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The 46/50 kDa phosphoprotein VASP purified from human platelets is a novel protein associated with actin filaments and focal contacts

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Vasoactive agents which elevate either cGMP or cAMP inhibit platelet activation by pathways sharing at least one component, the 46/50 kDa vasodilator-stimulated phosphoprotein (VASP). VASP is stoichiometrically phosphorylated by both cGMP-dependent and cAMP-dependent protein kinases in intact human platelets, and its phosphorylation correlates very well with platelet inhibition caused by cGMP- and cAMP-elevating agents. Here we report that in human platelets spread on glass, VASP is associated predominantly with the distal parts of radial microfilament bundles and with microfilaments outlining the periphery, whereas less VASP is associated with a central microfilamentous ring. VASP is also detectable in a variety of different cell types including fibroblasts and epithelial cells. In fibroblasts, VASP is concentrated at focal contact areas, along microfilament bundles (stress fibres) in a punctate pattern, in the periphery of protruding lamellae, and is phosphorylated by cGMP- and cAMP-dependent protein kinases in response to appropriate stimuli. Evidence for the direct binding of VASP to F-actin is also presented. The data demonstrate that VASP is a novel phosphoprotein associated with actin filaments and focal contact areas, i.e. transmembrane junctions between microfilaments and the extracellular matrix.

Key words: cAMP/cGMP/cytoskeleton/phosphorylation/protein kinase

Introduction

Numerous vasoactive agents regulate human platelet activation. Platelet agonists such as thrombin, thromboxane A_2, platelet activating factor, collagen and ADP elevate intracellular free Ca^{2+}, activate myosin light chain kinase, protein kinase C and protein tyrosine kinases, and platelet activation ultimately results in shape change, adhesion, aggregation and degranulation (Haslam, 1987; Siess, 1989; Rink and Sage, 1990). In contrast, many physiologically and pharmacologically important platelet inhibitors such as prostacyclin and endothelium-derived relaxing factor (EDRF) elevate platelet levels of cAMP, cGMP or both, activate cAMP- and/or cGMP-dependent protein kinases (cAMP-PK, cGMP-PK) and inhibit platelet activation by agonists (Walter, 1989; Walter *et al.*, 1991). The two cyclic nucleotides cAMP and cGMP may have distinct but also common sites of action in human platelets. Cyclic nucleotide-induced inhibition of platelet activation apparently involves an early step of the activation cascade such as inhibition of phospholipase C and subsequent Ca^{2+} discharge from intracellular stores (Siess, 1989; Rink and Sage, 1990; Walter *et al.*, 1991; Geiger *et al.*, 1992). Alternatively, Ca^{2+}-ATPases, myosin light chain kinase (Walter, 1989; Walter *et al.*, 1991) and cytoskeletal elements (Fox, 1987; Zhang *et al.*, 1988; Chen and Stracher, 1989; Fox and Berndt, 1989; Siess, 1989) have also been considered as potential targets, but the precise mechanism of action of cyclic nucleotide-elevating platelet inhibitors is not very well understood.

Recently, we purified and characterized a 46/50 kDa vasodilator-stimulated phosphoprotein (VASP) which is stoichiometrically phosphorylated by cAMP- or cGMP-dependent protein kinase in experiments with purified proteins as well as in intact human platelets treated with cAMP- or cGMP-elevating platelet inhibitors, respectively (Waldmann *et al.*, 1986, 1987; Halbrügge and Walter, 1989; Halbrügge *et al.*, 1990). VASP phosphorylation at one site by either protein kinase alters the properties of the protein in SDS–PAGE causing a shift in its apparent molecular mass from 46 to 50 kDa (Halbrügge and Walter, 1989). This phosphorylation-induced change in the apparent molecular mass of VASP has recently been used for the quantitative analysis of VASP phosphorylation in intact cells (Halbrügge *et al.*, 1990; Nolte *et al.*, 1991a,b).

VASP phosphorylation correlates very well with vasodilator-induced inhibition of platelet activation (Waldmann *et al.*, 1987; Halbrügge *et al.*, 1990; Siess and Lapetina, 1990; Walter *et al.*, 1991); however, the precise functional role of VASP has not been elucidated. Recently, the presence and regulation of VASP phosphorylation was also discovered in other human cell types including lymphocytes and granulocytes (Halbrügge *et al.*, 1992). Because of the potential importance of this 46/50 kDa phosphoprotein VASP for signal transduction pathways regulated by both cAMP- and cGMP-elevating hormones and vasoactive agents, we have now investigated the subcellular distribution of VASP in human platelets and a variety of other cell types. Evidence will be presented that VASP is a novel phosphoprotein associated with actin filaments and focal contact areas. Our data suggest these cellular structures and their associated proteins as potential targets for regulation by cAMP- and cGMP-dependent protein kinases.

