

A Talin Homologue of *Dictyostelium* Rapidly Assembles at the Leading Edge of Cells in Response to Chemoattractant

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Abstract. In an attempt to identify unknown actin-binding proteins in cells of *Dictyostelium discoideum* that may be involved in the control of cell motility and chemotaxis, monoclonal antibodies were raised against proteins that had been enriched on an F-actin affinity matrix. One antibody recognized a protein distinguished by its strong accumulation at the tips of filopods. These cell-surface extensions containing a core of bundled actin filaments are rapidly protruded and retracted by cells in the growth-phase stage. The protein of 269 kD turned out to resemble mouse fibroblast talin (Rees et al., 1990) in its primary structure. The fit is best among the first 400-amino acid residues of the NH₂-terminal region where identity between the two proteins is 44% and the last 200-amino acid residues of the COOH-terminal region with 36%

identity. In the elongated cells of the aggregation stage the *Dictyostelium* talin is accumulated at the entire front where also F-actin is enriched. Since this protein exists in a soluble state in the cytoplasm, mechanisms are predicted that cause accumulation at sites of the cell where a front is established. Evidence for receptor-mediated accumulation was obtained by local stimulation of cells with cAMP. When a new front was induced by the chemoattractant, the talin accumulated there within half a minute, indicating a signal cascade in *Dictyostelium* responsible for assembly of the talin beneath sites of the plasma membrane where chemoattractant receptors are strongly activated. The ordered assembly of the talin homologue together with actin and a series of other proteins is considered to play a key role in chemotactic orientation.

CELLS of *Dictyostelium discoideum* are highly motile and chemotactically responsive, resembling in size and behavior most closely the polymorphonuclear granulocytes of the mammalian organism. During locomotion of *Dictyostelium* cells their microfilament and microtubule systems are rapidly reorganized, which allows these cells to change polarity and direction of movement within seconds either spontaneously or in response to gradients of chemoattractant. Organization of the microfilament system in *Dictyostelium* cells is based on actin filaments that by a variety of associated proteins are cross-linked into networks or bundles (Condeelis, 1992, 1993) and are attached to the plasma membrane (Chia et al., 1993; Hitt et al., 1994). Stable structures like the actin cables of fibroblasts or the protein complexes assembled at sites of focal adhesion are not found in *Dictyostelium* cells; these structures might not be compatible with the dynamics of the microfilament system.

In addition to motor proteins of the myosin family, more than 20 actin-binding proteins from *D. discoideum* cells have been characterized (Schleicher and Noegel, 1992). Some of them show high F-actin cross-linking, capping, severing, or

G-actin sequestering activity in vitro, others are distinguished by their functions in vivo as evidenced by phenotypic changes in gene-replacement mutants.

Up to three actin-binding proteins known to exhibit strong activities in vitro have been eliminated in *D. discoideum* cells, including the two major F-actin cross-linking proteins α -actinin and 120-kD gelation factor, and the F-actin fragmenting protein severin. Although mutants lacking some or all of these proteins show alterations in cell behavior or multicellular development (Schleicher et al., 1988; André et al., 1989; Brink et al., 1990; Witke et al., 1992; Cox et al., 1992), their cells are still capable of moving towards chemoattractant (Gerisch et al., 1993). Even in a myosin II null background elimination of the three actin-binding proteins does not extinguish motility and chemotactic responsiveness (Wallraff, E., and G. Gerisch, unpublished results). Therefore, it is questionable to extrapolate from marked activities measured in vitro to the importance of a cytoskeletal protein within a living cell.

Results obtained with mutants defective in coronin are consistent with this notion. This actin-binding protein has been identified by its coprecipitation with actin-myosin complexes. Coronin would have escaped detection by standard activity assays since it does not show obvious effects on actin polymerization or crosslinkage, neither in fluorescent nor viscometry assays (De Hostos et al., 1991). In the living cell,

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however, the absence of coronin substantially slows down locomotion and causes a cytokinesis defect (De Hostos et al., 1993a).

As a result of these findings, we decided to screen for new proteins of *D. discoideum* that may be involved in the control of cell motility and chemotaxis by using affinity to actin as the basic criterion for proteins of potential interest. Starting with a triple mutant avoided the masking of proteins to be discovered by the major actin-binding proteins that are already known. Here we report on a new protein that is of interest for two reasons: it transiently assembles with the microfilament system at specific loci of the cells, and it is related to talin of mouse fibroblasts.

Materials and Methods

Growth of Dictyostelium Cells

D. discoideum AX2 wild type and mutant HG1397 cells were grown axenically at 23°C in liquid nutrient medium (Watts and Ashworth, 1970) on a gyratory shaker at 150 revs/min as described by Malchow et al. (1972). The cells were harvested during exponential growth at a density of not more than 5×10^6 per ml, washed, and starved in 17 mM K/Na phosphate buffer, pH 6.0, on the shaker in suspension at a density of 1×10^7 cells per ml to initiate development.

F-Actin Affinity Chromatography

Actin was prepared from rabbit skeletal muscle according to Spudich and Watt (1971) followed by gel filtration on Sephacryl S 300, and an F-actin column was prepared essentially as described by Miller and Alberts (1989). To identify *Dictyostelium* actin-binding proteins, growth-phase cells of mutant HG1397 were washed twice in 17 mM K/Na phosphate buffer, pH 6.0, and lysed in homogenization buffer (30 mM Tris-HCl, pH 7.6, 2 mM DTT, 4 mM EGTA, 0.2 mM ATP, 30% wt/vol of sucrose and protease inhibitors; Barth et al., 1994) by nitrogen excavation in a Parr bomb after equilibration at 900 psi for 15 min. The 100,000 g supernatant of the extract was applied to the F-actin column. After washing of the column with A buffer (50 mM Hepes/KOH, pH 7.5, 0.05% Nonidet P-40, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM DTT, supplemented with 10 µg/ml leupeptin, 10 µg/ml pepstatin, and 10 µg/ml aprotinin), the bound proteins were eluted with A buffer containing first 1 mM ATP and subsequently 0.1, 0.5, and 1 M KCl.

Monoclonal Antibody Production, Immunofluorescence, and Scanning Electron Microscopy

Monoclonal antibodies were raised by injecting BALB/c mice using Alugel S (Serva, Heidelberg Germany) as an adjuvant against the pooled fraction of proteins eluted from the F-actin column. Spleen cells were fused with PAIB₃Ag8-myeloma cells, and antibody 169-477-5, designated here as mAb 477, was identified in hybridoma culture supernatant by immunoblotting of homogenate and immunofluorescent labeling of *D. discoideum* growth-phase cells.

For fluorescent labeling, growth-phase or aggregation competent cells were seeded on glass coverslips and allowed to attach for 45 min. Cells were fixed either for 10 min with methanol at -20°C, or for 30 min with a mixture of 15% (vol/vol) of saturated picric acid in water and 1 or 2% of paraformaldehyde, 10 mM Pipes-HCl, pH 6.0, at room temperature. After picric acid/formaldehyde fixation, cells were postfixed with 70% ethanol. Fixed cells were washed three times for 5 min in PBS, pH 7.4, supplemented with 0.75% glycine, twice for 15 min in PBG (0.5% BSA and 0.045% fish gelatine in PBS) and incubated overnight with mAb 477 hybridoma culture supernatant. After washing six times with PBG the cells were incubated for 1 h with affinity-purified goat anti-mouse IgG (Dianova, Hamburg, Germany) either conjugated with TRITC (diluted 1:200) or with Cy3 (diluted 1:2,000). For comparison, cells were labeled with affinity purified polyclonal antibodies against 30-kD actin-bundling protein (courtesy of Dr. M. Fechheimer) followed by FITC-conjugated goat anti-rabbit IgG (Dianova). F-actin was labeled after picric acid/formaldehyde fixation with FITC-conjugated phalloidin (Sigma Chem. Co., St. Louis, MO).

