FAP52, a Novel, SH3 Domain-containing Focal Adhesion Protein*

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Src-homology 3 (SH3) domain is a 60–70-amino acid motif present in a large variety of signal transduction and cytoskeletal proteins. We used reverse transcriptase-polymerase chain reaction with degenerate and specific primers and chicken brain mRNA to clone a cDNA that codes for a novel SH3 domain-containing protein. The sequence predicts a 448-amino acid polypeptide with a molecular mass of 51,971 daltons. In the amino terminus, it shows a very high propensity for α-helicity, suggesting coiled-coil and possibly a higher order oligomeric arrangement. In the carboxyl terminus, there is a unique SH3 sequence. In Northern blotting, a major 3.7-kilobase and a minor 7.2-kilobase transcript was detected in most chicken tissues. In immunofluorescence microscopy and immunoelectron microscopy on cultured chicken fibroblasts, the protein was localized to focal adhesions in which it showed a distinct codistribution with the focal adhesion proteins vinculin, talin, and paxillin. Phosphoamino acid analysis showed that in cultured chicken heart fibroblasts, the protein contains phosphoserine, but no phosphothreonine or phosphotyrosine, and that the phosphorylation is not dependent on fibronectin. We propose this protein the name FAP52, for Focal Adhesion Protein of 52 kDa, and suggest that it forms part of the multicomponent complex constituting focal adhesion sites.

Focal adhesions (FAs) are specialized membrane domains in cultured cells that mediate the attachment of cells to the growth substratum and extracellular matrix. They consist of pericellular and transmembrane structures connected to the actin-based cytoskeleton. The biochemical composition of FAs includes, e.g. the regulatory components of the FAs, the well known Src-homology 2 (SH2) and Src-homology 3 (SH3) domains that are important, e.g. in the substrate recognition of kinases and in the protein targeting, respectively (4).

SH3 domain is a 60–70-amino acid-long protein motif, which occurs widely, often in conjunction with a SH2 domain, in proteins of the signal transduction pathways in which it is involved in mediating protein-protein interactions (5–7). It is also present in various cytoskeletal proteins, such as a spectrin and myosin I (8, 9). The high degree of divergence of the known SH3 domains suggests that our current catalog of SH3-containing proteins is by no means exhaustive. In an effort to find new SH3-containing proteins, we have used polymerase chain reaction (PCR) and degenerate primers, designed on the basis of the conserved sequences in a repertoire of known SH3-containing proteins (10). Here, we report the identification, cDNA cloning, sequence analysis, subcellular distribution, and phosphoamino acid analysis of a novel SH3 domain-containing protein, which, based on the immunofluorescence studies, appears to be a focal adhesion-associated protein. Due to its subcellular localization and molecular mass, we propose it a name Focal Adhesion Protein of 52,000 daltons, FAP52.

EXPERIMENTAL PROCEDURES

General Procedures and Computer Programs—The solutions, buffers, and procedures for standard purification and precipitation of DNA, for restriction enzyme digestion, and ligations were as described in Sambrook et al. (11). For sequence analysis by the dideoxynucleotide chain termination method of Sanger (12), the T7 sequencing kit (Pharmacia Biotech Inc.) was used. Synthetic oligonucleotides, obtained from the Oligonucleotide Core Facility of Biocenter Oulu, were synthesized on a 392 DNA synthesizer (Applied Biosystems). For sequence alignments, the program CLUSTAL W (13) was used. Secondary structure prediction was by PHD, an automatic mail server for secondary structure prediction (14–17).

cDNA Cloning and Sequencing—Reverse transcription (RT) and PCR with degenerate oligonucleotide primers were used to amplify fragments of mRNAs encoding for SH3-containing proteins expressed in a developing chicken brain. For that purpose, total RNA was prepared from chicken embryonal (gestation age 16 days) brain tissue by the guanidine isothiocyanate method (18). RNA was reverse-transcribed using highly degenerate downstream primers designed on the basis of the moderately well conserved tryptophan-containing region (6) in the C-terminal of many SH3 proteins (in α-spectrin this corresponds to the amino acids residues 1003–1009; Ref. 9). The primer sequence was 5′-VWV MVCYTYCCACCARTC-3′ (for the symbols, see Nomenclature Committee of the International Union of Biochemistry (19), with a degeneracy of 864.

