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# Analysis, purification and properties of a 50 000-dalton membrane-associated phosphoprotein from human platelets

MARIA HALBRÜGGE* and ULRICH WALTER
*Medizinische Universitätsklinik, Labor für Klinische Biochemie, Josef Schneider Strasse 2, 8700 Würzburg (F.R.G.)*

## ABSTRACT

Recently, the development of a monospecific antiserum against a 46 000/50 000-dalton membrane protein from human platelets which was stoichiometrically and reversibly phosphorylated in intact human platelets in response to vasodilators was reported. Using this antiserum, the subcellular distribution and the purification of this vasodilator-stimulated phosphoprotein (VASP) from human platelets has now been analysed. The VASP of human platelets is primarily a membrane-associated protein and can be purified to apparent homogeneity by salt extraction and sequential ion-exchange and dye-ligand chromatography with a purification factor of 1200 and a yield of 13%. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis under reducing and non-reducing conditions indicated that purified monomers of this VASP are linked by interchain disulphide bonding.

## INTRODUCTION

Vasodilators which elevate the intracellular concentration of cGMP (*e.g.*, sodium nitroprusside, endothelium-derived relaxing factor, nitroglycerine) or cAMP (*e.g.*, prostacycline, prostaglandin $E_1$) inhibit the aggregation of platelets by an unknown mechanism [1,2]. In intact human platelets, these cyclic nucleotide-elevating vasodilators stimulate the phosphorylation of certain proteins, which is mediated by the activation of cGMP- and cAMP-dependent protein kinase (cGK and cAK, respectively) [3,4]. A 50 000-dalton membrane protein was identified which was phosphorylated in intact human platelets in response to both cGMP- and cAMP-elevating vasodilators and in platelet membranes by endogenous cGK and cAK [3,4]. Because of the potential importance of this vasodilator-stimulated phoshoprotein (VASP) for the mechanism of action of vasodilators, VASP was recently purified from human platelets by analysing its phosphate incorporation during the purification procedure [5]. VASP was purified as a dephospho-protein with an apparent molecular mass of 46 000 dalton and can be converted to a 50 000-dalton protein by stoichiometric phosphorylation [5]. In addition, a monospecific antiserum was developed which can be used to determine the level of VASP and the phosphorylation of this protein in intact human platelets [6]. Because of the limitation of the phosphate incorporation procedure for the determination of VASP in crude cell fractions [5], we

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now report an immunological analysis of the purification procedure and some additional biochemical properties of VASP.

## EXPERIMENTAL

### *Materials*

Cyclic nucleotides and protein markers were purchased from Boehringer (Mannheim, F.R.G.) and [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol) and [$^{125}$I]protein A (30 mCi/mg) from Amersham Buchler (Braunschweig, F.R.G.). cGK from bovine lung and subunits of cAK from bovine heart were purified as described previously [7]. Q-Sepharose FF and the Mono S HR 5/5 columns were obtained from Pharmacia (Freiburg, F.R.G.), CM-cellulose (CM 52; Whatman) from Kontron (St. Leon Rot, F.R.G.) and Orange A (covalently linked to agarose) from Amicon (Witten, F.R.G.). Nitrocellulose paper was purchased from Schleicher & Schüll (Dassel, F.R.G.) and the Bio-Rad protein assay dye reagent concentrate from Bio-Rad Labs. (Munich, F.R.G.). All other chemicals were of highest purity commercially available.

### *Preparation of homogenates, cytosol and membranes of human platelets*

Human platelets were prepared from freshly obtained blood as described [8] and resuspended in 10 m*M* $NaH_2PO_4$ (pH 7.6) containing 2 m*M* EDTA, 2 m*M* EGTA, 1 m*M* phenylmethylsulphonyl fluoride (PMSF) and 100 U aprotinin/ml. The platelets ($5 \cdot 10^9$/ml) were lysed by freezing in liquid nitrogen and thawing. Cytosol and membrane fragments were separated by centrifugation for 3 h at 100 000 *g* at 4°C. The membrane fragments were resuspended in 20 m*M* $NaH_2PO_4$ (pH 7.6) containing 2 m*M* EDTA, 2 m*M* EGTA, 1 m*M* PMSF and 100 U aprotinin/ml (one fifth of the lysis volume). Homogenates, cytosol and membranes were diluted with half the volume of 200 m*M* Tris–HCl (pH 6.7) containing 15% (v/v) glycerol, 6% (w/v) sodium dodecyl sulphate (SDS), 10% (v/v) $\beta$-mercaptoethanol and a trace of bromophenol blue, immediately boiled and then analysed by Western blotting (Fig. 1).

