Molecular and Cellular Endocrinology 215 (2004) 143–148

MCE
Molecular and Cellular Endocrinology

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Characterization of the adrenal-specific antigen IZA (inner zone antigen) and its role in the steroidogenesis

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Abstract

Inner zone antigen (IZA) is a protein specifically expressed in the zona fasciculata and reticularis of the adrenal cortex. The cDNA encoding IZA was found to be identical to that encoding the previously reported putative membrane-associated progesterone receptor (MPR) and the TCDD-induced 25 kDa protein (25-Dx). From its structure, MPR was classed as a member of a protein family containing a haem-binding domain, and progesterone was proposed to be a ligand of this domain. Indeed, when GST-tagged IZA was expressed in *Escherichia coli* and purified, the purified GST–IZA had a brown colour with maximum absorbance at 400 nm. The addition of dithionate shifted the absorbance peak to 420 nm, suggesting a haem-binding function. The possible role of IZA in steroidogenesis has been addressed, and the inhibition of adrenal steroidogenesis by the addition of an anti-IZA monoclonal antibody has been reported. When COS-7 cells were transformed with plasmids for appropriate steroidogenic enzymes in the presence or absence of an IZA expression plasmid and tested for their steroidogenic activities, 21-hydroxylation of progesterone was found to be specifically activated by IZA overexpression, suggesting the involvement of IZA in progesterone metabolism. Taken together, the available evidence suggests that IZA may have an important role in the functions of the adrenal zona fasciculata and reticularis.

Keywords: Adrenal zone; IZA; Membrane-associated progesterone receptor; 25-Dx

1. Introduction

Recent genome information has revealed the presence of numerous unidentified genes and their products. In addition, domain classification in functionally characterized and uncharacterized proteins has also provided new insights into the roles of proteins. The haem-binding domain is one such domain that has been shown to be, conserved in a number of proteins (Mifsud and Bateman, 2003). Haem-binding capacity has been shown in some cases, but in many cases, the haem-binding like domains are believed to function as the binding site for other ligands. The inner zone antigen (IZA) (Raza et al., 2001) is of interest in this context. IZA contains a haem-binding like domain, and because it was previously identified as a putative membrane-associated progesterone receptor (MPR) (Meyer et al., 1996), its haem-binding like domain was thought to be a steroid-binding domain. However, in recent studies we concluded that IZA was indeed haem protein, involved in adrenocortical steroidogenesis. Here, we summarize the history of studies on IZA/MPR.

Abbreviations: ACTH, adrenocorticotropic hormone; CYP11B1, 11β-hydroxylase P450; CYP11B2, aldosterone synthase P450; CYP21, 21-hydroxylase P450; DAP, damage response protein related to membrane-associated progesterone receptors; GST, glutathione-S-transferase; IZA, inner zone antigen; MPR, membrane-associated progesterone receptor; ZF, zona fasciculata; ZG, zona glomerulosa; ZR, zona reticularis

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doi:10.1016/j.mce.2003.11.025

2. Rat IZA cDNA is identical to MPR/25-Dx cDNA

The rat adrenal cortex is composed of three distinct zones: the zona glomerulosa (ZG), the zona fasciculata (ZF), and the zona reticularis (ZR). Cells in ZG secrete aldosterone, whereas those in ZF and ZR (the inner zone) secrete glucocorticoid. Although only in the final step of steroid production is the aldosterone pathway different from the glucocorticoid pathway in the rat, through the presence of aldosterone synthase P450 (CYP11B2) in the ZG and 11β-hydroxylase P450 in inner zones (CYP11B1) (Nonaka et al., 1998), cells in the ZG and inner zones show very different morphological and functional characteristics. Steroid synthesis in ZG is mainly regulated by the renin–angiotensin II system and in inner zones by adrenocorticotropic hormone (ACTH) (Orth et al., 1992). However, how steroidogenesis is regulated in each zone remain poorly understood. Identification and characterization of molecules specifically expressed in distinct zones may provide more information to resolve these subjects, and IZA was found in these efforts (Mitani et al., 1994; Halder et al., 1998; Mukai et al., 2003).

