Villidin, a Novel WD-repeat and Villin-related Protein from *Dictyostelium*, Is Associated with Membranes and the Cytoskeleton

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Villidin is a novel multidomain protein (190 kDa) from *Dictyostelium* amoebae containing WD repeats at its N-terminus, three PH domains in the middle of the molecule, and five gelsolin-like segments at the C-terminus, followed by a villin-like headpiece. Villidin mRNA and protein are present in low amounts during growth and early aggregation, but increase during development and reach their highest levels at the tipped mound stage. The protein is present in the cytosol as well as in the cytoskeletal and membrane fractions. GFP-tagged full-length villidin exhibits a similar distribution as native villidin, including a distinct colocalization with Golgi structures. Interestingly, GFP fusions with the gelsolin/villin-like region are uniformly dispersed in the cytoplasm, whereas GFP fusions of the N-terminal WD repeats codistribute with F-actin and are associated with the Triton-insoluble cytoskeleton. Strains lacking villidin because of targeted deletion of its gene grow normally and can develop into fruiting bodies. However, cell motility is reduced during aggregation and phototaxis is impaired in the mutant strains. We conclude that villidin harbors a major F-actin binding site in the N-terminal domain and not in the villin-like region as expected; association of villidin with vesicular membranes suggests that the protein functions as a linker between membranes and the actin cytoskeleton.

INTRODUCTION

The actin-binding protein villin is an essential component of microvilli in intestinal epithelial cells (Bretscher and Weber, 1980). Its overall domain structure classifies the protein as a member of the gelsolin family, which is characterized by six highly homologous segments that exhibit extensive intramolecular sequence similarities (Eichinger *et al.*, 1991; Sun *et al.*, 1999). The distinct feature of villin is the additional actin-bundling activity conferred by the 8.5-kDa C-terminal headpiece with an intrinsic stretch of basic amino acid residues (Friederich *et al.*, 1992). A mutant mouse strain lacking villin has no overt defects and forms normal microvilli; however, knockout mice are susceptible to intestinal injury (Ferrary *et al.*, 1999). For a long time villin seemed to be specific for mammalian intestinal microvilli but there is accumulating

evidence that proteins with the combination of gelsolin-like segments and the villin headpiece are present in many cell types and in lower eukaryotes. One of the first examples was protovillin in *Dictyostelium* amoebae, which is highly homologous to villin but exhibits only capping activity (Hofmann *et al.*, 1992, 1993). The discovery of molecules like supervillin (Pestonjamas *et al.*, 1997; Wulfschlegel *et al.*, 1999), advillin (Marks *et al.*, 1998), dematin (Azim *et al.*, 1995), and villin-like proteins from plants (Klahre *et al.*, 2000) strongly suggests that this family is widely distributed and may be responsible for a number of distinct functions. A member of this family in nematodes, *unc-115*, codes for a multidomain protein with LIM domains and dematin-like regions including a typical headpiece. Animals carrying a mutation in *unc-115* exhibit an uncoordinated phenotype that is thought to be based on defects in axon morphogenesis resulting from impaired response of the actin cytoskeleton to axon guiding signals (Lundquist *et al.*, 1998).

Gelsolin-like domains occur in combination with a wide variety of other domains. In nearly all cases the gelsolin-consensus resides in the C-terminal region, whereas the N-terminal extensions contain the already mentioned LIM-

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domains in UNC-115, nuclear localization signals in super-villin, leucine-rich repeats in the *Drosophila flightless* protein, and WD repeats in the protein presented in this study (Campbell *et al.*, 1993; Wulfschlegel *et al.*, 1999). WD-repeat proteins are characterized by a weakly conserved core domain flanked by the dipeptides GH and WD (Smith *et al.*, 1999). WD-repeats often form a propeller structure that seems to be an ideal platform for protein-protein interactions. This structural motif also occurs in the β -subunit of heterotrimeric G proteins, a component of the transmembrane signaling machinery (Wall *et al.*, 1995). Several cytoskeletal proteins contain WD repeats or have the potential for a propeller structure as well, most notably the actin-binding protein coronin, which accumulates at the leading edge of migrating amoebae (de Hostos *et al.*, 1991; Gerisch *et al.*, 1995), and the Arp2/3 complex in which the p40 subunit forms a seven bladed propeller (Robinson *et al.*, 2001).

