Molecular and Cellular Endocrinology xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Development of monoclonal antibodies against human CYP11B1 and CYP11B2

Celso E. Gomez-Sanchez [a,b,*], Xin Qi [b], Carolina Velarde-Miranda [b], Maria W. Plonczynski [b], C. Richard Parker [c], William Rainey [d], Fumitoshi Satoh [e], Takashi Maekawa [e], Yasuhiro Nakamura [e], Hironobu Sasano [e], Elise P. Gomez-Sanchez [a,b,f]

[a] Endocrine Section, G.V. (Sonny) Montgomery VA Medical Center, USA
[b] Endocrinology, University of Mississippi Medical Center, USA
[c] Department of Obstetrics and Gynecology, University of Alabama, Birmingham, AL, USA
[d] Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI, USA
[e] Tohoku University, Department of Pathology, Tohoku University, Sendai, Japan
[f] Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, MS, USA

ARTICLE INFO

Article history:
Received 8 October 2013
Received in revised form 26 November 2013
Accepted 29 November 2013
Available online xxxx

Keywords:
CYP11B1
CYP11B2
Adrenal cortex
Monoclonal antibodies
17α-Hydroxylase
Immunofluorescence

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 17α-hydroxylase.

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 11β-hydroxyandrostenedione, except in species that do not express adrenal 17α-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 3β-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 11β-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 17α-hydroxypregnenolone which is oxidized to 17α-hydroxyprogesterone by 3β-hydroxysteroid dehydrogenase followed by 21-hydroxylation

* Corresponding author. Address: G.V. (Sonny) Montgomery VA Medical Center, 1500 E. Woodrow Wilson Dr. Jackson, MS 39216, USA. Tel.: +1 601 368 3844.
E-mail address: cgomez-sanchez@umc.edu (C.E. Gomez-Sanchez).

0303-7207/$ - see front matter © 2013 Published by Elsevier Ireland Ltd.
http://dx.doi.org/10.1016/j.mce.2013.11.022

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 17α-hydroxylase, corticosterone is the primary glucocorticoid.

Primary aldosteronism is the most common form of secondary hypertension affecting 4–10% of patients with essential hypertension (Funder et al., 2008) and is associated with significant cardiovascular morbidity and mortality (Funder et al., 2008; Milliez et al., 2005; Reincke et al., 2012). Of the multiple forms of primary aldosteronism, 30–50% are due to an aldosterone-producing adenoma and 50–70% are due to bilateral zona glomerulosa hyperplasia (idiopathic hyperaldosteronism), with 2–5% due to unilateral hyperplasia, adrenal carcinomas or familial forms of hyperaldosteronism (Funder et al., 2008; Young, 2007). The histopathology of adrenals causing hyperaldosteronism, including those with aldosterone-producing adenomas are complex (Neville and O'hare, 1985). Patients with aldosterone-producing adenomas frequently have peritumoral hyperplasia and micro and macronodules, some of which express CYP11B2 enzyme mRNA and protein (Boulkroun et al., 2011, 2010; Nishimoto et al., 2010; Nanba et al., 2013; Volpe et al., 2013).

The adrenal cortex of rats and mice show a clear delineation between expression of CYP11B1 in the ZF and CYP11B2 in the ZG (Mitani et al., 1994; Wotus et al., 1998). CYP11B2 is expressed in a layer of cells 4–10 deep immediately underneath the capsule. The ZF expressing CYP11B1 lies below this ZG and in the rat is separated by a layer of cells that do not express either enzyme which has been called the undifferentiated zone (ZU) or stem cell area (Mitani et al., 1994; Romero et al., 2007). The expression of CYP11B2 and the width of the ZG are dependent on the sodium in the diet (Romero et al., 2007). Studies with human adrenals using *in situ* hybridization demonstrate that CYP11B1 and CYP11B2 are not expressed in the same cells; however there is not the clearly delineated zonation pattern of expression seen in rats. Some cells expressing CYP11B1 are adjacent to the fibrous adrenal capsule (Pascoe et al., 1995; Enberg et al., 2004; Shigematsu et al., 2008).

The amino acid sequences of CYP11B1 and CYP11B2 are 93% homologous, making it difficult to generate specific antibodies to distinguish between these homologous enzymes. A rabbit polyclonal CYP11B2 antibody was described two decades ago (Ogishima et al., 1991), but was not pursued until more recently (Nishimoto et al., 2010) when it was used in conjunction with another polyclonal antibody generated against the homologous sequence of the CYP11B1 (Nishimoto et al., 2010). These antibodies were used to demonstrate that in normal human adrenals CYP11B2 enzyme-expressing cells form two patterns; some are scattered singly beneath the capsule and others are arranged in clusters named 'aldosterone-producing cell clusters' (Nishimoto et al., 2010). These antibodies have been used to perform 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. (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.

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

Fig. 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,

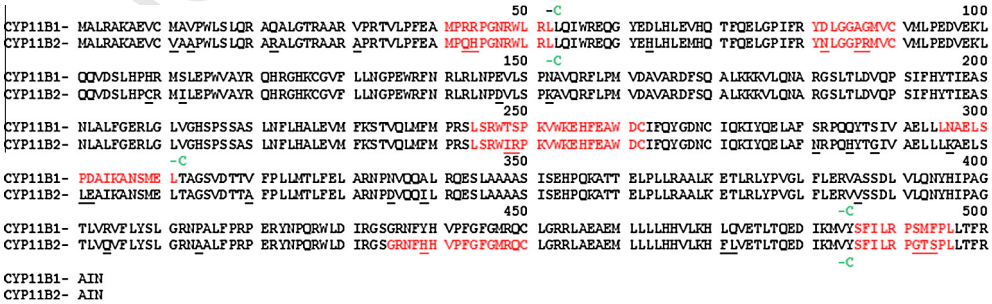

Fig. 1. Comparative alignment of the protein sequence between human CYP11B1 and CYP11B2. The underlined letters indicate the amino acid differences between the sequences. The red letters are the sequences used for synthesis of peptides that were conjugated for immunization. The green –C represents a cysteine that was added to the synthetic peptide for conjugation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Please cite this article in press as: Gomez-Sanchez, C.E., et al. Development of monoclonal antibodies against human CYP11B1 and CYP11B2. Molecular and Cellular Endocrinology (2013), http://dx.doi.org/10.1016/j.mce.2013.11.022