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The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function

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The vasodilator-stimulated phosphoprotein (VASP) is associated with actin filaments and focal adhesions, which form the interface between the cytoskeleton and the extracellular matrix. VASP is phosphorylated by both the cAMP- and cGMP-dependent protein kinases in a variety of cells, including platelets and smooth muscle cells. Since both the cAMP and cGMP signalling cascades relax smooth muscle and inhibit platelet activation, it was speculated that VASP mediates these effects by modulating actin filament dynamics and integrin activation. To study the physiological relevance of VASP in these processes, we inactivated the VASP gene in mice. Adult VASP-deficient mice had normal agonist-induced contraction, and normal cAMP- and cGMP-dependent relaxation of intestinal and vascular smooth muscle. In contrast, cAMP- and cGMP-mediated inhibition of platelet aggregation was significantly reduced in the absence of VASP. Other cAMP- and cGMP-dependent effects in platelets, such as inhibition of agonist-induced increases in cytosolic calcium concentrations and granule secretion, were not dependent on the presence of VASP. Our data show that two different cyclic, nucleotide-dependent mechanisms are operating during platelet activation: a VASP-independent mechanism for inhibition of calcium mobilization and granule release and a VASP-dependent mechanism for inhibition of platelet aggregation which may involve regulation of integrin function.

Keywords: Ena/focal adhesion platelet/integrin/smooth muscle/VASP

Introduction

Vasodilator-stimulated phosphoprotein (VASP) belongs to a family of proline-rich proteins that include the *Drosophila* protein Enabled (Ena), its mammalian orthologue Mena, and the Ena-Vasp like protein Evl (Gertler *et al*., 1996). These proteins are composed of a central proline-rich domain, and N- and C-terminal domains which are highly homologous and named Ena–VASP homology domains 1 and 2 (EVH1 and EVH2; Gertler *et al*., 1996). VASP was identified originally as a substrate of cAMP- and cGMP-dependent kinases (cAK and cGK, respectively; Halbrügge *et al*., 1990), which are major receptors for the cyclic nucleotides cAMP and cGMP in various mammalian cells (Lincoln and Corbin, 1992; Francis and Corbin, 1994). The production of cyclic nucleotides is stimulated by vasodilators such as nitric oxide (NO) and prostacyclin, and leads to smooth muscle relaxation and inhibition of platelet activation. An important but unanswered question is whether or not phosphorylation of VASP is essential for the biological responses of the cyclic nucleotide signalling cascades.

VASP was isolated initially from human platelets but is also expressed in a wide variety of other cells and tissues (Reinhard *et al*., 1992). In various cell types, VASP is concentrated along highly dynamic filamentous membrane structures, in focal adhesions and cell–cell contacts. A similar subcellular localization was also shown for Mena (Gertler *et al*., 1996). Evidence for a potential role for VASP and Mena in controlling microfilament assembly comes from observations that demonstrate that both proteins bind profilin and the surface protein ActA of the intracellular pathogen *Listeria monocytogenes* (Chakraborty *et al*., 1995; Gertler *et al*., 1996). Profilin is an actin-monomer sequestering protein that can also promote the polymerization of F-actin under some conditions (Pantaloni and Carlier, 1993). It is also found on one pole of intracellularly motile *Listeria*, where actin polymerization is induced and organized into an actin tail (Theriot *et al*., 1994). It was postulated that VASP and Mena are essential for connecting the surface-bound listerial polypeptide ActA with profilin, which enables *Listeria* to move through the cytoplasm ahead of the leading edge of the growing actin filaments (Charaborty *et al*., 1995; Reinhard *et al*., 1995; Gertler *et al*., 1996; Cossart and Lecuit, 1998).

Zyxin and vinculin contain ActA-related motifs, and are responsible for recruiting VASP and Mena to focal adhesions (Brindle *et al*., 1996; Gertler *et al*., 1996; Reinhard *et al*., 1996), which are subcellular structures that link the actin cytoskeleton to the extracellular matrix and transduce bidirectional signals (Burridge and Chranowska-Wodnicka, 1996). The major transmembrane components of focal adhesions are integrins, a large family

of adhesion molecules that bind both extracellular matrix proteins and counter receptors (Hynes, 1992). The cytoplasmic domain of integrins can bind to several components of focal adhesions, including structural proteins such as talin and α-actinin, linker proteins such as shc, and kinases such as focal adhesion kinase (FAK) and integrin-linked kinase (ILK). The presence of VASP and Mena in focal adhesions suggests that VASP, and possibly also Mena, could modulate integrin activity.

When platelets are activated with agonists such as collagen, collagen-related peptides (CRPs), prostaglandins or ADP, integrin αIIbβ3 switches from a resting to an activated conformation inducing the binding to fibrinogen. Fibrinogen-binding is important for crosslinking platelets at sites of vascular injury and promotes the aggregation of platelets and clot formation. The activation mechanism of αIIbβ3, which is also called 'inside-out' signalling, has been extensively studied but is not fully understood. Although agonist-induced activation of platelets occurs via several signal transduction pathways, the final activation occurs most probably through altered interactions between the cytoplasmic domain of αIIbβ3 and components of the focal contacts. Experimental evidence suggests that the membrane proximal region of both the α and β subunit of αIIbβ3 have a crucial role for modulating the conformational changes leading to activation or inactivation of the fibrinogen receptor. The presence of VASP in focal contacts, its ability to bind vinculin and zyxin, and the fact that phosphorylation of VASP in platelets correlates with inhibition of fibrinogen binding to the fibrinogen receptor, αIIbβ3 integrin (Horstrup *et al*., 1994), suggests a role for VASP in regulating fibrinogen receptor activation.

To assess directly the functional significance of VASP *in vivo* and *in vitro*, we inactivated the VASP gene in embryonic stem (ES) cells and generated VASP-deficient mice. Here we report that VASP-null mice are viable and fertile, and have no physical abnormalities. Agonist-induced smooth muscle contractility and cyclic nucleotide-dependent smooth muscle relaxation were not affected in intestinal and vascular explants of VASP-null mice, showing that VASP is not essential in the regulation of the smooth muscle tone. However, *in vitro* analysis of VASP-deficient platelets revealed that the cAMP- and cGMP-mediated inhibition of platelet aggregation was significantly reduced. In contrast, the cAMP- and cGMP-mediated inhibition of intracellular Ca^{2+} release and granule secretion was unaffected and is therefore a VASP-independent effect.

Results

Targeted disruption of the VASP gene

The structure of the mouse VASP gene is shown in Figure 1A. Exon 2 begins with the third nucleotide of the second amino acid and is 172 nucleotides in length. To obtain a targeted disruption of the VASP gene, we used a gene trap vector with a *geo* DNA (Figure 1A; see Materials and methods) inserted in-frame into exon 2. The *geo* cassette is flanked by 3 kb genomic DNA containing part of intron 1 and 2.8 kb genomic DNA containing part of exon 13 and additional 3′ genomic DNA, respectively. Upon homologous recombination, this knockout vector replaces almost the entire VASP gene (exons 2–13). Out of 13 ES cell clones that survived G418 selection, two targeted clones were identified by Southern blot analysis of *Bam*HI-digested genomic DNA (data not shown).

Both clones were used to generate chimeric males which transmitted the mutant allele to their progeny. Mice heterozygous for the mutation in the VASP gene appeared normal and were indistinguishable from their wild-type litter mates. Southern blot genotyping (Figure 1B) of 305 offspring from heterozygous intercrosses showed that 24.9% were wild-type, 48.3% were heterozygous and 26.8% were homozygous for the VASP mutation. The Mendelian transmission of the VASP-null mutation to offspring of heterozygous crosses indicates that the absence of VASP has no loss of essential function during development.

To confirm that the mutation leads to a loss of VASP function, the expression of mRNA and protein was examined. Northern blot analysis of RNA derived from several tissues, including duodenum, brain, kidney, heart, lung, liver, spleen, intestine, thymus and aorta (Figures 1C and 2A; data not shown), showed a reduced level of VASP mRNA in heterozygous individuals and a complete absence in homozygous mice.

Western blot analysis of platelets, liver and duodenum using anti-VASP polyclonal and monoclonal antibodies revealed that both phosphorylated (50 kDa) and dephosphorylated (46 kDa) VASP protein were absent from cells and tissues derived from homozygous mutant mice (Figure 1D). The same tissue blot was reprobed with an anti-cGKI antibody, to test whether cGKI which can bind to and phosphorylate VASP at specific serine/threonine residues has altered expression levels in the mutants. Figure 1D demonstrates that the absence of VASP had no effect on cGKI expression.