Results

Characterization of VASP antiserum M4

In immunoblots of platelet and fibroblast homogenates, a polyclonal rabbit antiserum raised against purified VASP (M4) specifically recognized a polypeptide doublet of 46/50 kDa (Figure 1, lanes 3–4). The 46 kDa VASP shifts to a

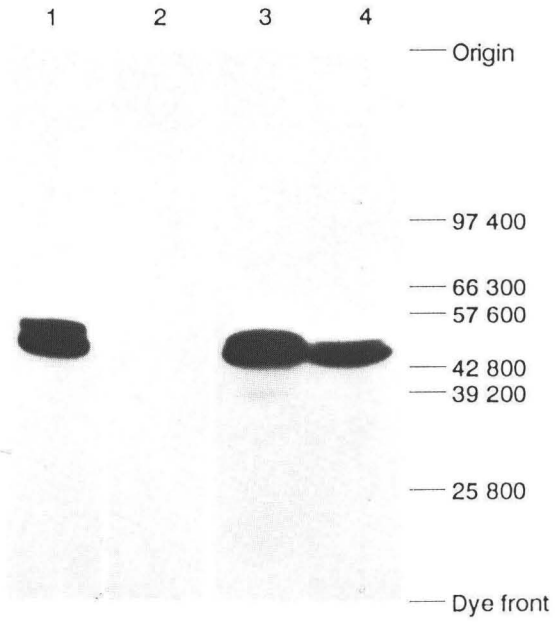

Fig. 1. Specificity of antiserum against VASP (M4) in immunoblots. Proteins in homogenates of human platelets (lanes 1 and 2: 135 μg; lane 3: 50 μg) and human dermal fibroblasts (lane 4: 200 μg) were probed with either affinity-purified M4 (lane 1), preadsorbed affinity-purified M4 (lane 2) or original M4 anti-VASP (lanes 3–4). Molecular masses (in Da) of standard proteins are indicated at the right margin. VASP (a closely spaced doublet with apparent molecular masses of 46 and 50 kDa) is specifically recognized by M4 and affinity-purified M4.

protein with apparent molecular mass of 50 kDa in SDS–PAGE when one specific site is phosphorylated by cAMP- or cGMP-dependent protein kinases (Halbrügge and Walter, 1989). Thus, the 46 kDa and 50 kDa protein bands recognized by antiserum M4 represent dephospho- and phosphoforms of VASP with respect to this particular phosphorylation site (Halbrügge *et al.*, 1990) and will subsequently be referred to as dephospho- and phospho-VASP, respectively, although VASP contains additional phosphorylation sites which were not analysed here. Immunoblotting experiments with antiserum M4 and affinity-purified M4 gave identical results (Figure 1, lanes 1, 3 and 4). Moreover, the patterns of subcellular VASP distribution obtained by immunofluorescence were identical with antiserum M4 and affinity-purified M4, whereas preimmune serum was negative (not shown). When affinity-purified M4 was preincubated with the 46/50 kDa area of nitrocellulose sheets to which platelet homogenate proteins had been transferred after SDS–PAGE, its VASP-staining capacity was completely lost as assayed by immunoblots (Figure 1, lane 2) or by indirect immunofluorescence (not shown). These results demonstrated that M4, used as complete serum or after affinity purification, selectively recognized VASP in its SDS-denatured form as well as *in situ*.

Localization of VASP to microfilaments and focal contact areas

Platelets that are allowed to settle onto coverslips adhere and begin to extrude pseudopods (Zobel and Woods, 1983; Behnke and Bray, 1988). These pseudopods have been shown to contain microfilament bundles (Debus *et al.*, 1981) and their distal portions coincide with areas of firm attachment to the underlying substratum (focal contacts; Zobel and Woods, 1983; Alexandrova and Vasiliev, 1984). Staining of spreading and fully spread platelets for actin shows three main areas of fluorescence, i.e. the radial microfilament bundles, the rim of the veil-like lamellae spread out between such bundles (Debus *et al.*, 1981; Figure 2a′–e′), and, in some platelets, a ring-like structure in the centre of the platelet (Figure 2e′). Indirect immunofluorescence with antiserum M4 showed VASP concentrated in two of these structures, the terminal portions of radial microfilament bundles and the peripheral microfilaments of veils (Figure 2a–e). The central ring, which in fully spread platelets appeared as an area of very prominent actin staining, was labelled less intensely and less homogeneously with M4, revealing a dotted distribution of VASP (Figure 2e and e′).

M4 also revealed the expression and subcellular distribution of VASP in tissue culture cells. In analogy to the staining patterns of adhering platelets, VASP appeared to be highly concentrated at the terminal portions of microfilament bundles (stress fibres) of stationary fibroblasts derived from man or rat, respectively (Figure 3a–f), and also at the peripheral regions of the protruding lamellae developed by locomoting or spreading cells, especially in ruffling areas (Figure 3d). In addition, well spread fibroblasts showed a distinct, quite regular distribution of VASP as periodically arranged dots along their stress fibres (Figure 3a, e and f).

Expression of VASP in different cell types

The expression of VASP was analysed by immunoblotting of homogenates from different blood and tissue culture cells and also by indirect immunofluorescence (Table I). With regard to human cells, VASP was found not only in platelets and differentiated blood cells like granulocytes and lymphocytes, but also in their tumour derivatives, leukaemia (HEL 92.1.7, HL-60 and K-562) and lymphoblastoma (HuT 78, Jurkat, Raji and U-937) cells. VASP was also located in certain tissue-forming cells analysed (endothelial cells, vascular smooth muscle cells and fibroblasts) and in cell lines derived from such cells (neuroblastoma–glioma hybrid cells N×G 108CC15 and epithelial cells A-431, LLC-PK1, BHK and PtK2). In addition, prominent VASP staining was detected at the site of intercalated discs of rat heart (data not shown).

Although the content of VASP in different cells has not been exactly quantified, a rough estimate derived from a comparison of lanes 3 and 4 in Figure 1 suggests that there is ~5- to 8-fold more VASP per mg of homogenate in platelets than in human dermal fibroblasts. Platelets have been shown previously to contain 2.45 μg/mg VASP (Halbrügge and Walter, 1990). Like fibroblasts, other cell types of human origin so far investigated contained less VASP than platelets (Halbrügge *et al.*, 1992).

