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Analysis of the Adaptor Function of the LIM Domain-Containing Protein FHL2 Using an Affinity Chromatography Approach

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Abstract Containing four LIM domains and an N-terminal half LIM domain, FHL2 has been predicted to have an adaptor function in the formation of higher order molecular complexes in the nucleus and the cytoplasm of cells. We expressed recombinant FHL2 in insect cells using the baculovirus system and used it to isolate direct or indirect interaction partners from the cytosolic fraction of fibroblasts by affinity chromatography. These were identified by their peptide mass fingerprints using MALDI-TOF mass spectrometry. Cytoskeleton-associated proteins present among the bound proteins were shown to co-localise with FHL2 in cell lamellipodia by indirect immunofluorescence staining. J. Cell. Biochem. 92: 612–625, 2004. © 2004 Wiley-Liss, Inc.

Key words: FHL2; LIM domain; lamellipodia; proteomics; MALDI-TOF; mass spectrometry

FHL2 is the second member of a small family of five proteins containing four and a half LIM domains [Fimia et al., 2000]. The acronym LIM derives from *L*in-11, *I*sl-1, and *M*ec-3, three transcription factors in which a specific motif, LIM, was originally described [Way and Chalfie, 1988; Freyd et al., 1990; Karlsson et al., 1990]. The LIM motif is composed of two adjacent zinc fingers and has been shown to mediate protein–protein interactions, hence the assumption that one of the functions of proteins containing LIM domains is to serve as adaptors in the formation of higher order molecular complexes needed for the accomplishment of many cellular processes [Khurana et al., 2002; Retaux and Bachy, 2002]. The concept is supported by the observation that a large variety of proteins containing LIM domains associate with the actin cytoskeleton, function as nuclear-cytoplasmic shuttlers, or regulate transcriptional activity [Khurana et al., 2002; Retaux and Bachy, 2002]. FHL2 has been localised in the cytoplasm and in the nucleus of cells [Genini et al., 1997; Wixler et al., 2000] and could, therefore, be involved in the structuring of higher order molecular complexes at these locations.

We have identified FHL2 as a binding partner for the cytoplasmic tail of several integrin subunits, including α3A and B, α7A and β1 [Wixler et al., 1999, 2000]. When associated into α3β1 and α7β1 heterodimers, these integrins function as receptors for laminins, a subset of extracellular matrix proteins important for the control of cellular functions like migration, differentiation, proliferation, and expression of specific genes [Aumailley and Rousselle, 1999; Aumailley et al., 2000]. However, integrins are devoid of enzymatic activity and signal transmission requires the association of cytosolic and cytoskeletal proteins with the cytoplasmic tail of the receptor [Zamir and Geiger, 2001]. Interestingly, FHL2 has been localised in some, but

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not all, cytoskeleton- and integrin-associated structures [Wixler et al., 2000; Li et al., 2001]. Thus FHL2 could function as an adaptor in the formation of the integrin signalling complexes. Moreover, based on yeast two-hybrid screens, FHL2 has been found to interact with many-other proteins including hCDC47, a member of the minichromosome maintenance protein family regulating DNA replication [Chan et al., 2000], the androgen receptor [Muller et al., 2000], presenilin 2 [Tanahashi and Tabira, 2000], RNA recognition motifs [Dye and Patton, 2001], DNA-binding nuclear protein hNP220 [Ng et al., 2002], the insulin-like growth factor-binding protein-5 [Amaar et al., 2002], TUCAN-CARDINAL [Stilo et al., 2002], the promyelocytic leukemia zinc finger protein [McLoughlin et al., 2002], the potassium channel subunit MinK [Kupershmidt et al., 2002], β-catenin [Martin et al., 2002; Wei et al., 2003], titin, and various metabolic enzymes [Lange et al., 2002], as well as with actin [Coghill et al., 2003].

These observations suggest that FHL2 may be an adaptor widely used in diverse pathways. To get experimental support for this hypothesis at the protein level, we have prepared recombinant FHL2 to investigate its role as an adaptor protein in the formation of multiprotein aggregates or "interactomes", constituted by a certain set of proteins with a variety of bilateral interactions. By using an affinity-based approach, we have isolated specific sets of proteins able to interact, directly or indirectly, with FHL2 and the individual constituents were identified by their peptide mass fingerprints using MALDI-TOF mass spectrometry. Interestingly, considering the integrin-binding properties of FHL2, cytoskeleton-associated proteins were present among the bound proteins and shown to co-localise with FHL2 in cell lamellipodia by indirect immunofluorescence staining.

MATERIALS AND METHODS

Immunofluorescence Staining of Cells

Affinity-purified antibodies against FHL2 were prepared from an antiserum raised in rabbit against recombinant FHL2 [El Mourabit et al., 2003]. For antibody purification, recombinant Strep II-tagged FHL2 was loaded on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gels and electrophoretically transfered to nitrocellulose. The polypeptide band corresponding to Strep II-tagged FHL2 was identified by Ponceau red staining, cut out and incubated with the antiserum against FHL2. Bound antibodies were eluted with 100 mM glycine, pH 2.5, and neutralised with 1 M Tris-HCl, pH 8.8.

Human Wi26 fibroblasts were seeded on glass coverslips and grown overnight in DMEM containing 10% foetal calf serum. The cells were then fixed with 2% paraformaldehyde in PBS for 15 min, permeabilised with 0.2% Triton X-100 for 1 min, and incubated with the affinity purified rabbit antibodies against FHL2 alone or in combination with mouse monoclonal antibody F-VII against human vinculin (a gift from Dr. M. Glukhova, Institut Curie, Paris, France). Cy3- and FITC-conjugated secondary antibodies against rabbit or mouse immunoglobulins (Jackson, distributed through Dianova, Hamburg, Germany) were used to detect the primary antibodies. Fibrillar actin was visualised with FITC-conjugated phalloidin (Sigma-Aldrich, Deisenhofen, Germany). The stainings were viewed and recorded using a laser scanning confocal microscope (Leica, Heidelberg, Germany). In the cases of double-staining, the recorded images were superimposed using the Adobe-Photoshop software.

Transient Expression of Green Fluorescent Protein (GFP)-Tagged FHL2 in Fibroblasts

The cDNA encoding FHL2 was inserted in the pcDNA3 vector containing the coding sequence for EGFP (kindly provided by Dr. A. Penhoat, INSERM, Lyon, France) using *EcoR I* and *BamH I* restriction sites. Wi26 fibroblasts were seeded on glass coverslips in DMEM containing 10% foetal serum and transiently transfected with the construct or with the vector alone using lipofectamine (Invitrogen, Groningen, The Netherlands). After 18 h of culture, the cells were fixed and permeabilised as above and observed by laser confocal microscopy at an excitation wavelength of 488 nm.