Analysis of VASP, Mena and Evl expression in VASP-null mice

Adult mice lacking VASP have no apparent alteration of phenotype, are fertile and have a normal life span. Since VASP is highly expressed in platelets, platelet counts and bleeding time were evaluated in normal and VASP-null mice. Wild-type mice had $3.82\times10^5 \pm 1\times10^5$ platelets/μl blood ($n = 5$) and a bleeding time of 283 ± 141 s ($n = 10$). VASP-null mice had $4.49\times10^5 \pm 0.5\times10^5$ platelets/μl blood ($n = 5$) and a bleeding time of 313 ± 123 s ($n = 12$). Clearly, the absence of VASP changed neither platelet counts nor *in vivo* bleeding time.

Next, we tested whether the absence of VASP changes the expression of Mena and Evl. Northern and immunoblot assays were performed with tissues derived from normal and VASP-null mice, respectively. Figure 2A demonstrates that the steady-state level of Mena and Evl mRNA is unaltered in VASP-null tissues. Western blot analyses revealed normal expression of Mena and Evl in brain or platelets derived from VASP-null mice (Figure 2B).

To exclude morphological abnormalities in organs of VASP-null mice, we performed histological analyses of various tissues including brain, kidney, heart, lung, liver, spleen, intestine and thymus. None of the organs tested showed obvious abnormalities (not shown). Since the VASP gene was replaced by the β-galactosidase gene in VASP mutant mice, we utilized lacZ staining to monitor VASP expression in tissues. Analysis of lacZ staining in

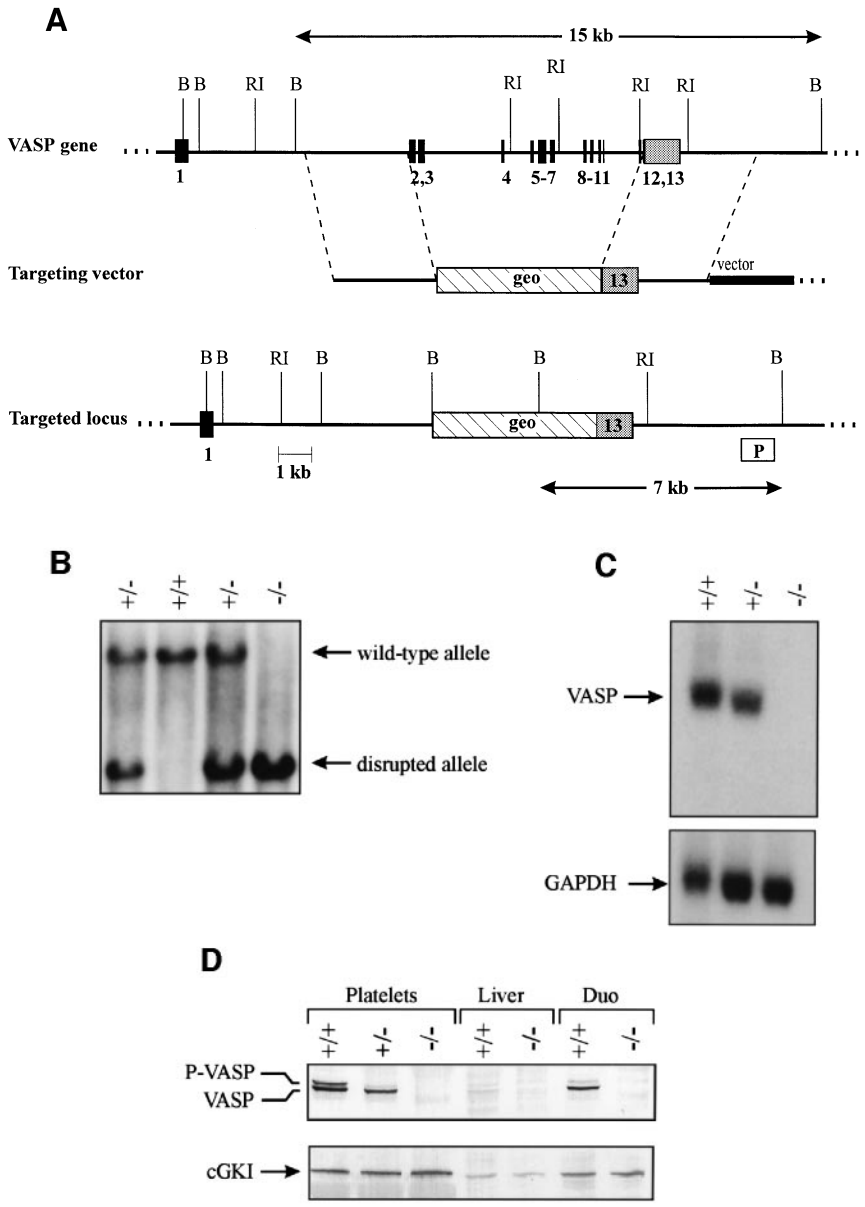

Fig. 1. Targeted inactivation of VASP. (**A**) The murine VASP gene (top) consists of 13 exons (black boxes) with exon 13 containing 3′ UTR sequences (shaded box). The targeting vector (middle) contains 5.8 kb of genomic DNA flanking the *geo* cassette. After homologous recombination (bottom), the *geo* cassette replaces the complete coding sequence of the VASP gene. Lines represent intronic sequences, P denotes the probe used for Southern blot analyses, which detects a 15 and 7 kb fragment in the wild-type and knockout allele, respectively. B, *Bam*HI; R, *Eco*RI. (**B**) Southern blot analysis of mouse tail DNA derived from a progeny of heterozygous breeding, following digestion with *Bam*HI and hybridization with probe P. (**C**) Northern blot analysis of total RNA isolated from the duodenum of $VASP^{+/+}$, $VASP^{+/-}$ and $VASP^{-/-}$ mice. The blot was hybridized with a mouse VASP cDNA probe (top). To verify equal loading, the same blot was probed with a mouse GAPDH cDNA probe (bottom). (**D**) Immunoblot analysis of VASP (top) and cGMP-dependent protein kinase I (cGKI) expression in platelets, liver, and duodenum (duo) from wild-type (+/+), and heterozygous (+/–) and homozygous (–/–) mutant mice. Polyclonal antibodies raised against VASP and cGKI were used. The VASP antibody detects the 50 kDa phosphorylated (P-VASP) and 46 kDa dephosphorylated VASP protein.

heterozygous and homozygous mutant mice revealed a wide distribution of VASP. Tissues derived from normal animals served as controls (Figure 3A, C and E). LacZ-positive cells were detected in brain (Figure 3B), long bones (Figure 3D), heart (Figure 3F), spleen, liver, thymus and lung (not shown). Interestingly, the pyramidal cell layer in the hippocampus showed high lacZ activity, while the cells in the dentate gyrus were apparently negative (Figure 3B). In developing long bones, high level of lacZ activity was detected in osteocytes at the area of trabecular bone formation, whereas the cartilaginous regions lacked lacZ-positive cells (Figure 3D).