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5 The abbreviations used are: FAs, focal adhesion(s); CEHF, chicken embryo heart fibroblasts; CNBr, cyanogen bromide; FCS, fetal calf serum; PCR, polymerase chain reaction; RT, reverse transcriptase; PAGE, polyacrylamide electrophoresis; SH2, Src-homology 2; SH3, Src-homology 3; Pipes, 1,4-piperazinediethanesulfonic acid; bp, base pairs; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; TBE, Tris-buffered saline.
RT was carried out by using avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim GmbH). For PCR, an aliquot of cDNA was amplified by using the downstream primers described above and upstream primers based on the consensus sequence of a conserved N-terminal portion of the SH3 domain (6) which in a-spectrin corresponds to the polyproline II-like (PPII) sequence expressed in the cortex (7). The primer was 5'-KTSHKDGCYDTTPAYTAYWY-3', with a degeneracy of 20,736. After an initial melting step of 5 min at 95 °C, 35 cycles were carried out (1 min, 95 °C; 1 min, 45 °C; 1 min, 72 °C), followed by a 15-min final extension step in the following mixture: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 15 mM MgCl₂, 200 μM dNTPs, with 2.5 units of AmpliTaq® DNA polymerase (Perkin-Elmer).

The PCR products were resolved in a 0.5% agarose gel electrophoresis by using 4% NuSieve GTG-agarose (FMC Bioproducts). The bands of the expected size of approximately 100 bp were cut out and the DNA purified by using a silica-based matrix (Promega). The purified DNA was treated with Klenow Fragment of DNA polymerase I (Boehringer Mannheim GmbH) and subcloned into Smal-cut pGEM7-vector (Promega). The construct was transfected into competent XL1-blue cells (Stratagene) by using the standard protocol, depending on the position of the insert from several clones were determined, and several inserts corresponding to the expected size of about 100 bp were sequenced.

Several of the sequenced inserts had a sequence comparable to a unique SH3 domain. The nucleotide sequence of one of them was extended by using PCR cloning from a chicken brain cDNA library (Chicken Brain 5'-Stretch cDNA in agt10, Clontech) and from our own chicken brain cDNA library (9). Sequencing in both sense (5'-TTAAGTGATGGTGATCTTAAA-3') and antisense (5'-TTTTCTAGATTACCTCCTCCTCTA-3') primers, corresponding to the middle portion of the clone obtained from the previous step, were designed. PCR cloning was carried out by using the template DNA and the above primers along with agt10 forward (5'-CTTCTTGAAGACTACAGCTCTGAGTGAAG-3') and agt10 reverse (5'-GAGGGTCATGGAATCTTCTGAAAGGGT-3') primers. Several bands were obtained. The gel-purified DNA was ligated into a T/A cloning vector (Invitrogen). Both strands were sequenced by using SP6 and T7 promoter primers (Promega). Three clones, two extending 3' and one 5' of the original sequence, were harvested. Their sequences were obtained by using specific primers (Fig. 1A).

For the PCR amplification of the 5'-end of the corresponding mRNA, the 5'-AmpliFINDER RACE kit (Clontech) was used. The oligonucleotide 5'-GCGAGCTTTTCTCTTTTGAG-3' was used for PCR amplification. The longest clone obtained extended about 600 nucleotides from the priming site and over-extended about 600 nucleotides from the priming site and over-extended 5'-terminal region of the nucleotide sequence, were obtained by using specific primers (Fig. 1A).

Northern Blot Analysis—Total RNA was prepared from the embryonic chicken brain, lung, intestine, gizzard, liver, cardiac muscle, skeletal muscle, skin, kidney, and eye by using guanidine isothiocyanate method (18). Poly(A)+ RNA was then purified by affinity chromatography on an oligo(dT) column (Pharmacia). mRNA from cultured chicken embryonic heart fibroblasts (CEHF) was purified by using Quick-Prep Micro mRNA purification kit (Pharmacia). Five μg of total RNA (tissue samples) and 5 μg of mRNA (CEHF), along with the RNA size markers (Promega), was then resolved in formaldehyde-agarose gel (2%). The bands of the original sequence were cut out and purified by using a silicon-based matrix (Promega). The purified DNA was resolved in a 4% NuSieve GTG-agarose (FMC Bioproducts). The bands of the expected size, were excised and frozen immediately. The frozen samples were sliced with a razor blade on ice, and the bands were homogenized in an extraction buffer (1% SDS, 40 mM dithiothreitol, 5 μg EDTA, 7.5 mM sodium phosphate, pH 7.4) supplemented with protease inhibitors as above with a Dounce homogenizer. The extracts were boiled in Laemmli's sample buffer for 5 min and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroblotting onto nitrocellulose filters (Schleicher & Schuell) as described by Towbin et al. (21). The filters were then incubated in TTBS buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) with Affi-K7, followed by the secondary antibody. The primary antibody was affinity-purified using Bio-Rad protein assay reagent. Constant amounts of protein were separated on 10% SDS-PAGE and subjected to Western blotting. Thereafter, the electrochemiluminescent signal was detected using a computerized imaging system (Amersham). The results were then developed with 3,3'-diaminobenzidine (0.5 mg/ml in TBS) with H₂O₂ and 0.03% NiCl₂ or with the ECL method (Amersham).