Phosphorylation of VASP in intact human fibroblasts in response to prostaglandin E_1 (PG-E_1) and a cGMP-analogue

The demonstration of VASP as part of the microfilamentous system not only in platelets but also in fibroblasts raised the question of whether VASP is phosphorylated by cAMP- and cGMP-dependent protein kinases in intact fibroblasts, as has

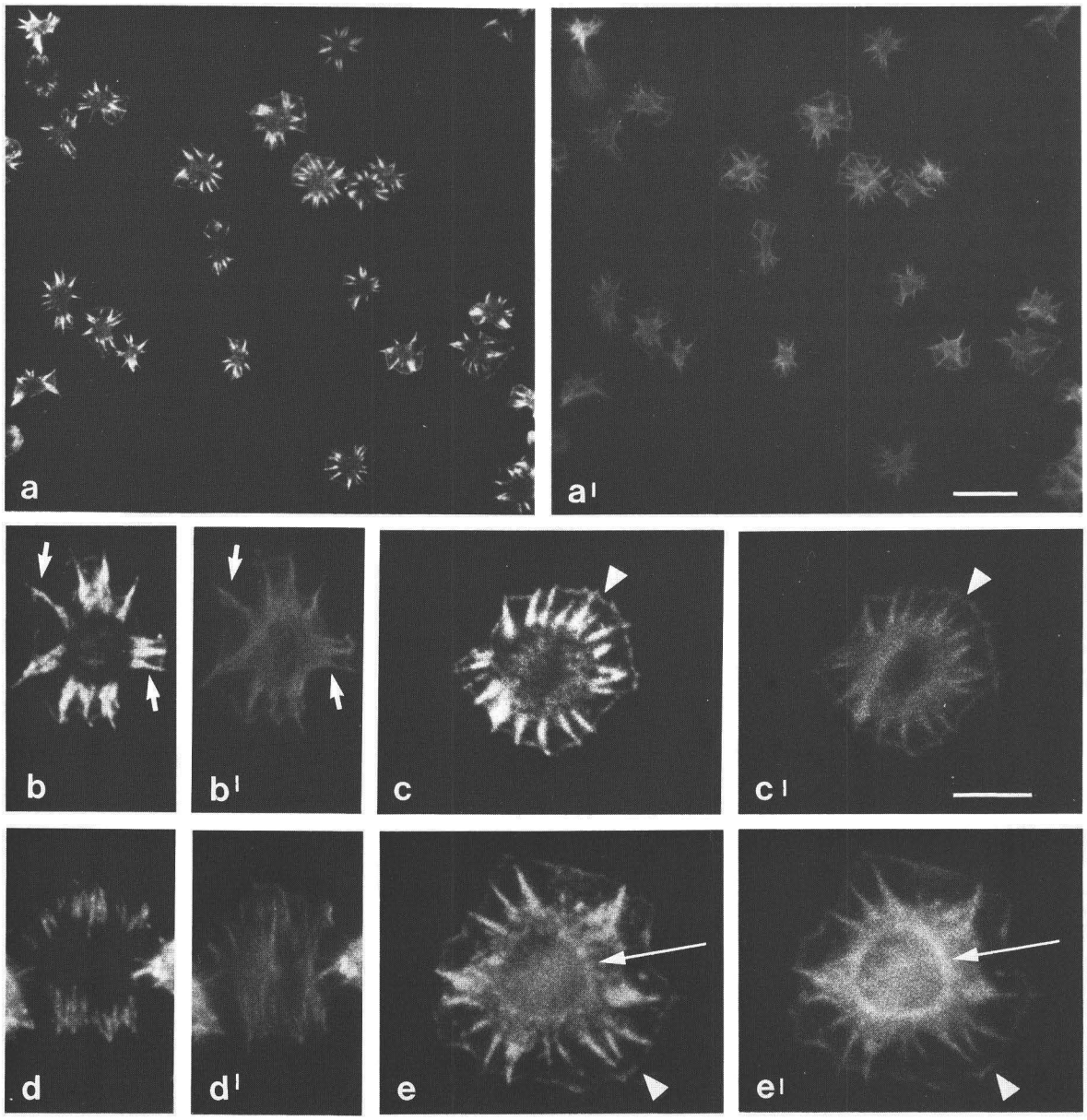

Fig. 2. Comparison of subcellular distribution of VASP and F-actin in human platelets spread on glass. Localization of VASP (a−e) and F-actin (a′−e′) was revealed by double labelling, i.e. indirect immunofluorescence and NBD-phallacidin staining, respectively. (**a** and **a′**): Overview, showing a population of platelets in different stages of spreading, with intense VASP staining of radial microfilament bundles. (**b,b′−e,e′**): Selected views of individual platelets in consecutive stages of spreading. (b and b′): Partially spread platelet. Microfilament bundles are arranged in spikes that stain for actin and VASP (arrows), but the lamella has not reached the tips of these bundles. (c and c′ and d and d′): Later stages in spreading. The lamella has extended to the distal portions of the radial microfilament bundles, VASP is co-localized with actin at the periphery (arrowheads in c,c′). Staining of the fully extended microfilament bundles is mostly confined to their distal portions, as is especially evident in d and d′. (e and e′): Fully spread platelet. The lamella has engulfed the microfilament bundles (arrowheads). In addition to the presence of actin in radial bundles and the peripheral regions of the lamella, actin filaments are organized in a central ring (long arrow in e′). This structure is not homogeneously decorated by M4. Bar in a′, valid for a and a′: 10 μm. Bar in c′, valid for b,b′−e,e′: 5 μm.

