The VacA toxin of *Helicobacter pylori* identifies a new intermediate filament-interacting protein

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Introduction

The VacA toxin is a major virulence factor of *Helicobacter pylori*, a bacterium that colonizes the stomach mucosa of the majority of the human population and induces gastroduodenal ulcers and stomach cancers in a sizeable proportion of infected patients (Warren and Marshall, 1983; Marshall et al., 1985; Parsonnet, 1998). VacA is produced as a 140 kDa precursor, which is released into the extracellular medium as a 95 kDa mature protein (Phadnis et al., 1994; Schmitt and Haas, 1994; Lupetti et al., 1996). In the growth medium of *H. pylori*, VacA is present as a 95 kDa protein as well as 37 and 58 kDa fragments associated by non-covalent interactions (Telford et al., 1994). In cells exposed to the toxin, there is an extensive rab7-dependent fusion and swelling of late endosomal compartments, which eventually acquire lysosomal protein markers (Papini et al., 1994, 1997; Molinari et al., 1997). This causes an alteration in cellular trafficking with mis-targeting of acid hydrolases (Satin et al., 1997) and an inhibition of antigen processing within the antigen-processing compartment (Molinari et al., 1998).

VacA forms trans-membrane anion-specific channels (Tombola et al., 1999; Iwamoto et al., 1999), which are likely to contribute to vacuolization by causing osmotic swelling of such acidic compartments where the toxin localizes following endocytosis (Garner and Cover, 1996; Ricci et al., 1997; Montecucco et al., 1999). VacA can also induce vacuoles from the cytosol upon transfection (de Bernard et al., 1997). The p37 domain plus a portion of the p58 domain is the minimum sized fragment of the toxin required to induce vacuoles upon expression in the cell cytosol (de Bernard et al., 1998; Ye et al., 1999). These results suggest that VacA interacts with cytosolic proteins and that this interaction contributes significantly to vacuolization.

The yeast two-hybrid screening technique (Vojteck et al., 1993; Serebriiskii et al., 1999) is a powerful method to identify partners in protein–protein interactions within cells. In order to identify putative VacA-interacting protein(s) of the cell cytosol, we performed an extensive yeast two-hybrid screening of a library derived from HeLa cells, which are the most sensitive to VacA (de Bernard et al., 1998), using VacA as bait.

Here, we present the results of such a screening, which led to the identification of a novel protein of 54 kDa (abbreviated as VIP54, for VacA-interacting protein of 54 kDa, or for vimentin-interacting protein of 54 kDa, see below). Immunofluorescence microscopy performed with highly specific anti-VIP54 antibodies and biochemical experiments indicate that VIP54 is a novel intermediate filament (IF) protein that interacts with vimentin. We also report on the distribution of this protein in nerve tissue and its expression upon tumourigenesis.