Immunofluorescence Microscopy—For immunofluorescence microscopy, the cells were grown on glass coverslips. They were briefly washed in Hank's salt solution and then fixed with 4% formaldehyde in a cytokeratin-stabilizing buffer (100 mM Pipes, pH 6.8, 5 mM EGTA, 2 mM MgCl₂, 20% Triton X-100) for 10 min at room temperature. After being washed in PBS, the cells were post-fixed in −20 °C methanol for 5 min, incubated with 10% PBS-glycine (PBS with 20 μM glycine) for 30 min, and washed with PBS. They were then overlaid with Affi-K7 at 4 °C for 30 min followed by Texas Red-conjugated anti-rabbit IgG (Jackson Immunoresearch). For double labeling experiments, the primary antibodies to talin (mouse monoclonal; Developmental Studies Hybridoma Bank, Department of Biology, University of Iowa), to paxillin (mouse monoclonal; Zymed Laboratories Inc.), to vinculin (mouse monoclonal; Biohit-Locus, Inc.), and the secondary antibodies tetramethylrhodamine isothiocyanate-conjugated (Caltag Laboratories) or fluorescein isothiocyanate-conjugated (Dako A/S) anti-rabbit IgG were used. The cells were viewed with a Nikon BH-2 fluorescence microscope equipped with appropriate filters.

Immunoelectron Microscopy—For ultrastructural localization studies, double labeling immunoelectron microscopy on whole mount cytoskeletal preparations of cultured cells was done by following the procedure described elsewhere (23). Briefly, CEHEFs grown onto gold grids were permeabilized and fixed by a treatment with 0.1% Triton X-100, 0.1% glutaraldehyde in cytoskeleton-stabilizing

rose affinity chromatography (20). The cells were used for the experiments between the second and eighth passages.

Preparation of Antibodies—To generate antibodies, the cDNA corresponding to the of nucleotides 482–1471 of the full-length sequence (Fig. 1B) was amplified by using PCR and subcloned into a pEB5-α prokaryotic expression vector (Novagen, Madison, WI). The insert was ligated into pEB5 and transformed into E. coli bacteria. The bacteria were cultured in a lysis buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 1% Triton X-100) and lysed by sonication. Laemmli’s sample buffer was added to the lysates, which were then cleared and resolved in 10% SDS-PAGE. After staining with Coomassie Blue, pieces of gel containing the expressed polypeptide band of 48 kDa, corresponding to the polypeptide expressed from the insert, were cut out and sonicated briefly in 50% methanol to remove the dye. Thereafter, the gel was washed and neutralized and the polypeptides electroeluted from the gel by using Bio-Rad model 422 electroeluter. The electroeluted protein was dialyzed against 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100 and used for immunizations. The authenticity of the protein used for immunizations was verified by partial amino acid sequence (Procie 494, Perkin-Elmer). One of the obtained antisera, named Anti-K7, was selected for further studies on the basis of its specificity as assessed by immunoblotting. It was further affinity-purified on CNBr-activated Sepharose 4B beads (Pharmacia) coated with the electroeluted 48-kDa polypeptide. The affinity-purified antisera is denoted Anti-K7.

