Endogenous cleavage of the Arg-379–Ala-380 bond in vitronectin results in a distinct conformational change which ‘buries’ Ser-378, its site of phosphorylation by protein kinase A*

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Activation of blood platelets by thrombin was previously shown to specifically release protein kinase A, which in human plasma singles out and phosphorylates one protein, identified as vitronectin. This protein is known to be involved in processes that follow platelet stimulation, specifically, in the binding of heparin (interfering with the heparin-mediated inhibition of thrombin and Factor Xa by antithrombin III), in the growth of endothelial cells and in fibrinolysis. This paper shows that phosphorylation of vitronectin by protein kinase A is stoichiometric (approx. 1 mol/mol), that it is targeted to one site (Ser-378) at the C-terminal edge of the heparin-binding domain, and that it distinguishes between the two physiologically occurring forms of vitronectin: the one-chain (75 kDa) form, and the nicked two-chain (65 + 10 kDa) form, held together by an interchain disulphide bridge. Protein kinase A phosphorylates the one-chain form but not the two-chain form, although Ser-378 and the complete recognition sequence of the kinase are still present in the clipped 65 kDa chain. Cleavage of the Arg-379–Ala-380 bond results therefore in a conformationally distinct form of vitronectin in which Ser-378 is ‘buried’. This is demonstrated by our finding that Ser-378 is present in the 65 kDa chain of clipped vitronectin but inaccessible to phosphorylation at physiological pH. Upon binding heparin, the phosphorylation site becomes exposed and able to undergo a stoichiometric phosphorylation at physiological pH.

INTRODUCTION

The concerted phosphorylation of proteins by cyclic AMP-dependent protein kinase (PKA) plays a key role in the intracellular implementation of hormonal stimuli mediated by the second messenger cyclic AMP (Sutherland & Rall, 1960; Walsh *et al.*, 1968; Cohen, 1988). We have recently provided evidence suggesting that, in addition to its well-established functions within the cell, PKA may also have physiological extracellular functions (Korc-Grodzicki *et al.*, 1988a,b). This was based on our finding that, upon stimulation of rabbit platelets with thrombin (one of their physiological agonists), they specifically release PKA (along with its co-substrate ATP), which selectively phosphorylates one plasma protein (Korc-Grodzicki *et al.*, 1988a). Subsequently we showed that human platelets similarly release PKA upon thrombin stimulation, and that in this case also PKA singles out and phosphorylates one of the numerous proteins in human plasma. The protein specifically phosphorylated in human plasma was immunochemically and biochemically identified as vitronectin (Korc-Grodzicki *et al.*, 1988b).

Vitronectin (also known as S-protein, serum spreading factor and epibolin) is a rather abundant glycoprotein in human plasma and serum (100–500 µg/ml) (Podack & Müller-Eberhard, 1979; Hayman *et al.*, 1983; Barnes *et al.*, 1983; Shaffer *et al.*, 1984). It promotes cell adhesion and spreading (Barnes & Silnutzer, 1983; Hayman *et al.*, 1985; Silnutzer & Barnes, 1985; Yatohgo *et al.*, 1988), binds to activated platelets (Thiagarajan & Kelly, 1988) and binds to endothelial cells in suspension (Preissner *et al.*, 1988). These processes can be inhibited by peptides containing an Arg-Gly-Asp sequence, suggesting that the binding takes place through integrin cell receptors for vitronectin (Pytela *et al.*, 1986; Suzuki *et al.*, 1987). In addition, vitronectin has been shown to bind heparin and to interfere with the heparin-mediated

inhibition of thrombin and Factor Xa by antithrombin III (Preissner *et al.*, 1985; Jenne *et al.*, 1985; Preissner & Müller-Berghaus, 1986, 1987; Podack *et al.*, 1986) and to inhibit the lytic activities of complement factors C7, C8 and C9 (Podack *et al.*, 1977, 1984; Preissner *et al.*, 1983).