Here we describe villidin, a multidomain protein from *Dictyostelium* that contains five gelsolin-like segments and a headpiece found in conventional villin. The N-terminal region of the protein harbors three PH-domains and WD-repeats that could form a seven-bladed propeller structure. GFP-fusion constructs suggest that the protein associates with the Golgi apparatus and the ER, and that the WD-repeat domain can bind to the actin cytoskeleton. A knock-out mutant shows reduced single cell motility and aberrant phototaxis at the multicellular stage, which implies an involvement of villidin in directed movement.

MATERIALS AND METHODS

Strains, Growth Conditions, and *Dictyostelium* Development

Dictyostelium discoideum strain AX2 and transformants of this strain expressing various fusion proteins of villidin with GFP were cultivated in nutrient medium as described (Watts and Ashworth, 1970), in suspension culture at 22°C. For development in liquid culture cells were washed in 17 mM phosphate buffer, pH 6.0 (Malchow *et al.*, 1972), resuspended at a density of 1×10^7 cells/ml in the buffer, and starved for 6 h. For development on solid substratum growing cells were harvested, washed, and deposited on phosphate agar plates at a density of 5×10^7 to 1×10^8 cells per plate.

Cloning of Villidin

During sequencing of DNA clones derived from a slug stage cDNA library, plasmid pFC3 was identified, which encoded protein sequences with high homology to the C-terminus of *Dictyostelium* protovillin (Hofmann *et al.*, 1992, 1993). To obtain full-length clones the library was screened with a 900-base pair *EcoRI* fragment of pFC3 as a probe. Several clones were isolated, among them vil20, a clone, which harbored a 5.0-kb insert coding for the entire villidin. The sequence data are available from GenBank/EMBL/DDBJ under accession no. AJ427856.

Protein Expression and Generation of Monoclonal Antibodies

The 900-base pair *EcoRI* fragment of pFC3 encoding the C-terminal 263 amino acids of villidin was cloned into the ATG-expression vector pIMS5, and the recombinant protein expressed in *Escherichia coli* XL1 blue (Simon *et al.*, 1988). The protein was purified and used for production of monoclonal antibodies as described (Schleicher *et al.*, 1984). Polyclonal antisera (6335, 6336) were raised by immunizing rabbits (Eurogentec, Ougree, Belgium). For immunofluorescence

studies we also used purified IgG of mAb 257-6 and the polyclonal antiserum pAb 6336 after affinity purification on pFC3 protein coupled to a 1-ml NHS-activated HiTrap column (Amersham/Pharmacia, Freiburg, Germany). Using vil20 cDNA as a template, a 300-base pair fragment that coded for the villidin headpiece domain only was cloned into the *EcoRI* site of pIMS5. The derived polypeptide contained 79 amino acids and the endogenous stop codon of villidin. Four additional amino acids (M-G-E-F) at the N-terminus were encoded by the vector. The headpiece was expressed and purified following standard procedures. Only the C-terminal region of the villidin gene could be expressed in bacteria in sufficient quantities. It was not possible to obtain recombinant proteins from other regions independent from the use of different expression systems and the construction of numerous DNA inserts. This rendered it impossible to assay domain functions in more detail.

Subcellular Distribution of Villidin

Axenicly grown log-phase wild-type cells were harvested, washed twice with phosphate buffer, and resuspended in phosphate buffer at a cell density of 8×10^7 cells/ml. To achieve an even distribution of cells, 10 ml of this suspension was layered onto a Petri dish containing phosphate agar, and the cells were allowed to settle for 10 min. After removing the clear supernatant the cells were starved at 21°C, harvested, washed twice with phosphate buffer, and resuspended in 10 mM Tris, pH 8.0, 1 mM EGTA, 1 mM DTT, 2 mM $MgCl_2$, protease inhibitor cocktail (P2714, Sigma, Deisenhofer, Germany), and the cysteine-protease inhibitor E64 (E0514, Sigma). The cells were opened either by repeated passaging through Nuclepore filters (pore size 5 μm ; Zind Verfahrenstechnik, Bodenheim, Germany) or by lysis with 1% Triton X-100 (membrane-grade).

