Actin filaments and microtubules are two major cytoskeletal systems involved in wide cellular processes, and the organizations of their filamentous networks are regulated by a large number of associated proteins. Recently, evidence has accumulated for the functional cooperation between the two filament systems via associated proteins. However, little is known about the interactions of the kinesis superfamily proteins, a class of microtubule-based motor proteins, with actin filaments. Here, we describe the identification and characterization of a novel kinesis-related protein named DdKin5 from Dictyostelium. DdKin5 consists of an N-terminal conserved motor domain, a central stalk region, and a C-terminal tail domain. The motor domain showed binding to microtubules in an ATP-dependent manner that is characteristic of kinesis-related proteins. We found that the C-terminal tail domain directly interacts with actin filaments and bundles them in vitro. Immunofluorescence studies showed that DdKin5 is specifically enriched at the actin-rich surface protrusions in cells. Overexpression of the DdKin5 protein affected the organization of actin filaments in cells. We propose that a kinesis-related protein, DdKin5, is a novel actin-bundling protein and a potential cross-linker of actin filaments and microtubules associated with specific actin-based structures in Dictyostelium.

Actin filaments and microtubules are two major cytoskeletal systems involved in a wide variety of cellular processes, including intracellular transport, cell division, cell migration, and cellular morphogenesis. These functional multiplicities result from changes in the assembly and organization of filamentous networks that are regulated by a large number of associated proteins, including cross-linking, severing, capping, and motor proteins. The kinesis superfamily is a class of microtubule-based motor proteins defined by a conserved motor domain responsible for ATP hydrolysis and microtubule binding. Since the first identification of kinesis (1), many related proteins (kinesis-related proteins, KRPs) have been identified from a wide range of eukaryotic cells and shown to be involved in cell division and intracellular transports of various cargoes, including vesicles, organelles, large protein complexes, and cytoskeletal filaments (reviewed in Refs. 2 and 3).

The cellular slime mold, Dictyostelium discoideum, is a useful model system to study cytoskeletons and motor proteins. It is suitable for biochemical and genetic approaches, including gene disruption and overexpression. Moreover, it shows a wide range of cellular processes, such as binary fission, intracellular transport, endocytosis, and chemotaxis. A number of studies have been made on the actin cytoskeleton and its regulation by associated proteins in Dictyostelium (reviewed in Refs. 4 and 5). In addition, some KRPs were recently identified as motors associated with membranous structures in Dictyostelium. One of them, K7, was shown to be involved in the development of Dictyostelium cells (6). DdUnc104, the closest relative of Unc104/KIF1 KRPs in Dictyostelium, was purified using an organelle transport assay and shown to be involved in membrane transport in cells (7).

Recently, evidence has accumulated for the functional cooperation between the two filament systems during various cellular processes (reviewed in Ref. 8). In addition, some KRPs have been shown to interact with actin filaments physically or functionally. In melanophores, pigment granules are transported along both microtubules and actin filaments by a combination of kinesis II, cytoplasmic dynein, and an actin-based motor protein, myosin V (9–12). Moreover, biochemical studies demonstrated the direct interaction between conventional kinesis and myosin V (13). In migrating cells, conventional kinesis is required for focal delivery of a factor that promotes focal adhesion disassembly (14). In cytokinesis, the proper placement of a cleavage furrow requires interactions between cortical actin filaments and spindle asters microtubules (8). The CH01 isoform of the mitotic kinesis-like protein 1 subfamily, which is required for the completion of cytokinesis, has been shown to interact directly with actin filaments via the middle of the tail (15). Nevertheless, as yet, little is known about the interactions between KRPs and actin filaments.

In this study, we describe the identification and characterization of a Dictyostelium KRP designated as DdKin5 (Dictyostelium discoideum kinesis 5). We found that the C-terminal tail domain of DdKin5 directly interacts with actin filaments and bundles them in vitro. The intracellular localization of DdKin5 suggested the association of DdKin5 with actin in vivo. Analyses of Dictyostelium cells overexpressing DdKin5 suggested the involvement of DdKin5 in the organization of actin cytoskeletons. We propose that DdKin5 is a novel actin-bundling protein and a potential cross-linker of actin filaments and microtubules in Dictyostelium.

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The abbreviations used are: KRP, kinesis-related protein; DTT, dithiothreitol; PBS, phosphate-buffered saline; GFP, green fluorescent protein; HRP, horseradish peroxidase; PBST, phosphate-buffered saline containing 0.05% Tween 20; BSA, bovine serum albumin; TRITC, tetramethylrhodamine isothiocyanate; GST, glutathione S-transferase.
**EXPERIMENTAL PROCEDURES**

**Strains, Culture, Development, and Transformation of Dictyostelium Cells**—The axenic strain Ax2 and the parental strain for the gene disruption mutant, Ax2, and mutants were cultivated in an HL5 nutrient medium (17) supplemented with penicillin and streptomycin either on Petri dishes or in a shaken suspension at 22 °C. For development, cells were seeded on DM agar plates (17) covered with *Escherichia coli* Br lawn and incubated at 22 °C. Transformation was performed by electroporation according to Howard et al. (18).