At least *in vitro*, vitronectin appears to be secreted (from cultured HepG2 cells) as an intact single-chain polypeptide with an apparent molecular mass of about 75 kDa (Suzuki *et al.*, 1985; Jenne & Stanley, 1987), but in circulating plasma vitronectin exists in two forms, a single-chain polypeptide (V_{75}) and a nicked polypeptide (V_{65+10}) in which the two chains are linked by an interchain disulphide bond (Barnes & Silnutzer, 1983; Dahlbäck & Podack, 1985). The cleavage site for the conversion of the V_{75} into the V_{65+10} appears to be between Arg-379 and Ala-380, which immediately follows the C-terminus of the heparin-binding domain (positions 341–379) (Suzuki *et al.*, 1984; Jenne & Stanley, 1985).

Since vitronectin and thrombin are implicated in both the haemostatic process and the modulation of complement function (Machovich, 1984; Preissner, 1989), our finding that stimulation of platelets by thrombin brings about a specific release of PKA which singles out and specifically phosphorylates vitronectin in plasma (Korc-Grodzicki *et al.*, 1988b) may constitute an important link in the series of molecular and cellular events that lead to haemostasis and repair of the endothelium.

This paper focuses on the phosphorylation of vitronectin by PKA, its stoichiometry and its specificity, identifying Ser-378 as the single site of phosphorylation. This site is shown to be accessible to phosphorylation in the intact one-chain form of vitronectin (V_{75}) but is ‘buried’ in the nicked two-chain form (V_{65+10}) of this protein. Finally, it is shown that the endogenous cleavage of the Arg-379–Ala-380 peptide bond in vitronectin results in a structural malleability and that, upon binding of

Abbreviations used: R, reducing; NR, non-reducing; V, vitronectin; PKA, protein kinase A; C, the free catalytic subunit of PKA.

* This paper is dedicated to Professor Edmond H. Fischer, a discoverer and pioneer of regulatory protein phosphorylation, on his 70th birthday.

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heparin, vitronectin undergoes a distinct conformational change that exposes the PKA phosphorylation site.

EXPERIMENTAL

Materials

Freshly frozen human plasma was obtained from the blood bank of the Tel Hashomer Medical Center, Ramat Gan, Israel. Mes, Hepes, ATP, thrombin, heparin, poly(ethylene glycol) (3350 Da), phenylmethanesulphonyl fluoride, benzamidine hydrochloride, GSSH and affinity-purified goat anti-(rabbit IgG) antibodies were purchased from Sigma Chemical Co. Rabbit anti-(human vitronectin) polyclonal antibodies were from Calbiochem. DEAE-Sepharcel and Blue Sepharose CL-6B were purchased from Pharmacia. Hydroxyapatite and molecular-mass markers for SDS/PAGE were from Bio-Rad Laboratories. [γ - 32 P]ATP (3000 Ci/mmol) was obtained from Amersham. Immobilon [poly(vinylidene difluoride)] membranes were supplied by Millipore Corp. Nitrocellulose paper (0.45 μ m pore size) was from Schleicher and Schuell.

Vitronectin

Vitronectin was prepared from freshly frozen human plasma using a purification procedure described elsewhere (Korc-Grodzicki *et al.*, 1990). Our preparations usually contained the two molecular forms of this protein: V_{75} , a one-chain (75 kDa) form, and V_{65+10} , a proteolytically clipped two-chain protein (65 kDa and 10 kDa) held together by a disulphide bridge (Dahlbäck & Podack, 1985; Jenne & Stanley, 1985). These two forms of vitronectin were partially resolved on Blue Sepharose with a shallow salt gradient (0.15–1 M-NaCl). In this preparation (not shown) the bulk of vitronectin emerged from the column as a broad peak containing V_{75} and V_{65+10} that was preceded by some fractions containing mainly V_{65+10} and followed by some fractions containing essentially pure V_{75} .