For separation on sucrose gradients, cells were starved for 6 h and opened by freeze-thaw; complete rupture of cells was controlled by light microscopy. The homogenate was loaded onto a discontinuous sucrose gradient consisting of 1.7-ml layers of 0.88, 1.02, 1.17, 1.32, 1.47, 1.62, and 2.49 M sucrose in 10 mM HEPES/NaOH, pH 7.4, 1 mM DTT, and freshly added protease inhibitors (see above). The gradient was centrifuged in a SW40 swinging bucket rotor (Beckman Coulter, Unterschleissheim, Germany) at 29,000 rpm for 20 h at 4°C. The gradient was fractionated from top to bottom and immediately assayed for acid and alkaline phosphatase activities using *p*-nitrophenylphosphate as described previously (Loomis, 1969; Loomis and Kuspa, 1984). Equal amounts of protein from the different fractions were subjected to SDS-PAGE, blotted onto nitrocellulose, and stained with antibodies as indicated.

Construction of Vectors Allowing Expression of GFP Fusion Proteins

Green fluorescent protein (S65T red-shifted GFP) was fused to full-length villidin (aa 1–1704), the first four WD repeats (GFP-vilK8, aa 1–270), the complete WD region containing PH domain 1 (vilK1-GFP, aa 1–597), the complete WD region containing PH domain 1 and the adjacent P/T/S-rich region (vilK2-GFP, aa 1–725), the intervening sequence containing PH domains 2 and 3 (vilK3-GFP, aa 728–1002), and the villin homology domain (GFP-vilK4, aa 992–1704) by cloning PCR amplified DNA fragments at first into the AT-vector pCR2.1 (Invitrogen, DeSchelp, Netherlands). After verification of the sequences, the fragments were recloned into pDEX-GFP or pBsr-GFP vectors (Westphal *et al.*, 1997; Mohrs *et al.*, 2000) and introduced into AX2 wild-type cells by calcium phosphate-mediated transformation (Nellen *et al.*, 1984). Transformants were selected for growth in the presence of G418 or blasticidin (Life Technologies, Eggenstein, Germany; ICN Biochemicals Inc., Aurora, OH), and GFP-expressing transformants were identified by visual inspection under a fluorescence microscope. GFP was located at the C-terminus in vilK1, vilK2, and vilK3. The full-length villidin, vilK8, and the vilK4 polypeptide carried the GFP at the N-terminus (see also Figure 5).

Figure 2. The villidin mRNA and the protein are upregulated during development. (A) Villidin transcript and (B) protein accumulate strongly during mound formation and culmination. The villidin message has a size of ~6 kb. For the Western blot whole cell homogenates (2×10^5 cells per lane) were probed with villidin-specific mAb 257-6.

present in members of this family. There is a typical villin headpiece at the very C-terminus.

Hybridization of restriction fragments on Southern blots under stringent conditions showed only those bands expected from the sequence indicating that there is a single gene encoding villidin. This is supported by data from the *Dictyostelium* genome sequencing project (<http://www.uni-koeln.de/Dictyostelium/>).

Expression of Villidin during Development

Most of the actin-binding proteins identified in *Dictyostelium* are present in growing cells and throughout all stages of development. A notable exception is interaptin, an ER-associated actin-binding protein that accumulates during late aggregation (Rivero *et al.*, 1998). We analyzed the expression of the villidin gene during development and found that the 6-kb message is present in low amounts in growing cells, rapidly accumulates between 6 and 10 h of development (onset of multicellularity), and decreases only after 14 h (Figure 2). Villidin gene transcription during development was not affected by exogenous pulses of cAMP (our unpublished results). At the protein level villidin was barely detectable in growing cells, but accumulated steadily up to the tipped mound stage (Figure 2). We estimated the levels of villidin in growing cells by using the PFC3 polypeptide for calibration and mAb 257-6 in quantitative immunoblots. Villidin is present at a concentration of $\sim 0.3 \mu\text{M}$, which shows its low abundance compared with actin ($175 \mu\text{M}$) or profilin ($50 \mu\text{M}$) concentrations (Haugwitz *et al.*, 1994).

