

Functional Differences between L- and T-Plastin Isoforms

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Abstract. Fimbrins/plastins are a family of highly conserved actin-bundling proteins. They are present in all eukaryotic cells including yeast, but each isoform displays a remarkable tissue specificity. T-plastin is normally found in epithelial and mesenchymal cells while L-plastin is present in hematopoietic cells. However, L-plastin has been also found in tumor cells of non-hematopoietic origin (Lin, C.-S., R. H. Aebersold, S. B. Kent, M. Varma, and J. Leavitt. 1988. *Mol. Cell. Biol.* 8:4659–4668; Lin, C.-S., R. H. Aebersold, and J. Leavitt. 1990. *Mol. Cell. Biol.* 10: 1818–1821). To learn more about the biological significance of their tissue specificity, we have overproduced the T- and L-plastin isoforms in a fibroblast-like cell line, CV-1, and in a polarized epithelial cell line, LLC-PK1. In CV-1 cells, overproduction of

T- and L-plastins induces cell rounding and a concomitant reorganization of actin stress fibers into geodesic structures. L-plastin remains associated with microfilaments while T-plastin is almost completely extracted after treatment of the cells with non-ionic detergent. In LLC-PK1 cells, T-plastin induces shape changes in microvilli and remains associated with microvillar actin filaments after detergent extraction while L-plastin has no effect on these structures and is completely extracted. The effect of T-plastin on the organization of microvilli differs from that of villin, another actin-bundling protein. Our experiments indicate that these two isoforms play differing roles in actin filament organization, and do so in a cell type-specific fashion. Thus it is likely that these plastin isoforms play fundamentally different roles in cell function.

THE actin-based cytoskeleton is involved in a variety of cellular processes such as cytokinesis, cell interactions, and transmembrane signaling in addition to its well-known functions in cell motility and cell organization. Depending on the functions performed by a given cell type and its subcellular domains the actin microfilaments display specific arrangements. They can be as diverse as the highly ordered actin filament core bundles found in brush border microvilli and stereocilia or the network of cross-linked microfilaments present in the subcortical actin cytoskeleton. These different organizations are achieved by different classes of actin-binding proteins associated with the filaments.

Fimbrin is a 68-kD protein that has the ability to bundle microfilaments *in vitro*. It was first identified as a component of the microvilli of intestinal cells and subsequently found in a variety of cell types where it is associated with polarized actin filaments in membrane ruffles, filopodia, stereocilia, and cell adhesion sites (Bretscher and Weber, 1980a, 1981; Bretscher, 1981; Glenney et al., 1981). However, determination of the primary structure of chicken intestinal fimbrin has revealed that this protein is a member of a family including

human L- and T-plastin previously identified as proteins induced by transformation in neoplastic human fibroblasts (Goldstein et al., 1985; Lin et al., 1988, 1990; de Arruda et al., 1990). L-plastin is found in normal or transformed hematopoietic cells where it can be phosphorylated in response to treatment with interleukins or phorbol esters (Goldstein et al., 1985; Matsushima et al., 1988; Zu et al., 1990). In adherent macrophages, this protein is constitutively phosphorylated on a serine residue present in the head piece domain (Messier et al., 1993). T-plastin is present in epithelial and mesenchymal cells but neoplastic cells derived from these tissues synthesize both T- and L-plastins. It has recently been reported that a third member, I-plastin, is a human fimbrin isoform restricted to absorptive intestinal and kidney cells, two cell types which display a highly organized brush border (Lin et al., 1993, 1994). These three proteins are highly homologous as I-plastin shares 73 and 75% identity with L- and T-plastin, respectively (Lin et al., 1988, 1990, 1994). Moreover, a yeast homologue of fimbrin (SAC6p) has been identified (Adams et al., 1991). The SAC6p gene product (SAC6p) is a dominant suppressor of a temperature-sensitive mutation of actin. It is 44 and 38% identical to the human L- and T-plastin, respectively. Mutants lacking SAC6p do not have normal actin cables, lose asymmetry in cortical actin distribution and are defective in both morphogenesis (Adams et al., 1989, 1991) and endocytosis (Kübler and Riezman, 1993).

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Fimbrins/plastins have a modular organization. They contain an amino-terminal domain (10 kD) with two potential Ca^{2+} binding sites flanked by EF-hand motifs, and two repeated domains which are homologous to the putative actin-binding domains of actin filament cross-linking proteins (Matsudaira, 1991). In vitro studies have shown that fimbrin purified from chicken intestinal epithelium has the ability to arrange F-actin filaments in bundles wherein all filaments exhibit the same polarity. This activity was dependent on ionic strength but was insensitive to calcium in physiological range (Bretscher, 1981; Glenney et al., 1981). Experiments performed with I-plastin, the human homologue of chicken fimbrin, have shown that the bundling properties of this protein are negatively regulated by calcium (Lin et al., 1994). Similar results have been obtained with L-plastin. Therefore, Ca^{2+} concentrations in the micromolar range indeed inhibit the association of L-plastin with actin filaments (Pacaud and Derancourt, 1993) and its ability to bundle these filaments (Pacaud and Harricane, 1987; Namba et al., 1992). In addition, L-plastin is capable of distinguishing between actin isoforms, as it has been shown to efficiently bundle β actin but not α or γ actin isoforms (Namba et al., 1992).

The observation that these highly homologous isoforms of plastin/fimbrin are present in various cell types and cell compartments, and the possibility that they associate with different actin filaments suggest that they are functionally different. To test this hypothesis, we have transfected fibroblast-like CV-1 cells and an epithelial cell line (LLC-PK1) with the cDNAs encoding the human L- or T-plastins, and analyzed their subcellular distribution and avidity for actin filaments after mild treatment with a non-ionic detergent. The transfected species were tagged with an epitope to discriminate from the endogenous protein. These experiments show that T- and L-plastin isoforms associate with different actin structures. Their ability to induce changes in microfilament organization is shown to depend on the cell type in which each isoform was overproduced. In addition, we show that the effect of T-plastin on filament organization differs from that of villin, a multifunctional actin-binding protein present in brush border microvilli (Bretscher and Weber, 1979, 1980b; for review see Friederich et al., 1990).

Materials and Methods

Cell Culture

CV-1 monkey kidney cells (CCL 70; American Type Cell Culture [ATCC], Rockville, MD) and LLC-PK₁ pig kidney cells (CL 101; ATCC) were grown in Dulbecco's minimum essential medium supplemented with 10% fetal calf serum, at 37°C, under a 10% CO_2 atmosphere. Caco-2 human colon adenocarcinoma cells (HTB 37; ATCC) were grown in the same conditions except that the medium was supplemented with nonessential amino acids.

Antibodies

The monoclonal antibody, P5D4, raised against the 11-amino acid carboxy terminus of the vesicular stomatitis virus glycoprotein G was previously described (Kreis, 1986) and was kindly provided by Dr. T. Kreis (University of Geneva, Switzerland). The monoclonal antibody, VII-E-7, raised against the 13-amino acid carboxy terminus of the Sendai virus L protein was previously described (Einberger et al., 1990) and was a kind gift of Dr. J. Neubert (Max-Planck-Institut für Biochemie, Martinsried, Germany). Rabbit polyclonal antibodies were raised against the 11-amino acid carboxy termi-

nus of the vesicular stomatitis virus glycoprotein G and against the 13-amino acid carboxy terminus of the Sendai virus L protein. An affinity-purified polyclonal anti-villin antibody (1-135) has been previously described (Maunoury et al., 1988). The monoclonal anti-human vinculin antibody was a kind gift of Dr. B. Geiger (Weizmann Institute of Science, Rehovot, Israel). Texas red-linked donkey anti-rabbit antibody and the fluorescein-linked sheep anti-mouse IgG were purchased from Amersham Corp. (Arlington Heights, IL).

Reagents

Rhodamine-conjugated phalloidin was obtained from Sigma Chemical Co. (St. Louis, MO). Restriction enzymes were purchased from Amersham Corp. and Ampli Taq Polymerase from Cetus Corp. (Berkeley, CA).

Recombinant DNA Constructs

Isolation of T-plastin cDNA. Total RNA was isolated from Caco-2 cells by the guanidium isothiocyanate method (Chirgwin et al., 1979) and enriched for poly(A)⁺ RNA by passage over oligo dT cellulose (Aviv and Leder, 1972). cDNA was synthesized from 1 μg of poly(A)⁺ RNA according to the method described by Gubler and Hoffman (1983) using an oligonucleotide complementary to the 3'-end of human L-plastin mRNA (5' TCACACCTCTTCATTCCTT 3') as a specific primer for the first strand (Lin et al., 1988). A λ gt 10 cDNA library was generated and screened with a 60 nucleotide-long ³²P-labeled RNA probe corresponding to the position 450–510 of T-plastin mRNA (Lin et al., 1988). The complete coding sequence and the 5'-noncoding sequence encoding the T-plastin were obtained from two overlapping clones. Partial sequence and restriction map indicated that this cDNA encodes the human T-plastin.

Isolation of L-plastin cDNA. Human plastin cDNA containing the complete coding sequence was obtained by amplification of the cDNA generated by reverse transcription of mRNA extracted from the Caco-2 cell line. The first strand reaction contained 5 μg mRNA, 20 μmol of random hexanucleotides, 200 μM of each dNTP, 20 units of placental RNAase inhibitor, 50 mM Tris-HCl (pH 8.3), 10 mM MgCl_2 , 50 mM KCl, and 40 units of AMV reverse transcriptase. The mixture was incubated at 42°C for 1 h, heated at 95°C for 10 min, treated with 10 μg of RNase DNase free, and then precipitated. The pellet was resuspended in 50 μl water and 10 μl were used for amplification. Amplification was carried out with two different sets of primers leading to two overlapping cDNA fragments encoding the complete human L-plastin. In the first amplification, the sense primer 5' AAGCTGTCTGCAGATCAGA 3' corresponded to the nucleotides 37–56 and the reverse primer 5' CCTGCTGCAGCATGCATTCT 3' was complementary to the nucleotides 1139–1158 of human L-plastin cDNA (Lin et al., 1988). In the second amplification, the sense primer 5' TGCTGCAGCAGGCGGAGAG 3' corresponding to the nucleotides 1147–1164 and the reverse primer 5' CGGGATCCTCACACCTCTTCATTCTCT 3' flanked at its 5'-end of an EcoRI site were complementary to the oligonucleotides 2003–2021 of human L-plastin cDNA. Amplification of the cDNA fragment was performed using a Perkin-Elmer thermal cycler (The Perkin-Elmer Corp., Norwalk, CT) and the cycling parameters were 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min for 25 cycles. The first amplified fragment was digested by PstI and the second by PstI-EcoRI endonucleases. The two fragments were ligated through the PstI site in the EcoRI-PstI sites of the Bluescript-KS plasmid (Stratagene, La Jolla, CA). The complete human L-plastin cDNA sequence was checked by double-strand sequencing using the T7 Sequencing Kit (Pharmacia Fine Chemicals, Piscataway, NJ). One discrepancy was found at nucleotide 1669 (the G was changed into a T) with the published sequence (Lin et al., 1988). This nucleotide substitution did not result in a change of the corresponding amino acid.