After washing twice with PBG and three times with PBS, the labeled cells were embedded in Gelvatol (Polyvinyl alcohol, type II; Sigma Chem. Co., St. Louis, MO) containing 2.5 µg/ml of DABCO (Janssen, Beerse, Belgium) and photographed with an AxioPhot fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Confocal images were obtained with an LSM 410 laser scanning microscope (Carl Zeiss) using a 100× objective of aperture 1.3. The images were recorded with a voxel size of 0.083 µm in the x and y axes, and 0.3 µm in the z axis. For the printouts the images were smoothed by subdividing the pixels and linear interpolation.

For scanning electron microscopy, cells spread on glass were fixed with glutaraldehyde/osmic acid and prepared by critical point drying as described by Claviez et al. (1986).

Chemotactic Stimulation

Chemotaxis experiments were performed essentially as described by Gerisch and Keller (1981). *D. discoideum* AX2 cells were starved for 6 h and transferred onto glass coverslips with an engraved grid (CELLocate; Eppendorf, Hamburg, Germany) in 17 mM K/Na phosphate buffer and photographed at intervals on an Axiovert microscope (Carl Zeiss). Cells were stimulated with glass micropipettes filled with 1×10^{-4} M cyclic AMP, fixed during reorientation with picric acid/formaldehyde followed by ethanol, and labeled with mAb 477.

DNA Cloning and Sequencing

A summary of the clones used to compile the complete coding sequence is presented in Fig. 1. First, a λgt11 cDNA library of *D. discoideum* growth-phase cells (Graham et al., 1988) (courtesy Dr. A. Kaplan, Washington University, School of Medicine St. Louis, MO) was screened with ¹²⁵I-labeled mAb 477. This screen identified one 1.3-kb clone, λc11. Total extract of the *Escherichia coli* induced by IPTG and subjected to SDS-PAGE showed after immunoblotting with mAb 477 a single band corresponding to the β-gal λc11 fusion protein, which was not seen in non-induced bacteria. The λc11 insert was used to rescreen the library resulting in clones of slightly larger size. One of them was used to screen a genomic library (Giorda et al., 1990) (courtesy Dr. H. L. Ennis, Roche Institute, Nutley, NJ), from which clones 24-5 and 16g were obtained. Since use of the 5' end of the sequence for screening failed to identify clones containing additional sequences, a PCR-based strategy was adopted to walk towards the 5' end of the gene.

A genomic map was constructed using ECL-labeled (Amersham Corp. Arlington Heights, IL) or ³²P-labeled clone 24-5 to identify restriction sites in the 5' direction that were several kilobases apart. This information was used for inverted and subsequently for direct PCR. For inverted PCR, 2 µg of genomic DNA was digested with appropriate enzymes and religated, added to a standard 100 µl PCR cocktail containing 100 pM of each sequence-specific primer. After 30 cycles of amplification under standard conditions in a thermocycler (Perkin Elmer Corp., Branchburg, NJ) the reaction product was purified via QIAquick-spin PCR purification kit (Qiagen Inc., Chatsworth, CA), digested with restriction enzymes and cloned into pUC19 (Yanisch-Peron et al., 1985). The appropriate restriction sites were deduced from Southern analysis or introduced via the primers used for the PCR reactions. Presence of the internal restriction sites predicted, the expected length of the PCR clones obtained, and identity of the sequence at the region overlapping with the previous clone were criteria to identify the correct DNA fragments.

DNA double strands were sequenced using the dideoxy chain termination method (Sanger et al., 1977) and T7 polymerase sequencing kit (Pharmacia, Uppsala, Sweden). All portions of the coding region were sequenced in both directions using oligonucleotide primers derived from the sequence. For each PCR step, two independent clones were sequenced. The sequence was analyzed using the FASTA and BESTFIT programs of the University of Wisconsin Genetics Computer Group Software (Devereux et al., 1984) and the MIPSX database (Max-Planck-Institut, Martinsried, Germany).

Nucleic Acid Hybridization

For Southern blots, genomic DNA was prepared according to Mehdy et al. (1983), restricted with appropriate enzymes and electrophoresed in 0.8% agarose gels. After denaturation the gel was neutralized with 0.5 M Tris, pH 7.5, 1.5 M NaCl. The DNA was transferred to nylon membrane (Pall Biodyne, East Hills, NY) in 20 × SSC (3 M NaCl, 0.3 M Na-citrate), and cross-linked to the membrane on a transilluminator for 2 min. DNA was hybridized using either the ECL system (Amersham Corp.) or nick-translated probes in buffer containing 50% formamide at 37°C.

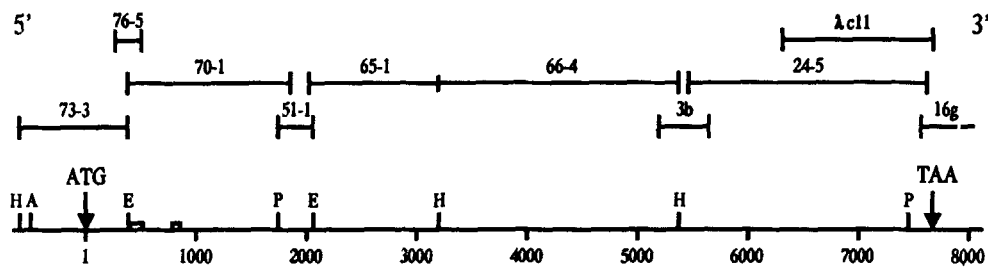


Figure 1. Organization of the genomic sequence of talin in *Dictyostelium*. The genomic sequence reveals the presence of two short introns of 117 and 73 bp in the 5' region, which are indicated by small boxes. Restriction sites: *AccI* (A), *EcoRI* (E), *HindIII* (H), and *PstI* (P). Clones used to compile the complete coding se-

quence are shown on top. Clone 73-3 contains the first in-frame *ATG* translation start codon of the open reading frame. Clone 16g contains the stop codon (*TAA*) followed by two putative polyadenylation signals. Scale is in nucleotides.

For Northern blots, total RNA was prepared by phenol/chloroform extraction. Axenically grown cells were washed in 17 mM phosphate buffer, pH 6.0, and either transferred onto HABG nitrocellulose filters (Millipore Corp., Bedford, MA) for development, or starved in suspension up to the aggregation-competent stage. RNA blots were probed with clones 24-5, 3b and 65-1 (Fig. 1).