### *Radioimmunolabelling of proteins on nitrocellulose (Western blot)*

Proteins of platelet extracts, of the pooled fractions after chromatography and purified VASP were separated by 9% SDS–polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose and radioimmunolabelled using a monospecific rabbit antiserum against VASP (diluted 1:800) as described in detail previously [6]. VASP was localized by autoradiography [6] and radioactivity bound to the 46 000- and 50 000-dalton forms was determined by cutting out and measuring the bands in a gamma-counter. Under the conditions used (purified VASP up to 500 ng; platelet homogenate or extract protein up to 200 $\mu$g), the radioactivity bound to VASP was proportional to the amount of purified VASP or total protein analysed.

### *Purification of VASP from platelet membranes*

Purification was performed at 4°C as described in detail previously [5]. Platelet membranes were prepared from $3 \cdot 10^{12}$ platelets and incubated with 250 m*M* NaCl in buffer A [20 m*M* $NaH_2PO_4$ (pH 7.6)–2 m*M* EDTA–2 m*M* EGTA–100 U aprotinin/ml–1 m*M* PMSF) for 30 min. After centrifugation at 100 000 *g* for 1 h, the supernatant was separated and dialysed for 4 h against buffer A (pH 8.0) to remove the

salt and change the pH. The dialysed extract was applied to 30 g of Q-Sepharose FF, pre-equilibrated with buffer A (pH 8.0), stirred for 30 min and the anion exchanger was removed by filtration. The pH of the filtrate was then adjusted to 5.5 by the slow addition of 0.5 *M* HCl with constant stirring. Subsequently the filtrate containing VASP was applied to the cation exchanger CM-cellulose (15 g) that had been pre-equilibrated with buffer A (pH 5.5) and the mixture was stirred for 30 min. Unbound protein was removed by filtration and, after washing, the adsorbent was placed in a column (18 cm × 2.0 $cm^2$). Elution was performed with 0–1 *M* NaCl in 105 ml buffer A (pH 5.5) using a flow-rate of 1 ml/min. VASP was detected by incorporation of $^{32}P$ (see the next section). VASP-containing fractions (*ca.* 24 ml) were pooled and dialysed overnight against buffer A (pH 7.5). The dialysed fractions were applied at a flow-rate of 0.35 ml/min to an Orange A column (8 cm × 1.3 $cm^2$) that had been pre-equilibrated with buffer A (pH 7.5). After washing the gel, elution was performed with a gradient of 0–1 *M* KCl in 70 ml of buffer A (pH 7.5) using a flow-rate of 0.5 ml/min. VASP-containing fractions (*ca.* 35 ml) were dialysed overnight against buffer A (pH 6.3) and subsequently applied to a Mono S HR 5/5 column that had been pre-equilibrated with buffer A (pH 6.3). After washing the column, bound protein was eluted using a gradient of 0–1 *M* NaCl in 48 ml of buffer A (pH 6.3) and a flow-rate of 0.75 ml/min as indicated in Fig. 2. Fractions of 1 ml were collected and the absorbance at 280 nm was recorded. VASP was detected by Coomassie brilliant blue staining and incorporation of $^{32}P$. Fractions 15–32 were concentrated by dialysis against buffer A (pH 7.5) containing 50% glycerol. The concentrated protein was stored at −20°C.

*Phosphorylation experiments with partially purified fractions*

For detection of VASP in the partially purified fractions, 10 μl of the fractions collected after chromatography on CM-cellulose, Orange A and Mono S were phosphorylated using the conditions described elsewhere [9], except that cGK (50 ng) was added to the reaction mixture (total volume 25 μl). Proteins were separated by 9% SDS-PAGE and stained with Coomassie brilliant blue. Incorporation of $^{32}P$ was detected by autoradiography.