Results

Identification of an *H.pylori* VacA cytotoxin-interacting protein

In the attempt to identify putative VacA-interacting protein(s), a HeLa cell library was screened using the yeast two-hybrid method. The two-hybrid L40 yeast reporter strain was first transformed with a plasmid encoding a fusion between full-length VacA and the bacterial protein LexA, which recognizes specific DNA sequences upstream of the two reporter genes HIS3 and LacZ (Vojteck et al., 1993). This screening strain was subsequently transformed with a HeLa cDNA plasmid library encoding C-terminal fusion proteins with the transcriptional activation domain of Gal4 (GAD). Library transformation of 1 × 10⁷ independent clones yielded six His-β-Gal-positive clones. As a control for specificity, library plasmids rescued from...
positive clones were transformed into a reporter yeast strain containing a bait encoding LexA fused with the p37 domain of VacA, which is unable to vacuolate HeLa cells (de Bernard et al., 1998). Figure 1A shows that all clones interacted specifically with VacA, but not with p37, and that the N-terminal region of p58 was involved directly in such interactions. Sequencing of all positive clones revealed that they possessed the same reading frame in fusion with GAD, and that all of them encoded the same protein. The sequence of this protein did not match with any other sequence available in databases. Immune antisera were chosen on the basis of their hydrophilicity and the absence of their sequences in databases. Immune antisera were raised against VIP54 to use as tools in examining the cellular localization of the protein, rabbits were immunized with keyhole limpet haemocyanin (KLH)-conjugated VIP54 peptides (underlined in Figure 2) chosen on the basis of their hydrophilicity and the absence of their sequences in databases. Immune antisera were affinity purified and tested on a variety of cultured cells. Several detergent extraction/fixation procedures were tried before choosing the one (described in Materials and methods) that best preserved cellular filaments (Arcangeletti et al., 1997). Immunofluorescence staining patterns of different cell lines showed a filamentous distribution of VIP54. Therefore, double immunofluorescence staining of VIP54 with filament proteins, including actin, desmin, cytokeratins and vimentin, was performed. VIP54 distribution closely resembles that of vimentin, and two examples of such an association are provided in Figure 4. There is an extensive co-localization of the two proteins, in most, but not all, cells and tissues tested. In particular, after extensive washing, the bound material was analysed by immunoblot staining using an anti-VacA polyclonal antibody. This assay showed that VacA was capable of binding the polypeptide (Figure 1B).

**Pull-down of VacA with the GST fusion protein**

In order to assess further the specificity of the interaction of the cloned protein fragment with VacA, the cDNA encoding the protein fragment was cloned from the pGAD vector into a pGEX vector and expressed in *Escherichia coli*. The GST fusion protein was bound to a glutathione–Sephrose matrix and VacA was loaded onto the column.

**Primary structure of the VacA-interacting protein**

Cloning and sequencing of the human VacA-interacting protein were performed as described in Materials and methods. The derived amino acid sequence is reported in Figure 2 together with the sequence of the mouse orthologue. The latter was obtained by screening a cDNA mouse phage library using the cDNA of the human protein, excised from the pGAD construct, as a probe. Accordingly, a clone containing the 5′ region, but lacking the 3′ region, was obtained. The sequence of the mouse protein was completed with the contribution of sequences from two sources; two sequences present in the expressed sequence tag (EST) database, and one working draft sequence (see legend to Figure 2). The VacA-interacting protein consists of 500 residues, corresponding to a mol. wt of 54 133 Da with a predicted isoelectric point of 5.0. For this reason, the protein will be abbreviated as VIP54 for VacA-interacting protein of 54 kDa. Secondary structure prediction methods suggest that several segments of VIP54 adopt an α-helical conformation, whereas the N- and C-terminal regions are predicted to be devoid of secondary structural elements. Even though there are a few heptad repeats of hydrophobic residues, the protein appears to lack the extended coiled-coil segments that are a hallmark of IFs and associated proteins (Gan et al., 1990; Fuchs and Weber, 1994; Steinbock and Wiche, 1999).

**Tissue distribution of VIP54**

Northern blot analysis of human organ mRNA, using a probe from pGADGH-VIP, demonstrated a ubiquitous expression of a 3500 bp mRNA (Figure 3). The highest levels of expression were found in brain, heart, skeletal muscle, kidney and liver. Intermediate levels of expression were detected in small intestine, placenta and lung, and low levels were found in colon, thymus, spleen and leukocytes.