Results

A Protein That Accumulates at the Tip of Filopods in Growth-phase Cells

A cytosolic fraction was prepared from HG1397, a triple mutant lacking three major actin-binding proteins that have strong F-actin cross-linking or fragmenting activity in vitro: α -actinin, 120-kD gelation factor, and severin (Schindl et al., 1995). Proteins of this fraction were applied to a phalloidin-stabilized F-actin column. Bound proteins were eluted with ATP and KCl and used to immunize mice for monoclonal antibody production. The antibodies raised were assayed by immunoblotting of total cellular proteins separated by SDS-PAGE, and screened by immunofluorescence microscopy for reactivity with proteins that have a peculiar location within the cortical network of actin filaments. Among the antibodies obtained, some reacted with a 30-kD actin-bundling protein (p 30) known to be accumulated in filopods and in the actin-rich front region of *D. discoideum* cells (Fechheimer, 1987), indicating that the search for antibodies against actin-binding proteins was efficient. One antibody, mAb 477 (Fig. 2 A), reacted specifically with a single protein band above the 200-kD position which was hardly distinguishable from that of myosin II heavy chains. Since in mutants that lack myosin II or express truncated heavy chains, the mAb 477 reactive band remained unchanged, it became obvious that the antibody recognized a yet unidentified protein. When homogenate of growth-phase cells was fractionated about 60% of the protein was recovered in the 100,000 g supernatant.

Immunofluorescence labeling of methanol-fixed growth-phase cells with mAb 477 revealed a pattern distinct from that of any known *Dictyostelium* protein. The label was strongly accumulated in fluorescent spots at the tiny tips of filopodial cell-surface extensions (Fig. 2 B and D). Sometimes the antibody label was also enriched along the length of filopods. Because of its conspicuous accumulation in filopods we had provisionally designated the protein as "filopodin" (Gerisch et al., 1993).

In addition to its strong accumulation in cell-surface extensions a uniform labeling of filopodin in the entire cyto-

plasm was observed, in accord with the presence of the protein in the cytosolic fraction. The actin-bundling protein known to be enriched in filopods (Fechheimer, 1987) did not show up at their tips (Fig. 2, C and E) and labeling with phalloidin (Fig. 2 F) illustrated that F-actin is much more abundant in structures of the cell cortex other than the mAb 477-labeled ones. Therefore, it is not just binding to actin filaments which determines positioning of the protein recognized by this antibody.

The Protein Enriched in Filopods Is a Dictyostelium Homologue of Talin

Screening of an expression library with mAb 477 revealed a cDNA clone encompassing an open reading frame for a polypeptide of 47 kD. The antibody specifically recognized in *E. coli* a β -gal fusion protein that migrated as a 180-kD polypeptide in SDS-PAGE. According to the sequence deduced from the cDNA, the cloned fragment exhibited similarities to the COOH-terminal region of mouse talin (Rees et al., 1990). To obtain the complete sequence of filopodin additional clones were identified using the initial clone to reprobe cDNA and genomic libraries in combination with a PCR-based strategy to walk in the 5' direction.

The derived amino-acid sequence of filopodin is shown in Fig. 3. Alignment of this sequence with that of mouse talin shows that these two sequences are 46% similar and 24% identical as averaged over their entire lengths. The fit is best for the amino-terminal 400-amino acid residues that are 66% similar and 44% identical, and for the carboxy-terminal 200-amino acid residues that are 52% similar and 36% identical (Fig. 4, top). The central regions show weaker relationship with 40% similarity and 19% identity. The predicted relative molecular mass of the polypeptide is 268,810 D, which is larger than 220 kD on SDS-PAGE. A similar difference has been found for mouse talin and is probably due to its amino-acid composition. The amino acid compositions of the *Dictyostelium* protein and mouse talin are similar; the alanine content of both proteins is high, 13 and 18%, respectively. The calculated isoelectric points of the two proteins, 6.05 and 6.10, are almost identical. The secondary structure prediction suggests a high content of α -helices for the central domain of the *Dictyostelium* protein, as for the rod domain of mouse talin. This is consistent with the finding that chicken gizzard talin has a high content of α -helices (Molony et al., 1987). Because of its similarities to the talin from mouse fibroblasts, we henceforth refer to filopodin as *Dictyostelium* talin.

The *Dictyostelium* protein shows sequence relationships in

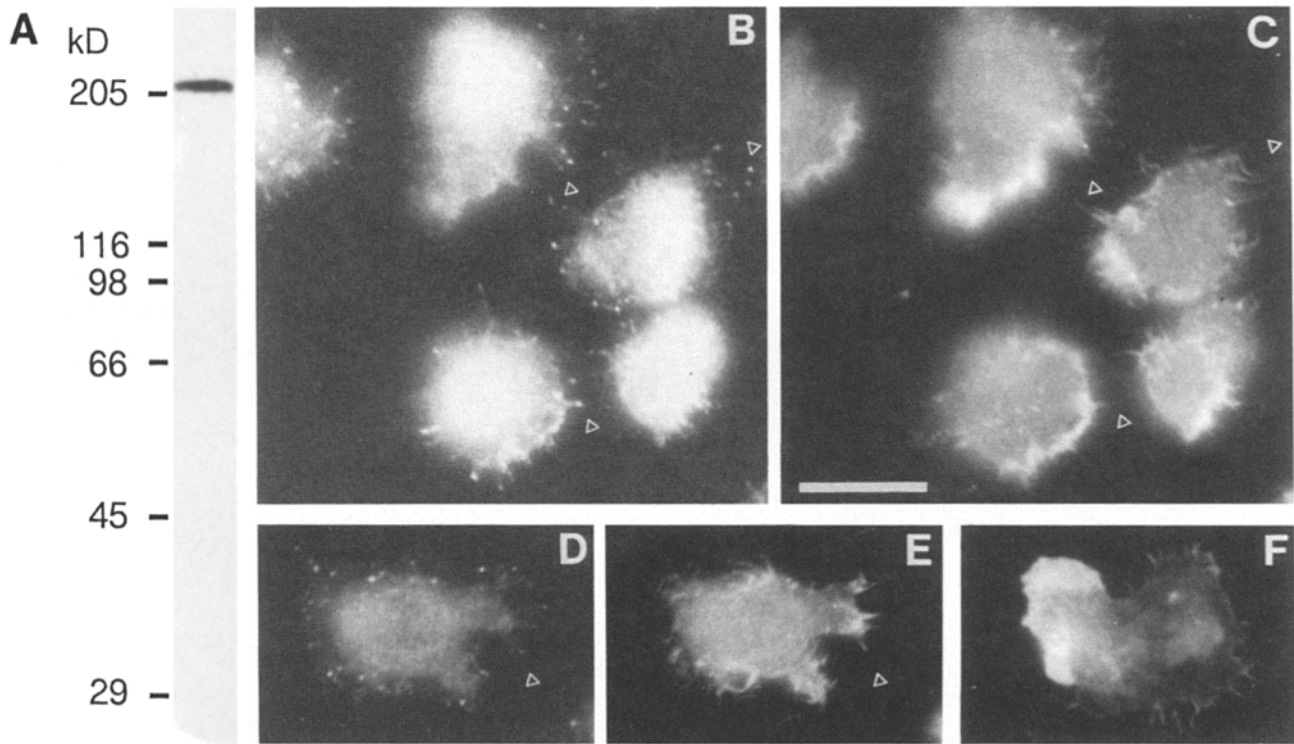


Figure 2. Apparent molecular mass and localization of the protein recognized by mAb 477. (A) Immunoblot of total proteins from growth-phase cells of *D. discoideum*, separated by SDS-PAGE. A single band is labeled, corresponding to a protein larger than 200 kD. (B-E) Growth-phase cells fixed with methanol at -20°C and double-labeled first with mAb 477 followed by Cy3-conjugated goat anti-mouse IgG, and second with rabbit antibodies against actin-bundling protein p30 followed by FITC-conjugated goat anti-rabbit IgG. Label of mAb 477 is shown in B and D, anti-p30 label in the same cells in C and E. The triangles indicate filopods labeled at their tips with mAb 477, and at their shafts with p30 antibody. In F a growth-phase cell labeled for F-actin with TRITC-conjugated phalloidin after fixation with picric acid/formaldehyde is shown. Bar, 10 μm .