Recombinant Production of Strep II-Tagged FHL2 in Insect Cells

The pACT2-FHL2 plasmid [Wixler et al., 1999] was used as a template to amplify by PCR the sequence coding for full-length human FHL2. The sense (5′-accgaagcttatgactgagcgctttgactgc-3′) and anti-sense (5′-ataagaatgcggccgctgggatgtctttcccacagtc-3′) primers introduced single restriction sites for *Hind III* and *Not I*,

respectively, in order to allow in-frame insertion of a StrepII tag sequence at the 3′ end of the FHL2 sequence in the pCEP-PUCs vector [Smyth et al., 2000] (kindly provided by Dr. N. Smyth, Center for Biochemistry, Cologne). The construct was sub-cloned into the transfer vector pVL1393 (kindly provided by Dr. M. Scheffner, Center for Biochemistry, Cologne) using the sense (5′-gccggatccatgactgagcgctttgactgc-3′) and anti-sense (5′-ccggaattcctacttgaattg-3′) oligonucleotides inserting restriction sites for *BamH I* and *EcoR I*, respectively. The construct was confirmed to be correct by nucleotide sequence analysis. This plasmid was co-transfected with BaculoGold™ (PharminGen, Becton Dickinson, Heidelberg, Germany) into *Spodoptera frugiperda* (Sf9) cells using CellFectin (Life Technologies, Berlin, Germany). The cells were grown at 27°C in modified Grace insect medium (TNM-FH medium, Sigma-Aldrich) containing 10% foetal calf serum. After 72 h of culture, the medium containing the recombinant viruses was collected and either used immediately or stored at −80°C. For protein production, *Trichoplusia ni* cells (High5; kindly provided by Dr. M. Scheffner) were infected with the recombinant viruses and grown in TNM-FH medium at 27°C. After 48 h of culture, infected cells were collected and homogenised in 100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% IGEPAL (Sigma-Aldrich), 100 μg/ml aprotinin, 100 μM leupeptin, 1 mM Pefabloc (Roche, Mannheim, Germany). The lysates were centrifuged at 20,000*g* for 30 min and the supernatant was used for purification of the recombinant protein.

Purification of StrepII-FHL2

Extracts of infected High5 cells were incubated at 4°C with StrepTactin Sepharose (IBA, Göttingen) which had been pre-swollen in 100 mM Tris-HCl, pH 8.0, and packed in a chromatography column. The column was extensively washed with 1 M NaCl in 100 mM Tris-HCl, pH 8.0, and then eluted with 3 mM desthiobiotin (Sigma-Aldrich). Aliquots of eluted fractions were analysed by SDS–PAGE and Coomassie Brilliant Blue staining of the gels.

Circular Dichroism Spectroscopy

Purified StrepII-tagged FHL2 was concentrated using a vacuum centrifuge, resuspended in and dialysed against 50 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS). The concentration of the solution (100 μg/ml) was calculated from the absorption at 280 nm as described [Gill and von Hippel, 1989]. Circular dichroism spectra in the far UV region were recorded at 25°C in a thermostated quartz cell of 1 mm optical path length in a Jasco spectropolarimeter (Model 715 CD). Mean molar ellipticities [θ] (expressed in degrees cm^2 $dmol^{-1}$) were calculated on the basis of a mean residue molecular mass of 110 Da. Five spectra were accumulated to improve the signal/noise ratio. The spectra recorded with buffer alone were subtracted. Secondary structure analysis was performed using the variable selection method SelCon [Sreerama and Woody, 1994].

SDS–PAGE and Immunoblotting

Protein samples were mixed with SDS-containing sample buffer and electrophoretically separated on polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue or used for electrophoretic transfer to nitrocellulose membranes for immunoblotting. The blots were incubated with the following primary mouse monoclonal antibodies: AC40 against actin, BM 752 against α-actinin (both from Sigma-Immunochemicals), 4F12 against cortactin (Upstate Biotechnologies, distributed by Biomol, Hamburg, Germany), 349 against paxillin (Transduction Laboratories, distributed by Becton Dickinson, Heidelberg, Germany), 23A8 against Rac-1 (Upstate Biotechnologies) and 26C4 against RhoA (Santa Cruz, Heidelberg, Germany). Secondary antibodies coupled to horseradish peroxidase (Dako, Hamburg, Germany) and chemiluminescence were used for signal detection with X-ray films.

Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight (MALDI-TOF) Mass Sprectrometry

Polypeptide bands were excised from stained gels and processed for trypsin digestion after reduction and alkylation as previously described [El Mourabit et al., 2003]. Aliquots of the digests (0.4 μl) were mixed with 1.2 μl of 2,5-dihydroxybenzoic acid (5 mg/ml) in 0.1% TFA/acetonitrile (2:1) and spotted onto a 800 μm anchor target (Bruker Daltonics, Bremen, Germany). Positive ion spectra were acquired on a Reflex IV MALDI-TOF mass spectrometer (Bruker Daltonics) in the reflectron mode as previously described. A peptide calibration standard (Bruker Daltonics) was used for external calibration of the mass range from m/z

1,046 to m/z 3,147. Identification of proteins was performed with the Mascot software.

For protein mass determination, samples were desalted and concentrated by micro reversed phase chromatography on C4-Zip tips (Millipore, Schwalbach, Germany). Briefly, the tips were activated in 0.1% TFA/acetonitrile (1:2), equilibrated in 0.1% TFA and proteins were adsorbed to the resin by repeated pippeting. Salts were removed by extensive washing in 0.1% TFA and 2.5 μl of a saturated solution of sinapinic acid in 0.1% TFA/acetonitrile (1:2) was used to elute the tips onto a stainless steel MALDI target. Linear spectra were obtained in the positive ion mode on a Reflex IV MALDI-TOF mass spectrometer (Bruker Daltonics). The M + H and M + 2H signals of bovine serum albumin were used for external calibration of the spectra.

Affinity Chromatography on FHL2-Sepharose

An affinity matrix was prepared by coupling purified Strep II-tagged FHL2 (10 mg) to 500 mg of CNBr-activated Sepharose 4B (Pharmacia Biotech) according to manufacturer's instructions. Wi26 fibroblasts ($\sim 5 \times 10^7$ cells) were lysed and homogenised in ice-cold 25 mM $NaHCO_3$, pH 7.8, containing 340 mM sucrose, 1 mM Pefabloc, 3 mM NEM, and 1 mM each $ZnCl_2$, $MgCl_2$, KCl, and $MnCl_2$. The cell lysate was centrifuged (15,000g, 45 min at 4°C) and the supernatant gently stirred with the affinity matrix for 16 h. The matrix (2 ml) was packed in a column and washed successively with the lysis buffer alone or containing 0.15 M NaCl (each 20 ml). The proteins were eluted with 0.5 and 1 M NaCl in the same buffer (4 and 6 ml, respectively) All operations were performed at 4°C. Eluted fractions (1 ml) were precipitated with 10% trichloracetic acid and analysed by SDS–PAGE and staining of the gels by Coomassie Brilliant Blue.

