2 11β-hydroxysteroid dehydrogenase (11βHSD2) is expressed at high levels in mineralocorticoid target cells where it plays a critical role in conferring aldosterone selectivity on inherently nonselective mineralocorticoid receptors (MRs) (for review, see Ref. 1). The main target of aldosterone is the renal collecting duct. Previous studies have shown that both the native 11βHSD2 in collecting duct cells (2, 3) as well as the cloned enzyme expressed in oocytes or mammalian cells (4–7) have an unusually high affinity for its substrate and catalyze irreversible dehydrogenation (*i.e.* inactivate glucocorticoids).

For efficient protection of the MR, the subcellular location of 11βHSD2 should allow rapid inactivation of glucocorticoids before they can occupy the MR, which, in the absence of hormone, is mainly cytoplasmic (8). However, the subcellular localization of 11βHSD2 is still debated. Our recent data using a 11βHSD2-green fluorescent protein (GFP) fusion protein revealed exclusive endoplasmic reticulum (ER) localization both in CHO cells (9) as well as in MDCK cells, a cell line originating from the distal nephron (10). These observations are in agreement with earlier results indicating that 11βHSD2 is a microsomal enzyme (3, 11). On the other hand, our findings disagree with the conclusion of a recent study suggesting that in addition to a cytoplasmic localization, about 40% of 11βHSD2 resides in the nucleus (12).

The goal of this study was to reexamine the subcellular localization of the endogenous 11βHSD2 in renal aldosterone target cells using an antibody against the rabbit 11βHSD2 in combination with a nuclear marker and confocal laser scanning microscopy. The data presented here clearly demonstrate that 11HSD2 is extranuclear in mineralocorticoid target cells and confirm that the previously observed ER localization of the 11βHSD2-GFP fusion protein is not due to inappropriate localization due to the GFP tag or expression in nonaldosterone target cells.

Materials and Methods

Generation of antibody against the rabbit 11βHSD2

A mouse polyclonal antibody was generated against a fusion protein containing the C-terminal region of the rabbit 11βHSD2 (amino acids 139–406). The rabbit 11βHSD2 complementary DNA (6) was used as template to amplify the corresponding region using an upper primer (5′-CGGAATTCTGACCAAGCCAGCAGACATTAG-3′) and a lower primer (5′-GCTGGTACCTGTGTGTGCAGCTCATCG-3′), which add *Kpn*I and *Eco*RI restriction sites. After digestion with *Kpn*I and *Eco*RI, the PCR product was ligated into the pPROEx-1 vector (Life Technologies, Gaithersburg, MD), which contains an N-terminal polyhistidine tag and a TEV protease cleavage site. Direct sequencing was performed to verify that the insert is in the correct reading frame and is identical to the appropriate region of the rabbit 11βHSD2 (6). The construct was transfected into *Escherichia coli* DH5α, and production of the fusion protein was induced by isopropyl β-D-thiogalactopyranoside for 3 h. The fusion protein was solubilized in 8 M urea from bacterial inclusion bodies, purified on Ni-NTA resin according to the manufacturer's instructions (Life Technologies), and then cleaved with recombinant TEV protease (Life Technologies) to remove the polyhistidine tag. BALB/c female mice were immunized with 100 μg of the cleaved fusion protein in complete Freund's adjuvant ip once and subsequently twice with 50 μg

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fusion protein in incomplete Freund's adjuvant at 2-week intervals. Blood was obtained from the tail, and the serum titer was determined by enzyme-linked immunosorbent assay using the purified fusion protein as antigen.