Immunoblotting and Immunoprecipitation—For immunoblotting, CEHEFs were washed with PBS and then scraped into radiolaboratory precipitation buffer (158 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 0.2 mM sodium orthovanadate, 1% Triton X-100 and 10 mM Tris-HCl, pH 7.2) supplemented with the protease inhibitors NaF (50 mM), aprotinin (200 μg/ml), leupeptin (1 μg/ml), phenylmethylsulfonyl fluoride (0.25 mM), and benzamidine (0.5 mM). For immunoblotting of tissues, small pieces of 18-day-old chicken embryos were freshly excised and frozen immediately. The frozen samples were sliced with a razor blade on ice, and the pieces were homogenized in an extraction buffer (1% SDS, 40 mM dithiothreitol, 5 μg EDTA, 7.5 mM sodium phosphate, pH 7.4) supplemented with protease inhibitors as above with a Dounce homogenizer. The extracts were boiled in Laemmli’s sample buffer for 5 min and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblotting with the rabbit anti-K7 antisera. The immunoprecipitations were carried out by using Bio-Rad protein assay reagent. Constant amounts of protein were separated by 10% SDS-PAGE and subjected to Western blotting. Thereafter, the electrochemiluminescent signal was detected using a computerized imaging system (Amersham). The results were then developed with 3,3’-diaminobenzidine (0.5 mg/ml in TBS) with H₂O₂ and 0.03% NiCl₂ or with the ECL method (Amersham).
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ing buffer for 2 min, followed by an incubation with 8% paraformaldehyde in cytoskeleton-stabilizing buffer for 8 min. For the double immunolabeling, the cells were incubated first with the monoclonal anti-paxillin antibody (diluted in 5% FCS in PBS-glycine) for 45 min and then with a protein A-gold complex (size 10 nm, Janssen Life Sciences Products) for 30 min. The cells were then incubated with protein A (Pharmacia) in PBS (0.1 mg/ml) for 10 min. This was followed by an incubation with Affi-K7 (diluted in 5% FCS in PBS-glycine) for 45 min and then with a protein A conjugated to gold particles of 15 nm in diameter (a kind gift from Dr. Varpur Marjomäki, University of Jyväskyla, Jyväskyla, Finland) for 30 min. After immobilanlating, the cells were first postfixed with 2.5% glutaraldehyde in phosphate buffer for 10 min and then negative-stained by, first, washing briefly in distilled water and then immersing in a drop of 1% aqueous uranyl acetate for 1 min. For a control staining, rabbit preimmune serum was substituted for Affi-K7 antibodies. The cells were examined in a Philips 410 LS transmission electron microscopy.

Phosphoamino Acid Analysis—Phosphoamino acid analysis of attacking and well spread cells on both plain culture dishes and on culture dishes precoated with plasma fibronectin (10µg/ml) both in the presence and absence of FCS was carried out as follows: Subconfluent CEHFs were trypsinized and washed with phosphate-free buffer. Phosphoproteins were labeled by plating the cells and incubating in Eagle's CEHFs were trypsinized and washed with phosphate-free buffer. Phosphoamino acid analysis of attacking and well spread cells on both plain culture dishes and on culture dishes precoated with plasma fibronectin (10µg/ml) both in the presence and absence of FCS was carried out as follows: Subconfluent CEHFs were trypsinized and washed with phosphate-free buffer. Phosphoproteins were labeled by plating the cells and incubating in Eagle's medium containing 3% FCS and 10% [32P]orthophosphate (370 MBq/ml; Amersham) at 37°C for 6 h. The cells were harvested and lysed by scraping in radioimmuno precipitation buffer. Thereafter, FAP52 was immunoprecipitated by adding 100 µl of Affi-K7 to 5 ml of the clarified lysate and incubating at 4°C for 6 h. Seventy µl of a 50% slurry of anti-rabbit IgG-Sepharose (Sigma) was added to the mixture that was then incubated at 4°C for 1 h on a rocking platform. Immunoprecipitates on the Sepharose-beads were washed in radioimmuno precipitation buffer. The proteins in the immunoprecipitates were resolved in 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Immobilon, Millipore) as described by Boyle et al. (24). The phosphoproteins on the membrane were visualized by autoradiography. The band corresponding to FAP52 was cut out and subjected to acid hydrolysis (6 N HCl for 1 h at 110°C), whereafter the phosphoamino acids were separated in two dimensions on thin-layer cellulose plates as described (24).

RESULTS

Isolation and Sequencing of cDNA Clones—The RT-PCR cloning strategy and the clones obtained are shown in Fig. 1A. Sequencing of the amplification products revealed several novel SH3-encoding cDNAs, as judged by the comparison of the deduced amino acid sequences against the data banks. One of them was selected for further cloning (striped boxes). Oligonucleotide primers based on this sequence and chicken brain cDNA libraries were used to extend the sequence to 5'- and 3'-directions. Two 3'-extending clones, which were colinear and which both presented a putative stop codon (arrows to the right in Fig. 1A), were obtained. One clone extended 1100 nucleotides upstream from the priming site but lacked a possible start site of translation (an arrow to the left in Fig. 1A). By using RACE and RT-PCR with thermostable reverse transcriptase, several colinear clones extending upstream and containing a putative start site for translation were obtained. One of them, a 600-bp cDNA obtained by RACE, was selected for further characterization (a thick bar in Fig. 1A).

The nucleotide and the predicted amino acid sequence of the longest open reading frame are shown in Fig. 1B. The predicted protein has 448 amino acids and a molecular mass of 51,971 daltons (Da). The open reading frame contains a methionine codon starting at the nucleotide 29, and it extends to TGA termination codon at the position 1570. Stop codons were present in all three reading frames preceding the initiation methionine. Only a partial agreement with the Kozak consensus sequence is seen in codon representing the putative translation start site. However, in codon usage and putative open reading frame analysis by the programs Codon Preference and Testcodon (GCC), an unequivocal open reading frame extending from the codon 224 to 1570 is predicted. In the coding region, there are no obvious signal sequence or transmembrane domains. The SH3 domain resides in the 3'-terminal end of the protein, encompassing the residues 390–448.