RESULTS

FHL2 Localises to Lamellipodia

We and others have previously shown that FHL2 occasionally associates with some, but not all, cell adhesion structures [Wixler et al., 2000; Li et al., 2001]. We have now conducted a more detailed analysis of the subcellular localisation of FHL2 by immuno-staining of Wi26 fibroblasts with an antibody raised against recombinant FHL2. Observation of the immunofluorescence signals by laser scanning confocal microscopy shows a prominent staining of the nucleus (Fig. 1A), in agreement with previous studies [Genini et al., 1997; Wixler et al., 2000]. In addition, the antibody distinctly decorates lamellipodia (Fig. 1A, arrow heads), cellular protrusions known to be transient and dynamic. At a higher magnification, the structures in lamellipodia stained by the antibody appear as thin filaments of irregular length (Fig. 1B–D, arrows). To obtain independent experimental evidence for the specific localisation of FHL2 within lamellipodia, Wi26 fibroblasts were transiently transfected with EGFP-tagged FHL2. The subcellular localisation pattern of the fusion protein was determined by laser scanning confocal microscopy. Here again, besides being targeted to the cell nucleus, EGFP-tagged FHL2 was seen as thin elongated filaments present in lamellipodia (Fig. 2).

To determine whether FHL2 colocalises with known cytoskeletal proteins at these locations, we applied double immunofluorescence staining of FHL2 and actin or vinculin. Observation of the staining patterns by laser confocal microscopy indicated that the small filaments containing FHL2 (Fig. 3A,D) were in register with the actin microfilaments within lamellipodia (Fig. 3B,C) while FHL2-positive patches were also labelled by antibodies against vinculin (Fig. 3E,F), a marker of cell adhesions. The co-localisation of FHL2 and vinculin was observed only in adhesion structures present in lamellipodia but not in vinculin-positive patches at other locations in the cells (Fig. 3F).

Expression and Purification of Strep II-Tagged FHL2

In order to identify the proteins, in particular those related to the cytoskeleton and adhesion structures, that could be associated with FHL2 in the cytoplasm of cells, we took a proteomics approach. For this purpose, substantial amounts of FHL2 were needed and we resorted to recombinant expression of the protein. The full-length FHL2 cDNA with a carboxy-terminal StrepII tag was cloned in the genome of the baculovirus and the resulting recombinant viruses were used to infect High5 insect cells. Extracts of infected cells were loaded on a StrepTactin affinity matrix and recombinant Strep II-tagged FHL2 was eluted with 3 mM desthiobiotin. Analysis of the eluted fractions by SDS–PAGE showed a prominent band at about

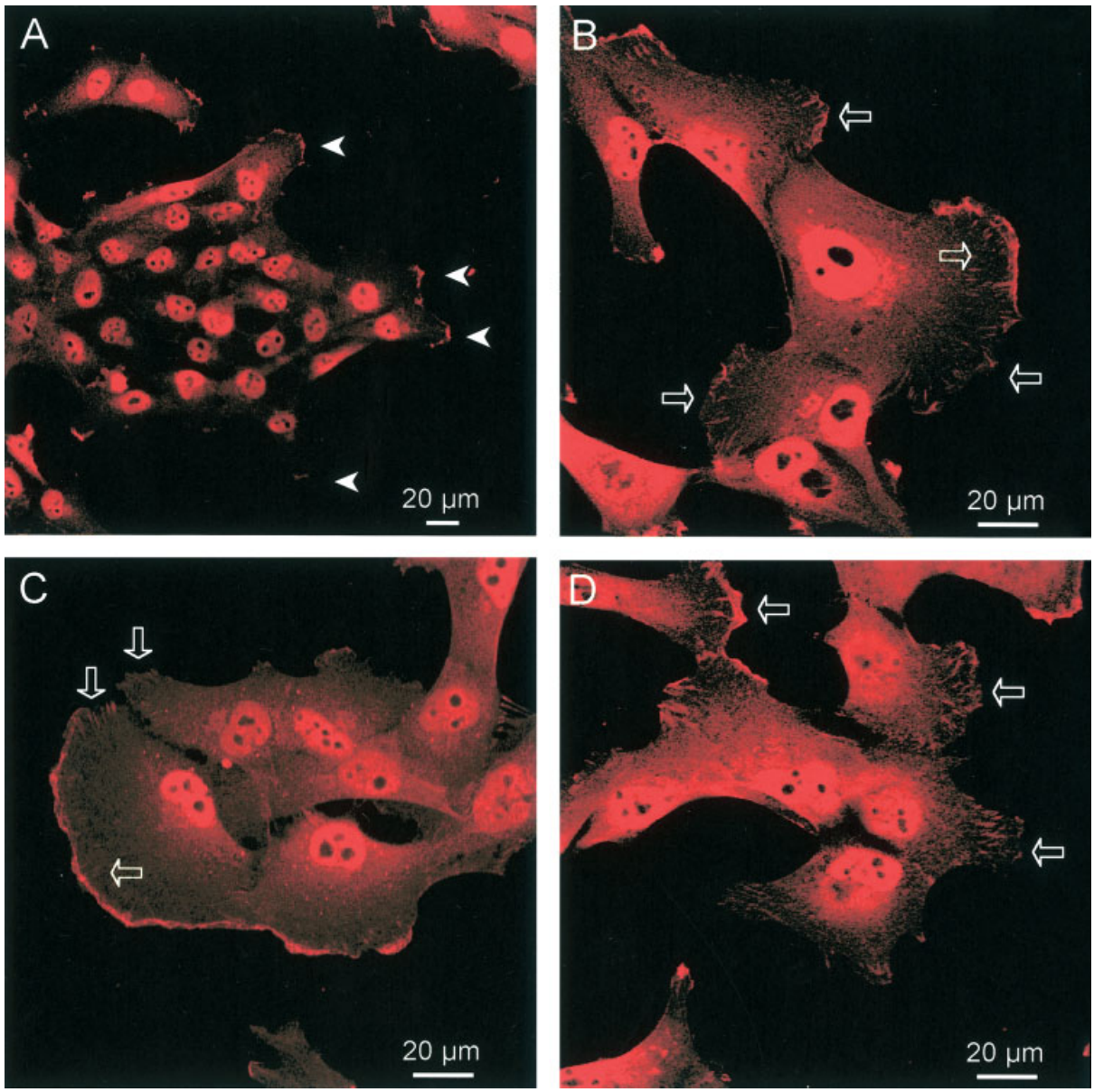

Fig. 1. Indirect immunofluorescence staining of FHL2 in Wi26 fibroblasts. Wi26 fibroblasts grown overnight were processed for indirect immunofluorescence staining with an affinity-purified antibody against FHL2 followed by Cy-3 conjugated secondary antibodies. The stainings were viewed using confocal laser scanning microscopy. At low magnification (**A**) a prominent nuclear staining is observed as well as decoration of structures in lamellipodia (arrow heads). At higher magnification (**B**–**D**), these appear as thin elongated filaments (arrows).