**Cloning of DdKin5 cDNAs**—Partial genomic DNA sequences encoding a novel KRP (referred as DdKin5) were obtained from the Genome Sequencing Centre Jena in the Dictyostelium genome project. Genomic DNA clones carrying the entire *DdKin5* gene were obtained by inverse PCR from genomic DNAs that were digested with EcoT22I and NdeI and circularized with ligase (Fig. 8A, EcoT22I and NdeI fragments). The 3' end of the *DdKin5* gene was confirmed by 3'-rapid amplification of the cDNA end using the total RNA extracted from vegetative Ax2 cells as a template. cDNA clones encoding the full-length DdKin5 protein were obtained by reverse transcription-PCR using the total RNA as a template. Several clones were sequenced for all the PCR products. While cDNA end using the total RNA extracted from vegetative Ax2 cells as a template, cDNA clones encoding the full-length DdKin5 protein were obtained by reverse transcription-PCR using the total RNA as a template. Several clones were sequenced for all the PCR products.

**Antibodies**—DNA encoding the fragment of DdKin5 (residues 342–583) was amplified by PCR from the *DdKin5* cDNA and cloned into pET-15b His tag expression vector (Novagen). *E. coli* BL21(DE3) (Novagen) cells transformed with the vector were grown at 37 °C to *A*_{600} = 0.6, at which point protein expression was induced by the addition of 10 μM isopropyl-1-thio-galactopyranoside. Following further shaking for 13 h at 22 °C, the cells were harvested and resuspended in LB (50 mM NaH_{2}PO_{4}, pH 8.0, 100 mM NaCl, 0.5 mM EDTA, 1 mM β-mercaptoethanol) containing 1 mM phenylmethylsulfonyl fluoride. Following sonication, lysates were centrifuged at 25,000 × *g* for 20 min. The pellet was washed twice with LB containing 0.5% Triton X-100 and extracted with an extraction buffer (8 M urea, 50 mM NaH_{2}PO_{4}, pH 8.0, 500 mM NaCl, 0.5 mM EDTA, 10 mM DTT) at 37 °C for 1 h. After centrifugation, the solubilized extract was successively dialyzed against LB containing 4 M urea for 1 h, LB containing 2 M urea for 1 h, LB for 1 h, and LB overnight. After centrifugation, the protein was mixed with nickel-nitrilotriacetic acid-agarose (Qiagen) for 1 h at 4 °C and eluted with LB containing 250 mM imidazole. The eluted protein was dialyzed against PBS and used for immunization of a rabbit to raise antiserum against the protein (Asahi Technoglass).

**For Western blotting, gels were transferred to Immobilon polyvinylidene difluoride membranes (Millipore) by electrophoresis. Blots were blocked for 30 min with 5% skim milk (Difco) in Tris-buffered saline containing 0.05% Tween 20 and incubated with crude anti-DdKin5 antisera diluted 1:10,000 in 10 mM NaH_{2}PO_{4}, pH 8.0, 0.1 M NaCl, 0.5% Triton X-100, 5% glycerol, and 0.05% NaN_{3} at 4 °C overnight. The membrane was washed with 0.05% Tween 20, 1% BSA in PBS, and incubated with a horseradish peroxidase-conjugated secondary antibody. After washing twice with 3% BSA in Tris-buffered saline containing 0.05% Tween 20, the membrane was exposed to autoradiography film.**

**Immunofluorescence Microscopy**—To reduce background labeling in immunofluorescence studies, the anti-DdKin5 antisera was preadsorbed with fixed Dicti5-n5 cells according to Fukui et al. (23) with some modifications. Briefly, 1.5 × 10^{6} Dicti5-n5 cells were harvested, fixed with methanol at −20 °C for 5 min, and washed three times with PBS. Washed cells were divided into three fractions, one of which was resuspended with 500 μl of the anti-DdKin5 antisera diluted 1:3000 in PBST. The mixture was incubated for 1 h at room temperature with agitation and centrifuged to remove fixed cells. After two more repetitions of the incubation, the mixture was supplemented with 3% BSA and fixed with methanol at −20 °C for 5 min and washed three times in PBS. After being blocked with 3% BSA in PBST, cells were incubated with the preadsorbed anti-DdKin5 antisera for 1 h at room temperature. After being washed in PBS three times, cells were incubated with Alexa 488-conjugated goat anti-rabbit antibody (Molecular Probes) diluted 1:4000 in PBST containing 3% BSA and 2 ng/ml TRITC-conjugated phallolidin (Sigma) for 1 h at room temperature. Cells were washed four times in PBS and observed as described previously (24).