Figure 3. Distribution of villidin after fractionation of cellular extracts on a sucrose gradient. Cells were developed for 8 h, disrupted by freeze-thawing and loaded onto a discontinuous sucrose gradient. After centrifugation, fractions 1-11 were collected from top to bottom and analyzed by measurement of acid phosphatase activity and the relative amounts of villidin and the Golgi marker comitin after densitometric scanning of immunoblots.

Intracellular Distribution of Villidin

To determine the intracellular distribution of villidin, cells were disrupted by passage through Nuclepore filters or treatment with detergent. Lysis of cells by shear forces leaves the membranes essentially intact, whereas lysis with Triton X-100 solubilizes the membranes, and only the Triton-insoluble cytoskeleton is pelletable. In both cases villidin was found in the soluble fraction as well as in the pellets. Treatment of the pellets with 100–200 mM NaCl released villidin. Because of its high sensitivity to proteolytic degradation in the particulate fraction, the total yield of villidin decreased during the successive extractions. Subcellular localization of villidin was further studied by fractionation of total cell homogenates on sucrose gradients using various markers to distinguish between membranes of different origins (Figure 3). The first fractions had high acid phosphatase and α -L-fucosidase activities characteristic of lysosomes (our unpublished results), whereas subsequent fractions contained alkaline phosphatase characteristic of membranes of intermediate and high densities. Villidin was found associated with the higher-density membranes together with comitin, a marker for Golgi membranes (Weiner *et al.*, 1993). The lysosomal integral membrane protein LmpB (Janssen *et al.*, 2001) and the cell adhesion molecule csA (Faix *et al.*, 1990) were found in fractions of higher densities corresponding to endosomes, Golgi, and plasma membranes (our unpublished results).

Figure 4. Full-length GFP-tagged villidin labels Golgi and ER membranes. (A) A confocal Z-series shows a cell with two nuclei and highest concentrations of GFP-villidin in perinuclear regions. The confocal frames have a distance of 1.2 μm from top to bottom; the bar represents 5 μm . (B) To identify the subcellular distribution of GFP-villidin (a, b, c) in more detail, cells were double-stained with Cy3-labeled anti-mouse IgG for the Golgi-marker comitin (a', mAb 190-340-2), or the ER marker proteins PDI (b', mAb 221-135-1) and interaptin (c', mAb 260-60-10). The panels a'', b'', c'' show the overlays of both labels. Bar, 10 μm .

To investigate the importance of distinct villidin domains for intracellular localization, we constructed GFP fusions with the full-length protein and various subdomains and expressed them under the control of the actin15 promoter, which is active in growing and developing cells. Cells expressing the full-length construct (GFP-vilfl) showed a tubular and punctate distribution throughout the cytoplasm with enhanced localization to perinuclear regions. A series of confocal images through a cell shows the association of the most intense staining with the two nuclei (Figure 4A). The expression of full-length villidin in growth phase cells, which contain a rather low level of endogenous villidin, did not result in an obviously aberrant phenotype.

To distinguish the various membrane structures, the GFP-vilfl-expressing cells were counterstained for the Golgi

marker comitin (Figure 4Ba; Weiner *et al.*, 1993), and the ER markers protein disulfide isomerase PDI (Figure 4Bb; Monnat *et al.*, 1997) and interaptin (Figure 4Bc; Rivero *et al.*, 1998). Comitin and GFP-vilfl show a clear colocalization in the Golgi apparatus, with the comitin somewhat more concentrated in the center of the membrane stacks. The PDI antibody stained the ER as a tubular network with regions of different intensities and a continuous staining around the nucleus. Codistribution of the ER marker and villidin occurs especially in areas of a denser meshwork and around the nucleus. Although interaptin was restricted to the rough ER around the nucleus, GFP-vilfl was present over a wider area. Further investigation of the membranes of the contractile vacuole and the endo/lysosomal system of *Dictyostelium* using mAb 221-35-2 directed against the A subunit of the

Figure 5. Subcellular distribution of GFP-tagged villidin domains in living cells. A series of distinct villidin domains was fused to GFP and expressed in AX2 cells during growth (left panels) or after 6 h of development (middle and right panels). The N-terminal constructs (vilK1-GFP and vilK2-GFP) shared the complete WD region including the first PH domain and accumulated in F-actin-rich regions. In contrast, the constructs GFP-vilK8 with the first four WD repeats, vilK3-GFP, which contained only the PH domains 2 and 3, and the whole gelsolin/villin-like domain (GFP-vilK4) showed no specific enrichment in regions with high cytoskeletal dynamics. Bars, 10 μ m.

