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

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The VacA toxin produced by *Helicobacter pylori* acts inside cells and induces the formation of vacuoles arising from late endosomal/lysosomal compartments. Using VacA as bait in a yeast two-hybrid screening of a HeLa cell library, we have identified a novel protein of 54 kDa (VIP54), which interacts specifically with VacA, as indicated by co-immunoprecipitation and binding experiments. VIP54 is expressed in cultured cells and many tissues, with higher expression in the brain, muscle, kidney and liver. Confocal immunofluorescence microscopy with anti-VIP54 affinity-purified antibodies shows a fibrous pattern typical of intermediate filaments. Double label immunofluorescence performed on various cell lines with antibodies specific to different intermediate filament proteins revealed that VIP54 largely co-distributes with vimentin. In contrast to known intermediate filament proteins, VIP54 is predicted to contain ~50% of helical segments, but no extended coiled-coil regions. The possible involvement of this novel protein in interactions between intermediate filaments and late endosomal compartments is discussed.

Keywords: cytotoxin/*Helicobacter*/intermediate filaments/vacuoles/vimentin

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 1×10^7 independent clones yielded six His-β-Gal-positive clones. As a control for specificity, library plasmids rescued from

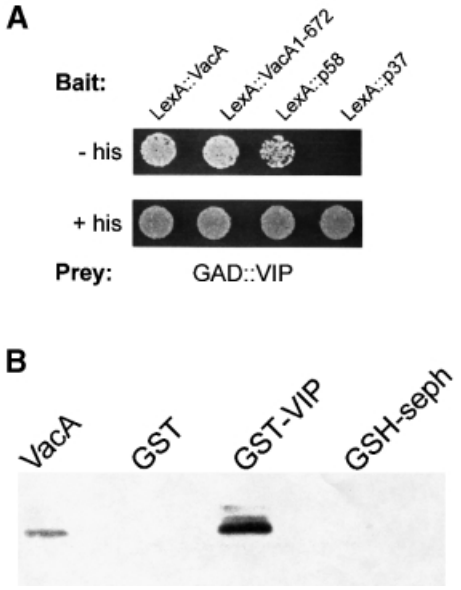

Fig. 1. Identification of a VacA-interacting protein. (**A**) VacA-interacting protein(s) were identified in a HeLa cell library using the yeast two-hybrid method. *HIS3* reporter gene activation (growth on histidine-lacking medium) caused by specific interactions between protein(s) encoded by gene(s) present in the library and VacA or VacA fragment 1–672 or p58 is shown. L40 reporter yeast cells co-transformed with Gal4 RNA polymerase II activation domain (GAD::) fusions in the 2μ/LEU2 pGAD plasmid and LexA DNA-binding domain fusions in the 2μ/TRP1 pLexA plasmid (LexA::) for the proteins indicated were spotted on synthetic complete medium lacking tryptophan and leucine (+his) and on synthetic complete medium lacking tryptophan, leucine and histidine (–his). A LexA bait fusion (LexA::) with the p37 domain of VacA was used as a control for non-specific interactions. (**B**) Binding of VacA to a GST–VIP fusion protein. A 100 μg aliquot of GST–VIP54 was mixed with 10 μg of purified VacA in the presence of 20 μl of glutathione–Sepharose. After incubation, the matrix was washed extensively and matrix-bound proteins were solubilized in LSB, separated by SDS–PAGE and immunoblotted. The nitrocellulose membrane was probed with a polyclonal anti-VacA antibody followed by an anti-rabbit IgG peroxidase-conjugated antibody. Proteins were revealed with ECL (Pierce). Controls were performed by using glutathione–Sepharose alone and conjugated with GST. The first lane shows purified VacA as reference.

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 and it is therefore a novel protein (see below).

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–Sepharose matrix and VacA was loaded onto the column. 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).

