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学位論文題目	Identification and Characterization of Occludin: A Novel Adhesion Molecule of Tight Junctions
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In multi-cellular organisms, establishment of compositionally destinct fluid compartments by epithelium and endothelium is crucial for the development and function of most organs. Tight junction (TJ), the most apical element of epithelial and endothelial junctional complexes in vertebrates, is directly involved in this compartmentation by sealing cells to create the primary barrier to the diffusion of solutes through the paracellular pathway. TJ is also thought to function as a boundary between the apical and basolateral plasma membrane domains, which differ in protein and lipid compositions and physiological functions, to create and maintain epithelial and endothelial cell polarity.

Other intercellular junctions such as adherens junctions, desmosomes, and gap junctions bear specific types of integral membrane proteins which play crucial roles in each junction. To clarify the structure and function of TJ at the molecular level, an integral membrane protein should be identified. In TJ, despite of intensive studies, an integral membrane protein remained elusive for quite some time. Here, by the use of the monoclonal antibody technique, he first identified an integral membrane protein localizing at TJ, which was designated as "occludin". Then he cloned its cDNA, which enabled us to clarify some unique functions of this molecule; its association with the some peripheral membrane proteins of TJ, its localization signal at TJ, and its cell adhesion ability.

In the Chapter 1, he identified and integral membrane protein of TJ and analyzed its primary structure by cDNA cloning. Recently, we found that ZO-1, a tight junction-associated protein, was concentrated in the so called isolated adherens junction fraction from the liver (Itoh, M., A. Nagafuchi, S. Yonemura, T. Kitani-Yasuda, Sa. Tsukita, and Sh. Tsukita. 1993. J. Cell Biol 121:491-502). Using this fraction derived from chick liver as an antigen, he obtained three monoclonal antibodies specific for a ~65kD protein in rats. This antigen was not extractable from plasma membranes without detergent, suggesting that it is an integral membrane protein. Immunofluorescence and immunoelectron microscopy with these mAbs showed that this ~65kD membrane protein was exclusively localized at tight junctions of both epithelial and endothelial cells: At the electron microscopic level, the labels were detected directly over the points of membrane contact in tight junctions. To further clarify the nature and structure of this membrane protein, he cloned and sequenced its cDNA. He found that the cDNA encoded a 504 amino acid polypeptide with the calculated molecular mass of 55.9kD. A search of the data base identified no proteins with significant homology to this membrane protein.

A most striking feature of its primary structure was revealed by a hydrophilicity plot: Four putative membrane-spanning segments were included in the N-terminal half. This hydrophilicity plot was very similar to that of connexin, an integral membrane protein in gap junctions. These findings revealed that an integral membrane protein localizing at tight junctions is now identified, which they designated as 'occludin'.

In the Chapter 2, he investigated the roles of the COOH-terminal cytoplasmic domain of occludin using its cDNA. Immunofluorescence and laser scan microscopy revealed that chick full-length occludin introduced into human and bovine epithelial cells was correctly delivered to and incorporated into preexisting TJ. Further transfection studies with various deletion mutants showed that the long COOH-terminal cytoplasmic domain consisting of 255 amino acids (domain E), especially its COOH-terminal ~150 amino acids (domain E358/504), was necessary for the localization of occludin at TJ. Secondly, domain E was expressed in E.coli as a fusion protein with glutathione-S-transferase (GST), and this fusion protein was shown to be specifically bound to a complex of ZO-1 (220kD) and ZO-2 (160kD) among various membrane peripheral proteins. In vitro binding analyses using GST-fusion proteins of various deletion mutants of domain E narrowed down the sequence necessary for the ZO-1/ZO-2 association into the domain E358/504. Furthermore, this region directly associated with the recombinant ZO-1 produced in E.coli. He concluded that occludin itself can localize at TJ and directly associate with ZO-1. The coincidence of the sequence necessary for the ZO-1 association with that for the TJ localization suggests that the association with underlying cytoskeletons through ZO-1 is required for occludin to be localized at TJ.