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1  HSI SLK I N I V G A N T V K L R F A P D N C I Q E C C T H I F E K T N E G G P O K G L Y Q A H I E K Q B A R W L A M E K T L Q F Y D I N S D Q Q L Y K K H R P Q F K L L D G T I K T G L V
101  D E S Q V S E I V N S I C K X O G I N P E E Y S I N S A C A M L N T Q I L S E G I S E N D I T V L M K O F F N D A N I D R N D P V Q L H I L L F V C R D G I I E G Y P P R E E S L A I S
201  A L Q C V Q L G D Y N P K P H P G F L T L K D Y L P L W L K S K G V E K D I F K H E K V L S M T E Y N A K Y T R V Q C R S L K T Y G M T S F D V K I R E Y G K K H V D H I G I T R E Q K L
301  L M L T E T K E V I N T H P L O H I R W A A T D K S F T L D F G D H E T E Y L L Q T F N P R Q I S Q L I O G Y I E I I M A R K D S S K V I E K E D T A N G V E E V M A V K G S V A N S S Y G
401  Y G A G G G A N G L Q P S Q O I P I D L K S A L R A T D L L I G E L G F R S S G A T P Q N F T R S P T L T P O O F K H L I S H T N M A I A A G G L F Q M T P P P G O I A A F Q G A I
501  T K R A Q I I M A E L N T V G T A A N A G Y F P N A S F S O E I I G V A T K L S E M A R L L A I G S T I Q O T C D E K S O K A A Q T E I P V Q S L V T I A A A C D N E Y T V D S S K L L I
601  E C A N N S A I A D N G L V V G N S V E P I D S L L L G Q I Q N T L K S T S L S D S E L L S T T E N L A S T S C H P E R K Q I T N I T O S A L N O S N A L L T A P K S G E I P P G O Y N L M A
701  R V S D I I E S V N L I N Y A M C S E R E Y K I S I T S N G V E V G E E I L A G N L T E E F A T V A N D L T N A I M T R S N L K N P O T V M E S Y O V A G H A N R L I T C T K A V A S R A D T
801  Q S Q R L F N S T N A V F S V A N L S N H C R S Y I K N P E Q A K T P Q I V E T A G H L Q P L T Q M S T D A G K I A C I T S L R D Y S K E M I A Q V S S I S T S R T S S Q V L P D A N G I T L
901  L K G A D V S D A L S K L M V G I R K R V L D P K S E A T O M E L L T L A Q K G S L P P H L V S T K R F A P K I S O P W Q K R L I F S S A A A G S V Q K L M A G E A T R R I C O H I E T E
1001  A L E V F D S T I A D L E T T E I A I A G G F L D A V S G I T R E G A E L L N V A I X D L N K V N N E L V D I R V N P A R L G D L V K S A T E S A S V A I S A K T L I C A T T C G Q V Q T K L M G
1101  I T Q K L I M B Q L T R A S R V S R N D R S E L L D R R S N O V S I S T A A L V G S T A N V O C K E L D E A S A D I S N L L K M G S L E S I L S Q P T E F A F Y E E I A S E T K A
1201  L N A S O Q V Y A M A N N K L G L G A X I T A S A L S T V S H A Q N A I V L T E N A T N A I L A S T V A L G Q Q I I G L L D P S K A R I A N Y K D P I Y D Q N I N O A K S V E D H V
1301  V G O R S L G O G D N N T I C D E A V D R I I E A T R S L K O T I L F D T S O L Q N A H I E D L H Q Q S L L A I T Q A S K L G S I T S M L V N S K N S D L V G S G S T A E R I I T M I E A A K K
1401  V N C S I S T N P D I L L P A K S I L D A S Q L T A N Q A D V N H V L S H A A T I A A C T Q L L G I T R A S Q F N E Q Q B Q Q V R D G I V K S T O O L A R A T S S L A R A V K S V T S R
1501  E P G A K A M I S Q S L Q L E S A I N H L I T S S V P A S E R G I G I A D F N K L M S T C R S V S T A S S Q L I S A S C S Q K P D I E L S S I L S E N A V L M T N S L K D I I K V T S B O P
1601  O V N F C E A I E I A Q R A I S D S S V A L S V A G S F D S A N N K E G L S V S E S Q R V L V V T K I O T I N D I L L A S R O S P E A I G I S A M A L S F I A P S L N V T T P A L A T A
1701  P D A Q A N D I V T E S R A V G S I L K L C Q A S L I A S N S P S K E T Q I I V N K C V D A S E A M S L W A G I S S G V N L Y K E L D E S L D R I R K S V V O T S A K D A P K D S N R G Y O E
1801  Y K E E L S N I T M L A L S L X T I V A T D G N L V S I S T I S K I A N Y I S D T A H V S A I L A T T S B O O K I R D S E I I T S S R Q V I V S T G D V N H I K V N S T D K A N S G A R V N D S
1901  Y R A N D I T R F L Q S L K O G A I G E I L S O A A T D Q I R K V I S D L G Y S L F A A A G L N D Q S S Q S T W E V T Q K H N K M Q D T T Q A K L I V S S S Q L V G S R P T O R
2001  K L G A T T V A N T V S S L V K T A M D I A S V L A D T T S Q Q I L S A S K A L S I S S Q Q V L A T X D A Q P F K D A T A F R S I L G K S A E R V A E D V G P L T S V Y T I S D A G K I X
2101  E L K S I V Q V A N Y H E K P O T V L S N X O A T E I I F A G A R D L A K S I E I V S Y T S S O G L Y K S S Q V V S V Q S F I S N S G V I A L L O N D L K S V L E N V Q T G O
2201  M L A L L Q C V D Q D N G S T S I A D A T R S I S D C V N S I V L K S L P G O Q N I V V E E D N V L E D L R A I A R D E L S A C A R S I E E A T A K L I A A R P O S K S N G K L D A B O V A A
2301  T I V A S S A I A R A A K L V N S A A V A G S K R E D Q I A S G S V K A P T S N G L I S A A G V G A A T H R L V E A A M S A T G K A B E E L I A T A S V A A T A L L V S A S R A X
2401  S G D Y O S Q A R H S L T A A R Q V A S A T S O L V A A A A A T I P D E Q Q E E Q P Q P M T O S K V E L B O Q R I L K L E K E L E T A R R Q M A S R Q N Y N N 2491

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Figure 3. Deduced sequence of the talin homologue of *D. discoideum*, consisting of 2,490 amino acids plus the terminal methionine. These sequence data are available from GenBank/EMBL/DDJB under accession number U14576.

its NH_2 -terminal portion not only to talin, but also to ezrin (Gould et al., 1989) and other members of the band 4.1 family, such as protein-tyrosine-phosphatase (Gu et al., 1991; Yang and Tonks, 1991), radixin (Funayama et al., 1991), and moesin (Lankes and Furthmayr, 1991). The COOH-terminal half of the *Dictyostelium* sequence is homologous to the sla2 protein from *Saccharomyces cerevisiae*, as is the COOH-terminal portion of mouse talin (Holtzman et al., 1993). Sla2 is a 109-kD protein of the actin skeleton required for normal cytokinesis of the yeast. A similar protein corresponding to the COOH-terminal half of mouse fibroblast and *Dictyostelium* talin has also been found in *Caenorhabditis* (Fig. 4, bottom).