30 kDa (Fig. 4A), while mass spectrometry analysis gave a molecular mass of 33,791 Da for the recombinant product, consistent with the apparent mass in SDS–PAGE and close to the mass of 33,543 Da calculated for the construct. In addition, the polypeptide band of 30 kDa was excised from the Coomassie-stained SDS–PAGE gel and analysed by MALDI-TOF mass spectrometry after in-gel trypsin digestion. Peptide mass fingerprints confirmed the identity of the recombinant product with FHL2 (Fig. 4B). The purity of the protein was estimated to be >98% from densitometry measurement of band intensity in SDS–PAGE gels.

Analysis of Recombinant FHL2 by Circular Dichroism Spectroscopy

A LIM domain is predominantly folded into β sheet structures with a short α helix at the C-terminal end [Perez-Alvarado et al., 1994; Kontaxis et al., 1998]. To test whether the

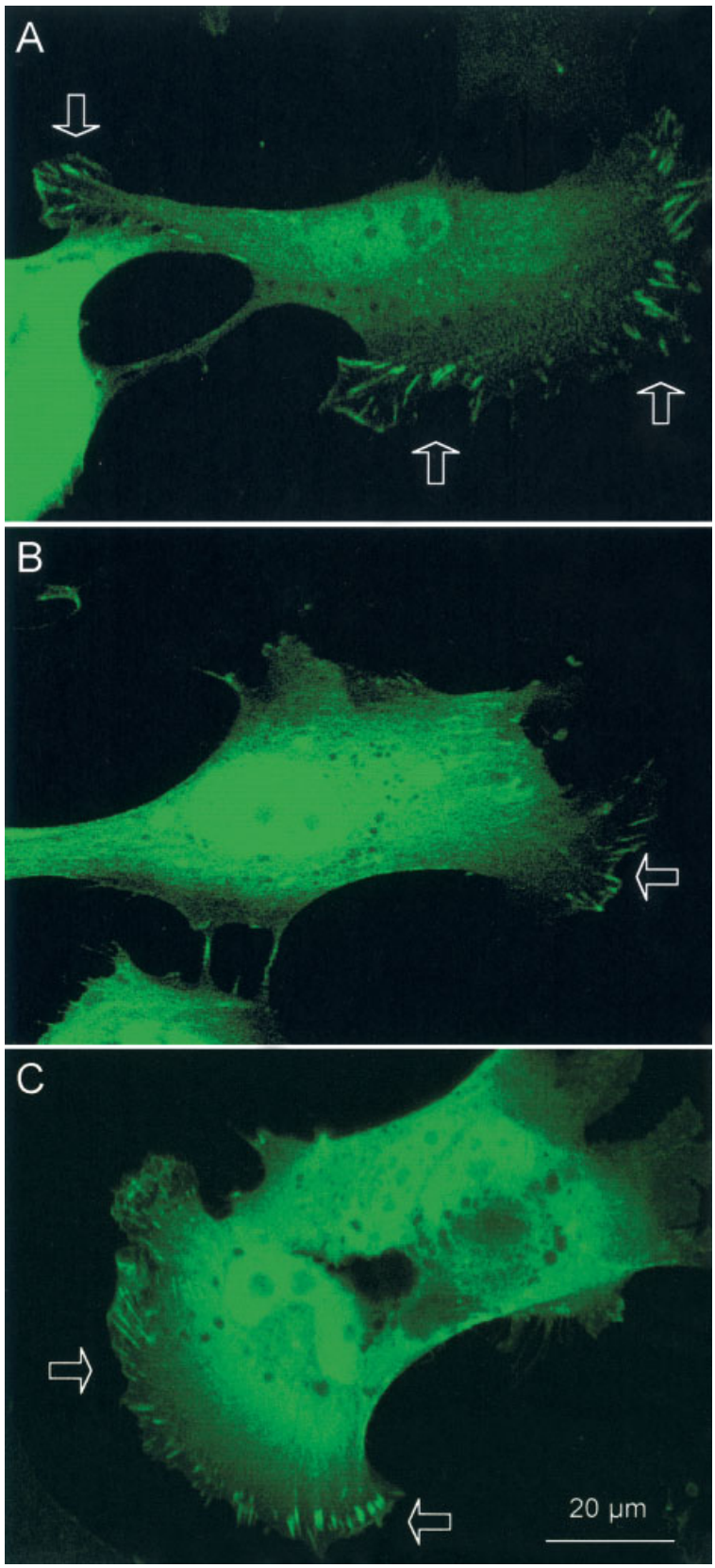

Fig. 2. Expression and localisation of EGFP-tagged FHL2 in Wi26 fibroblasts. Wi26 fibroblasts were transiently transfected with EGFP-tagged FHL2 and observed using confocal laser scanning microscopy. **A**–**C** show different images of fibroblasts 18 h post-tranfection. Arrows indicates the localisation of EGFP-tagged FHL2 in thin filaments within lamellipodia.

recombinant FHL2 was in a correct conformation, the secondary structure was analysed by circular dichroism spectroscopy. The spectrum recorded at 25°C was bimodal and showed a shoulder at 222 nm and a distinct minimum at 195 nm (Fig. 5). Determination of secondary structures by least-squared regression analysis [Sreerama and Woody, 1994] indicated that β sheets accounted for 37% and α-helical structures for 19%. These values are in good agreement with the prediction for LIM domains, indicating that recombinant FHL2 is correctly folded.

Isolation and Identification of Proteins Retained on FHL2-Sepharose

Purified recombinant StrepII-FHL2 was immobilised on Sepharose and the matrix used to isolate cellular proteins by FHL2 affinity. The cytosol fraction of a lysate of Wi26 fibroblasts was incubated with the FHL2 matrix and after extensive washing, bound proteins were stepwise eluted with 0.5 and 1 M NaCl. Analysis of the eluted fractions by SDS–PAGE showed that a set of more than 20 proteins were retained on the affinity matrix, a small portion of which were displaced with 0.5 M NaCl, and a larger portion with 1 M NaCl (Fig. 6). When a similar cell lysate was chromatographed on an affinity matrix prepared with an unrelated Strep II-tagged intracellular protein, GIPC, only four polypeptides were specifically eluted with 1 M NaCl, and none of these overlapped with those eluted from the FHL2 matrix (Coevoet et al., unpublished observation). The polypeptide bands eluted with 1 M NaCl from the FHL2 matrix were excised from the gel and analysed after in-gel trypsin digestion by MALDI-TOF mass spectrometry (Fig. 7 and Table I). The experimental peptide mass spectrum from each selected gel band was matched against theoretical trypsin cleavage patterns available in database. Unambiguous protein identification was based on several criteria, including a score of at least 60 between matching peptides and input peptides with the Mascot software and no large discrepancy between the predicted mass of the identified protein and its experimental molecular mass deduced from SDS–PAGE gels (Table I). We also verified that the deduced peptides correctly matched the sequence of the protein and that coverage was over 10% (Table I). According to these selection criteria, proteins retained on the FHL2 matrix and eluted with 1 M NaCl are metabolic enzymes, chaperones, molecules involved in ubiquitination, and signalling pathways or in the organisation and the dynamics of the cytoskeleton, in particular actin and vinculin (Table I).