Immunoblotting

Rabbit cortical collecting duct (CCD) cells and outer medullary collecting duct cells were isolated by immunodissection as previously described (13). Total tissue homogenate was also prepared from rabbit kidney cortex, kidney papilla, distal colon, pancreas, heart, adrenal glands, and submandibular glands by snap-freezing the tissues after dissection and homogenizing in 1% SDS-containing solubilization buffer (14). Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL), with dithiothreitol then added to a final concentration of 1 mM. Protein (10–25 μg) was electrophoresed on a 12.5% SDS-polyacrylamide gel and 4% stacking gel, and transferred to polyvinylidene difluoride Immobilon (Millipore, Bedford, MA) membrane with 10 mM 3-[cyclohexylamino-L-propanesulfonic acid (CAPS), pH 11.0 as transfer buffer. The membrane was blocked for 1 h in 5% nonfat milk in TBST (10 mM Tris-HCl, pH 7.4; 150 mM NaCl; and 0.02% Tween-20) and then probed with a 1:10,000 dilution of the mouse 11βHSD antibody or preimmune serum at room temperature for 1 h. Membranes were washed with TBST, incubated with a 1:20,000-fold dilution of alkaline phosphatase-conjugated rabbit antimouse IgG at room temperature for 1 h, and washed again four times with TBST, and antibody binding was localized by incubating with Vistra ECF substrate (Molecular Dynamics, Sunnyvale, CA) and scanning on a FluorImager-575 (Molecular Dynamics).

Immunohistochemistry and laser scanning confocal microscopy

Male New Zealand rabbits were anesthetized. The kidneys were perfused with periodate-lysine-paraformaldehyde (PLP) fixative, kept in PLP for an additional 2 h at room temperature (15), and finally embedded in paraffin. Four-micron thick sections were deparaffinized, and endogenous peroxidase activity was blocked by incubation with 3% H_2O_2. Immunohistochemistry was performed using a 1:500 dilution of the 11βHSD2 antiserum or preimmune serum, followed by a 1:100 dilution of a horseradish peroxidase-conjugated antimouse IgG (Zymed, San Francisco, CA). 11βHSD2 immunoreactivity was visualized by the Tyramide Signal Amplification system (TSA-Indirect, New England Nuclear, Boston, MA) (16), with Cy-5-conjugated streptavidin (1:500) or horseradish peroxidase-conjugated streptavidin (1:500) followed by diaminobenzidine. For nuclear staining, sections were incubated with the DNA dye, propidium iodide, at 1 μg/ml after treatment with deoxyribonuclease-free ribonuclease (5 μg/ml, 37 C for 30 min). Sections were mounted in Vectashield (Vecta Laboratories, Burlingame, CA). For fluorescence confocal microscopy, a Bio-Rad MRC-1024 Laser Scanning Confocal System (Bio-Rad, Richmond, CA) was used, with a ×63 planapo objective (numerical aperture 1.4) and 647 nm excitation and 680 ± 16-nm bandpass filter for Cy-5 fluorescence, and 568-nm excitation and 605 ± 16-nm bandpass filter for propidium iodide fluorescence. A Z-series of images was generated using a mechanical step size of 0.18 μm. Images were subjected to 15 cycles of a constrained maximum likelihood estimation algorithm (AutoQuant Imaging, Watervliet, NY) (17).

Results

Western blotting

We have generated a polyclonal antibody against the rabbit 11βHSD2 by immunizing mice with a fusion protein encompassing amino acids 139–406 in the C-terminal region of 11βHSD2. Enzyme-linked immunosorbent assay indicated that the mouse sera had a very high titer (1:50,000), whereas preimmune sera were negative. Sera from three of the four mice immunized with 11βHSD2 reacted with a single band on Western blots of rabbit CCD cells, with an apparent molecular mass of approximately 41 kDa (Fig. 1, *left panel*, lane 1). This reaction is specific, as none of the preimmune sera gave signals (Fig. 1, *left panel*, lane 2). The 11βHSD2 antibody also recognized a single protein band with the same size in total kidney cortex, outer medulla, and submandibular gland (Fig. 1, *right panel*). On the other hand, no signal could be detected in the rabbit proximal tubule, colon, renal papilla, liver, heart, or small intestine. Although not an aldosterone target tissue, 11βHSD2 protein was abundant in the adrenal gland (Fig. 1, *right panel*, last lane). Previous results showed that 11βHSD2 messenger RNA (mRNA) is expressed in sheep and rat (5, 18, 19), albeit not in mouse (20), adrenal.