The predicted amino acid sequence was analyzed for a similarity with other known sequences. An alignment with three sequences that showed the highest degree of similarity is shown in Fig. 1C. H74, a sequence deduced from a mouse brain cDNA for a protein with an unknown function, shows a 70% identity with the FAP52 sequence. EM13 and EG13 are proteins isolated from Echinococcus multilocularis and Echinococcus granulosus, respectively, on the basis of their immunogenicity (25). Their sequences show approximately 50% identity with FAP52. The highest similarities between the proteins are present in the C-terminal SH3 domains.

The EM13 and EG13 sequences in the data base differ from FAP52 sequence in having truncated N- and C termini. However, a closer scrutiny of the EG13 nucleotide sequence discloses an upstream open reading frame of 21 amino acids (amino acids 16–36) that closely corresponds to the N-terminal sequence of FAP52. In the C termini of both EM13 and EG13, a frameshift of one nucleotide, reveals a stretch of 6 amino acids that corresponds to the C-terminal part of the SH3 domain of FAP52 and H74 and recovers the last SH3 β-strand (see below). On the basis of this comparison, we suggest that FAP52, H74, EM13, and EG13 are members of the same family of proteins.

Secondary structure prediction by using the multiple alignment and the network server PHD proposes a very high probability for α-helix in the N-terminal two-thirds of the proteins. There are six long α-helices, each about 40 amino acids long, and several shorter regions with a high degree of α-helicity (Fig. 1C). These are flanked by short linker regions with a predicted coil structure. The confidence scores above 9 indicate a >90% reliability of the prediction. The α-helices appear to be too long to fold with a globular structure. The regular arrangement of the hydrophobic amino acids with a heptad periodicity suggests a coiled-coil arrangement. However, in the heptads, not only the positions α and β but also other positions tend to be conserved, suggesting that the helices may form higher order oligomeric structures rather than simple coiled-coil dimers.

Following the α-helical N-terminal portion, there is a region of about 130 amino acids that shows the lowest degree of sequence similarity between the four proteins and no clear secondary structure. It probably represents a nonglobular linker region between the N-terminal α-helical domain and the C-terminal SH3 domain. In summary, the analysis of the sequence suggests a three-domain structure with an α-helical, rod-like N-terminal domain and a C-terminal SH3 domain interspersed by a nonglobular linker region.

Expression of FAP52 in Chicken Tissues—Northern blot analysis revealed two transcripts of sizes 3.7 and 7.2 kb. They were present in all the tissues studied: gizzard, liver, cardiac muscle, skeletal muscle, brain, lung, intestine, kidney, skin, and eye (Fig. 2, lanes b–k) and also in CEHF cells (Fig. 2, lane l). The major transcript in all the tissues was of 3.7 kb.

Immunolocalization of FAP52—Affinity-purified antibodies to FAP52 (Affi-K7) were used to localize FAP52 in cultured CEHFs, both by using immunofluorescence and immunoelectron microscopy. The specificity of the antibodies was demonstrated by immunoblotting of the total lysates of CEHFs and various chicken tissues and by immunoprecipitation of FAP52 from total CEHF lysate by using Affi-K7 (Fig. 3). In blotting, only a single band of about 63 kDa was seen in CEHFs and in all the tissues tested (Fig. 3, lanes a–e). A band of a similar molecular mass along with a faster migrating band of 55 kDa
corresponding to the heavy chain of immunoglobulins was also seen in immunoblot of an Affi-K7 immunoprecipitate of CEHFs (Fig. 3, lane g). No corresponding bands were detected in an immunoprecipitate of CEHF lysate (Fig. 3, lane f) and in immunoblotting of tissues (data not shown) when preimmune serum was substituted for Affi-K7, attesting to a specific recognition of a band of 63 kDa by the antibodies and a lack of cross-reacting species in the tissues and cells tested. Notably, in SDS-PAGE, FAP52 migrates considerably slower than is expected on the basis of its molecular mass of 51,971 Da.

In immunofluorescence microscopy of permeabilized cells with Affi-K7, a distinct decoration of elongated, plaque-like structures was seen (Fig. 4, a–c and g). They were located at the ventral surface and predominantly along the perimeter of the cell, as judged by a differential focusing. No such staining was seen in unpermeabilized cells, indicating that the immunological recognition occurred at the cell surface and was not due to cytoplasmic fluorescence.
noreferreradhesions, the specific plasma membrane domains that
are responsible for the anchorage of the cells to their growth
substratum (26).

