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Development of Monoclonal Antibodies against Human CYP11B1 and CYP11B2

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Abstract

1. The final enzymes in the biosynthesis of aldosterone and cortisol are by the cytochrome P450 CYP11B2 and CYP11B1, respectively. The enzymes are 93% homologous at the amino acid level and specific antibodies have been difficult to generate.
2. Mice and rats were immunized with multiple peptides conjugated to various immunogenic proteins and monoclonal antibodies were generated. The only peptide sequences that generated specific antibodies were amino acids 41-52 for the CYP11B2 and amino acids 80-90 for the CYP11B1 enzyme.
3. The mouse monoclonal CYP11B2-41 was specific and sensitive for use in western blots and produced specific staining of the zona glomerulosa of normal adrenal glands. The rat monoclonal CYP11B1-80 also detected a single band by western blot and detected only the zona fasciculata. Triple immunofluorescence of the adrenal demonstrated that the CYP11B1 and the CYP11B2 did not co-localize, while as expected the CYP11B1 co-localized with the 17 α -hydroxylase.

Keywords

CYP11B1; CYP11B2; Adrenal cortex; Monoclonal antibodies; 17 α -Hydroxylase; Immunofluorescence

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1. INTRODUCTION

The adrenal cortex has three anatomically and functional distinct zones. The outer zona glomerulosa (ZG) comprises small cells arranged in clusters that synthesize aldosterone, primarily under the control of the renin-angiotensin system. Interior to the ZG, is the zona fasciculata (ZF) comprised of larger cells in sheaves that synthesize glucocorticoids, primarily cortisol or corticosterone, depending on the species. The zona reticularis is the inner most ring of smaller cells next to the adrenal medulla that synthesize adrenal androgens, including dehydroepiandrosterone sulfate, androstenedione and 11 β -hydroxyandrostenedione, except in species that do not express adrenal 17 α -hydroxylase, including mice and rats (Miller and Auchus, 2011). The initial steps in steroidogenesis are common to all steroids and occur in all zones of the adrenal. These include the facilitated transfer of cholesterol by the steroidogenic acute regulatory (StAR) protein to the mitochondria, where cholesterol is hydroxylated twice and cleaved by the CYP11A1 (cholesterol side chain cleavage enzyme) to generate pregnenolone. Pregnenolone leaves the mitochondria where it is oxidized and isomerized by microsomal 3 β -hydroxysteroid dehydrogenase type 2 (and probably type 1 in the zona glomerulosa (Doi et al., 2010)) to form progesterone, which is then 21 hydroxylated by the CYP21A2 enzyme to form deoxycorticosterone (DOC). At this point, due to zone-specific enzyme expression, steroid synthesis diverges in the zones of the adrenal cortex. In the ZG, DOC is transferred into the mitochondria where the CYP11B2 enzyme successively hydroxylates it at the 11 β -position to form corticosterone, then at the 18-position to generate 18-hydroxycorticosterone, and then again at the 18-position to generate an ephemeral and theoretical germinal diol that spontaneously and rapidly dehydrates to aldosterone (Okamoto et al., 2005, Kojima et al., 1984, Curnow et al., 1991). Because humans express CYP17A1 in the fasciculata pregnenolone is converted into 17 α -hydroxypregnenolone which is oxidized to 17 α -hydroxyprogesterone by 3 β -hydroxysteroid dehydrogenase followed by 21-hydroxylation by CYP21A2 to 11-deoxycortisol. 11-Deoxycortisol and DOC then enter the mitochondria where they are acted upon by the CYP11B1 enzyme that is specific to the ZF to generate cortisol and corticosterone, specifically. There is no significant further metabolism of cortisol or corticosterone in the zona fasciculata. In species with no adrenal 17 α -hydroxylase, corticosterone is the primary glucocorticoid. immunohistochemistry in adrenals with aldosterone- and cortisol-producing adenomas (Nishimoto et al., 2010, Nanba et al., 2013, Volpe et al., 2013).

