FAP52 Regulates Actin Organization via Binding to Filamin*

Marko Nikki‡, Jari Meriläinen§, and Veli-Pekka Lehto‡¶†

From‡ the Department of Pathology, University of Oulu, FIN-90014 University of Oulu, Finland, §Thermo Labsystems OY, Laboratory Technologies Division, FIN-00811, Helsinki, Finland, and ¶the Department of Pathology, University of Helsinki, FIN-00290 Helsinki, Finland.

*This work was supported by the Finnish Academy and the Finnish Cancer Research Fund.

‡To whom correspondence should be addressed: Dept. of Pathology, University of Oulu, P.O.Box 5000 (Aapistie 5), FIN-90014 University of Oulu, Finland. Tel.: (358) 8-5375950; Fax: (358) 8-5375953; E-mail: lehto@csc.fi.

†The abbreviations used are: FAP52, focal adhesion protein, 52 kD; ABP-280, actin-binding protein, 280 kDa; IFM, immunofluorescence microscopy; FCH, FER-CIP4 homology; SH3, Src-homology 3; IEM, immunoelectron microscopy; GT, glutathione; IPTG, isopropyl-β-D-thiogalactopyranoside; OL, overlay; PMSF, phenylmethylsulphonyl fluoride; BSA, bovine serum albumin; FCS, fetal calf serum; NC, nitrocellulose; HRP, horseradish peroxidase; aa, amino acid(s); GST, glutathione-S-transferase; PCR, polymerase chain reaction; HA, hemagglutinin; CEHF, chicken embryo heart fibroblast; RT, room temperature; PBS, phosphate-buffered saline; BPBS, 0.1% BSA in PBS; GA, glutaraldehyde; IP, immunoprecipitation; IB, immunoblotting; CBB, coomassie brilliant blue; TBS, Tris-buffered saline, SPR, surface plasmon resonance; ECL, enhanced chemiluminescence.

Running title: FAP52 binds filamin
SUMMARY

FAP52, a focal adhesion-associated phosphoprotein, is a member of a FAP52/PACSIN/syndapin family of proteins. They share a multidomain structure and are implicated in actin-based and endocytotic functions. Here we show, by using both native and recombinant proteins, that FAP52 selectively binds to the actin-crosslinking protein filamin (ABP-280). This was based on an affinity purification followed by a sequence determination by mass spectrometry, co-immunoprecipitation, overlay binding, and surface plasmon resonance analysis. Binding studies with deletion mutants showed that the sites of the interaction map to the highly $\alpha$-helical N-terminal part of FAP52, and to the C-terminal region of filamin which also contains binding sites to some transmembrane signaling proteins. In immunofluorescence and immunoelectron microscopy of cultured fibroblasts, a different overall subcellular distribution was seen for filamin and FAP52 except for a stress fiber–focal adhesion junction in which they showed a notable overlap. Overexpression of the full-length and mutant forms of FAP52 led to an extensive reorganization of actin and filamin in cultured fibroblasts. Thus, the results show that FAP52 interacts with filamin and we propose that this interaction is important in linking and coordinating the events between focal adhesions and the actin cytoskeleton.

**KEY WORDS:** Focal adhesion, cytoskeleton, actins, filamins, immunofluorescence, filopodia.
INTRODUCTION

FAP52 is a recently described focal adhesion-associated protein which in immunofluorescence microscopy (IFM)\(^1\) colocalizes with vinculin, paxillin and talin (1). The latter are well-known focal adhesion proteins which are also closely associated with the actin cytoskeleton (2). More recently, two homologues of FAP52, PASCIN2 and syndapin II, were identified (3, 4). They all share a modular structure typified by a well-conserved FER-CIP4 homology (FCH) domain in the very N-terminus, followed by a highly \(\alpha\)-helical region, and a C-terminal Src-homology 3 (SH3) domain. In the middle, there is a “linker” region which shows a high degree of sequence variation and does not conform to any specific domain signature. Thus far, no specific function is assigned to FAP52. PACSIN 2, its closest homologue, participates in the organization of the actin cytoskeleton and in the regulation of vesicular traffic (5). Syndapin II, on the other hand, along with other syndapin isoforms, is involved in endocytosis and actin dynamics (4). Both syndapin II and PACSIN 2 bind N-WASP, an important regulator of actin cytoskeleton organization (4, 5). Thus, all the three members of the family share a property of being closely associated with the actin cytoskeleton-associated proteins or functions.

In this study, we set out to identify binding partners of FAP52. A single polypeptide band of 280 kDa was purified from cultured cells by applying copurification and coimmunoprecipitation techniques, and shown to be filamin (actin-binding protein, 280 kDa; ABP-280) by using electrospray mass spectrometric analysis of tryptic peptides. Interaction between FAP52 and filamin was further verified by using direct binding assays. In IFM and immunoelectron microscopy (IEM) of cultured cells, distinctly demarcated codistribution of FAP52 and filamin was seen at sites where the actin stress fibers abut the focal adhesions. In cells overexpressing FAP52, a distinct reorganization of actin and filamin was seen. On the basis of these results we suggest that filamin serves as a link between FAP52 and the actin...
cytoskeleton and that this interaction is important in linking focal adhesions to actin cytoskeleton.
EXPERIMENTAL PROCEDURES

General procedures—The standard solutions, buffers, and procedures for the purification and precipitation of DNA, restriction enzyme digestions, ligation reactions, SDS-PAGE runs and stainings were as described in Sambrook et al. (6). DNA sequencing was carried out on an automated ABI Prism 377XL DNA Sequencer (Perkin Elmer).

Materials—E. coli strain BL21(DE3) cells, expression vectors pGEX-2T and pGEX-2TK, glutathione (GT)-Sepharose 4B, thrombin, Rainbow High Molecular Weight Markers, and synthetic oligonucleotides were purchased from Amersham Pharmacia Biotech, isopropyl-β-D-thiogalactopyranoside (IPTG) from MBI Fermentas, Site-Directed Mutagenesis kit, pfu polymerase and T4 DNA ligase from Stratagene, restriction enzymes from MBI Fermentas and Promega Co., and chicken gizzard filamin from Progen Biotechnik GmbH. For blot overlay (OL) assay, chicken gizzard filamin was purified according to the method of Feramisco and Burridge (7). HeLa cells were obtained from the American Type Culture Collection, Immu-Mount mounting medium from Shandon, Inc., Fugene 6-transfection reagent, reduced GT, protease inhibitors aprotinin, leupeptin and phenylmethylsulphonyl fluoride (PMSF) from Boehringer Mannheim Gmbh, ampicillin, bovine serum albumin (BSA) and benzamidine from Sigma Chemicals Co., and Triton X-100 from Merck. Cell culture media and fetal calf serum (FCS) were from Hyclone Laboratories, Inc., and nitrocellulose (NC) membranes from Schleicher & Schuell, Inc.