Table 1 list the major reports concerning IZA and related products, MPR and 25-Dx. Laird et al. (1988) isolated a monoclonal antibody that reacted with the homogenate prepared from the adrenal inner zones, but not that from the ZG (Fig. 1A and B). The antigens recognized by this antibody were named as IZAgs. This was the first IZA study, which also reported the involvement of IZA in steroidogenesis and it was shown that the addition of IZA antibody to rat adrenal microsomal and mitochondrial fractions caused a dose-dependent inhibition of 21-hydroxylation of progesterone and 18-hydroxylation of deoxycorticosterone, respectively. Barker et al. (1992) reported the presence of two isoforms of IZA with different molecular mass in a denatured SDS-PAGE gel, one was 27–28 kDa and the other was 55–60 kDa. As mentioned later, it was considered that the larger molecule might be a dimer of 28 kDa IZA. Thereafter, wide distribution of IZA in mammals was reported (Ho and Vinson, 1993; Ho et al., 1994). Finally, Raza et al. (2001) succeeded in purifying rat IZA protein using an immobilized monoclonal antibody column, and the N-terminal sequence was determined, showing that completely matched with the previously reported sequences for rat 25-Dx (GenBank^{TM} accession number U63315) and MPR (GenBank^{TM} accession number AJ005837). 25-Dx cDNA was identified as one of the dioxin inducible genes in the rat liver (Selmin et al., 1996). MPR protein was originally purified from the progesterone-binding factor-rich fraction of porcine liver (Meyer et al., 1996), and its cDNA was also isolated from porcine vascular smooth muscle cells (Falkenstein et al., 1996). Although the N-termini of MPR/IZA and 25-Dx are identical, the C-terminal parts show differences (Fig. 1C). To determine which molecule was expressed in the adrenal inner zone, we amplified cDNA of each molecule by reverse transcription-polymerase chain reaction using specific primers. However, only the cDNA for MPR was amplified from RNA prepared, even from liver mRNA (Raza et al., 2001). Because the point at which MPR and 25-Dx cDNA sequence diverge overlap with the acceptor site of mRNA splicing, one reason for the reportedly different C-terminus in 25-Dx cDNA may be splicing error. However, there are several substitutions and deletions of nucleotides in the 3′-end of 25-Dx cDNA, and two sequences corresponding to 25-Dx cDNA have been deposited in data bases (GenBank^{TM} accession numbers U63315 and NM_021766). Nevertheless, the total cDNA, initiating nucleotide and polyA lengths are the same, suggesting a single source of these. No cDNA corresponding to rat 25-Dx has been identified in other mammals, including human, mouse and pig, indicating that some unexpected recombinations may occur during 25-Dx cDNA cloning.

A search for molecules similar to IZA in GenBank^{TM} reveals the presence of an interesting feature designated Haeme-1 domain (Fig. 1D). This constitutes a 50-amino acid sequence that is conserved in a domain family, which includes the haem-binding domains of cytochrome b5 and related proteins, as well as domains of unknown function in some chitin synthases (Mifsud and Bateman, 2003). This is also present in IZA. Originally, this Haem-1 domain in IZA was though to bind to steroids, because MPR was thought to be a progesterone receptor.

3. IZA/MPR dimers

Two isoforms of IZA/MPR have been identified in denatured SDS-PAGE gels (Barker et al., 1992; Meyer et al., 1996; Raza et al., 2001). The smaller 28 kDa IZA was observed in microsomal- and mitochondria-rich fractions, whereas the larger 58 kDa form was found in the cytosolic fraction of rat adrenal inner zones (Raza et al., 2001). Levels of 28 kDa IZA protein were elevated by ACTH pretreatment, and similarly. cAMP treatment of cultured adrenocortical cells resulted in an increase in 28 kDa IZA,

Table 1
History of IZA

IZA	25-Dx	MPR
Mab-IZA, ↓CYP21 and ↓CYP11B1 activity (Laird et al., 1988) ACTH, ↑IZA (Barker et al., 1992) Widely distributed in mammals (Ho et al., 1994) IZA cDNA (Raza et al., 2001)	25-Dx cDNA (Selmin et al., 1996) Yeast *dap1* cDNA (Hand et al., 2003)	MPR cDNA (Falkenstein et al., 1996)

↓, down regulation; ↑, up regulation.