Cyclic nucleotide-induced relaxation of smooth muscle is not impaired in VASP-null mice

Western and Northern blot analyses clearly showed VASP expression in murine smooth muscle organs (Figures 1C, D and 2A), and lacZ assays demonstrated lacZ-positive smooth muscle cells in VASP mutant mice, in agreement with recent studies showing VASP expression in rat vascular smooth muscle cells (Markert *et al*., 1996; Mönks *et al*., 1998). To test whether VASP is an important component in the regulation of the smooth muscle tone, we isolated smooth muscle strips of the gastric fundus, and aortic rings from wild-type and VASP-null mice, and

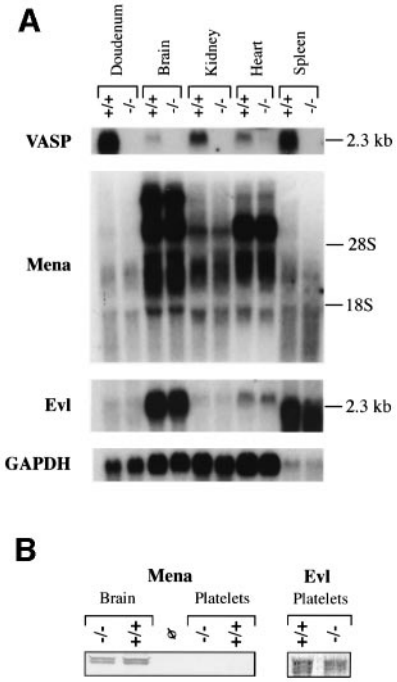

Fig. 2. Expression of VASP, Mena and Evl in normal and VASP-null tissues. (**A**) Total RNA was isolated from 6-week-old normal (+/+) and VASP-null (–/–) mice and probed with specific cDNA probes as indicated. In contrast to VASP and Evl, Mena is expressed in several splice variants (see also Gertler *et al*., 1996). Normal mice express VASP mRNA in all organs tested. Note that VASP expression is low but clearly visible in brain. In none of the organs derived from VASP-null mice, Mena or Evl is upregulated. (**B**) Immunoblot showing expression of Mena and Evl protein. Mena is highly expressed in brain but is absent from normal platelets. Evl is expressed in normal (+/+) and VASP-null (–/–) platelets. Note that Evl expression is apparently unaltered in VASP-null platelets.

analysed their response to contractant and relaxant agents. Wild-type and VASP$^{-/-}$ fundus strips responded to endothelin-1 with contractions amounting to 7.6 ± 0.8 mN [*N* (number of animals) = 11, *n* (number of strips) = 29] and 8.1 ± 0.8 mN (*N* = 11, *n* = 31), respectively, indicating that agonist-induced contractions of intestinal smooth muscle are normal in the absence of VASP. Electrical field stimulation (EFS; 1–16 Hz) produced similar frequency-dependent relaxations in preparations from both wild-type and VASP-null mice. The relaxant response was blocked by the NOS inhibitor, N$^{\omega}$-nitro-L-arginine (L-NOARG) or the guanylyl cyclase inhibitor, ODQ (data not shown), showing that the EFS-induced relaxations are dependent on the release of NO. To test the relaxant effects of cGMP and cAMP, we used the potent cGMP analogue 8-(4-chlorophenylthio) guanosine-3′,5′-cyclic monophosphate (8-pCPT-cGMP) (Menshikov *et al*., 1993), and the cAMP kinase activator, Sp-5,6-DCL-cBIMPS (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′-cyclic monophosphorothioate, Sp-isomer; Sandberg *et al*., 1991), respectively. At the concentrations used in this study (0.1, 1 and 10 μM), we did not observe significant differences between muscle strips isolated from wild-type and VASP-null mice (Figure 4A). In addition, the relaxant effects of 8-Br-cGMP 10 nM–10 μM or forskolin 1 nM–1 μM were similar in both genotypes (data not shown).

The effect of VASP on vascular smooth muscle contractility was tested in aortic rings isolated from normal and VASP-null mice. Phenylephrine (3 μM) induced a contraction of 2.3 ± 0.2 mN (*N* =11, *n* =33) in the wild-type aortas, which was not significantly different from that induced in the wild-type preparations, 2.4 ± 0.3 mN (*N* =11, *n* = 33). Wild-type and VASP-null aortic rings exhibited similar relaxant responses to 8-pCPT-cGMP and Sp-5,6-DCL-cBIMPS 10, 30 and 100 μM (Figure 4B), acetylcholine 1 nM–2 μM, and to sodium nitroprusside 0.1 mM (data not shown).

These data suggest that VASP is not essential for agonist-induced contraction and vasodilator-induced relaxation of intestinal and vascular smooth muscle under the conditions described above.

VASP modulates cAMP- and cGMP-effects on platelet aggregation

To study the role of VASP in the cAMP- and cGMP-induced inhibition of platelet activation, the effects of cyclic nucleotides on collagen-induced aggregation was analysed in wild-type and VASP-deficient platelets. Addition of 5 μg/ml of collagen aggregated wild-type and VASP-deficient platelets, as measured by recording light transmission in an aggregometer (Figure 5A–D). When collagen is added to the platelets, light transmission abruptly decreases, resulting in an initial transient peak (Figure 5A–D) which is due to the change in platelet shape from disks to spiny spheres (Zucker, 1989). Figure 5 shows that the extent of the shape change was identical in normal and VASP-null platelets but the shape change occurred much earlier in VASP-null platelets. This faster shape change reduced the time necessary for the collagen-induced aggregation of VASP-null platelets when compared with wild-type platelets. Whereas VASP-null platelets reached 50% of the maximum aggregatory response to 5 μg/ml collagen in 89 ± 4 s (*n* = 26), wild-type platelets needed 106 ± 4 s (*n* = 26) which was significantly longer ($p < 0.003$). The extent of aggregation was not significantly different between normal and VASP-null platelets (Figures 5 and 6).

Incubation of wild-type platelets with 10 μM 8-pCPT-cGMP significantly ($p < 0.002$) inhibited aggregation by 77.8% (*n* = 6; Figures 5A and 6A). In contrast, treatment of VASP-null platelets with 10 μM 8-pCPT-cGMP induced only a 21.6% (*n* = 6) reduction of collagen-induced aggregation (Figure 6A); this did not differ significantly from the aggregatory response occurring in the absence of cGMP. Increasing the 8-pCPT-cGMP concentrations to 20 μM (*n* = 4) and 100 μM (*n* = 5), significantly reduced the aggregatory response of wild-type as well as VASP-null platelets (Figures 5B and 6A). Even at 100 μM 8-pCPT-cGMP, VASP-null platelets exhibited a significant reduction of the cGMP-effects as compared with platelets isolated from litter-matched wild-type mice (*n* = 5; $p < 0.05$; Figure 5).When cyclic nucleotides were tested at 20 μM, no significant differences were observed between normal and VASP-null platelets (Figure 6A).

The effects of cAMP-dependent phosporylation of VASP on platelet aggregation was tested by using Sp-5,6-DCL-cBIMPS. The concentration of Sp-5,6-DCL-cBIMPS that induced a significant suppression of aggregation in wild-type platelets was 6 μM. Sp-5,6-DCL-cBIMPS inhibited aggregation by 73.6% in wild-type (*n* = 6) and