Antibodies—Rabbit polyclonal antibody to FAP52, denoted Affi-K7, was produced and affinity-purified as described previously (1). Mouse monoclonal anti-filamin antibody was obtained from Chemicon International, Inc., mouse monoclonal anti-GST and rabbit polyclonal anti-HA epitope antibodies from Santa Cruz Biotechnology, Inc., rhodamine-phalloidin, Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 546 goat anti-mouse IgG from Molecular Probes, Inc., and mouse monoclonal anti-paxillin antibody and rabbit anti-
mouse IgG from Zymed Laboratories, Inc. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG, goat anti-rabbit IgG-agarose and goat anti-mouse IgG-agarose were purchased from Sigma Chemicals Co. For IEM, Protein A-gold particles (Ø 5 nm and 10 nm), prepared by Slot and Geuze (8), were used.

**DNA constructs and protein expression**—All the cDNA constructs and the corresponding polypeptides were designated as “FAP52” or “Fil” for FAP52 and filamin, respectively, followed by the subscripted number of the first and the last amino acid (aa) residue expressed.

Full-length FAP52 and its fragments were produced as fusion proteins with glutathione-S-transferase (GST) in BL21(DE3) cells by employing the expression vectors pGEX-2T and pGEX-2TK. The 1.35-kb cDNA encoding the full-length FAP52 was inserted into the BamHI/EcoRI cloning site in the vector as described previously (1). For the cloning and expression of the 3′- and 5′-truncated mutants of FAP52, polymerase chain reaction (PCR) with the oligonucleotide primers with add-on sequences for a BamHI and EcoRI restriction sites and the full-length cDNA of FAP52 as a template, were utilized. The resulting cDNAs were cut with the matching restriction enzymes and ligated to BamHI/EcoRI-cut pGEX-2T or pGEX-2TK vectors.

For the production of the C-terminal fragments of filamin, pGEX-4T-1 vectors with the cDNAs encoding for the fragments Fil1524-2283, Fil2283-2490 and Fil2495-2647, were employed (a kind gift from Dr. T.P. Stossel, Division of Hematology, Brigham and Women’s Hospital, Boston, USA; 9). Further C-terminal truncation mutants were generated from the construct encoding for the fragment Fil1524-2283 by using a Site-Directed Mutagenesis kit and oligonucleotide primers to generate stop codons to appropriate sites of the cDNAs.

GST fusion proteins were expressed in *E. coli* BL21(DE3) cells according to standard protocols, purified from the cell lysates on GT-Sepharose 4B beads, and liberated from the
beads by incubation with reduced GT. For some experiments, FAP52 or its mutant form was released from its fusion partner by incubation with thrombin. For the control experiments, GST was expressed from the plasmid pGEX-2TK and purified as above.

For the expression in cultured animal cells, cDNA-constructs encoding the full-length FAP52 or its fragments were produced by PCR with engineered restriction sites. They were cut with EcoRI and EcoRV and subcloned into the EcoRI/EcoRV cloning site of pRK5-vector (a kind gift from Dr. Joseph Schlessinger, New York University, Medical Center, New York, NY). A hemagglutinin (HA)-epitope tag was engineered to the C-terminus of the constructs by primer design. The following constructs were generated: FAP52-HA (FAP52₁-₄₄₈ plus HA-tag); FAP52Nt-HA (FAP52₁-₂₉₃ plus HA-tag); FAP5₂₅₃-HA (FAP5₂₃₉₀-₄₄₈ plus HA-tag).

**Cell cultures, transfections and immunofluorescence microscopy**—Cultures of chicken embryo heart fibroblast (CEHF) were established and the cells were grown as described previously (1). HeLa-cells were grown according to the same protocol with an exception that Dulbecco’s modified Eagle’s medium was used. For transfections, the cells were grown on glass coverslips to a confluence of 50-70%. Transfections were carried out by using a Fugene 6-transfection reagent and following the manufacturer’s instructions.

For IFM, CEHF-cells grown on glass coverslips were fixed and stained essentially as described (1). In cases with staining of actin filaments, post-fixation was carried out with ethanol for 1 min. As primary antibodies, Affi-K7 (rabbit), rabbit polyclonal anti-HA, mouse monoclonal anti-filamin or mouse monoclonal anti-paxillin were used at appropriate dilutions. Fluorochrome-conjugated secondary antibodies or phalloidin (Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 546 goat anti-mouse IgG or rhodamine-phalloidin) were applied as secondary antibodies. Viewing was under a Zeiss LSM510 laser scanning microscope.
equipped with a Zeiss Axiovert 110M inverted microscope (Carl Zeiss Microscopy). The images were processed by using an LSM 3D software, version 5.2. (Carl Zeiss Microscopy).

**Immunoelectron microscopy**—For IEM, CEHF-cells were grown close to confluence. After washing with Hank’s salt solution and a fixation with 4% paraformaldehyde in phosphate-buffered saline (PBS) containing 2.5% sucrose for 1 h, they were harvested with a cell scraper and pelleted. The pellet was resuspended in 2.3 M sucrose and frozen in liquid nitrogen. Cryosections (thickness 80 nm) were cut by using a Leica Ultracut UCT microtome and placed on Butvar-coated nickel grids. They were washed with PBS and blocked with 5% BSA with 0.1% ColdWaterFishSkin gelatin in PBS for 10 min. For double labeling, the sections were first incubated with the monoclonal mouse anti-filamin antibody in 0.1% BSA in PBS (BPBS) for 60 min, followed by an incubation with the rabbit anti-mouse IgG in BPBS for 30 min, and with Protein A-gold (∅ 10 nm) in BPBS for 30 min. Between each of these steps, the sections were washed with PBS-glycine (6 x 5 min). After this, the sections were fixed in 1% glutaraldehyde (GA) in PBS and blocked with 1% BSA in PBS-glycine for 30 min. They were then incubated with Affi-K7 followed by Protein A-gold (∅ 5 nm), both in BPBS. Washings were with PBS-glycine as above. The sections were then post-fixed in 2% GA for 5 min, washed with PBS and distilled water, counterstained with neutral 2% uranyl acetate for 5 min, and coated with 1.8% methyl cellulose with 0.3% uranyl acetate. The sections were analyzed using a Philips 410 LS transmission electron microscope.

**Preparation of cell lysates**—Whole-cell lysates were prepared from the cells grown to confluence. They were first rinsed with PBS and then scraped into a lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1.5 mM MgCl2, 0.2 mM Na3VO4, and 1% Triton X-100) supplemented with protease inhibitors (0.1 µM aprotinin, 0.1 mM leupeptin, 1 mM PMSF and 5 mM benzamidine), and incubated on ice bath for 20 min. The lysates were cleared by centrifugation at 12 000 x g for 5 min at 4°C.
**Immunoprecipitation**—For immunoprecipitation (IP), Affi-K7 or anti-filamin antibodies were added to 500 µl of the cell lysate (0.15 mg/ml). For control reactions, an equal volume of PBS was added instead of the antibody. Then, 50 µl of anti-rabbit IgG-agarose or anti-mouse IgG agarose, respectively, in PBS, was added, and incubated on a nutator for 16 h at 4°C. The beads were collected by centrifuging at 500 x g for 2 min, and then washed three times with PBS. The volume was adjusted to 60 µl with PBS. After addition of the Laemmli’s sample buffer (15 µl) and boiling for 2 min, the eluates were run on a 7.5% or 10% SDS-PAGE and then analyzed by immunoblotting (IB).