Genomic Southern blots probed with clones covering the entire coding region indicated that the *Dictyostelium* talin is encoded by a single gene, *talA*. Northern blots hybridized with different clones of the coding sequence recognized a single transcript of 8 kb, which was detected during growth and aggregation, and in the slug and culmination stages at roughly the same amounts.

Three-dimensional Distribution of the *Dictyostelium* Talin in Growth-phase Cells

Since talin is an adhesion plaque protein in fibroblasts, we

| | |
|-------------|---|
| N-terminus: | |
| D.d. | MSLSLKNIVGANTVYKTRFAPDMITDEQCTHIFKTNK...GPNGLYQARI...EQQSRMLAMEYTLQFYDMSDQGVYKHKRPFKLDGDTI 95 |
| M.m. | HYALLSLKLS...GNYVYKTFQEPFHYDNCNMTREKTPDALGPPHMDGLFSDQDPKFI...MLRAGKALQYPLMANGDHYKTRKGRPKLKEDEQY 97 |
| D.d. | KTQLVDSQKYSVNSIKKNGIKNPFEEISLH...NSAGALNNTQI...SEQGISSEINIVYAKKP 158 |
| M.m. | KTIYVQDSKTYDMLNTICARIGITNIDREYVRELMEKRDDEGTGTRKDKVTLRDEKINRKLKQKLTQOELMGLDGRFTLRQGVKHEHTLILRRKF 197 |
| D.d. | FENDANIDRNPVQGLLFLVOCRDGIIIEGKYPTREESLSALQCVQVGLGYNPTKEPEGLTLDKVLSPQMLSKVKEKDIKPKHKLNSVTEVNAKY 258 |
| M.m. | FYSQVYSDPQVQLNLLVQARDI...LQNSHPVSEDKACPEAFQFQIQDGRPHRQKHAAGFLDLKDFLPEYVYQK...EERRTPQAKKNGQMSIEARY 296 |
| D.d. | RIVGLCSLSTYQKTSFQVYK...ZREYKAKKNDHDI...LITRQKMLMLTETKVTNHPKIKH...KMAATDKSFTLDFGSDHETVLLQTPNPKISQLTGGYI 358 |
| M.m. | RYYKLAASLSTYQVSPFLKKEKQDGRKMLVPLR...LITRQKMLMLTETKVTNHPKIKH...KMAATDKSFTLDFGSDHETVLLQTPNPKISQLTGGYI 396 |
| D.d. | EIIHAKRDSK...VIEKEDTANGVYEVYAKK 389 |
| M.m. | DIILKFKKXKXNHLQEGDEESTMLDQVYKPK 428 |
| C-terminus: | |
| D.d. | AATIVDASSATKAVAKLVNSAAVAGSKRRDDI...ASGIVYK...DPTNSGLI...SAAKGVCAATHRLV...EAAKKSATQKAE...ELIATARSVAATALLVSA 2346 |
| M.m. | EQIIEAKNSI...AATSAALVYKASAKQRELLAQKGVSA...PAHALDQDQSGGLI...SAAKRVYAA...NINLEAANA...VQGAASQKLI...SAAKRVYAA...STQGLVSA 2441 |
| D.d. | SRAKSDQYQAAHSHLS...TAAQVASATSDLVAAKAAATIFDQDQEEQEDQFNFTGSKYKLEQQK...ILKLEKLETARPKMNSRQKYNKN 2491 |
| M.m. | CVYKADQDSE...AMKRLQAAGNKKRPAQDNI...VYKAAAFEDQENETVYVYKRVYQIAQ...TAAQV...REKRELEAKR...LAQTRQOQVFLPFLSPLR 2538 |
| M.m. | DKR 2541 |
| D.d. | AATIVDASSATKAVAKLVNSAAVAGSKRRDDI...ASGIVYK...DPTNSGLI...SAAKGVCAATHRLV...EAAKKSATQKAE...ELIATARSVAATALLV 2394 |
| S.c. | ALMILDAVVALVAAKQ...QNEIATITTS...IFLQYK...KNSRMTGLI...SAAKAVAGATN...ITPASKFLIT...SEDNEN...TSPQFV...YAKRVAA...STQGLV 669 |
| D.d. | SASRAKSDQYQAAHSHLS...TAAQVASATSDLVAAKAAATIFDQDQEEQEDQFNFTGSKYKLEQQK...ILKLEKLETARPKMNSRQKYNKN 2491 |
| S.c. | AASRYKSTHEK...AQDLEKHSK...DVTACKSLGNV...KHSIEDHST...SQQQPLDFTSENTL...KTAEMTQD...VILKLEKLETARPKMNSRQKYNKN 965 |
| S.c. | DDD 968 |
| D.d. | AATIVDASSATKAVAKLVNSAAVAGSKRRDDI...ASGIVYK...DPTNSGLI...SAAKGVCAATHRLV...EAAKKSATQKAE...ELIATARSVAATALLV 2395 |
| C.e. | NESTLAWQALNSV...DNGVYASR...LQETI...VAAK...KAGS...PAE...YK...N...Q...T...GLL...SAAK...K...V...A...V...L...V...S...D...Q...V...Y...Q...F...G...R...E...N...L...V...A...G...E...L...A...S...T...Q...G...L...V 821 |
| D.d. | ABRAKSDQYQAAHSHLS...TAAQVASATSDLVAAKAAATIFDQDQEEQEDQFNFTGSKYKLEQQK...ILKLEKLETARPKMNSRQKYNKN 2491 |
| C.e. | SSRVNADKDSK...LDA...L...V...A...K...V...N...T...Q...V...A...V...N...Q...T...L...D...E...G...S...L...D...F...Y...L...S...L...A...K...K...R...E...S...V...K...L...E...Q...L...K...R...A...K...L...A...K...R...Q...Y...H...M...Q...L...V 918 |
| C.e. | NRVSP 923 |

Figure 4. Comparison of the *Dictyostelium* talin sequence (*D.d.*) with the NH₂-terminal and COOH-terminal regions of mouse talin (*M.m.*), and with the COOH-terminal regions of *Saccharomyces cerevisiae* Sla2p (*S.c.*) and a *Caenorhabditis elegans* protein (Waterston, R., accession number M98552) (*C.e.*). Similarities of the two NH₂-terminal sequences are most abundant in the stretch between residues 295 and 399 of the mouse sequence. In the *Dictyostelium* protein, a stretch of 37-amino acid residues corresponding to positions 131 to 167 of the mouse sequence is missing. Identical residues are indicated by lines, similar residues by double and single dots according to the algorithm of the UWGCG BESTFIT program.

explored accumulation of the *Dictyostelium* homologue in filopods attached or non-attached to a substratum. Accurate images of the pattern of immunolabeling in growth-phase cells were obtained by confocal microscopy after fixation of the cells with a mixture of picric acid and formaldehyde, which preserves the shape of *D. discoideum* cells more reliably than methanol does and allows satisfactory labeling with most of the antibodies we have tested (Brink, 1989; De Hostos et al., 1991, 1993b). Fig. 5 (A to F) shows confocal sections of a growth-phase cell where extensions of the cell surface that are in contact with the substratum can be distinguished from extensions that are free. Strong labeling is recognized at the tips of both extensions, indicating that it is not the contact with a surface that induces the talin to assemble.