Phosphoamino Acid Analysis—The phosphorylation state of
FAP52 in growing cultures of CEHFs was studied by immuno-
precipitating FAP52 with Affi-K7 from a lysate of $^{32}$P-labeled
cells (Fig. 6A) and then subjecting it to a phosphoamino acid
analysis (Fig. 6B). In cultures grown on fibronectin-coated
dishes in the presence of the label for 6 h, FAP52 is present in
a phosphorylated state as shown by immunoprecipitation and
autoradiography in which a distinctly labeled band of 63 kDa
could be seen (Fig. 6A, lane b). No band was seen in the control
precipitation with the preimmune serum (Fig. 6A, lane c).
Similar results were obtained on cells grown on nonprecoated
dishes (data not shown). Two-dimensional thin-layer electro-
phoresis showed that FAP52 is exclusively phosphorylated on
serine (s arrow) residues with no signs of phosphorylation on
threonine (t arrow) or tyrosine (y arrow) (Fig. 6B). Neither
could we show any phosphorylation to tyrosine by using immu-
noprecipitation with Affi-K7 followed by immunoblotting with
anti-phosphotyrosine antibodies (data not shown).
DISCUSSION

In this study, our aim was to identify novel SH3 domain-containing proteins. Our strategy was based on the fact that, despite their overall low degree of similarity, there are fairly well conserved regions in the N and C termini of the various SH3 domains (6). Primers for RT-PCR cloning were designed on the basis of the most highly conserved sequences, which in the N terminus encompass the residues 1–10 and in the C terminus the residues 35–40 (for a justification of the domain boundaries and of the numbering, see Ref. 6). These sites contribute to a smooth patch on the domain surface, which has been shown to correspond to the ligand binding site of the SH3 domain (27). It is interesting, however, that the amino acid residues in the ligand-binding site are not completely conserved, suggesting that SH3 domains recognize a family of related domains or proteins in different species and tissues. Thus, a strategy of using degenerate primers with a high degree of degeneracy can be expected to allow for identification of novel sequences with distinct ligand binding properties. This is an important consideration since, due to generally low binding affinities to their ligands (27–29), identification and purification of SH3 domain-containing proteins by using conventional protein affinity purification techniques has met with little success.

In this study, one of the clones obtained encodes for a 52-kDa protein with a SH3 domain in its C terminus. Antibodies were raised against a polypeptide that corresponds to the N-terminal and middle portions of the protein. In immunofluorescence and immunoelectron microscopy, the protein was found to be localized in focal adhesions as judged by its colocalization with the focal adhesion proteins vinculin, talin, and paxillin. Thus, the protein appears to be a novel SH3-containing protein that is associated with the focal adhesions. Hence, we propose it the name FAP52 for Focal Adhesion Protein of 52 kDa.

The 2947-bp composite cDNA, obtained in this study, contains a 1347-bp coding region flanked by an 223-bp 5'-noncoding region and an 1377-bp 3'-noncoding region. Northern blot analysis revealed a major mRNA species of 3.7 kb. Therefore, 0.7 kb of noncoding region remains unaccounted for. We believe that it includes additional 3'-noncoding sequences, since we have not been able to identify a polyadenylation signal in the 3'-noncoding sequence. We also saw a mRNA of 7.2 kb in the Northern blots. Whether it represents a larger isoform or a cross-hybridizing species remains to be studied.

SH3 domains are found especially in proteins involved in signal transduction pathways and in cytoskeletal proteins (for recent reviews, see Refs. 4 and 30). They are distinct protein modules that can be, e.g. crystallized independently of the rest of the protein (10). X-ray crystallography and NMR spectroscopy has shown that the domain encompasses a relatively flat surface, which forms the ligand-binding site flanked by two loops (27). The N and C termini of the domain are in close apposition, which makes the ligand-binding site bulge out from the surface of the rest of the protein.