already been demonstrated for platelets (Waldmann *et al.*, 1987; Halbrügge *et al.*, 1990). Therefore, the state of VASP phosphorylation was determined in homogenates of human dermal fibroblasts which had been incubated with either PG-E_1 or with 8-(4-chlorophenylthio)-guanosine-3′,5′cyclic monophosphate (8-pCPT-cGMP). PG-E_1 was chosen since this prostaglandin raises cAMP in many cell types including fibroblasts (Smith, 1989) and causes VASP phosphorylation mediated by cAMP-PK in platelets and lymphocytes (Halbrügge *et al.*, 1990, 1992). 8-pCPT-cGMP was chosen since this cell membrane permeant cGMP-analogue is a selective cGMP-PK activator which has no major effects on other protein kinases or phosphodiesterases and which was shown to cause VASP phosphorylation mediated by cGMP-PK in platelets (Geiger *et al.*, 1992). After a 5 min incubation of fibroblasts with 5 μM PG-E_1, almost all of VASP was converted from the 46 kDa dephosphoform to the 50 kDa phosphoform, whereas a 30 min incubation with 8-pCPT-cGMP shifted about half of VASP to its 50 kDa phosphoform (Figure 4). Incubation with a combination of PG-E_1 and 8-pCPT-cGMP was no more effective than PG-E_1 alone (Figure 4). The fluorescence analysis of VASP