**Affinity chromatography and pull-down experiments**—Bacterially produced GST-FAP52 or GST was immobilized on GT-Sepharose 4B beads in PBS. The beads (25 µl) were incubated with the CEHF lysate (0.15 mg protein in 500 µl of lysis buffer) overnight at +4°C on a nutator. They were then washed three times with PBS and the volume was adjusted to 60 µl with PBS. After addition of 15 µl of a sample buffer and boiling, the proteins were separated on a 7.5% SDS-PAGE gel which was then stained with coomassie brilliant blue (CBB).

For the pull-down experiments, 5 µg of the GST fusions of fragments of FAP52 or filamin were immobilized on 20 µl of GT-Sepharose 4B beads in Tris-buffered saline (TBS). Twenty µg of the chicken gizzard filamin or the bacterially produced FAP52, respectively, in 500 µl of TBS, was added, and the mixture was then incubated for 2 h at +4°C on a nutator. The beads were washed three times with TBS and, after addition of 20 µl of a sample buffer, subjected to SDS-PAGE separation, followed by IB with mouse anti-filamin or Affi-K7, respectively.

**Immunoblotting**—For IB, the electrophoretically separated proteins were transferred to NC membranes which were then blocked in 3% nonfat dried milk powder in TBS. Thereafter,
the filters were incubated with Affi-K7 or mouse anti-filamin for 1 h at room temperature (RT). After an overnight washing with TBS at RT, the filters were incubated either with the HRP-conjugated goat anti-rabbit IgG or the HRP-conjugated goat anti-mouse IgG, respectively. After a 5-hour washing with TBS, the blot was developed by the ECL method as described previously (1).

**Blot overlay assay**—For blot OL assays, bacterially produced FAP52 and GST, or GST-fusions of filamin mutants were separated on SDS-PAGE and transferred to NC membranes. The filters were blocked by incubating with 3% nonfat dried milk powder in TBS overnight at 4°C or for 2 h at RT, and then incubated with the purified filamin or recombinant FAP52 in 1% nonfat dried milk powder in TBS for overnight at 4°C. For the visualization of the protein bands, the overlays were washed with TBS for 3 h and then incubated with either the mouse anti-filamin antibodies or Affi-K7, respectively, as above, followed by a HRP-conjugated goat anti-mouse IgG or anti-rabbit IgG, respectively. The blots were developed by using the ECL detection system.

**Electrospray ionization mass spectrometry**—For mass-spectrometry, pieces corresponding to the protein bands of interest were excised from the CBB-stained polyacrylamide gels and processed as described by Shevchenko et al. (10). Briefly, the proteins were in-gel reduced, alkylated, and digested with trypsin at 37°C. The supernatant obtained was acidified with formic acid, loaded onto a Poros R2 (Perseptive Biosystems) microcolumn, and desalted according to Gobom et al. (11). The peptides eluted with 40% methanol/5% formic acid were introduced into a nanoelectrospray needle (Protona). Nanoelectrospray tandem mass spectrometry was performed on an API III mass spectrometer (Perkin-Elmer Sciex) equipped with a nanoelectrospray source, as described elsewhere (12).

**Surface plasmon resonance analysis**—Surface plasmon resonance (SPR) measurements were performed on a BIACORE 3000 analyzer under a control of a BIACORE control
software, version 3.1.1. (Biocore AB). For experiments with the GST fusions of FAP52, of 
Fil1524-2283, or mutants thereof as a ligand, anti-GST antibody (Biacore AB) was immobilized 
on a carboxymethyl-coated CM5 sensor chip by employing the GST capture and the amine 
coupling kits (Biacore AB). Full-length GST-FAP52 or its mutants (0.15 mg/ml in 10 mM 
sodium acetate, pH 5.5), or GST-Fil1524-2283 or its mutants (0.15 mg/ml in HBS-EP; 0.01 M 
HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% (v/v) polysorbate 20; Biacore AB) were 
then injected onto the chip for 30 s with a flow rate of 30 µl/min. The chip was then 
equilibrated with HBS-EP.

For binding assays, chicken gizzard filamin or recombinant FAP52, both in HBS-EP, were 
then passed over the chip at concentrations ranging from 0 nM to 800 nM at a flow rate of 30 
µl for 1 min for filamin or 3 min for FAP52. The binding of the analyte to the ligand was 
detected from the change in the sensorgram. In order to correct the sensorgram for a 
background binding and bulk refractive index changes, a different flow cell without GST-
fusion protein was used as a reference. As controls, GST-coated chips were used over which 
filamin or FAP52 was passed at a concentration of 100 nM. Kinetics was analyzed by 
BIAevaluation software, version 3.1.
RESULTS

Identification of filamin as a FAP52-binding protein—In order to identify binding partners of FAP52, we carried out IP experiments from the lysates of cultured CEHF by using affinity-purified rabbit anti-FAP52 antibodies (Affi-K7). IP followed by an SDS-PAGE and silver staining revealed reproducibly a 280-kDa polypeptide band that was seen in the experimental (Fig. 1A, lane 2) but not in the control precipitations (Fig. 1A, lane 1). A band of a similar molecular weight was also detected in the affinity binding (“pull-down”) experiments in which the cell lysate was passed over a column in which GST-FAP52 was coupled onto GT-conjugated Sepharose beads (Fig. 1B, lane 2). No such band was seen in control experiments with GST-coated beads (Fig. 1B, lane 1). In "pull-down", but not in IP experiments, also another prominent band of about 220 kDa was seen.

The 280-kDa band was excised from the CBB-stained gel and subjected to in-gel trypsin digestion. The extracted peptides then underwent analysis by nanoelectrospray tandem mass spectrometry. The partial primary structures of several peptides in the mass spectrum of the 280-kDa band were compared with the sequences in the databases. In 5 cases there was a match with GST, in 4 cases with FAP52, in one case with myosin, and in one case with filamin (ABP-280; 13). We also analysed the 220-kDa band seen in Fig. 1B. Five peptides out of seven showed a match with myosin heavy chain. We concluded that the myosin sequence found among the peptides derived from the 280-kDa band is actually originating from the more abundant material in the 220-kDa band.

Since the filamin-like sequence derived from the band that was not seen in the controls and since the partial sequence and the Mr of the band match with those of filamin, we decided to explore the possibility of an interaction between filamin and FAP52. The presence of FAP52-, GST- and myosin sequences among the peptides derived from the 280-kDa band
was deemed unauthentic and being most probably due to an abundance of these proteins in the type of experiments that were carried out.