V/H+-ATPase (Jenne *et al.*, 1998) as well as the incubation with vacuolin mAb 221-1-1 (Rauchenberger *et al.*, 1997) showed that villidin is not associated with these subcellular compartments (our unpublished results).

Among the WD repeats at the N-terminus of villidin, the first four show the highest homologies to the WD consensus sequence. A corresponding GFP-fusion protein (GFP-K8) showed, however, an overall distribution in growth phase cells and in cells after 6 h of starvation (Figure 5, A and B). Surprisingly, cells expressing the vilK1-GFP or vilK2-GFP products, which carry the WD-repeats and the first PH domain but lack the gelsolin and villin related domains, were strongly enriched at fronts of moving cells (Figure 5, C and D, and E and F). Cells expressing vilK3-GFP or GFP-vilK4 fusion products that lack the WD-repeats but have the

second and third PH domains or the gelsolin and villin related domains were not found at the fronts of cells but were uniformly distributed throughout the cytoplasm (Figure 5, G and H, and I and J). Several of the fusion products were seen in nuclei, possibly as the result of carrying a short stretch of basic amino acids in the linker peptide that connected GFP to the villidin domains (Westphal *et al.*, 1997).

Despite considerable efforts it was impossible to express recombinant villidin domains in bacteria for functional analyses. Therefore, we used *D. discoideum* GFP mutants as expression system and analyzed the interaction of full-length villidin and its domains with the actomyosin complex by preparation of Triton-insoluble cytoskeletons and subsequent immunoblotting with GFP antibodies. The data are consistent with the findings at the single cell level (see

Triton-insoluble cytoskeleton:

Figure 6. Cosedimentation of different villidin domains with the Triton-insoluble cytoskeleton. *D. discoideum* mutants that expressed GFP-fusions with full-length villidin (GFP-vilfl), the first four WD repeats (GFP-vilK8), the complete WD region including the first PH domain (vilK1-GFP) and the adjacent Pro/Ser/Thr-rich protein stretch (vilK2-GFP), or the gelsolin/villin segments (GFP-vilK4) were solubilized with Triton X-100 and centrifuged at $10,000 \times g$. Supernatants and pellets were analyzed in immunoblots using the GFP-specific mAb K3-184-2. Only the constructs that contain the complete WD region and its overlapping PH domain cosediment with the Triton-insoluble cytoskeleton.

Figure 5): Cosedimentation with actin occurs in the presence of full-length villidin and the constructs vilK1-GFP and vilK2-GFP, which carry the complete WD region and the first PH domain. Constructs with protein domains further upstream (vilK8-GFP, first 4 WD repeats) or downstream (GFP-vilK4, villin-like segments and villin-headpiece) do not cosediment with the Triton-insoluble fraction (Figure 6). Usually the sedimentation of villidin, vilK1-GFP, or vilK2-GFP was not complete even when additional F-actin was added to the Triton extract. This suggests that the interaction with F-actin is either weak, requires additional, yet unknown proteins, or that the soluble population of villidin is part of protein complexes that compete with its actin-binding function.

Redistribution of the N-terminal Villidin-GFP Fusions during Endocytosis and Phagocytosis

Fluid-phase uptake in *Dictyostelium* occurs by two mechanisms, macropinocytosis and micropinocytosis, and both events appear to involve the actin cytoskeleton. We followed the redistribution of vilK2-GFP in an actively endocytosing cell that formed several macropinosomes in the time frame taken. At the site where a macropinosome is formed the N-terminal villidin domain is at first only locally enriched. As soon as the pinosome emerges, vilK2-GFP is present in the extending area that engulfs the fluid (10 s), and it further accumulates when the pinosome fuses (20 and 30 s). It stays on the pinosome during internalization (40 s) and then disappears after 50 and 60 s (our unpublished results). vilK2-GFP was also relocated to the active site during phagocytic uptake of yeast particles (Figure 7, arrowhead), where it locally accumulated once the yeast particle had contacted the plasma membrane (15 and 30 s). The protein stayed on the phagocytic cup during uptake of the yeast particle and started to dissociate at the late stages of uptake so that only