To estimate the extent of talin accumulation in the filopod tips, the size of the thin, tapered filopods has to be related to the size of the diffraction patterns that limit optical resolution. In fact, the scanning electron micrograph of Fig. 5 G illustrates that the diameters of filopods are smaller than the diameters of their optical representations in the confocal sections A, E, or F. The false color code in these scans indicates

that the brightness in the pixels over the bulk cytoplasm and the filopod tips is about the same. The tip diameter of the filopods is about 150 nm (Claviez et al., 1986). Under the conditions of confocal microscopy used the size of an optical resolution element is $0.25 \times 0.25 \mu\text{m}$ in the x and y axes, and about $0.8 \mu\text{m}$ in the z direction. From these data a 14-fold higher fluorescence intensity in the small volume of the filopod tips than in the bulk cytoplasm is calculated, which means that the talin is enriched in the tips by more than one order of magnitude.

Filopods are extended and retracted in *Dictyostelium* cells in periods of less than a minute (Gerisch, 1964). Because of its rapid, strictly localized, and reversible accumulation, *Dictyostelium* talin can be taken as a marker for the reorganization of the actin skeleton that occurs during changes in cell shape and locomotion.

Chemoattractant Induces Local Talin Accumulation in Aggregating *Dictyostelium* Cells

During a starvation period of about 6 h, cells of *D. discoideum* develop from the growth phase to the aggregation-competent stage. In the course of preaggregative development, the cells not only express new proteins which are involved in aggregation, e.g., the csA cell-adhesion protein (Gerisch, 1987), but also change their shape and motility behavior. Relevant to this paper is the chemotactic responsiveness of aggregating cells to cAMP which is due to the developmentally regulated expression of the cAR1 type of cAMP receptors (Klein et al., 1987) and the $\alpha 2$ subunit of G proteins (Pupillo et al., 1989; Kumagai et al., 1991).

Expression of the talin in *Dictyostelium* proved not to be under developmental control, but its distribution became altered when the cells acquired, with the onset of aggregation, the capability to assume a strongly elongated cylindrical shape. In this polarized state the cells possess a well circumscribed front and only few filopods along their length. In the elongated cells of the aggregation stage, the talin was accumulated in the entire front region. This localization was particularly evident when the cells had started to aggregate into streams by end-to-end contacts (Fig. 6, A-D). In the multicellular slug the protein formed a cap on one end of the cells which probably pointed into the direction of movement (Fig. 6, E and F).

By local stimulation with cAMP through a micropipette, the polarity of aggregation-competent *D. discoideum* cells can be changed at will (Gerisch et al., 1975; Swanson and Taylor, 1982). One or two new fronts are elicited at the site of strongest stimulation, while the previous front is paralysed (Segall and Gerisch, 1989). To study redistribution of the talin during chemotactic orientation, we have recorded aggregation-competent cells at intervals before and during their stimulation through a micropipette, and finally have fixed and labeled them with antibody to visualize the talin at early phases of reorientation.

Two examples of chemotactically responding cells are shown in Fig. 7. In both cases a new front was induced towards the micropipette at the flank of the cells, where normally no distinct accumulation of the talin is found. After about half a minute of stimulation the new fronts were brilliantly labeled with the anti-talin antibody, much stronger than any other part of the cells including the previous front region.

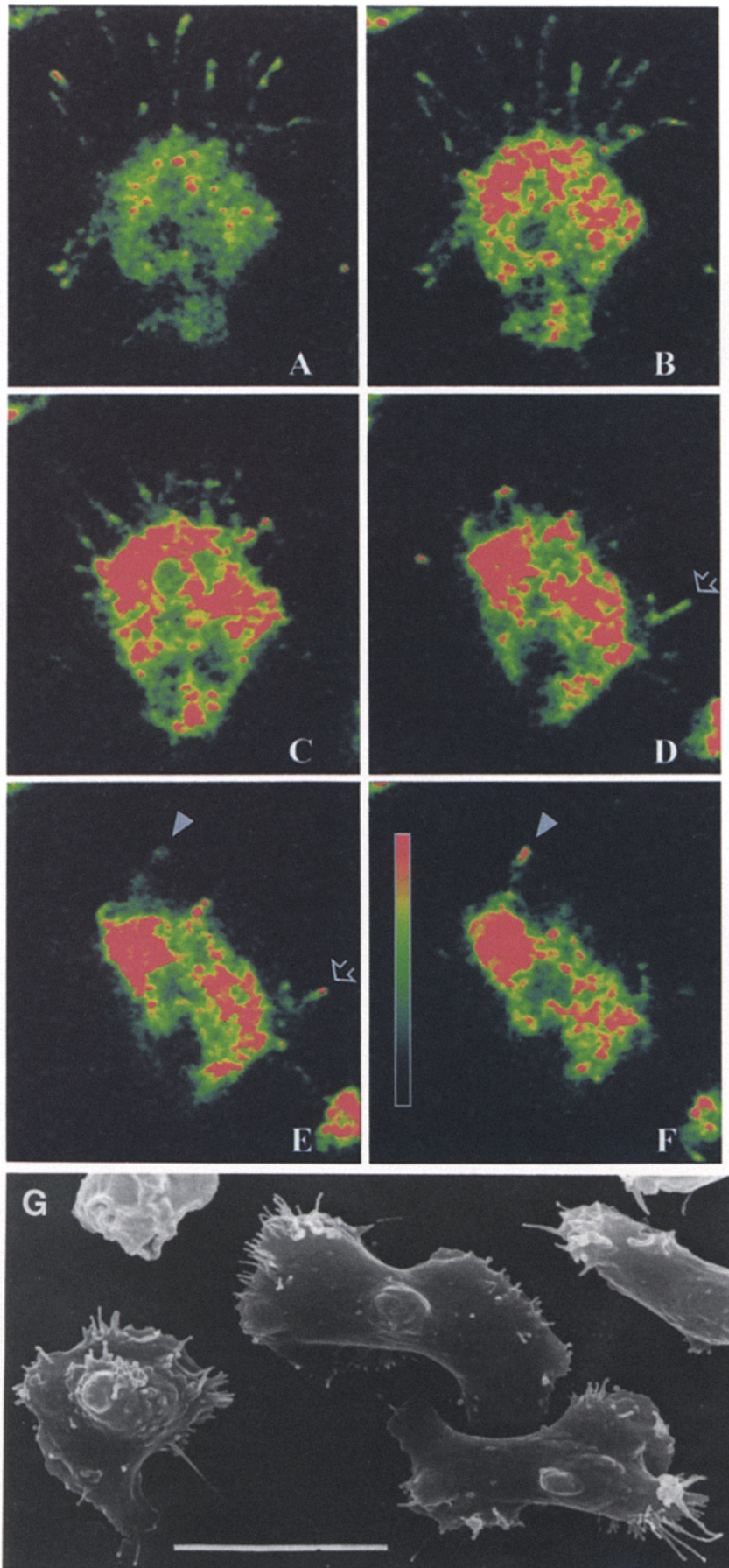


Figure 5. Three-dimensional distribution of talin in a growth-phase cell attached to a glass surface (*A-F*) as compared to filopod size and shape in similar cells of *D. discoideum* (*G*). The cell imaged in *A-F* was labeled with mAb 477 and Cy3-conjugated goat anti-mouse IgG. Confocal sections were taken from the bottom of the cell close to the substratum (*A*) to the free upper surface (*F*) at distances of $0.6\ \mu\text{m}$, except for *C* to *D* taken at $1.2\ \mu\text{m}$. Arrows indicate filopods that are not attached to the substratum. False colors from dark green to red indicate increasing intensities of labeling. The scanning electron micrograph of *G* has been adjusted to the same magnification as the confocal images. Bar, $10\ \mu\text{m}$.