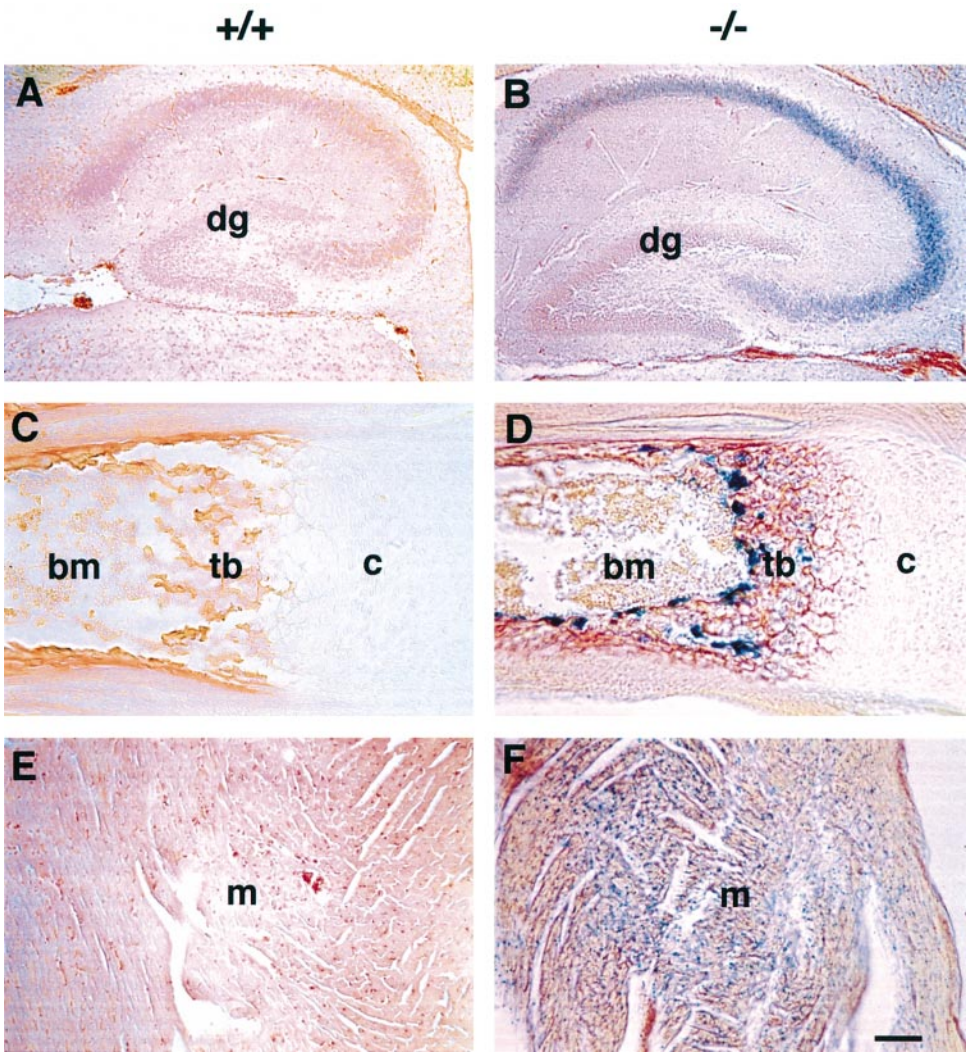

Fig. 3. LacZ expression in tissues of VASP-null mice. Tissue sections from normal (+/+) and VASP-null (–/–) mice were stained for β-galactosidase. High activity of the reporter gene was detected in the pyramidal cell layer of the hippocampus (**B**), in osteocytes of the developing metatarsal bone (**D**) and in cardiac muscle cells (**F**) of VASP-null (–/–) mice. Note that β-galactosidase activity was absent in the dentate gyrus of VASP-null mice. As expected, tissues derived from normal mice contained no β-galactosidase-positive cells (**A**, **C** and **E**). dg, dentate gyrus; c, cartilage; tb, trabecular bone; bm, bone marrow; m, myocardium. Bar represents 200 μm.

30.6% in VASP-null platelets ($n = 6$), respectively (Figures 5C and 6B). At 6 μM and 10 μM the inhibitory effects of Sp-5,6-DCL-cBIMPS were significantly different between the wild-type and mutant platelets ($p < 0.05$; Figure 6B).

These data show that the negative effect of cyclic nucleotides on platelet aggregation is at least partly mediated via VASP.

VASP is involved in αIIbβ3 integrin activation

When platelets are exposed to agonists such as collagen, the fibrinogen receptor αIIbβ3 integrin becomes activated and capable of binding to fibrinogen. This interaction crosslinks platelets and leads to aggregation. To test whether the activation of αIIbβ3 is altered in the absence of VASP, we analysed fibrinogen binding to resting platelets and to collagen-activated platelets that had been pretreated with or without cyclic nucleotide analogues.

Figure 7A shows that neither normal nor VASP-null platelets bound fibrinogen prior to activation. When activation was induced with collagen, VASP-null platelets bound significantly more fibrinogen than wild-type platelets. The difference in fibrinogen binding between normal and VASP-null platelets could be observed at all collagen concentrations tested, but was most pronounced at the high concentration (10 μg/ml) (Figure 7A). Pre-treatment of platelets with 100 μM 8-pCPT-cGMP or 100 μM Sp-5,6-DCL-cBIMPS significantly reduced collagen-induced binding to fibrinogen in normal (Figure 7B and C) but not in VASP-null (Figure 7D and E) platelets.

These data indicate that VASP is involved in the regulation of the inactivation of αIIbβ3 integrin.

VASP is not involved in the regulation of $[Ca^{2+}]_i$ and serotonin secretion

Collagen, thrombin, ADP and other platelet agonists induce a transient increase in intracellular calcium concentration ($[Ca^{2+}]_i$). These calcium transients, which trigger a cascade of biochemical and structural changes, mediate full platelet activation including aggregation and degranulation. The elevation of $[Ca^{2+}]_i$ results from the release of calcium from intracellular stores and from calcium influx through the cell membrane. Activation of the platelet

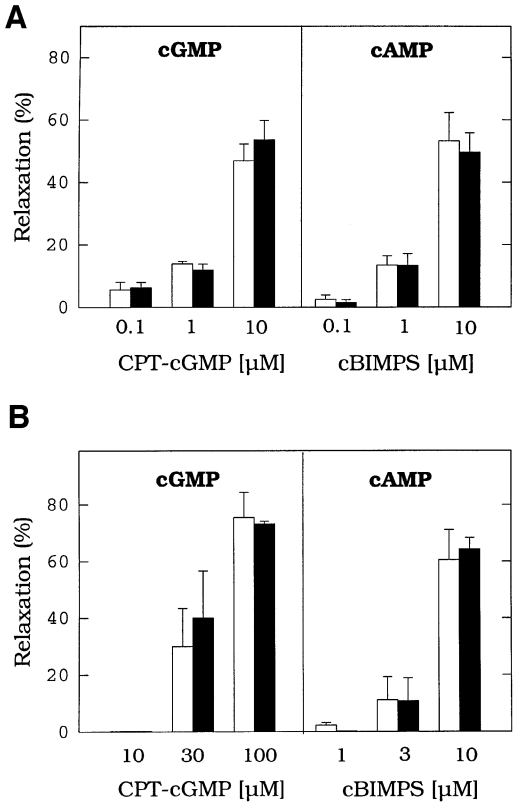

Fig. 4. Cyclic nucleotide-induced smooth muscle relaxation in the presence and absence of VASP (**A**) Relaxant effect of 8-pCPT-cGMP (left, cGMP) and Sp-5,6-DCL-cBIMPS (right, cAMP) on precontracted (10 nM endothelin-1) gastric fundus isolated from normal (white bars) and VASP-null litter-mates (black bars). (**B**) Effect of cyclic nucleotides on normal (white bars) and VASP-null (black bars) aortic rings precontracted with 3 μM phenylephrine. Data are presented as mean ± SEM.

Table I. Cyclic nucleotide-dependent inhibition of collagen- and U46619-induced increases in $[Ca^{2+}]_i$ shown as percentage of control lacking cyclic nucleotide

	8pCPT-cGMP		cBIMPS	
	10 μM	100 μM	6 μM	100 μM
Collagen				
wild-type	3 ± 3	41.1 ± 7.4	4.5 ± 1.7	56.4 ± 4.8
VASP$^{-/-}$	4.6 ± 3.1	39.5 ± 6.1	2.9 ± 1.7	49.9 ± 5
U46619				
wild-type	3.3 ± 1.9	43.6 ± 3.7	4.2 ± 1	50.7 ± 4.7
VASP$^{-/-}$	2.9 ± 1.9	41.2 ± 7.3	5.7 ± 2.2	49.6 ± 6.3

Table II. Inhibition of serotonin secretion shown as percentage of control lacking cyclic nucleotide

	8pCPT-cGMP		cBIMPS	
	10 μM	100 μM	6 μM	100 μM
wild-type	4 ± 4	22 ± 2.6	1.5 ± 1.5	69.2 ± 4.9
VASP$^{-/-}$	8 ± 4.7	33.9 ± 6.2	13.8 ± 9.4	75.9 ± 5

cAMP or cGMP signalling cascades inhibits agonist-induced calcium mobilization (Rink and Sage, 1990). Several studies have shown that cGMP- and cAMP-dependent phosphorylation of VASP correlates well with the inhibition of calcium mobilization in human platelets (Geiger *et al*., 1994; Eigenthaler *et al*., 1993), which has suggested that VASP might be involved in the regulation of $[Ca^{2+}]_i$.

To test this hypothesis, we measured $[Ca^{2+}]_i$ in intact mouse platelets. Wild-type and VASP-null platelets responded to collagen (5 μg/ml) with similar transient increases in $[Ca^{2+}]_i$ of 180 ± 28.8 nM and 205 ± 33.8 nM, respectively. Incubation with 10 μM 8-pCPT-cGMP elicited only a marginal effect on collagen-induced calcium transients in both wild-type and VASP-null platelets that was not significantly different between the two genotypes (Table I). After preincubation with 100 μM 8-pCPT-cGMP, the collagen-induced increase in $[Ca^{2+}]_i$ was reduced to 111 ± 30 nM in the wild-type platelets, and to 128 ± 31 nM in the mutant platelets (Table I). Also, the cAMP-dependent inhibition of calcium mobilization at 6 μM and 100 μM cBIMPS was not affected by the loss of VASP (Table I). Similarly, the effects of high and low cyclic nucleotide concentrations on $[Ca^{2+}]_i$ elevation induced by the thromboxane A2 mimetic U46619 were apparently identical in normal and VASP-null platelets (Table I).