Figure 7. Redistribution of vilK2-GFP during phagocytosis. Uptake of rhodamine labeled yeast cells by a phagocytosing cell (arrowhead) is followed over a period of 135 s. Confocal images were taken every 15 s. VilK2-GFP assembles at the site of cup formation (30 s) and is released when the uptake is nearly completed (90–105 s). At the same time this cell is involved in pinocytic uptake (arrow/105"). Bar, 10 μm.

Figure 8. Generation of villidin[−] mutants. (A) A replacement vector was constructed by replacing an internal 1.4-kb *SspI*/*EcoRV* fragment of the villidin gene with the blasticidin resistance cassette. (B) Southern blot analysis showed that in the isolated mutants a gene replacement had occurred because in *Bgl*II-digested mutant DNA the endogenous *Bgl*II fragment carried a novel *Bgl*II site that had been introduced by the blasticidin resistance cassette (shown for two independent mutants).

a discontinuous ring remained during completion of engulfment (90 s). Once the particle had been engulfed completely, vilK2-GFP was present close to the plasma membrane (105 s) and then disappeared. In the cell shown we also observed the formation of a pinocytic cup (Figure 7/105", arrow). A comparison of both events showed that vilK2-GFP accumulation at the pinocytic site occurs in a wider zone than at the phagocytic cup. In both instances the localization of vilK2-GFP and actin overlapped at the sites of pinosome and phagosome formation (our unpublished results). Interestingly, we could not detect a similar distribution with full-length GFP-villidin. It remains to be shown whether the localization of the WD-repeats occurs in a regulated manner and might be masked in the whole molecule.

Inactivation of the Villidin Gene and Characterization of the Villidin-minus Mutant

For inactivation of the villidin gene we generated a vector in such a way that we replaced a central endogenous fragment of 1.4 kb by the 1.4-kb blasticidin resistance cassette (Figure 8A). Transformants were selected and analyzed by colony blotting using mAb 257-6 for absence of the protein. Several independent villidin negative clones were isolated. To investigate the gene inactivation event at the DNA level, we isolated chromosomal DNA from several independent transformants and digested it with *Bgl*II. *Bgl*II does not cleave within the villidin coding sequences and gives rise to a single fragment of ~12.5 kb carrying the gene. In the mutants we observed two hybridizing fragments of 8.2 and 3.8 kb (Figure 8B). The *Bgl*II site in the gene had been introduced by the blasticidin resistance cassette after successful gene replacement. At the mRNA level the villidin transcript was no longer detectable nor was an altered transcript present; lack of the protein was confirmed by Western blot analysis (our unpublished results). At the immunofluorescence level villidin[−] mutants were no longer labeled with affinity-purified polyclonal antibody (Figure 9A). In wild-type cells, villidin-specific antibodies showed a meshwork-like staining with many punctate enrichments distributed throughout the cytoplasm and around the nucleus.

Growth of *Dictyostelium* cells can be analyzed under various laboratory conditions and a detailed analysis has often revealed defects in cytoskeletal mutants (Rivero *et al.*, 1999b). When we followed the growth of villidin[−] mutants

on a lawn of bacteria and determined the increase in colony diameter as a relative measure of growth rate, we did not observe differences relative to wild-type cells. Similarly, mutant cells were indistinguishable from wild-type cells with regard to generation times and maximal cell densities when grown in axenic media or in shaking suspension in the presence of *E. coli* B/r. These results indicate that phagocytosis and pinocytosis are unimpaired. The results were confirmed by quantitative assays using fluorescently labeled dextran for pinocytosis assays and fluorescently labeled yeast cells for phagocytosis assays (our unpublished results). Growth behavior under increased osmotic stress (115 mM sorbitol or 30 mM NaCl) was also comparable to wild-type cells.