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 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,

A

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hVIP54     1          SPSEPGSSCSPTPPPVPRRGTHTTVSQVQPPPSKASAPEPPAEEEVATGTTS
mVIP54       MDTLYTGS....L.P.............A......P..S....PS....-T....AE.N.

hVIP54    61 ASDDLEALGTLSLGTTEEKAAAEAAVPRTIGAELMELVRRNTGLSHELCRVAIGIIVGHI
mVIP54       TP.....QDA..PE.........TV.............................VV....

hVIP54   121 QASVPASSPVMEQVLLSLVEGKDLSMALPSGQVCHDQQRLEVIFADLARRKDDAQQRSWA
mVIP54       ..T......T..............T.................................

hVIP54   181 LYEDEGVIRCYLEELLHILTDADPEVCKKMCKRNEFESVLALVAYYQMEHRASLRLLLLK
mVIP54       .....D............................SD.........................

hVIP54   241 CFGAMCSLDAAIISTLVSSVLPVELARDMQTDTQDHQKLCYSALILAMVFSMGEAVPYAH
mVIP54       ..........................................V.................

hVIP54   301 YEHLGTPFAQFLLNIVEDGLPLDTTEQLPDLCVNLLLALNLHLPAADQNVIMAALSKHAN
mVIP54       .............S........M..........M..........T.PE.........R.T.

hVIP54   361 VKIFSEKLLLLLNRGDDPVRIFKHEPQPPHSVLKFLQDVFGSPATAAIFYHTDMMALIDI
mVIP54       .......................R.................S.S..................

hVIP54   421 TVRHIADLSPGDKGPFGAGQRPWPGVPRLLEPGSTPSREPHPVERSGVPALTSSWASGCP
mVIP54       ...Q.....................................................

hVIP54   481 RPLHPALQLVIDSAFGGRSV
mVIP54       ....................
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B

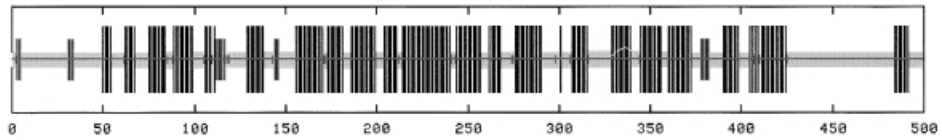

Fig. 2. Sequence homology between human and mouse VIP54. (**A**) The human and mouse sequences of the protein, determined as described in Materials and methods, are compared. The human VIP54 nucleotide sequence is included in the DDBJ/EMBL/GenBank nucleotide sequence database with accession No. AJ242655. 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.

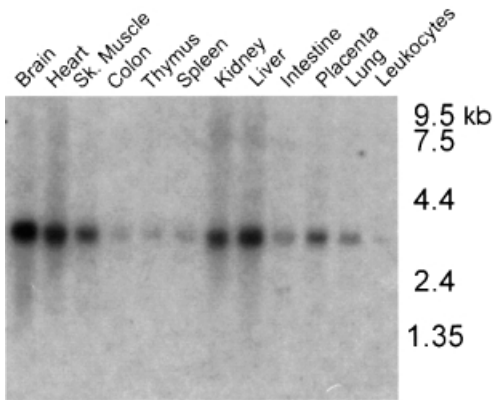

Fig. 3. Tissue expression of VIP54. The expression of VIP54 among various human tissues was examined by Northern blot analysis of poly(A) RNA isolated from various human tissues (Clontech) with a probe derived from the construct pGAD-VIP54. Molecular weights are indicated.