Chromatography was performed by high-pressure liquid chromatography (HPLC) on a TSK-GEL for 20 min and eluted with 0.1 M ammonium acetate in 50% methanol at 37 °C. The column was eluted with a linear gradient of 0–20% methanol in 5 min and collected into 2–3 × 10^{6} cells/ml. Twenty-five μl of a solution was spotted onto ethanol-washed glass coverslips and incubated at 22 °C until the cells started spreading, at which point they were overlaid with thin agarose sheets (23) and fixed in ethanol con- taining 2% formaldehyde for 15 °C for 5 min. Spreading was observed as described for vegetative cells, except that agarose sheets were peeled off and cells were washed once before the incubation with the

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3. Iwai and K. Sutoh, unpublished observation.
antiserum and that coverslips were finally mounted using 90% glycerol in 0.17 M potassium/sodium phosphate buffer (pH 6.3), and resuspended at a density of 1.6 × 10^9 cells/ml in a 2× cytoskeleton buffer (20 mM PIPES-KOH, pH 7.0, 30 mM KCl, 4 mM MgCl₂, 2 mM DTT) containing a complete protease inhibitor mixture without EDTA (Roche Applied Science). Cells were divided into two fractions, one fraction was used lysed by addition of an equal volume of 1% Triton X-100 containing 10 mM EGTA or 0.2 mM CaCl₂ at room temperature. Lysates were agitated and immediately centrifuged for 30 s at full speed in a microcentrifuge. The supernatants were collected, and the pellets were resuspended in an equivalent volume of a 1× cytoskeleton buffer. They were mixed with an equal volume of a 2× SDS-PAGE sample buffer and boiled for 5 min. Proteins were resolved by 17 mM potassium/sodium phosphate buffer (pH 6.3), and resuspended in an actin-binding buffer containing 50 mM KCl and 50 mM EGTA or 0.2 mM CaCl₂ at room temperature. Lysates were agitated and immediately centrifuged for 1 h at 4°C. The resin was precipitated again, and bound protein was eluted with an elution buffer (50 mM imidazole and 1 mM DTT) for 2 h at room temperature, mixtures were centrifuged at 10,000 × g for 20 min. The supernatants were collected, and the pellets were resuspended in an equal volume of the actin-binding buffer. For coedestion assays of His-ST with F-actin were performed as described previously (27) with slight modifications. Briefly, various concentrations of GST-T (0.5–5 µM) were mixed with 2 µM F-actin in an actin-binding buffer containing 0.2 mg/ml BSA and various concentrations of KC(10–200 mM). After incubation for 30 min at room temperature, mixtures were centrifuged at 160,000 × g for 20 min. The supernatants were collected, and the pellets were resuspended in an equal volume of the actin-binding buffer. For coedestion assays of His-ST with F-actin at low speed, 5 µM His-ST was mixed with 5 µM G-actin in an actin-binding buffer containing 50 mM KCl. After incubation for 2 h at room temperature, mixtures were centrifuged at 10,000 × g for 20 min. The supernatants were collected, and the pellets were resuspended in an equivalent volume of the actin-binding buffer. All samples were mixed with an equal volume of a 2× SDS-PAGE sample buffer and boiled for 5 min. They were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue for densitometry.

FIG. 1. Deduceds amino acid sequence of DdKin5. DdKin5 consists of 990 amino acids residues with a molecular mass of 110 kDa. Putative α-helical coiled-coil regions predicted by the Paircoil program (61) are underlined. Putative leucine zipper motifs are double underlined. The basic C-terminal tail region is a dashed underlined. The nucleotide sequence data base has been submitted to the GenBank™ nucleotide sequence data base with accession number AB102778.

tone powder of rabbit skeletal muscle and purified as described previously (26). Coedestion assays of GST-T with F-actin were performed as described previously (27) with slight modifications. Briefly, various concentrations of GST-T (0.5–8 µM) were mixed with 2 µM F-actin in an actin-binding buffer containing 0.2 mg/ml BSA and 10 mM KCl. Otherwise, 4 µM GST-T was mixed with 4 µM F-actin in an actin-binding buffer containing 0.2 mg/ml BSA and various concentrations of KC (10–200 mM). After incubation for 30 min at room temperature, mixtures were centrifuged at 160,000 × g for 20 min. The supernatants were collected, and the pellets were resuspended in an equal volume of the actin-binding buffer. For coedestion assays of His-ST with F-actin at low speed, 5 µM His-ST was mixed with 5 µM G-actin in an actin-binding buffer containing 50 mM KCl. After incubation for 2 h at room temperature, mixtures were centrifuged at 160,000 × g for 20 min. The supernatants were collected, and the pellets were resuspended in an equivalent volume of the actin-binding buffer. All samples were mixed with an equal volume of a 2× SDS-PAGE sample buffer and boiled for 5 min. They were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue for densitometry.