Several years ago, we failed several times to obtain workable rabbit polyclonal antibodies against the human CYP11B2 enzyme using the same sequence originally described by Ogishima *et al* (Ogishima et al., 1991)(unpublished). Therefore, as there was significant need for high quality antibodies against these enzymes, we initiated a program to generate monoclonal antibodies using multiple peptide epitopes for human CYP11B1 and CYP11B2. Herein we describe the successful generation of specific human CYP11B1 and CYP11B2 monoclonal antibodies that can be used for both immunohistochemistry and western immunoblot analysis. immunohistochemistry in adrenals with aldosterone- and cortisol-producing adenomas (Nishimoto et al., 2010, Nanba et al., 2013, Volpe et al., 2013).

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2. MATERIALS AND METHODS

2.1. Materials

Iscove cell culture media was purchased from Life Technologies (Grand Island, NY), Fetal Clone I serum was from Thermo Fisher (Waltham, MA). PEG 1450 was from ATCC (Manassas, VA), human IL6 and IL21 were from Peprotech (peprotech.com).

2.2. Design of peptide conjugates for the generation of antibodies specific for the CYP11B1 and CYP11B2 enzymes

Figure 1 is a comparison of the sequences between the human CYP11B1 and CYP11B2. As the amino acid sequences differ only by 7%, peptides for immunization were designed to comprise those areas where there are amino acid differences. The synthesis of the peptides that were at least 85% pure was done commercially. A cysteine was added to sequences that did not have a terminal cysteine for conjugation at either the N- or C-terminal of the peptide so that the non-conserved amino acid was distal to the conjugation site (Fig 1). Conjugation was done using either N-(iodoacetyl)-caproic acid-NHS or maleimidocaproic acid-NHS to keyhole limpet hemocyanin, porcine thyroglobulin or chicken serum albumin at a molar ratio of ~20:1 using standard techniques. The peptides were also conjugated to chicken ovalbumin at a lower molar ratio ~5:1 to coat microplates for ELISA screening.

2.3. Preparation of eGFP fusion protein with CYP11B1 and CYP11B2

The plasmids pEGFP-hCYP11B1 and pEGFP-hCYP11B2 were prepared from the plasmid pSV-hCYP11B1 and pSV-hCYP11B2 (Kawamoto et al., 1992) by digesting with EcoR1 and Kpn1 and ligating to those sites in pEGFP-C1 (Clontech, Mountain View, CA). The mitochondrial signal peptide was removed from the resulting plasmid. The individual plasmids were transfected into H293TN cells cultured in 145 mm plates using PEI87 (Thomas et al., 2005) and a day later, cells were scrapped and lysed with RIPA buffer with protease and phosphatase inhibitors (Thermo Fisher, Waltham, MA). The extract was further mixed with Laemmli buffer and subjected to PAGE electrophoresis. The location of the band was validated using an antibody against GFP (Neuromab, Davis, CA).

2.4. Immunization of mice and rats

For the CYP11B1, five different peptides as shown underlined in figure 1 were conjugated to chicken serum albumin, keyhole limpet hemocyanin or porcine thyroglobulin. The individual conjugates (~20 µg) injected subcutaneously at approximately 3 week intervals into 4 female Sprague-Dawley rats each. The first inoculation comprised an emulsion with complete Freund's adjuvant; the 3 subsequent boosters contained incomplete Freund's adjuvant. One week after the last injection, ~20µg of conjugated peptides were injected intraperitoneally without adjuvant, and then blood and the spleen were obtained 3 days later under isoflurane anesthesia. The spleens were crushed using sterile frosted glass slides and the spleen cells divided in 3 aliquots and frozen using 7% DMSO in 50% RPMI/porcine serum.

Five different peptides corresponding to the hCYP11B2 sequence underlined in figure 1 were similarly conjugated as above and ~10µg injected subcutaneously to 4-5 female Swiss-Webster mice as described for the rats. Spleen cells were divided into 2 aliquots.

Animal studies were approved by the IACUC of the G.V. (Sonny) Montgomery VA Medical Center.