**VIP54 co-localizes with vimentin- and desmin-containing intermediate filaments**

In order to raise antibodies against VIP54 to use as tools in examining the cellular localization of the protein, rabbits were immunized with keyhole limpet haemocyanin (KLH)-conjugated VIP54 peptides (underlined in Figure 2) chosen on the basis of their hydrophilicity and the absence of their sequences in databases. Immune antisera were affinity purified and tested on a variety of cultured cells. Several detergent extraction/fixation procedures were tried before choosing the one (described in Materials and methods) that best preserved cellular filaments (Arcangeletti et al., 1997). Immunofluorescence staining patterns of different cell lines showed a filamentous distribution of VIP54. Therefore, double immunofluorescence staining of VIP54 with filament proteins, including actin, desmin, cytokeratins and vimentin, was performed. VIP54 distribution closely resembles that of vimentin, and two examples of such an association are provided in Figure 4. There is an extensive co-localization of the two proteins, in most, but not all, cells and tissues tested. In particular,
Fig. 2. Sequence homology between human and mouse VIP54. (A) The human and mouse sequences of the protein, determined as described in Materials and methods, are compared. The human VIP54 nucleotide sequence is included in the DDBJ/EMBL/GenBank nucleotide sequence database with accession No. AJ242655. Numbering of the human sequence is based on that of the corresponding mouse sequence. Different amino acids in the mouse sequence are indicated. Identical amino acid residues (459 of a total of 500) are shown as dots, whereas similar ones are shadowed. The dash in the first line of the mouse sequence indicates a residue that is present in the human protein but not present in the mouse protein. The mouse sequences from position 384 to 432 and from 432 to the C-terminus were derived from the EST data bank (accession Nos AI158261 and AA840455) and from a working draft sequence (accession No. AC007439), respectively. Peptides used to produce anti-VIP54 antisera are underlined. (B) Prediction of the secondary structure of VIP54 performed with a set of different programs made available by Pôle Bio-Informatique Lyonnais (http://pbil.ibcp.fr). Larger bars indicate putative α-helices, smaller bars β-strands and spaces between bars are predicted to be structureless.

Fig. 3. Tissue expression of VIP54. The expression of VIP54 among various human tissues was examined by Northern blot analysis of poly(A) RNA isolated from various human tissues (Clontech) with a probe derived from the construct pGAD-VIP54. Molecular weights are indicated.

in the human stomach mucosa, we found that, in agreement with previous studies (Steinert and Roop, 1988), vimentin is present only in parietal cells and not detectable by immunofluorescence in epithelial cells, whereas the anti-VIP54 antibody stains parietal cells strongly and epithelial cells weakly (not shown). An association between VIP54 and vimentin-containing IFs was not anticipated based on the structure of VIP54 with its absence of the characteristic coiled-coil segments present in all IF proteins. In adult rat skeletal muscle (EDL), there is very little vimentin, and a higher expression of desmin, which associates mainly with the Z lines. The anti-VIP54 antibody strongly stains this tissue, with an extensive overlap with desmin distribution (not shown). This indicates that VIP54 can also interact with desmin, a finding that is not surprising since vimentin and desmin are highly homologous (Steinert and Roop, 1988).

The association with vimentin- and desmin-containing filaments is emphasized in cells treated with colchicine,
which causes the collapse of these filaments and their aggregation into perinuclear curved caps (Lazarides, 1982; Lawson, 1983). Figure 5 shows that VIP54 and vimentin retract together into characteristic arrays in colchicine-treated cells, with almost complete staining overlap. In addition, the same filament collapse with coincident staining patterns of the two proteins was found in cells microinjected with the anti-vimentin monoclonal antibody (not shown). Similarly, in colchicine-treated C2C12 cells, which express desmin, VIP54 follows the collapse of desmin-containing IFs (not shown). It should be noted that the colchicine-induced filament protein redistribution does not affect cytokeratin-containing filaments (Figure 5D, E and F).

**Constant ratio of VIP54 and vimentin**
All cell lines analysed by immunofluorescence were also analysed by Western blot, by incubating blotted cellular proteins with anti-vimentin and anti-VIP54 antibodies. Figure 6 shows that the anti-VIP54 antibody identified a single band with a mol. wt of 54 kDa and that a constant ratio of staining of VIP54 and vimentin is apparent in the nine cell lines tested herein, which included baby hamster- and dog-derived cells. This result is strongly indicative of a stoichiometric interaction between the two proteins. We also found that VIP54 and vimentin are present in the human hepatoma cell line Hep3B, but they are both absent in HepG2, another human hepatoma cell line (Knowles et al., 1980).
M.de Bernard et al.