Construction of the T-plastin-VSV G Fusion cDNA. T-plastin was tagged by incorporating an oligonucleotide encoding the 11 carboxy-terminal amino acids of the vesicular stomatitis virus glycoprotein G upstream the termination codon (Kreis, 1986). This oligonucleotide is flanked at the 5'-end by SmaI and SfiI sites and at the 3'-end by XbaI, BglII and KpnI restriction sites (see Fig. 3 A). To ligate the 3'-end of the T-plastin cDNA coding sequence with this oligonucleotide, the T-plastin cDNA starting at the unique BglII site and ending upstream of the translation termination codon was amplified. The oligonucleotides used for the amplification were designed to allow the two fragments to be ligated across the SmaI site and to allow the deletion of the translation termination codon. The sense primer (5' TCCTGGAAGATCTTGGG 3') corresponded to the nucleotides 1613–1629 and the reverse primer (5' ATCCGGGACACTCTCTTCAT-TCCCCTG 3') was complementary to the bases 1959–1978 of plastin cDNA and flanked at its 5'-end by a SmaI site. The amplification was carried out

according to the conditions described above and the amplified fragment was digested by BglII and SmaI restriction endonucleases and purified on a 1.5% agarose gel. The BglII-SmaI fragment of the T-plastin cDNA was replaced by the corresponding BglII-SmaI amplified fragment. The sequence of the amplified fragment was checked by double-strand sequencing. The T-plastin cDNA excised from the plasmid after digestion with EcoRI-SmaI was then ligated to the SmaI-XbaI oligonucleotide encoding the tag sequence and present in the KS plasmid. The T-plastin cDNA fused to the tag sequence was then inserted into the HindIII-XbaI restriction sites of the expression vector pCB6, downstream from the cytomegalovirus promoter.

Construction of the L-plastin-SVL Fusion cDNA. L-plastin was tagged by incorporating an oligonucleotide encoding the 13 carboxy-terminal amino acids of the Sendai virus L protein (Einberger et al., 1990). This oligonucleotide is flanked at its 5'-end by a SmaI restriction site and at its 3'-end by XbaI and BglII restriction sites (see Fig. 3 B). To ligate the 3'-end of the L-plastin cDNA coding sequence with this oligonucleotide, the L-plastin cDNA starting upstream of the HindIII site in position 1503 and ending upstream of the translation termination codon was amplified. The sense primer (5' GTTCCTGTTGACTGGA 3') corresponded to the nucleotides 1430-1445 of L-plastin cDNA and the reverse primer (5' GGCCCGGGCACCCTCTT 3') was complementary to the nucleotides 2018-2010 of L-plastin cDNA and flanked at its 5'-end by a SmaI site. The amplification was carried out according to the conditions described before. After digestion by HindIII and SmaI endonucleases and purification, the fragment was ligated to the tag sequence through the SmaI site in the KS plasmid (KS-L-Tag). The sequence of the amplified fragment was checked by double-strand sequencing. To obtain the complete L-plastin cDNA fused to the tag, the KpnI-ClaI fragment was inserted into the plasmid KS-L-tag digested by KpnI-ClaI (see Fig. 3 B). The L-plastin cDNA fused to the tag sequence was then inserted in the KpnI-XbaI site of the expression vector pCB6, downstream from the cytomegalovirus promoter.

Bacterial Expression of Plastin Isoforms and Bundling of F-actin

The L- and T-plastin cDNAs, the T-plastin-VSV-G fusion cDNA and the L-plastin-SV-L fusion cDNA were cloned into the BamHI-EcoRI sites of the plasmid expression vector pGEX-2T (Smith and Johnson, 1988). A BamHI site was introduced upstream of the ATG initiation codon so that plastin isoforms could be expressed as fusion proteins with the glutathione S-transferase. Pure plastin was then recovered after cleavage of the fusion protein with human thrombin (Sigma Chem. Co.) according to the procedure described by Smith and Johnson (1988).

The ability of the four recombinant proteins to bundle F-actin was tested in vitro with rabbit F-actin and human platelet F-actin. Rabbit skeletal muscle actin was prepared according to the method of Spudich and Watt (1971) and was a kind gift from Dr. J. Vandekerckhove (University of Ghent, Belgium). Human platelet actin was prepared from a platelet acetone powder essentially following the procedure described by Weber et al. (1992). The actin containing fractions, recovered after filtration through a Sephadex G-150 column, were subjected to a cycle of polymerization and depolymerization according to Spudich and Watt (1971) to obtain pure actin.

G-actin from rabbit muscle or human platelets (12.5 μ M) was polymerized for 3 h in the presence of 1 mM MgCl₂ and 40 mM KCl. F-actin (1.25 μ M) was mixed with various amounts of purified L- and T-plastins and incubated overnight in a buffer containing 10 mM N-(2-hydroxyethyl) piperazine-N'-(2-ethane-sulfonic acid) (Hepes), pH 7, 40 (α -actin) 100 (β -, γ -actin) mM KCl, 1 mM MgCl₂, 3 mM EGTA, 1 mM dithiothreitol, and 0.5 mM ATP. The reaction mixtures were loaded onto a carbon-coated grid and negatively stained with 1% uranyl acetate. Filaments were observed with a Zeiss 109 electron microscope.

Northern Blot Analysis

Total cellular RNAs were prepared from CV1 and LLC-PK1 cells by the guanidium isothiocyanate method (Chirgwin et al., 1979) and enriched for poly(A)⁺ RNAs with the Dynabeads mRNA purification kit (DynaL A. S., Oslo, Norway). mRNAs were fractionated through formaldehyde-agarose gels, and the blots were hybridized with ³²P-labeled RNA probes. Blots were prehybridized at 55°C for 16 h in 50% formamide, 4 \times SSC, 0.05 M Na₂HPO₄, pH 7.4, 1 \times Denhardt's solution, 500 μ g/ml tRNA. Hybridization with ³²P-labeled RNA probes (2 \times 10⁶ cpm/ml) was carried out for 18 h at 55°C in the same solution. Blots were washed twice in 2 \times SSC, 0.1% SDS, once in 0.2 \times SSC, 0.1% SDS at 65°C for 30 min and finally once in 0.1 \times SSC, 0.1% SDS for 30 min at 70°C.

Transient cDNA Expression in CV1 and LLC-PK1 Cells

Exponentially grown cells were seeded 24 h before DNA transfection on 3.5-cm plastic culture dishes. DNA transfer was performed following the procedure of Chen and Okayama (1987). Cells were first incubated in the presence of the calcium-phosphate DNA precipitate for 18 h under 3% CO₂. After washing with PBS, fresh medium was added. Production of the proteins encoded by the transfected cDNAs was analyzed after a 40 h incubation under 10% CO₂.

Microinjection

Cells were plated on 1.2-cm diameter glass coverslips. Microinjection was performed 48 h after plating using a semi-automated microinjection system (Eppendorf) and microinjection capillaries. DNA (0.1 mg/ml) mixed with 0.25% FITC-coupled dextran (150,000; Sigma Chem Co.) was injected in sterilized water. Cells were analyzed 18-20 h after microinjection.

Immunoblotting

The transfected cells plated on 3.5-cm diameter dishes were washed with PBS, lysed, and scraped off with a rubber policeman in presence of 100 μ l Laemmli buffer (Laemmli, 1970). Proteins were separated by electrophoresis on a 7% SDS-polyacrylamide gel under reducing conditions. Electrophoretic transfer to nitrocellulose and immunolabeling of the proteins were performed essentially according to the method described by Burnette (1981) and modified by Coudrier et al. (1983).

Indirect Immunofluorescence

Transfected cells were fixed with 3% paraformaldehyde. CV1 and LLC-PK1 cells were permeabilized for 5 min with 0.2% and 0.5% Triton X-100, respectively. Fimbrin isoforms encoded by the transfected DNA were analyzed by indirect immunofluorescence in two sequential incubation steps. Cells were first incubated with monoclonal anti-peptide VSV-G or anti-peptide SV-L or polyclonal anti-villin antibodies depending on the transfected DNA. Second, cells were incubated with fluorescent mouse or rabbit anti-IgG antibodies (conjugated to either rhodamine or fluorescein). Fluorescent double labeling of the cell surface and plastin isoforms and double-labeling of F-actin and plastin isoforms were performed as previously described (Friederich et al., 1989). Depending on the experiment, cells were viewed either with a Zeiss axiophot microscope or a CLSM Leica confocal laser microscope.

Detergent Extraction

Two approaches were used to determine the association of the produced proteins with the cytoskeleton (Kreis, 1987).

Indirect Immunofluorescence of Detergent-extracted Cells. Cells were briefly washed with PBS containing 0.1 mM CaCl₂ and 0.1 mM MgCl₂. The coverslips were then dipped four times for 2 s in the case of CV1 cells and for 10 s in the case of LLC-PK1 cells in four different beakers each containing 30 ml extraction buffer (50 mM 2-[N-morpholino] ethane sulfonic acid, 3 mM EGTA, 5 mM MgCl₂, 0.5% wt/vol Triton X-100, pH 6.4). Cells were then fixed immediately with a 3% paraformaldehyde solution.

Cell Fractionation into Detergent-soluble and Insoluble Fractions. CV1 and LLC-PK1 transfected cells (3.5-cm dishes) were extracted on the dish for 10 and 40 s respectively at room temperature with 300 μ l of the extraction buffer described above. Detergent-soluble fractions were precipitated 4 h in 85% acetone at -20°C and the pellets recovered after centrifugation 10 min at 4°C. Detergent-insoluble material was solubilized in Laemmli buffer as described above. Soluble and insoluble material was resuspended in the same volume of Laemmli buffer and the same volume was loaded onto the separation gel.

Scanning Electron Microscopy

Scanning electron microscopy was carried out on cultures grown on glass cover slips. After fixation with 2% glutaraldehyde in cacodylate buffer, cultures were postfixed in 2% OsO₄ aqueous solution and dehydrated in a graded series of ethanol incubations (from 70-100%). Wet coverslips were transferred into Freon 113 and dried after substitution with liquid CO₂ in a Balzers critical point drier. Dried cultures were coated with gold by means of a Polaron gold sputter coater. Samples were viewed with a Jeol 35CS scanning electron microscope.