In sequence comparison, the SH3 domain of FAP52 shows a close conformation with the consensus sequence completed from more than 70 distinct SH3 sequences (6). Unlike most other SH3 domains, which have a well conserved double tryptophan in the C-terminal part of the domain, however, SH3 domain of FAP52 has only one tryptophan followed by cysteine. In this respect, FAP52 shows a similarity with the SH3 domain of Abl oncoprotein. Moreover, unlike most other SH3 domain-containing proteins, FAP52 does not contain any other recognizable sequences characteristic of signal transduction cascade proteins, such as kinase, SH2, PTPase, Cdc25, or GAP domains (30). In this sense, it resembles α-spectrin, myosin I, ABP-1, SLA1 and BEM1, and p47 box and p67 box components of the neutrophil oxidase (6). Interestingly, most of these “SH3-only” domain proteins, such as the yeast proteins ABP-1, SLA-1, and BEM1, seem to have a function closely associated with the control of the cell morphology or to be involved in the organization of the actin cytoskeleton (31–33). Thus, the domain structure of FAP52 is well in harmony with its localization in focal adhesions, which represent principal plasma membrane anchorage sites of actin filaments in cultured cells (26, 34). On the basis of the speculation concerning the domain structure of FAP52, it could be surmised that FAP52 serves as an “adapter” with SH3 mediating the linkage to the plasma membrane and the helical N terminus interacting with actin or some other focal adhesion components.

Comparison of FAP52 to other sequences in the data bases showed a high degree of similarity to H74, a mouse protein of unknown function, and to EM13 and EG13, major antigenic proteins of E. multilocularis and E. granulosus, respectively. Apart from an association of EM13 with the surface membrane of E. multilocularis (25), nothing is known of the function of EM13 or EG13 that could be instructive of the function of FAP52. More recently, we noticed that, albeit a low overall similarity, FAP52 shows a striking similarity in domain structure to a Schizosaccharomyces pombe protein Cdc15 (35), a cell cycle-dependent protein that serves as a key component in the reorganization of F-actin during cytokinesis. As in FAP52, there is a coiled-coil region also in the N terminus of Cdc15, and an SH3 domain in the C terminus. Interestingly, studies with genetically defective S. pombe suggest that an interaction with a fission yeast profilin and myosin regulatory chain, both of which interact with actin, may be important for the actin-associated functions of cdc15 (35). In regard to the similarity in their domain structure and their association with actin-containing structures, we suggest that also FAP52 and Cdc15 may represent members of the same family of proteins.

Focal adhesions are specialized membrane domains at the ventral surface of cultured cells. They are best known for their role as “feet,” which provide the attachment sites by which the cells anchor themselves to the growth substratum (36). At the cytoplasmic face of the plasma membrane, focal adhesions serve as attachment points of the actin filament bundles to the plasma membrane (34). Apart from this structural role, focal adhesions have recently emerged as major sites of signal transduction pathways stimulated by cell-matrix interactions (37–40). Accordingly, in addition to the well characterized struc-
tural components, such as α-actinin (41), vinculin (42), talin (43), and paxillin (34), also a growing number of proteins with enzymatic or as yet unidentified functions have been described in focal adhesions (44). These include v-src in src-transformed cells (45), protein kinase C (46), focal adhesion kinase (p125<sup>FAK</sup> (47), 48)), FAK-related protein without a kinase domain (FRNK (49)), tenuin (50), the myristoylated, alanine-rich protein kinase C substrate of protein kinase C (MARCKS (51)), calpain II (52), zyxin (53), and cCRP (54). The role of focal adhesions as nodes in signal transduction pathways is highlighted in the src-transformed cells in which the focal adhesion-associated v-src oncprotein pp60<sup>src</sup> is responsible for the highly enhanced tyrosine phosphorylation of paxillin and tensin (37). Also talin, vinculin, and β<sub>3</sub> integrin subunit have been reported to be tyrosine-phosphorylated in src-transformed cells, supposedly reflecting the presence of activated Src in focal adhesions (55–58). Interestingly, some of the interactions involved are dependent on the SH3 domain of pp60<sup>src</sup>, as evidenced by the binding of a recombining fusion protein containing the SH3 domain of pp60<sup>src</sup> to paxillin (59).

Phosphoamino acid analysis showed that FAP52 is present in a phosphorylated state in growing cells. Only phosphorylation on serine, with no detectable phosphorylation on tyrosine or threonine, could be found in cells that were allowed to attach on either uncoated or fibronectin-precoated culture dishes for several hours. This is drastically different from the rapid phosphorylation on tyrosine of, e.g., FAK, which occurs in the early phase of the formation of focal adhesions brought about plating the cells onto fibronectin-coated dishes (48). This implies that, at least in its phosphorylated state, FAP52 plays a role in the later rather than the early phases of the emergence of focal adhesions.

In conclusion, we describe in this paper the identification, sequence analysis, and partial characterization of FAP52, a novel focal adhesion-associated SH3 domain-containing protein. Based on its modular structure, SH3 domain in its C terminus and a highly α-helical N terminus with a predicted propensity to form coiled-coil structures, and on its phosphorylation on serine residues, we suggest that FAP52 is involved, in a modifiable manner, in the protein-protein interactions within focal adhesions. For the elucidation of its functional role, identification of its binding partner(s) is needed.