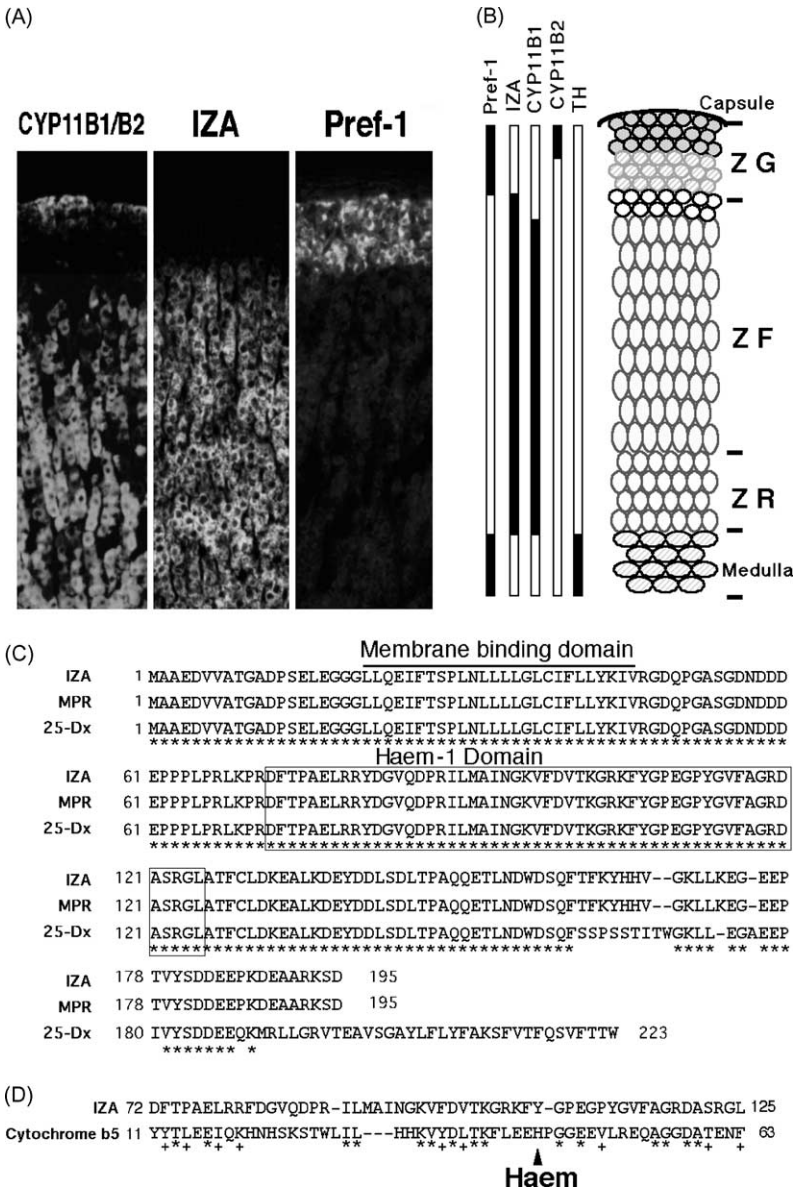

Fig. 1. (A) Immunohistochemical analyses of the rat adrenal cortex using anti-CYP11B1/B2, IZA and Pref-1 antibodies. (B) Rat adrenal zones (TH: tyrosine hydroxylase). (C) Primarily sequences of rat IZA, MPR and 25-Dx proteins. A putative membrane binding domain is indicated by a bar. The Haem-1 domain is indicated by a box. Amino acid residues conserved in all three molecules are marked by asterisks. (D) Alignment of the Haem-1 domain of rat IZA and the haem-binding domain of human cytochrome b5. Identical and similar amino acids conserved in two molecules are marked by asterisks and plus signs, respectively. The histidine residue, in cytochrome b5, coordinating with haem is indicated.

concomitantly in this case with a decrease in 55 kDa IZA, suggesting a conversion of the 55 kDa IZA to 28 kDa IZA, associated with enhanced steroidogenesis (Barker et al., 1992). Meyer et al. (1996) reported that both isoforms of MPR in the porcine liver had same N-teminal sequence. CHO cells overexpressing 28 kDa MPR also produced the larger isoform of MPR (Falkenstein et al., 1999). In addition to dimer formation, we also observed the formation of an IZA trimer when IZA protein was overexpressed in *Escherichia coli*. These results suggested that IZA/MPR might form multimers via a reducing reagent resistant bond, and the change from monomer to multimer might result in changes in intracellular distribution of these molecules and also of their functions.