Results

T- and L-plastins in Fibroblast-like (CV-1) and Epithelial (LLC-PK1) Cell Lines

Isoform Synthesis in CV-1 and LLC-PK1 Cells. Because it was previously reported that T- and L-plastins display a cell type-specific distribution (Lin et al., 1988; de Arruda et al., 1990), we studied the presence of these two isoforms in CV-1 and LLC-PK1 cells, using Northern blot analysis and immunochemical detection of cell extracts. Fig. 1 A, lane 3 shows that L-plastin mRNA (3.7 kb) and T-plastin mRNA (3.4 kb) are both detected in the LLC-PK1 cell line, although the level of T-plastin is higher. In addition, this cell line contains I-plastin m-RNA (data not shown). In contrast, only T-plastin mRNA could be detected with the mRNA extracted from the CV-1 confluent cells (Fig. 1 A, lane 2). When the mRNA was extracted from nonconfluent CV-1 cells, a faint band corresponding to the L-plastin mRNA could be detected after a longer exposure—24 h instead of 12 h (Fig. 1 A, lane 1).

Western blot analysis was performed with a polyclonal antibody raised against T-plastin produced in bacteria. This antibody detected a single band of 68 kD in the CV-1 cell extract (Fig. 1 B, lane 1) while an additional band approximately twice the size of plastin and of variable intensity could be seen in the LLC-PK1 cell extract (Fig. 1 B, lane 2).

Immunofluorescent Localization of Endogenous Plastins in CV-1 Cells. Fig. 2 shows an immunofluorescence

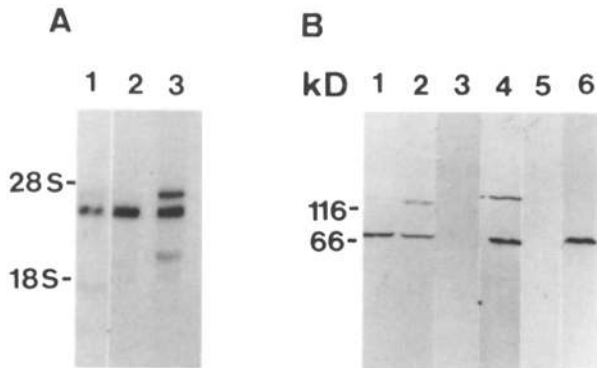


Figure 1. (A): Northern blot analysis. 0.8 μ g of poly(A)⁺ mRNA prepared from nonconfluent CV-1 cells (lane 1), confluent CV-1 cells (lane 2), and LLC-PK1 cells (lane 3) were separated by gel electrophoresis, transferred onto nitrocellulose membrane and hybridized sequentially with cRNA probes coding for T- and L-plastins. The 28S and 18S are indicated on the left. (B) Immunoblot analysis of protein lysates from CV-1 and LLC-PK1 cells and CV-1 cells transfected with plastin cDNAs. The presence of plastin in CV-1 (lane 1) and LLC-PK1 cells (lane 2) was revealed by immunoblotting with the polyclonal anti-plastin antibodies. The protein encoded by the L-plastin cDNA was detected with the monoclonal anti-peptide SV-L antibody (lane 4) and the protein encoded by the T-plastin cDNA was detected with the monoclonal anti-peptide VSV-G antibody (lane 6). Monoclonal anti-peptide SV-L antibody (lane 3) and monoclonal anti-peptide VSV-G antibody (lane 5) were tested on untransfected CV-1 cells.

double-labeling pattern of CV-1 cells stained with a polyclonal anti-plastin antibody (Fig. 2, A and B) and phalloidin (Fig. 2, A' and B'). Diffuse plastin staining was observed at the cell cortex, likely associated with the cortical actin cytoskeleton (Fig. 2 B). Strong staining was also observed in filopodia (Fig. 2 B) as well as at the tip of actin cables in the focal points (Fig. 2 A). In stress fibers, discontinuous staining could be seen along some actin filaments (Fig. 2 A).

Monitoring the Synthesis of T- and L-plastins in Cells Transfected with the cDNAs

cDNA Constructs and Protein Analysis. To study the intracellular distribution of plastin isoforms, recombinant cDNAs encoding the L- and T-isoforms were inserted in the eukaryotic expression vector pCB6, under the control of the cytomegalovirus promoter. Because both proteins are present in the cell lines studied and because polyclonal antibodies raised against one isoform cross-react with the other, epitope tagging was used to discriminate between the endogenous and transfected proteins. The eleven carboxy-terminal amino acids of the vesicular stomatitis virus glycoprotein G preceded by a proline-rich secondary structure breaker (GPPGP) were linked in frame to the carboxy terminus of T-plastin upstream of the stop codon. The thirteen carboxy-terminal amino acids of the Sendai virus L protein preceded by three amino acids (PGP) were linked in frame to the carboxy-terminus of L-plastin upstream of the stop codon (Fig. 3, A and B). CV-1 cells transfected with these two constructs synthesized a fusion protein detectable by immunoblot analysis with monoclonal antibodies directed against the tag corresponding to the carboxy terminus of the viral protein L (Fig. 1 B, lane 4) or of the glycoprotein G (Fig. 1 B, lane 6). However, the monoclonal antibody directed against the carboxy-terminus of the viral L protein recognized, in addition to a protein of 68 kD corresponding to L-plastin, a band of about twice its size (lane 4) absent from cell lysates prepared from untransfected cells (lane 3). This band of higher molecular weight appears to be specific for L-plastin, since it is detected with the polyclonal antibody in LLC-PK1 cells and not in CV-1 cells which contain a low level of this isoform. Moreover, it is only detected with the monoclonal antibody directed against the SV-L tag in cells transfected with the cDNA encoding the L-plastin.

Actin-bundling Activity of L- and T-isoforms. To test whether the addition of an epitope at the carboxy-terminal end of these proteins affected the cross-linking activity of the plastin isoforms, we tested the ability of the T- and L-plastin isoforms with and without the tag to bundle actin filaments. Electron microscopy of negatively stained samples showed that T-plastin with or without the tag formed bundles with rabbit α -actin (Fig. 4, B and C) as well as with platelet actin (data not shown). As previously reported (Namba et al., 1992), we could not observe filament bundles with L-plastin incubated with rabbit α -actin (data not shown). However, when L-plastins with or without the tag were incubated with platelet actin made up of the β - and γ -isoforms, we could observe actin bundles (Fig. 4, E and F). In the absence of these actin bundling proteins, only single filaments were observed (Fig. 4, A and D). These experiments indicate that the epitope tags did not affect the bundling activity of the plastin isoforms.

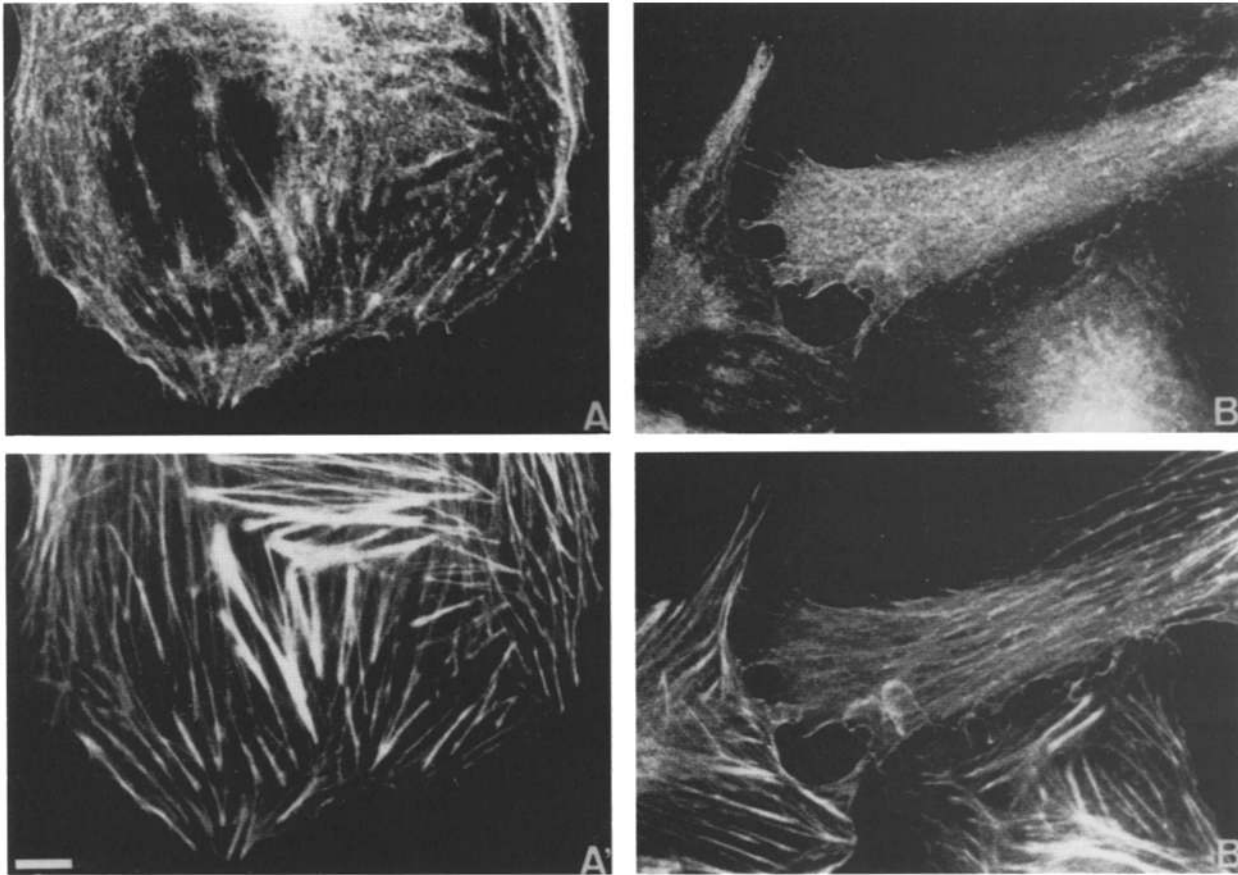


Figure 2. Immunolocalization of plastin in CV-1 cells. Fluorescent double labeling with anti-plastin antibodies and fluorescein-coupled anti-rabbit IgG antibodies (*A* and *B*) and rhodamine-coupled phalloidin (*A'* and *B'*) was performed on CV-1 cells fixed and permeabilized with Triton X-100. (*A* and *A'*) Focal plane at the bottom of the cells. Note that plastin colocalizes with the tips of the stress fibers and a discontinuous staining is also observed along connecting fibers. (*B*) Note that diffuse cortical staining is observed. Bar, 10 μm .