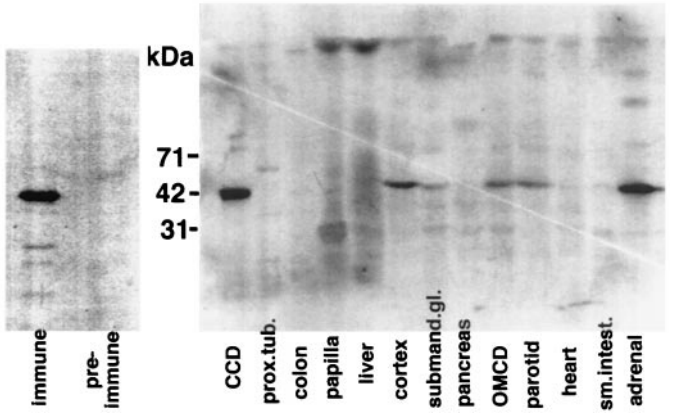

FIG. 1. Western blot of rabbit tissues probed with an 11βHSD2 antibody. The *left panel* shows 10 μg/lane protein from CCD cells, with the first lane probed with a 1:10,000 dilution of immune serum, and the second lane probed with a 1:10,000 dilution of preimmune serum. The *right panel* shows 10–25 μg solubilized protein from various rabbit tissues probed with a 1:10,000 dilution of immune serum, as described in *Materials and Methods*. prox. tub., Proximal tubule; submand. gl., submandibular glands; OMCD, outer medullary collecting duct; sm. intest., small intestine.

Immunohistochemistry

As shown in Fig. 2, 11βHSD2 antibody reacted specifically within the kidney, whereas the preimmune serum was negative (not shown). 11βHSD2 immunoreactivity was strongest in the connecting tubule and CCD (Fig. 2), and became weaker toward the papilla; the tip of the papilla was negative (not shown). Within the CCD, reactivity was heterogeneous, with strong staining of principal cells and undetectable reaction in intercalated cells (also see Fig. 3). Renal blood vessels did not react with the 11βHSD2 antibody. The intrarenal distribution of 11βHSD2 protein corresponded well with the pattern of mRNA distribution in rabbit kidney (6).

Immunoreaction of the 11βHSD2 antibody was very weak in the rabbit colon (not shown). This observation together with the lack of an immunoreactive band on Western blots of rabbit colon suggest that the expression level of 11βHSD2 in the colon is much lower than that in the human, where both immunohistochemistry and Northern analysis detected 11βHSD2 (4, 21, 22). In addition, no 11βHSD2 immunoreactivity could be detected in rabbit brain, in accordance with reports that failed to detect 11βHSD2 mRNA by Northern analysis in the brains of different species (4, 5). On the other hand, *in situ* hybridization showed signals in the nucleus

tractus solitarius and subcommissural organ in rats (23). Therefore, we looked specifically at these regions of the brain, but did not detect immunoreactive 11βHSD2. It should be noted that there are no data showing the presence of immunoreactive 11βHSD2 protein in the brain of any species.