These data show that although phosphorylation of VASP parallels the cyclic nucleotide-induced inhibition of calcium mobilization, VASP is not involved in the cGMP- or cAMP-dependent inhibition of agonist-induced calcium transients in platelets.

In addition to platelet adhesion and aggregation, granule release is one of the critical events in primary hemostasis. Calcium mobilization and protein kinase C (PKC) activation are generally believed to be prerequisites for granule release by platelets (Siess, 1989). However, $[Ca^{2+}]_i$, protein phosphorylation and granule release exhibit no linear proportionality, at least in human platelets (Dalla Via *et al*., 1996). As a measure of granule release from activated platelets, we analysed collagen-induced serotonin secretion. Although 10 μM 8pCPT-cGMP and 6 μM Sp-5,6-DCL-cBIMPS significantly inhibited wild-type platelet aggregation, these analogue concentrations had only marginal effects on platelet serotonin secretion. However, higher concentrations of both cyclic nucleotide analogues significantly inhibited collagen-induced serotonin secretion (Table II), and the effects of 100, 60 and 30 μM 8-pCPT-cGMP as well as 100, 60 and 30 μM Sp-5,6-DCL-cBIMPS, respectively, did not differ between wild-type and VASP-null platelets (Table II; and data not shown).

Discussion

VASP has no essential function during development

In the present study we have generated mice lacking VASP and analysed the functional consequences in smooth muscle and platelets. VASP-null mice have a normal life span, show normal hemostasis, display no obvious tissue abnormalities and are fertile. VASP-null embryos analysed at various developmental stages displayed no abnormalities

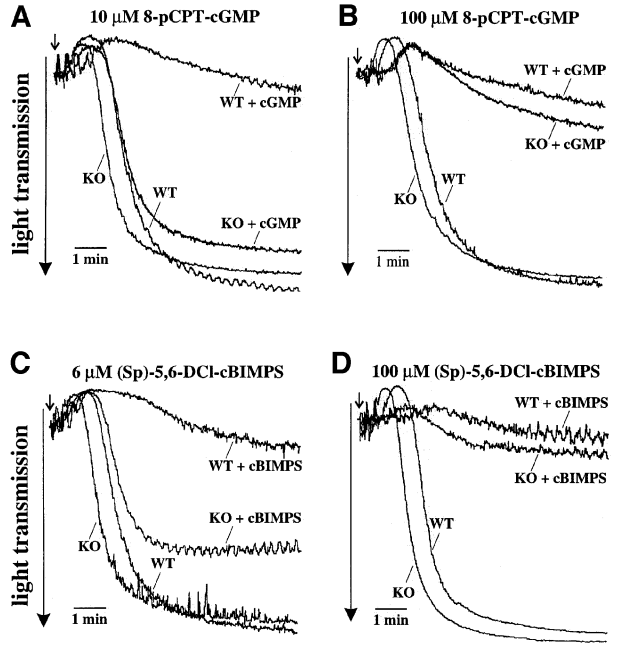

Fig. 5. Collagen-induced aggregation of normal (WT) and VASP-null (KO) platelets in the presence and absence of cyclic nucleotides. (A and B) Effect of 10 μM (**A**) and 100 μM (**B**) 8-pCPT-cGMP on the aggregatory response. (C, D) Effect of 6 μM (**C**) and 100 μM (**D**) Sp-5,6-DCL-cBIMPS on collagen-induced aggregation. Representative recordings of changes in light transmission are shown. Collagen (5 μg/ml) was used to stimulate platelet aggregation. Note that the change in platelet shape demonstrated by the decrease of light transmission occurs earlier in VASP-null platelets. Arrows show the addition of collagen.

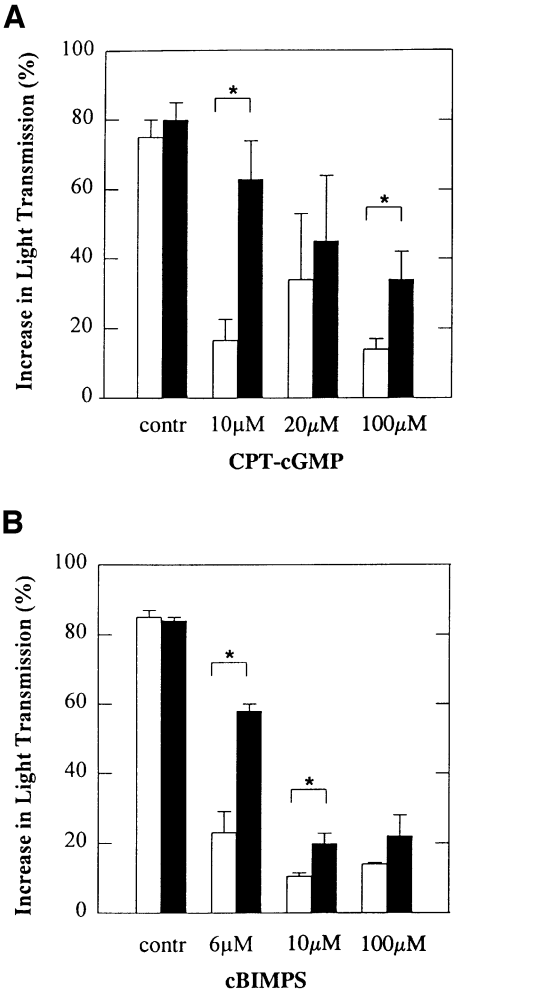

Fig. 6. Effect of cyclic nucleotides on collagen-induced aggregation of normal (white bars) and VASP-null (black bars) platelets. (**A**) Aggregatory response to 5 μg/ml collagen in the absence (control) and presence of 8-pCPT-cGMP (cGMP). (**B**) Sp-5,6-DCL-cBIMPS (cAMP)-induced inhibition of aggregation as compared to control (control). Data are shown in mean ± SEM of four experiments. Asterisks indicate statistical difference ($p < 0.05$). Data represent the extent of aggregation 20 min after addition of collagen.

and we found no indications of any defects resulting from abnormalities in cell migration. We also isolated fibroblasts from VASP-null mice and did not find obvious abnormalities in adhesion or migration (not shown).

Considering results of previous studies that suggest VASP plays an important role in microfilament dynamics (Reinhard *et al*., 1995), cell migration (Reinhard *et al*., 1992; Markert *et al*., 1996; Mönks *et al*., 1998) and signalling of cGMP- and cAMP-dependent kinases (Halbrügge *et al*., 1990), we were surprised that in our study the lack of VASP affected neither development nor postnatal life. A likely explanation for this unexpected finding is that the absence of VASP function may be substituted by other members of the Ena/VASP family of proline-rich proteins. Currently, this family includes Mena (Gertler *et al*., 1996), the mouse orthologue of *Drosophila* Ena (Gertler *et al*., 1990, 1995), and Evl (Gertler *et al*., 1996). All three proteins share the same domain structure composed of homologous N- and C-terminal domains (termed EVH1 and EVH2, respectively) which are separated by a proline-rich central domain. Beside structural similarities, VASP and Mena seem to have similar functions and properties. First, all known proteins which bind VASP also bind Mena (Gertler *et al*., 1996). The proline-rich central domain of both proteins can bind to profilin. The EVH domains of VASP as well as Mena bind to the proline-rich motifs of zyxin, vinculin and ActA (Brindle *et al*., 1996; Gertler *et al*, 1996; Reinhard *et al*., 1996; Niebuhr *et al*., 1997). Secondly, both have a similar subcellular localization in focal contacts and along actin microfilaments. These similarities together with the unaltered expression of Mena and Evl in tissues derived from VASP-null mice suggest that these proteins can substitute each other. Support for redundancy rather than compensation within the Ena/VASP family comes from expression studies in our VASP-null mice, which revealed that in all tissues analysed the expression of neither Mena nor Evl is changed. Finally, the Ena mutation in *Drosophila* can be fully rescued by either human VASP (Ahern-Djamali *et al*., 1998) or murine Mena (F.Gertler, personal communication). Gene targeting will give a clear answer. First, intercrosses with mice carrying null mutations in VASP, Mena and Evl will allow to address the question of redundancy; such experiments are in progress. Secondly, the establishment of mice with null mutations in genes encoding other target proteins of cGKI and cAK than VASP will allow us to address their role in regulating smooth muscle function and calcium transients.

