Molecular Biology of the Cell
Vol. 11, 1315–1327, April 2000

A New Paxillin-binding Protein, PAG3/Papα/KIAA0400, Bearing an ADP-Ribosylation Factor GTPase-activating Protein Activity, Is Involved in Paxillin Recruitment to Focal Adhesions and Cell Migration

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Submitted September 1, 1999; Revised December 6, 1999; Accepted January 12, 2000
Monitoring Editor: Richard Hynes

Paxillin acts as an adaptor molecule in integrin signaling. Paxillin is localized to focal contacts but seems to also exist in a relatively large cytoplasmic pool. Here, we report the identification of a new paxillin-binding protein, PAG3 (paxillin-associated protein with ADP-ribosylation factor [ARF] GTPase-activating protein [GAP] activity, number 3), which is involved in regulation of the subcellular localization of paxillin. PAG3 bound to all paxillin isoforms and was induced during monocyte maturation, at which time paxillin expression is also increased and integrins are activated. PAG3 was diffusely distributed in the cytoplasm in premature monocytes but became localized at cell periphery in mature monocytes, a fraction of which then colocalized with paxillin. PAG3, on the other hand, did not accumulate at focal adhesion plaques, suggesting that PAG3 is not an integrin assembly protein. PAG3 was identical to KIAA0400/Papα, which was previously identified as a Pyk2-binding protein bearing a GAP activity toward several ARFs in vitro. Mammalian ARFs fall into three classes, and we showed that all classes could affect subcellular localization of paxillin. We also examined possible interaction of PAG3 with ARFs and showed evidence that at least one of them, ARF6, seems to be an intracellular substrate for GAP activity of PAG3. Moreover, overexpression of PAG3, but not its GAP-inactive mutant, inhibited paxillin recruitment to focal contacts and hampered cell migratory activities, whereas cell adhesion activities were almost unaffected. Therefore, our results demonstrate that paxillin recruitment to focal adhesions is not mediated by simple cytoplasmic diffusion; rather, PAG3 appears to be involved in this process, possibly through its GAP activity toward ARF proteins. Our result thus delineates a new aspect of regulation of cell migratory activities.

INTRODUCTION

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) (reviewed in Hynes, 1992; Lauffenburger and Horwitz, 1996; Sheetz *et al.*, 1998). Integrins recruit a characteristic set of cytoplasmic proteins, with scaf-

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Abbreviations used: aa, amino acids; Ab, antibody; AlF, aluminum fluoride; ARF, ADP-ribosylation factor; ECM, extracellular matrix; EGFP, enhanced green fluorescent protein; GAP, GTPase-activating protein; GST, glutathione *S*-transferase; HA, influenza hemagglutinin; IgG, immunoglobulin G; PAG, paxillin-associated protein with ARF GAP activity; PAK, p21 GTPase-activated kinase; PH, pleckstrin homology; SH3, Src homology 3; TPA, 12-*O*-tetradecanoyl-phorbol acetate.

folding as well as signaling properties, at their cytoplasmic regions during this process (Clark and Brugge, 1995; Burridge and Chrzanowska-Wodnicka, 1996). It is well documented in fibroblasts that integrin macroaggregates grow and shrink over time during cell migration, although the position of each macroaggregate remains fixed as the cell translocates (Regen and Horwitz, 1992). 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 (Burridge and Chrzanowska-Wodnicka, 1996). 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 (reviewed in Hall, 1994; Hall, 1998). 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 (Ridley and Hall, 1992). Furthermore, the Rho A protein has been shown to participate in regulation of the phosphorylation status of myosin light chain and thus to regulate the contractility of the actomyosin network (for reviews, see Burridge and Chrzanowska-Wodnicka, 1996; Lauffenburger and Horwitz, 1996). Rho A can also activate phosphatidylinositol 4-phosphate 5′-kinase to produce phosphatidylinositol 4,5-bisphosphate, which interacts with gelsolin, profilin, and vinculin and helps regulate actin polymerization and cytoskeleton–membrane attachment (for review, see Burridge and Chrzanowska-Wodnicka, 1996; Siddiqui and English, 1997). Moreover, Rho A is able to activate phospholipase D to produce phosphatidic acid and to regulate actin polymerization (Ha *et al*., 1994). Despite 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.* (1998), 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 cells. 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 (Taylor *et al*., 1992; Donaldson and Klausner, 1994; Nuoffer and Balch, 1994; Zhang *et al*., 1994; Schekman and Orci, 1996). Members of the family include six isoforms of ARF and the ARF-like proteins (Tsuchiya *et al*., 1991; Clark *et al*., 1993). The six ARF isoforms are highly homologous to one another and classified as class I, II, or III based on sequence similarity (Tsuchiya *et al*., 1991). Class I includes ARF1–3; 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 (Stearns *et al*., 1990; Serafini *et al*., 1991; Donaldson *et al*., 1992; Kahn *et al*., 1992); ARF1 also functions in endoplasmic reticulum-to-Golgi transport (Balch *et al*., 1992; Dascher and Balch, 1994), the *trans*-Golgi network (Stamnes and Rothman, 1993), endosome–endosome fusion (Lenhard *et al*., 1992; West *et al*., 1997; Ooi *et al*., 1998), protein secretion, and fluid-phase endocytosis (Zhang *et al*., 1994), as well as phospholipase D activation (Brown *et al*., 1993; Kahn *et al*., 1993; Cockcroft, 1996). The GTP-bound form of ARF1 recruits protein coats, including the clathrin-associated adaptor proteins AP-1 and AP-3 and the non-clathrin coatomer, to membranes and initiates budding of the membrane vesicles (Lenhard *et al*., 1992; Donaldson and Klausner, 1994; Boman and Kahn, 1995; Dittie *et al*., 1996; Ooi *et al*., 1998; Springer *et al*., 1999). 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 that 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 (D'Souza-Schorey *et al*., 1995; Peters *et al*., 1995; Radhakrishna *et al*., 1996, 1999; Radhakrishna and Donaldson, 1997; Song *et al*., 1998). 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 (Kahn and Gilman, 1986).

Paxillin, one of the integrin assembly proteins, is highly tyrosine phosphorylated upon integrin activation (Burridge *et al*., 1992) and acts as an adaptor protein in integrin signaling (reviewed in Turner, 1998). Paxillin can interact directly with several integrin assembly proteins, including vinculin, talin, integrin β1, 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 (Graham *et al*., 1994) and its binding to papillomavirus E6 proteins. Paxillin binding activity toward different types of E6 proteins correlates with degrees of disruption of the actin cytoskeletal architecture induced by infection with each type of papillomavirus (Tong and Howley, 1997; Tong *et al*., 1997). Human paxillin is composed of multiple isoforms (α, β, and γ) with different biochemical properties and different patterns of expression (Mazaki *et al*., 1997).

We 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 (Mazaki *et al*., 1998). 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. We have, therefore, hypothesized that some intracellular active process, rather than a process of simple diffusion, may exist that helps transport paxillin to sites of integrin macroaggregates at the plasma membrane (Mazaki *et al*., 1998). Paxillin is a soluble protein; thus we 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 the process of integrin activation. We have shown that human monocytes express all three isoforms of paxillin, and expression of all isoforms is augmented upon cell maturation (Mazaki *et al.*, 1997). Here, we 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 our analysis, the same molecule was also identified as a Pyk2-binding protein and named Papα (Andreev *et al.* 1999). PAG3/Papα/KIAA0400 contains a zinc finger motif that is highly homologous to that of mammalian ARF1 GAP (Cukierman *et al.*, 1995) and yeast ARF GAP protein Gcs1(Poon *et al.*, 1996). The zinc finger motif is essential for the ARF1 GAP activity (Cukierman *et al.*, 1995). Andreev *et al.* (1999) have shown that this protein exhibits 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. We show here that PAG3/Papα/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. We analyzed intracellular interactions among paxillin, PAG3, and ARFs. We 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, we discuss the relationship of ARF-mediated intracellular regulations with the subcellular localization of paxillin and with cell migratory activities.