*Association between filamin and FAP52 in cells in vivo*—The association between filamin and FAP52 was further explored by IP of FAP52 from a lysate of CEHF-cells and then analyzing the precipitate by IB for coprecipitation of filamin. In a reciprocal experiment with HA-tagged FAP52 (FAP52-HA) -transfected HeLa-cells, anti-filamin immunoprecipitate was analyzed for co-IP of FAP52-HA. As shown in Fig. 2A, filamin was present in the precipitate obtained by using Affi-K7 but not in the control precipitates (Fig. 2A, lanes 2 and 1, respectively). On the other hand, FAP52-HA was seen in the anti-filamin but not in the control immunoprecipitates (Fig. 2B, lanes 2 and 1, respectively). These data show that FAP52 and filamin coimmunoprecipitate from the cell lysates and suggest that there could be a direct interaction between FAP52 and filamin.

*Demonstration of a direct interaction between FAP52 and filamin*—To determine whether the observed co-IP of FAP52 and filamin is due to a direct interaction between them or is mediated by some other protein(s), we carried out direct binding assays. In OL assay, bacterially produced FAP52 or GST-fusions of the C-terminal fragments of filamin were run in SDS-PAGE, transferred to an NC membrane, and then overlaid with purified filamin or FAP52, respectively, followed by an incubation with relevant antibodies and an enhanced chemiluminescence (ECL) detection. As seen in Fig. 3A, filamin binds to FAP52 (Fig. 3A, lane 2), but not to GST that was used as a control (Fig. 3A, lane 1). In Fig. 3A, lanes 3 and 4, positions of GST and FAP52, respectively, in a parallel SDS-PAGE, stained with CBB, are shown.

In reciprocal experiment, binding of FAP52 to filter-anchored filamin was tested. For that purpose, bacterially produced C-terminal fragments of filamin in the fusion with GST were used. In OL, binding of FAP52 is seen to GST-Fil\textsubscript{1524-2283} (Fig. 3B, lane 1) but not to GST-
Fil2283-2496 (lane 2) or to GST-Fil2495-2647 (lane 3). In Fig. 3B, lanes 4-6, a parallel SDS-PAGE run of the same fusion proteins is shown. The polypeptides were transferred to NC filter and visualized by the anti-GST antibody and the ECL detection system. These data show that there is a direct interaction between FAP52 and filamin and that in filamin the interaction site resides in the fragment encompassing the residues 1524-2283.

The interaction between filamin and FAP52 was further explored by SPR analysis. In these experiments, anti-GST antibody was immobilized on a chip and used to capture the GST-FAP52 fusion protein. The sensorgram (Fig. 3C) obtained upon injection of chicken gizzard filamin at concentrations ranging from 0 to 800 nM onto the GST-FAP52-coated chip, shows a high-affinity binding between filamin and FAP52. The association and dissociation rate constants $k_a$ and $k_d$ were determined to be $1.06 \times 10^5$ M$^{-1}$ s$^{-1}$ and $1.02 \times 10^{-3}$ s$^{-1}$, respectively, yielding a dissociation rate constant ($K_D = k_d/k_a$) of $9.64 \times 10^{-9}$ M. No binding was observed in the control experiments in which the GST-coated chip was overlaid with filamin (Fig. 3C, insert).

Mapping of the interaction site in FAP52—Pull-down assay and SPR were used to map the filamin-binding region in FAP52. For that purpose, a large number of truncation mutants of FAP52 were expressed as GST-fusions in bacteria, purified and used in the assays.

For the pull-down assays, GST-FAP52 or its mutants were immobilized on GT-Sepharose beads and incubated with chicken gizzard filamin. After collecting and washing of the beads, they were run on SDS-PAGE. The electrophoretically separated proteins were then transferred to an NC membrane, followed by an incubation with the mouse anti-filamin and the ECL detection. Fig. 4A shows that anti-filamin reactive material could be harvested not only from the beads carrying the full-length GST-FAP52 (lane 5) but also those carrying the peptides 1-184, 1-219, and 1-359 (lanes 2-4). No binding of filamin was seen to beads.
carrying the more N-terminal peptides 1-145 (*lane 1*) or the C-terminal peptides 146-448, 185-448, 220-448, 281-448, 360-448 and 390-448 (*lanes 6-11*).

For SPR analysis, the GST fusion of FAP52 or its mutants were immobilized onto a sensor chip and chicken gizzard filamin was then passed over the chip at a concentration of 100 nM. Fig. 4B shows that filamin binds to the GST-FAP521-448 and to the N-terminal peptides 1-184, 1-219 and 1-359. No binding was observed to peptides 1-145, 146-448, 185-448, 220-448, 281-448, 360-448 or 390-448. Fig. 4C shows an SDS-PAGE gel of the fusion proteins used in the experiments.

Both the OL and the SPR results indicated, in total conformity, that the residues 146-184 have an essential role in the binding of filamin. The capability of this region alone to bind filamin was tested with SPR, in an experiment in which filamin was passed over the GST-FAP52146-184-coated chip. No binding was observed (Fig. 4B, *insert*). Thus, it can be concluded that the region spanning the aa 146-184 is necessary but not sufficient for the binding of filamin. For the binding, also a sequence N-terminal of the aa 145 is needed as indicated by binding with an N-terminal 1-184 peptide.

*Mapping of the interaction site in filamin*—The FAP52-binding site in filamin was determined by pull-down experiments and by SPR by employing sequentially truncated mutants of the C-terminal half of filamin in fusion with GST.

For the pull-down experiments, filamin mutants in fusion with GST were immobilized onto the GT-Sepharose beads which were then incubated with the recombinant FAP52. Binding of FAP52 to the beads was analyzed by SDS-PAGE followed by a transfer to an NC filter, IB with Affi-K7, and ECL detection. Fig. 5A shows the binding of FAP52 to the filamin peptides 1524-1858, 1524-1956, 1524-2064, 1524-2132, 1524-2172 and 1524-2283 (*lanes 3-8*). There was no binding of FAP52 to the peptides 1524-1780 and 1524-1664 (*lanes 1 and 2*).
For SPR analysis, GST-Fil1524-2283 or its truncated mutants were captured onto the sensor chip, and the recombinant FAP52 was passed over the chip. In Fig. 5B, binding of FAP52 to the truncated filamins is shown. A strong binding is seen to the peptides 1524-1780, 1524-1858, 1524-1956, 1524-2064, 1524-2132, 1524-2172 and 1524-2283, and a weak binding to 1524-1664. In Fig. 5C, SDS-PAGE analysis of the purified, bacterially produced GST fusions of the filamin fragments used in the assay is shown.