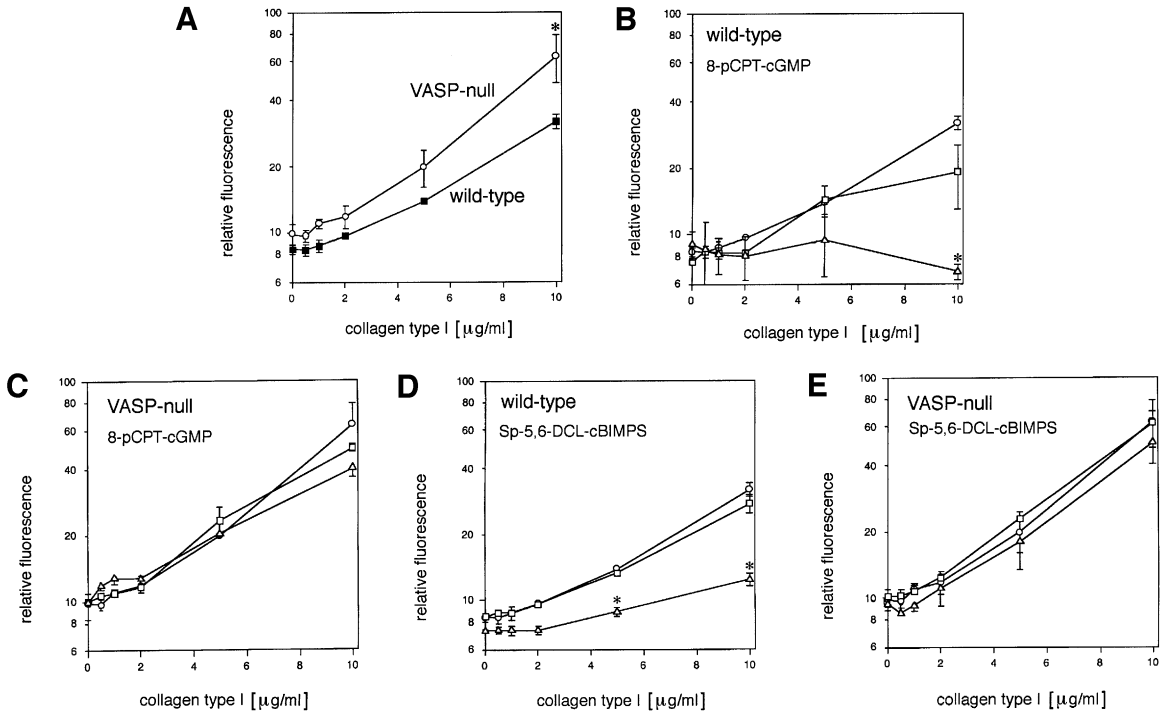

Fig. 7. Binding of fibrinogen to normal and VASP-null platelets measured by flow cytometry using FITC-labelled fibrinogen. (**A**) VASP-null platelets (○) activated with collagen bind significantly (marked with *) more fibrinogen than wild-type platelets (■). (B–D) Pretreatment of platelets with 100 μM 8-pCPT-cGMP (**B**, **C**) or 100 μM Sp-5,6-DCL-cBIMPS (**D**, **E**) reduced collagen-induced fibrinogen binding of wild-type platelets (B, D) but not VASP-null platelets (C, E). Data shown are mean ± SEM of three experiments. Asterisks indicate statistical difference ($p < 0.05$). Symbols in B–D: ○, no cyclic nucleotide treatment; □, 1 μM 8-pCPT-cGMP or Sp-5,6-DCL-cBIMPS treatment; △, 100 μM 8-pCPT-cGMP or Sp-5,6-DCL-cBIMPS treatment.

Regulation of smooth muscle function and Ca^{2+} transients is normal in the absence of VASP

The identification of VASP resulted from a search for proteins which are phosphorylated upon treatment of platelets with cyclic nucleotide-elevating vasodilators. Vasodilators that elevate either cGMP (endothelium-derived relaxing factor, organic nitrates, sodium nitroprusside) or cAMP (prostacyclin, prostaglandin E2, forskolin) inhibit the contraction of smooth muscle cells and the aggregation of platelets mainly through cGKI- or cAK-mediated phosphorylation of specific proteins such as VASP, IP3 receptor, phospholamban, rapB1, etc. (see Siess, 1989). The tight association of VASP phosphorylation and kinase-mediated events at the molecular and cellular level led to the hypothesis that VASP may transmit the cyclic nucleotide-mediated inhibitory signals. We have shown recently that ablation of the cGKI gene profoundly interferes with NO/cGMP-dependent smooth muscle cell relaxation *in vivo* and *in vitro* (Pfeifer *et al.*, 1998). Smooth muscle strips derived from cGKI-null mice do not respond to NO/cGMP, and $[Ca^{2+}]_i$ is not changed by the addition of cGMP in cGKI-null cells. In addition, cGMP-induced phosphorylation of VASP is suppressed in cGKI-null platelets (S.Massberg, M.Sausbier, P.Klatt, M.Bauer, A.Pfeifer, W.Siess, F.Krombach, R.Fässler, P.Ruth and F.Hofmann, manuscript submitted). The lack of selective inhibitors for VASP function and/or phosphorylation makes it difficult to test a direct role of VASP in regulating smooth muscle function and vasculature tone. Our VASP-deficient mouse is, therefore, an ideal model to test proposed functions of VASP in tissue explants and cells such as platelets. We isolated tissue strips from stomach, aorta and penile erectile tissue (not shown) of normal and VASP-null mice, treated them with various concentrations of cGMP or cAMP analogues and recorded the smooth muscle tone. The results are unambiguous and show that smooth muscle contractility is independent of VASP. Tissue strips derived either from normal or VASP-null mice contracted with similar rate when treated with agonists (endothelin, phenylephrine) and relaxed identically when they were pre-treated with cGMP or cAMP analogues.

The analysis of $[Ca^{2+}]_i$ in intact murine platelets using collagen as well as U46619, a thromboxane receptor agonist, clearly showed that the agonist-induced elevation of $[Ca^{2+}]_i$ in platelets could be inhibited to the same extent in normal and in VASP-null platelets by cGMP and cAMP. These data exclude VASP as a candidate protein involved in the cyclic nucleotide-mediated regulation of Ca^{2+} transients and are consistent with an earlier observation showing that human cGKIβ overexpression increases phosphorylation of VASP without altering thrombin-evoked calcium response in human embryonic kidney 293 cells and Swiss mouse 3T6 fibroblasts (Meinecke *et al.*, 1994). Furthermore, these results are in agreement with the view that other proteins mediate the vasodilator-induced effects. Several proteins are phosphorylated by cAK or cGKI, including the IP3 receptor and phospholamban, which regulate $[Ca^{2+}]_i$ that is crucial for the initiation of contraction (Lincoln and Cornwell, 1993; Francis and Corbin, 1994).

An alternative explanation for the normal smooth muscle function in the absence of VASP could still be the presence of Mena and/or Evl. Smooth muscle tissues such as aorta,

intestine and uterus express high levels of different Mena splice variants (data not shown). In platelets, however, Mena was not detected and hence cannot substitute for the lack of VASP in regulating calcium transients. We found expression of Evl in normal and VASP-null platelets. It remains to be shown, however, whether Evl and Mena can be phosphorylated by cGKI or cAK.

VASP is involved in cyclic nucleotide-induced inhibition of platelet aggregation

Platelet activation is characterized by a cascade of events beginning with adhesion and spreading to matrix proteins, the formation of calcium transients, granule release and aggregation. Cyclic nucleotides activate cyclic nucleotide-dependent kinases (cAK and cGKI) which inhibit all steps of this cascade and simultaneously phosphorylate a number of proteins including VASP. Therefore, it has been speculated that VASP may execute at least some inhibitory effects of the cyclic nucleotide-regulated kinases.

