氏　　名　近藤明子

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学位論文題目　Intracellular dynamics of a focal adhesion protein and its relationship to cell migratory activity: an analysis of PAG3,a novel paxillin-binding ARFGAP protein

論文審査委員　主　査　教　授　重本　隆一
教　授　永山　國昭
教　授　小幡　邦彦
佐邊　壽孝（（財）大阪バイオサイエンス研究所）

論文内容の要旨

Cell adhesion and migration play essential roles in a wide variety of physiological and pathological aspects of the organization of multicellular organisms, such as embryogenesis, organogenesis, wound repair, inflammatory processes, and cancer invasion and metastasis. Adhesion and migration are primarily mediated by integrin binding to extracellular matrices (ECMs). Integrins recruit a characteristic set of cytoplasmic proteins, with scaffolding as well as signaling properties, at their cytoplasmic regions during this process. It is well documented in fibroblasts that integrin macroaggregates grow and shrink over time during cell migration, though the position of each macroaggregate remains fixed as the cell translocates. It is believed that there must be mechanisms that orchestrate the dynamics of protein recruitment and assembly at the cytoplasmic tails of integrins, but the molecular processes remain to be established. The precise subcellular locations where integrins initially assemble with their cytoplasmic binding proteins are also not known.

The small GTP-binding proteins of the Rho family have been shown to play pivotal roles in regulating the dynamic properties of the actin-based cytoskeletal organization, which is also essential for cell migratory activity. For example, Rho A has been shown to be involved in the formation of actin stress fibers and focal adhesion assembly in Swiss 3T3 cells. Furthermore, Rho A protein has been shown to participate in regulation of the phosphorylation status of myosin light chain, and thus regulate the contractility of the actomyosin network. Rho A can also activate phosphatidyl inositol 4-phosphate 5'-kinase to produce phosphatidyl inositol 4,5-bisphosphate, which interacts with gelsolin, profilin, and vinculin; and helps to regulate actin polymerization and cytoskeleton-membrane attachment. Rho A is moreover able to activate phospholipase D to produce phosphatidic acid, and to regulate actin polymerization. In spite of these extensive studies, however, the precise mechanism of how Rho A, as well as other Rho-family proteins, regulates focal adhesion assembly and its connection to actin fibers that ultimately leads to the regulation of cell migratory activity, remains to be established.

Recent studies by Norman et al., on the other hand, have shown that ADP-ribosylation factor 1 (ARF1), which belongs to another small GTP-binding protein family, participates in paxillin recruitment to sites of focal contacts in Swiss 3T3. They also showed that ARF1 can potentiate the Rho A-stimulated stress fiber formation, and suggested that ARF1 and Rho A activate complementary pathways that together lead to the formation of paxillin-rich focal adhesions at the ends of prominent actin stress fibers.

ARF-family proteins have been implicated in the regulation of membrane and vesicle traffic in mammalian cells. Members of the family include six isoforms of ARF, and the ARF-like proteins. The six ARF isoforms are highly homologous to one another, and classified as class I, II or III based on sequence similarity. Class I includes ARF1, 2 and 3; class II, ARF4 and 5; and class III, ARF6. Among them, ARF1 has been most thoroughly studied. ARF1 has been shown to regulate membrane traffic at multiple sites within the cell. ARF1 colocalizes primarily with Golgi-associated proteins and acts at the Golgi; ARF1 also functions in ER-to-Golgi transport, trans-Golgi network,

endosome-endosome fusion, protein secretion and fluid-phase endocytosis, as well as phospholipase D activation. The GTP bound form of ARF1 recruits protein coats, including the clathrin-associated adaptor proteins AP-1 and AP-3, and the nonclathrin coatomer, to membranes and initiates budding of the membrane vesicles. Subsequent hydrolysis of GTP to GDP by ARF1 may trigger disassembly of the coat from the vesicle, which is necessary for the vesicle to fuse to the target membranes. On the other hand, ARF6, the ARF which is most distantly related to ARF1, shows a rather wide distribution in the cytoplasm and localizes to an endosomal compartment and membrane ruffling regions. ARF6 primarily regulates endosomal trafficking as well as receptor-mediated endocytosis at the cell periphery, actin rearrangements beneath the plasma membrane, and cell spreading. Unlike other small GTP-binding family proteins such as Ras-family and Rho-family proteins, it is noteworthy that the intrinsic GTPase activity of ARF proteins is almost undetectable in vitro.