2.5. Screening for antibody generation

96-well high-binding ELISA plates (Greiner) were coated with 100 ng/100 μ l of the corresponding peptide conjugated to ovalbumin in carbonate buffer 0.1M, pH 9.4. Serum was diluted in ELISA buffer (triethanolamine 0.05M, sodium chloride 0.1M, tween-20 0.1% and sodium azide 0.1% pH 7.4) starting at 1/300 dilution with a volume of 100 μ l/well. After incubating for 1 hr, the plates were washed 4 times in phosphate buffered saline with 0.1% tween-20 and incubated with a goat anti-mouse or anti-rat Europium-labeled antibody prepared in-house (Morra Di Cella et al., 2002). After 30 min, plates were washed, enhancement solution added, and 20 min later read in a FLUOstar Omega from BMG Labtech for time-resolved fluorometry. The supernatant of the wells that had positive antibodies were also screened using the corresponding peptide to the homologous enzyme that was used for immunization to identify sera from animals with no or minimal crossreactivity.

2.6. Fusion of spleen cells

The spleen cells from animals with the highest titer and lowest crossreactivity were fused. The mouse and rat cells were fused to a mouse myeloma SP2-mIL6-hIL21 cell which was modified by us from mouse myeloma SP2 (ATCC, Manassas, VA). Fusion was performed using PEG 1450 (ATCC), then the cells were plated into 10 $\times$ 96-well plates and cultured in Iscove with 15% Fetal Clone I and 20 ng/ml of hIL6 and 10 ng/ml of hIL21. At approximately 10-14 days the wells were initially screened using the peptide-ovalbumin conjugate as above and the positive wells rescreened using the corresponding peptide conjugate of the opposite enzyme. Those positive clones that did not crossreact were then further screened using western blot with the recombinant EGFP-CYP11B corresponding protein. Only those clones that gave a single band on western blot were subcloned using methylcellulose media (Davis, 1986) and the positive clones were evaluated further using western blots with extracts of H293TN cells transfected with EGFP fusion proteins with the CYP11B1, CYP11B2 plasmids, HAC15 and HAC15R cells (Wang and Rainey, 2011, Wang et al., 2012) and by immunohistochemistry using human autopsy adrenal glands embedded in paraffin.

2.7. Western blots

Media from the wells of positive clones was screened by western blot using a Surf-Blot (Idea Scientific Company, Minneapolis, MN). A one well PAGE 12% gel was loaded with either 0.8-1 mg of EGFP-CYP11B2 or EGFP-CYP11B1 protein, run as usual, then transferred to a PVDF membrane. The membrane was placed on the 21 channel Surf-Blot and aliquots of the media from positive clones diluted 1:10 were placed and incubated for 1 h. The antibody was aspirated and the membrane washed twice, removed and washed two more times, then incubated with either a goat anti-mouse HRP labeled antibody or a goat anti-rat HRP labeled antibody (Jackson ImmunoResearch Inc., Allentown, PA) diluted 1:10k. After washing, the membranes were then incubated with Super Signal West Pico substrate and exposed to film. The control well contained a GFP antibody from Neuromab (Davis, CA).

2.8. Immunohistochemistry

Paraffin embedded adrenals were cut at 6 microns and the sections dried, then melted on to the slides at 56C for 4 hrs. After deparaffination, the slides were subjected to antigen retrieval using Trilogy (Cell Marque Corporation, Rocklin, CA) in a steamer for 30 min. Endogenous peroxidases were inactivated with 0.1% phenylhydrazine for 15 min. The slides were blocked using Tris 0.05M, 5% goat serum and 0.5% SDS pH 7.4 for 1 hr. The CYP11B2 antibody (hCYP11B2-41-17B clone 1/1000 dilution) was incubated in PBS with

0.2% Tween-20, 5% goat serum pH7.4 for 24 hr. The slides were then washed 5 times in PBS with 0.2% Tween-20 and incubated with second antibody. For the mouse anti-CYP11B2, we used goat Polink-2 Plus HRP (GBI labs, Mukilteo, WA) and developed using DAB. Samples were counterstained with Meyer Hematoxylin (Vector Laboratories, Burlingame, CA). For the rat anti-CYP11B1, endogenous alkaline phosphatase was inhibited using levamisole, then the slides were incubated with rat anti-CYP11B1 (hCYP11B1-80-2-2, 1/300 dilution). Polink-2 Plus AP Rat (GBI labs, Mukilteo, WA) was the secondary antibody, binding was detected with GBI-Permanent Red substrate (GBI labs, Mukilteo, WA), and the sections were counterstained with Meyer Hematoxylin before mounting.