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,

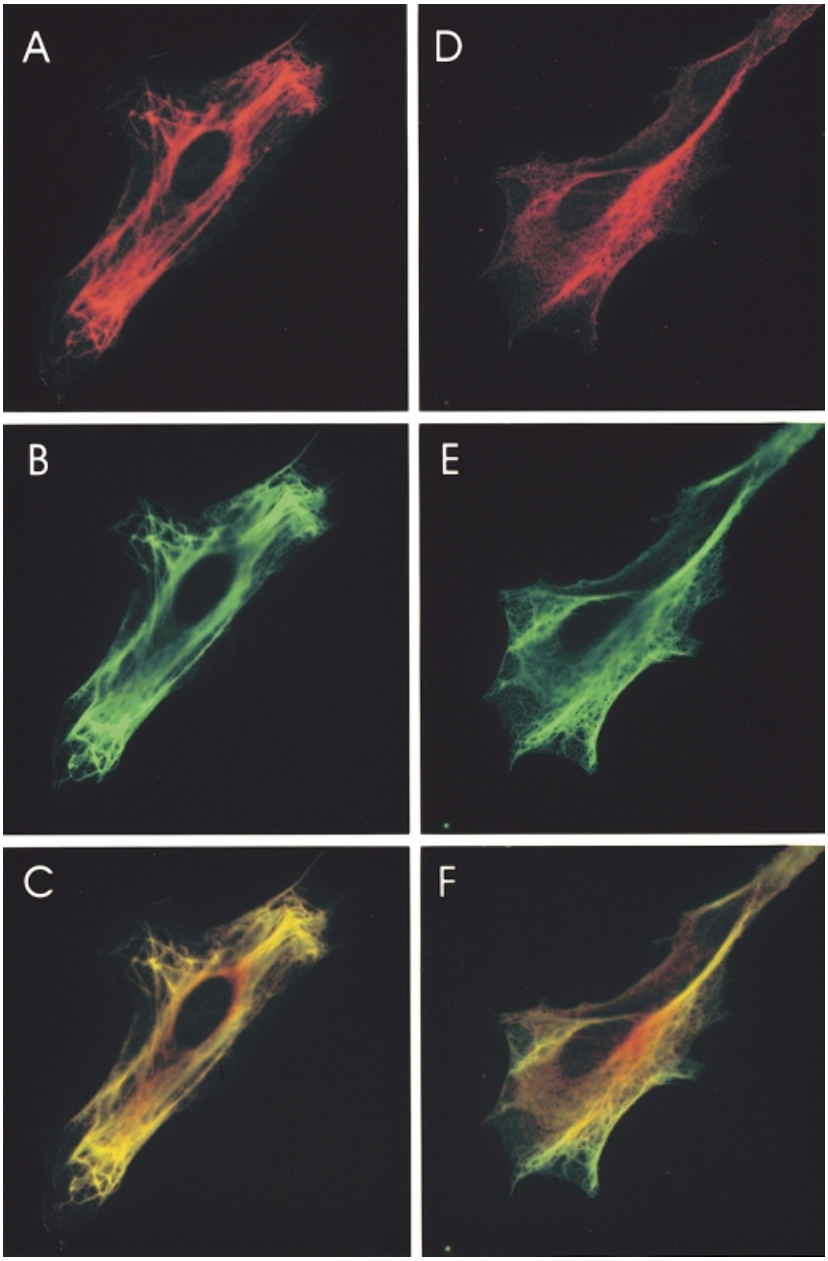

Fig. 4. Co-localization of vimentin and VIP54. MDCK cells (**A**, **B** and **C**) and BHK cells (**D**, **E** and **F**) were processed for immunofluorescence as reported in Materials and methods. Cells were stained with an affinity-purified polyclonal antibody against VIP54 (A and D) and with a monoclonal antibody against vimentin (B and E). Secondary antibodies were a Texas red-conjugated anti-rabbit IgG and an FITC-conjugated anti-mouse IgG. Overlays of the two staining patterns are shown in yellow (C and F).

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).