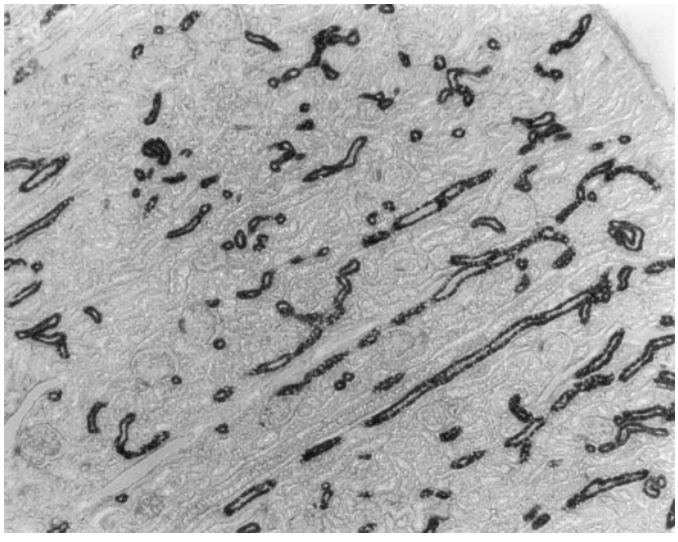

FIG. 2. Immunohistochemical localization of 11βHSD2 in rabbit kidney. PLP-fixed rabbit kidney sections were stained with the anti-11βHSD2 mouse serum at a 1:500 dilution. Immunoreaction was visualized using horseradish peroxidase-labeled antimouse IgG and the TSA-Indirect system, as described in *Materials and Methods*. Images were captured on a PXL cooled CCD camera attached to an Olympus IMT2 microscope (Olympus, New Hyde Park, NY), using a ×20 objective. Intense positive reaction of cortical collecting duct cells can be observed. Magnification, ×256.

Subcellular localization of 11βHSD2 using confocal laser scanning microscopy

To determine the exact subcellular localization of 11βHSD2, we performed confocal laser scanning microscopy on renal sections double stained with the 11βHSD2 antibody and the DNA dye, propidium iodide. As shown in Fig. 3, in the renal aldosterone target cells, 11βHSD2 is found exclusively in the cytoplasm (*green* fluorescence), exhibiting a reticular staining pattern characteristic of the endoplasmic reticulum. Nuclei are stained *red* with the DNA dye, propidium iodide, and there is no indication of intranuclear 11βHSD2 staining, which would appear as *orange* fluorescence (combination of *green* and *red* fluorescence). As the collecting duct cells shown in Fig. 3 are not exactly in the same plane, two optical sections are shown; in Fig. 3A, the plane corresponds to the plane of the center of the upper cell's nucleus, whereas in Fig. 3B, the plane is at the center of the lower cell's nucleus. With the optical section through the center of the nucleus, in both cells the nuclei are negative for 11βHSD2. The 11βHSD2-negative cell shown in the *lower right corner* of Fig. 3A (*red* fluorescence only corresponding to the nuclei) is most likely an intercalated cell. The other nucleus in the *upper right corner* of Fig. 3A is in a neighboring non-CCD tubule.

Discussion

11βHSD2 is a key factor in conferring aldosterone selectivity on the MR, by inactivating intracellular glucocorticoids before they can occupy the MR. However, 11βHSD2 can be an effective "guardian" of the MR only if it is localized in a subcellular compartment that allows quick and efficient metabolism of glucocorticoids. One factor that is likely to be