Catalytic subunit of PKA

Pure catalytic subunit of PKA was prepared by the method of Beavo *et al.* (1974) and assayed as described by Kupfer *et al.* (1979). The resulting enzyme preparations had a specific activity of 8–13 units/mg. One unit of enzyme activity is defined as the amount of enzyme that catalyses the transfer of 1 μ mol of [32 P]phosphate from [γ - 32 P]ATP to histone H2B/min at pH 6.5 and 30 °C (Jiménez *et al.*, 1982).

Identification and assay of vitronectin by phosphorylation with the catalytic subunit of PKA

Portions of the samples to be assayed (platelet-free plasma, fractionated or purified vitronectin) were added to a reaction mixture (final volume 50 μ l) containing the following constituents at the indicated final concentrations: pure catalytic subunit of PKA (2.5 μ g/ml), magnesium acetate (10 mM), [γ - 32 P]ATP (10 μ M, 50 Ci/mmol) and Hepes buffer (50 mM, pH 7.5). Phosphorylation was allowed to proceed for 15 min at 30 °C and arrested by addition of 12 μ l of sample buffer to yield the following final composition: SDS (2%, w/v), 2-mercaptoethanol (0.75 M), glycerol (10%, v/v), Tris/HCl buffer (25 mM, pH 6.7) and Bromophenol Blue (0.1 mg/ml). The samples were subjected to SDS/PAGE under reducing (R) or non-reducing (NR) conditions and autoradiography or counting for radioactivity. Where specified, the vitronectin samples were incubated with heparin (as indicated) before their phosphorylation with PKA.

Procedures carried out as described in the literature

Protein concentration was determined by the method of Bradford (1976). SDS/PAGE was carried out according to the

procedure described by Laemmli (1970) under either reducing conditions (in the presence of 2-mercaptoethanol) or non-reducing conditions (in the absence of 2-mercaptoethanol). The gels were fixed and stained with a Coomassie Blue solution (0.25%) in 50% (v/v) methanol/7% (v/v) acetic acid. Destaining was carried out with a 20% (v/v) propan-2-ol solution containing acetic acid (7%, v/v). Gels assayed for radioactively labelled proteins were dried under vacuum and autoradiographed at –20 °C using Agfa X-ray film. Alternatively, the lanes of such gels were cut out and their radioactivity was counted in a scintillation counter with a toluene-based scintillant. Immunoblotting was carried out as described by Towbin *et al.* (1979). The CNBr cleavage was based on the method of Gross & Witkop (1962). 32 P-labelled vitronectin was subjected to SDS/PAGE(R) in order to resolve the 75 kDa and 65 kDa polypeptide chains. These polypeptides were eluted from the gel with a Tris/glycine buffer, pH 8.3, in a Biotrap BT1000 electroelution apparatus (Schleicher and Schuell). The eluate was freeze-dried and redissolved in formic acid (70%) containing CNBr (1 mg/ml). After various time intervals at 22 °C, the sample was diluted with distilled water, freeze-dried and washed twice more. The products of the CNBr fragmentation were dissolved in sample buffer and analysed by high Tris/SDS/PAGE(R) (Fling & Gregerson, 1986) and subsequent autoradiography.

Amino acid sequencing

Sequence analysis of vitronectin or its fragments was performed after resolution by SDS/PAGE(R) (with a 7–20% linear gradient of polyacrylamide) and transfer by Western blotting on to an Immobilon membrane (Matsudaira, 1987) in 50 mM-Hepes buffer, pH 7.5, containing 20% (v/v) methanol. A constant current of 300 mA was applied for 3 h with cooling. The Immobilon paper was washed extensively with distilled water, stained with Coomassie Blue (0.25%) in 50% (v/v) methanol/7% (v/v) acetic acid and washed with a 1:1 (v/v) mixture of methanol and 20% (v/v) acetic acid to remove the excess dye. The stained bands of interest were cut out from the paper and stored desiccated at –20 °C until sequencing by an Applied Biosystems 475A sequencer.