In order to test this hypothesis in platelets derived from VASP-null mice we chose the collagen-induced platelet aggregation assay, which most closely resembles the *in vivo* situation where platelets are exposed to a subendothelial, collagen-rich matrix upon vessel damage. Addition of collagen to platelets leads to $\alpha 2\beta 1$ integrin-mediated adhesion and spreading, and activation of GPVI, a collagen binding molecule on the platelet surface which is crucial for initiating activation (Sixma *et al*., 1997; Kehrel *et al*., 1998). Our aggregation studies of normal and VASP-deficient platelets revealed three major results: first, VASP-null platelets aggregate significantly more quickly upon stimulation with collagen. This accompanies a more rapid change in platelet shape which suggests that the collagen receptor is more easily activated and/or the microfilament dynamics is more active when VASP is lacking. Secondly, suppression of platelet aggregation by cyclic nucleotides which activate cGKI or cAK, respectively, is significantly less pronounced in the absence of VASP. Thirdly, suppression of calcium transients and serotonin secretion by cyclic nucleotides is not affected by the lack of VASP. These data show unambiguously that VASP modulates collagen-induced aggregation but not calcium transients and granule secretion during platelet activation. We have recently generated mice lacking cGKI. Platelets from cGKI-null mice are unresponsive to cGMP and exhibit a complete loss of the cGMP-dependent inhibition of agonist-induced aggregation, granule release and calcium mobilization (S.Massberg, M.Sausbier, P.Klatt, M.Bauer, A.Pfeifer, W.Siess, F.Krombach, R.Fässler, P.Ruth and F.Hofmann, manuscript submitted), corroborating the central role of the NO/cGMP signalling cascade in platelets.

The faster aggregation of VASP-null platelets after collagen treatment is unexpected and may point to a new role of VASP. It has been proposed that VASP functions to enhance the rate of actin polymerization at the surface of motile *Listeria*. This hypothesis, combined with presence of VASP in regions of dynamic actin reorganization, has led to a model in which the normal cellular function of VASP is to promote actin assembly during cell motility and spreading. Interestingly, the results of our gene inactivation analysis suggest that one fundamental role of VASP may be to retard actin assembly. VASP-deficient platelets exhibited a reproducible increase in the kinetics of aggregation, a process which depends upon actin assembly at the cellular leading edge. Removal of VASP also correlated with impaired inhibition of aggregation by cyclic nucleotide elevating agents. These observations are most consistent with a model in which one function of VASP is to inhibit aggregation, and that this inhibition is greatly enhanced by cGKI or cAK phosphorylation of VASP. Therefore, depending on the cellular context, it is possible that VASP and its relatives may function either to impede or promote actin assembly. Further work will be required to understand how this family of proteins modulates actin dynamics.

Interestingly, while lack of cGKI in platelets results in complete unresponsiveness to cGMP analogues, lack of VASP only interferes with inhibition of aggregation by low concentrations of cGMP/cAMP. This clearly shows that cGMP-dependent regulation of calcium transients and granule release is VASP-independent whereas modulation of aggregation by cGMP/cGKI involves VASP. The different effects of cAMP and cGMP analogue concentrations on collagen-induced platelet aggregation in wild-type and VASP-null platelets were most obvious at low concentrations, while high concentrations of cAMP and cGMP analogues inhibited aggregation with almost the same efficiency in the absence and presence of VASP. These results suggest that VASP is one of the first targets of the cyclic nucleotide signals and only after reaching higher cAMP and cGMP concentrations calcium transients and granule release are affected. At such concentrations, cyclic nucleotides perform their inhibitory effects on agonist-induced platelet aggregation via an additional, VASP-independent mechanism(s).

Does VASP modulate integrin activation?

Already when VASP was purified it was noted that >90% of the protein was recovered in the membrane fraction (Halbrügge and Walter, 1989). Immunostaining with specific antibodies for VASP revealed its presence along actin filaments and in focal contacts, which are protein-rich structures that link the extracellular matrix through integrins to the actin cytoskeleton and to signal transduction pathways (Jockusch *et al*., 1995; Yamada and Geiger, 1997). The signal tranduction pathway of focal contacts is operating in two directions: ligand binding to integrins changes the activities of cytoplasmic kinases, GTPases and phospholipases, leading to the activation of various signalling pathways, and is called outside-in signalling. Conversely, the affinity of integrins for ligands can be modulated through a process termed inside-out signalling or integrin activation (Schwartz *et al*., 1995). Experimental evidence suggests that the activation of integrins is due to a conformational change in the receptor. Although the biochemical basis for this change is not clear, recent experiments showed that the disruption of a salt bridge between the membrane proximal portions of the αIIb and the $\beta 3$ cytoplasmic domain of the fibrinogen receptor on platelets leads to activation of the receptor and promotes constitutive outside-in signalling (Hughes *et al*., 1996). Platelets are normally inactive and thus nonadhesive within the circulation, but become activated by appropriate stimuli such as collagen, prostaglandins and ADP.

The correlation of VASP phosphorylation with reduced binding of αIIb$\beta 3$ to fibrinogen in cyclic nucleotide treated

platelets (Horstrup *et al*., 1994) suggested a role for VASP in modulating integrin activation. The increased binding activity of the fibrinogen receptor on activated VASP-null platelets, the cyclic nucleotide-mediated inhibition of fibrinogen binding and platelet aggregation is implicating VASP in the modulation of inside-out signalling of integrins. The molecular mechanism for this VASP function could be that the phosphorylation of VASP alters the interaction with vinculin and zyxin and consequently the linkage between integrins and actin filaments. So far nobody has reported a direct interaction between VASP and the cytoplasmic domain of integrins. Since immunolocalization of vinculin, talin and other focal contact proteins was normal in fibroblasts derived from VASP-null mice (data not shown) the absence of VASP may lead to subtle changes which escape detection by immunostaining. The availability of VASP-null mice, however, will allow us to investigate VASP-deficient focal contacts and their interactions with integrins at the morphological, biochemical and cell biological level.

Materials and methods

Generation of VASP-null mice

A cDNA encompassing the coding sequence of VASP was generated from an intestinal cDNA by RT–PCR using the primer pair CATGAG-CGAGACGGTCATCTG (forward) and TCAAGGAGAACCCCGCTT-CCTC (reverse). The primers were constructed on the basis of the published mouse VASP cDNA sequence (Zimmer *et al*., 1996). The amplified cDNA fragment was sequenced and used to isolate several overlapping cosmid clones from a genomic library made from D3 ES cells (a gift from J.S.Mudgett, Merck Sharp & Dohme, NJ). Sequence analysis of subcloned fragments derived from one of the cosmid clones confirmed that exon 1 contains 5′ untranslated sequence and the nucleotide codons for the first 2 amino acids, and exon 2 encodes for the following 57 amino acids. Using PCR, a promotorless β-galactosidase neomycine fusion gene (*geo*) lacking the first 2 amino acids and containing the bovine growth hormone poly(A)$^+$ signal was inserted into exon 2 (for cloning details contact reinhard.fassler@pat.lu.se). Upon splicing of exon 1 with exon 2 the geo fusion mRNA will consist of the first 2 amino acids derived from exon 1, and the *geo* which replaces the coding sequence between exon 2 and exon 13. The final targeting vector was composed of 3 kb homologous DNA 5′ of *geo* and 2.8 kb of homologous DNA 3′ of *geo*.

The targeting construct was linearized and electroporated into R1 ES cells (provided by A.Nagy, Mount Sinai Hospital, Toronto, Canada). After selection in G418 individual clones were isolated and analysed by Southern assay as described (Fässler and Meyer, 1995). Two targeted ES cell clones were expanded and injected into blastocysts to generate germline chimeras. Chimeric mice were mated with C57BL/6 females to test for germline transmission or with 129sv females to obtain inbred lines carrying the mutated VASP allele.

RNA isolation, Northern blot analysis and immunoblotting

Total RNA from adult mouse tissue (duodenum, brain, aorta, liver, spleen, heart and kidney) was isolated as described (Chomczynsky and Sacchi, 1987). For Northern blot analysis, 20 μg of total RNA was separated on a 1% agarose/2.2 M formaldehyde gel and transferred to Hybond N+ membrane (Amersham), UV cross-linked and probed in Church buffer. The following random labelled probes were used: mouse VASP cDNA (nt 1284–1621), Mena cDNA (nt 1317–2289), Evl cDNA (nt 558–1791; provided by Frank Gertler, MIT, Cambridge, MA) and mouse cDNA for GAPDH.

Immunoblotting was carried out as described previously (Pfeifer *et al*., 1998). Polyclonal and monoclonal antibodies against VASP (both from Dianova, Hamburg, Germany), cGKI (Pfeifer *et al*., 1998), and Mena (Gertler *et al*., 1996) were used. The analysis of Mena and Evl expression was carried out using polyclonal and monoclonal antibodies, respectively, both of which were obtained from F.Gertler.