For double staining the sections were incubated with both primary antibodies together overnight, processed using a kit from GBI (Polink DS-MRt-Human C Kit), and stained using Emerald green (HRP) and Permanent-Red (AP) substrate using a process similar to the single staining above.

2.9. Immunofluorescence

Triple immunofluorescence was done using both monoclonal antibodies and a rabbit anti 17 α -hydroxylase antibody (Sakuma et al., 2013). Samples were processed as above, but a mixture of the 3 antibodies was used (rat hCYP11B1-80-2-2 1/50, mouse hCYP11B2-41-17B 1/150 and rabbit 17 α -hydroxylase 1/300) and incubated overnight. After washing, a mixture of highly absorbed antibodies were used, goat anti-mouse IgG Alexa Fluor 488, goat anti-rat IgG Alexa Fluor 594 and goat anti-rabbit IgG Alexa Fluor 647 (Jackson ImmunoResearch Inc. Allentown, PA) at a dilution 1/1,000 was done for 1 hr, the slides were then washed and mounted with Vectashield HardSet Mounting Medium with DAPI (Vector Labs, Burlingame, CA). The slides were then photographed using an Eclipse Nikon Microscope with a Rover camera and pseudocolored.

RESULTS

3.1

A total of 16 different myeloma cell fusions using either mouse or rat spleens generated antibodies that tested positive using ELISA based screening. Subsequent screening using immunoblot analysis demonstrated that four clones produced specific antibodies (two specific for CYP11B2 and two for CYP11B1). (Fig 2). The two mouse monoclonal antibodies were produced using the CYP11B2-41-thyroglobulin antigen (MPQHGPGRWL RL-C). Antibody specificity for CYP11B2 was demonstrated using ELISA, western blot (Fig 3) and immunohistochemistry. Antibody number 17 (IgG1- κ) was slightly better for immunohistochemistry and antibody 13 (IgG1- κ) was slightly better for western analysis. The two rat monoclonal antibodies against CYP11B1 were produced by animals inoculated with the CYP11B1-80-thyroglobulin conjugate (YDLGGAGMVC). The antibodies did not cross-react with CYP11B2 by ELISA, western blot analysis (Fig 4) and immunohistochemistry. Both antibody clones (2 & 7) worked well for immunohistochemistry and number 7 (after subcloning) was clearly better for western blot.

Figure 3 shows a western blot using antibody CYP11B2-41-13 demonstrating a strong band at an apparent mass of 75 kD for the eGFP-CYP11B2, while using five times more protein for the eGFP-CYP11B1 did not reveal a band, nor did lysates from H293TN cells transfected with the expression plasmid for CYP11B1 or their isolated mitochondria. CYP11B2-41-13 detected protein in lysates from H293TN cells transfected with CYP11B2 expression vectors and protein detection was even more prominent using isolated mitochondria. Control HAC15R adrenal cells and those stimulated by angiotensin II,

forskolin or potassium demonstrated an increase in the expression of CYP11B2, with potassium increasing CYP11B2 most in this system.

The rat monoclonal antibody against the CYP11B1 was not as sensitive in western analysis, but gave strong single bands using isolated mitochondria from HAC15 cells (Fig 4). The antibody gave a single band at a molecular mass of ~ 75 kDa with the eGFP-CYP11B1 and did not react with the eGFP-CYP11B2 (Fig 4). The anti-CYP11B1 antibody detected a single band at approximately 50 kDa from mitochondria of HAC15 cells (Fig 4).

