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学位論文名	Molecular identification of adrenal inner zone antigen as a heme-binding protein （副腎内層特異的抗原（IZA）はヘム結合蛋白質である）
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論文内容の要旨

Introduction :

Inner zone antigen (IZA) is a protein first recognized by monoclonal antibody raised against the rat adrenal cortex. It has also been reported as a putative membrane progesterone receptor in porcine vascular smooth muscle cells or a TCDD-induced 25-KDa protein in the rat forebrain. In human, there are two kinds of IZAs ; the human IZA1 is the homolog of rats IZA in liver and kidney, and the human IZA2, the isoform of the human IZA1 present in placenta. A yeast homolog of IZA was recently reported as "Damage response protein related to membrane-associated progesterone receptors I protein" (Dap1p). So, IZA has been studied by many investigators from a variety of viewpoints and a variety of biological functions have been attributed to it. However, its precise physiological role is still unclear. Our preliminary results suggested that IZA contains a heme-chromophore. Recently, Mallory et al. also reported that Dap1p is a heme-binding protein. We attempted to further characterize the molecular natures of this protein.

Methods and Results :

IZA was expressed as either $(His)_6$-fused protein or GST-fused protein in *E.coli* and purified to homogeneity using affinity chromatographic method. The purified protein was tinged with brown color, the color clearly being distinct from that of cytochrome b5. The UV and visible light absorption spectra of $(His)_6$-tagged h-IZA1 showed the oxidized form of heme-chromophore with a sharp gamma-absorption peak at 402 nm and broad absorptions between 497 nm and 616 nm. When the sample was treated with sodium dithionite, the spectra were converted to those of the reduced heme-chromophore with distinct alpha-, beta-, and gamma-peaks at 567 nm, 538 nm and 420 nm, respectively. The addition of CO to the reduced sample changed the spectra into a CO-binding form with alpha- and gamma-peaks at 559 nm and 426 nm, respectively. These data suggest that IZA probably contains a protoheme-chromophore. The nature of heme bound to IZA was further studied by measuring EPR spectra. The spectra of rat GST-fused IZA either at 5 K or at 15 K revealed high-spin type

signals with g values near 6.0 and 2.0. However, unlike those of oxidized myoglobin, the EPR signals of IZA showed strong anisotropy, and furthermore, the signals observed near g=6.0 appeared to be a mixture of two components. When ^{14}NO was added to the reduced form of IZA, the EPR spectra revealed a ^{14}NO -bound penta-coordinated heme, indicating the amino acid residue which coordinated to the heme-iron was disrupted upon binding NO. Taken together these EPR properties suggest that the IZA like myoglobin contains a high-spin type heme.

To determine which amino acid residue interacts with heme in IZA, a variety of mutant IZAs were produced in which amino acids suspected to bind the heme-ligand had been disrupted. The purified mutants were then measured for their heme absorptions. Because an imidazole group often plays a role in binding heme in many heme-proteins, we first introduced mutations into His165 and His166 in h-IZA1, even though they are located outside the predicted heme/steroid binding domain. H165N-IZA and H166N-IZA, however, were found capable of binding heme as strongly as the wild-type. Amino acid side-chain groups other than imidazole that could interact with heme molecule are thiol and phenol. Noting that Tyr107, Tyr113, Tyr139, and Cys129 are present in the heme/steroid binding region, and moreover, are conserved in h-IZA1, h-IZA2 and rat IZA, mutants Y107F, Y113F, Y139F, and C129A were produced. The heme-absorptions of these mutants, however, again seemed not significantly diminished compared to that of the wild-type. We further tested mutants, such as Y43F, Y164F, Y180F, and P109A, but none of these single-amino acid mutants seemed to lose heme binding capability completely. When two phenol groups, Tyr107 and Tyr113 were disrupted, the mutant appeared substantially to lose its capacity to bind heme. However, another double mutant, Y164F/H166N-IZA, retained heme-binding capacity. When mutations were introduced into a four consecutive amino acids-stretch from Asp99 to Lys102, the mutant bound heme at a level of 10% of the wild-type. It should be noted that three amino acid residues in this tetrapeptide, Asp99, Thr101 and Lys102, are conserved between IZA and cytochrome b5.

To determine the intracellular localization of IZA, we expressed IZA, cytochrome b5, an endoplasmic reticulum-associated protein, and CYP11B1, a mitochondrial inner membrane-associated protein, in HeLa cells. The cells were stained with the specific antibodies directed against the respective proteins. The result showed that the intracellular location of IZA was completely consistent with that of coexpressed cytochrome b5, suggesting that IZA is associated with the endoplasmic reticulum membrane. Given that IZA is abundantly present in the endoplasmic reticulum of zona fasciculata cells, it would be reasonable to speculate that IZA might be somehow involved in the physiology of the adrenal cortex. Therefore, we tried to test if IZA functions as a regulator of biosynthesis of corticosteroids, but were not able to obtain a conclusive result.

Conclusion :

Taken together, we have shown here that IZA, the adrenal inner zone antigen, is a heme-binding protein present in the endoplasmic reticulum membrane. The primary structure of its heme-binding region looks weakly similar to that of cytochrome b5, presumably forming a hydrophobic pocket. The heme in IZA is type b, and binds to the protein in high-spin type. To identify an amino acid residue(s) that possibly plays a role to bind the heme, extensive site-directed mutation studies were conducted, though the results remain somewhat ambiguous. It is possible to conclude that the heme/steroid binding region in IZA constitutes a hydrophobic pocket that could accommodate a heme molecule, and, in this pocket, two Tyr residues, Tyr107 and Tyr113, and a peptide stretch D99-K102 play important roles in attaching the heme-iron at one side of the protoporphyrin ring. Though our results presented here could not conclusively point a precise physiological role(s) played by IZA in the adrenal cortex, we surmise that this heme-containing and microsomal protein might have a role to supply heme molecules to cytochrome P450-involved reactions and eventually influence adrenal steroidogenesis.

論文審査の結果の要旨

本論文では副腎皮質の束状層と網状層で高い発現を示す因子（IZA）のタンパクとしての特性を解明した。cDNAの情報から、IZAは膜結合性プロゲステロン受容体として報告されているものと同一であり、ステロイドを結合するタンパクである可能性が示唆されていた。しかし、IZAタンパクを実際に精製すると、IZAはプロゲステロン受容体ではなく、ヘム結合タンパクであることが明らかとなった。さらに、ヘムを結合しないIZA変異体の作成に成功し、変異体の解析を行った結果、IZAはプロゲステロンの代謝酵素の一種であるCYP21の活性を促進させることが示唆された。副腎皮質においてプロゲステロンは種々のステロイドの中間産物であり、ステロイド合成酵素はヘムを有している点で、IZAがヘムタンパクであることを証明したことは非常に重要である。以上の結果は学位論文に値すると考えられる。