MATERIALS AND METHODS

Cells and Antibodies

COS-7 cells were grown with Dulbecco's modified Eagle's medium (with 4.5 g of glucose/l; Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (HyClone, Logan, UT). U937 cells were cultured with RPMI 1640 with 10% FCS. Human peripheral monocytes were prepared from peripheral venous blood collected from normal donors. Monocyte cells in the blood sample were isolated by centrifugation on Ficoll-Paque Plus (Pharmacia Biotech, Uppsala, Sweden), followed by sedimentation through Percoll (Pharmacia Biotech) according to the manufacturer's instructions, and cultured with RPMI 1640 supplemented with 10% FCS. For differentiation, monocyte cells were treated with 1.6×10^{-7} M 12-*O*-tetradecanoylphorbol acetate (TPA; Sigma, St. Louis, MO) for 3 d (Gidlund *et al.*, 1981).

Anti paxillin antibody (Ab 199–217), which recognizes the α, β, and γ isoforms, was described previously (Mazaki *et al.*, 1997). Rabbit polyclonal anti-PAG3 antisera was raised against glutathione *S*-transferase (GST)-fusion forms of PAG3 (M2) protein produced in *Escherichia coli* (see below), which contained amino acids 863-1006 of PAG3. The antisera was affinity purified using GST-PAG3 (M2) protein before use. Other antibodies were purchased from commercial sources: anti-paxillin (mouse monoclonal; Transduction Laboratories, Lexington, KY), anti-influenza hemagglutinin (HA, clone 16B12; Berkeley Antibody, Richmond, CA), anti-green fluorescent protein (GFP; Clontech, Palo Alto, CA), anti-Pyk2 (mouse monoclonal; Transduction Laboratories), and anti-phosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY). Secondary antibodies to rabbit or mouse immunoglobulin G (IgG) each conjugated with peroxidase, Cy2, and Cy5 were from Jackson ImmunoResearch (West Grove, PA).

Plasmids and Recombinant Proteins

All procedures for nucleic acid manipulation were done according to standard methods (Sambrook *et al.*, 1989) unless otherwise described.

pAcG2TK/paxillin α was constructed by ligating the *Bam*HI–*Eco*RI cDNA fragment isolated from pGEX/paxillin α (Mazaki *et al.*, 1997), encoding the entire region of human paxillin α, into the *Bam*HI–*Eco*RI site of pGEX-2TK (Pharmacia Biotech) to be fused in frame to the COOH terminus of GST. The recombinant protein produced in *E. coli* by the induction with isopropyl-β-D-thiogalactopyranoside was purified using glutathione-Sepharose beads (Pharmacia Biotech) and phosphorylated in vitro using a catalytic subunit of cAMP-dependent kinase (Sigma) and [γ-^{32}P]ATP according to a method described previously (Kaelin *et al.*, 1992). A TPA-induced monocyte U937 λgt 11 cDNA library (Clontech) was then screened using the phosphorylated GST-TK-paxillin α protein as a probe.

KIAA0400 cDNA was a gift from Dr. T. Nagase (Kazusa DNA Research Institute, Chiba, Japan). The cDNA fragment was amplified by a PCR method using oligonucleotides 5′-GCT GAA GGT CAA CGA AAT CA-3′ and 5′-TGC TAT TTT GCA GCA CAG AC-3′, and the resultant fragment was ligated into the *Bam*HI–*Not*I site of pEBG expression vector (Mayer *et al.*, 1995) to be fused in frame to the COOH terminus of GST.

For construction of mutants of PAG3 cDNA, each corresponding cDNA fragment was amplified from the original KIAA0400 cDNA by PCR and ligated into the pGEX-2TK (for M1 and M2 mutants) or pEBG (M3 and M4 mutants) vectors in frame with the COOH terminus of GST. Production of the M3 and M4 mutants in *E. coli* was accompanied by severe degradation of the recombinant proteins; thus the mammalian expression system was used for these proteins. Oligonucleotides used were as follows: 5′-CGGGATCCGCGCTCTATAACTGTGTGGCTGACA-3′ and 5′-CGGGATCCTCAGTCAGCGATAAAGTGCACAAAT-3′ for the M1 mutant encompassing amino acids 951-1006 (951–1006 aa); 5′-CGGGATCCCCGAGCAAGCCTGCCCCGCC-3′ and 5′-CGGAATTCTCAGTCAGCGATAAAGTGCA-3′ for M2 (863–1006 aa); 5′-CGGGATCCGTTCACGTTGAATATGAATGGCGAC-3′ and 5′-AAGGAAAAAAGCGGCCGCTCAGTCAGCGATAAAGTGCAC-3′ for M3(685–1006 aa); and 5′-CGGGATCCATGCCGGACCAGATCTCCGT-3′ and 5′-AAGGAAAAAAGCGGCCGCTCAGTGAGAATTAAATCTTCCAGATAA G-3′ for M4 (1–684 aa). Proteins encoded by the pGEX2TK vector were produced in *E. coli* by induction with isopropyl-β-D-thiogalactopyranoside, and proteins encoded by the pEBG vector were produced in COS-7 cells. These proteins were then subjected to purification using glutathione beads as previously described (Mazaki *et al.*, 1997).

The enhanced GFP (EGFP) fusion protein with PAG3 was made by isolating the *Sma*I–*Sma*I cDNA fragment from pGEX2TK/PAG3 containing the entire region of PAG3 and ligating it into the *Sma*I site of the pEGFP-C1 vector (Clontech), fusing it in frame with the COOH terminus of EGFP. For the construction of the C436A mutant of PAG3 (CA mutant) in which the critical cysteine residue for the GAP activity at amino acid 436 was mutated into alanine to diminish the GAP activity as in the case of ARF1 GAP (Cukierman *et al.*, 1995), the 680-bp *Aat*II-*Stu*I fragment corresponding to that of the original cDNA but encoding the mutation was made by PCR using 5′-GGCAATGACGTCGCCTGTGACTGTGGGGCG-3′ and 5′-AAAAGGCCTTCCCCCGCAGGAGCAACTTGAG-3′. The *Aat*II-*Stu*I fragment of the pEGFP-C1/PAG3 was then replaced with the resulting fragment.

Recombinant proteins were made in the baculovirus system by ligating *Bgl*II–*Eco*RI cDNA fragments, each encoding the entire coding region of human paxillin α, β, and γ isolated from pBabePuro/paxillin plasmids (Mazaki *et al.*, 1997) into the *Bgl*II–*Eco*RI site of the pVL1392 vector (PharMingen, San Diego, CA). pAcG2T/paxillin α

(N) and pAcG2T/paxillin (LIM), each encoding a GST fusion protein with the NH_2-terminal half of paxillin α (1–324 aa) and the four repeats of paxillin LIM domains (325–557 aa), were constructed by ligating each cDNA fragment amplified from pGEX2T/paxillin α (Mazaki *et al.*, 1997) using oligonucleotides of 5′-ATGGATCCATG-GACGACCTCGACGCCCTGCTG-3′ and 5′-ATGAATTCCTGCA-GAGTCCGCGACTGTGGCG-3′ and 5′-ATGGATCCGGGGCCTGC-AAGAAGCCCATCGC-3′ and 5′-ATGAATTCCTAGCAGAA-GAG-CTTGAGGAAGC-3′ into the *Bam*HI–*Eco*RI site of the pAcG2T baculovirus vector. Each recombinant protein was produced according to the manufacturer's instructions (PharMingen).

cDNAs in pcDNA 3 vector each encoding HA-ARF1, HA-ARF5, HA-ARF6, HA-ARF1N126I, HA-ARF5N126I, and HA-ARF6N122I were gifts from Dr. K. Nakayama (Tsukuba University, Tsukuba, Ibaraki, Japan).

Nucleotide sequences were confirmed with all the plasmids after the construction.

Protein Binding Analysis

Cell lysates were prepared with 1% NP-40 buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 1% aprotinin, 2 μg/ml leupeptin, and 3 μg/ml pepstatin A) as described previously (Sabe *et al.*, 1994). Protein concentrations were determined using a Dc protein assay kit (Bio-Rad, Hercules, CA) with BSA (Sigma) as a standard.

For protein binding analysis, 500 μg of cell lysate were mixed with 5 μg of GST fusion protein bound to glutathione-beads, unless otherwise mentioned, incubated for 1 h at 4°C, and then washed four times with 1% NP-40 buffer. Proteins retained on the beads were then separated by SDS-PAGE and subjected to immunoblotting analysis visualized by an enzyme-linked chemiluminescence method, as previously described (Sabe *et al.*, 1994; Mazaki *et al.* 1998). Each figure shows representative results from at least two independent experiments.