RESULTS

PKA differentiates between the intact form and the nicked form of vitronectin

When purified human vitronectin is subjected to SDS/PAGE(R), then stained with Coomassie Blue or immunoblotted with rabbit anti-(human vitronectin) antibody, it appears as two protein bands (75 kDa and 65 kDa; Fig. 1, lanes A and B) (cf. also Barnes & Silnutzer, 1983; Dahlbäck & Podack, 1985). However, only the 75 kDa band becomes phosphorylated when the preparation is incubated with the C subunit of PKA and [γ - 32 P]ATP (Fig. 1, lane C). The 75 kDa band seen in SDS/PAGE(R) represents the native (one-chain) vitronectin (V_{75}) (Scheme 1), and the 65 kDa band is derived from an endogenous cleavage of the Arg-379–Ala-380 peptide bond at the C-terminal edge of its heparin-binding domain (Dahlbäck & Podack, 1985; Jenne & Stanley, 1985; Suzuki *et al.*, 1985; Ruoslahti *et al.*, 1987). This cleavage thus converts V_{75} into a nicked form V_{65+10} , composed of a 65 kDa chain and a 10 kDa chain that are held together by an interchain disulphide bridge (Scheme 1).

In view of the substrate-recognition properties of PKA (Zetterqvist *et al.*, 1976), Ser-378 in the sequence Arg-Arg-Pro-Ser-Arg-Ala was tentatively identified as a plausible site for phosphorylation in vitronectin by this enzyme (Korc-Grodzicki

Fig. 1. Characterization of purified vitronectin by protein staining, immunoblotting and enzymic labelling with the C subunit of PKA

Samples (10 μ g) of vitronectin purified as described elsewhere (Korc-Grodzicki *et al.*, 1990) were subjected to SDS/PAGE(R). Left panel (7 % gels): lane A, the gel was fixed and stained with Coomassie Blue as described in the Experimental section; lane B, the sample was transferred on to nitrocellulose paper by a Western blot and detected by double-antibody labelling with rabbit anti-(human vitronectin) antibodies and goat anti-(rabbit IgG) antibodies conjugated with peroxidase. Right panel: a similar vitronectin preparation (final concentration 2.7 μ M) was phosphorylated with the catalytic subunit of PKA (25 nM) and [γ - 32 P]ATP (50 μ M; 0.5 Ci/mmol) at either pH 7.5 (lane C) or pH 6.0 (lane D). In both cases the reaction was allowed to proceed for 15 min at 30 $^{\circ}$ C, then arrested by addition of sample buffer and boiling (3 min at 95 $^{\circ}$ C). After SDS/PAGE(R) (with a 7–20 % linear gradient of polyacrylamide) the gels were subjected to autoradiography.

et al., 1988b). Yet, although the formation of V_{65+10} should retain this residue in the penultimate position at the C-terminus of the heavy chain (V_{75}), and within a sequence sufficient for recognition by PKA, the phosphorylation of the clipped form of vitronectin does not occur under the standard conditions of our assay, performed at pH 7.5.

PKA phosphorylation site is 'buried' in V_{65+10}

The discriminatory behaviour of PKA in the phosphorylation of V_{75} but not of V_{65+10} (Fig. 1) led us to consider the possibility that the PKA phosphorylation site becomes cryptic or 'buried' in clipped vitronectin. In order to test this hypothesis, we attempted to perform the phosphorylation reaction under deforming (reversible) conditions for vitronectin by lowering the pH to 6.0, which promotes the binding of vitronectin to immobilized heparin (Barnes *et al.*, 1985). Under these conditions, both V_{75} and V_{65+10} were phosphorylated (Fig. 1, lanes C and D). Moreover, the phosphorylation was stoichiometric: 0.98 ± 0.1 mol/mol of V_{75} and 1.15 ± 0.15 mol/mol of V_{65+10} . The ability to induce phosphorylation of the clipped form of vitronectin by lowering the pH of the reaction mixture to 6.0 and the stoichiometric phosphorylation of the 75 kDa band, as well as the 65 kDa band, then observed after SDS/PAGE(R) indicates that the cleavage of the Arg-379–Ala-380 bond alters the conformation of V_{65+10} in a manner that makes Ser-378 'buried' or inaccessible to the kinase.