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REFERENCES

1. Jockusch, B. B., Beubeck, P., Giehl, K., Kromer, M., Moschner, J., Rothgel, M., Schuler, K., Stanke, G., and Winkler, J. (1995) Annu. Rev. Cell Dev. Biol. 11, 379–416
2. Burridge, K., and Chrzanowska-Wodnicka, M. (1996) Annu. Rev. Cell Dev. Biol. 12, 463–519
3. Yamada, K. M., and Miyamoto, S. (1995) Curr. Opin. Cell Biol. 7, 681–689
4. Pawson, T. (1990) Nature 343, 573–580
5. Koch, C. A., Anderson, D., Moran, M. F., Ellis, C., and Pawson, T. (1991) Science 252, 668–674
6. Musacchio, A., Gibson, T., Lehti, V.-P., and Saraste, M. (1992) FEBS Lett. 307, 55–61
7. Pawson, T., and Schlessinger, J. (1993) Curr. Biol. 3, 434–442
8. Lehto, V.-P., Wasenius, V.-M., Salven, P., and Saraste, M. (1998) Nature 334, 388
9. Wasenius, V.-M., Saraste, M., Salven, P., Eramaa, M., Holm, L., and Lehto, V.-P. (1999) J. Cell Biol. 146, 79–93
10. Musacchio, A., Noble, M., Paupitz, R., Wierenga, R., and Saraste, M. (1992) Nature 356, 851–855
11. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
12. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
13. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680
14. Rost, B., Sandberg, C., and Schneider, R. (1994) Comput. Appl. Biosci. 10, 53–60
15. Rost, B., and Sander, C. (1994) Proteins 19, 55–72
16. Rost, B., and Sander, C. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7558–7662
17. Rost, B., and Sander, C. (1993) J. Mol. Biol. 232, 584–599
18. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5294–5299
19. Nomenclature Committee of the International Union of Biochemistry (NC-IUB) (1985) Eur. J. Biochem. 150, 1–5
20. Engvall, E., and Ruoslahti, E. (1977) Int. J. Cancer 20, 1–5
21. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
22. Viliikind, U., and Swierenga, S. H. (1989) Histochemistry 91, 81–88
23. Sormunen, R., and Lehto, V.-P. (1995) Eur. J. Cell Biol. 68, 387–397
24. Boyle, W. J., van der Geer, P., and Hunter, T. (1991) Methods Enzymol. 201, 110–149
25. Frosch, P. M., Geier, G., Kaup, J. F., Muller, A., and Frosch, M. (1993) Mol. Biochem. Parasitol. 58, 301–310
26. Burridge, K., Fath, K., Kelly, T., Nuckolls, G., and Turner, C. (1988) Annu. Rev. Cell Biol. 4, 487–525
27. Musacchio, A., Saraste, M., and Wilmanns, M. (1994) Nat. Struct. Biol. 1, 546–551
28. Vignera, A. R., Arrondo, J. L. R., Musacchio, A., Saraste, M., and Serrano, L. (1994) Biochemistry 33, 10925–10933
29. Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W., and Schneider, S. L. (1994) Cell 76, 933–945
30. Cohen, G. B., Ren, R., and Baltimore, D. (1995) Cell 80, 237–248
31. Chennevert, J., Corrado, K., Bender, A., Pringle, J., and Herskowitz, I. (1992) Nature 356, 77–79
32. Holzman, D. A., Yang, S., and Drubin, D. G. (1993) J. Cell Biol. 122, 635–644
33. Kavanaugh, W. M., and Williams, L. T. (1994) Science 266, 1862–1865
34. Turner, C. E., Glenney, J. R., Jr., and Burridge, K. (1990) J. Cell Biol. 111, 1059–1068
35. Fankhauser, C., Reymond, A., Cerutti, L., Utzig, S., Hofmann, K., and Simanis, V. (1995) Cell 82, 435–444
36. Burridge, K., Petch, L. A., and Romer, L. H. (1992) Curr. Biol. 2, 537–539
37. Burridge, K., Turner, C. E., and Romer, L. H. (1992) J. Cell Biol. 119, 893–903
38. Lo, S. H., and Chen, L. B. (1993) Cancer Metastasis Rev. 12, 268, 851–855
39. Schaller, M. D., Borgman, C. A., Cobb, B. C., Reinals, A. B., and Parsons, J. T. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5192–5196
40. Schaller, M. D., Borgman, C. A., and Parsons, J. T. (1993) Mol. Cell. Biol. 13, 785–791