Histology and β-galactosidase staining

For histology and histochemistry, 1-week-old and 3-month-old normal, and heterozygous and homozygous VASP mutant mice were killed. Tissues (brain, kidney, hearth, lung, liver, spleen, intestine, thymus and aorta) were isolated and fixed overnight at 4°C in phosphate-buffered saline (PBS, pH 7.2) containing 4% paraformaldehyde, dehydrated in ascending ethanol series, cleared in xylol and embedded in paraffin. Sections (6 μm) were counterstained with hematoxylin and eosin.

For lacZ staining tissues were fixed overnight at 4°C in 0.1 M phosphate buffer (pH 7.3) containing 0.2% glutaraldehyde and 2% paraformaldehyde. After three washes for 30 minutes each in solution A (0.1 M phosphate buffer (pH 7.3) containing 0.2% NP-40, 5 mM EGTA, 2mM MgCl and 0.1% Na-deoxycholate), tissues were stained overnight at 37°C in solution A supplemented with 0.1% X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside; Sigma/Aldrich), 10 mM potassium ferricyanide and 10 mM potassium ferrocyanide. Staining was stopped by briefly rinsing tissues in PBS. After postfixation in 10% buffered formalin, tissues were dehydrated in ethanol, embedded in paraffin and sectioned (20–30 μm). The sections were counterstained with eosin.

Platelet counts and tail bleeding assay

Platelet counts were determined on blood obtained from vena cava inferior of 4-week-old mice by standard techniques. To determine bleeding time, a 1 cm piece of the tail was cut with a razor blade from anesthetized 4-week-old mice and the bleeding was monitored by absorbing the blood onto Kimwipe paper. The endpoint of bleeding was defined as permanent interruption of blood flow for more than 10 s.

Functional smooth muscle studies

Adult normal and VASP-null mice (15–25 g) of both sexes were killed by CO_2 asphyxia and the thoracic aorta and stomach were dissected out. The tissue specimens were placed in an ice-cold Krebs solution (119 mM NaCl, 4.6 mM KCl, 15 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4 and 5.5 mM glucose). The aorta was cut into 1–2 mm ring segments. The stomach was opened along curvatura minor, the mucosa removed by sharp dissection, and 4–5 mm muscle strips were prepared from the gastric fundus. Silk-ligatures were tied at both ends of each strip. The preparations were transferred to thermostatically controlled (37°C) 2.5 (aorta) or 5 (gastric fundus) ml tissue baths containing Krebs' solution for recording of mechanical activity as described (Pfeifer *et al*., 1998).

The aorta rings were repeatedly stretched for 30 min until a stable resting tension of 2–3 mN was obtained. The contractile capacity was examined by adding an isotonic high K^+ Krebs' solution (60 mM KCl, 60 mM NaCl, 15 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4 and 5.5 mM glucose). Relaxations were studied in preparations precontracted by phenylephrine (0.1–3 μM), corresponding to 50–70% of the contraction obtained by high K^+ Krebs' solution.

The fundus strips were stretched to a tension of 4 mN, and thereafter allowed to equilibrate for 30 min, and were then exposed to a Ca^{2+}-free Krebs' solution (119 mM NaCl, 4.6 mM KCl, 15 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 0.1 mM EGTA and 5.5 mM glucose) to define the basal contraction level to which all calculations were related. To study relaxant responses the strips were precontracted by endothelin-1 (0.3–3 μM; Peninsula, Belmont, CA), which induces a stable tension level (Pfeifer *et al*., 1998).

Electrical field stimulation (EFS) was applied by means of platinum wire electrodes as previously described (Pfeifer *et al*., 1998). All experiments were carried out in the presence of indomethacin (1 μM; Dumex, Copenhagen, Denmark), phentolamine (1 μM, Ciba-Geigy/Novartis, Basel, Switzerland), propranolol (1 μM; Sigma/Aldrich) and scopolamine (1 μM; Sigma/Aldrich). Other drugs used were 8-Br-cGMP, forskolin N^{ω}-nitro-L-arginine (Sigma Chemical Company, St Louis, MO), acetylcholine (Sigma/Aldrich) and ODQ (Tocris Cookson Ltd, UK).

Platelet aggregation

Whole blood was collected from normal and VASP-null mice anesthetized with pentobarbital by puncturing the inferior vena cava with heparinized syringes at a final concentration of 25 U heparin/ml blood. The blood from 3–4 VASP-null mice and wild-type littermates was pooled for each platelet aggregation experiment. Blood was diluted with 500 μl of HEPES–Tyrodes-buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCL, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$, pH 7.3), and platelet-rich plasma (PRP) was obtained by centrifugation for 7.5 min at 250 *g*. Thereafter, prostacyclin at a final concentration of 300 nM was added to the PRP, and platelets were pelleted by centrifug-

ation at 1200 *g* for 5 min. The platelet pellet was resuspended in HEPES–Tyrodes-buffer and incubated for 30 min at 37°C. Platelet suspension was adjusted to 300 000 platelets/μl. Optical aggregation experiments were conducted in a four-channel aggregometer (Aggrecorder II PA-3220; Kyoto Daiichi Kagaku, Kyoto, Japan). Preincubation in HEPES–Tyrodes-buffer without and with cGMP analogues (8pCPT-cGMP; Biolog Life Science Institute, Bremen, Germany) or without and with cAMP analogues (Sp-5,6-DCL-cBIMPS; Biolog Life Science Institute) was performed for 20 min at room temperature. Immediately prior to the aggregation experiments, platelets were preincubated for 1 min at 37°C in HEPES–Tyrodes-buffer containing 1 mM $CaCl_2$.

Flow cytometric analysis of fibrinogen-FITC binding to platelets

Diluted PRP (5×10^7 platelets/ml) was treated with or without 1 or 100 mM of 8pCPT-cGMP or Sp-5,6-DCL-cBIMPS, respectively. Afterwards platelets were incubated for 3 min at room temperature with 150 μg/ml fibrinogen-FITC (saturating concentration), activated with 2, 4, 6, 8 or 10 μg/ml bovine collagen type I (Kehrel *et al*., 1993) for 3 min at room temperature, fixed for 30 min with 1% formaldehyde (final concentration) in PBS, washed with PBS and analysed in a flow cytometer as described previously (Kehrel *et al*., 1998).

Determination of cytosolic [Ca^{2+}]

Washed platelets were incubated with 8 μg/ml Fura-2AM dye (Sigma/Aldrich) for 30 min at 37°C in the dark. Platelets were centrifuged and resuspended in Ca^{2+}-free HEPES–Tyrodes-buffer containing 0.1 mM EGTA (for measurements of Ca^{2+} mobilization induced by 10 μg/ml collagen) or supplemented with 1 mM $CaCl_2$ (for measurement of $[Ca^{2+}]_i$ elevation induced by 5 μM U46619). Fluorescence was monitored at 37°C with a LS50B dual wavelength fluorescence spectrophotometer (Perkin-Elmer, Überlingen, Germany). To obtain F_{max} and F_{min}, digitonin (30 μg/ml final concentration) and EGTA (pH 8.4; 10 mM final concentration) were added. Cytosolic [Ca^{2+}] was determined as described (Sage, 1995).

Serotonin secretion

PRP from normal and VASP-null mice was prepared as described above, diluted in HEPES–Tyrodes-buffer (10^8 platelets/ml) and loaded with 2 μCi/ml 5-[^{3}H]hydroxytryptamine binoxalate (NEN, Boston, MA) for 1 h at 37°C. Prostacyclin was added at a final concentration of 300 nM and platelets were pelleted by centrifugation at 750 *g* for 5 min. The pellet was resuspended in HEPES–Tyrodes-buffer (2.5×10^8 platelets/ml) and incubated with different concentrations of 8pCPT-cGMP or Sp-5,6-DCL-cBIMPS (Biolog Life Science Institute), respectively, for 20 min at 37°C. Platelets were then stimulated with 2.5 μg/ml collagen (Helena Laboratories, Beaumont, TX) for 5 min at 37°C. After centrifugation at 1500 *g* for 5 min, the supernatant was counted in a Beckman scintillation counter to determine the amount of secreted 5-[^{3}H]hydroxytryptamine binoxalate.

Statistical evaluation

Statistical results are expressed as mean ± SEM. When statistical differences between two means were determined, an unpaired Student's *t*-test was performed, whereas for multiple comparisons, a one-way analysis of variance (ANOVA) was used. A probability of $p < 0.05$ was regarded as significant. *N* equals the number of animals and *n* the number of smooth muscle strips. Statistical calculations are based on *N*.

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