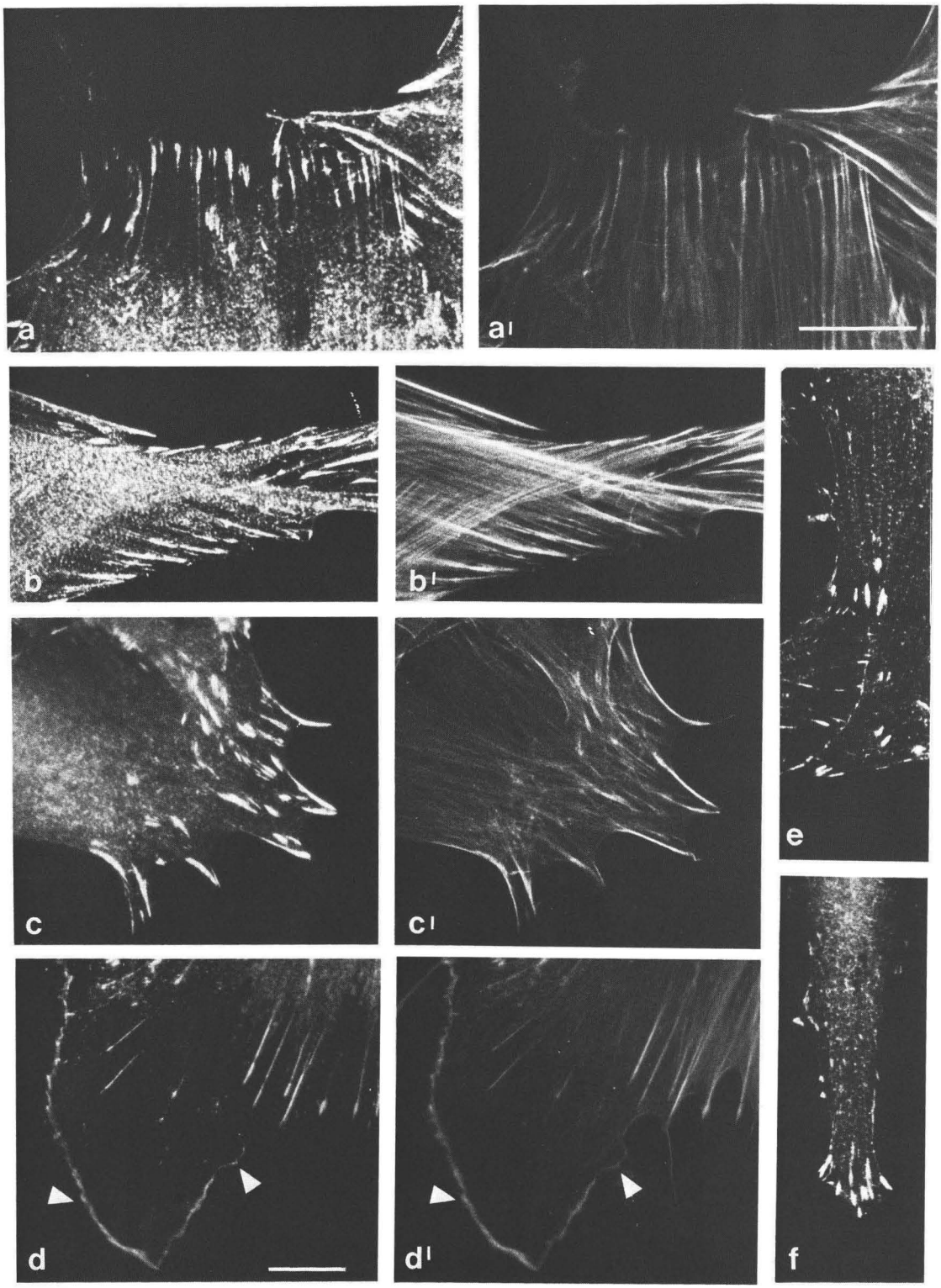

Fig. 3. Comparison of the subcellular distribution of VASP (**a**−**f**, revealed by M4 staining) and F-actin (**a′**−**d′**, decorated with NBD-phallacidin), respectively, in rat (a,a′−c,c′, e and f) and human (d and d′) fibroblasts. All comparisons (a,a′−d,d′) were made with double fluorescence. VASP is highly concentrated in virtually all focal adhesion sites of well spread stationary fibroblasts, at the periphery (a,a′−c,c′) as well as at the ventral membrane (c and c′). In addition, stress fibres show anti-VASP decoration in distinct periodicities in their proximal portions (e and f). (d and d′): Locomoting or spreading fibroblast, showing VASP and F-actin co-localized in the ruffling periphery of a protruding lamella (arrowheads). Bar in a′ (valid for a,a′−c,c′, e and f), 10 μm; bar in d′ (valid for d and d′), 20 μm.

distribution and actin organization did not reveal any dramatic differences between controls and cells treated with either PG-E_1 or 8-pCPT-cGMP (not shown). Also, conditions which affected the phosphorylation state of VASP (e.g. treatment of cells with PG-E_1 and 8-pCPT-cGMP) did not alter the Triton X-100 solubility of VASP since VASP was recovered nearly quantitatively in the soluble fraction of both control and treated cells (not shown).

Table I. Detection of VASP in different cell types by immunoblotting or indirect immunofluorescence

Cell type	Immunoblot	Immunofluorescence
Primary cells and tissues		
Platelets (human)	+	+
Lymphocytes (human)[a]	+	n.d.
Granulocytes (human)[a]	+	n.d.
Endothelial cells (human)[a]	+	+
Dermal fibroblasts (human)	+	+
Muscle fibroblasts (rat)	+	+
Embryo fibroblasts (chicken)	n.d.	+
Vascular smooth muscle cells (porcine)[b]	+	+
Heart (rat)[b]	n.d.	+
Cell lines		
Human[a]		
HEL 92.1.7	+	n.d.
HL-60	+	n.d.
Jurkat	+	n.d.
K-562	+	n.d.
U-937	+	n.d.
HuT 78	+	n.d.
Raji	+	n.d.
A-431	+	n.d.
Porcine		
LLC-PK1	+	+
Rodent		
BHK	+	+
NxG 108CC15	+	n.d.
Marsupial		
PtK2	+	+

+, presence of 46/50 kDa VASP in immunoblots or observation of typical immunofluorescene pattern, respectively
n.d., not determined
[a] Halbrügge *et al.*, 1992
[b] K.Abel, T.Markert and U.Walter, unpublished experiments

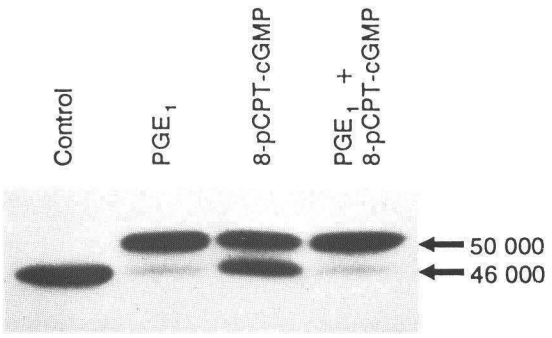

Fig. 4. Autoradiograph showing the phosphorylation of VASP in intact human fibroblasts. Monolayers of fibroblasts were incubated with buffer (control), 5 μM PG-E_1 for 5 min, 0.47 mM 8-pCPT-cGMP for 30 min, and with both 5 μM PGE_1 and 0.47 mM 8-pCPT-cGMP (25 min with the cGMP-analogue alone followed by a 5 min incubation with the analogue + PG-E_1) as indicated. Fibroblasts were harvested in SDS sample buffer and analysed for VASP phosphorylation by immunoblotting. The shift of the apparent molecular mass from 46 to 50 kDa indicates phosphorylation of VASP.

Cosedimentation of VASP with F-actin

The co-localization of VASP with microfilaments, as revealed by indirect immunofluorescence, prompted the question of whether VASP binds directly to F-actin. To address this question, purified VASP and F-actin were co-

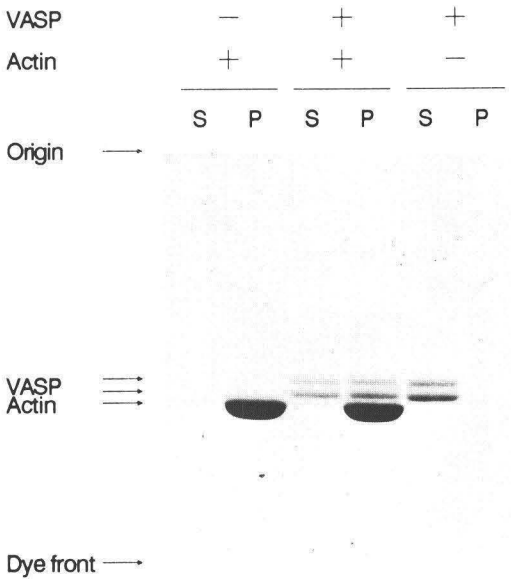

Fig. 5. Binding of VASP to F-actin in a cosedimentation assay. VASP and F-actin were mixed (final concentrations: 110 μg/ml VASP, 500 μg/ml actin), incubated for 30 min at room temperature and then centrifuged at 100 000 *g* for 2 h. Equivalent amounts of supernatants (S) and resuspended pellets (P) were separated by SDS–PAGE and stained with Coomassie blue. The composition of the samples analysed is indicated at the top of each lane.