Protein Transient Expressions and Confocal Immunofluorescence Microscopy

COS-7 cells ($0.5–1 \times 10^5$) in a 35-mm culture dish were transfected with 4 μg of plasmid DNAs by the calcium phosphate precipitation method as previously described (Bonifacino *et al.*, 1989) or with 1 μg of plasmid DNAs using FuGENE 6 according to the manufacturer's instructions (Boheringer Mannheim, Indianapolis, IN), and 16–20 h later cells were trypsinized and replated onto glass chamber slides (polystyrene vessel tissue culture–treated glass slide; Becton Dickinson, San Jose, CA). Thirty-six to 48 h after transfection, cells were fixed in 3.7% paraformaldehyde (Sigma) in PBS for 10 min at room temperature, washed twice with PBS, and then incubated for 5 min in 0.1% BSA/PBS. To activate GTP-binding proteins, cells were treated for 1 h with 30 mM NaF and 50 μM $AlCl_3$ (aluminum fluoride [AlF]) at 37°C as previously described (Radhakrishna *et al.*, 1996; Ooi *et al.*, 1998) before fixation. A 1-h incubation with AlF was chosen by our preliminary time course study (from 10 to 120 min) as optimal for activation of transfected ARFs in COS-7 cells. Cells were then subjected to successive incubations with primary and secondary antibodies in the presence of 0.2% saponin (Sigma) and 0.1% BSA/PBS. For observation of adhesion plaques, fixed cells were permeabilized by incubating for 5 min with 0.2% Triton X-100/PBS and successive incubations with primary and secondary antibodies each diluted in 2% BSA/PBS. Incubation with each antibody was carried out at room temperature for 1 h, and samples were rinsed with PBS after each antibody incubation. After a final rinse with PBS, coverslips were mounted with 50% glycerol/PBS. Cells were visualized and confocal images acquired using a confocal laser scanning microscope (model 510; Carl Zeiss, Oberkochen, Germany). Each figure of microscopic analysis showed representative results that were observed in a majority of the cDNA-transfected cells (>100–200 cells) in three independent experiments.

Cell Adhesion and Haptotaxis Migration Assays

Cell adhesion and migration assays were performed using modified Boyden chambers (tissue culture treated, 6.5-mm diameter, 10-μm thickness, 8-μm pores, Transwell; Costar, Cambridge, MA), as previously described (Klemke *et al.*, 1998). In brief, only the underside of the polycarbonate membrane on the upper chambers was coated with 10 μg/ml collagen type I (Upstate Biotechnology), fibronectin (Sigma), vitronectin (Wako Chemicals, Tokyo, Japan), or BSA in PBS for 2 h at 37°C, rinsed with PBS, and then placed into the lower chamber filled with 400 μl of a migration assay medium (fibroblast basal medium supplemented with 0.5% BSA; Clonetics, San Diego, CA). COS-7 cells (2×10^5 cells per 60-mm dish) or 2×10^5 U937 cells were transfected with 2.6 μg of pEGFP-C1/PAG3 or pEGFP-C1/PAG3 (CA) using FuGENE 6, and 40 h after transfection cells were trypsinized, washed once with Dulbecco's modified Eagle's medium containing 0.5% (wt/vol) soybean trypsin inhibitor (Sigma) and twice with a migration assay medium, and suspended in the same medium at 1×10^6 cells/ml. Cells (1×10^5) were then applied onto the upper migration chambers and allowed to migrate into the underside of the upper chamber for 3 h at 37°C with 5% CO_2. After the nonmigrated cells on the upper membrane surface were removed with a cotton swab, cells that migrated to the underside of the upper chamber were fixed with 3.7% paraformaldehyde in PBS. To measure the adhesive activity, another 1×10^5 cells were plated onto culture dishes, which was coated with each ECM and also blocked with heat-inactivated BSA (inactivated at 70°C for 1 h), incubated for 30 min at 37°C, and then fixed as above. cDNA transfection efficiency was measured by fixing cells without trypsinization. Cells positive for EGFP-PAG3 or EGFP-PAG3 (CA) were detected by fluorescence from the EGFP and counted using a laser scanning microscope with a 20× objective (model 510; Carl Zeiss). Before data acquisition, the threshold for the detection of the laser scanning microscope was adjusted to eliminate the background autofluorescence signals of mock-transfected cells. Percent cell adhesion and percent cell migration were calculated by dividing the numbers of transfection-positive adhered cells or migrating cells by the number of applied transfection-positive cells, which was calculated by the cDNA transfection efficiency. Each determination represents the average of three independent experiments, and error bars represent SEM.

RESULTS

Isolation of a New Paxillin-binding Protein, PAG3

A λgt 11 cDNA expression library prepared from phorbol ester–stimulated U937 cells was screened by the far Western protein-blotting method using GST-TK-paxillin α protein as a probe. By screening 5×10^6 plaques, we isolated two cDNA clones that strongly bound to the probe. Sequencing analysis revealed that these two clones encoded the same protein but did not cover the entire coding region of the protein. We then searched a computer database of expressed sequence tags and found that KIAA0400 cDNA (Ishikawa *et al.*, 1997) and s19 cDNA (Yamabhai and Kay, 1997) showed a close similarity to our clones (Figure 1A; our unpublished data). The s19 cDNA clone did not contain the entire protein coding region. KIAA0400 cDNA, on the other hand, contained a complete protein coding region and encoded a protein with 1006 amino acids, which contained multiple protein modules, such as a pleckstrin homology (PH) domain, three repeats of an ankyrin motif, and an Src homology 3 (SH3) domain. This protein also contained a zinc finger with a structure of CxxC-x16-CxxC, which showed a close similarity to that of ARF1 GAP (Cukierman *et al.*, 1995; Figure 1B). In addition to screening paxillin-binding pro-