Villidin protein accumulation is most prominent during development. An analysis of the development of the mutants on phosphate agar or on nitrocellulose revealed that the mutants were moderately delayed by ~2 h in early development. Analysis of the expression pattern of development-specific proteins and mRNAs did not indicate significant alterations. However, in a cAMP gradient villidin[−] cells moved significantly more slowly than wild-type cells. Aggregation competent cells were positioned near a capillary that was filled with the chemoattractant cAMP and cell motility measured over a 30-min period. Under these conditions motility of villidin-minus cells was about half that of wild-type cells (our unpublished results).

Because villidin is prominent during the slug stage, we investigated this stage in more detail. In Figure 9B the migration rates of wild-type and mutant slugs are analyzed as well as the phototactic response. We found that wild-type slugs formed long trails and moved accurately toward the light source, whereas mutant slugs left shorter trails (8.3 mm compared with 15.8 mm for wild type after 2 d of phototactic migration). The mean deviation from the source of light was 17.4° for wild type and 30.8° for mutant slugs. It appears that the rate of migration as well as phototactic orientation are both impaired by loss of villidin.

DISCUSSION

The actin cytoskeleton in nonmuscle cells is regulated by a large number of highly specialized actin-binding proteins that share a limited set of functional domains. The protein

Figure 9. Analysis of villidin-minus mutants. (A) Immunofluorescence staining of villidin in wild-type cells was relatively weak. To obtain reliable immunofluorescence data, we mixed villidin- with AX2 wild-type cells as internal controls. The phase contrast (a) shows one villidin- cell together with two wild-type cells. All cells can be visualized with mAb 102-425-1 against severein (b) and DAPI (c). Staining with affinity-purified polyclonal antibody against villidin (d) shows that the protein is absent in the mutant cell and localized at vesicles and in perinuclear regions (arrows) in AX2 wild-type cells. (B) Migration and phototaxis of villidin-deficient slugs was drastically altered compared with AX2 wild-type slugs. AX2 and mutant (vil-) cells were deposited on a

phosphate agar plate and incubated in a phototaxis chamber for 2 d. Slug trails were transferred to a nitrocellulose filter, stained with Coomassie brilliant blue, and analyzed. As starting point of the slug migration the center of application of the cells was chosen. Only slug trails that could be followed unambiguously were included. For AX2 wild type we analyzed 94/92 slug trails and for the villidin- mutant 334/332 slug trails. Shown is the mean length of slug trails in mm (slug migration) and the mean deviation from the direct migration path toward the light source in degrees (deviation from light source).

villidin, described in this study, carries multiple domains, some of which are related to those in actin-binding proteins. The most characteristic features of villidin are the homologies to villin at the C-terminus and to WD-repeat proteins at the N-terminus. Subcellular localization of villidin indicates an interaction with Golgi and ER membranes as well as distinct associations of the WD repeats with F-actin-rich structures. Knockout mutants suggest an involvement of villidin in cell motility and phototactic movement at the multicellular stage during *D. discoideum* development.

The gelsolin/villin family of proteins is rapidly expanding and numerous closely related proteins have been described that share features with gelsolin or villin (Kwiatkowski, 1999). In some cases additional domains are present as in supervillin from bovine neutrophils (Pestonjasp *et al.*, 1997) or the *flightless* protein from *Drosophila* (Campbell *et al.*, 1993). *D. discoideum* GRP125 (Stocker *et al.*, 1999) and villidin (this study) harbor extensions as well but lack one of the six gelsolin segments found in other members of the family. A third type of protein that includes abLIM or TALB combines a villin headpiece with domains previously identified in other actin-binding proteins (Tsujioka *et al.*, 1999). It is typical for these more complex gelsolin/villin-like proteins that their actin-binding functions are changed.

Recently the *in vitro* activity of different headpieces was studied and set in correlation to differences in the three-dimensional structures (McKnight *et al.*, 1997; Vardar *et al.*, 2002). It turned out that the villin-like headpieces of supervillin and villidin do not bind to muscle actin, a behavior that we have confirmed for the interaction of villidin headpiece with *D. discoideum* actin. According to McKnight and coworkers (Vardar *et al.*, 2002), a functional headpiece requires a positively charged patch for interaction with a negatively charged region in actin (−11 charges/monomer).

The supervillin headpiece contains no positive patch region, and the corresponding area in the villidin headpiece is negatively charged (−11 net charge) thus rendering interaction with actin as a target protein very unfavorable.