Subcellular fractionation and immunoprecipitation of VIP54

The interaction between VIP54 and vimentin was investigated further by biochemical methods. It is well established that vimentin filaments are preserved after extraction of cells with high salt and Triton X-100 (Granger and Lazarides, 1980; Lazarides, 1982). As documented in Figure 7A, vimentin and VIP54 remained together in the insoluble IF-containing fraction, although the relative amount of VIP54 appeared to be reduced with the extraction procedure. To examine the interaction between VIP54 and vimentin in more detail, anti-vimentin monoclonal antibody-coated Sepharose beads were added to highly diluted cell lysates and the bound proteins were analysed by immunoblotting (Figure 7B). The coated beads were
VIP54 is a new intermediate filament-interacting protein

A

Fig. 7. Association of vimentin and VIP54. MDCK cells from one culture dish were lysed as described in Materials and methods. (A) Proteins present in the soluble (S) and insoluble (P) fractions were separated by SDS–PAGE, analysed by Western blotting with a monoclonal anti-vimentin antibody and the polyclonal anti-VIP54 antibody, and developed by chemiluminescence. (B) Total cell lysates were processed for immunoprecipitation as described in Materials and methods and incubated with protein A–Sepharose in the presence (+) or absence (−) of 20 μl of the anti-vimentin monoclonal antibody. Immunoprecipitated proteins were solubilized in sample buffer and analysed by Western blotting as in (A).

capable of precipitating VIP54 together with vimentin. However, such evidence of an interaction between the two proteins does not discriminate between a direct and an indirect interaction.

VIP54 is highly expressed in astrocytes and neuroblastoma, but not in CNS neurons

Vimentin is used frequently as a marker for de-differentiation in several types of tumours (Ramaekers et al., 1982; Thomas et al., 1999). Given the high expression of VIP54 in the brain, its distribution in this tissue was investigated further. Figure 8 compares the VIP54 distribution in a mixed cell population isolated from cerebellum with that in neuroblastoma cells (HTB11). While astrocytes contain a large amount of VIP54 (Figure 8A), the protein is not present in neurons, which are identified with an anti-neurofilaments monoclonal antibody (Figure 8B). In contrast, VIP54 is present in large amounts in primary neuroblastoma cells (Figure 8D), established in culture after surgical removal of the tumour. VIP54 and vimentin also co-localize in this kind of cell. Such findings indicate that VIP54 may be useful in studies of the central nervous system (CNS) and that it is a potential marker of tumours originating from CNS neurons.

Discussion

Here we report the identification and characterization of a novel protein of IFs. The protein (named VIP54) is highly conserved between human and mouse, suggesting that it plays an essential role in the cell.

VIP54 localizes on vimentin-containing filaments and, in the absence of vimentin, with desmin-containing filaments. Even though VIP54 contains some heptadic repeats of hydrophobic residues, it does not conform to the structure of IF proteins, characterized by the presence of two long coiled-coil stretches. Nonetheless, immuno-fluorescence, co-immunoprecipitation and cell extraction experiments indicate that VIP54 interacts with vimentin. The molecular basis of their interaction remains to be clarified by more detailed biochemical investigations. The unusual structural features of VIP54 suggest that it is associated with vimentin-containing IFs, rather than being an integral IF protein involved in fibre assembly.

Vimentin and other IF proteins are very useful antigens in the histological classification of human tumours (Ramaekers et al., 1982). An analysis of VIP54 expression in human tumours was beyond the scope of the present work. However, the findings that the protein is confined to the glia, that it is completely absent from CNS neurons and that it is highly expressed upon transformation in neuroblastomas suggest that VIP54 may be a novel and useful marker for tumour development and/or progression. Experiments with other human tumours are under way, and preliminary data support this indication.