Since Ser-378 is located at the C-terminal end of the heparin-binding domain and since heparin has been shown to induce significant conformational changes in vitronectin (Tomasini & Mosher, 1988; Hildebrand *et al.*, 1988), we investigated the effect of heparin on the accessibility of this residue to phosphorylation by PKA. As shown in Fig. 2, heparin (50 μ g/ml) has a pronounced effect on the phosphorylation of the 65 kDa

Scheme 1. Representation of the structure and conformation of V_{75} , V_{65+10} and V_{65+10} with heparin, under conditions of physiological pH and ionic strength

In V_{75} the positively charged heparin-binding domain (A) and the negatively charged N-terminal domain (B) are held together by electrostatic forces. Note that, in V_{75} , Ser-378 is exposed, but it becomes inaccessible to phosphorylation ('buried') as a result of the endogenous cleavage (between Arg-379 and Ala-380) that converts it into a two-chain form, and that, upon binding of heparin, Ser-378 becomes exposed again and can then be stoichiometrically phosphorylated by PKA.

band originating from V_{65+10} (as is shown below). In V_{65+10} heparin 'exposes' the phosphorylation site for PKA at physiological pH and makes it as accessible to the kinase as it is at pH 6.0 in the absence of heparin (Fig. 2, compare lanes G and H with lanes A and B).

In order to establish whether the 65 kDa band observed after SDS/PAGE(R) and shown to undergo a heparin-dependent phosphorylation represents a physiologically relevant V_{65+10} form of vitronectin, we took advantage of a preparation in which the fractions containing the 75 kDa and 65 kDa bands observed in SDS/PAGE(R) were partially resolved during chromatography on Blue Sepharose, as described in the Experimental section (cf. also Dahlbäck & Podack, 1985). These fractions were phosphorylated (at pH 7.5) in the absence and in the presence of heparin, and then subjected to SDS/PAGE under reducing or non-reducing conditions. As shown in Fig. 3, heparin mainly

Fig. 2. Effect of heparin on the phosphorylation of vitronectin by PKA, measured at different pH values

Vitronectin (final concentration $1.5 \mu\text{M}$) was preincubated in the absence or in the presence of a 3-fold molar excess of heparin, for 2 h at 22°C in the following buffers: 50 mM-Mes, pH 6.0 or 6.5 (lanes A-D); 50 mM-Hepes, pH 7.0 or 7.5 (lanes E-H). Phosphorylation of vitronectin was allowed to proceed (15 min at 30°C) with the catalytic subunit of PKA ($2.5 \mu\text{M}$), $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ($10 \mu\text{M}$; 0.5 Ci/mmol) and magnesium acetate (10 mM) (all final concentrations). The reaction was arrested by addition of sample buffer and boiling (3 min at 95°C). The samples were then subjected to SDS/PAGE(R) (10% gels) and autoradiography.

Fig. 3. Characterization, by SDS/PAGE under reducing and non-reducing conditions of the molecular species of vitronectin whose phosphorylation by PKA is heparin-dependent

The preparation containing mainly a 75 kDa band and the preparation containing mainly a 65 kDa band [both in SDS/PAGE(R)] were diluted (final concentration $1.5 \mu\text{M}$ in vitronectin) and preincubated (60 min at 22°C) with or without a 3-fold molar excess of heparin (buffer: 50 mM-Hepes, pH 7.5). The four samples were then phosphorylated with PKA and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ under our standard phosphorylation conditions, and after 15 min the phosphorylation was arrested by boiling the samples in the presence of 2% SDS under reducing or non-reducing conditions, i.e. in the presence or in the absence of 2-mercaptoethanol (0.75 M). The SDS/PAGE gels (10%) were dried, and then subjected to autoradiography.

enhances the extent of phosphorylation of V_{65+10} , i.e. of the band that migrates as a 75 kDa protein under non-reducing conditions and as a 65 kDa band under reducing conditions (Fig. 3, compare lanes E and F with lanes G and H). However, the V_{75} form of vitronectin, which migrates as a 75 kDa band under both non-reducing and reducing conditions, is in this experiment essentially unaffected by the presence of heparin (Fig. 3, compare lanes A and B with lanes C and D).