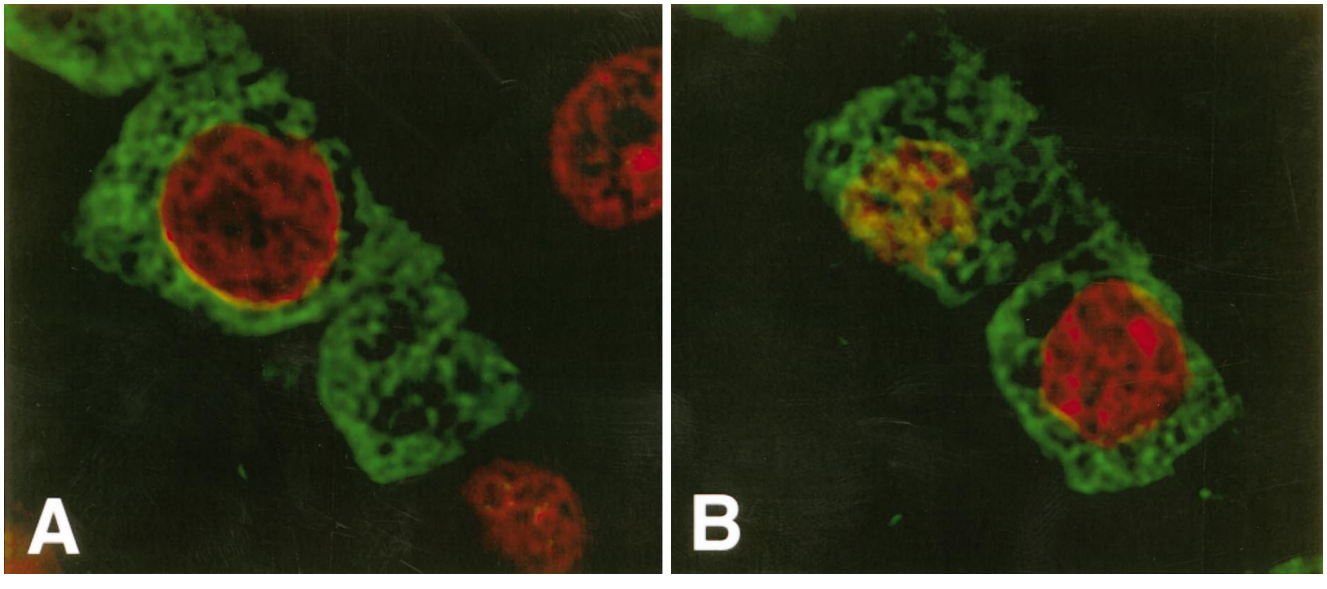

FIG. 3. Confocal laser scanning microscopy of 11βHSD2 immunofluorescence in rabbit CCD. Rabbit kidney sections were stained with a 1:500 dilution of the mouse anti-11βHSD2 serum, followed by a 1:100 dilution of horseradish peroxidase-conjugated antimouse IgG. The 11βHSD2 immunoreaction was visualized with the TSA-Indirect system and Cy-5-conjugated streptavidin (the Cy-5-associated fluorescence is displayed in *green* pseudocolor). The sections were treated with ribonuclease and stained with 1 μg/ml propidium iodide (*red* fluorescence). A CCD is shown with three cells in both panels; two of the cells are positive for 11βHSD2 (*green*). In A, the focal plane is through the center of the upper cell's nucleus, whereas in B, the plane is through the center of the lower cell's nucleus. The cell in the *lower right corner* of A, which is negative for 11βHSD (*red* fluorescence only), corresponds to an intercalated cell. Magnification, ×3460.

important for this task is the vicinity of 11βHSD2 to the unliganded MR. The bulk of evidence suggest that in the absence of mineralocorticoids, the MR is in the cytoplasm, and upon hormone binding it translocates to the nucleus (8, 24, 25). However, the subcellular localization of 11βHSD2 is still controversial. The deduced amino acid sequence of the cloned 11βHSD2 clearly predicts a membrane protein (4–6). Indeed, earlier cell fractionation studies found the highest enzymatic activity in the microsomal fraction in the native epithelium (3, 26) as well as in cells expressing recombinant 11βHSD2 (27).

Previously we examined the subcellular localization of 11βHSD2 using a 11βHSD2-GFP chimera. Our data showed that when expressed in either CHO cells (9) or renal epithelial cells (10), 11βHSD2-GFP localized exclusively to the endoplasmic reticulum. As the possibility that the subcellular localization of a 11βHSD2-GFP fusion protein expressed in host cells differs from that of the endogenous enzyme could not be excluded, we reinvestigated this issue in the rabbit kidney using a novel antibody against 11βHSD2.

The major finding of the present study is that endogenous 11βHSD2 is extranuclear in renal collecting duct cells, which are the main target cells of aldosterone. The data presented here are in good agreement with our previous observations (9, 10) as well as with data obtained for human kidney (22), although in the latter study no costaining with nuclear markers was performed. On the other hand, our results conflict a report by Shimojo *et al.*, who found that in addition to cytoplasmic staining, about 30–40% of the total 11βHSD2 immunoreactivity in human tissues localizes to the periphery of nuclei (12). The reason for this discrepancy is unclear, although it seems unlikely to be related to species differences (*i.e.* rabbit *vs.* human), as Kyossev's data were also obtained with human kidney (22).