Considering the co-localisation of these two proteins with FHL2 in thin filaments of lamellipodia and their detection in the eluted fractions, those fractions were immunoblotted with antibodies against several actin-binding proteins which may not have been detected by Coomassie staining of the gels. Paxillin and α-actinin, two proteins associated with focal contacts and actin stress fibers [Isobe et al., 1988; Turner, 2000], were not detected, while cortactin, which associates with actin in cell protrusions [Wu and Parsons, 1993], was present in the eluted material (Fig. 8) and detected with a similar signal intensity as vinculin, which had been identified already by MALDI-TOF mass spectrometry. Furthermore the eluted fractions were immunoblotted with antibodies against the small GTPases Rac 1 and RhoA known to be involved in the formation of lamellipodia and actin stress fibers, respectively [Mackay and Hall, 1998]. Rac 1, but not RhoA, was detected in the eluted fractions (Fig. 8).

DISCUSSION

By way of its four and a half LIM domains, FHL2 could be involved in the formation of multiprotein aggregates or "interactomes", made up by a defined set of proteins with a variety of bilateral interactions. Some of these may play a role in integrin signalling and association with the cytoskeleton. This hypothesis is supported by the fact that multiple cytoplasmic and nuclear proteins were found to interact with FHL2 in yeast two-hybrid screens and assays. However, while several studies suggest that the nuclear form of FHL2 is involved in transcriptional regulation [Fimia

Fig. 3. Subcellular localisation of actin, vinculin, and FHL2 determined by double-immunofluorescence staining. Wi26 fibroblasts were double-stained with a rabbit antibody against FHL2 followed by second antibodies against rabbit immunoglobulins conjugated to Cy3 (red, **A**) or FITC (green, **D**) and with FITC-conjugated phalloidin (green, **B**) or a mouse monoclonal antibody against vinculin followed by Cy3-conjugated second antibodies against mouse immunoglobulins (red, **E**). Images were recorded by laser scanning confocal microscopy using single channel and superimposed (merge, **C** and **F**) using the AdobePhotoshop software.

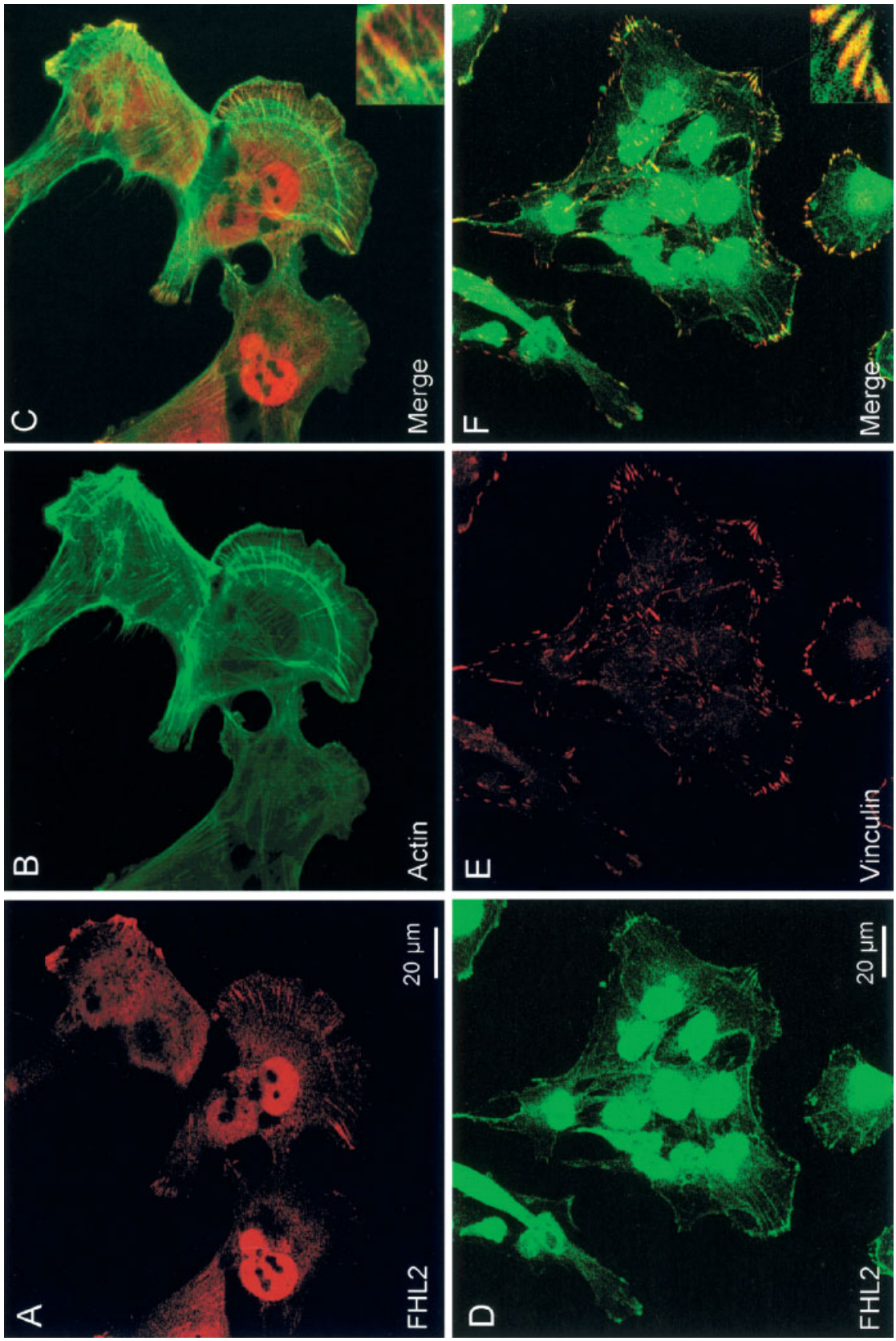

Fig. 3.