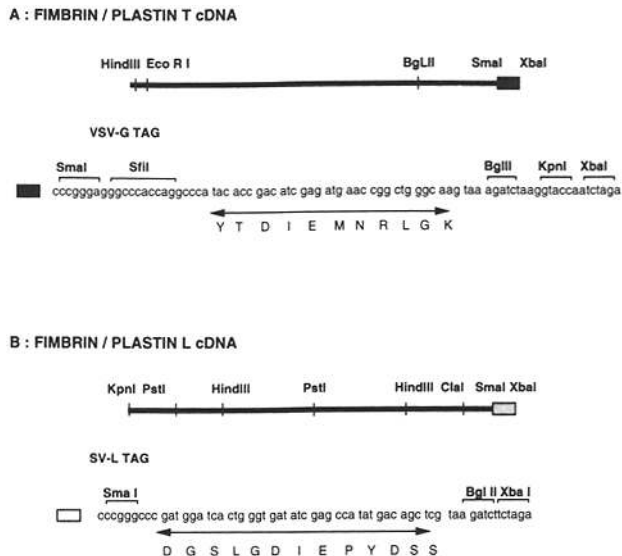


Figure 3. T- and L-plastin cDNA constructs. (*A*) Schematic representation of the cDNA construct encoding tagged T-plastin. The T-plastin cDNA was fused through the SmaI site to an oligonucleotide encoding eleven amino acids of the vesicular stomatitis virus glycoprotein G tail, referred to as the VSV-G tag and represented by a black box (Kreis, 1986). Only the restriction enzyme sites that

Different Behavior of T- and L-plastin Isoforms in Fibroblast-like CV-1 Cells

L- and T-plastin Isoforms Are Differentially Extracted with Detergent in Transfected CV-1 Cells. To understand the functional differences between the plastin isoforms, we have compared their subcellular distributions and their effects on actin cytoskeleton organization in transfected CV-1 cells. Transfection of CV-1 cells with either L-plastin (Fig. 5 *A*) or T-plastin cDNAs (Fig. 5 *B*) led to the synthesis of high levels of protein as indicated by the relative immunofluorescence intensity between transfected and untransfected cells using the polyclonal antibody. The measurement of the fluorescence intensity was determined with images of cells reconstructed from the software devised on the confocal microscope. A fivefold excess of T-plastin was synthesized in cells transfected with T-plastin cDNA compared with endogenous

were used for the constructions are indicated. (*B*) Schematic representation of the cDNA construct encoding the tagged human L-plastin. The L-plastin cDNA was fused through the SmaI site to an oligonucleotide encoding 13 amino acids of the Sendai virus protein L tail, referred to as the SV-L tag and represented by a light box (Einberger et al., 1990).

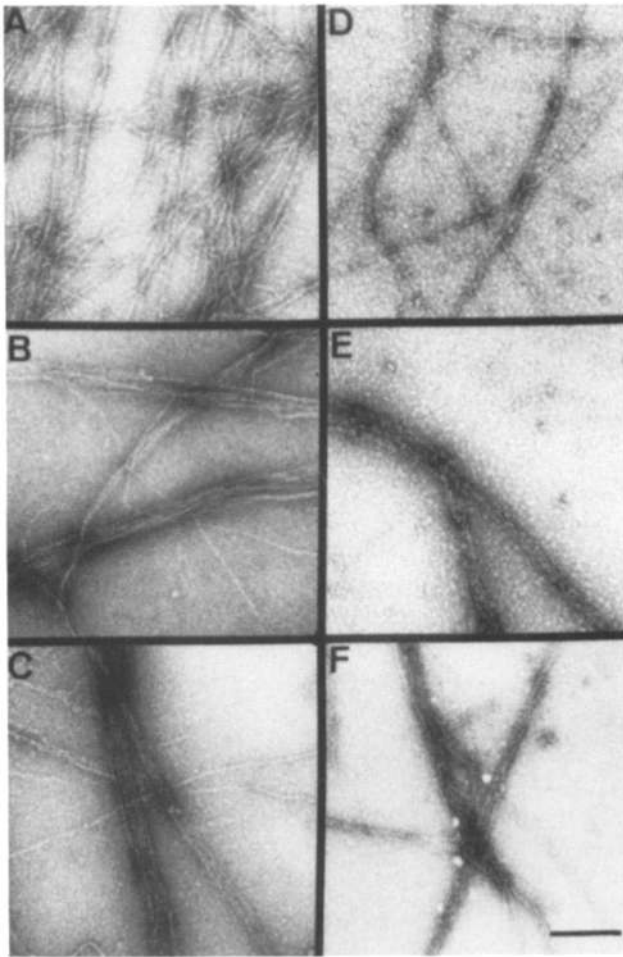


Figure 4. Actin bundling activity of recombinant plastins. Electron micrographs of negatively stained rabbit muscle F-actin (A–C) or platelet F-actin (D–F). F-actin from rabbit muscle (A) was incubated with recombinant T-plastin (B) or with recombinant tagged T-plastin (C). Platelet F-actin (D) was incubated with recombinant L-plastin (E) or with recombinant tagged L-plastin (F). Bar, 0.2 μm .

plastins, while a threefold excess of L-plastin was synthesized over endogenous. Due to an excess of unbound plastin in the cytosol, a clear colocalization of these isoforms with F-actin structures was only visible in the cortical region of the cells.

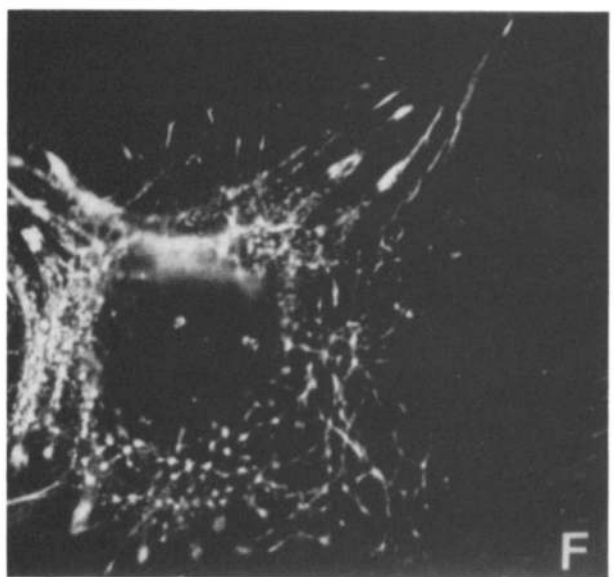
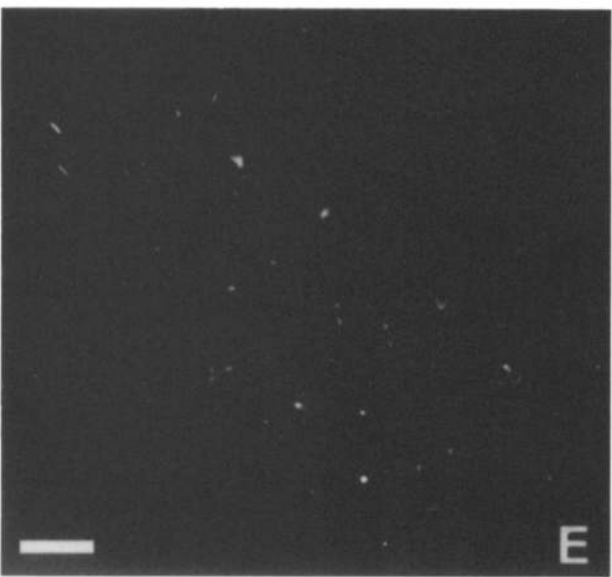
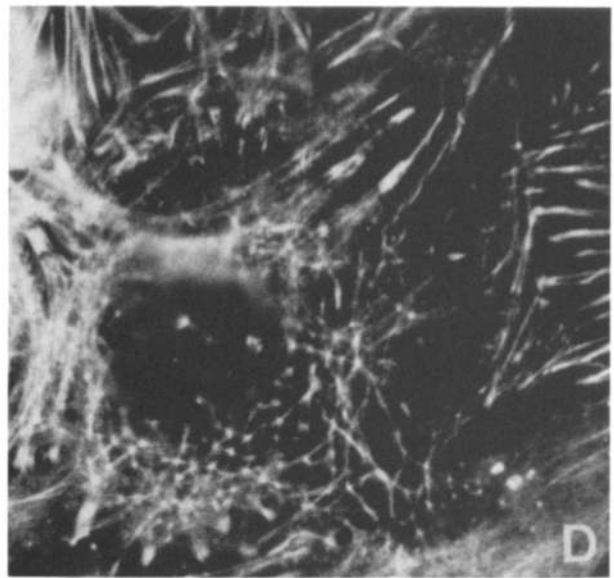
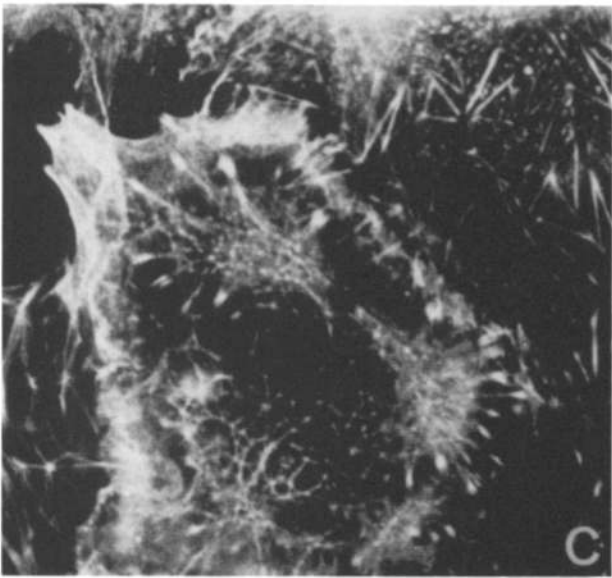
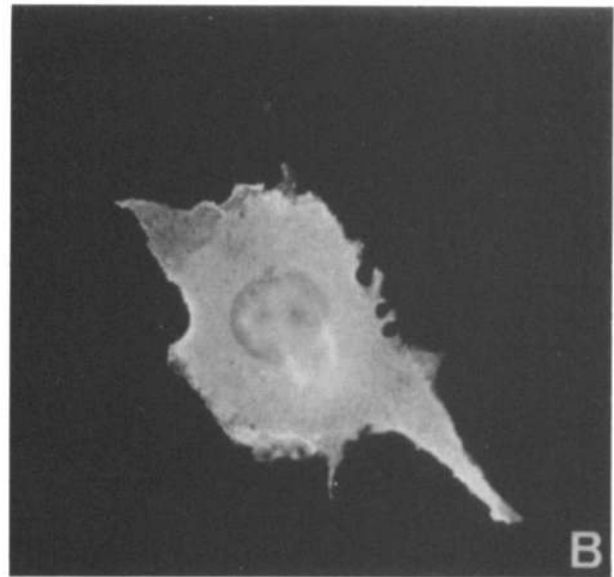
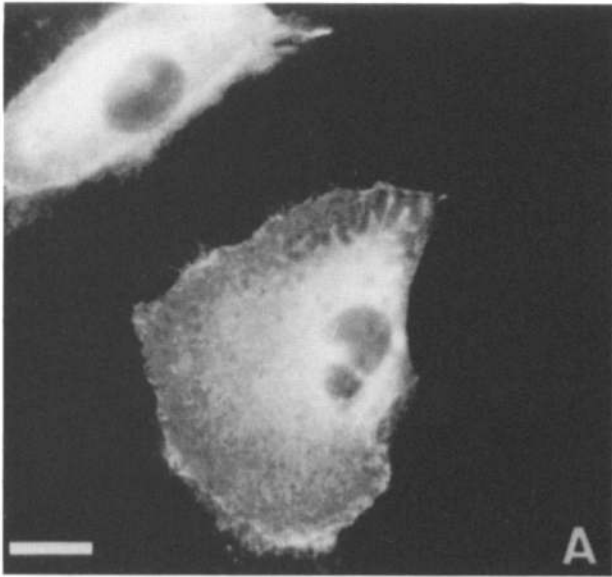
To evaluate the association of these isoforms with the different actin structures we used a qualitative approach whereby transfected cells are preextracted with a non-ionic detergent (Triton X-100) before fixation. This procedure en-