In a more quantitative assessment of the heparin effect it was found that heparin accelerates the rate of phosphorylation of

Fig. 4. Effect of heparin on the rate of PKA phosphorylation of the 75 kDa and 65 kDa bands of vitronectin

A vitronectin preparation containing roughly equal amounts of these two bands [as measured by SDS/PAGE(R)] was used. Two portions of this preparation ($1 \mu\text{M}$) in $475 \mu\text{l}$ of Hepes buffer, pH 7.5, containing benzamide hydrochloride ($1 \mu\text{M}$) and GSSH ($1 \mu\text{M}$) were preincubated in the absence ($\square$) and in the presence ($\blacksquare$) of heparin ($10.5 \mu\text{M}$) for 60 min at 22°C . The volume of the reaction mixture was adjusted to $500 \mu\text{l}$ by addition of magnesium acetate, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and subunit C (final concentrations of 10 mM, $10 \mu\text{M}$ and $2.5 \mu\text{M}$ respectively). At various time intervals following the addition of subunit C, $50 \mu\text{l}$ portions were withdrawn, mixed with $12 \mu\text{l}$ of a buffer solution containing SDS (10%) and 2-mercaptoethanol (3.75 M) and boiled for 3 min. The samples were subjected to SDS/PAGE(R), fixed, stained and dried under vacuum. The rate of phosphorylation was determined by excising the $\gamma\text{-}^{32}\text{P}$ -labelled 75 kDa and 65 kDa bands of vitronectin and counting in liquid-scintillation fluid. The results are plotted as the percentage of maximal (stoichiometric) phosphorylation of the 75 kDa (a) and the 65 kDa (b) bands.

Fig. 5. Effect of ionic strength on the heparin-stimulated phosphorylation of vitronectin

Triplicate samples ($50 \mu\text{l}$) containing vitronectin ($2 \mu\text{M}$) were preincubated with a 3-fold molar excess of heparin in the presence of various concentrations of NaCl. The phosphorylation reaction was initiated by the addition of subunit C, magnesium acetate and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at the same final concentrations as indicated in the Experimental section. The reaction was arrested after 15 min by mixing the samples with $14 \mu\text{l}$ of a solution containing SDS (10%) and 2-mercaptoethanol (3.75 M) and boiled for 3 min. The incorporation of $[\text{P}]\text{phosphate}$ was determined quantitatively by counting for radioactivity the 75 kDa and 65 kDa Coomassie Blue-stained bands of vitronectin excised from a dry 10% SDS/PAGE(R) gel. Phosphorylation of the 75 kDa band ($\bullet$) and the 65 kDa bands ($\blacksquare$) is shown.

both V_{65+10} and V_{75} , but that the rate enhancement is 4.7-fold and only 1.7-fold respectively (Figs. 4a and 4b). It should be emphasized that the heparin-stimulated phosphorylation of the two molecular forms of vitronectin results from a heparin effect on vitronectin itself, and not on PKA. In similar control experiments (results not shown) we found that heparin actually reduces the rate of phosphorylation of histone H2B and

Fig. 6. Characterization of the PKA-labelled site in vitronectin by phospho amino acid analysis (a) and CNBr fragmentation (b)