Paxillin, one of the integrin-assembly proteins, is highly tyrosine phosphorylated upon integrin activation, and acts as an adaptor protein in integrin signaling. Paxillin can interact directly with several integrin-assembly proteins, including vinculin, talin, integrin b1, focal adhesion kinase, Pyk2, c-Src and Csk. The importance of paxillin in protein assembly and signaling has also been suggested by the lack of tyrosine phosphorylation in neutrophils isolated from a patient with a leukocyte adhesion deficiency, and its binding to Papilloma virus E6 proteins. Paxillin binding activity towards different types of E6 proteins correlates with degrees of disruption of the actin cytoskeletal architecture induced by infection with each type of Papilloma viruses. Human paxillin is composed of multiple isoforms (α, β and γ) with different biochemical properties and different patterns of expression.

They have shown in fibroblasts that the cytoplasmic pool of paxillin primarily resides in the perinuclear region, a fraction of which seems to overlap with the Golgi apparatus. As will be described in this paper, there also appears to be a relatively large cytoplasmic pool in other types of cells, such as epithelial cells. They have, therefore, hypothesized that some intracellular active process, rather than a process of simple diffusion, may exist that helps to transport paxillin to sites of integrin macroaggregates at the plasma membrane. Paxillin is a soluble protein; thus they attempted to purify paxillin binding proteins that may be involved in localization of paxillin in the cytoplasm.

The process of monocyte maturation in vitro provides a good model to explore the biochemical events involved in process of integrin activation. They have shown that human monocytes express all three isoforms of paxillin, and expression of all isoforms is augmented upon the cell maturation. Here, they report the isolation of a paxillin-binding protein, named PAG3 (Paxillin-associated protein with Arf GTPase-activating protein (GAP) activity, number 3), from mature U937 monocyte cells. PAG3 corresponds to KIAA0400 previously isolated by Ishikawa et al., 1997; and during their analysis, the same molecule was also identified as a Pyk2 binding protein and named Papα. PAG3/Papα/KIAA0400 contains a zinc finger motif that is highly homologous to that of mammalian ARF1 GAP and yeast ARF GAP protein Gcs1. The zinc finger motif is essential for the ARF1 GAP activity . Andreev et al. have shown that this protein exhibits a GAP activity against several isoforms of ARFs in vitro; and also demonstrated that this protein inhibits ARF-dependent generation

of post-Golgi vesicles and secretion of a truncated form of placental alkaline phosphatase. They show here that PAG3/Pap α/KIAA0400 also binds to all three isoforms of human paxillin (α, β and γ), and is highly induced during monocyte maturation, during which integrins are activated and the cells become adherent and motile. They analyzed intracellular interactions among paxillin, PAG3 and ARFs. They also suggest that the GAP activity of PAG3 is involved in the recruitment of paxillin to focal contacts of adhesion plaques, and cell migratory activity. Finally, they discuss the relationship of ARF-mediated intracellular regulations to the subcellular localization of paxillin, and to cell migratory activities.