RESULTS

Identification of DdKin5 as a Kinesin-related Protein

We cloned and determined the full sequence of the Ddkin5 gene from D. discoideum. Fig. 1 shows the deduced amino acid sequence of DdKin5. Sequence analysis showed that the DdKin5 gene encodes a protein with a molecular mass of 110 kDa that consists of an N-terminal kinesin motor domain, a central stalk region, and a C-terminal tail region. The motor domain of DdKin5 showed the highest degree of homology with members of conventional kinesins, sharing 53% identity with Neurospora kinesin (28) and 52% identity with human kinesin heavy chain (29). However, outside the motor domain, DdKin5 did not show significant homology to conventional kinesins, other KRP’s, or any other proteins in the data base. The central stalk region was predicted to contain four α-helical coiled-coil regions (Fig. 1, underlined). Two of them were predicted to contain leucine zippers (Fig. 1, double underlined) corresponding to short coiled-coils (30). Because the coiled-coil stalk region in the Drosophila kinesin heavy chain is important for the dimerization of the protein (31), it is likely that these multiple coiled-coils and leucine zippers also facilitate the dimerization of DdKin5. The nonhelical region between putative coiled-coil

Expression of Proteins in Dictyostelium—For the expression of GFP-tagged proteins in Dictyostelium cells, DNAs encoding the full-length DdKin5 (residues 1–990) or truncated fragments (residues 1–949, 950–990, or 805–990) were amplified by PCR from DdKin5 cDNA clones and ligated with the coding sequence of the GFP S65T mutant (19) at the 3′ end. GFP and DdKin5 or its fragments were separated by the linker GPGKDP, which had resulted from the cloning procedures. They were cloned into an extrachromosomal vector pBIG (20) to express GFP-tagged proteins under the control of the actin-15 promoter. For the expression of FLAG-tagged proteins, DdKin5 was encoding the full-length DdKin5 and a truncated fragment (residues 342–990) were amplified by PCR and ligated with the FLAG-coding sequence at their 5′ ends. They were cloned into pBIG to express FLAG-tagged proteins under the control of the actin-15 promoter. Ddkin5-null cells were used for transformation with all these vectors. Transformsants were selected and maintained at 6 μg/ml G418, except for two of GFP-tagged fragments (residues 950–990 and 805–950) selected at 10 μg/ml G418. Cells were observed as described in the section “Immunofluorescence Microscopy,” except that they were stained with 5 or 10 ng/ml TRITC-conjugated phalloidin for 30 min at 37°C. For counting the nuclei per cell, cells were adhered onto glass coverslips for 5 min (for cells grown in suspension) or 1 h (for cells grown on a surface) and strongly compressed by thin agarose sheets (23). They were fixed with methanol at −15°C for 5 min and stained with 50 ng/ml 4′,6-diamidino-2-phenylindole for 30 min at 37°C. Nuclei in >300 cells grown on a surface or >100 cells grown in suspension were counted for each cell line. Differences between Axx2 and other mutants were analyzed by the Mann-Whitney test.

Expression of Proteins in E. coli and Purification—For the expression of the GST-tagged DdKin5 tail domain (GST-T), DNA encoding the DdKin5 tail domain (residues 950–990) amplified as above was cloned into the pGEX-4T-3 GST tag expression vector (Pharmacia). For the expression of the His-tagged DdKin5 tail domain with a coiled-coil (His-ST), DNA encoding the DdKin5 tail domain with the coiled-coil (residues 805–990) amplified as above was cloned into the pET-15b His tag expression vector (Novagen). E. coli BL21(DE3) (Novagen) cells transformed with these vectors were grown at 37°C to an A₆₀₀ = 1, at which point the protein expression was induced by the addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. After further shaking for 3 h at 22°C, cells were harvested and resuspended in lysis buffer (50 mM Tris-HCl or NaH₂PO₄, NaOH, pH 7.5, 300 mM NaCl, 1 mM EDTA) containing 5 mM DTT and 1 mM phenylmethylsulfonyl fluoride. Following sonication, lysates were centrifuged at 25,000 × g for 20 min. For the purification of GST-T, the supernatant was mixed with glutathione-Sepharose 4B (Pharmacia) for 1 h at 4°C. The resin was precipitated and washed twice with the lysis buffer. The resin was precipitated again, and bound protein was eluted with an elution buffer (50 mM Tris-HCl, 200 mM NaCl, 1 mM DTT). Eluted proteins were dialyzed against an elution buffers (10 mM PIPES-KOH, pH 7.0, 2 mM MgCl₂, 1 mM EDTA, 0.5 mM DTT) and finally centrifuged at 200,000 × g for 30 min to remove aggregates. The concentrations of purified proteins were determined by Coomassie protein assay reagent (Pierce) using BSA as a standard.