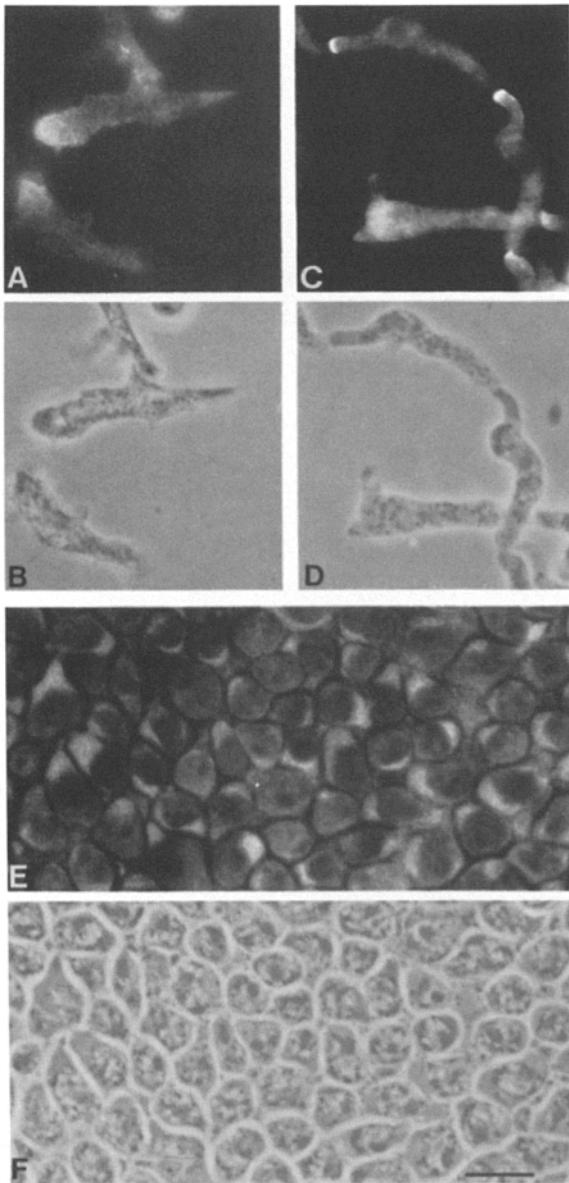


Figure 6. Distribution of *Dictyostelium* talin in developing cells. (A–D) Aggregating cells after 6 h of starvation; (E and F) cells from a squeezed slug. Cells were either fixed with picric acid/formaldehyde (A and B), or at -20°C with methanol (C–F), and labeled with mAb 477 and Cy3-conjugated goat anti-mouse IgG. (A, C and E) Immunofluorescence images; (B, D, and F) corresponding phase-contrast micrographs. Strong labeling of the talin at the leading edges of aggregating cells is shown to be independent of the type of fixation (A, C). In slug cells the talin is enriched in caps which coincide with hyaline regions free of organelles (E, F). The bar indicates $10\ \mu\text{m}$.

Discussion

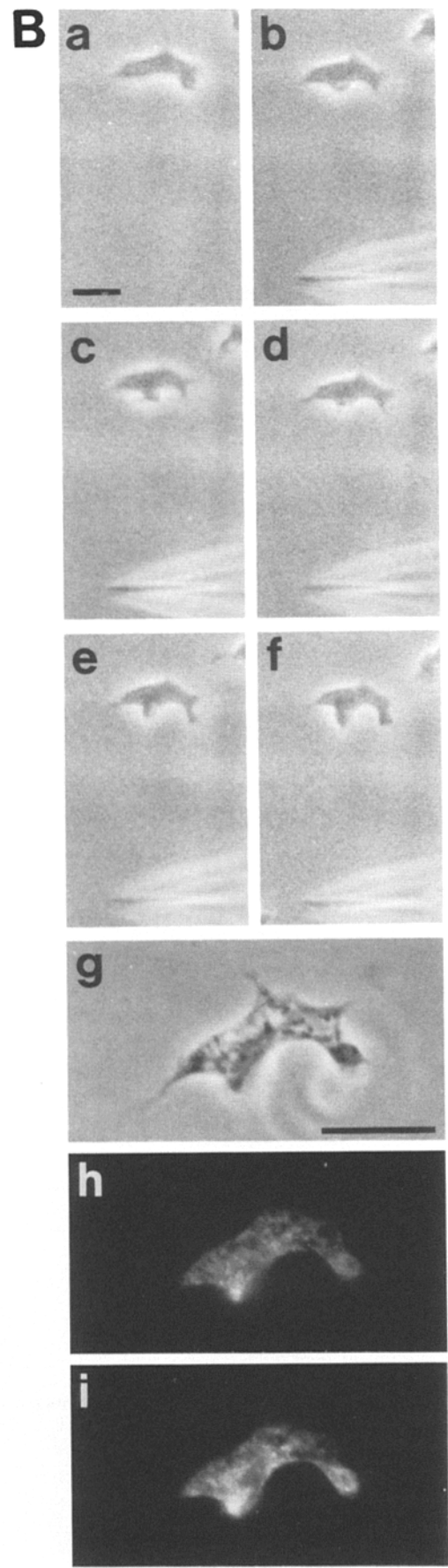
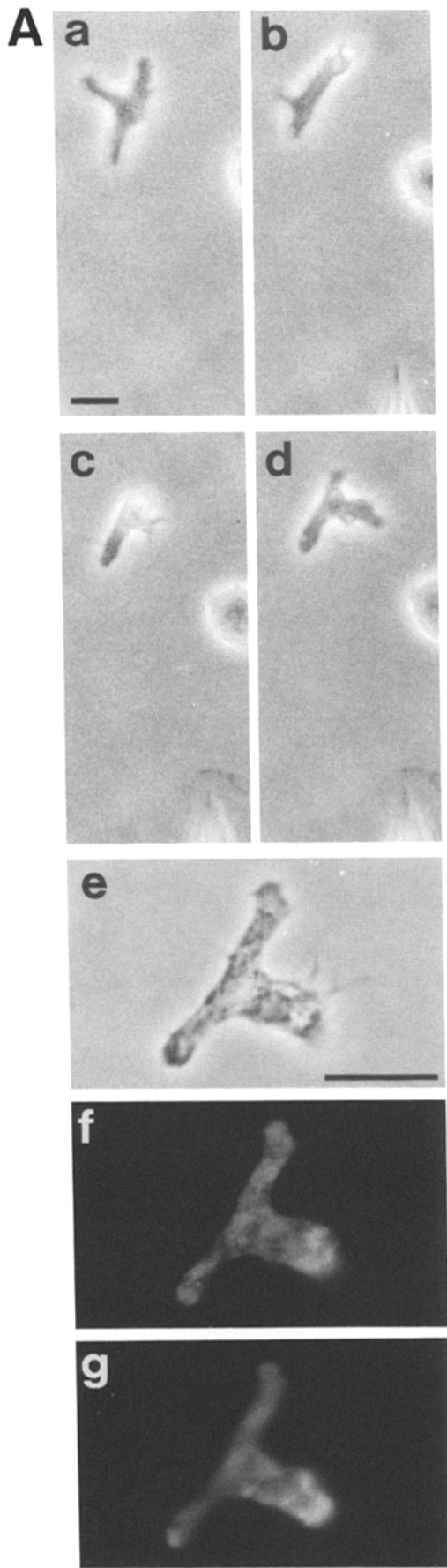
Talin of vertebrate cells is a dumbbell-shaped antiparallel homodimer (Goldmann et al., 1994). The polypeptide sequence indicates three regions in each subunit: an NH_2 -terminal head segment of about 47 kD which can be cleaved off by calpain II, a central region with high α -helix forming capacity, and a COOH -terminal domain which is rich in charged amino acids. The sequence of the central region in-

dicates a weakly periodic pattern of α -helical motifs that presumably form a tightly packed hydrophobic central strand, thus giving rise to the rod shape of the molecule (McLachlan et al., 1994).