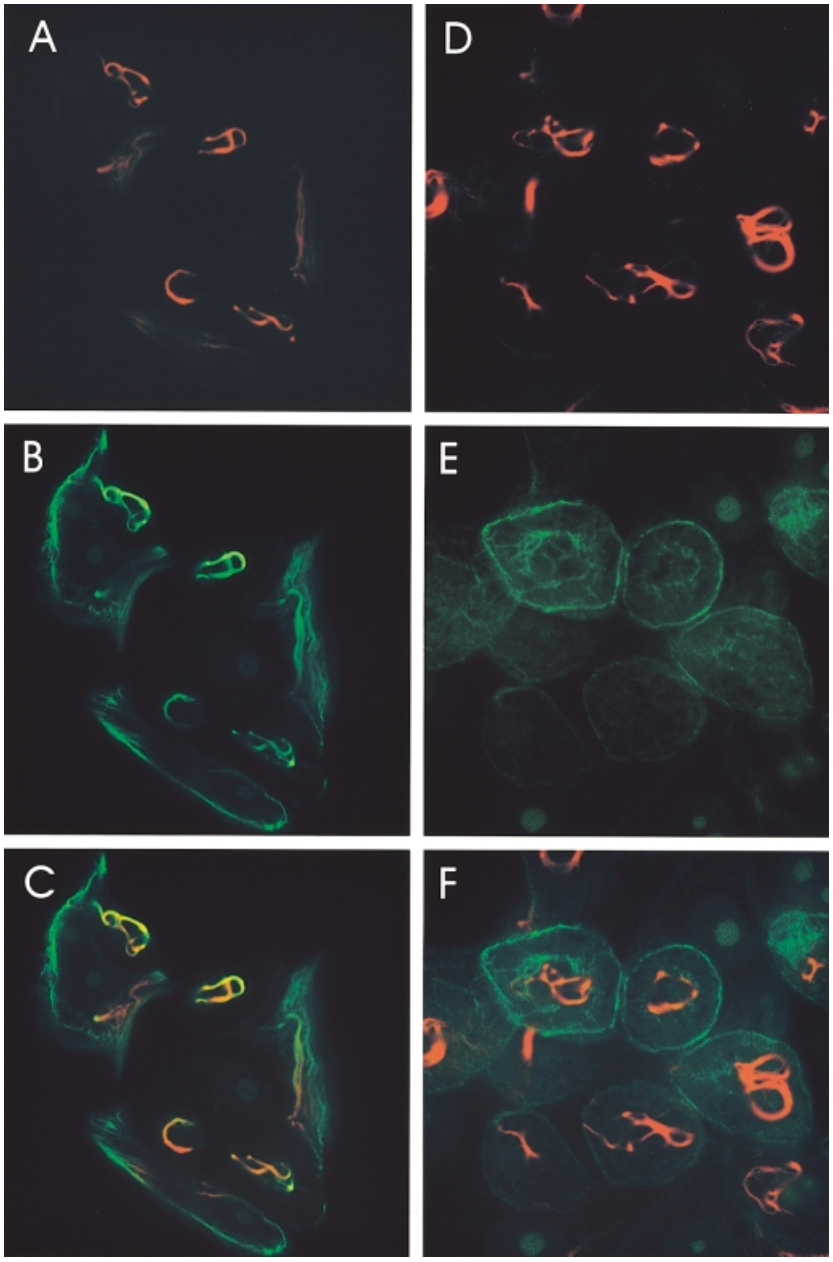

Fig. 5. Effect of colchicine on vimentin and VIP54 distribution in MDCK cells. MDCK cells were treated with colchicine (10 μg/ml) for 16 h and then processed for immunofluorescence. Cells were stained with polyclonal antibody against VIP54 (**A** and **D**) and with monoclonal antibody against vimentin (**B**) or anti-cytokeratin (**E**). Secondary antibodies were a Texas red-conjugated anti-rabbit IgG and an FITC-conjugated anti-mouse IgG. Overlays of the two staining patterns are shown in (**C**) and (**F**).

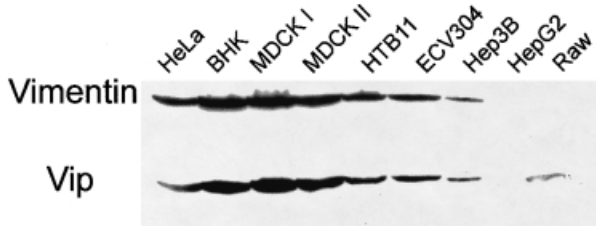

Fig. 6. Vimentin and VIP54 co-distribute in different cell lines. A total of 4×10^5 cells seeded the previous day in 24-well plates were detached with 5 mM EDTA pH 7.4. Cellular pellets obtained after a brief spin at 14 000 r.p.m. were dissolved in LSB, subjected to SDS–PAGE and analysed by Western blotting using a monoclonal anti-vimentin antibody and the affinity-purified polyclonal anti-VIP54 antibody. Proteins were revealed by chemiluminescence (Pierce).

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

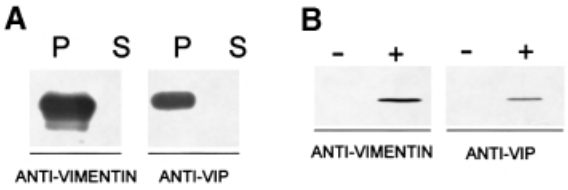

Fig. 7. Association of vimentin and VIP54. MDCK cells from one culture dish were lysed as described in Materials and methods. (**A**) Proteins present in the soluble (S) and insoluble (P) fractions were separated by SDS–PAGE, analysed by Western blotting with a monoclonal anti-vimentin antibody and the polyclonal anti-VIP54 antibody, and developed by chemiluminescence. (**B**) Total cell lysates were processed for immunoisolation 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. Immunoisolated 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, immunofluorescence, 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 immunofluorescent 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 pGEM1Myc3 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 *Xma*I and *Bam*HI sites of the yeast two-hybrid vector pBTM116