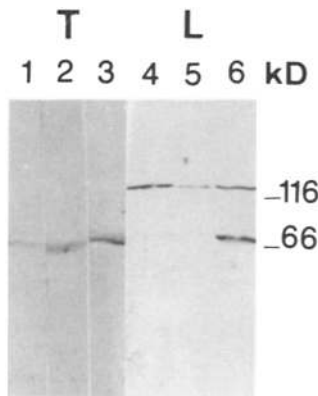
ables the extraction of membrane and cytosolic proteins while tightly cytoskeleton-associated proteins remain attached (Kreis, 1987). This simple assay allows one to evaluate the avidity of actin-binding proteins for microfilaments. Following the extraction, cells transfected with the plastin cDNAs were analyzed by double immunofluorescence with anti-tag antibodies and fluorochrome-conjugated phalloidin. Phalloidin staining in cells transfected with T- or L-plastin cDNAs indicated a conspicuous reorganization of stress fibers into a polygonal network forming geodesic structures (Fig. 5, C and D), reminiscent of structures observed at the early stages of cell spreading (Lazarides, 1976). Often, these structures assembled above the nucleus (data not shown). However, a major difference in the association of the T- and L-plastin isoforms with actin filaments was obtained after detergent extraction. In CV-1 cells transfected with T-plastin cDNA, the corresponding protein was nearly completely extracted, while in few cells, residual labeling appeared to be restricted to focal contacts (Fig. 5 E). In contrast, CV-1 cells transfected with L-plastin cDNA showed a strong association of this protein with the vertices of the actin network, as well as a strong labeling at the tip of the fibers extending beyond the focal plane (Fig. 5 F). In addition, a discontinuous fluorescent staining of the filaments which attach to the vertices was noted (data not shown).

These observations were confirmed by fractionating transfected CV-1 lysates into detergent soluble and insoluble material (Fig. 6). Immunoblotting performed with antibodies directed against the two different tags on soluble and insoluble fractions indicated that T-plastin was almost completely recovered in the soluble fraction (lane 2) while the major part of L-plastin remained associated with the insoluble pellet (lane 4). In the latter case, an important proportion of L-plastin migrated as a dimer.

Overproduction of Plastin Isoforms Is Frequently Accompanied by a Diminution of Focal Contacts. Immunofluorescent detection of transfected CV-1 cells indicated that high levels of plastin isoforms led to a partial rounding-up of the cells. In addition, both proteins were found concentrated on the ventral face of these cells, presumably in focal contacts. To test whether the plastin isoforms colocalized with and had an effect on focal contacts, cells microinjected with cDNAs encoding these two isoforms were stained with anti-tag antibodies and with an antibody recognizing vinculin, a protein known to be associated with focal contacts (Geiger et al., 1980). Double immunofluorescent staining was analyzed by confocal microscopy and focal contacts were visualized by reflection interference microscopy. In most cells transfected with the T-plastin cDNA and extracted prior to fixation (Fig. 7, A, C, and E), double labeling of the cells with anti-tag (Fig. 7 A) and anti-vinculin antibodies

Figure 5. Differential association of plastin isoforms with microfilaments. CV-1 cells were microinjected with cDNAs coding for T- or L-plastin. 24 h after microinjection, cells were fixed and permeabilized with Triton-X100 (A and B) or extracted with non-ionic detergent before fixation (C–F). (Left) cells transfected with T-plastin cDNA. (A) Labeling of nonextracted cells with anti-peptide VSV-G antibody and fluorescein-coupled anti-mouse IgG antibodies revealed a strong diffuse signal due to overproduction of T-plastin. (C and E) Double-labeling with anti-peptide VSV-G antibody and fluorescein-coupled anti-mouse IgG antibodies (E) and rhodamine-coupled phalloidin (C). (Right) Cells transfected with L-plastin cDNA. (B) Diffuse staining of nonextracted cells with anti-peptide SV-L antibody. (D and F) double labeling with anti-peptide SV-L antibody and fluorescein-coupled anti-mouse IgG antibodies (F) and rhodamine-coupled phalloidin (D). L-plastin colocalized with focal contacts and the vertices of microfilaments. Bar, 10 μm .





detergent soluble (2 and 5) and insoluble fractions (1 and 4) as well as lysates from nonextracted cells (3 and 6) were separated on a 7% polyacrylamide gel and the proteins were transferred onto nitrocellulose filters.

(Fig. 7 C) indicated that these two proteins colocalized in focal contacts. The fluorescent spots matched the areas corresponding to the adhesion plaques observed by reflection-interference microscopy (Fig. 7 E). The most striking effect observed, however, was a significant decrease in the number and size of the focal contacts in many transfected compared to untransfected cells (data not shown). In addition, these focal contacts were detected mainly at the periphery of the cells. Similar observations were made with cells overproducing L-plastin (Fig. 7, B, D, and F).

Specific Changes Associated with T- and L-plastin Production in a Polarized Epithelial Cell Line (LLC-PK1)

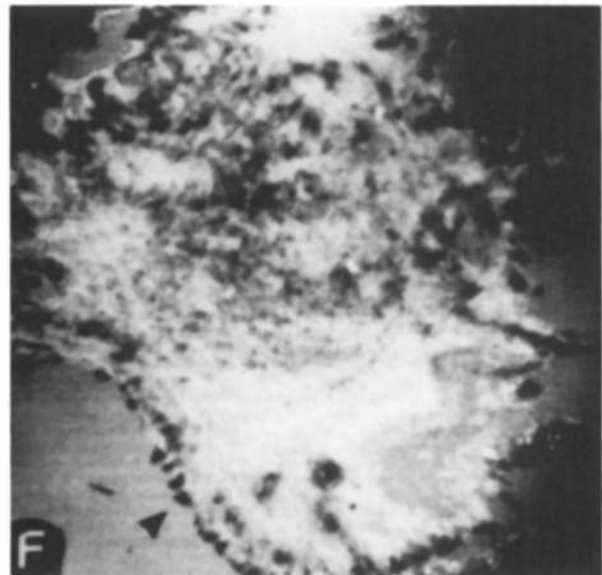
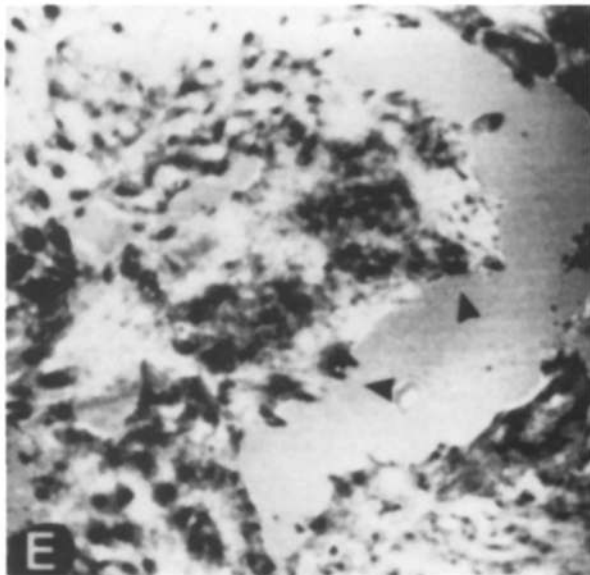
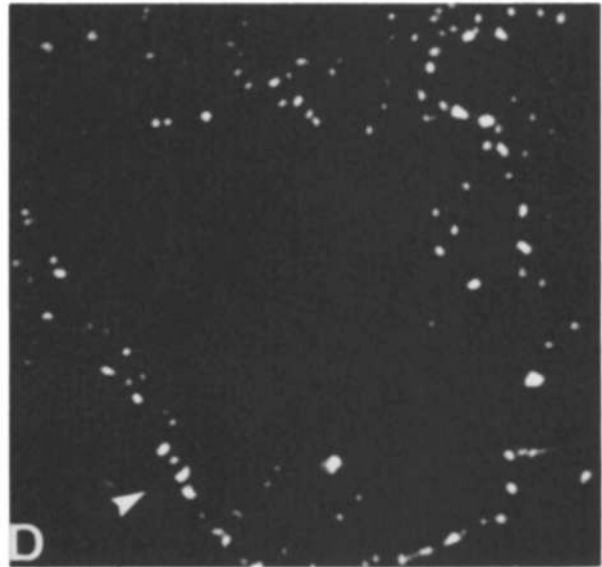
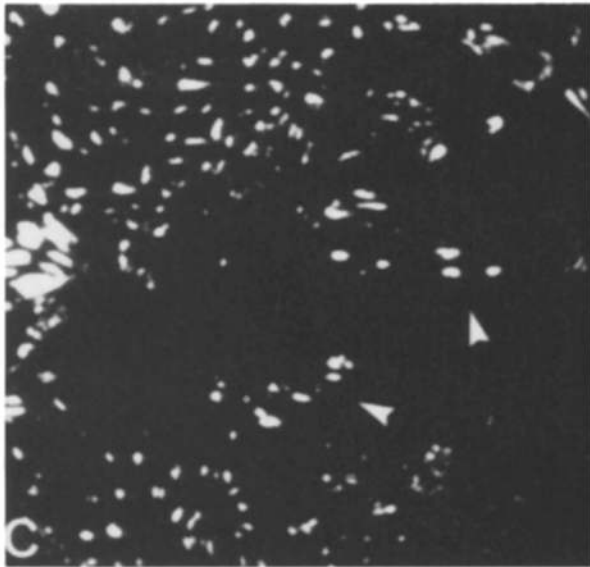
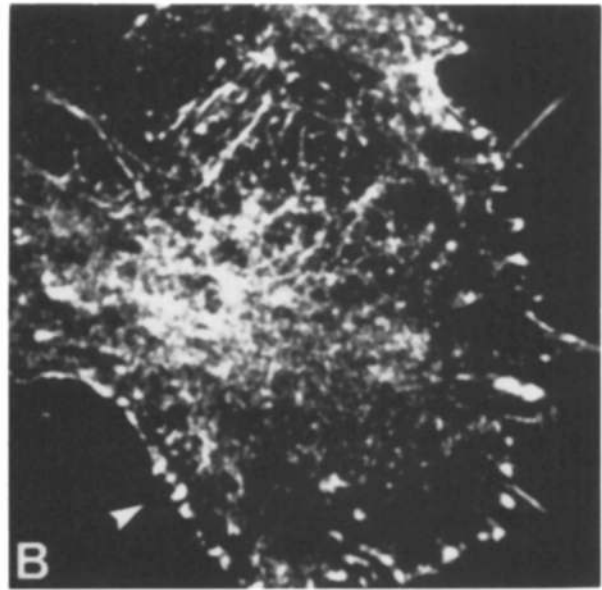
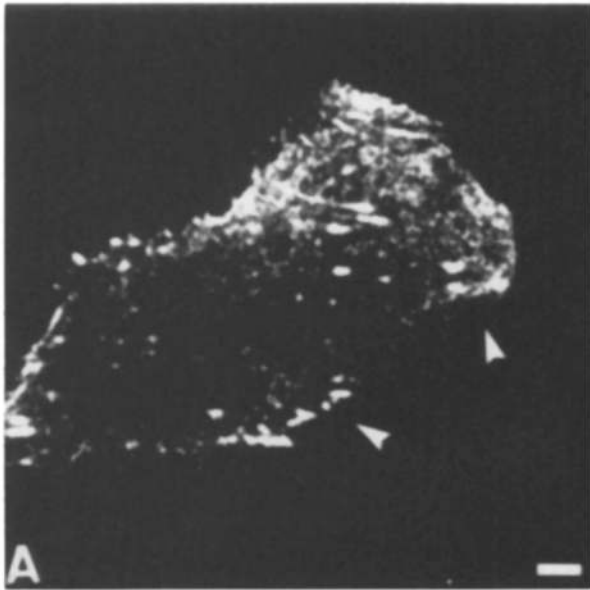
We have assessed the effects of L- and T-plastins in the polarized epithelial cell line, LLC-PK1 which is derived from kidney proximal tubule. These cells display a functional asymmetry specified by the underlying cytoskeleton. In particular, these cells assemble highly organized microvilli on their apical surface when they reach confluency. These microvilli, which form a structure called the brush border, contain uniformly polarized bundles of actin filaments to which fimbrin is associated. We have compared the effects of L- and T-plastins to that of villin, another actin-bundling protein associated with the actin cytoskeleton of the brush border microvilli and which has been shown previously to play an important role in brush border assembly (Friederich et al., 1989). In the experiments reported below, low density LLC-PK1 cells were transfected and just reached confluency upon analysis. At this state of confluency, cells displayed short microvilli on their surface, as illustrated in scanning electron microscopy experiments (see Fig. 11).