incubated for 30 min at room temperature, and the mixture was subsequently centrifuged at 100 000 *g* for 2 h. F-Actin was pelleted nearly quantitatively by this high speed centrifugation, and a major part of total VASP co-sedimented with F-actin, whereas in the absence of F-actin no VASP was detected in the 'pellet' fraction (Figure 5). Interestingly, the ratio of the 46 kDa and 50 kDa forms of VASP found in the F-actin pellet was similar to that found in the supernatant (Figure 5), suggesting that both the 46 and 50 kDa forms of VASP cosedimented equally well with F-actin. It is unlikely that the cosedimentation of actin and VASP was due to contaminating proteins since the preparations of both actin and VASP were >95% pure and since no contaminating proteins (which could have mediated VASP binding to F-actin) were observed in the pellet fractions when analysed by SDS–PAGE and Coomassie blue staining (Figure 5). However, our findings do not exclude the possibility that, in cells, the association of VASP with actin filaments is mediated by an interaction with other components.

Discussion

VASP is a novel actin filament- and focal contact-associated protein

The results of this study together with our published biochemical data indicate that VASP, a substrate for cAMP- as well as cGMP-dependent protein kinases, is a widely distributed, novel protein associated with actin filaments and focal contact areas. Many actin-binding proteins have already been described (Vandekerckhove, 1990; Hartwig and Kwiatkowski, 1991), some of which have an apparent molecular mass similar to that of VASP in SDS–PAGE. Therefore, it has to be considered whether VASP may be identical to one of these or related proteins, in particular tropomodulin, band 4.9 or a 53 kDa actin filament bundling

protein from porcine brain. In contrast to VASP, tropomodulin is not a substrate for cAMP-PK (Fowler, 1987), nor does it bind to F-actin (Fowler, 1990). VASP and band 4.9 differ with respect to their Triton X-100 solubility (this study; Faquin *et al.*, 1988) and their properties as protein kinase C substrates (Horne *et al.*, 1985; Palfrey and Waseem, 1985; Faquin *et al.*, 1986, 1988; Waldmann *et al.*, 1987). Furthermore, the hydrodynamic properties of VASP indicate a highly asymmetrical structure (unpublished results) and differ from the hydrodynamic properties reported for tropomodulin (Fowler, 1987), band 4.9 (Siegel and Branton, 1985) and the 53 kDa actin filament bundling protein from porcine brain (Maekawa *et al.*, 1983). Sasaki *et al.* (1990) purified a 48 kDa protein from platelets which bound to an actin–Sepharose column; however, the limited data available on this protein do not allow a direct comparison with VASP.

Our recent, unpublished data strongly indicate that VASP differs not only from biochemically characterized actin filament- and focal contact-associated proteins, but also from all proteins with established primary structure. Comparison of partial VASP sequences (obtained from protein microsequences and a partial cDNA clone) to sequences currently available in nucleic acid and protein databases, revealed no identity with any protein (C.Haffner *et al.*, unpublished results), including the 47 kDa N-terminal proteolytic fragment of talin (Rees *et al.*, 1990).

The immunofluorescence analyses reported here demonstrate that VASP is associated with the microfilament system of a wide variety of cell types. Like VASP, several other microfilament proteins such as talin (Burridge and Connell, 1983a,b) and α-actinin (Lazarides and Burridge, 1975) were also found to be concentrated at focal adhesion sites, i.e. at cell–matrix junctions, and in the cortical actin filament webs at the periphery of highly dynamic lamellae. VASP is also present in cell–cell contacts (unpublished). This concentration in cell–matrix as well as in cell–cell junctions, together with the distinct and periodical localization of VASP in stress fibres (which contain microfilament proteins arranged in sarcomere-like units) is especially reminescent of the distribution of α-actinin. Indeed, we found that these VASP-positive spots, like α-actinin (Sanger *et al.*, 1983), showed a cell-line specific spacing, and double labelling with antibodies against VASP and α-actinin produced superimposable patterns of immunofluorescence (unpublished observations). However, there are also differences in the intracellular localization of these two proteins. In particular, the intracellular localization of VASP in spread platelets (this study) differs from that of α-actinin (Debus *et al.*, 1981), and VASP is not associated with the cleavage furrow in mitotic cells or with the peripheral belt of epithelial cells (unpublished), whereas all these structures have been found to contain α-actinin (Fujiwara *et al.*, 1978; Sanger *et al.*, 1987; Schulze *et al.*, 1989).

Phosphorylation of VASP by cAMP- and cGMP-dependent protein kinases in fibroblasts

Analysis of the phosphorylation-induced change in VASP mobility in SDS–PAGE was used to determine the state of VASP phosphorylation in intact human fibroblasts. In addition, the effects of agents which have been shown (Halbrügge *et al.*, 1990; Geiger *et al.*, 1992) to activate selectively either cAMP-PK (e.g. PG-E_1) or cGMP-PK (e.g. 8-pCPT-cGMP) were analysed. As demonstrated in Figure 4, activation of cAMP-PK by PG-E_1 converted VASP almost quantitatively from the 46 kDa dephosphoform to the 50 kDa phosphoform, whereas maximal VASP phosphorylation due to activation of cGMP-PK by 8-pCPT-cGMP was ~50%. These data not only demonstrate VASP phosphorylation in intact fibroblasts, but also provide functional evidence for the presence and regulation of cGMP-PK in these cells. The latter is not trivial since the limited expression and cellular distribution of cGMP-PK, as well as low cellular concentrations of cGMP-PK, have hindered investigations of the physiological role of this kinase (Walter, 1989). Only preliminary immunocytochemical experiments (Walter, 1989) had suggested that fibroblasts may contain type I cGMP-PK.