The results of the pull-down experiments suggest the residues 1780-1858, corresponding to the repeat 16, as the binding region for FAP52. A closely similar binding data was obtained from the SPR analysis, except that also Fil1524-1780, which in the pull-down assay did not bind FAP52, showed a strong binding. The different binding of Fil1524-1780 in the two assays is probably not due to artefactual conformational changes since in both cases the filamin-GST chimera was anchored to the substrate via a “linker” (anti-GST antibody in SPR and GT in pull-down assay). Rather, it may reflect the different sensitivities of the techniques. The lacking or low binding of Fil1524-1664 in both assays clearly indicates, however, that the principal binding of FAP52 to filamin is in the region aa 1664-1858 spanning the repeats 15-16.

*Colocalization of FAP52 and filamin in cultured fibroblasts*—Biochemical studies described indicated a direct interaction between FAP52 and filamin. Thus, it was expected that there is at least a partial colocalization between FAP52 and filamin in cells *in vivo*. This was studied by using a confocal laser scanning IFM and IEM. We applied double immunostaining with Affi-K7 and the anti-filamin antibodies. Also antibodies to focal adhesion protein paxillin and a filamentous actin-binding compound phalloidin were used for comparative purposes.

Confocal IFM of well-spread (Fig. 6A) and spreading CEHFs (Fig. 6B) showed that FAP52 is typically associated with the focal adhesions (verified by a double staining for
paxillin; not shown) and filamin along the actin fibers (verified by visualizing actin fibers with phalloidin; not shown). At a closer scrutiny and in merged images, distinct overlaps of the staining patterns could be seen. One was in the areas where the filamin-decorated filaments merge with the focal adhesions (Fig. 6A, arrows). The overlap area seems to correspond to the more proximal part of the focal adhesion or a putative “juncture” region in which the fibers are linked to the focal adhesions. Another distinct overlap was seen in the spreading cells with a smooth-contoured forward-moving front region (Fig. 6B, arrow). In them, in addition to the focal adhesion structures, a distinct colocalization was seen in distinct spots which were located at regular intervals along the very edge of the cell (Fig. 6B, arrowheads).

**Immunoelectron microscopy**—Fig. 7 is an electron micrograph of a cryosection of cultured CEHFs. The sections were double-stained with antibodies to FAP52 and filamin. The localization of the bound antibodies was revealed by secondary antibodies conjugated to small (∅ 5 nm) and large (∅ 10 nm) gold particles, respectively. The larger particles were seen most prominently along filamentous structures that could be discerned even though the overall contrast after uranyl staining is quite low (Fig. 7, arrows). The smaller particles, corresponding to FAP52, on the other hand, were only seen in the peripheral parts of the cells, and especially in elongated structures which, by their morphology and localization, represent focal adhesions (Figs. 7, arrowheads). In Fig. 7, B and C, which represent higher magnifications of the areas indicated by rectangles in Fig. 7A, a colocalization of FAP52 (smaller particles) and filamin (larger particles) was seen (circled areas). Interestingly, the gold particles were often linearly arranged, strongly suggesting that they are associated with an underlying filamentous structure (Fig. 7, B and C, bar-ended arrows).

**Rearrangement of actin and filamin cytoskeleton in cells overexpressing FAP52**—Direct interaction between FAP52 and filamin, as shown by biochemical techniques, and a close
colocalization between FAP52 and filamin, as shown by confocal microscopy and IEM, prompted us to study the changes in the distribution of filamin in cells overexpressing FAP52 or its specific regions/domains. Due to the actin-cross linking properties of filamin, also the effect of the forced expression of FAP52 on the organization of actin cytoskeleton was investigated. For these purposes, confocal microscopy of CEHF s transiently transfected either with HA-tagged FAP52 (FAP52-HA) or its truncated forms were used. Double-staining with appropriate antibodies and phalloidin was used to analyze the distributions of FAP52-HA, filamin and actin.

A typically elongated or polygonal fibroblastic morphology was seen in most naive (untransfected) CEHF cells (Fig. 8A, arrows). Cells overexpressing HA-FAP52, on the other hand, displayed several types of abnormal morphologies. Some of them were overly elongated and showed several cell surface protrusions (Fig. 8A, arrowheads). More typical were cells with an outlook suggesting abnormal or arrested spreading (Fig. 8B). In some cells, prominent ruffling edge-type formations were seen (Fig. 8, C and D, arrows). A co-accumulation of actin and FAP52 was also seen in the ruffling edge formations (Fig. 8, C and D).

In most transfectants, a distinctly abnormal filamin distribution was seen; instead of a linear organization along actin fibers, numerous dot-like densities were seen especially close to the cell surface (Fig. 8B; red). Almost invariably, the dots also contained FAP52 (Fig. 8B; green). Instead of stress fibers, only thin and shorter filaments were seen running from one dot to another. This was also seen in double-staining with phalloidin; actin was only seen as distinct dots which also contained FAP52 and as thin filaments interconnecting the dots as a meshwork (Fig. 8D).

We also carried out transfection experiments using the HA-tagged N-terminal peptide of FAP52 (aa 1-293; FAP52Nt-HA) encompassing the filamin-binding site. For the most part
similar changes in actin and filamin organization were seen as with the overexpression of the full-length protein. A specific change seen with the N-terminal peptide was, however, the presence of transfectants with numerous and often very long filopodial extensions on the cell surface (Fig. 8, E and F). In more quantitative terms, the proportion of the cells with numerous filopodia was 30% in untransfected cells, and 60% and 64% in FAP52-HA and FAP52_{NT}-HA transfected cells, respectively, with cells with very long extensions (which were sometimes severed from the cell body; Fig. 8F, arrow) seen exclusively in FAP52_{NT}-HA transfectants. No major changes were seen upon transfection with the constructs encoding the SH3 domain of FAP52 (not shown).

Due to the presence of FAP52 in the focal adhesions in naive cells, we also wanted to see what happens to focal adhesions in cells overexpressing FAP52-HA or FAP52_{NT}-HA. Surprisingly, even in cells in which there were major distortions of the cell morphology and actin network, there were still distinct focal adhesions to be seen as revealed by a staining with anti-paxillin. They were not normal, however. Instead of a radially oriented axis and regular spacing, they displayed a more haphazard orientation. In most cases they were also smaller and fewer in number than their counterparts in the naive cells (61 vs. 40 focal adhesions per cell in nontransfected vs. transfected cells; n = 61 and 57, respectively; Fig. 8G).

Based on these studies, it is clear that the overexpression of the full-length FAP52 and its N-terminal, filamin-binding site-containing peptide brings about major derangements of the actin/filamin organization accompanied by unscheduled and exaggerated filopodia formation and minor derangements in the focal adhesion formation and architecture.
DISCUSSION

In this study, we provide evidence that FAP52 binds filamin and suggest that, via filamin, FAP52 is involved in the regulation of the actin cytoskeleton. Filamin, as a putative ligand for FAP52, was discovered and identified by using affinity purification and mass spectrometry. An in vivo association between FAP52 and filamin was verified by reciprocal co-IP studies, and a direct interaction between the proteins was demonstrated by using blot OL assays and SPR analysis. Also the results from the studies on the distribution of FAP52 and filamin in cultured cells are consistent with an interaction between the proteins. Confocal microscopy of the cells overexpressing FAP52 or its filamin binding fragments also showed that an aberrant expression of FAP52 leads to a gross derangement of the actin cytoskeleton organization. This is taken as an evidence that FAP52, via its interaction with filamin, is involved in the regulation of the actin cytoskeleton organization.