Effect of T-plastin and Villin on the Organization of Actin Cytoskeleton in LLC-PK1 Cells. Cells transfected with

cDNAs encoding L-plastin, T-plastin, or villin (Friederich et al., 1989) were analyzed by double immunofluorescence staining with anti-tag or anti-villin antibodies and rhodamine-conjugated phalloidin so as to compare the effect of overproduction of these bundling proteins on the organization of the microvillar actin cytoskeleton (Fig. 8). L-plastin colocalized with various actin structures including actin filaments of microvilli, but did not induce any changes in the organization of actin cytoskeleton (data not shown). In contrast, LLC-PK1 cells transfected with the T-plastin cDNA exhibited actin-containing structures on their surface which were thicker and longer than those of the adjacent nontransfected cells (Fig. 8, A and B). It is interesting to note that these actin-containing extensions were primarily located at cell-cell contacts. In villin transfected cells, we could observe very long actin-containing structures which were, in contrast to those found in T-plastin producing cells, distributed all over the apical surface (Fig. 8, C and D). To evaluate the association of L- and T-plastins with microvillar microfilaments, the transfected cells were preextracted before fixation as previously described. While T-plastin was essentially associated with the cytoskeleton of the microvilli present at the cell surface (Fig. 8 B), L-plastin was completely extracted (data not shown). This was confirmed by immunoblot analysis of soluble and insoluble fractions obtained after treatment of the cells with the non-ionic detergent Triton X-100. L-plastin was found almost exclusively in the soluble fraction (Fig. 9, lane 6), while a significant amount of T-plastin was found in the insoluble pellet (Fig. 9, lane 2).

Villin and T-plastin but Not L-plastin Induce Modification of Brush Border Microvilli. Analysis of LLC-PK1 cells transfected with villin or T-plastin cDNAs revealed a modification of the actin cytoskeleton on the apical surface of these cells (Fig. 8). The modifications of the cell surface were analyzed by two different methods: (a) immunofluorescence analysis of the apical surface of the LLC-PK1 cells transfected with plastin or villin cDNAs. Fluorescent double-labeling of transfected cells was carried out with fluorochrome conjugated wheat germ agglutinin, which allowed the observation of cell surface organization (Fig. 10, A, C, and E) along with anti-tag or anti-villin antibodies (Fig. 10, B, D, and F). These experiments indicated that L-plastin had no effect on cell surface organization, as cells transfected with L-plastin were covered by short microvilli identical to that observed on the cell surface of adjacent control cells (Fig. 10, A and B). In contrast, T-plastin positive cells exhibited thick microvilli on their surface primarily concentrated at the cell-cell contact (Fig. 10, C and D). However, cell surface organization induced by overproduction of T-plastin was different from that observed in villin positive cells (Fig. 10, E and F). In LLC-PK1 cells, villin induced the formation of long membrane extensions over their entire apical surface (Fig. 10, E and F). (b) Scanning electron microscopy. To monitor the changes in the shape of the microvilli observed after transfection of LLC-PK1 cells, sub-

Figure 7. Confocal immunofluorescence double labeling of plastin isoforms and vinculin in focal contacts. CV-1 cells were microinjected with either T-plastin cDNA (A, C, and E) or L-plastin cDNA (B, D, and F), detergent extracted and subjected to immunofluorescence analysis. (A and B) Cells stained with polyclonal anti-peptide VSV-G (A) or anti-peptide SV-L antibodies (B) and fluorescein-coupled anti-rabbit IgG antibodies. (C-D) Cells stained with a monoclonal anti-vinculin antibody and rhodamine-coupled anti-mouse IgG antibodies. (E and F) The same cells were examined by reflection-interference microscopy to localize focal contacts. Bar, 5 μ m.



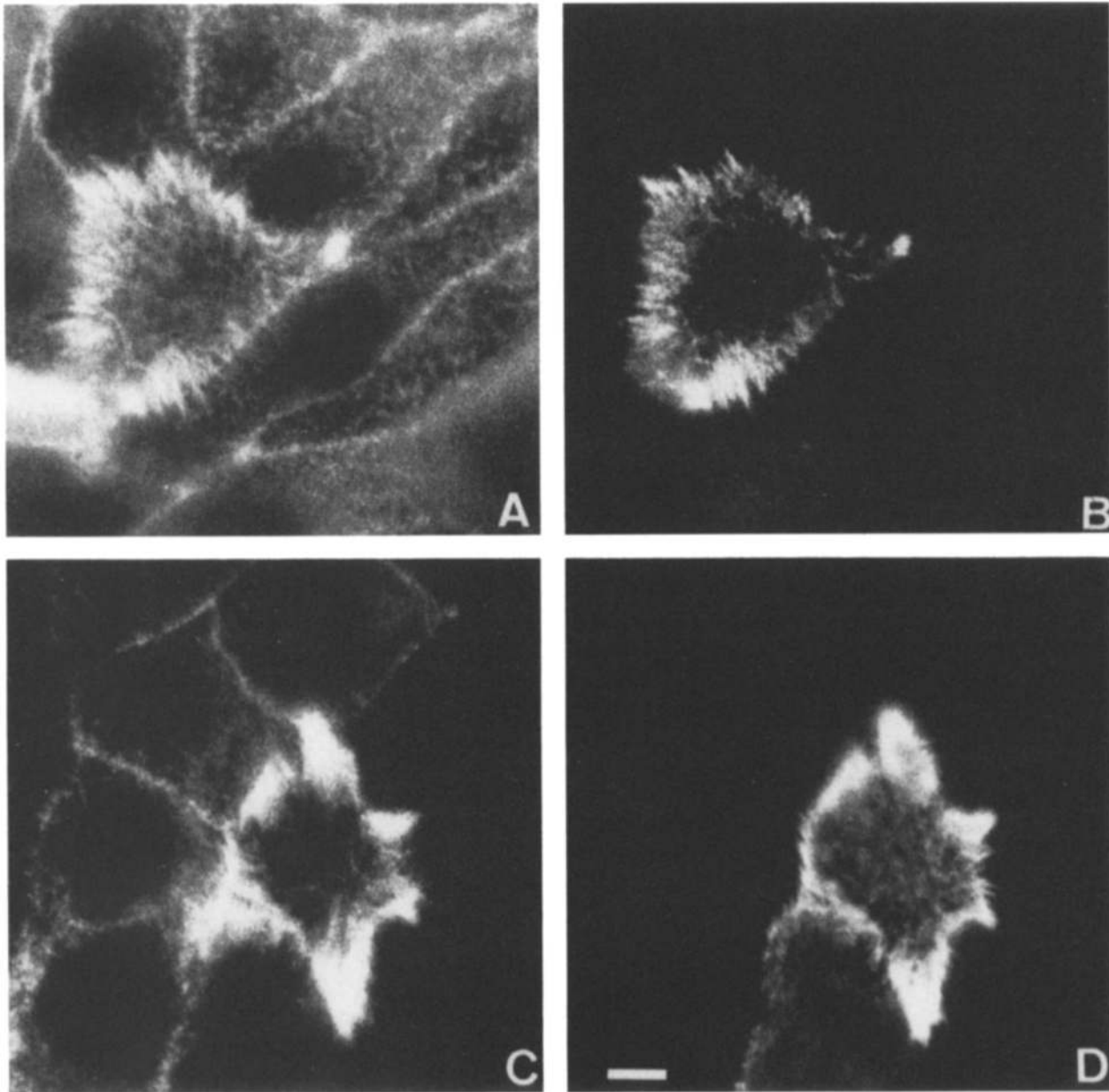


Figure 8. T-plastin and villin modify the microvillar actin-cytoskeleton. LLC-PK1 cells were transfected with cDNA encoding either T-plastin or villin, extracted with non-ionic detergent, fixed and labeled for immunofluorescence. (A and B) Double staining was performed with anti-peptide VSV-G antibody and fluorescein-coupled anti-mouse IgG antibodies (B) and rhodamine-coupled phalloidin (A). (C and D) Double labeling of villin transfected cells was carried out with a polyclonal anti-villin antibody and fluorescein-coupled anti-rabbit IgG antibody (D) and rhodamine-coupled phalloidin (C). Bar, 8 μm .