In the Chapter 3, he investigated the cell adhesion ability of occludin. Chick occludin was overexpressed in insect cells by recombinant baculo virus infection. When the cells were observed by confocal immunofluorescence microscopy, the majority of expressed chick occludin occurred inside cells. Thin section electron microscopy of these cells identified peculiar electron-dense membrane structures which consist of thin parallel or concentric lamellae. These multilamellar structures were shown to consist of many disk-like structures each of which has a loop of membrane with its both ends. These structures were heavily labeled with anti-chick occludin monoclonal antibodies. These observation led us to speculate that the disk-like structure was transformed from each cisterna whose luminal space was completely collapsed by accumulation of occludin molecules. Furthermore he analyzed the arrangements of intramembranous particles in the multilamellar structures by freeze-fracture technic. Two distinct types of fracture images were observed: In one type, numerous particles ~10μm in diameter were densely packed in a ramdom or linear

pattern, whereas in the other type short straight grooves were occasionally observed. He concluded that under the condition of this study, occludin shows a tendency to polymerize into a short strand inside the membrane. These findings provided the first evidence that occludin is an adhesion molecule working at TJ in a homophylic manner.

This study on identification and characterization of occludin, a novel adhesion molecule of TJ, opened a new way to analyze the structure and function of TJ at the molecular level. Further analyses of occludin will lead us to a better understanding how the permeability and polarization of epithelial and endothelial cell sheets are regulated in the near future.

審査結果の要旨

タイトジャンクションは、脊椎動物の上皮細胞、血管内皮細胞に特有の細胞間接着装置で、1）細胞層が異なる溶液組成よりなる2つの領域を隔てること、2）細胞層が極性を形成、維持すること、においてきわめて重要な役割を果たしている。ところがタイトジャンクションの接着分子はこれまで知られておらず、そのような膜貫通型タンパク質を同定することは、タイトジャンクションの構造と機能を分子レベルで解明するための大きな課題であった。

申請者、古瀬幹夫は、ニワトリ肝臓から調製した細胞間接着装置分画を用いてモノクローナル抗体を作製してスクリーニングすることにより、タイトジャンクションに局在する膜貫通型タンパク質オクルディンを初めて同定し、cDNAの塩基配列を決定した。次に、遺伝子工学的手法を用いて、オクルディンのC末端側細胞質領域が既知の膜裏打ちタンパク質と結合することを示し、タイトジャンクションの分子構造に関するモデルを提出した。また、膜裏打ちタンパク質との結合が、オクルディンがタイトジャンクションへ局在するのに必要であることも明らかにしている。さらに、細胞内でオクルディンを大量に発現させる系において、オクルディンが2枚の膜を密着させる活性をもつことを見い出し、オクルディンがタイトジャンクションの接着分子であることを証明した。

本論文によるオクルディンの発見は、その接着分子が長らく不明であったために分子レベルでの解析が進んでいなかったタイトジャンクションの構造と機能に関する研究に新たな道を開いたものとして高く評価できる。また、細胞生物学にとどまらず、タイトジャンクションに関わりが深いと考えられている医学的な問題についても今後新しい知見をもたらすことが期待される。研究内容の一部は、Journal of Cell Biology に2報の原著論文として既に掲載されている。

以上より、審査委員会は、本論文を学位授与に充分値するものと判定した。

また、審査委員会は、古瀬幹夫氏の提出論文の内容および医学生物学一般の知識について、試問を行った。その結果、古瀬氏は、本人の行った研究に関連する知識を充分に有しているのみならず、その位置付けも充分に理解していると判定された。また、医学生物学全般における知識に関しても問題はなかった。既に、Journal of Cell Biology に2篇の比較的長い論文を投稿し、掲載されていること、さらに、1篇の英文論文を投稿中であること、および試問の結果を総合すると、古瀬氏の英語の能力も充分なレベルにあると判断された。

以上により、審査委員会は、古瀬幹夫氏の論文内容、学識、語学力ともに博士（学術）を授与するに充分であると判定した。