3.2. Immunohistochemistry

Figure 5 is a composite of immunohistochemistry of 5 different autopsy adrenals from 4 adults, and one 5 day old infant (5F). CYP11B2 immunoreactivity is a brown-black color in panels A-F and green-blue color in panels I-J. Panel A-D are the same adrenal at two different regions and magnifications. Immunohistochemistry for CYP11B2 labeled cells adjacent to the adrenal capsule and indicated a variable distribution. As previously described, CYP11B2 immunoreactive cells formed two patterns, one of scattered cells (Fig 5 A&B and E), the other more tightly clustered cells that have been called “aldosterone-producing cell clusters” (Fig 5, panels C, D) (Nishimoto et al., 2010). The infant adrenal had significantly more CYP11B2 immunoreactive cells, with both patterns, though the CYP11B2 immunoreactive cell clusters predominated. There were far more CYP11B1 immunoreactive cells (pink), Fig 5 Panels G-K, corresponding to the zona fasciculata. Unlike in the round rat and mouse adrenals in which CYP11B2 immunoreactive cells form a relatively even concentric ring around CYP11B1 immunoreactive cells separated by a ring of cells that express neither CYP11B1 nor CYP11B2, in the adult human the CYP11B1 immunoreactive cells extended up to the capsule in many places and mingled with CYP11B2 immunoreactive cells (Fig 5, I,J,K). Double staining demonstrated that CYP11B2 and CYP11B1 were expressed in different cells; however subcapsular region was comprised of both CYP11B1 and CYP11B2 immunoreactive cells.

3.3. Immunofluorescence

The immunofluorescent staining was similar to the immunohistochemistry and revealed that CYP11B2 was not expressed in the same cell as CYP11B1 or 17 α -hydroxylase, while CYP11B1 co-localized with the 17 α -hydroxylase (Fig 6). Occasional cells expressing the CYP11B2 were found distant from the adrenal capsule mixed with cells expressing 17 α -hydroxylase and CYP11B1.

DISCUSSION

4.1

Until recently a significant obstacle in the study of the human adrenal cortex has been the lack of specific and versatile antibodies against the last and unique enzymes required for the synthesis of cortisol and aldosterone. We have produced mouse monoclonal antibodies against human CYP11B2 that are specific and do not cross-react with the CYP11B1 enzyme. Our previous experience using analogous sequences to obtain monoclonal antibodies against the rat CYP11B2 (Wotus et al., 1998) could not be used as this section of the molecule between amino acids 175-192 is identical in human CYP11B1 and CYP11B2. Five different peptides comprising sequences that differed between the CYP11B2 and CYP11B1 by at least 1 nonconserved amino acid were used for immunization. Though every single animal responded with relatively high serum antibody titers reacting with the peptide conjugated to ovalbumin, only the conjugate of amino acids 41-52 elicited suitable monoclonal antibodies. Specificity of the antibody was tested using an ELISA with a conjugate corresponding to the homologous sequence from the CYP11B1, a fusion protein

of GFP-CYP11B2 compared to GFP-CYP11B1 and H293TN cells transfected with the cDNA encoding either CYP11B2 or CYP11B1. No evidence of cross-reaction was found. The antibodies worked very well for immunohistochemistry and we showed that the distribution of CYP11B2 is next to the capsule as has been shown by others (Nishimoto et al., 2010, Nanba et al., 2013, Aiba and Fujibayashi, 2011). The CYP11B2 immunoreactive glomerulosa cells were present in two different patterns, as scattered cells or in clusters of cells that have been called aldosterone-producing cell clusters. It is not known if the two patterns share similar regulation or function. The samples from adults had relatively few CYP11B2 immunoreactive cells close to the capsule as has been reported, while the infant adrenal had many more (Aiba and Fujibayashi, 2011). Newborn infants have higher plasma levels of aldosterone and a physiological partial resistance to aldosterone due to the low expression of the mineralocorticoid receptor (Martinerie et al., 2009, Martinerie et al., 2009). Aiba and Fujibayashi have described a decrease in the expression of the CYP11B2 in older human adrenals, as well an increase in cells expressing neither CYP11B2 nor CYP11B1 (Aiba and Fujibayashi, 2011). Aldosterone and renin decrease slightly with age when subjects are on a normal sodium diet, but the decrease is more apparent when subjects are challenged with upright posture or mild sodium depletion (Weidmann et al., 1975).