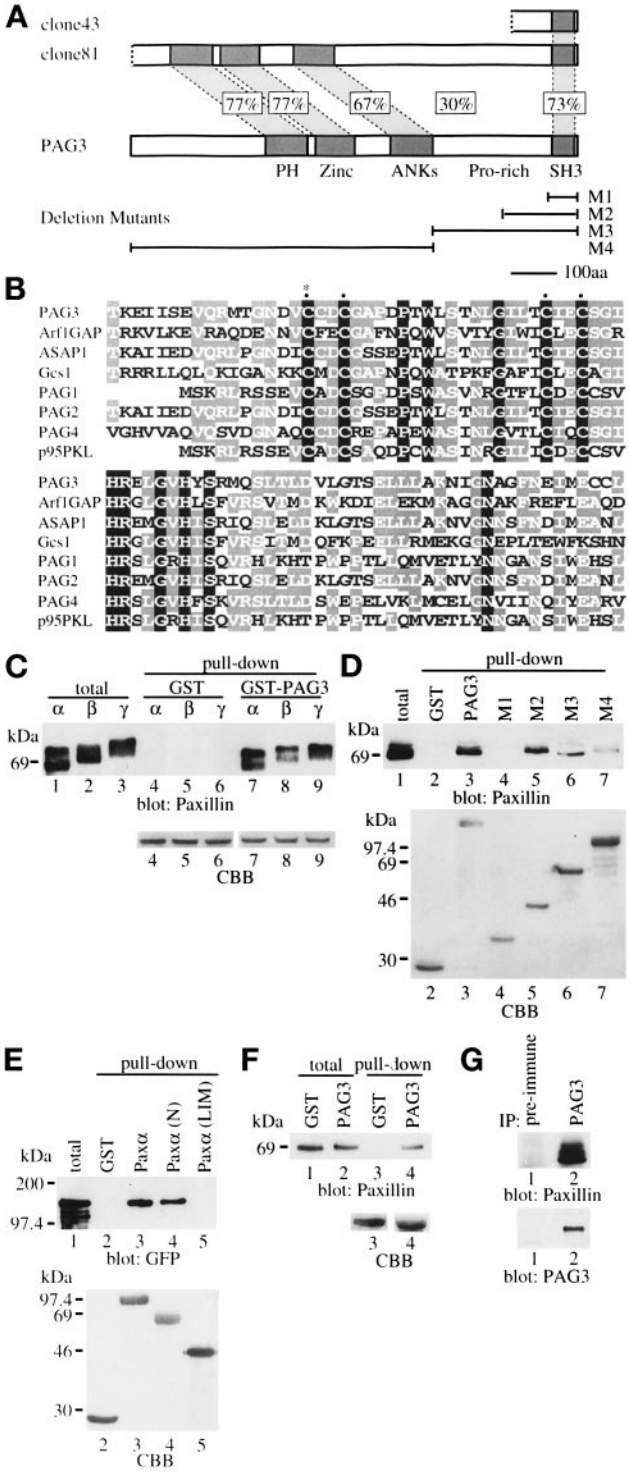

Figure 1. Structure of PAG3 and its binding toward paxillin. (A) Schematic diagram of PAG3 and its comparison with original two clones isolated using GST-paxillin α as a probe (clones 43 and 81). (B) Comparison of the zinc finger domain of PAG3 (residues 420–505) with those of ARF1 GAP (residues 6–91), ASAP1 (residues 453–538; Brown *et al.*, 1998), GCS1 (residues 10–95), PAG1 (residues 1–83), PAG2 (which corresponds to clone 81), PAG4 (residues 404–489), and p95PKL (residues 1–83). The cDNA for PAG2 was not complete; therefore, residue numbers were not assigned. Identical residues are framed and shadowed. The positions of four conserved cysteines of the zinc finger motif are marked by dots, and the residue mutated in the PAG3 CA mutant is marked by an asterisk. ASAP1 corresponds to the s19 clone, and PAG1, PAG2, and PAG4 correspond to our collection of paxillin-binding proteins (our unpublished data). (C) PAG3 binding to paxillin isoforms. Each 2.5 μg of GST alone (lanes 4–6) or a GST fusion form of PAG3 (lanes 7–9) expressed in COS-7 cells and purified on glutathione beads were incubated with 15 μg of Sf-9 cell lysates, each producing recombinant paxillin isoforms of α, β, and γ. After incubation, beads were washed, and proteins were retained on the beads were subjected to immunoblotting analysis using anti-paxillin antibody, Ab 199–217. Each 0.5 μg of the same cell lysates was included as "total" (lanes 1–3). (D) Paxillin binds to the COOH-terminal region of PAG3. Each 2.5 μg of GST alone (lane 2) or GST fusion forms of wild-type and deletion mutants of PAG3 (lanes 3–7) purified on glutathione beads as described in MATERIALS AND METHODS were incubated with 15 μg of Sf-9 cell lysate producing recombinant paxillin α to test binding, as above. Lane 1 included 0.5 μg of the same cell lysate. For the deletion mutants of M1–M4, see A. (E) PAG3 binds to the NH_2-terminal region of paxillin. Each 5 μg of GST alone (lane 2) and GST fusion forms of paxillin wild type and mutants (lanes 3–5) as described in MATERIALS AND METHODS were purified on glutathione beads and incubated with 500 μg of COS-7 cell lysate expressing the EGFP fusion form of PAG3 to test binding. Thirty micrograms of the total COS-7 cell lysate were included in lane 1. Immunoblot was done with anti-GFP antibody. (F) Association of PAG3 and paxillin in vivo. Each 1×10^6 COS-7 cells were transfected with 10 μg of pEBG or pEBG/PAG3 plasmid, and GST (lane 3) or GST-PAG3 (lane 4) was pulled down from each 1 mg of the cell lysate using glutathione beads to analyze its association with endogenous paxillin. Each 30 μg of the total cell lysates were included in lane 1 (cells with pEBG) and lane 2 (cells with pEBG/PAG3). Immunoblot was done with anti-paxillin antibody, Ab 199–217. (G) In vivo association of endogenous PAG3 and paxillin. Each 1 mg of cell lysate prepared from TPA-treated U937 cells was subjected to immunoprecipitation using anti-PAG3 antibody (lane 2) or the preimmune serum (lane 1) coupled with protein A-Sepharose beads. Precipitated proteins were then subjected to immunoblotting analysis using anti-paxillin antibody, Ab 199–217, and anti-PAG3 antibody. In C–F, amounts of each fusion protein used for pull-down assays are shown by Coomassie brilliant blue staining (CBB).

teins by the far Western method, we had also purified several paxillin-binding proteins from HeLa cell extracts (our unpublished results). The cDNAs corresponding to these proteins all contained the conserved CxxC-x16-CxxC motif of ARF GAP1 (also see Figure 1B). We thus named these paxillin-binding proteins bearing ARF GAP motifs as PAGs. We named KIAA0400 cDNA PAG3 and report its analysis here.

Binding of PAG3 to paxillin was then confirmed using in vitro and in vivo binding assays. We constructed a cDNA encoding GST fused to PAG3 and expressed it in COS-7 cells. The recombinant GST fusion protein purified on glutathione beads was then incubated with each isoform of recombinant human paxillin, α, β, and γ, produced by the baculovirus system. As shown in Figure 1C, each isoform was equally bound to the GST fusion form of PAG3 in vitro. We next constructed a series of deletion mutants of GST-PAG3 and tested binding toward recombinant paxillin α. As shown in Figure 1D, the COOH-terminal region of PAG3

contained in the M2 mutant was sufficient for binding; this mutant contained a region similar to our original short cDNA clone. The M2 mutant contained a proline-rich sequence and an SH3 domain, which existed proximal to the COOH-terminal of PAG3. The SH3 domain alone of PAG3 (M1 mutant), however, was not sufficient for binding (Figure 1D). Paxillin can be divided into the NH_2-terminal half-region and the COOH-terminal LIM domain. We found that PAG3 bound to the NH_2-terminal half-region of paxillin but not to the LIM domain (Figure 1E). Furthermore, to examine the in vivo binding of PAG3 and paxillin, COS-7 cells were transfected with GST-PAG3, and the GST fusion protein was isolated with glutathione beads. As shown in Figure 1F, a significant amount of endogenous paxillin coprecipitated with the beads. Endogenous association of PAG3 with paxillin was also confirmed in TPA-treated U937 cell lysates by the coprecipitation of paxillin with anti-PAG3 antibody (Figure 1G).

During our analysis of this protein, the same cDNA clone was identified as a Pyk2-associated protein named PAPα and has been shown to exhibit GAP activity against ARF1, ARF5, and ARF6 in vitro (Andreev *et al.*, 1999). Therefore, KIAA0400/PAPα/PAG3 cDNA indeed encoded a protein with ARF GAP activity. In this paper, we refer to KIAA0400/PAPα/PAG3 as PAG3 for short.

PAG3 Is Induced and Binds to Paxillin during Monocyte Maturation

Expression of PAG3 in U937 monocyte cells was examined. We made polyclonal antibodies against the M2 portion of PAG3, which were then affinity purified. We found that PAG3 was expressed at only marginal levels in the undifferentiated U937 cells but became highly expressed after stimulation with TPA for 3 d (Figure 2A). We also found that PAG3 was tyrosine phosphorylated in U937 cells with a similar level regardless of the TPA treatment (Figure 2B). Similar induction of PAG3 was seen with human peripheral blood monocytes stimulated with TPA (Figure 2A). PAG3 protein expression was also detected in several cultured cell lines (Figure 2A).

We also examined the binding of PAG3 to proteins in monocyte cell lysates. The GST fusion form of PAG3 pulled down both Pyk2 and paxillin. We found that although binding of PAG3 to Pyk2 was almost unchanged before and after monocyte maturation, binding to paxillin was increased severalfold upon monocyte maturation (Figure 2C). We also found that both paxillin and Pyk2 bound to the M2 and M3 mutants of PAG3 (our unpublished results), suggesting that paxillin and Pyk2 may bind to the same region of PAG3.

Colocalization of PAG3 and Paxillin in the Cytoplasm and Peripheral Membranes

Colocalization of endogenous PAG3 and paxillin was then investigated with U937 monocyte cells (Figure 3A). In unstimulated cells, both paxillin and PAG3 showed very weak staining. With TPA-stimulated and adhered cells, significant fractions of both PAG3 and paxillin were detected at the leading edges of the peripheral membrane, and colocalization of both proteins was seen at several, but not all, regions of the cell periphery.