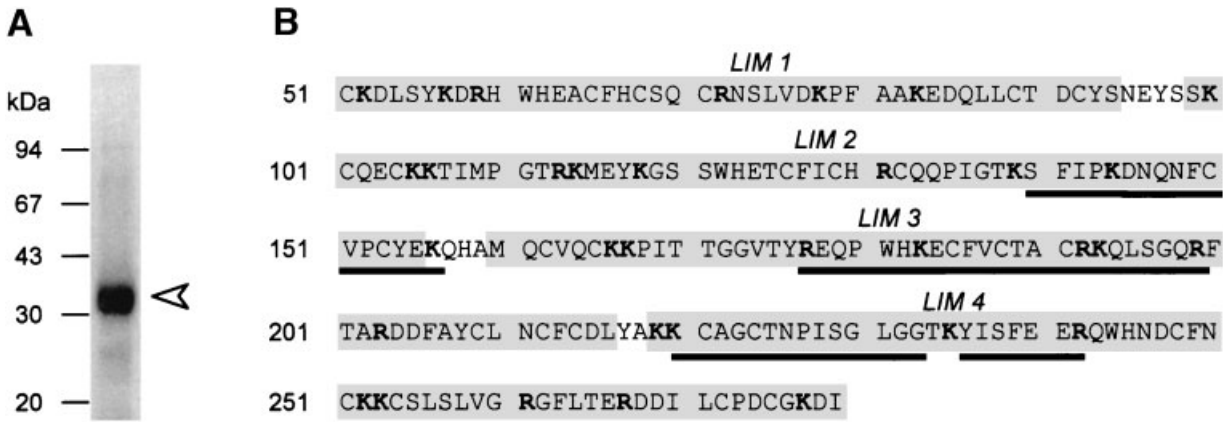

Fig. 4. Purification of StrepII-tagged FHL2 and identification by peptide mass fingerprints. **A**: Extracts of High5 cells transfected with baculoviruses encoding StrepII-tagged FHL2 were applied to on a StrepTactin column (1 ml). After washing, the column was eluted with 3 mM desthiobiotin and 500 µl fractions were collected. Aliquots (10 µl) of the fractions were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 10% polyacrylamide gel under non-reducing conditions. Staining of the gel with Coomassie Brilliant Blue shows a major band of the expected 30 kDa size (arrow head) in the peak fraction. Molecular masses of protein standards are indicated. **B**: The 30 kDa band shown in (A) was excised and submitted to MALDI-TOF mass spectrometry after in-gel trypsin digestion. The identified peptides are underlined in the FHL2 sequence. The different LIM domains (*1/2 LIM, LIM 1 to 4*) are in grey boxes and bold R and K residues correspond to potential trypsin cleavage sites.

et al., 2000; Muller et al., 2000; Morlon and Sassone-Corsi, 2003], little is known about the role of FHL2 in the cytoplasm. To test whether FHL2 could function as an adaptor in cytoplasmic multiprotein complexes, we applied a proteomics approach based on the affinity of cytosolic proteins for FHL2. Compared to yeast two-hybrid screens, which are limited to the identification of proteins that directly interact with FHL2, this approach allows the identification of more peripheral interacting partners present in complexes formed around FHL2.

Affinity chromatography requires substantial amounts of the purified primary ligand protein. We recently used the baculovirus/insect cell expression system for efficient production of recombinant FHL2 with an amino-terminal tag [El Mourabit et al., 2003]. However, in this case two forms of FHL2, full-length and with a carboxy-terminal truncation, were generated and purified [El Mourabit et al., 2003]. A related cleavage of FHL2 was observed also in mammalian cells and may represent a way of inactivating the FHL2 adaptor function [El Mourabit et al., 2003]. In the present work, we used a carboxy-terminal tag, which allowed the purification of the full-length form of FHL2 to homogeneity. In addition, circular dichroism spectroscopy of recombinant FHL2 indicated that the protein was correctly folded.

Purified StrepII-tagged FHL2 was used to construct an affinity matrix, which was employed to isolate such cytosolic proteins that bind directly or indirectly to FHL2. Several classes of molecules were retained with high affinity on the FHL2 matrix and were identified by MALDI-TOF mass spectrometry. Of particular interest, actin and vinculin, two proteins important for the formation of cell adhesion structures, were present in the material eluted from the FHL2 matrix with 1 M NaCl. This agrees with the recent observation that actin binds to FHL2 in yeast two hybrid interaction assays [Coghill et al., 2003]. In a cellular context, both endogenous FHL2 and transfected EGFP-tagged FHL2 localise to thin filamentous structures in fibroblast lamellipodia, cellular protrusions forming at the leading edge of migrating cells. At this location, cellular adhesions are thought to be nascent and, at a later stage, mature into more stable structures allowing the transmission of the forces required for cell migration and of integrin-mediated signalling [Cukierman et al., 2002]. In these dynamic cellular protrusions, FHL2 partially co-localises with actin microfilaments and with vinculin, while this is not the case at other cellular locations such as in mature focal contacts or along actin stress fibers. The induction of cellular protrusions and the organisation of the actin-based cytoskeleton are regulated by proteins belonging to the Rho family of low molecular weight GTP-binding proteins, i.e., RhoA, Cdc42, and Rac-1 [Mackay and Hall,

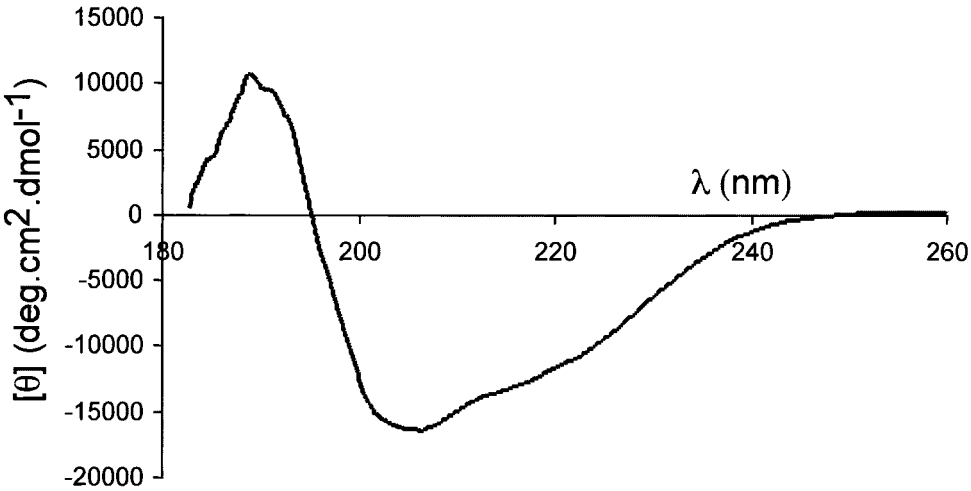

Fig. 5. Circular dichroism spectroscopy of Strep II-tagged FHL2. The circular dichroism spectra in the far UV region were recorded in a thermostated quartz cell of 1 mm optical path length. The spectra were measured in TBS at 25°C. Five spectra were accumulated to improve the signal/noise ratio. Circular dichroism spectra recorded with buffer alone were used for baseline subtraction.