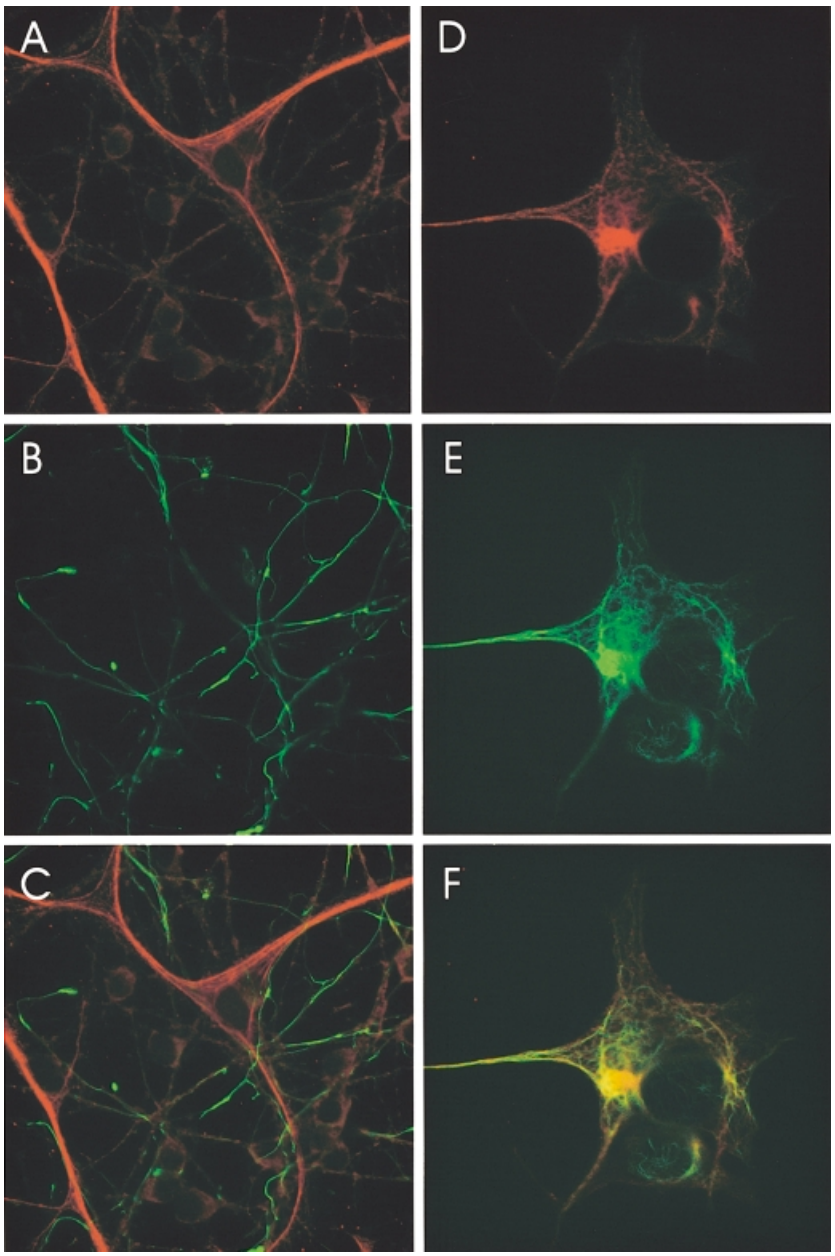

Fig. 8. Expression of VIP54 in the CNS and in neuroblastoma. Eight-day-old cerebellar cells were processed for immunofluorescence and labelled with anti-VIP54 polyclonal antibody (**A**) and with an anti-neurofilament monoclonal antibody to identify the neurons (**B**). The overlay in (**C**) clearly 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′-TCCTTGTTAGGGAATTCATTAAAGCCTTTTCCT, containing the recognition sequences (underlined) for *Xma*I and *Eco*RI, 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 *Xma*I and *Eco*RI was gel purified and ligated into the *Xma*I and *Eco*RI site of pCDNA3. The plasmid was then digested with *Eco*RI and *Bam*HI and mixed in a ligation reaction with the *vacA* fragment from bp 490 to 2844 excised from the plasmid pGEMp95 by digestion with *Eco*RI and *Bam*HI. The resulting entire *vacA* sequence was excised from the pCDNA3 vector by digestion with *Xma*I and *Bam*HI 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 *Eco*RI and *Bam*HI 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′-GAATGCCCGGGGGCCGCCTTTTTCACAACCGTGA and 5′-GAATGGGATCCTTAAGC-