Filamin emerged as a putative binding partner of FAP52 from a “pull-down” experiment employing GST-FAP52 and from co-IP experiments from the solubilizate of cultured fibroblasts. Positive identification was based on a direct sequencing of the 280 kDa-band that was repeatedly seen in the “pull-down” experiments. Occasionally, also a smaller band of about 220 kDa was seen which turned out to be heavy chain of myosin. In subsequent studies we were, however, unable to demonstrate a direct interaction between myosin and FAP52. This observation implies, however, that FAP52 could be involved in a multimolecular complex encompassing also myosin.

Filamin is an actin cross-linking protein and is involved in the maintenance of the cytoskeletal architecture and in the adhesion and migration of cells (14). Most of the protein, which occurs as a dimer, is composed of 23 approximately 96-aa long repeats. They are flanked by an N-terminal actin-binding domain and a C-terminal homodimerization domain which both are important for the organization of the cortical actin network (14, 15); they
determine the capacity of filamin to direct the positioning of actin and to form orthogonal actin/filamin networks (13, 16). Filamin is a multifunctional protein and a great number of proteins binding to its repeat domains have been described. Most of them are either receptors or signaling proteins and are thought to be involved in synchronizing cell surface events with changes in the cytoskeletal architecture (14, 17). Especially pertinent in regard to the present study is a binding of filamin to some integrins which are closely linked both to cell adhesion and actin organization. Thus, filamin binds to the cytoplasmic tail of integrin β1 (18), the domain which is responsible for the focal adhesion targeting (19) and cytoskeletal linkage of the molecule. It also binds to integrin β7 cytoplasmic domain (18). In platelets, glycoprotein Ib-IX (GPIb-IX), the receptor for von Willebrand factor, is constitutively associated with filamin (20). Filamin also binds to the cytoplasmic tail of β2-integrin close to the binding site of α-actinin, another actin-cytoskeleton associated protein (21).

In cultured fibroblasts, FAP52 is seen in focal adhesions in which it colocalizes with paxillin, talin and vinculin (1). It is a phosphoprotein, being phosphorylated on serine but not on threonine or tyrosine residues. Up till now, no “ligands” for FAP52 have been described. FAP52 has a distinctly multi-domain structure. In the N-terminal part, encompassing the aa residues 1-145, there is a FCH domain (22), and, encompassing aa residues 146-280, a highly α-helical region. In the C-terminus, there is an SH3-domain. In the middle part of the molecule, encompassing the aa residues 281-389, there is a flexible “linker” region which does not form any recognizable fold (1).

The putative binding site for filamin, as shown by the OL and SPR assays employing truncated FAP52, resides in the N-terminal half of the protein with the region spanning from aa 146 to aa 185 as its indispensable estate. Interestingly, in the secondary structure prediction analysis, this region shows a high degree of α-helicity and a propensity to a coiled-coil arrangement (1). In this respect it seems to differ from the other known filamin-binding
peptides, which, according to our own analysis (not shown) do not display any notable $\alpha$-helicity. It is noteworthy, however, that there is no obvious similarity between the filamin-binding regions of the various known filamin ligands except for a cluster of charged residues which, according to helical wheel analysis, could serve as a platform to filamin binding in integrins and GpIb$\alpha$ (21). This variability, on the other hand, is quite expected in the light of the scattered nature of the binding sites in filamin.

In filamin, the binding site for FAP52 was localized to a region aa 1524-1858. This area corresponds to the repeats 14-16 and the calpain sensitive hinge region 1. Importantly, binding of furin (23), tumor necrosis factor receptor-associated factor 2 (TRAF2; 17), dopamine D-receptor (24, 25), and calcium-sensing receptor (26, 27) have been localized to the same area. Among some other filamin-binding proteins, GpIb$\alpha$ binds to the repeats 17-19 (28, 29), SEK-1 to the repeats 21-23 (30) and presenilin-1 to the repeats 22-24 (31). Thus, it is apparent that sufficient unique information resides in the repeats to create specific binding sites for a number of proteins. Binding of FAP52 close to the hinge region (between the repeats 15 and 16) could effect the flexibility of the filamin dimer, and, thus, could have an effect on the overall organization of the filamin-crosslinked actin network.

In cultured fibroblasts filamin is mostly seen associated with the stress fibers (32, 33). FAP52, on the other hand, is associated with the focal adhesions. In double staining experiments they mostly showed a complementary pattern in that FAP52 was seen in the focal adhesions at which actin filaments abut head on. At a close examination and in merged image it was obvious, however, that at the juncture of the focal adhesions and the microfilaments, there is an area in which the distributions of FAP52 and filamin overlap. It was seen especially in the focal adhesions in well spread and stationary cells. On the other hand, there were also focal adhesions which did not show any distinct overlap with filamin.
staining. This suggests that FAP52-filamin association is only seen in structures representative of a certain subclass of focal adhesions.

There are several detailed studies on the localization of filamin in cultured cells at both light- and electron microscopic level. Based on them, filamin has been shown to be associated with stress fibers and membrane ruffles (32, 34), microspikes (32), and in a delicate actin-based subcortical net and polygonal actin filament nets (33). In stress fibers, filamin is distributed in a discontinuous manner ("spotty" or "patchy") at shorter or longer intervals, depending on the cell type (33, 35, 36).

The present results, based on a careful analysis of confocal micrographs, clearly show that FAP52 is not only a focal adhesion-associated protein but that it is also present in the juncture area connecting focal adhesions and stress fibers and even to varying lengths to the distal ends of stress fibers proper. This could be taken to implicate a "supportive" function in this hinge region to FAP52 and/or to the components it might bind to. Given that there is a juncture of some sorts between the focal adhesion and its abutting stress fiber, it is reasonable to speculate that there are overlapping elements which either guide the well-orchestrated coevolvement and/or reinforce the structure of these two complex architectural organizations. Interestingly, remarkably little is known of the molecular mechanisms of the link between these two structures especially when compared with the detailed knowledge on the structure and composition of the focal adhesions and stress fibers in separation.