A partial endoplasmic reticular, partial nuclear localization of 11βHSD2 is particularly puzzling, because its amino acid sequence clearly predicts a membrane protein, and there is no precedent that the same protein occurs simultaneously in both a membrane and the nucleus. According to current knowledge, entry into the nuclear compartment occurs exclusively through the nuclear pores, either by diffusion of small hydrophilic molecules or by the aid of nuclear localization signals. However, 11βHSD2 contains hydrophobic membrane-spanning domains and does not have nuclear localization signals. In theory, cleavage of the hydrophobic N-terminal region might allow a truncated molecule to diffuse through the nuclear pores, yet it seems unlikely that such cleavage would occur only on 30–40% of 11βHSD2 molecules. Even more to the point, should such a truncated 11βHSD2 diffuse into the nucleus through the nuclear pore, its intranuclear distribution pattern should be homogeneous instead of the rim-like staining observed by Shimojo *et al.* (12). In addition, recent data by Krozowski and co-workers show that an N-terminal deletion mutant of 11βHSD2 is still associated with microsomal membranes (27). Thus, the apparent rim-like staining for 11βHSD2 in the above report remains puzzling, all the more so as such a nuclear subcompartment, occupying the outer 30–40% of the nucleoplasm is presently unknown. Therefore, the possibility that the observed partial nuclear localization is due to an experimental artifact has to be considered.

There are several factors that could have led to such an artifact. First, the rim-like 11βHSD2 staining in the nucleus observed by confocal microscopy (12) might have been due to solubilization and diffusion of the antigen during tissue preparation or mounting. In particular, autolysis might have occurred due to the lack of immediate fixation of the tissue, as specimens were obtained at autopsy. Second, even with confocal microscopy, out of focus light cannot be totally excluded. The theoretical axial resolution at the emission wavelength of propidium iodide is approximately 0.6–0.7 μm and can be even larger depending on the objective used. Therefore, overprojection of nuclear and endoplasmic reticular elements is likely to occur. Such overprojection might be exacerbated if the tissue had shrunk or otherwise became distorted during processing. With a thicker optical slice, the overprojection artifact will be different depending on whether the plane is through the center of the nucleus. Furthermore, at any given optical section, one can observe cells in which the focus is through the center of the nuclei, and cells in which the focus is below or above the nucleus, as illustrated in Fig. 3 of the present study. Thus, if the calculation of nuclear *vs.* cytoplasmic fluorescence in a given field is made from several cells (as it was made in the study of Shimojo *et al.*), cytoplasmic staining of those cells where the plane of the optical section is not through the center of the nuclei could be misinterpreted as intranuclear staining. Finally, the antibody used in the studies by Shimojo *et al.* recognizes two bands on Western blot in the kidney (12), raising the possibility that the intranuclear staining reflects reaction with an unrelated antigen. Taken together, the above arguments call into question the conclusion that 11βHSD2 staining in the human kidney is in part intranuclear.

Our finding that 11βHSD is exclusively cytoplasmic [*i.e.* localizes to the endoplasmic reticulum in both mineralocorticoid target cells (present study) as well as nontarget cells (9, 10)] also contradicts the idea that the subcellular localization of 11βHSD2 differs in aldosterone target *vs.* nontarget cells (28). One reason for this discrepancy might be that whereas our data in aldosterone target *vs.* nontarget cells were obtained by confocal laser scanning microscopy at high resolution, Petrelli *et al.* determined 11βHSD2 enzymatic activity in cell fractions from colon *vs.* placenta obtained by differential centrifugation without solubilization of the nuclear membrane (28). However, 11βHSD2 is present in the nuclear envelope (9, 10), which cannot be separated from the nuclear fraction by differential centrifugation. Thus, in our opinion the main reason for the discrepancy between our findings and those of Petrelli *et al.* is technical differences between the two studies.

In conclusion, our data obtained with a 11βHSD2-specific antibody and confocal microscopy demonstrate that 11βHSD2 in renal mineralocorticoid target cells is in the cytoplasmic compartment, with no evidence for nuclear staining. Localization of 11βHSD2 to the ER, which is distributed over the entire cytoplasm, seems ideal for fulfilling its biological function, *i.e.* the quick and efficient inactivation of intracellular glucocorticoids before they can occupy the

MR, which is mostly cytoplasmic in the absence of hormone (8). If 11βHSD2 were located in the nucleus, MRs in the cytoplasm would be more likely to be occupied by glucocorticoids before 11βHSD2 could inactivate these steroids, and thus, aldosterone specificity would be compromised.

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