F-actin Coedestion Assays—Actin was extracted from the ace-
regions from residues 733 to 780 is rich in asparagine and regions from residues 733 to 780 is rich in asparagine and serine residues. Although the significance of the amino acid clusters often found in Dictyostelium is not well known (32), a serine-rich region was recently found to mediate membrane fusion in Dictyostelium cells (33). The C-terminal tail region is rich in basic residues (Fig. 1, dashed underline; calculated pl = 11.57), implying some interactions with negatively charged structures. Based on the homology in the motor domain and the whole structural organization, we placed DdKin5 as a far distant relative of conventional kinesins. However, the lack of homology outside the motor domain implied the involvement of DdKin5 in novel cellular functions different from those of other members of conventional kinesins.

The motor domains of many KRPs show ATP-dependent binding to microtubules responsible for microtubule-based motile activities (1). To characterize the microtubule binding properties of the motor domain of DdKin5, we first expressed the motor domain in E. coli cells but found that most of the expressed protein was insoluble or inactive (data not shown). Therefore, we expressed the first 542 residues of DdKin5 as a fusion with GFP in Dictyostelium cells (Fig. 5A, MS-GFP). The protein included the motor domain and a part of the putative coiled-coil regions with GFP at the C terminus, but not the tail domain, to exclude the possibility that the tail domain may have inhibitory effects on the activities of the motor domain (34). Extracts of cells expressing MS-GFP were incubated with microtubules in the presence of 2 mM AMPPNP and centrifuged to precipitate microtubules with bound MS-GFP. About 70% of MS-GFP was found in the microtubule pellet (Fig. 2, +MT), whereas all the MS-GFP remained in the supernatant in the absence of microtubules (Fig. 2, −MT). The microtubule pellet was resuspended to release MS-GFP and centrifuged. About half of MS-GFP was released from microtubules in the presence of 10 mM ATP (Fig. 2, +ATP). About 10% was released in the absence of nucleotide (Fig. 2, −nucleotide), possibly because of the higher salt concentration in the buffer used for the release. However, the amount of the released protein in the absence of nucleotide was certainly smaller than that in the presence of ATP, suggesting the dependence of the release on ATP. These experiments indicate that the motor domain of DdKin5 binds to microtubules in an ATP-dependent manner, which is characteristic of KRPs. Although we performed the microtubule gliding assay using the expressed protein to determine the motor activity of DdKin5, we could not observe the gliding of microtubules, possibly because of the low yields of the protein.

Intracellular Localization of DdKin5 and Its Fragments—To determine the intracellular localization of DdKin5, antisera was raised against a bacterially expressed DdKin5 fragment and preadsorbed with methanol-fixed Ddkin5-null cells (described below) to reduce the background reactivity. Immunofluorescence microscopy using the anti-DdKin5 antisera in vegetative cells showed that DdKin5 was specifically enriched at actin-rich cell surface protrusions such as pseudopodia and crowns and also weakly distributed throughout the cytoplasm (Fig. 3A). When starved, Dictyostelium cells undergo chemotactic migration toward extracellular chemotactant eAMP, becoming highly polarized and exhibiting actin assembly primarily in the pseudopods at their leading edges (35). In chemotaxing cells, DdKin5 was specifically enriched at the actin-rich leading edges and also weakly distributed throughout the cytoplasm (Fig. 3B). Fluorescence was almost absent in a negative control, Ddkin5-null cells (Fig. 3C).

To support the finding that DdKin5 associates with actin filaments in Dictyostelium cells, the cells were lysed with Triton X-100 in the absence or presence of Ca\(^{2+}\) and fractionated by centrifugation at low speed. In the absence of Ca\(^{2+}\), a ~110-kDa protein with a predicted molecular mass of DdKin5 was detected with the anti-DdKin5 antisem in both the Triton-insoluble cytoskeletal fraction and the Triton-soluble fraction (Fig. 4, −Ca\(^{2+}\); DdKin5 was not partitioned into the Triton-insoluble fraction in the presence of Ca\(^{2+}\) (Fig. 4, +Ca\(^{2+}\)); under these conditions, actin filaments were severed by Ca\(^{2+}\)-dependent actin-severing proteins (36) and almost absent in the low-speed pellet fraction (Ref. 37 and Fig. 4), whereas microtubules were resistant to Ca\(^{2+}\), as previously described (38), and still precipitated as the Triton-insoluble fraction (Fig. 4). These results, together with the colocalization of DdKin5 with actin, indicate that DdKin5 associates with actin filaments and also resides as a cytoplasmic pool in cells. No associations of DdKin5 with microtubules in cells were observed in these studies. Similarly, conventional kinesin was localized to membranous structures but not to microtubules in immunofluorescence studies (39), and the majority of the protein was recovered in the soluble fraction after cell lysis (40).