VIP54 was identified via a yeast two-hybrid screening of a HeLa cell library using the vacuolating toxin released from H. pylori as bait. The specificity of the VIP54–VacA interaction was deduced from the repeated and unique finding of VIP54 in the genetic screenings and from its capacity to bind VacA in a VIP54-loaded column. VacA causes a defined alteration of intracellular protein trafficking by causing a massive enlargement of late endocytic compartments. Do such properties of VacA indicate that a link between late endosomes and IFs exists in intoxicated cells? IFs and their associated proteins have recently been established as dynamic components of the cytoplasmic and nuclear cytoskeleton. The association of IFs with the nuclear membrane and with the plasmalemma is clearly documented (Georgatos et al., 1985; Georgatos and Blobel, 1987). Although the exact nature of such membrane–IFs interactions is not yet established, clearly IFs do possess elements capable of mediating membrane interactions. Therefore, on the basis of present knowledge, the possibility that vimentin-containing filaments modify the status of endosomal/lysosomal compartments via VIP54-mediated interactions deserves further investigation. In particular, such interactions would promote the fusion of late endosomal membranes and, consequently, would contribute to cell vacuolization. In vacuolated cells, VIP54 immuno-fluorescent staining is largely lost. The compression and deformation of cytoplasm caused by vacuoles hamper experiments of co-localization of VIP54 and VacA in vacuolated cells. Also, attempts to demonstrate the presence of VIP54 on purified late endosomes have failed (not shown): the protein is probably lost during cell fractionation due to its lower affinity interaction with the membrane and its higher affinity interaction with vimentin. Clearly, further studies are required to determine the role of VacA–VIP54–vimentin interaction in vacuole biogenesis.

Materials and methods

Materials

Cell culture media, protein A–Sepharose and monoclonal antibodies against human cytokeratin were purchased from Sigma. Fetal calf serum (FCS) was purchased from PAA Laboratories. Monoclonal antibodies against porcine vimentin were purchased from Santa Cruz Biotechnologies; polyclonal anti-VacA antibody was a kind gift from Dr Daniela Burroni (CHIRON, Siena); rhodamine- and fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit and anti-mouse antibodies and Texas red-conjugated goat anti-rabbit antibodies were purchased from Dako; and horseradish peroxidase (HRP)-conjugated goat anti-rabbit and rabbit anti-mouse antibodies were purchased from Calbiochem. DNase and RNase, bovine serum albumin (BSA) and protease inhibitor cocktail tablets were purchased from Roche. Vector pGEM1Myt3 was produced by Dr Harald Stenmark and was a generous gift of M.Zerial (EMBL, Heidelberg); restriction enzymes were from Promega; and vector pGEX–4T3 was from Pharmacia. MDCK type II cells were a kind gift from Professor R.Foisner (University of Vienna, Austria).

Plasmids

The open reading frame encoding mature wild-type VacA was cloned into XnotI and BamHI sites of the yeast two-hybrid vector pBTM116

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Fig. 8. Expression of VIP54 in the CNS and in neuroblastoma. Eight-day-old cerebellar cells were processed for immunofluorescence and labelled with anti-VIP54 polyclonal antibody (A) and with an anti-neurofilament monoclonal antibody to identify the neurons (B). The overlay in (C) clearly shows the lack of VIP54 in neurons. Primary cells established in culture from a surgically removed neuroblastoma were stained with the anti-VIP54 (D) and anti-vimentin (E) antibodies used in Figure 4, and the two patterns are merged in (F).

[pLexA (Vojtek et al., 1993)], in-frame with the LexA protein; the vacA gene from bp 316 to 490 was amplified from plasmid pGEMp95 (de Bernard et al., 1997) by PCR using the following oligonucleotides: 5′-GAATGCCCGGGGGCCGCCTTTTTCACAACCGTGA and 5′-TC-CTTGTAGGGAATTCAAAAAGCCTTTTCCT, containing the recognition sequences (underlined) for XmaI and EcoRI, respectively. A 10 ng aliquot of plasmid and 50 pmol of each primer was used. The reaction mixtures were pre-incubated for 2 min at 94°C, and 27 amplification cycles were performed as follows: denaturation at 94°C for 1 min; annealing at 52°C for 2 min; extension at 72°C for 2 min; with the final elongation step of 72°C for 10 min. The PCR fragment digested with XmaI and EcoRI was gel purified and ligated into the XmaI and EcoRI site of pCDNA3. The plasmid was then digested with EcoRI and BamHI and mixed in a ligation reaction with the vacA fragment from bp 490 to 2844 excised from the plasmid pGEMp95 by digestion with EcoRI and BamHI. The resulting entire vacA sequence was excised from the pCDNA3 vector by digestion with XmaI and BamHI and mixed in a ligation reaction with the pLexA vector previously digested with the same enzymes to obtain the plasmid pLexA-VacA.