4.2

To facilitate double immunostaining or triple immunofluorescence using our mouse anti-human CYP11B2 antibody, we generated a rat monoclonal antibody to CYP11B1. Immunization of rats resulted in the generation of high titer antibodies with all five antigens (Fig 1), however of the many hybridomas generated, only two were specific for CYP11B1 for an ELISA with the homologous sequence of the CYP11B2, as well as by western analysis using the eGFP fusion proteins and cells transfected with hCYP11B1 and hCYP11B2 cDNAs. The CYP11B1 antibody is less sensitive for western protein detection than the CYP11B2 antibody as mitochondrial protein was required for clear detection, rather than that obtainable from whole cell homogenates. It however gives a single band at the appropriate mass in the western blot. It also works very well for immunohistochemistry or immunofluorescence (Fig 5 and 6). CYP11B1 immunoreactivity was detected in both the zona fasciculata and reticularis and extends to the capsule in many adult adrenal glands (Fig 5 F-K). In sections where CYP11B2 is expressed there are some mingling with cells that do not express either CYP11B1 or CYP11B2 enzyme (Fig 5 J&K). As expected, CYP11B2ir does not co-localize with 17 α -hydroxylase or CYP11B1, while 17 α -hydroxylase and CYP11B1 were expressed in the same cell (Fig 6).

There were cells that did not express either CYP11B1 or CYP11B2. The human adrenal differs from mouse and rat adrenals in which the zona glomerulosa and zona fasciculata are clearly distinct and separated by what has been called an undifferentiated zone believed to comprise adrenal stem cells (Mitani et al., 1994, Mitani et al., 2003). These cells that express neither CYP11B1 nor CYP11B2 are more abundant in adrenals obtained from rats on a standard (0.3-0.4% sodium) or high sodium diet and mingle with cells expressing the CYP11B2 enzyme (Romero et al., 2007). The width of the zona glomerulosa and number of cells expressing the CYP11B2 increases very significantly when rats are sodium chronically depleted (Mitani et al., 1994, Romero et al., 2007, Mitani et al., 2003, Mitani et al., 1998). However even after a protracted high sodium diet in the rat in which aldosterone is maximally suppressed, there is still measureable plasma aldosterone and nests of adrenal glomerulosa cells expressing the CYP11B2 enzyme in the midst of cells that do not (Romero et al., 2007). These resemble the aldosterone-producing cell clusters found in the adult human adrenal and probably represent a similar response to high sodium diet where aldosterone production is lower, but remains clearly detectable. While manipulation of sodium intake is possible in experimental animals, human adrenals are obtained randomly at

autopsy, often from patients who died after acute or chronic illnesses involving stress and electrolyte derangements which produce significant morphological changes in the adrenal (Neville and O'hare, 1985).

4.3

In conclusion, we have described the production, testing and characterization of two new monoclonal antibodies against human CYP11B1 and CYP11B2 enzymes that are specific and do not cross-react. Monoclonal antibodies have a clear advantage over polyclonal antibodies; they can be produced in a uniform fashion from hybridoma cells, while polyclonals can change from bleeding to bleeding and animal to animal. These two antibodies will be very useful in the study of adrenal disorders including the causes of primary aldosteronism and Cushing syndrome.

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HIGHLIGHTS

Mouse anti-human CYP11B1 and rat anti-human CYP11B2 monoclonal antibodies were made.

CYP11B2 is expressed in scattered cells and clusters called APCC.

CYP11B1 and CYP11B2 enzymes do not co-localize in the normal adrenal cortex.

CYP11B1 and 17 α -hydroxylase co-localize in cells of the zona fasciculata.