*SDS-PAGE under reducing and non-reducing conditions*

Reducing and non-reducing SDS-PAGE were performed as described previously [10]. For non-reducing SDS-PAGE, 200 m*M* Tris–HCl (pH 6.7) containing 15% (v/v) glycerol, 6% (w/v) SDS, a trace of bromphenol blue and no β-mercaptoethanol was added to the purified preparation. Samples were heated for 5 min at 100°C and 3 min at 80°C for reducing and non-reducing SDS-PAGE, respectively. Proteins were separated by 9% SDS-PAGE and stained with Coomasie brilliant blue.

As standards cGK (monomer = 74 000 dalton; dimer = 148 000 dalton), RI (monomer = 47 000 dalton; dimer = 95 000 dalton) and RII (monomer = 56 000 dalton) were analysed [11,12].

*Measurement of protein*

The protein content was measured according to the method described by Bradford [13] using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The recent purification of the 46 000/50 000-dalton VASP from human platelets and the development of a monospecific polyclonal antibody against VASP [5,6] made this study of the subcellular distribution and determination of the purification procedure for VASP possible. Previously, VASP determination was based on the amount of $^{32}P$ incorporated from [$\gamma$-$^{32}P$]ATP into the 50 000-dalton protein catalysed by cGK. However, determination of VASP by this method was difficult and unsatisfactory, especially for crude cellular fractions, for a number of technical reasons, including interference by ATPases, phosphatases and other phosphoproteins with an apparent molecular mass of 50 000 dalton [5]. Further, the phosphorylation procedure could obviously measure only dephospho-VASP. In contrast, a radio-immunolabelling procedure using the antiserum and purified VASP standards was developed which could measure both the dephospho- (46 000 dalton protein) and phospho (50 000-dalton protein) forms of VASP in addition to the phosphorylation of VASP due to the conversion of the 46 000- to the 50 000-dalton protein [6].

With this procedure, the subcellular distribution of VASP in homogenate, membranes (particulate fraction) and cytosol (soluble fraction) prepared from human platelets was measured (Fig. 1). This Western blot analysis demonstrates that VASP of untreated human platelets is primarily present as the dephospho-form (46 000 dalton) and recovered in the membrane fraction (>95%) after cell lysis. A previous study showed that VASP of intact human platelets can be quantitatively converted to the

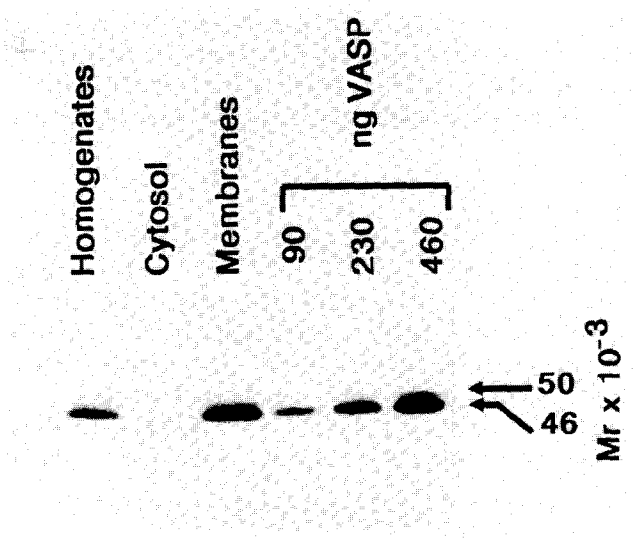


Fig. 1. Autoradiography showing the subcellular distribution of VASP in human platelets determined by an immunological analysis. Homogenates, cytosol and membranes (each 100 $\mu$g of protein) and 90–460 ng of purified VASP (lanes as indicated from left to right) were analysed by Western blotting using a monospecific antiserum against VASP (see Experimental). Arrows indicate the 46 000- and 50 000-dalton forms of VASP. Mr = Molecular mass.