In fibroblasts, talin is part of a complex of proteins assembled at sites of focal contact to anchor actin cables to integrins. Together with talin a number of soluble proteins including vinculin and α -actinin are assembled in the adhesion plaques (Burrige et al., 1988; Beckerle and Yeh, 1990; Geiger et al., 1990). The NH_2 -terminal head domain of mouse talin shows sequence similarities to the spectrin-binding protein band 4.1 of erythrocytes (Conboy et al., 1986) and to ezrin from microvilli (Gould et al., 1989). Since these proteins are linked to the plasma membrane, talin has been proposed to contain a membrane-binding site that is located in the head domain. In fact, direct evidence has been provided for binding of the NH_2 -terminal domain of platelet talin to acidic phospholipids (Niggli et al., 1994). Integrin- and vinculin-binding sites have been mapped to the rod region (Horwitz et al., 1986; Gilmore et al., 1993). For the binding of talin to actin, which is of low affinity, the head domain is also not required (Muguruma et al., 1990).

Our data provide the first evidence in a non-vertebrate cell for a full-length talin homologue that comprises not only the COOH -terminal region but also the NH_2 -terminal domain (Fig. 4). The presence of a talin homologue in *Dictyostelium* calls for a search for other adhesion plaque proteins as, for instance, vinculin, and integrins. Proteins similar to these fibroblast proteins may also in *Dictyostelium* form a functional complex together with actin. However, it is possible as well that talin has a separate function in *Dictyostelium*, not requiring the series of proteins present in adhesion plaques. Several observations are in line with this possibility. α -Actinin, an actin-binding component of adhesion plaques is also present in *Dictyostelium* cells but does not co-accumulate with talin at the tips of filopods (Brink, 1989). In fibroblasts, talin assembles at two types of loci: first beneath ribs of lamellipods that are rich in actin filaments, and de novo at sites of focal contact (Burrige and Connell, 1983a,b; DePasquale and Izzard, 1991). It is conceivable that the sites of talin accumulation in *Dictyostelium* are equivalent to the first type in fibroblasts, whereas the second type is missing. Even during the formation of adhesion plaques in fibroblasts, talin does not need vinculin in order to find its position, which means the accumulation of talin precedes that of vinculin.

The *Dictyostelium* homologue of talin we have identified proved to be a soluble cytoplasmic protein that transiently associates with the cytoskeleton during cell locomotion. Control of this association by chemoattractant was demonstrated by locally stimulating cells in the early aggregation stage with cAMP. This stimulation elicits a new leading edge followed by suppression of the previous one. After less than a minute of stimulation strong accumulation of the talin was found at the new front. Two principal questions are raised by these findings: how does the talin become located to the tips of filopods or, in aggregating cells, to the leading edge and, second, what is its function there? Talin is the only known *Dictyostelium* protein that is localized to the tips of filopods. Other proteins that transiently associate with the actin cortex in *Dictyostelium* cells either bind to cell surface extensions of any shape, like coronin (De Hostos et al.,



1991), or ABP50 (Dharmawardhane et al., 1991), or they assemble along the length of the filopods, like the 30-kD actin-bundling protein (Fechheimer, 1987). Based on these data we propose that the talin homologue of *Dictyostelium* contains a particular recognition site for signals that precisely target the protein to its assembly position. In growth-phase cells of *Dictyostelium* this position is the narrow space between the ends of actin bundles and the curvature of the plasma membrane (Claviez et al., 1986). In thrombin-activated platelets changes in cell shape are accompanied by the shift from a uniform distribution to a submembranous location of talin (Beckerle et al., 1989). This redistribution is known to be accompanied by the phosphorylation of talin (Bertagnoli et al., 1993). If phosphorylation is a general mechanism of regulating the association of talin with other proteins (Tidball and Spencer, 1993), it may also be responsible in *Dictyostelium* for the chemoattractant-elicited recruitment of talin from the cytoplasm which is reminiscent of the redistribution in platelets.

Analysis of the role of talin in *Dictyostelium* cells has to wait for gene disruption or other mutants. Nevertheless, several functions relevant to *Dictyostelium* are suggested by the activities of vertebrate talin. Because of its actin- and lipid-binding activity, vertebrate talin has the potential of linking actin filaments directly to membranes. As a dimer, talin is capable of bridging the barbed ends of actin filaments, and is also capable of nucleating actin filament formation (Kaufmann et al., 1992). These activities of talin may be relevant to the formation of filopods or, in aggregating *Dictyostelium* cells, to the extension of the leading edge. It appears to be important that forces generated by actin polymerization are transmitted to the membrane in order to push a filopod rapidly out of the plane of the cell surface. If so, the bundles of growing actin filaments need to be tightly fixed at their ends to small areas of the plasma membrane, and talin might be involved in this anchorage. Evidence has been provided that nucleation of actin polymerization is important for the protrusion of a leading edge (Condeelis, 1992, 1993). A rise in nucleating activity has been found within 5–10 s upon stimulation of *D. discoideum* cells with cAMP (Hall et al., 1989), and more than a single protein including talin might be responsible for this increase.

Finally, we wish to emphasize a technical point. The detection of talin in *Dictyostelium* demonstrates the usefulness of mutants that lack the most avid actin-binding proteins (Schindl et al., 1995) for the identification of new proteins associated with the actin cytoskeleton.

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Figure 7. Distribution of talin after orientation of *D. discoideum* cells towards the chemoattractant cAMP. Two experiments (A and B) are shown; in each of them a stimulated cell had formed a new front. Fluorescent labeling with mAb 477 visualizes accumulation of the talin at the new fronts induced by the attractant. In A, the unstimulated cell moving to the top was photographed at 40 s before insertion of the pipette (a). Photographs of b to d were taken at 2, 27, and 38 s after the insertion. Immediately thereafter the cell was fixed for labeling with mAb 477. (e) Phase-contrast image; (f and g) fluorescence images showing talin accumulation at the newly formed leading edge. For f the focus was near to the substratum, for g to the upper surface of the cell extending into the free fluid space. In B, the cell had moved to the right-hand side before stimulation. Upon stimulation it extended two competing pseudopods towards the micropipette, one from its initial front region. Photographs were taken at 20 s before insertion of the micropipette (a), and at 1, 10, 21, 28, and 44 s after insertion (b–f). The cell fixed and labeled with mAb 477 is shown in phase contrast (g) and in fluorescence images focused near to the substratum (h) and close to the cell's upper surface (i). Talin is most strongly accumulated in the new front region, but also enriched in the competing pseudopod at the right-hand side. Bars, 10 μ m.

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