Appropriate elevation of cAMP-PK activity in fibroblasts and epithelial cells is known to result in reversible disruption of stress fibres (Lamb *et al.*, 1988; Roger *et al.*, 1988; Turner *et al.*, 1989) and in the loss of talin and vinculin from focal contacts (Turner *et al.*, 1989). The phosphorylation and concomitant inhibition of myosin light chain kinase by cAMP-PK is thought to be of fundamental importance for the observed breakdown of the cellular actin skeleton (Lamb *et al.*, 1988; Fernandez *et al.*, 1990). However, the cAMP-PK-mediated phosphorylation of VASP in fibroblasts described in this study is nearly quantitative and complete after a 5 min incubation with PG-E_1, i.e. under conditions where major alterations of the actin skeleton are not detectable. Also, cGMP-PK-mediated VASP phosphorylation was not accompanied by gross morphological alterations in the cells studied.

Alternatively, VASP phosphorylation may have functional consequences other than structural changes of the actin cytoskeleton. Cyclic nucleotide elevating agents which stimulate VASP phosphorylation in human platelets concomitantly inhibit both the protein kinases (myosin light chain kinase and protein kinase C) and the Ca^{2+} mobilization associated with platelet activation (Waldmann *et al.*, 1987; Walter, 1989; Halbrügge *et al.*, 1990; Nolte *et al.*, 1991a,b; Geiger *et al.*, 1992). This multifocal inhibition would be easiest to achieve if phospho-VASP inhibited an early step of platelet activation, e.g. at the level of phospholipase C (Walter, 1989; Walter *et al.*, 1991; Geiger *et al.*, 1992). Furthermore, our present evidence demonstrating a cytoskeletal association of VASP would position VASP at a site which is perhaps critical for regulation of phospholipase C. Recently, thrombin activation of platelets was shown to increase the total cytoskeleton associated enzyme activities of phospholipase C, inositol-lipid kinases and diacylglycerol kinase (Grondin *et al.*, 1991). A similar cytoskeleton association and regulation of such enzyme activities involved in lipid metabolism has been reported for EGF-treated A-431 cells (Payrastre *et al.*, 1991). Therefore, the cytoskeleton, and in particular cytoskeleton anchorage sites at the plasma membrane, are prominent candidates for forming a matrix for close contacts between various signal transduction components (Grondin *et al.*, 1991; Payrastre *et al.*, 1991) modulated by phosphorylation. Future work will have to clarify the functional role of VASP phosphorylation and the physiological significance of the specific association of VASP with actin filaments and focal contact areas.

Materials and methods

Cells

Human dermal fibroblasts, kindly provided by Dr Höhn (Department of Human Genetics, University of Würzburg, FRG), were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Eggenstein, FRG) supplemented with 10% fetal calf serum (FCS, Gibco BRL) and used as passages 7–12 for experiments. Rat muscle fibroblasts (Schulze *et al.*, 1989) and chicken embryo fibroblasts (Westmeyer *et al.*, 1990) were cultured as described. BHK and PtK2 cells were cultured in DMEM supplemented with 10% FCS. LLC-PK1 cells were grown in minimal essential medium (MEM, Gibco BRL) supplemented with 7.5% FCS. Culture media were supplemented with 1% antibiotic–antimycotic solution (Sigma, Deisenhofen, FRG). All cells were cultured at 37°C with 5% CO_2.

Experiments with fibroblasts

Human dermal fibroblasts were grown for 2–3 days in 58×15 mm tissue culture dishes (Nunc, Wiesbaden-Biebrich, FRG) until they reached a density of $0.7-2.0\times10^4$ cells/cm^2. For immunofluorescence analysis, cells were grown in parallel on coverslips. One hour prior to experiments, cells were incubated with a medium containing 10 μM indomethacin in order to inhibit endogenous prostaglandin synthesis. Cells were incubated at 37°C with 5 μM PG-E_1 (Sigma) and/or 470 μM 8-pCPT-cGMP (BioLog, Bremen, FRG) for 5 min or 30 min, respectively. Agents examined were added to the cells in phosphate buffered saline (PBS, 10 mM sodium phosphate pH 7.4, 150 mM NaCl) supplemented with 1 mM $CaCl_2$, 2 mM $MgCl_2$ and 10 μM indomethacin. Cells incubated with the vehicle medium alone were used as controls. At the end of incubations, cells were lysed in SDS sample buffer or fixed for immunofluorescence as indicated below.

Antiserum

M4 rabbit antiserum against human VASP was raised as described previously (Halbrügge *et al.*, 1990). Affinity-purified M4 was prepared by adsorption and elution of antibodies using protein antigens immobilized on nitrocellulose blots essentially as described (Smith and Fisher, 1984). In short, antiserum M4 (600 μl, diluted 1:10 in PBS, 0.5% Tween 20) was adsorbed to the 46/50 kDa area of a nitrocellulose sheet to which platelet homogenate proteins (1.1 mg) separated by SDS–PAGE had been transferred. Antibodies were eluted from nitrocellulose strips by three successive 30 s washes (1.5 ml each) with glycine–HCl buffer, pH 2.3. This eluate, which was immediately neutralized by the addition of 0.25 vol of 250 mM sodium phosphate, is referred to as 'affinity-purified M4'. An aliquot of affinity-purified M4 (37 μl), diluted 1:60 in buffer A (PBS, 0.5% Tween 20, 100 μg/ml BSA, 0.01% NaN_3), was successively preadsorbed to the 46/50 kDa area of four different nitrocellulose blots of platelet homogenate proteins (2.3 mg per sheet) for several hours each. This solution depleted of VASP antibodies was used for control immunofluorescence and immunoblot experiments.