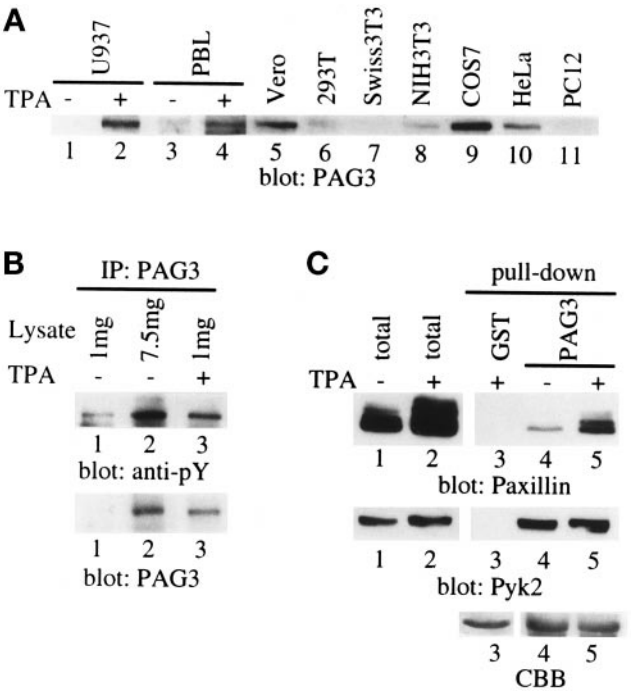

Figure 2. Induced expression of PAG3 and its increased binding to paxillin during monocyte maturation. (A) Cell lysates from U937 monocytes, human peripheral monocytes (PBL), and several other cell lines were separated on SDS-PAGE and subjected to immunoblot analysis using anti-PAG3 antibody. Monocyte cells undifferentiated (−TPA) or differentiated by treatment with TPA for three days (+TPA) are shown. (B) PAG3 protein was immunoprecipitated from U937 cells treated with (lane 3) or without (lanes 1 and 2) TPA and subjected to sequential immunoblot analysis using anti-phosphotyrosine antibody (4G10; anti-pY) and anti-PAG3 antibody. Different amounts of cell lysates (1 mg in lane 1, 7.5 mg in lane 2, and 1 mg in lane 3) were initially used for comparison of the tyrosine phsosphorylation levels. (C) Cell lysates from TPA-treated or untreated U937 cells were incubated with GST-PAG3 purified on glutathione beads to analyze PAG3 binding toward paxillin and Pyk2. Immunoblots were done with same membrane filter by sequential hybridization with anti-paxillin antibody (Ab 199–217, Pax) and anti-Pyk2 antibody (Pyk2). In C, amounts of each fusion protein used for pull-down assays (lane 3, GST; lanes 4 and 5, GST-PAG3) are shown by Coomassie brilliant blue staining (CBB).

To analyze further the precise subcellular localization of the proteins as well as their intracellular interactions, we used COS-7 epithelial cells rather than monocyte cells for technical reasons. Moreover, we found that COS-7 cells expressed significant amounts of endogenous PAG3 (see Figure 2A) and paxillin but only marginal levels of Pyk2 (our unpublished results), thus reducing any possible effects of Pyk2 toward PAG3 in COS-7 cells.

Immunostaining revealed that both endogenous paxillin and PAG3 were widely distributed in the cytoplasm of COS-7 cells, with large regions of overlap (Figure 3B). Codistribution of these two proteins at the cell periphery was also seen when the focus was adjusted across the center of the nucleus (cell body). Moreover, punctate staining in the cytoplasm was seen with PAG3 and, albeit less clearly, with paxillin. Both proteins may be colocalized within some of

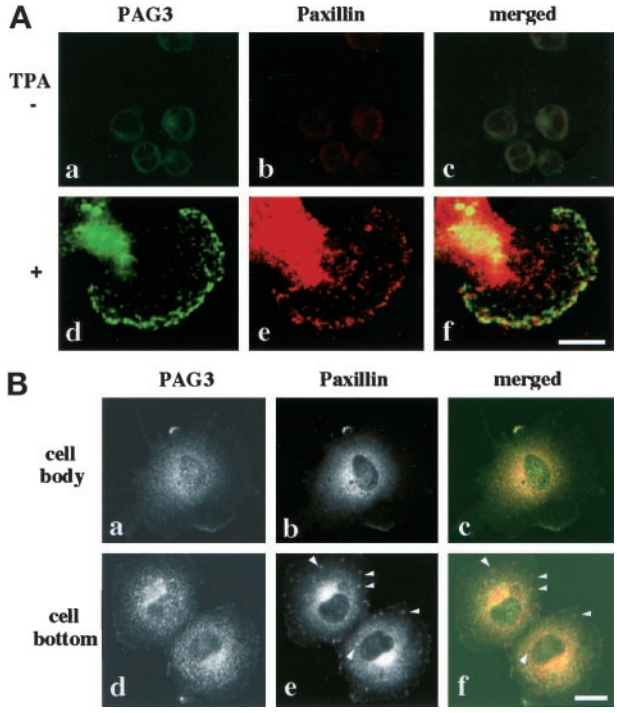

Figure 3. Colocalization of endogenous PAG3 and paxillin in the cytoplasm and at the cell periphery in U937 monocyte cells (A) and COS-7 epithelial cells (B). U937 monocyte cells undifferentiated (A, a–c) or differentiated by TPA treatment for 3 d (A, d–f) are shown. Cells fixed with 3.7% paraformaldehyde were double immunolabeled with rabbit polyclonal anti-PAG3 antibody (a and d) and mouse monoclonal anti-paxillin antibody (b and e), followed by Cy2-conjugated donkey anti-rabbit IgG and Cy5-conjugated donkey anti-mouse IgG, and examined by confocal laser scanning microscope. Focuses were adjusted 5.0 μm above the surface of the glass chamber plate in A (a–c) and 3.0 μm above this in B (a–c), each across the center of the nucleus in the majority of the cells, or 0.5 μm above this in A (d–f) and B (d–f), which are near the bottom layers of cells. In e and f in B, paxillin localized at the cytoplasmic pool is still seen in addition to that localized to focal adhesion plaques, and several focal adhesion plaques are marked by arrowheads. The right column represents the merging of the left and the middle images. For clearer images of PAG3 and paxillin distribution, some photographs in B (a, b, d, and e) are shown as black-and-white images. Bars, 20 μm.

these punctate structures, but a clear assessment of the colocalization was difficult because of the weak signals and diffuse distributions of these punctate structures (see below). On the other hand, although PAG3 was still detected near the cell bottom, no significant accumulation of PAG3 was detected at focal adhesion plaques where paxillin was condensed (Figure 3B).

Colocalization of PAG3 with ARFs and Their Possible Functional Interaction

PAG3 has been shown to exhibit GAP activity in vitro against several ARFs, including ARF1, ARF5, and ARF6 (Andreev *et al.*, 1999). We then examined colocalizations between PAG3 and ARFs. Each HA-tagged ARF isoform cDNA was expressed in COS-7 cells. As described above, endogenous PAG3 in COS-7 cells was widely distributed in the cytoplasm and also localized at the cell periphery (see Figure 3B). Comparison with different ARF isoforms in COS-7 cells revealed that the subcellular localization of PAG3 appeared to overlap with those of all classes of ARFs: ARF1 (class I), ARF5 (class II), and ARF6 (class III) (Figure 4A). However, colocalization with PAG3 was more readily observed for ARF6. On the other hand, only a fraction of PAG3 was colocalized with ARF1, which was concentrated in the perinuclear area and colocalized with β-COP (Stearns *et al.*, 1990; Serafini *et al.*, 1991; Donaldson *et al.*, 1992; Kahn *et al.*, 1992). ARF5 also seemed to be well colocalized with PAG3 but to a lesser extent than ARF6.

We next explored possible functional interaction of PAG3 with ARF activities. AlF is a G protein activator that can affect the behavior of ARFs, including ARF1 and ARF6 (Radhakrishna *et al.*, 1996; Ooi *et al.*, 1998). AlF treatment gives rise to distinct cell phenotypes depending on the ARF cDNAs transfected: it increases the number and size of ARF1-associated punctate structures in the ARF1-transfected cells (Ooi *et al.*, 1998) and induces membrane protrusion in the ARF6-transfected cells (Radhakrishna *et al.*, 1996). Because these phenotypes induced by the AlF treatment are conspicuous and easily recognized, especially with ARF6-transfected cells, we tested in this system whether overexpression of PAG3 could counteract the ARF activities. We made a mutant PAG3 cDNA (CA mutant) in which the critical cysteine residue for the GAP activity was mutated into alanine to diminish the activity. As shown in Figure 4B, we found that although overexpression of the CA mutant did not suppress the phenotype of membrane protrusion seen in the ARF6-transfected and AlF-treated cells, overexpression of wild-type PAG3 could suppress the phenotype. After the AlF treatment, membrane protrusions were seen with >90% of cells expressing both ARF6 and the PAG3 CA mutant. On the other hand, such membrane protrusions were not observed with majority of the ARF6-expressing cells (>50–60% of cells) when the wild-type PAG3 was overexpressed. It is also interesting to note that the CA mutant was then clearly colocalized with ARF6 at the membrane protrusions of the cell periphery (Figure 4B). Similar colocalization of endogenous PAG3 with ARF6 at membrane protrusions was also observed in the ARF6-transfected and AlF-treated cells, which were not transfected with PAG3 plasmid (our unpublished results). In ARF1-transfected cells, on the other hand, we indeed observed a number of very small ARF1-containing punctate structures that emerged after the AlF treatment, which could not be suppressed by the overexpression of the PAG3 CA mutant. However, the numbers and sizes of the ARF1-positive punctate structures were varied among individual cells even before the PAG3 expression, and thus we could not assess the suppressive effect of PAG3 on the ARF1 activity clearly (our unpublished results). Unlike in the case of ARF6, the majority of endogenous PAG3 was not colocalized with ARF1 in the ARF1-transfected and AlF-treated cells (our unpublished results).