The DNA corresponding to the vacA gene from bp 316 to 2152 was inserted into the pLexA plasmid as follows: the vector pCDNA3 containing the entire sequence of vacA was digested with EcoRI and BamHI and the excised fragment (from bp 490 to 2844) was substituted with the vacA fragment from bp 490 to 2152 previously obtained by digestion with the same enzymes on a pGEM plasmid containing the vacA deletion (de Bernard et al., 1998). The truncated vacA gene was then cloned in the pLexA plasmid using the same strategy as used for the whole vacA gene. This truncated vacA gene encodes a VacA fragment that is referred to as VacA 1–672 in the text.

The vacA gene from bp 316 to 1272, corresponding to the 37 kDa N-terminal domain of VacA (Manetti et al., 1995), was cloned in the pLexA vector as follows: DNA was amplified from plasmid pLexA-VacA using synthetic oligonucleotides: 5′-GAATGCCCGGGGGCCGCTTTTCACAAACCGTGA and 5′-GAATGATCTTCAAGCGC-
A mouse pheage library was screened according to Sambrook et al. (1989) with a $^{32}$P-labelled DNA probe derived from a DNA fragment excised from pGADGH-VIP with EcoRI and BamHI. The probe was labelled using a random primer labelling kit (Roche). A total of 10 plaques were transferred to nitrocellulose membranes (Schleicher & Schuell) and probed with a polyclonal antiserum raised against the three positive clones obtained were sequenced, and all of them contained the region labelled using a random primer labelling kit (Roche). The three positive clones were subcloned into the vector pGEX-4T3 previously digested with EcoRI and BamHI, respectively.

Cloning of VIP54

Excised from pGADGH-VIP with EcoRI and BamHI, the probe was used as a probe to screen a plasmid DNA library prepared from Chinese hamster ovary (CHO) cells (Blumenthal et al., 1987) using the oligonucleotide based on the human VIP54 sequence.

**Pull-down of VacA**

Construct pGADGH-VIP was digested with EcoRI and XhoI, and the resulting fragment was ligated into the vector pGEX-4T3, respectively.

**Preparation of antisera**

The following peptides: CPAEEELAVRTGTSASSADDLE, CFADIRR-KDAQQRS and CLNRRGDPPVRDFKHEPQ (corresponding to the underlined segments in Figure 3 plus a terminal cysteine) were conjugated to KLH using an Inject Activated Immunogen Conjugation Kit (Pierce) and injected into rabbits. The anti-peptide antibodies generated were purified by affinity chromatography on a SulfoLink coupling gels (Pierce) onto which each peptide was conjugated. Of all the antibodies obtained by this procedure, those specific for the most N-terminal peptide (see Figure 2) gave the best immunostaining results, as shown in the figures. Comparative stainings were obtained with the other antibodies.