Fluorescent staining of cells

Cells grown on coverslips were fixed in 3.7% formaldehyde for 20 min on ice, permeabilized by 0.2% Triton X-100 in PBS for 10 min and incubated for 30 min at room temperature with M4 (diluted 1:500 in PBS) or affinity-purified M4 (diluted 1:60 in buffer A). Affinity-purified M4, preadsorbed as described above, was used without further dilution. Subsequently, cells were incubated for 30 min at room temperature with 4.3 μg/ml rhodamine-conjugated affinity-purified donkey anti-rabbit IgG (Dianova, Hamburg, FRG) and, where indicated, with ~0.33 μM NBD-phallacidin (Molecular Probes, Eugene, OR, USA). Cells were washed repeatedly with PBS between individual incubation steps.

For immunofluorescence studies with platelets, venous blood from healthy volunteers was collected into 5 ml coagulation tubes (Monovette SNC; Sarstedt, Mümbrecht, FRG) and centrifuged for 15 min at 360 *g*. The supernatant (platelet rich plasma) was placed onto ethanol-cleaned glass coverslips (Superior, Bad Mergentheim, FRG). Platelets were allowed to settle and spread for 30–40 min in a moist chamber at room temperature. Platelets not attached were removed by rinsing the coverslips twice with PBS. In some cases, platelet rich plasma was diluted 1:20 with HEPES buffer (10 mM HEPES pH 7.4, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose) and coverslips were rinsed twice and incubated for 5 min at 37°C in the same buffer prior to fixation. Spread platelets were fixed and permeabilized by sequential incubation with 3.7% formaldehyde in PBS for 10 min on ice and acetone for 10 min at −20°C, with PBS washings between incubations. Subsequent processing of all platelet samples was then identical to that described above for tissue culture cells.

All specimens were examined with Leitz Aristoplan or Zeiss Axiophot microscopes equipped with epifluorescence optics. Photographs were taken with Tri-X Pan 400 (Kodak).

Immunoblotting

Samples were separated by SDS–PAGE using 3% stacking and 8% separating gels. Proteins were electrophoretically transferred to nitrocellulose (0.45 μm, Schleicher & Schuell, Dassel, FRG) for 1.5–3.0 h at 3–4 V/cm in a buffer containing 25 mM Tris (pH 10), 192 mM glycine and 20% methanol. Nitrocellulose sheets were blocked for 6 h or overnight in blocking medium (PBS, 1% haemoglobin, 0.3% Triton X-100, 0.05% Tween 20, 0.01% NaN_3), incubated for 1.5 h at room temperature with antiserum M4 (1:1500 in blocking medium) or affinity-purified M4 with or without prior preadsorption. For use in immunoblots, affinity-purified M4 and preadsorbed affinity-purified M4 were applied at identical final dilutions (1:120 in buffer A). Antibodies bound to immobilized proteins were detected by incubation with 0.1 μCi/ml [^{125}I]protein A (>30 mCi/mg; Amersham Buchler, Braunschweig, FRG). Following the incubations with antiserum and [^{125}I]protein A, nitrocellulose sheets were washed repeatedly with PBS supplemented with 0.3% Triton X-100 and 0.05% Tween 20. Immunoreactivity was detected by autoradiography of nitrocellulose blots.

Protein purification

VASP was purified as described previously (Halbrügge and Walter, 1989) except that Q-Sepharose FF chromatography was performed at pH 7.5 and the CM-cellulose chromatography step was omitted. Protein concentrations were determined using the Bradford reagent for purified VASP and the Lowry reagent for cell homogenates with BSA as a standard. Actin was purified from porcine skeletal muscle acetone powder essentially as described (Jockusch and Isenberg, 1981). Actin concentration was calculated from the absorbance at 290 nm (Gordon *et al.*, 1976).

Cosedimentation assay

G-Actin (1 mg/ml) was allowed to polymerize for 30 min at room temperature by the addition of 2 mM $MgCl_2$ and 20 mM KCl (final concentrations). VASP was centrifuged at 195 000 *g* for 3 h at 4° C in order to remove insoluble particles. After a 30 min co-incubation of F-actin and VASP at room temperature (500 μg/ml actin, 110 μg/ml VASP) in an imidazole buffer (5 mM imidazole, 1 mM Tris–HCl [pH 7.2]; 60 mM KCl; 2 mM $MgCl_2$; 0.1 mM $CaCl_2$; 1.25 mM EGTA; 0.35 mM ATP; 0.5 mM DTE), the assay mixture was centrifuged at 100 000 *g* for 2 h at room temperature in Ultra-Clear tubes (Beckman, Frankfurt, FRG) coated with 5% BSA. Control sedimentations were performed in which either VASP or actin were omitted. Supernatants were carefully aspirated, pellets were resuspended in the original assay volume, and equal aliquots of both the supernatants and resuspended pellets were then analysed by SDS–PAGE.

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