ARF Activities Affect Subcellular Localization of Paxillin

ARF1 has been shown to be involved in the recruitment of paxillin to focal contacts (Norman *et al.*, 1998). Our results shown above, together with results showing that PAG3 may be a GAP for several ARFs (Andreev *et al.*, 1999), then

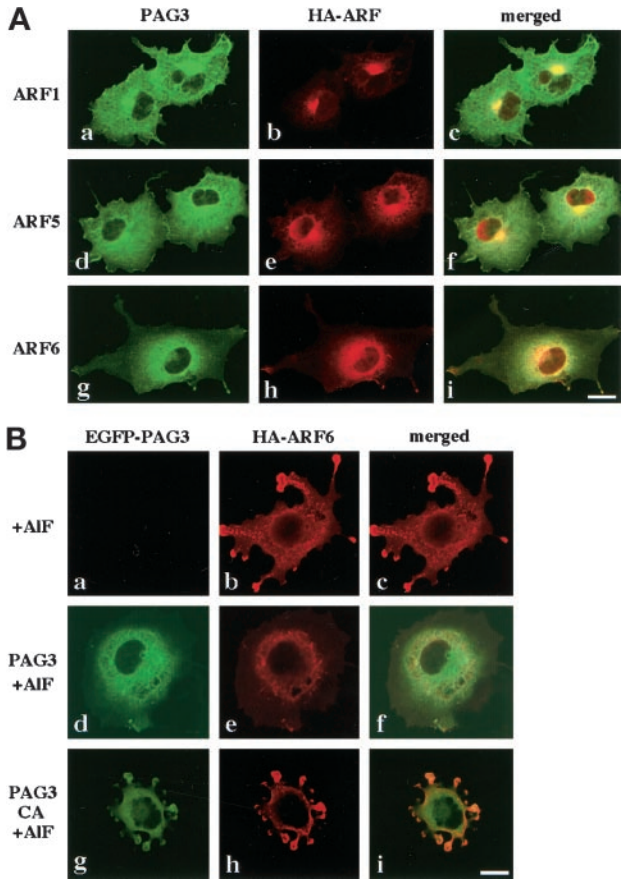

Figure 4. PAG3 and ARF isoforms. (A) Subcellular localization of PAG3 overlaps with ARF1, ARF5, and ARF6, but colocalization with PAG3 is more readily observed for ARF5 and ARF6 rather than for ARF1. COS-7 cells were transiently transfected by the calcium precipitation method with each plasmid encoding one of the HA-tagged wild-type ARF isoforms (a–c, ARF1; d–f, ARF5; g–i, ARF6) and then fixed, and each ARF protein was visualized by immunolabeling for the HA epitope using mouse monoclonal anti-HA antibody and Cy5-conjugated donkey anti-mouse antibody (b, e, and h). Endogenous PAG3 was visualized by anti-PAG3 antibody and Cy2-conjugated donkey anti-rabbit antibody (a, d, and g). (B) Overexpression of PAG3, but not its GAP-inactive mutant, counteracted ARF6 activity in the ARF6-transfected and AlF-treated cells. COS-7 cells were transiently transfected by FuGENE 6 with plasmids encoding HA-tagged wild-type ARF6 (a–c) or cotransfected with plasmids encoding HA-tagged wild-type ARF6 and EGFP-tagged wild-type (d–f) or with plasmids encoding HA-tagged wild-type ARF6 and the CA mutant of PAG3 (g–i). Each 0.5 μg of PAG3 and 0.5 μg of ARF plasmids were used, as determined by our preliminary titration experiments. Cells were then treated with AlF for 1 h at 37°C as described in MATERIALS AND METHODS and fixed. PAG3 proteins were visualized by the fluorescence from the EGFP tag (d and g). ARF6 (b, e, and h) was visualized as in A. In A and B, focuses were adjusted 3.0 μm above the surface of the glass chamber plate, across the center of the nucleus in the majority of the cells, and each right column represents the merging of the left and the middle images. Bars, 20 μm.

prompted us to examine whether different classes of ARFs could affect the paxillin subcellualr localization. We again used AlF treatment. With this system, changes in cellular

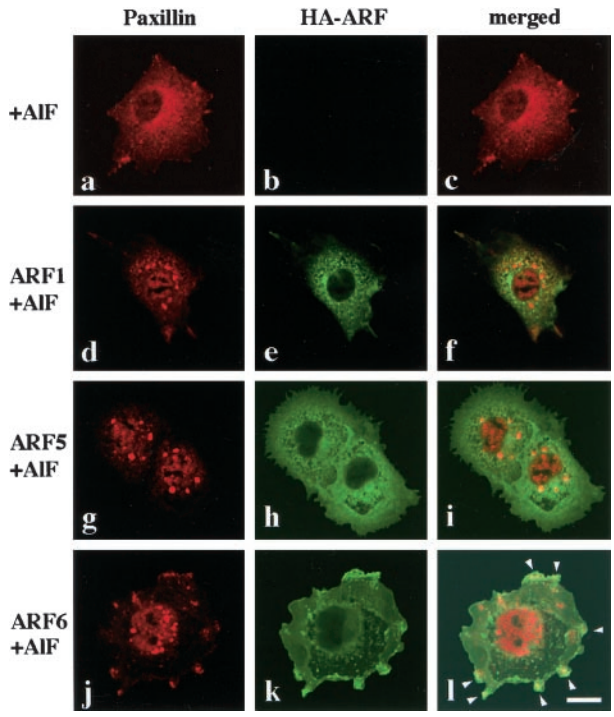

Figure 5. ARF activities affect subcellular localization of paxillin. COS-7 cells untransfected (a–c) or transiently transfected by FuGENE 6 with each plasmid encoding HA-tagged wild-type ARF1 (d–f), ARF5 (g–i), or ARF6 (j and k) were treated with AlF for 1 h at 37°C. Cells were then fixed, and endogenous paxillin (a, d, g, and j) was visualized using rabbit polyclonal anti-paxillin antibodies, coupled with Cy5-conjugated donkey anti-rabbit antibody, and HA-ARF proteins (e, h, and k) were visualized mouse monoclonal anti-HA antibody, coupled with Cy2-conjugated donkey anti-mouse antibody. Focuses were adjusted 3.0 μm above the surface of the glass chamber plate. The right column represents the merging of the left and the middle images (c, f, i, and l). Arrowheads in panel l indicate areas where paxillin is colocalized with ARF6. Bar, 20 μm. Expression of these wild-type HA-ARF proteins in the absence of the AlF treatment did not affect significantly the subcellular localization of paxillin (our unpublished data): see Figure 3B (b) for the subcellular distribution of paxillin in AlF-untreated cell for the comparison.

organization could be observed within a relatively short time (10–120 min), thus enabling the clear comparison of exogenous ARF activities. In our system, exogenous expression of any of ARF1, ARF5, and ARF6 per se did not affect significantly the subcellular localization of endogenous paxillin (our unpublished results). When these cells were treated with AlF, on the other hand, subcellular distribution of paxillin was drastically changed (Figure 5). Of these, a significant fraction, but not all, of paxillin then colocalized with ARF6 at several membrane protrusions. Paxillin also appeared to colocalize with ARF1 and ARF5 at some of their punctate structures.