4. Similarity and difference between IZA and cytochrome b5

Both IZA and MPR protein were purified from the membrane fractions of rat adrenals and porcine liver, consistent with the presence of a hydrophobic membrane associating region in their primary structures (Fig. 1C). Similarly, cytochrome b5 is a microsomal haem protein having a

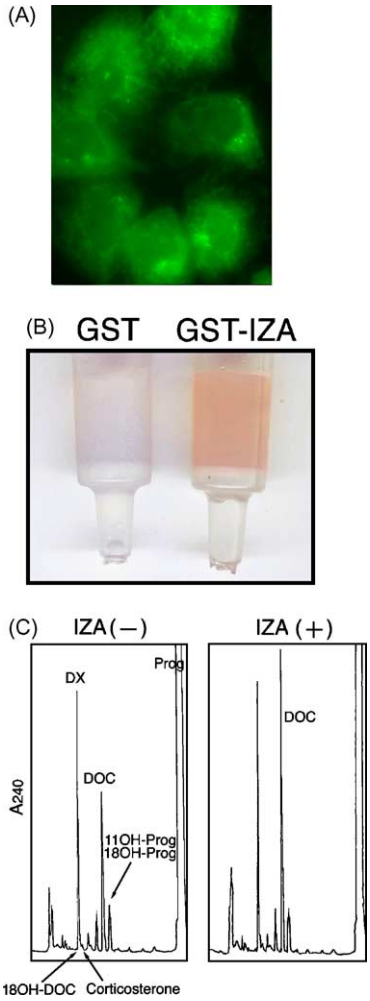

Fig. 2. (A) Cytochemical analysis of HeLa cells expressing green fluorescent protein-tagged IZA. (B) The colour of glutathione columns bound to GST and GST–IZA. (C) HPLC analyses of progesterone metabolites secreted from COS-7 cells expressing CYP21 and CYP11B1. COS-7 cells were transformed with CYP21, CYP11B1 and adrenodoxine expression vectors with (right panel) or without (left panel) IZA expression vector. After 24 h, cells were incubated with 100 μM of progesterone for 48 h. Metabolites were extracted from the culture medium with 5 n moles of dexamethasone (DX) and subjected to HPLC analyses. Peaks of deoxycorticosterone (DOC), 11-hydroxy- or 18-hydroxy-progesterone (11OH-Prog or 18OH-Prog), 18-hydroxy-DOC and corticosterone are indicated.

hydrophobic-membrane-anchoring region at its C-terminus. When green fluorescent protein-tagged IZA was expressed in HeLa cells (Fig. 2A), fluorescent signals formed tubular like structures of different sizes. The intensity of signals was higher near the nucleus than peripherally, suggesting that IZA was indeed a microsomal protein like cytochrome b5.

The most important similarity lies in the Haem-1 domain. As shown in Fig. 1D, this 54-amino acid sequence shares weak similarity with the N-terminal half of the haem-binding domain in cytochrome b5. In cytochrome b5, haem is bound coordinately by two histidine residues: one (arrowhead in Fig. 1D) is in the Haem-1 domain, but is not conserved in IZA, the other is out side the Haem-1 domain. Therefore, in IZA, there can be no coordinate histidine–haem. Neither is C-terminal half of the haem-binding domain of cytochrome b5 similar to IZA. Such differences in primary structure between IZA and cytochrome b5 may be one reason for supposing that IZA is not a haem protein (Mifsud and Bateman, 2003). However, purified IZA protein that had been expressed in *E. coli* as glutathione-S-transferase (GST) fusion protein has a brown colour (Fig. 2B). Its absorption spectrum had a maximum peak at 400 nm, and this peak was shifted to 420 nm after reduction by dithionate, indicating that IZA does indeed contain haem. However, we also observed that neither NADPH–P450 reductase nor b5 reductase could reduce IZA. Moreover, the brown colour of IZA, is typically seen in penta-coordinate haem proteins, in contrast to the red colour of cytochrome b5 consistent with a hexa-coordinate haem protein. These findings suggest that the properties and functions of haem in IZA may differ from those in cytochrome b5.

5. Physiological functions of IZA/MPR

MPR was isolated as one of the non-classical steroid receptors. Overexpression of MRP in CHO cells lead to increase in progesterone binding in the microsomal fraction (Falkenstein et al., 1999). This MPR-enriched microsomal fraction also showed moderate affinity for testosterone and weak affinity for corticosterone and cortisol, but did not bind to oestradiol or aldosterone. An antibody against MPR inhibited progesterone-induced increase in cellular Ca^{2+} in human sperm (Falkenstein et al., 1999). Krebs et al. (2000) reported the repression of oestradiol-priming MPR expression in the hypothalamus of ovariectomized rats by pretreatment with progesterone. MPR expression is also higher in the hypothalamus of female mice lacking classical nuclear progesterone receptors than in their wild-type litter-mates (Krebs et al., 2000). This evidence suggests that MPR plays a role in non-classical steroid signalling.