confluent cells were microinjected with cDNAs encoding L-plastin, T-plastin, or villin cDNAs and analyzed by scanning electron microscopy. Cells microinjected with either villin cDNA or T-plastin cDNA were unambiguously identified, as cells presenting a striking change in their morphology could be detected in the microinjected area of the coverslip. Villin induced the formation of long and parallel microvilli held in ordered patches (Fig. 11 V). T-plastin also induced elongation of the microvilli. However, their shape differed from that observed with villin, for they were shorter and intermingled (Fig. 11 T). As already observed by immu-

nofluorescence microscopy, changes in the morphology and density of the microvilli appeared more pronounced at the periphery of the cells. No changes in the morphology of the microvilli was observed when cells were microinjected with the L-plastin cDNA (data not shown).

Discussion

cDNA isolation of chicken intestinal fimbrin has revealed that different isoforms of this protein exist and that their cellular distribution is more specific than what was first anti-

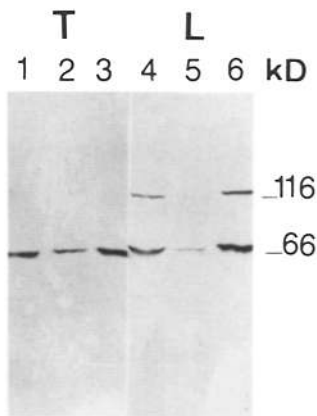


Figure 9. Immunoblot analysis of cell fractions obtained by detergent extraction. LLC-PK1 cells transfected with T- or L-plastin cDNAs were detergent extracted and analyzed by immunoblotting as described in the legend of Fig. 4. (1-3) Lysates from cells transfected with T-plastin cDNA. (4-6) Lysates from cells transfected with L-plastin cDNA. (1 and 4) Total cell lysates. (2 and 5) Insoluble fractions. (3 and 6) Soluble fractions.

pated from immunochemical studies. To test whether the tissue-specific expression of T- and L-plastin genes reflects functional differences, we have transfected fibroblast-like CV-1 cells and an epithelial cell line LLC-PK1 with cDNAs encoding these isoforms. Our results provide evidence that these two isoforms have different effects on actin filament structures despite their high degree of primary structure conservation (80% identities).

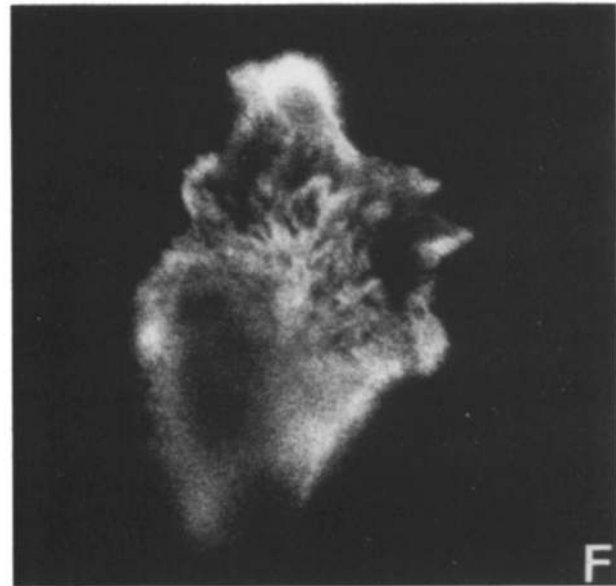
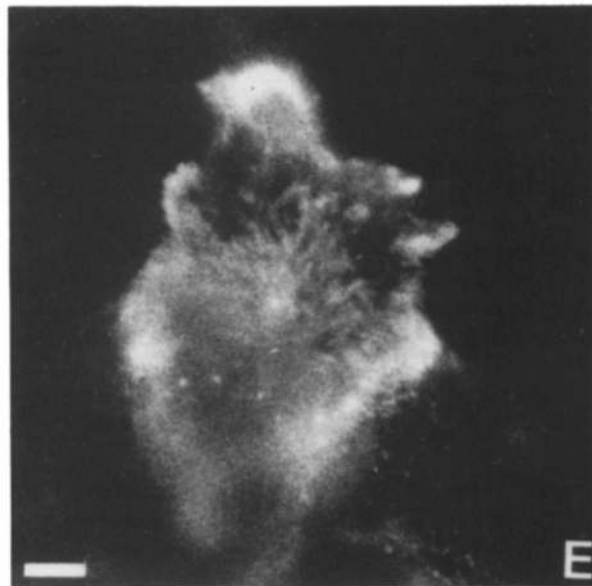
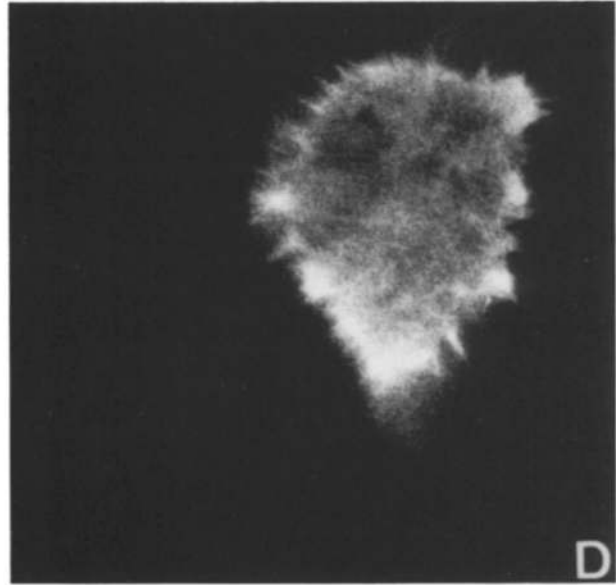
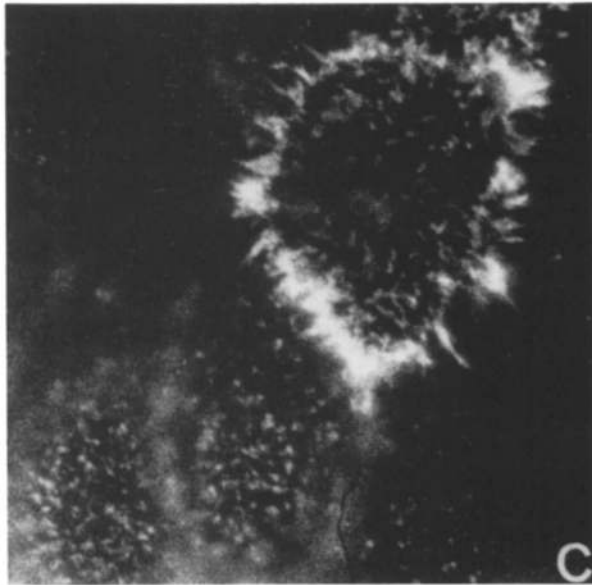
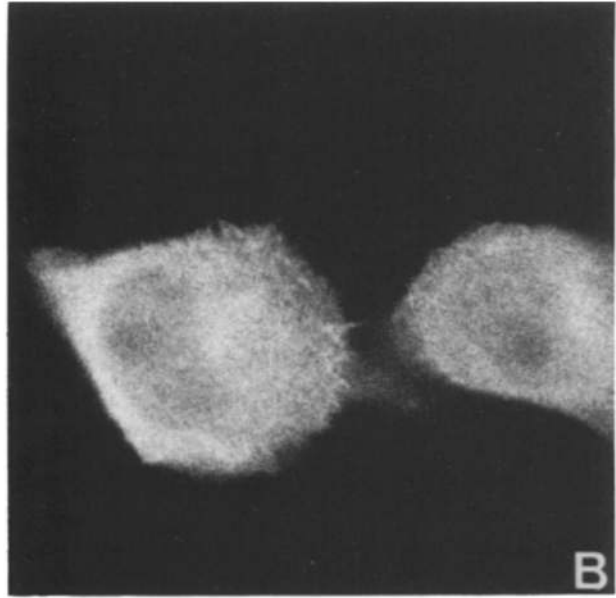
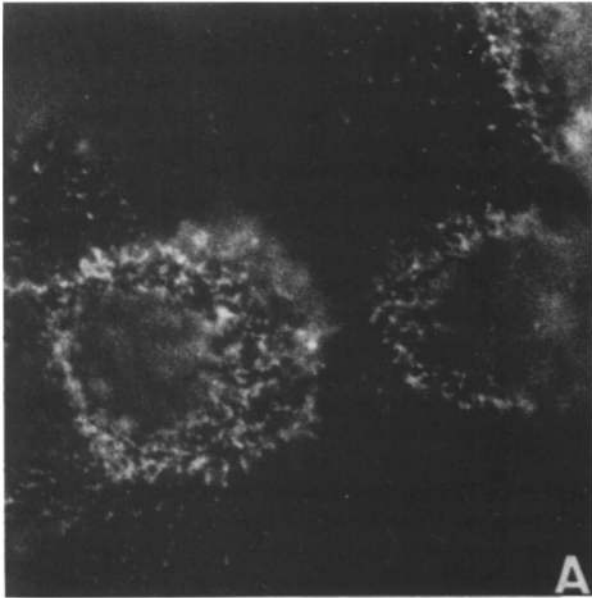
Although overproduction of T- or L-plastin in CV-1 cells did not lead to such striking modifications of the cortical actin cytoskeleton as was observed with villin (Friederich et al., 1989, 1992), high production levels of these proteins caused a partial loss of adherence and rounding-up of the cells. Concomitantly, actin filament bundles became organized into polygonal networks, structures reminiscent of those observed in rat embryo cells at the initial stage of spreading (Lazarides, 1976) or found in an epithelioid cell line (Rathke et al., 1979). However a major difference in the behavior of these two proteins was observed when cells were treated with non-ionic detergent before fixation. While L-plastin remained associated with this polygonal network, T-plastin was completely extracted from the stress fibers. L-plastin was found concentrated in foci and a discontinuous labeling could also be observed along some connecting fibers. It has been previously shown that the vertices also contain α -actinin, while myosin and tropomyosin are excluded (Lazarides, 1976; Rathke et al., 1979). Since a higher amount of T-plastin than L-plastin is normally present in CV-1 cells, this difference in the association of T- and L-plastins with actin filaments could be due to the fact that T-plastin competes for a limited number of binding sites with the endogenous protein. However, this hypothesis is unlikely since in CV-1 cells cotransfected with villin and T-plastin cDNAs, T-plastin is recruited to villin-induced F-actin spikes (Friederich et al., 1989) and is no longer detergent-extracted (data not shown). This experiment demonstrates that the avidity of overproduced T-plastin for F-actin structures in CV-1 cells is increased in presence of villin and depends on a specific organization of microfilaments.