GCTTTCATTTTT, containing the recognition sequences (underlined) for *Xma*I and *Bam*HI, respectively. A 10 ng aliquot of the plasmid pLexA-VacA and 50 pmol of each primer were used. The reaction mixture was pre-incubated for 6 min at 94°C, and 25 amplification cycles were performed as follows: denaturation at 94°C for 1 min; annealing at 62°C for 1 min; extension at 72°C for 2 min; with a final elongation step of 72°C for 10 min. The fragment, digested with *Xma*I and *Bam*HI, was gel purified and ligated with the vector pLexA, previously digested with the same restriction enzymes. The *vacA* gene from bp 1273 to 2844, corresponding to the 58 kDa C-terminal domain (Manetti *et al*., 1995), was cloned in the pLexA vector following the strategy previously used in the cloning of the 37 kDa domain, except that the PCR step was performed with the following oligonucleotides: 5′-GAATGCCCGGGTAAAAACGACAAACAAGAGAGCA and 5′-GAATGGGATCCTTAATTAGGAGTAGCAC, containing the recognition sequences (underlined) for *Xma*I and *Bam*HI, respectively.

Cloning of VIP54

A mouse phage 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 *Eco*RI and *Bam*HI. The probe was labelled using a random primer labelling kit (Roche). The three positive clones obtained were sequenced, and all of them contained the region encoding the N-terminus sequence of the protein. RT–PCR was performed with a Gibco kit on total RNA prepared from HeLa cells (Chomczynski and Sacchi, 1987) using the oligonucleotide based on the human VIP 3′ sequence: 5′-AACTCGTTTCTCTTGCACAT. To obtain double-stranded cDNA, a PCR was carried out using the single strand produced by RT–PCR as template and the following oligonucleotides, which were based on the mouse VIP 5′ sequence and the human VIP 3′ sequence: 5′-GAATGGAATTCCACCTCCATGGACACACTCTATACTGGCTCC and 5′-GAATGCTCGAGACGGCGGACTCATCGCATGGGG, containing the recognition sequence for *Eco*RI and *Xho*I, respectively. PCR was carried out by mixing 5 μl of single-stranded cDNA with 10 pmol of each primer. The reaction mixture was pre-incubated for 2 min at 94°C, and 35 amplification cycles were performed as follows: denaturation at 94°C for 1 min; annealing at 58°C for 1.5 min; extension at 72°C for 2 min; with a final elongation step of 10 min at 72°C. The fragment was gel purified before ligation into the vector pGEM1Myc3 previously digested with *Eco*RI and *Xho*I.

Pull-down of VacA

Construct pGADGH-VIP was digested with *Eco*RI and *Xho*I and the resulting fragment was ligated into the vector pGEX-4T3 previously digested with the same restriction enzymes. Purified DNA was introduced into *E.coli* AB1899 by $CaCl_2$ transformation, and protein expression was carried out with isopropyl-β-D-thiogalactopyranoside (IPTG) induction. The protein was purified by affinity chromatography on a glutathione–Sepharose matrix (Pharmacia). The pull-down experiment was performed at 4°C by mixing 100 μg of GST–VIP with 10 μg of VacA in phosphate-buffered saline (PBS) 0.5% Tween-20 in the presence of 20 μl glutathione–Sepharose. After 12 h, the matrix was washed three times with PBS 0.5% Tween-20 and solubilized in LSB (Tris acetate pH 6.8, 8% SDS plus bromophenol blue) plus protease inhibitors (Roche) and 5% β-mercaptoethanol. Samples were subjected to SDS–PAGE and analysed by Western blotting.

Preparation of antisera

The following peptides: CPAEEEVATGTTSASDDLE, CFADLARRKDDAQQRS and CLNRGDDPVRIFKHEPQ (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 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 $MgCl_2$, 1 mM EGTA, 100 mM NaCl, 0.1 mM dithiothreitol (DTT), 0.5% Triton X-100, 0.5 mg/ml DNase, 0.2 mg/ml RNase and protease inhibitors. Cell lysates were adjusted to 1% Triton X-100 and 500 mM NaCl, and soluble 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 immunoisolation 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 immunoblotting. 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% CO_2 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 $4 \times 10^4/cm^2$ 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 microtube; 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 micropipette was controlled using 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 [10 mM PIPES pH 6.9, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 1.2 mM phenylmethylsulfonyl fluoride (PMSF)] after careful washing with CSK buffer. All processing steps were done at room temperature. Cells were washed three times in PBS for 5 min, incubated 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 fluoresceinated 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 (Pierce).

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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