As mentioned above, studies on steroid metabolism in adrenocortical cells also showed the involvement of IZA in progesterone metabolism. To re-examine this possibility, COS-7 cells were transformed with CYP21 and CYP11B1 in the presence or absence of IZA overexpression vector, and progesterone was added into the culture medium as substrate (Fig. 2C). Overexpression of IZA resulted in a significant increase in the production of deoxycorticosterone, but had little effect on 11β-hydroxylation of progesterone. The enhancement of CYP21 activity by IZA was observed only when a high concentration of progesterone was added into the culture medium. A high concentration of progesterone also inhibited CYP21 activity and CYP11B1-mediated conversion of deoxycorticosterone to corticosterone. Moreover, when the level of progesterone in the rat adrenal was maintained at its usual low level, even higher accumulation of deoxycorticosterone and corticosterone was observed. These results

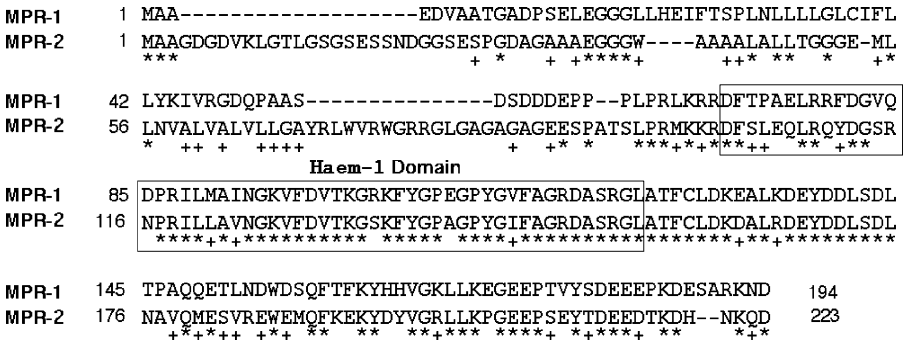

Fig. 3. Two isoforms of human MPR proteins. MPR-1 is identical to IZA/MPR, and MPR-2 is called Dg6.

lead to the conclusion that the level of progesterone has to be kept low in order to maintain corticosterone production, which may be supported by the IZA enhanced CYP21 activity. This carries the further implication that since IZA is expressed in only the inner zones of the adrenal cortex, 21-hydroxylation in the glomerulosa is likely to be much less efficient.

6. Wide distribution of IZA/MPR(s)

Though in the adrenal cortex, IZA is specifically expressed in the inner zones, of the adrenal cortex, it nevertheless appears in many other tissues, including vascular smooth muscle cells (Falkenstein et al., 1996), kidney (Ho et al., 1994; Raza et al., 2001), liver (Ho et al., 1994; Meyer et al., 1996; Raza et al., 2001), testis (Ho et al., 1994; Raza et al., 2001), brain (Runko et al., 1999; Krebs et al., 2000), ovary (Ho et al., 1994) and sperm (Falkenstein et al., 1999). Because these tissues do not express CYP21, other targets or functions of IZA/MPR have to be considered. Recently, a yeast homologue of IZA/MPR (named DAP1 (for damage response protein related to membrane-associated progesterone receptors)) was identified (Hand et al., 2003). The disruption of the *dap1* gene leads to sensitivity to the DNA-damaging reagent methyl methane sulphonate, elongated telomeres, suggesting that *dap1* is required for cell cycle progression following DNA damage. In addition, *dap1*-deficient cells have decreased levels of ergosterol but increased levels of the ergosterol intermediates squalene and lanosterol, indicating that *dap1* may play an important role in sterol synthesis in yeast cells. Moreover, these are two IZA/MPR genes in mammals (Gerdes et al., 1998; Bernauer et al., 2001), one on the X chromosome in the human genome, and the other gene (called *dg6*) on chromosome 4 (Fig. 3). Both gene products contain the putative transmembrane domain and the highly conserved sequence of 58 amino acids that includes the Haem-1 domain. These observations suggest the presence of broad targets for IZA/MPR family proteins in eukaryotic cells. Their molecular mechanisms of action and their structural analysis need to be addressed in future studies.

Acknowledgements

We thank Dr. Shiro Kominami and Dr. Takeshi Yamazaki (Hiroshi University, Japan) and Dr. Hiroshi Horii (Osaka University, Japan) for their generous advice. Our research on IZA was supported by Grants-in-Aids for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology and from Ministry of Health, Labour and Welfare Japan, and a grant from The Uehara Memorial Foundation. A part of this work was supported by grants from CREST Project of JPST for “Endocrine Disruption on Action of Brain Neurosteroids”. GPV and FR are grateful for BBSRC project grant support.

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