The lack of the first gelsolin-like segment in villidin may keep this domain from interacting with actin. As shown for severein, a typical member of the gelsolin family, the first and second segments are required for F-actin capping function, and the concerted action of the first and the third segment are essential for nucleation of actin polymerization (Eichinger *et al.*, 1991). The lack of the first segment in villidin would be expected to preclude it from a role in capping or nucleating activity. The data obtained with GFP-tagged villidin C-terminal domains confirm that these domains cannot bind F-actin on their own.

Cell fractionation studies showed that up to 50% of total villidin is present on internal membranes, and the distribution of the GFP-tagged full-length molecule shows clearly that Golgi-structures and ER-membranes are strongly labeled. The distribution to the Golgi was most prominent in GFP fusions of full-length villidin and the constructs that span the first 600 amino acids (vilK1 and vilK2). The major characteristics of this region are the WD repeats and the first of three PH domains. Most intriguing was the strong localization of GFP-tagged vilK1 and vilK2 in F-actin-rich regions in moving fronts, phagocytic and pinocytic cup structures. This was not found with GFP-tagged full-length villidin or constructs expressing only the gelsolin/villin-homologous domain at the C termini. We draw two major conclusions from these observations: i) full-length villidin is primarily associated with internal membranes (ER, Golgi, vesicular membranes), and ii) removal of the C-terminal two thirds of the protein uncovers a function of the WD repeats which causes a localization to F-actin-rich regions.

The best known example for the interaction between a WD protein and the actin cytoskeleton is coronin (de Hostos *et al.*, 1991, 1993). Coronin contains five WD repeats that bind to F-actin if additional amino acids upstream or downstream are present (Mishima and Nishida, 1999). In a similar manner one would expect that the putative propeller of the kelch repeat domain in the C-terminal half of the actin-fragmin kinase interacts with the actin-fragmin complex, thus enhancing phosphorylation of actin at Thr 203/204 (Eichinger *et al.*, 1996a; Steinbacher *et al.*, 1999). Similarly, the p40 subunit of the Arp2/3 complex forms a seven-bladed propeller, which possibly binds along actin filaments via a short insert between blades six and seven (Robinson *et al.*, 2001). It remains to be shown in detail how the WD repeats in villidin interact with the cytoskeleton, but there is increasing evidence that specialized propeller structures are able to bind to actin and actin-related proteins.

Disruption of the villidin gene caused rather mild phenotypic changes during growth and early development. This is not a surprising observation for cytoskeletal proteins as there is substantial redundancy, and it often requires the disruption of several genes before cytoskeletal reactions are significantly altered (Witke *et al.*, 1992; Eichinger *et al.*, 1996b). The finding that a protein associated with intracellular membranes affects motility both at the single-cell level and at the multicellular slug stage might be due to villidin's involvement in intracellular membrane flow. NEM-sensitive factor (NSF) has been recently shown to affect cell locomotion in *Dictyostelium*, and these findings point out an interdependence between membrane recycling, cell polarity, and locomotion (Thompson and Bretscher, 2002). Villidin as a membrane and F-actin-associated protein might then also affect these processes in a similar way. Recent experiments on infection of the villidin-minus mutant with *Legionella* showed a reduced uptake of the pathogen already during phagocytosis (Steinert and Schleicher, unpublished results). It remains to be shown whether villidin-dependent qualitative changes in the membrane of the phagocytic cup might here play a role as well.

Interestingly, phenotypic changes are usually found at the multicellular stage during *D. discoideum* development because the consequences of an unbalanced cytoskeleton are amplified in multicellular structures. Similar to the GRP125-minus mutant (Stocker *et al.*, 1999), the villidin-minus cells show a phototactic defect during the slug stage. Phototaxis is a process that involves multiple cellular functions. Genetic analysis of slug behavior suggests that as many as 55 genes are involved and that several of the encoded proteins regulate signal transduction pathways involving the intracellular messengers cAMP, cGMP, IP₃, and Ca²⁺ (Fisher, 1997; Fisher *et al.*, 1997). Phototactic migration is dependent on motility of individual cells that requires the proper functioning of the cytoskeleton. Villidin appears to play a role in motility related processes leading to phototactic movement.

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