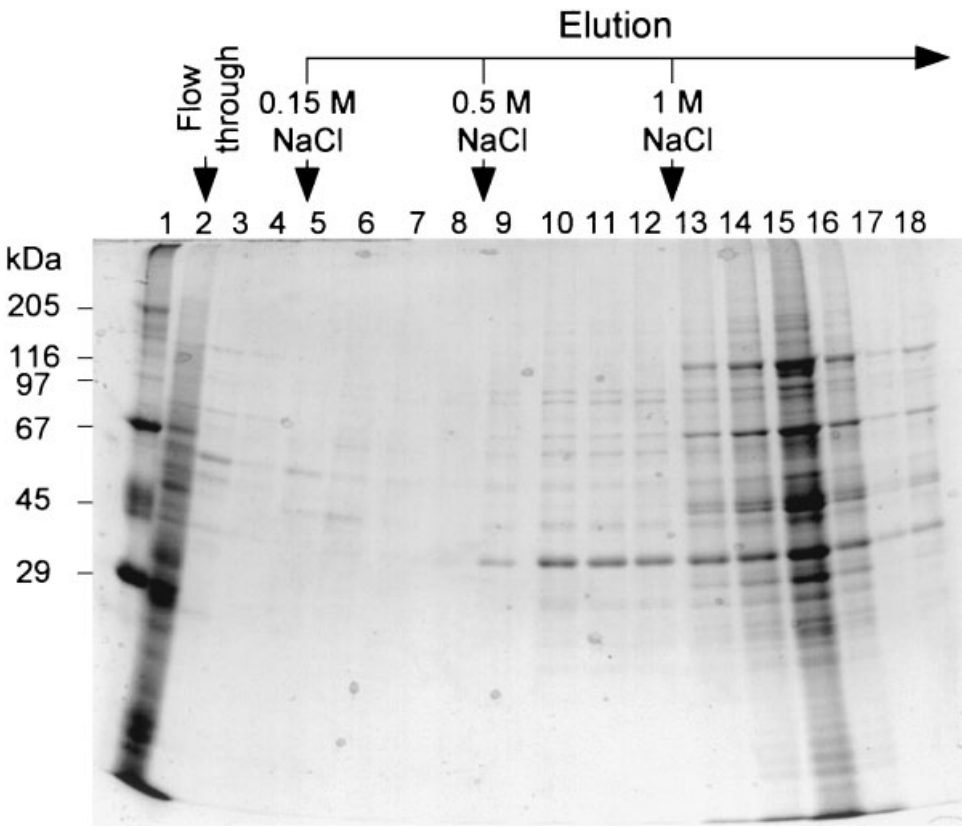

Fig. 6. SDS–PAGE analysis of cellular proteins retained on FHL2-Sepharose. The cytosolic fraction of a lysate of Wi26 fibroblasts ($\sim 5 \times 10^7$ cells) was incubated with Strep II-tagged FHL2 coupled to Sepharose and the mixture packed in a chromatography column. After extensive washing, the proteins were eluted with 0.5 and 1 M NaCl as indicated. Eluted fractions (1 ml) were precipitated with 10% trichloracetic acid and analysed by SDS–PAGE. **Lane 1**: Molecular mass markers. **Lane 2**: Flow through. **Lanes 3** and **4**: First and last fractions of the wash with buffer. **Lanes 5–8**: Two first and two last fractions of the wash with 0.15 M NaCl in buffer. **Lanes 9–12**: Fractions eluted with 0.5 M NaCl in buffer. **Lanes 13–18**: Fractions eluted with 1 M NaCl. The gel was stained with Coomassie Brilliant Blue.

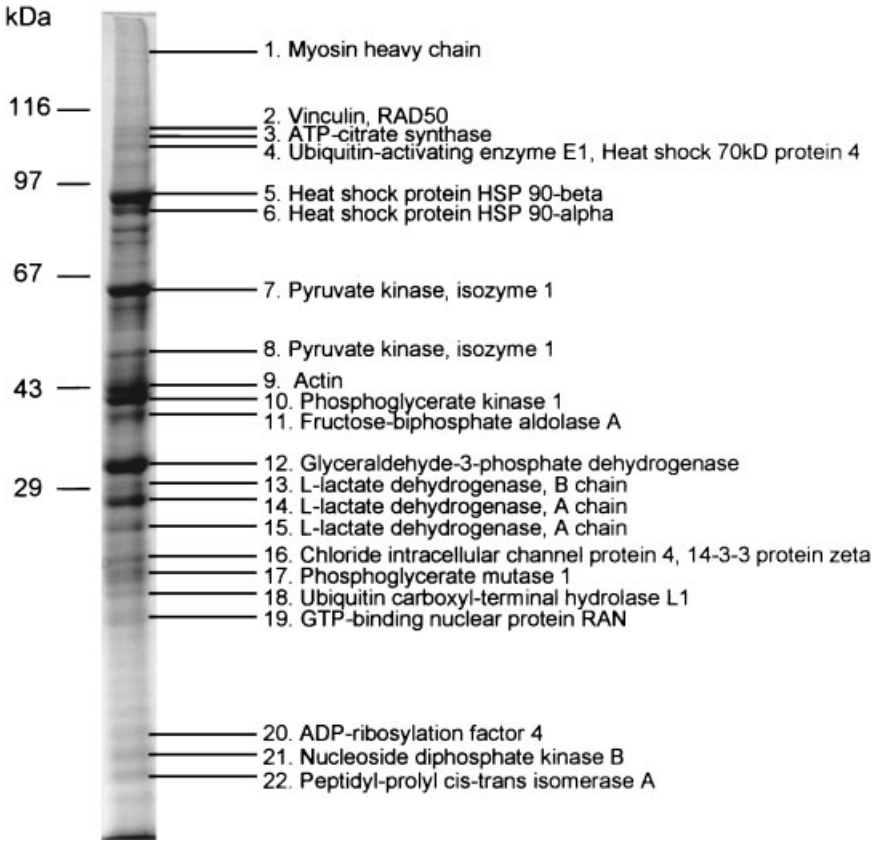

Fig. 7. Identification of the proteins retained on FHL2-Sepharose and eluted with 1 M NaCl. Protein bands were excised from lane 15 in the gel shown in Figure 6, digested with trypsin and analysed by MALDI-TOF mass spectrometry. Only bands not present in the fractions eluted with 0.5 M were analysed. Molecular mass markers are indicated at the left and the identity of the proteins identified by peptide mass fingerprints using MALDI-TOF mass spectrometry is given at the right.