The close association with filamin strongly suggests a role for FAP52 in the actin organization. This was clearly demonstrated by the overexpression experiments in which major rearrangements of the actin cytoskeleton were seen. They include the extensive filopodia formation in response to an overexpression of the full-length FAP52 and an emergence of a deranged net of actin fibers instead of stress fibers in cells overexpressing the N-terminal domain of FAP52. In this respect, it is interesting that an overexpression of the
Ras-related small GTPase RalA, which also binds filamin, elicits formation of filopodia on the surfaces of the Swiss 3T3 cells, and also recruits filamin to the sites of filopod formation (9). These data along with the known actin-organizing properties of the homologs of FAP52 point to the role of FAP52, via its binding to filamin, as a major regulator of the actin cytoskeleton.
Acknowledgments—We thank the Bioanalytical Research Group (Dr. Matthias Wilm) at
the EMBL for mass spectrometry analysis, Dr. Päivi Pirilä and Dr. Kalervo Hiltunen for
advice and invaluable help with surface plasmon resonance analysis, and Ms Marjaana
Vuoristo, Ms Marja Tolppanen, Ms Marja-Liisa Martti, Ms Tarja Piispanen and Mr Hannu
Wäänänen for skillful technical assistance.
REFERENCES

1. Meriläinen, J., Lehto, V.-P., and Wasenius, V.-M. (1997) *J. Biol. Chem.* **272**, 23278-23284

2. Critchley, D. R. (2000) *Curr. Opin. Cell Biol.* **12**, 133-139

3. Ritter, B., Modregger, J., Paulsson, M., and Plomann, M. (1999) *FEBS Lett.* **454**, 356-362

4. Qualmann, B., and Kelly, R. B. (2000) *J. Cell Biol.* **148**, 1047-1062

5. Modregger, J., Ritter, B., Witter, B., Paulsson, M., and Plomann, M. (2000) *J. Cell Sci.* **113**, 4511-4521

6. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring HarborLaboratory, Cold Spring Harbor, NY

7. Feramisco, J. R., and Burridge, K. (1980) *J. Biol. Chem.* **255**, 1194-1199

8. Slot, J. W., and Geuze, H. J. (1985) *Eur. J. Cell Biol.* **38**, 87-93

9. Ohta, Y., Suzuki, N., Nakamura, S., Hartwig, J. H., and Stossel, T.P. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 2122-2128

10. Shevchenko, A., Jensen, O. N., Podtelejnikov, A. V., Sagliocco, F., Wilm, M., Vorm, O., Mortensen, P., Shevchenko, A., Boucherie, H., and Mann, M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 14440-14445

11. Gobom, J., Nordhoff, E., Mirgorodskaya, E., Ekman, R., and Roepstorff, P. (1999) *J. Mass Spectrom.* **34**, 105-116

12. Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T., and Mann, M. (1996) *Nature* **379**, 466-469

13. Gorlin, J. B., Yamin, R., Egan, S., Stewart, M., Stossel, T. P., Kwiatkowski, D. J., and Hartwig, J. H. (1990) *J. Cell Biol.* **111**, 1089-1105

14. Stossel, T.P., Condeelis, J., Cooley, L., Hartwig, J. H., Noegel, A., Schleicher, M., and Shapiro, S. S. (2001) *Nature Rev.* **2**, 138-145

15. Weiishing, R. R. (1985) *Can. J. Biochem. Cell. Biol.* **63**, 397-413
16. Cunningham, C. C., Gorlin, J. B., Kwiatkowski, D. J., Hartwig, J. H., Janmey, P. A., Byers, H. R., and Stossel, T. P. (1992) *Science* **255**, 325-327

17. Leonardi, A., Ellinger-Ziegelbauer, H., Franzoso, G., Brown, K., and Siebenlist, U. (2000) *J. Biol. Chem.* **275**, 271-278

18. Pfaff, M., Liu, S., Erle, D. J., and Ginsberg, M. H. (1998) *J. Biol. Chem.* **273**, 6104-6109

19. Yläne, J., Chen, Y., O'Toole, T. E., Loftus, J. C., Takada, Y., and Ginsberg, M. H. (1993) *J. Cell. Biol.* **122**, 223-233

20. Andrews, R. K., and Fox, J. E. (1991) *J. Biol. Chem.* **266**, 7144-7147

21. Sharma, C. P., Ezzell, R. M., and Arnaout, M. A. (1995) *J. Immunol.* **154**, 3461-3470

22. Aspenström, P. (1997) *Curr. Biol.* **7**, 479-487

23. Liu, G., Thomas, L., Warren, R. A., Enns, C. A., Cunningham, C. C., Hartwig, J. H., and Thomas, G. (1997) *J. Cell Biol.* **139**, 1719-1733

24. Li, M, Bermak, J. C., Wang, Z. W., Zhou, Q. Y. (2000) *Mol. Pharmacol.* **57**, 446-452

25. Lin, R., Karpa, K., Kabbani, N., Goldman-Rakic, P., and Levenson, R.. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 5258-5263

26. Awata, H., Huang, C., Handlogten, M. E., and Miller, R. T. (2001) *J. Biol. Chem.* **276**, 34871-34879

27. Hjalm, G., MacLeod, J., Kifor, O., Chattopadhyay, N., and Brown, E. M. (2001) *J. Biol. Chem.* **276**, 34880-34887

28. Meyer, S. C., Zuerbig, S., Cunningham, C. C., Hartwig, J. H., Bissell, T., Gardner, K., and Fox, J. E. (1997) *J. Biol. Chem.* **272**, 2914-2919

29. Takafuta, T., Wu, G., Murphy, G. F., and Shapiro, S. S. (1998) *J. Biol. Chem.* **273**, 17531-17538

30. Marti, A., Luo, Z., Cunningham, C. C., Ohta, Y., Hartwig, J. H., Stossel T. P., Kyriakis, J. M., and Avruch, J. (1997) *J. Biol. Chem.* **272**, 2620-2628
31. Zhang, W., Han, S. W., McKeel, D. W., Goate, A., and Wu, J. Y. (1998) J. Neurosci. 18, 914-922

32. Heggeness, M. H., Wang, K., and Singer, S. J. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3883-3887

33. Langanger, G., de Mey, J., Moeremans, M., Daneels, G., de Brabander, M., and Small, J. V. (1984). J. Cell. Biol. 99, 1324-1334

34. Nunnally, M. H., D'Angelo, J. M., and Craig, S. W. (1980) J. Cell. Biol. 87, 219-226

35. Schloss, J. A., and Goldman, R. D. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4484-4488

36. Mittal, B., Sanger, J. M., and Sanger, J. W. (1987) Cell Motil. Cytoskeleton. 8, 345-359
FIGURE LEGENDS

FIG. 1. A polypeptide of Mr 280 kDa coimmunoprecipitates with FAP52 and binds to GST-FAP52 in affinity column chromatography. A, SDS-PAGE of the Affi-K7 (lane 2) and control immunoprecipitates (lane 1) from the CEHF extracts. Silver staining. B, SDS-PAGE of polypeptides from the extract of CEHF cells bound to the GT-Sepharose beads carrying GST-FAP52 (lane 2) or, as a control, GST (lane 1). CBB staining. On the left, the position of the molecular weight marker of 220 kDa is shown.