論文の審査結果の要旨

多細胞生物において細胞接着や細胞移動は、発生、再生、炎症、癌細胞の浸潤や転移など様々な局面で起こる基本的な現象である。これらの現象には、integrin と細胞外マトリックスの結合によって形成される integrin 接着点が深く関与していると考えられる。integrin はその細胞内領域で特定の蛋白質群と結合し、それらの蛋白質はシグナル伝達や scaffold としての役割を持っている。しかしながら、integrin が細胞内のどこでこれらの蛋白質を集積させ始めるのかや、その分子メカニズムは不明である。一方、低分子量G蛋白結合蛋白質の Rho family に属する Rho A について、actin stress fiber や接着点の形成、actomyosin network の収縮性制御、actin の重合や細胞骨格と膜接着の調節などに関与していることが報告されているが、接着点の形成やその actin fiber との結合を通じた細胞移動の調節メカニズムの詳細は不明であった。

最近、もう一つの低分子量G蛋白結合蛋白質の family に属する ARF1 が paxillin を integrin 接着点に集積させ、Rho A と相補的に働いて actin stress fiber の末端に paxillin に富む接着点を形成することが報告された。paxillin は、integrin に結合する蛋白質の一つで、他の integrin 結合蛋白質である、vinculin、talin、focal adhesionkinase、Pyk2 等と直接相互作用し、integrin の活性化に伴ってチロシンリン酸化される事が知られている。ARF family は膜や小胞の輸送に関与するといわれているが、そのうち ARF1 は、プレゴルジ体に存在し、小胞の出芽や細胞膜との融合を制御している。一方 ARF1 と構造的に最も遠い ARF6 は、細胞質に広く分布し、特に運動が活発に起こり新しい接着点を作るラッフル膜に局在し、エンドゾームから形質膜への小胞輸送に関与しているほか、形質膜下の actin 細胞骨格の再構築を調節しているとされる。これらの ARF には、他の低分子量G蛋白結合蛋白質と異なり GTPase 活性がほとんどなく、ARF の活性は ARF を GDP 結合型から GTP 結合型へ活性化する ARF GEP、および GTP 結合型から GDP 結合型へ不活性化する ARF GAP によって制御されている。

申請者らは、過去に線維芽細胞において paxillin の主な局在は、ゴルジ体と重なる核周囲の細胞質領域であることを示した。申請者は、可溶性蛋白質である paxillin が、ここから integrin 接着点に移行するメカニズムを探るために、paxillin と結合する蛋白質を Far Western protein-blotting 法によってスクリーニングし、U937 単球細胞より PAG3 (Paxillin-associated protein with Arf GTPase-activating protein activity, number 3)を単離、同定した。PAG3 は、他のグループによってほぼ同時に見い出された Pyk2 結合蛋白質と同じ分子であり、zinc finger motif を含んでいた。これは、ARF1 GAP に見られる GAP 活性に必須の zinc finger motif と高い相同性を持っており、実際に PAG3 が、ARF GAP 活性を持ち ARF の機能を阻害することが他のグループによって示されている。申請者は、PAG3 がヒトの３つの paxillin isoform のすべてに結合し、単球が成熟し接着性や運動性を獲得するにしたがって paxillin とともに発現が亢進する事を見いだした。さらに PAG3 を COS 細胞に強制発現させると paxillin の接着点への集積と細胞の運動活性が阻害されること、この効果は GAP 活性を持たない変異 PAG3 では認められないことを明らかにした。これらの結果は、paxillin の接着点への集積は、単なる拡散によるのではなく、PAG3 の ARF への GAP 活性を通したメカニズムによって行われていることを示している。このように申請者の研究成果は細胞移動の調節を理解する上で新しい局面を拓くものであり、学位論文として十分ふさわしい内容であると審査委員会において全委員一致で判定した。

本論文に関する学問的背景や関連分野の研究動向についての審査委員による口頭試問を行った結果、いずれも的確な応答が得られ、学位授与にふさわしい知識と学力を有していると判定された。本論文は分かりやすい英語で書かれており、また、この内容の一部は、申請者を筆頭著者とする英語論文として国際誌

である Mol. Biol. Cell に掲載予定となっていることから、英語力も十分なものであると判定した。以上、総合的に判断し学位を取得するに足る水準に達していることが全審査委員一致で認定された。