Then, we expressed GFP-tagged DdKin5 and a series of truncated fragments in Dictyostelium cells to identify the domain responsible for the association of DdKin5 with actin filaments (Fig. 5A). The proteins were expressed in the Ddkin5-null background to rule out any heterodimer formation with endogenous DdKin5. The full-length DdKin5 tagged with GFP at the N terminus (Fig. 5A, GFP-Ddkin5) showed a similar localization pattern to that of endogenous DdKin5 (Fig. 5B), indicating that the GFP tag at the N terminus rarely affected the intracellular localization of DdKin5. We found that truncation of the C-terminal 41 amino acid residues from GFP-Ddkin5 (Fig. 5A, GFP-ΔΔ) resulted in a diffuse distribution throughout the cytoplasm (Fig. 5C), suggesting that the C-terminal tail domain is necessary for the association of DdKin5 with actin filaments. The C-terminal tail domain tagged with GFP (Fig. 5A, GFP-T) was distributed throughout the cytoplasm and also specifically colocalized with actin (Fig. 5D), indicating that only 40 C-terminal amino acid residues are sufficient for the association of DdKin5 with actin filaments. The tail fragment consisting of the N-terminal GFP tag, one of the putative coiled-coil regions, and the C-terminal 40 residues (Fig. 5A, GFP-ST) was often aggregated into compact clusters with actin (Fig. 5E). Cells overexpressing GFP-ST showed an increase in the proportion of multinucleate cells when they were grown in shaken suspension culture (Fig. 5G), presumably because GFP-ST accumulated with actin in the cleavage furrow to obstruct the furrow ingression during cytokinesis (Fig. 5F).
Characterization of the Actin Binding Properties of the DdKin5 Tail Domain—The above results led us to conclude that the C-terminal tail domain is necessary and sufficient for the association of DdKin5 with actin filaments in cells. Given the existence of basic residues in the tail domain, it is possible that the domain directly interacts with the negatively charged actin molecule (41). To investigate the direct interactions of the tail domain with actin filaments in vitro, we expressed the tail domain tagged with glutathione S-transferase (GST) at the N terminus in E. coli (Fig. 5A, GST-T) and purified it to homogeneity to perform cosedimentation assays. GST alone showed no significant association with actin, as previously described (27). About 70% of 4 μM GST-T was found in the precipitation with actin filaments in the presence of 4 μM actin at a low salt concentration (Fig. 6A, +actin), whereas all the GST-T remained in the supernatant in the absence of actin (Fig. 6A, −actin). Using a constant final concentration of actin (2 μM) and varying concentrations of GST-T, the fractional saturation was plotted against the concentration of free GST-T to determine the dissociation constant (Fig. 6B). The mean $K_d$ was measured to be 0.58 μM under the low-salt condition. In addition, the binding of GST-T to actin filaments was sensitive to the concentration of salt (Fig. 6C). These experiments indicate that the tail domain of DdKin5 directly interacts with actin, primarily by electrostatic interactions.

Many actin cross-linking proteins direct cross-linking or bundling of actin filaments by utilizing two discrete actin-binding sites (42). The tail domain, the actin-binding site in DdKin5, is located adjacent to putative coiled-coil regions and possibly becomes two discrete actin-binding sites when the protein forms a dimer. Furthermore, the tagged tail domain including putative coiled-coil regions often aggregated into clusters with actin in Dictyostelium cells, as described above. These findings prompted us to investigate whether DdKin5 bundles or otherwise cross-links actin filaments. We first expressed the tail domain including coiled-coil regions as a six histidine-tagged protein in E. coli (Fig. 5A, His-ST) and purified it to homogeneity to perform cosedimentation assays at low speed. GST was not used as a tag in these studies to exclude artificial dimer formation of the protein via GST moiety (43). After incubation with actin filaments under a medium salt condition (50 mM KCl), His-ST was subjected to low-speed centrifugation to precipitate the cross-linked filaments. Most of His-ST and actin were found in the precipitation (Fig. 7A, +actin and +His-ST), implying that His-ST cross-linked actin filaments. On the other hand, actin or His-ST alone remained F-actin. The corresponding overlaid (DdKin5, green; F-actin, red; colocalization, yellow) and phase-contrast images are also shown for A and B. Bars, 10 μm.
Disruption of the Ddkin5 Gene and Overexpression of the Ddkin5 Protein—To examine the physiological functions of Ddkin5 in Dictyostelium cells, the Ddkin5 gene was disrupted using homologous recombination to generate the Ddkin5-null strain (Fig. 8A). By PCR, two independent clones were identified to have the homologous recombination event. They lost the expression of Ddkin5, as revealed by Western blot analysis with the anti-Ddkin5 antiserum (Fig. 8B). The Ddkin5-null cells showed normal morphology at the vegetative stage and grew normally in an axenic suspension culture (Fig. 8C). Furthermore, they showed normal pinocytosis, phagocytosis, exocytosis, and contractile vacuole functions under hypo-osmotic conditions, and cell-cell adhesions (data not shown). In the course of development upon starvation, these Ddkin5-null cells streamed to aggregate normally and formed fruiting bodies comparable with those of wild-type, Ax2 cells (Fig. 8D). These results suggest that the Ddkin5 gene is not essential for the normal growth and development of Dictyostelium cells under laboratory conditions.