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CYP11B1- MALRAKAEVC MAVFWLSLQR AQAIGTRAAR VPRTVLPFEA NPRRPGNRWL RLLQIWRBQG YEDLHLEVHQ TQQLGLPIFR YDLGGAGMVC VMLEPDAVEKL 100
CYP11B2- MALRAKAEVC VAAFWLSLQR AQAIGTRAAR AFRTVLPFEA NPQRPGNRWL RLLQIWRBQG YEDLHLEMDQ TQQLGLPIFR YDLGGAGMVC VMLEPDAVEKL
CYP11B1- QQVDSLPHFR MSLEPWATR QERGHRCGVF LLNGPEWRFH RLRLNFEVLS FWAVQRFLPM VDAVARDFSQ ALGGKVLQNA RGSLLDVQP SIFHYTIEAS 200
CYP11B2- QQVDSLPHCR MILEPWATR QERGHRCGVF LLNGPEWRFH RLRLNFEVLS FWAVQRFLPM VDAVARDFSQ ALGGKVLQNA RGSLLDVQP SIFHYTIEAS
CYP11B1- HIALFGERLG LVGHSPSSAS LNFLEALEVM FISTVQLMFH FRSLSRMTSP KVAKEHFEAM DCIFQYGDHC IQKITQE LAF SRPQQTYSIV AELLIMAELS 300
CYP11B2- HIALFGERLG LVGHSPSSAS LNFLEALEVM FISTVQLMFH FRSLSRMTSP KVAKEHFEAM DCIFQYGDHC IQKITQE LAF SRPQQTYSIV AELLIMAELS
CYP11B1- PDAIKANSME LTAGSVDTTV FPLIMTLFEL ARHFMVQAL RQESLAAAS ISEHFQKATT ELPLRLAALK ETLRLTPVGL FLERVAASDL VLQNYHIPAG 400
CYP11B2- LAIAKANSME LTAGSVDTTA FPLIMTLFEL ARHFMVQAL RQESLAAAS ISEHFQKATT ELPLRLAALK ETLRLTPVGL FLERVAASDL VLQNYHIPAG
CYP11B1- TLVRFYLISL GRNPALEFRP ERYNFORWLD IRGSGRNFTY VPFQFGMRQC LGRRLAEAM LLLHVLVLEH LQVETLTQED IRDAVSEPLR PSMFTLTER 500
CYP11B2- TLVRFYLISL GRNPALEFRP ERYNFORWLD IRGSGRNFTY VPFQFGMRQC LGRRLAEAM LLLHVLVLEH LQVETLTQED IRDAVSEPLR PQTSPLTTER
CYP11B1- AIN
CYP11B2- AIN

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Figure 1.
Comparative alignment of the protein sequence between human CYP11B1 and CYP11B2. The underlined letters indicate the amino acid differences between the sequences. The red letters are the sequences used for synthesis of peptides that were conjugated for immunization. The green –C represents a cysteine that was added to the synthetic peptide for conjugation.

Figure 2.

Representative immunoblots used for antibody screening of wells that had tested positive using ELISA. EGFP-CYP11B1 or EGFP-CYP11B2 (0.8-1 mg) was separated in a single lane 12% PAGE Criterion gel and transferred to a PVDF membrane and used in a 21 lane SurfBlot. Lane 20 of the CYP11B1 blot was probed with an anti-GFP antibody. The CYP11B1-80-thyroglobulin conjugate rat monoclonal antibodies had two positive clones by immunoblot (#2 and #7). The CYP11B2-41-thyroglobulin conjugate mouse monoclonal antibodies had two positive clones by immunoblot (#13 and #17). Supernatant from the cultured hybridomas were used at 1/50 dilution.

Figure 3. Immunoblot analysis using the CYP11B2-41-13 antibody to detect GFP fusion proteins for CYP11B2 and CYP11B1, lysated from 293TN cells transfected with cDNA for either CYP11B1 or CYP11B2 enzymes and HAC15 cells stimulated for 24 h with Angiotensin II, Forskolin or potassium.

Figure 4.

Immunoblot analysis using the CYP11B1-80-7 antibody to detect EGFP fusion proteins for CYP11B2 and CYP11B1 from homogenate of H293 transfected cells, as well as from isolated mitochondria from HAC15 cells from untreated cells and those incubated with angiotensin II, forskolin and potassium. Lanes were loaded with 30 μ g. The EGFP-CYP11B1 gave a strong band, while the EGFP-B2 did not give a band even after long overexposure.

Figure 5.

CYP11B1 and CYP11B2 use for adrenal immunohistochemistry: Panel A and C are two different areas of the same adrenal, one showing scattered cells and the other clusters stained with the CYP11B2-41-17B antibody. Panel B and D are similar but at higher magnification. Panel E shows scattered cells with variable degree of staining. Panel F is an adrenal from a 5 day infant. Panel G and H are staining at two magnifications with the CYP11B1-80-2-2 antibody. Panels I-K are double staining for CYP11B1 and CYP11B2.

Figure 6. Triple immunofluorescence for CYP11B1, CYP11B2 and 17 α -hydroxylase. The top panels are individual fluorescence for each protein and the bottom panels are the merged images as indicated.