FIG. 2. Filamin coimmunoprecipitates with FAP52, and, in the reciprocal experiment, HA-tagged FAP52 coimmunoprecipitates with filamin. A, SDS-PAGE and IB with anti-filamin of the extracts of CEHF incubated with either Affi-K7 (lane 2) or, for a control reaction, PBS (lane 1), followed by anti-rabbit IgG-agarose. B, SDS-PAGE and IB with Affi-K7 of the extracts of HeLa-cells expressing FAP52-HA incubated with either mouse anti-filamin (lane 2) antibodies or, for control reaction, PBS (lane 1), followed by anti-mouse IgG-agarose. The molecular weight markers are shown on the left.

FIG. 3. Filamin binds FAP52 in OL assay and in SPR analysis. A, FAP52, derived from the bacterially produced GST-FAP52 by a thrombin cleavage (lane 2), and, as a control, bacterially produced GST (lane 1), were run on 12% SDS-PAGE, transferred to NC filter, and then overlaid with the chicken gizzard filamin. Binding of filamin was probed with the anti-filamin antibodies and the ECL detection system. The positions of GST and FAP52 in a parallel, CBB-stained 12% SDS-PAGE gel are shown in the lanes 3 and 4, respectively. B, the bacterially produced truncation mutants of filamin in fusion with GST were run on 10% SDS-PAGE and transferred to an NC filter. The filter was then overlaid with the recombinant FAP52. The binding of FAP52 was probed by incubating with Affi-K7 and by ECL.
detection. The position of the same polypeptides (lanes 4-6, respectively) in the filter is shown by parallel 10% SDS-PAGE and IB with the anti-GST antibody followed by ECL detection system. Migration of the molecular weight markers is shown on the left. C, SPR analysis. GST-FAP52 was immobilized on a CM5 sensor chip, and chicken gizzard filamin was passed over the chip in mobile phase at concentrations ranging from 0-800 nM. In insert, sensorgram of a control experiment with GST anchored to the chip. Arrows indicate the beginning and the end of filamin injection.

**FIG. 4.** **Determination of the filamin-binding region in FAP52 by pull-down experiments and SPR.** 
A, SDS-PAGE and IB with anti-filamin of the full-length and the mutant FAP52 (as GST fusions) -coupled GT-Sepharose beads that were incubated with filamin. B, SPR analysis of a binding of filamin to the anchored full-length and truncated FAP52 (GST fusions). Filamin was passed over the chip at a concentration of 100 nM. In insert, sensorgram of a control experiment with the GST anchored to the chip. Arrows indicate the beginning and the end of filamin injection. C, SDS-PAGE (12%) analysis of the fusion proteins used in the pull-down experiments and SPR analysis.

**FIG. 5.** **Determination of the FAP52-binding region in filamin by pull-down experiments and SPR.** A, SDS-PAGE and IB with Affi-K7 of GT-Sepharose beads coupled with the filamin peptides and incubated with the recombinant FAP52. B, SPR analysis of the binding of FAP52 to the anchored filamin peptides (GST fusion) and GST. The recombinant FAP52 was passed over the chip at a concentration of 100 nM. Arrows indicate the beginning and the end of filamin injection. C, SDS-PAGE (10%) analysis of the fusion proteins used in the pull-down experiments and SPR analysis.
FIG. 6. **FAP52 and filamin colocalize in cultured CEHFs.** In double staining, FAP52 (green) shows a partial colocalization with filamin (red). FAP52 is in the elongated focal adhesions while filamin is seen as filamentous structures. In the merged image, a distinct overlap of the staining is seen in the area corresponding to the centrally oriented part of the focal adhesions (arrows, arrowheads). Bars, 10 µm.

FIG. 7. **Colocalization of FAP52 and filamin as revealed by gold-IEM of cryosections of cultured CEHF.** A, the large particles (filamin; arrows) are localized in fibrous structures, whereas the small particles (FAP52; arrowheads) are seen closer to the edges of the cells. B and C, blowups of the rectangled areas in A. A linear orientation of the particles is clearly seen (shown by bar-ended arrows). In circled areas, close proximity of the large (filamin) and the small particles (FAP52) is seen. Bars, 100 nm.

FIG. 8. **Double immunofluorescence microscopy shows an altered cellular morphology and filamin, actin and focal adhesion organization in cells overexpressing FAP52.** A, some of the transfected cells (arrowheads) exhibited an elongated cell shape with cell surface protrusions. Expression of HA-tagged FAP52 (green) and filamin (red) is detected. Untransfected cells are shown by arrows. B, an abnormal dot-like staining for filamin (red) was seen in many of the cells overexpressing FAP52. The dots also contain FAP52 (green), as seen in the merged image. C, a ruffling edge-type staining (arrows) of some cells overexpressing FAP52 (green). In the central part of the cell, FAP52 forms a dot-like staining pattern. The dots are distributed along the actin fibers (red), as seen in the merged image. D, codistribution of FAP52 (green) and actin (red) in the areas of the ruffling edge formation (arrows). E and F, cells overexpressing FAP52Nt-HA (green) typically exhibit, at their edges, long filopodia which are sometimes severed from the cell body (F, arrow). (G) the cells
overexpressing FAP52 (green) exhibit smaller and more randomly localized focal adhesions
(red) than naive cells. Bars, 10 µm.
Fig. 1

**A**

| kDa | 1 | 2 |
|-----|---|---|
| 220 |   | 280 kDa |

IP: - Affi-K7

**B**

| kDa | 1 | 2 |
|-----|---|---|
| 220 |   | 280 kDa |

Pull-down: GST GST-FAP52
Fig. 2

A

| kDa | 1 | 2 |
|-----|---|---|
| 220 |   | + |

IP: Affi-K7  
IB: Anti-filamin  

B

| kDa | 1 | 2 |
|-----|---|---|
| 66  |   | + |

IP: Anti-filamin  
IB: Affi-K7
Fig. 3

A

| kDa | 1   | 2   | 3   | 4   |
|-----|-----|-----|-----|-----|
| 66  |     |     |     |     |
| 46  |     |     |     |     |
| 30  |     |     |     |     |

OL: Filamin  
IB: Anti-Filamin  
CBB

B

Filamin peptide in fusion with GST

| kDa | 1   | 2   | 3   | 4   | 5   | 6   |
|-----|-----|-----|-----|-----|-----|-----|
| 97  |     |     |     |     |     |     |
| 66  |     |     |     |     |     |     |
| 46  |     |     |     |     |     |     |
| 30  |     |     |     |     |     |     |

OL: FAP52  
IB: Affi-K7  
IB: Anti-GST

C

RU vs. Time (s) for different concentrations of a protein in a binding assay.
Fig. 4

A

FAP52 peptide in fusion with GST

IB: anti-filamin

B

C

Staining: CBB
Fig. 5

A

Filamin peptide in fusion with GST

IB: Affi-K7

B

Filamin peptide in fusion with GST

C

Filamin peptide in fusion with GST

Staining: CBB
Fig. 6

A

filamin  FAP52  merge

B

filamin  FAP52  merge
Fig. 8