Overexpression of PAG3 Causes Loss of Paxillin Recruitment to Focal Contacts

To explore further the cellular function of PAG3, we next examined the effects of PAG3 overexpression on paxillin

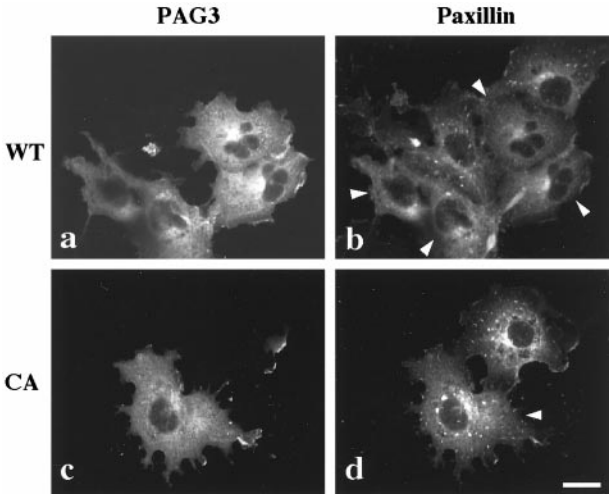

Figure 6. Overexpression of PAG3, but not its GAP-inactive mutant, inhibits paxillin recruitment to focal contacts. COS-7 cells were transiently transfected by the calcium precipitation method with plasmids encoding EGFP-tagged PAG3 (a and b) or the CA mutant (c and d). Cells were then fixed, and EGFP-PAG3 (a and c) and endogenous paxillin (b and d) were visualized as in Figures 4 and 3, respectively. Focuses were adjusted 0.5 μm above the surface of the glass chamber plate to visualize the focal contacts. The right column is of the same field as the left column, and arrowheads indicate cells expressing EGFP-PAG3 proteins. Bar, 20 μm.

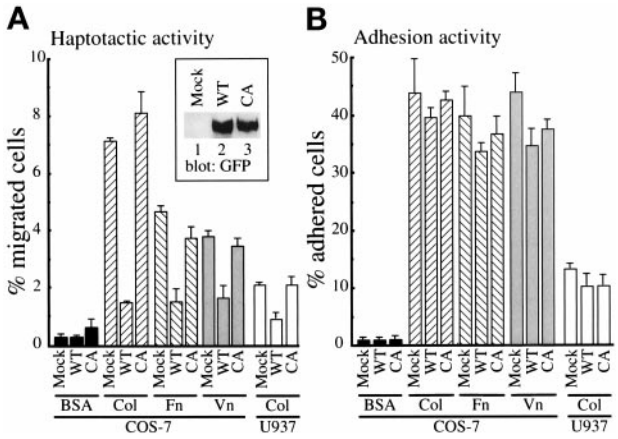

Figure 7. Overexpression of PAG3, but not its GAP-inactive mutant, decreases the cell migratory activity without significant effect on the cell adhesive activity. COS-7 cells or U937 cells were transiently transfected with plasmids encoding EGFP-tagged PAG3 (WT) or the CA mutant (CA) or subjected to the transfection procedure without plasmid DNA (Mock). U937 cells were treated with TPA for 3 d. Cells were collected by trypsinization and then subjected to haptotaxis migration assays (A) or adhesion assays (B) on collagen (Col), fibronectin (FN), or vitronectin (VN) in the absence of serum as described in MATERIALS AND METHODS. BSA coating was used as a negative control. Transfected cells adhered 30 min after replating were enumerated by counting cells positive for the EGFP fluorescence, and cells migrating during 3 h were enumerated by counting cells on the underside of the membrane that were positive for the EGFP fluorescence. Percentages of cell adhesion and migration were calculated as described in MATERIALS AND METHODS. Each bar represents the mean ± SEM of triplicate experiments. The inset in A is the anti-GFP antibody immunoblot showing the expression levels of EGFP-tagged PAG3 (WT) or the CA mutant (CA) in COS-7 cells used in these experiments. Expression of EGFP-tagged PAG3 protein was >10–20 times higher than that of endogenous PAG3 (our unpublished data).

localization. As shown in Figure 6, when PAG3 was overexpressed, no significant staining of paxillin was observed at focal adhesion plaques. Overexpression of the CA mutant of PAG3 did not exert such an effect; thus, this effect appeared to be mediated by the GAP activity of PAG3.

Overexpression of PAG3 Decreases Cell Migratory Activity

Cell adhesion and migratory activities are primarily mediated by integrin adhesion to the ECM. Our results described above prompted us to investigate how much PAG3 is involved in these cell activities on ECMs. Cell adhesion activity was measured with cDNA-transfected COS-7 cells by replating the cells on cell culture dishes coated with various ECMs. Cell migration activity was measured using modified Boyden chambers (Klemke *et al.*, 1998). As shown in Figure 7, only marginal differences were observed among untransfected cells and cells overexpressing PAG3 or the CA mutant with regard to the cell adhesion activity on collagen, fibronectin, and vitronectin. The rate of the cell spreading was also not affected significantly by the overexpression of PAG3 or the CA mutant compared with the untransfected cells (our unpublished results). On the other hand, a drastic effect was observed when cell migration activity was measured. The overexpression of PAG3 caused a severalfold decrease in the cell migratory activity on these ECMs compared with those overexpressing the CA mutant or with the mock-transfected cells (Figure 7). cDNA transfection efficiencies measured by counting the transfection-positive cells identified by the fluorescence from the EGFP tag and levels of exogenous protein expression measured by immunoblotting analysis were essentially the same between PAG3 and the CA mutant (our unpublished results; Figure 7A). These data provide quantitative evidence further supporting our analysis. Finally, we also examined exogenous expression of EGFP-PAG3 with U937 monocyte cells differentiated by TPA. Again, expression of EGFP-PAG3 seemed to act to decrease the heptotactic activity compared with expression of the CA mutant or the mock transfection, whereas the cell adhesion activity was almost unaffected (Figure 7).

DISCUSSION

Integrins play essential roles in a number of dynamic aspects of cell regulation, including migration and *trans*-invasion. Integrin function requires assembly of a number of different proteins at the integrin cytoplasmic domains. Therefore, it is believed that mechanisms that orchestrate protein assembly at the cytoplasmic tails of integrins might exist, and considerable effort has been devoted to identification of such mechanisms (reviewed in Burridge and Chrzanowska-Wodnicka, 1996). We have shown previously in fibroblasts that the cytoplasmic protein paxillin, which functions as an integrin adaptor, appears to localize to the perinuclear area of the cell

(Mazaki *et al.*, 1998). In this paper, we isolated a new paxillin-binding protein, PAG3, and provided evidence that cellular ARF activities are involved in the subcellular localization and the focal contact recruitment of paxillin, as well as in the regulation of cell migratory activities.

We identified PAG3 from mature monocyte cells and showed that PAG3 expression is highly induced during monocyte maturation, accompanied by its tyrosine phosphorylation. Upon monocyte maturation, integrins are activated, and cells become adherent, motile, and *trans*-invasive into tissues. We have shown that expression of all the three isoforms of paxillin also increased upon monocyte maturation and become highly phosphorylated (Mazaki *et al.*, 1997). Our results in this paper revealed that both PAG3 and paxillin are recruited to the cell periphery in mature monocytes adhering to the ECM. We also demonstrated that PAG3 binding to paxillin is increased during monocyte maturation, whereas the binding to Pyk2 is almost unchanged. Moreover, we demonstrated that PAG3 overexpression can inhibit paxillin recruitment to focal contacts and cell migratory activity. Thus, PAG3 seems to play an important role in the integrin activation and function, which take place during monocyte maturation. Andreev *et al.* (1999) have also suggested that PAG3 can be tyrosine phosphorylated by Pyk2 and by Src family kinases. These kinases exist in monocyte cells, and it would also be interesting to analyze the physiological role of tyrosine phosphorylation of PAG3 in monocyte cells. However, because of their small cytoplasm, hematopoietic cells including monocytes in general are not suitable for the analysis of intracellular protein localization or organelle structure. A relatively low efficiency of DNA transfection also hampers precise analysis. Thus, we have not yet described the precise mechanism of PAG3 function in monocyte cells.