**Subcellular fractionation and immunoprecipitation**

Subcellular fractionation and preparation of intermediate filaments were carried out as described (Eger et al., 1997) using MDCK type I cells. Briefly, MDCK cells from one 10 cm culture dish were lysed in 500 µl of ice-cold 50 mM HEPES pH 7.0, 5 mM MgCl₂, 1 mM EGTA, 100 mM NaCl, 0.1 mM dithiothreitol (DTT), 0.5% Triton X-100, 0.5 mg/ml DNsase, 0.2 mg/ml RNase and protease inhibitors. Cell lysates were adjusted to 1% Triton X-100 and 500 mM NaCl, and solved and insoluble fractions were separated by centrifugation for 20 min at 14 000 r.p.m. in a microfuge at 4°C, and then dissolved in sample buffer. For the immunostaining of vimentin and associated proteins, total cell lysates containing soluble and finely dispersed insoluble protein complexes were diluted 20-fold in lysis buffer containing 1% Triton X-100 and 500 mM NaCl, and vigorously homogenized. A monoclonal antibody to vimentin conjugated to protein A–Sepharose was added and incubated for 12 h at 4°C. The resin was washed three times in the same buffer, resuspended in sample buffer and subjected to SDS–PAGE and immunoblottting. Control experiments were performed with protein A–Sepharose in the absence of the monoclonal antibody.

**Cell culture and microinjection**

Cells were cultured as monolayers in plastic flasks under 5% CO₂ at 37°C. Media used were Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FCS for HeLa, BHK, MDCK type I and type II and Row cells; RPMI 1640 10% FCS for HTB-11 cells; modified Eagle’s medium (MEM) 1 mM Na-pyruvate 10% FCS for Hep-G2 and Hep-3B cells; and M199 5% FCS for ECV 304 cells. At 24 h before the experiments, cells were released with trypsin/EDTA and seeded in 24-well titration plates in DMEM 10% FCS at a density of 4 × 10⁴/cm² or, when required, on round glass coverslips.

For immunoblot analysis, cells were released with 5 mM EDTA pH 7.0 after two washes with PBS and collected in a microtubule; after a short spin, the cellular pellet was resuspended in LSB, subjected to SDS–PAGE and transferred to nitrocellulose membranes.

Cells were microinjected using Eppendorf microcapillaries. The microcapillary was placed into an Eppendorf micromanipulator attached to a Zeiss inverted microscope. Injection pressure was controlled using an Eppendorf microinjector model 5242. A monoclonal antibody against vimentin was concentrated to 20 mg/ml before injection and clarified by centrifugation for 15 min at 14 000 r.p.m. Microinjected cells were fixed 4 h after injection.

Cerebellar cells were grown in primary culture after enzymatic and mechanical dissociation from 6- to 7-day-old Wistar rats according to the procedure of Levi et al. (1984). Cells were plated on poly-l-lysine-coated glass coverslips, kept in basal Eagle’s medium supplemented with 10% FCS, 25 mM KCl, 2 mM glutamine, and were processed for immunofluorescence 8 days after seeding.

**Immunofluorescence microscopy**

Cells were detergent extracted simultaneously and fixed for 20 min by 10% Triton X-100 and 1% paraformaldehyde in CSK buffer. Cells were released with trypsin/EDTA and incubated overnight with 1% BSA for 20 min and processed for fluorescence labelling: primary antibodies were diluted in PBS 3% BSA and incubated with cells for 1 h. After several washes in PBS 1% BSA, rhodaminated and floresceinlabeled secondary antibodies diluted in PBS 3% BSA were added for 30 min and washed as before. Cell samples were mounted on 90% (v/v) glycerol, 0.2% (w/v) N-propylgallate in PBS and cells were viewed using the MRC-1024 Laser Scanning Confocal Imaging System (Bio-Rad).

Western blotting

After SDS–PAGE, proteins were transferred onto Protran nitrocellulose membranes (Schleicher & Schuell). Membranes were saturated with 5% dried milk in PBS 0.2% Tween-20 and incubated overnight with primary antibody diluted in the same buffer. After several washes with PBS 0.2% Tween-20, incubation with secondary antibody diluted in the same buffer plus 5% dried milk was carried out for 45 min. Membranes were washed in PBS 0.2% Tween-20, rinsed twice in PBS, and proteins were detected using the enhanced chemiluminescence (ECL) system (Fierce).

**Other methods and procedures**

Northern blot analyses were carried out according to published procedures (Sambrook et al., 1989) with a Human Multiple Tissue Northern Blot, purchased from Clontech (#7780-1).

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**VLP54: a new intermediate filament-interacting protein**

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