TABLE I. Identification of Proteins by Peptide Mass Fingerprints

Band	Protein	Accession number	Calculated mass	Score	Coverage (%)	Comments
1	Myosin heavy chain, non-muscle	P35579	226,531	162	13.93	Cytoskeleton
2	Vinculin	P18206	123,668	181	27.10	Cytoskeleton
	RAD50	Q92878	153,891	75	12.88	Genome metabolism
3	ATP-citrate synthase	P53396	120,825	150	24.52	Glycolysis
4	Ubiquitin-activating enzyme E1	P22314	117,848	92	20.88	Ubiquitination pathways
	Heat shock 70 kDa protein 4	P34932	94,299	92	20.35	Molecular chaperone
5	Heat shock protein HSP 90-β	P08238	83,133	200	34.16	Molecular chaperone
6	Heat shock protein HSP 90-α	P07900	84,542	200	30.50	Molecular chaperone
7, 8	Pyruvate kinase, isozyme M1	P14618	57,805	214	58.68	Glycolysis
9	Actin	P02570	41,736	134	45.86	Cytoskeleton
10	Phosphoglycerate kinase 1	P00558	44,596	240	68.53	Glycolysis
11	Fructose-biphosphate aldolase A	P04075	39,289	132	54.30	Glycolysis
12	Glyceraldehyde-3-phosphate dehydrogenase	P04406	35,922	133	46.70	Glycolysis
13	L-lactate dehydrogenase, B chain	P07195	36,507	92	35.43	Glycolysis
14, 15	L-lactate dehydrogenase, A chain	P00338	36,557	206	60.12	Glycolysis
16	Chloride intracellular channel protein 4	Q9Y696	28,772	119	53.75	Chloride channel
	14-3-3 protein zeta/delta	P29312	27,745	84	42.45	Signal transduction
17	Phosphoglycerate mutase 1	P18669	28,672	179	64.03	Glycolysis
18	Ubiquitin carboxyl-terminal hydrolase L1	P09936	24,824	143	72.19	Ubiquitination pathways
19	GTP-binding nuclear protein RAN	P17080	24,423	158	45.83	GTP-binding protein
20	ADP-ribosylation factor 4	P18085	20,379	74	53.07	GTP-binding protein
21	Nucleoside diphosphate kinase B	P22392	17,298	109	71.71	DNA and ATP-binding
22	Peptidyl-prolyl *cis–trans* isomerase A	P05092	17,881	146	68.90	Protein folding

Analysis by MALDI-TOF mass spectrometry of the proteins eluted from the FHL2 affinity matrix with 1 M NaCl and fractionated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).
Accession numbers and calculated masses are given according to SWISS-PROT. Coverage indicates the percentage of the full-length sequence covered by the matching peptides.

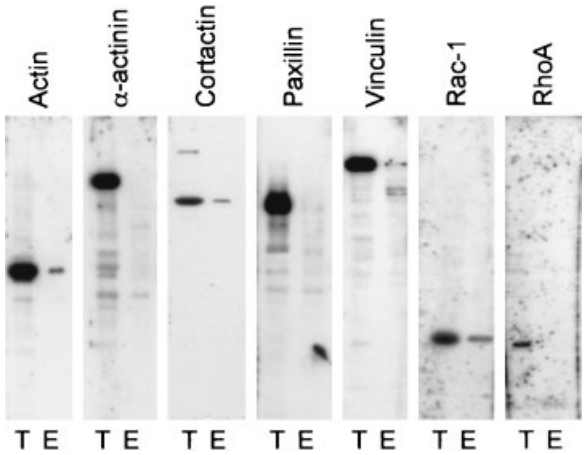

Fig. 8. Immunoblotting of proteins eluted from the FHL2-Sepharose column with 1 M NaCl. Aliquots (10 µl) of the cytosolic fraction before chromatography (T) and of the material eluted from the affinity column (E) were fractionated by SDS–PAGE and transfered to nitrocellulose for immunoblotting with monoclonal antibodies against the proteins indicated above the blots.

1998]. In particular, activation of Rac-1 induces the formation of membrane ruffles and lamellipodia, while the activation of RhoA promotes the formation of contractile actin stress fibers. The presence of Rac-1 among the proteins retained on the FHL2 matrix and the localisation of FHL2 in lamellipodia, suggest that FHL2 could be involved in the nucleation of nascent cell adhesion structures by bringing key effector proteins in close proximity. Interestingly, another member of the FHL family, FHL3, has been recently shown to bind actin and to inhibit the actin cross-linking activity of α-actinin [Coghill et al., 2003]. The latter binds fibrillar actin and, in a cellular context, it is observed in association with actin stress fibers. The cytosol fraction that was used for the chromatography was enriched in actin monomers and oligomers by centrifugation which could explain the absence of α-actinin in the actin-containing fractions eluted from the affinity matrix. This agrees well with the fact that there is no FHL2 staining along actin stress fibers, but solely where actin microfilaments are present, i.e., in lamellipodia. Thus FHL2 and FHL3 may both be involved in the control of actin bundling.

In addition, it is interesting to note that several proteins isolated are metabolic enzymes involved in glycolysis or coupled pathways. Other such enzymes (phosphofructokinase, creatine kinase, and adenylate kinase) were recently identified as binding partners for FHL2 using pull-down assays [Lange et al., 2002]. The enzymes were shown to co-localise with FHL2 in the sarcomeres of cardiomyocytes where FHL2 may act as a specific adaptor to couple metabolic enzymes to sites of high energy consumption [Lange et al., 2002]. FHL2 may have a similar function in nascent cellular adhesions where energy is needed for acto-myosin contraction, turn-over of lamellipodia, and maturation of cellular adhesions.

Four of the bound proteins are chaperones (heat shock 70 kDa protein 4, heat shock protein HSP 90-α and β) or involved in protein folding (peptidyl-prolyl *cis–trans* isomerase A). Those may have been retained by recognising minor folding defects present in recombinant FHL2 or introduced by purification and coupling to the Sepharose beads. Alternatively, such proteins may be physiologically needed in higher order molecular complexes to ensure correct folding of the constituents. Several of the FHL2 binding partners previously identified in yeast two-hybrid interaction assays were not detected in our approach. This may have several reasons. First, we only used the cytosolic fraction, which probably excludes identification of factors such as hCDC47, a member of the minichromosome maintenance protein family regulating DNA replication [Chan et al., 2000], or the DNA-binding nuclear protein hNP220 [Ng et al., 2002]. Second, other binding partners, such as the androgen receptor [Muller et al., 2000], presenilin 2 [Tanahashi and Tabira, 2000], or the insulin-like growth factor-binding protein-5 [Amaar et al., 2002] may not be soluble in the cytosol or may have been retained in amounts too low for detection. Nonetheless, an affinity approach at the protein level is essential to determine the composition of molecular complexes as those needed in signalling pathways and is a useful complement to genetic studies.

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