The change in the adherence of the cells was manifested by a decrease in the number and size of focal contacts as illustrated by reflection interference microscopy and immunofluorescence analysis of vinculin as a marker for focal contacts. The mechanism by which elevated levels of plastin isoforms leads to a decrease in cell adhesion is not clear. It

is interesting to note that overproduction of gelsolin in NIH 3T3 fibroblasts causes enhanced motility (Cunningham et al., 1991) while an over-production of vinculin and α -actinin, two proteins which participate in membrane-filament interactions in focal contacts, suppresses cell motility and tumorigenicity (Rodriguez Fernandez et al., 1992; Glück et al., 1993). Our studies and other's show that plastin is present in focal contacts and podosomes (Bretscher and Weber, 1980a; Carley et al., 1985; Messier et al., 1993), but no direct association of these proteins with any of the focal components so far identified has been demonstrated. This suggests that vinculin and α -actinin regulate attachment and spreading of a cell to its substratum by different mechanisms than those involving plastins. It is conceivable that the striking diminution of focal contacts frequently observed in transfected cells could result from a change in microfilament organization. The overproduction of these isoforms might induce a remodeling of the actin cytoskeleton by changing the pattern of tension/compression in the cells as proposed in the tensegrity model hypothesis (Ingber, 1993).

The specificity with which these two isoforms interact with microfilaments was also observed in the polarized epithelial cell line, LLC-PK1. L-plastin did not induce a striking change in morphology of the microvilli in LLC-PK1 cells and moreover, was extracted by detergent unlike what was observed in CV-1 cells. On the contrary, the T-plastin isoform remained associated with the highly organized actin bundles of the microvilli even after detergent extraction. In addition, T-plastin was able to increase the length and density of the brush border microvilli, particularly at the periphery of the cell. Such changes are reminiscent of previous observations indicating that the morphogenesis of microvilli in the brush border is initiated near the junctional complex (Chambers and Grey, 1979). However, the microvilli induced by the T-plastin isoform did not resemble those induced by villin, another actin bundling protein of the brush border microvilli. Although the villin-induced microvilli in transfected LLC-PK1 cells were lying prone, their morphology was reminiscent of that observed with kidney brush border microvilli (Coudrier et al., 1988). This may be due to the fact that the level of villin in cells transfected with T-plastin cDNA was not sufficient to contribute to the formation of long microvilli. Indeed, it has been previously shown that both, the sequential recruitment of the proteins participating in the formation of the core actin filaments at the apex of the cells, and the level of these proteins are important factors for the control of brush border assembly (Heintzelman and Mooseker, 1992). Another alternative is that T-plastin does not have the ability to organize microvillar microfilaments to form a highly organized brush border. This is supported by a recent report indicating the existence of a third plastin isoform specific for intestinal and renal brush border (Lin et al., 1994). Our results would then suggest that the T-isoform may only partially substitute for the function of the I-isoform.

The ability of both T-plastin and villin to elongate microvilli and the differential behavior of plastin isoforms according to cell type raise the questions of how actin bundling proteins can affect microfilament length and what determines the specificity of plastin isoforms? Villin displays different *in vitro* activities on actin organization and polymerization (bundling, nucleating, capping or severing). We do not know whether its morphogenic effect is only due to



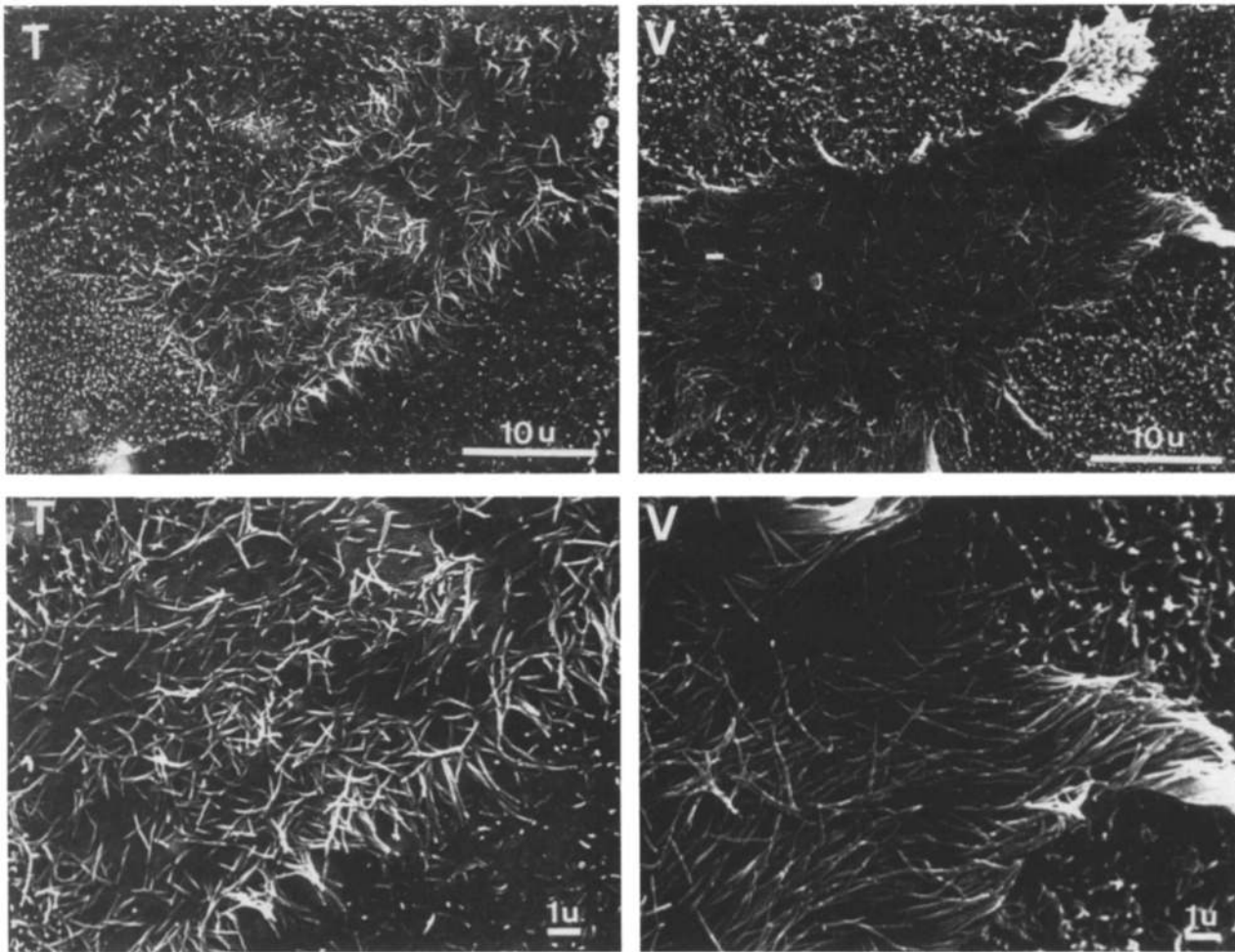


Figure 11. Scanning electron microscopy of LLC-PK1 cells microinjected with T-plastin cDNA or villin cDNA. (Left) Cells were microinjected with T-plastin cDNA (T). (Right) Cells were microinjected with villin cDNA (V). Bars: (top) 10 μm ; (bottom) 1 μm .

the bundling activity. Since only a bundling activity has been detected, *in vitro*, for fimbrin, the elongation of microvilli observed in transfected LLC-PK1 cells may result from a stabilization of preexisting microvillar microfilaments rather than from an initiation of polymerization.

Several hypotheses can be envisaged to explain the functional specificity of T- or L-plastin isoforms. Divergences in the primary structure of the actin-binding sites may account for these differences. Although the core domain of plastin isoforms contains two regions which share homologies with the putative actin-binding site of gelation proteins; the actin-binding sites of plastin have not yet been functionally mapped. These sites may lie in regions with divergences in primary structure and could interact with different sites on actin or with different actin isoforms. In this respect, *in vitro* experiments performed with T- and L-plastins show that the bundling activity of these proteins indeed depend on actin isoforms. In addition, these activities may be differen-

tially regulated by calcium, since the most important divergence in the primary structure between these two isoforms is observed in the headpiece domain which is related to the calcium binding site of calmodulin.

It is also possible that these two isoforms associate with actin filaments in different ways. T-plastin would preferentially associate with actin bundles while L-plastin which remains associated with the stress fibers in CV-1 cells would allow a more flexible interaction between microfilaments. Such dual function on actin organization has been observed with some α -actinin isoforms which display cross-linking activity (Meyer and Aebi, 1990).

The fact that each isoform displays a strict tissue specificity, suggests that the actin cytoskeleton to which they are associated is involved in different cellular functions. The biological significance of the differences observed between these two isoforms remains to be further investigated.

Figure 10. Monitoring of the cell surface in transfected LLC-PK1 cells. LLC-PK1 cells were transfected with L-plastin cDNA (A and B), T-plastin cDNA (C and D) and villin cDNA (E and F). (Left) The plasma membrane of living cells was labeled with rhodamine-coupled wheat germ agglutinin before permeabilization. (Right) After fixation and permeabilization, cells were stained for L-plastin (B), T-plastin (D) and villin (F) as described in Fig. 6. A change in cell surface is observed in cells transfected with either T-plastin cDNA or villin cDNA but not in cells transfected with L-plastin cDNA. Bar, 5 μm .

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