50 000-dalton phospho-form by treatment with cyclic nucleotide-elevating vasodilators [6]. In this study, the radioactivity bound to both the 46 000- and 50 000-dalton protein was used for the determination of VASP (see Experimental). From experiments shown in Fig. 1 using various amounts of purified VASP standards, it could be estimated that platelet homogenates contain 2.45 $\mu$g of VASP per milligram of homogenate protein. This corresponds to an intracellular concentration of about 20 $\mu M$ VASP, assuming an $M_r$ of 46 000 for VASP and a mean platelet volume of 5.2 fl [14]. As more than 95% of platelet VASP was recovered in the membrane fraction (Fig. 1), this fraction was used as the starting material for the purification of VASP. About 65% of this membrane-bound VASP could be extracted with a buffer containing 250 m$M$ NaCl, resulting in a 3.3-fold purification of VASP (Table I).

TABLE I

IMMUNOLOGICAL ANALYSIS OF THE PURIFICATION OF VASP FROM HUMAN PLATELET MEMBRANES

In this experiment, membranes of $3 \cdot 10^{12}$ platelets (corresponding to 10–15 l of human blood) were used. For the determination of specific and total activity of VASP, aliquots of the various purification fractions (membranes, NaCl extract and the pooled fractions obtained after chromatography on Q-Sepharose FF, CM-cellulose and Mono S) were analysed by Western blotting using a monospecific antiserum against VASP and [$^{125}$I]protein A (see Experimental and Fig. 1). VASP activity (determined by the binding of [$^{125}$I]protein A) is indicated here as specific activity (cpm/mg protein) or total activity (cpm) for the pooled fractions of the various purification steps.

| Purification step | Total protein (mg) | Specific activity $\times 10^{-3}$ (cpm/mg) | Total activity $\times 10^{-3}$ (cpm) | Purification (-fold) | Yield (%) |
|---|---|---|---|---|---|
| Membranes | 2268.00 | 233 | 528444 | 0 | 100.0 |
| NaCl extract | 448.00 | 771 | 345408 | 3.3 | 65.4 |
| Q-Sepharose FF | 70.40 | 2607 | 183533 | 11.2 | 34.7 |
| CM-Cellulose | 4.31 | 17659 | 75934 | 75.8 | 14.4 |
| Orange A + Mono S | 0.25 | 278830 | 69708 | 1196.7 | 13.2 |

Solubilized VASP was further purified by adsorbing unwanted proteins of the NaCl extract on the anion exchanger Q-Sepharose FF followed by cation-exchange chromatography on CM-cellulose, resulting in 76-fold purification with a yield of 14% (Table I). Final purification of VASP was obtained by sequential chromatography on the dye-ligand Orange A and the cation exchanger Mono S (Table I). Orange A chromatography was chosen because we found in preliminary experiments that certain phosphoproteins including VASP bind to this dye and that this chromatographic step separated VASP from other proteins. After Orange A chromatography, determination of the specific acitivity and purification factor for VASP was not possible because the VASP-containing fractions were too dilute to allow an accurate protein measurement. However, during the last step of the purification procedure, chromatography on Mono S at pH 6.3, VASP was concentrated and could be eluted as a sharp peak (Fig. 2) at about 120–150 m$M$ NaCl. Analysis of an aliquot obtained from the peak fractions by SDS-PAGE and Coomassie brilliant blue staining showed that VASP was purified primarily as the dephospho-form (46 000-dalton protein) and