To further elucidate the cellular functions of Ddkin5, N-terminal epitope-tagged Ddkin5 (Fig. 5A, FLAG-Ddkin5) or its truncated mutant lacking the motor domain (Fig. 5A, FLAG-ΔM) were overexpressed in Dictyostelium cells under control of the constitutively active actin-15 promoter. In these studies, the proteins were expressed in a Ddkin5-null background to exclude any heterodimer formation with endogenous Ddkin5. Transformed cells were cultured on a plastic surface at a low level of antibiotic G418 and used within 3 weeks, because these cells exhibited a reduced expression of the proteins otherwise. Cells overexpressing the mutant protein that lacks the motor domain (ΔM0) often showed large lamellipodia and membrane ruffles enriched with actin filaments (Fig. 9A, ΔM0), whereas Ddkin5-null cells showed a normal distribution of actin filaments with actin-rich cell surface protrusions (Fig. 9A, Null) comparable with those of wild-type, Ax2 cells (Fig. 9A, Ax2). Cells overexpressing full-length Ddkin5 (Ddkin5+) were often larger than other types of cells. They showed not only large lamellipodia and membrane ruffles observed in ΔM0 cells but also multiple crowns enriched with actin filaments along the periphery of cells (Fig. 9A, Ddkin5+). Just like cells overexpressing GFP-ST, Ddkin5+ and ΔM0 cells also showed defects in cytokinesis when grown in shaken suspension, presumably because of the accumulation of actin in the cleavage furrow

Ddkin5. The putative coiled-coil regions are shown by hatched areas, and the tail domain, by the black area. The lower diagrams are all constructs used in this study. GFP- and FLAG-tagged proteins were expressed in Dictyostelium. GST- and His-tagged proteins were expressed in E. coli. B, localization of GFP-Ddkin5, the GFP-tagged full-length Ddkin5. C, localization of GFP-ΔT, the GFP-tagged truncated mutant lacking the tail domain. D, localization of GFP-T, the GFP-tagged tail domain. E, localization of GFP-ST, the GFP-tagged tail domain with a part of the stalk. F, localization of GFP-St during cytokinesis. Two daughter cells are migrating toward the upper right and the lower left of the panel and accumulating GFP-ST with actin in the cleavage furrow at the center of the panel. All cells were stained for F-actin, and the corresponding overlay (GFP, green; F-actin, red; colocalization, yellow) images are also shown. Bars, 10 μm. G, numbers of nuclei in wild-type Ax2 cells (white bars) and cells overexpressing GFP-ST (gray bars) were grown in shaken suspension. The difference between two cell lines was significant (p < 0.01).
Some Dictyostelium mutant cells such as myosin II-null show defects in cytokinesis in shaken suspension but not in contact with surfaces, a milder condition for growth (44, 45). It must be noted that, even under this milder condition, DdKin5 cells showed a slight impairment of cytokinesis, whereas /H9004 cells showed normal cytokinesis (Fig. 9B). These results suggest that overexpression of DdKin5 or its deletion mutant affects the organization of actin cytoskeletons, and cells overexpressing full-length DdKin5 show severer defects than those overexpressing the mutant that lacks the motor domain.