PAG3 is identical to the recently described protein Papα (Andreev *et al.*, 1999). PAPα has been identified as a Pyk2-binding protein and was shown to exhibit strong in vitro GAP activity toward ARF1 and ARF5 but 10^2- to 10^3-fold less activity toward ARF6. ARF1 colocalizes with β-COP and regulates its subcellular localization. The subcellular localization of endogenous PAG3 includes β-COP-containing structures, as previously shown (Andreev *et al.*, 1999), although PAG3 exhibited a much broader distribution in the cytoplasm. The subcellular localization of ARF6 also includes the pre-Golgi structure, but inhibition of ARF6 activity does not affect the cellular distribution of β-COP (Peters *et al.*, 1995; our unpublished results). Likewise, our unpublished results and those of others (Andreev *et al.*, 1999) showed that PAG3 does not affect the subcellular localization of β-COP, although we confirmed that inhibition of ARF1 does affect it in our cell culture (our unpublished results), as shown previously (Peters *et al.*, 1995). We also showed that PAG3 is clearly colocalized with ARF6 in the AlF-treated ARF6-transfected cells. Moreover, we demonstrated that PAG3, but not its GAP-inactive mutant, can be inhibitory of the AlF induction of ARF6 activity in vivo. Taken together, our results suggest that ARF6 is an in vivo target of the GAP activity of PAG3, even though a previous study demonstrated that PAG3 exhibits only a weak activity toward ARF6 in vitro (Andreev *et al.*, 1999). In this regard, it has been reported that coatomer protein directly participates in the GTPase reaction of ARF1GAP, accelerating GTP hydrolysis by ARF1 an additional 1000-fold (Goldberg, 1999). The previous study by Andreev *et al.* (1999), however, was done without the addition of a coatomer protein. Our analysis implies that PAG3 function involves ARF6, and it remains to be determined how efficiently PAG3 can interact with ARF1 in vivo.

The function of ARF5, a class II ARF isoform, has not been well studied. Therefore, we did not assess the interaction between ARF5 and PAG3 in detail. Our preliminary data indicate that the subcellular distributions of ARF5 and PAG3 overlap, but there is one significant difference: an ARF5 dominant-negative mutant caused redistribution of β-COP (our unpublished results), whereas PAG3 did not. However, different GAP proteins may be involved in the recruitment of different coatomer proteins to the same ARF (Springer *et al.*, 1999). Thus, as in the case of the relationship of PAG3 with ARF1 as described above, our results do not preclude interaction of the PAG3 with ARF5 in vivo.

We showed that all the three classes of ARF activities can influence the subcellular localization of paxillin. Among the ARFs, colocalization with PAG3 is readily observed for ARF6, especially in the ARF6-transfected and AlF-treated cells. ARF6 primarily functions at or near the cell periphery. Our previous analysis in fibroblast cells, however, indicates that a major fraction of cytoplasmic paxillin in fibroblasts is localized at a perinuclear region that largely overlaps with the Golgi marker Golgi 58K protein (Mazaki *et al.*, 1998) and the pre-Golgi coatomer protein β-COP (our unpublished results). On the other hand, cells such as epithelial cells exhibit a relatively diffuse distribution of paxillin in the cytoplasm (see Figure 3B). We have identified several paxillin-binding proteins bearing ARF GAP activities (PAGs), and found that one of them, PAG1, is active primarily toward ARF1 (our unpublished results). We have also obtained a result showing that both ARF1 and PAG1 activities are involved in the regulation of the perinuclear localization of paxillin and β-COP. Therefore, PAG3 is not the sole protein that binds to and regulates the subcellular localization of paxillin. Similarly, ARF6 is not the only ARF protein that regulates the subcellular localization of paxillin, because ARF1 may also play this role, as previously described (Norman *et al.*, 1998).

Turner *et al.* (1999) have reported a protein named p95PKL (paxillin-kinase linker) that binds to the LD4 domain of paxillin and mediates paxillin association with p21 GTPase-activated kinase (PAK) and the guanine nucleotide exchanger (PIX). p95PKL contains a zinc finger motif that is closely homologous to those in ARF1 GAP, Gcs1, and PAGs (see Figure 1). At present, it is not known whether p95PKL exhibits ARF GAP activity, but there are several differences between p95PKL and PAG3. For example, PAG3 contains a PH domain, and its GAP activity requires the presence of phospholipids such as phosphatidylinositol 4,5-bisphosphate (Andreev *et al.*, 1999). On the other hand, p95PKL does not possess a PH domain. PAK and PIX are involved in the cellular activities of Cdc42 and Rac1, which are important for cell polarity and migration (reviewed in Hall, 1998). Therefore, the data by Turner *et al.* (1999) seem to imply a possible mechanism of intracellular dynamics of paxillin and cell migratory activities in which ARF activities also play roles. Possible interaction of PAG3 with PAK and/or PIX should also be investigated.

ARF family proteins have been implicated in the regulation of membrane and vesicle traffic in mammalian cells. How are the membrane trafficking processes regulated by ARF activity related to the subcellular localization of paxillin, as well as cell adhesive and migratory activity? Paxillin and PAG3 are colocalized at several areas within a cell, but PAG3 is not observed in focal contacts of adhesion plaques. We also showed that overexpression of PAG3, but not its GAP mutant, inhibits paxillin recruitment to focal adhesion plaques. These results are consistent with the hypothesis that PAG3 may be involved in the intracellular transport of paxillin but is not one of the components of integrin assembly proteins. Punctate staining of PAG3 in the cytoplasm also suggests that PAG3 may associate with cytoplasmic vesicles. Because membrane traffic is primarily involved in the intracellular transport of membrane or secretory proteins, one simple explanation could be that cytoplasmic paxillin is already associated with a membrane protein inserted in transport vesicles. This membrane protein may be an integrin, as also suggested previously (Norman *et al.*, 1998). However, Miyamoto *et al.* (1995) have suggested that paxillin is recruited to the cytoplasmic regions of integrins only after the cell surface integrins are cross-linked and thus seems to be nonessential for the translocation of integrins to the plasma membrane. Alternatively, because PAG3 appears to associate directly with membranes through its PH domain (Andreev *et al.*, 1999), binding of paxillin with PAG3 may enable an association and/or interaction of paxillin with the membrane structures and may be thus involved in its putative intracellular transport along membrane trafficking pathways. Consistent with this, a recent model suggests that ARF GAP proteins localize on the budding vesicles (Springer *et al.*, 1999). Moreover, it is also possible that ARF activity regulates cytoskeletal organization more directly, besides its function on membrane transport, and may thereby be involved in the paxillin recruitment to focal contacts, as has also been suggested (Norman *et al.*, 1998).

PAG3 has multiple domains for protein–protein interaction, and our preliminary results indicate that PAG3 can bind stably with several unidentified proteins (our unpublished results), in addition to paxillin and Pyk2. For example, $p130^{Cas}$, when overexpressed, was also able to bind to PAG3 (our unpublished results). We showed that PAG3 acts on ARF6, and PAG3 overexpression causes inhibition of cell migration. We therefore tested whether the expression of dominant active form of ARF6 (ARF6 Q67L) could restore cell migratory activity in PAG3-transfected cells and found that this is not the case (our unpublished results). Thus, several unknown proteins seem to be involved in the physiological function of PAG3, and PAG3 may not act only on ARF6. Moreover, we have not obtained firm evidence showing that PAG3 is indeed involved in the dynamic process of the hypothesized intracellular transport of paxillin. We also do not know to what extent the inhibition of paxillin recruitment to focal contacts caused by the overexpression of PAG3 relates to the inhibition of cell migratory activities.

In conclusion, our present study, together with the previous study of Norman *et al.* (1998), strongly indicates that the subcellular distribution of paxillin and its focal contact recruitment are not due to free cytoplasmic diffusion but are under the control of activities of ARF family GTP-binding proteins. The binding of paxillin with PAG3, a protein with ARF GAP activity, implies that the intracellular transport of paxillin may simultaneously regulate its transport activity. Further analysis to determine the precise mechanism of PAG3 function will provide further insight not only into the regulation of paxillin subcellular localization but also into the regulation of cell migratory activity through the regulation of ARF activities.

ACKNOWLEDGMENTS

We are grateful to K. Nakayama for the generous gift of ARF cDNAs and useful discussions, T. Nagase for KIAA0400 cDNA, and T. Sasaki for the Pyk2 antibody. We also thank Mihoko Sato and Manami Hiraishi for technical assistance, Mayumi Yoneda for secretarial work, and Heidi Greulich for critical reading of the manuscript. We are also indebted to Yasuhiro Minami and Victor Hsu for valuable discussions and comments. This work was supported in part by the Japan Science and Technology Corporation, a grant in-aid from the Ministry of Education, Science, Sports and Culture of Japan, and grants from the Mitsubishi Foundation, the Takeda Medical Foundation, The Mochida Memorial Foundation for Medical and Pharmaceutical Research, and the Novartis Foundation for the Promotion of Science.

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