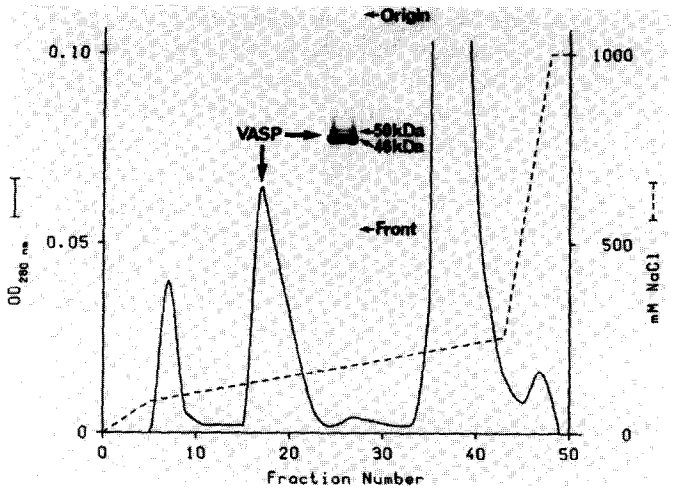


Fig. 2. Mono S chromatography of VASP. VASP was partially purified from human platelet membranes by salt extraction and chromatography on Q-Sepharose FF, CM-cellulose and Orange A. In the final purification step, VASP was chromatographed on a Mono S HR 5/5 column at pH 6.3. Elution was performed with a gradient of 0–1 *M* NaCl in buffer A (dashed line) as described under Experimental. Absorbance at 280 nm was measured (solid line) and 1-ml fractions were collected. Fractions 15–23 were pooled, dialysed and concentrated. Purified VASP (1.5 μg of protein) was analysed by SDS-PAGE and stained with Coomassie brilliant blue (inset). The proteins of this purified preparation migrated primarily as a 46 000-dalton (46 kDa) form and to a minor extent as a 50 000-dalton (50 kDa) form.

to a small extent as the phosho-form (50 000-dalton protein), as demonstrated in the inset in Fig. 2.

Aliquots of the pooled fractions of the various purification steps were then analysed for protein content and for VASP content by Western blotting (Fig. 3). In this experiment, an amount of protein from each purification step was selected that gave a roughly equal signal for VASP in the radioimmunolabelling procedure (Fig. 3). The autoradiogram demonstrates that the purifiation procedure removes some proteolysis products with lower molecular mass than intact VASP and that 0.1 μg of purified VASP gives about the same signal as VASP present in 100 μg of platelet membranes. This suggests that an enrichment of about 1000-fold is required in order to purify VASP from human platelet membranes to apparent homogeneity. This was indeed observed when the entire purification procedure was quantitatively analysed by Western blotting, which indicated a 1200-fold purification of VASP with a yield of 13% (Table I). This contrasts with the purification analysis by the phosphate incorporation method, which required a 200-fold enrichment to obtain VASP of apparent homogeneity with a yield of 3% [5]. The difference between the two methods is probably due to the fact that crude platelet fractions contain 50 000-dalton phosphoproteins in addition to VASP. This results in an overestimation of VASP in crude extracts and subsequent underestimation of the final yield and purification factor with the phosphorylation method. Using the more specific and reliable Western blot method, it can now be estimated that VASP represents about 0.1–0.2% of platelet protein, indicating that VASP is not a rare protein in human platelets. The

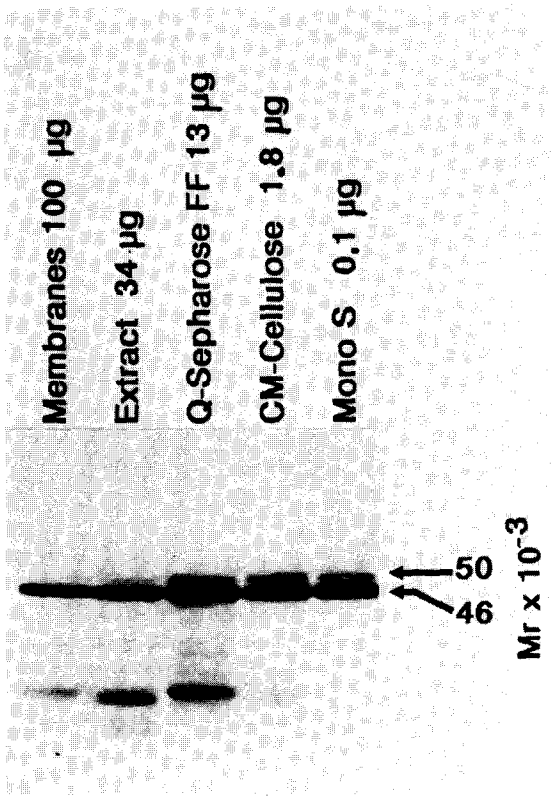


Fig. 3. Autoradiography showing the immunological analysis of VASP purification. Platelet membranes (100 µg of protein), the NaCl extract (34 µg of protein) and the pooled fractions after chromatography on Q-Sepharose FF (13 µg of protein), CM-cellulose (1.8 µg of protein) and Mono S (0.1 µg of protein) were analysed by SDS-PAGE (lanes as indicated from left to right) followed by Western blotting using a monospecific antiserum against VASP. The 46 000- and 50 000-dalton forms of VASP are indicated by arrows.