**DISCUSSION**

We have identified and characterized a KRP, DdKin5, from *D. discoideum*. The C-terminal tail domain of DdKin5 showed direct interaction with actin filaments in vitro primarily by electrostatic forces, and this tail domain including putative coiled-coil regions could bundle the filaments in vitro under the medium salt condition (50 mM KCl). Given the cellular concentration of actin filaments (70 μM; Ref. 46), the measured $K_d$ of the tail-actin interaction, and the ionic strength of cytosol in Dictyostelium (40–80 mM; Ref. 47), it is physiologically relevant that DdKin5 binds to and bundles the filaments in vivo. Immunofluorescence and cell fractionation studies suggested the association of DdKin5 with actin filaments in cells. In Dictyostelium, it has been reported that the expression of the C-terminal fragment of the 34-kDa actin-bundling protein lacking the inhibitory domain induces the formation of paracrystalline actin inclusions that resemble Hirano bodies (48). Similarly, the tail domain of DdKin5 including the stalk region with the GFP tag often aggregated into clusters with actin when overexpressed in cells, suggesting the actin-bundling activity of the domain in vivo. Outside the kinesin motor domain, DdKin5 did not show significant homology to any other proteins, including actin-bundling proteins ever found; therefore, these results indicate that DdKin5 is a novel actin-bundling protein.
In *Dictyostelium*, more than 10 actin cross-linking proteins have been identified and shown to localize to various actin-based structures, including the cell cortex, the cleavage furrow, pseudopodia, filopodia, crowns, and the phagocytic cup (5), suggesting their various functions in cells. One of them, ABP-120, is localized to the cell cortex and incorporated into newly formed protrusions following chemotactic stimulation to cross-link actin filaments into meshwork in the protrusions (49–51).

Immunofluorescence studies indicated that DdKin5 is specifically enriched at the actin-rich cellular protrusions in both vegetative and chemotaxing cells. It is likely that DdKin5 also participates in the bundling of actin filaments in such actin-based structures in *Dictyostelium* cells. Overexpression of DdKin5 caused abnormal organization of actin, suggesting the involvement of DdKin5 in the organization of actin cytoskeletons. Actin cross-linking proteins in *Dictyostelium* cells share both unique and overlapping functions in growth and development, as shown by phenotypic analyses of single and double mutants (52). Disruption of the *Ddkin5* gene did not affect cellular functions in growth and development, possibly because DdKin5 also shares overlapping functions with other actin cross-linking proteins.

Many KRP's have been shown to drive microtubule-based motility using a variety of *in vitro* motility assays (1). Because the motor domain of DdKin5 expressed in *E. coli* cells was rarely soluble or active, we expressed the domain in *Dictyostelium* cells. The expressed fragment in cell extracts bound to microtubules in the presence of AMPPNP and was released from microtubules in the presence of ATP. Although we did not observe the gliding of microtubules on the expressed fragment, possibly because of the low yields of the protein, the motor domain at least interacted with microtubules in an ATP-de-
pendent manner, like those of other KRP s. Given the actin-bundling activity of the tail domain, DdKin5 is likely to be a factor that connects microtubules to actin filaments physically or functionally in Dictyostelium cells.

Cells overexpressing the full-length DdKin5 showed severer defects in organization of actin and cytokinesis than those overexpressing the mutant that lacks the motor domain. Although the direct participation of DdKin5 in cytokinesis remains to be clarified, our result rather suggests that the motor domain of DdKin5 may play some roles in the organization of actin cytoskeletons, possibly by connecting microtubules to actin filaments. In Dictyostelium cells overexpressing the motor domain of cytoplasmic dynein, microtubule arrays were found to collapse and become highly motile, suggesting a role of dynein as a cortical anchor for microtubules (53, 54). Moreover, some plus-end-directed motile activities were observed to have an effect on microtubules at the cell cortex in these cells. Considering that conventional kinesins are plus-end-directed motors (55), DdKin5 could associate with cortical actin filaments via an ATP-dependent manner, suggesting that DdKin5 may connect microtubules to actin filaments. Because DdKin5 associates with actin filaments and bundles them, DdKin5 remains to be clarified, our result rather suggests that the motor domain of DdKin5 may play some roles in the organization of actin cytoskeletons, possibly by connecting microtubules to actin filaments. In Dictyostelium cells overexpressing the full-length DdKin5, actin bundles were found to translocate along the microtubules in vitro (60). DdKin5 could directly bundle and transport actin filaments to the cell cortex to organize actin-based structures in highly motile cells such as Dictyostelium cells.

In summary, we identified a novel KRP, DdKin5, from Dictyostelium cells. The C-terminal tail domain of DdKin5 directly interacts with actin filaments and bundles them in vitro, indicating that DdKin5 is a novel actin-bundling protein. The motor domain of DdKin5 shows binding to microtubules in an ATP-dependent manner, suggesting that DdKin5 may connect microtubules to actin filaments. Because DdKin5 associates with actin-based structures in cells, it is likely that DdKin5 is involved in the organization of actin cytoskeletons in such structures in Dictyostelium cells.

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Addendum—We found that Klopfenstein et al. (62) referred to DdKin5 as a conventional kinesin with unknown functions and termed the protein DdKHC2 in their review.
A Novel Actin-bundling Kinesin-related Protein from *Dictyostelium discoideum*
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