phosphorylation of VASP in intact platelets by the cytosolic cAK and cGK and the extractability of VASP from platelet membranes by salt strongly suggest that VASP of human platelets is a membrane-associated protein of the cytosolic compartment. Surprisingly, SDS-PAGE of VASP under reducing and non-reducing conditions indicated the presence of at least one interchain disulphide bonding in VASP (Fig. 4).

In the presence of β-mercaptoethanol VASP migrated as 46 000-dalton protein and in its absence 92 000-dalton protein, consistent with the occurrence of a disulphide bond-linked dimer of VASP under non-reducing conditions. This behaviour as a monomer or dimer under these two different experimental conditions was also observed with the regulatory subunit RI of the cAK (monomer 47 000 dalton, dimer 94 000 dalton) and with cGK (monomer 74 000 dalton, dimer 148 000 dalton), two proteins with established interchain disulphide bonding [11,12]. In contrast, the regulatory subunit RII of the cAK, a protein without interchain disulphide bonding [12], migrated as a monomer (56 000 dalton) in the presence and absence of

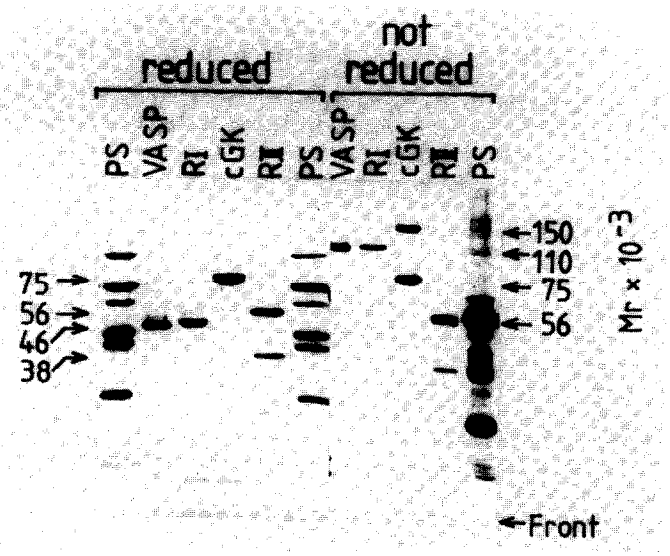


Fig. 4. SDS-PAGE of purified VASP and protein kinases under reducing and non-reducing conditions. Purified preparations of VASP, cAK regulatory subunit type I (RI), cGK and cAK regulatory subunit type II (RII) were heated for 5 min at 100°C in the presence (reduced) and for 3 min at 80°C in the absence (not reduced) of β-mercaptoethanol (see Experimental). The proteins (1.5 μg of each protein) were separated by SDS-PAGE (lanes as indicated from left to right) and stained with Coomassie brilliant blue. Arrows indicate the positions and the molecular masses of the apparent proteins and their subunits. PS = Protein standards.

β-mercaptoethanol (Fig. 4). The 38 000-dalton protein present in purified RII subunit is an RII fragment due to partial proteolysis. Although the occurrence of disulphide bonding is highly unusual for an intracellular protein owing to the high reducing capacity of the intracellular compartment, interchain disulphide bonding has been observed with some cytosolic enzymes such as RI, cGK and some other proteins [11,12]. Whether the interchain disulphide bonding of VASP indeed occurs *in vivo* and whether this is of physiological significance remains to be established. Preliminary gel filtration experiments [15] suggest that partially purified and homogeneous VASP under non-denaturing conditions is significantly larger than a dimer. Therefore, gel filtration and ultracentrifugation analysis of VASP before and after phosphorylation are required in order to determine the size and structure of VASP.

The availability of VASP as purified protein, the development of the specific antiserum and the increasing information about the biochemical properties of VASP should be helpful in elucidating the structure and function of VASP.

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