Purified vitronectin (4.5 μ M) was radiolabelled with [γ - 32 P]ATP and PKA, then subjected to SDS/PAGE(R) as described in the Experimental section. (a) The phosphorylated 75 kDa and 65 kDa bands were cut out together, and eluted from the gel by digestion (24 h at 37 °C) with trypsin (1 mg/ml in 50 mM-ammonium bicarbonate buffer, pH 8.3). The eluted material was then hydrolysed with 6 M-HCl (2 h at 110 °C; Cooper *et al.*, 1983), dried and dissolved in pyridine/acetic acid/water (1:10:189, by vol.). Marker phospho amino acids were mixed with the hydrolysed sample and the sample+marker mixture was subjected to electrophoresis (1000 V for 60 min) on a 20 cm $\times$ 20 cm thin-layer cellulose chromatography plate. The marker phospho amino acids were detected (dotted) by staining with ninhydrin (0.6% in acetone) and the 32 P-labelled phospho amino acids by autoradiography. (b) Another sample of the phosphorylated vitronectin mentioned above was used to resolve the 75 kDa and 65 kDa bands [by SDS/PAGE(R)]. The 65 kDa polypeptide band was isolated by electroelution from a SDS/PAGE(R) gel, as described in the Experimental section. The radioactive eluate was freeze-dried and dissolved in 0.5 ml of a solution of CNBr (1 mg/ml) in 70% formic acid. Samples were removed at the indicated times, washed as described in the Experimental section and analysed by SDS/PAGE(R) with a linear-gradient 8–20% polyacrylamide gel (Fling & Gregerson, 1986). The radioactively labelled fragments were detected by autoradiography.

Table 1. N-Terminal sequence of the thrombin-cleavage products of vitronectin

Apparent fragment size (kDa)	Amino acids identified and the corresponding sequence in vitronectin*
58	<div>1</div> <div>D Q E S C K G</div> <div>24 21 21 18 - 6 33</div>
17	<div>305</div> <div>T S A G T R Q P Q F I</div> <div>31 17 111 71 20 10 30 53 24 70 35</div>
10	<div>380</div> <div>A M W L S F</div> <div>52 30 30 56 7 10</div>
10	<div>380</div> <div>A T W L S F</div> <div>56 34 31 60 9 13</div>

* The numbering of amino acid positions given (above the amino acid residues) follows the sequence of vitronectin deduced by Jenne & Stanley (1985). The value under each amino acid residue represents the number of pmol of amino acid phenylthiohydantoin derivative recovered.

Kemptide (Kemp *et al.*, 1977) (Leu-Arg-Arg-Ala-Ser-Leu-Gly), which are commonly used as substrates of PKA. In the presence of heparin the rate of phosphorylation of the protein is reduced approx. 1.6-fold and that of the peptide is reduced approx. 3.6-fold.

Fig. 7. Heparin-triggered cleavage of 32 P-labelled vitronectin by α -thrombin and its use to identify the site of the PKA phosphorylation

Vitronectin (2.8 μ M) was incubated at 37 °C with α -thrombin (50 units/ml) in the absence and in the presence of heparin (50 μ g/ml) (lanes A and B and lanes C and D respectively, a). Immediately after the addition of α -thrombin (lanes A and C) and after 30 min incubation (lanes B and D), the samples were boiled in the presence of SDS (2%) and 2-mercaptoethanol (0.75 M). The proteins were analysed by SDS/PAGE(R) (with a linear gradient of 7–20% polyacrylamide), fixed and stained as described in the Experimental section. In a similar experimental setting (b) vitronectin was first radiolabelled by phosphorylation at pH 6.0, as described in the legend to Fig. 1. The material was then extensively dialysed against 50 mM-Hepes buffer, pH 7.5 containing GSH (1 mM) to remove excess [γ - 32 P]ATP. Portions of 32 P-labelled vitronectin were subsequently incubated as described above with α -thrombin in the absence and in the presence of heparin (lanes E–G and H–J for 2, 15 and 30 min respectively). The phosphorylated cleavage products were analysed by SDS/PAGE(R) and autoradiography. 10% gels were used in (a) and 7–20% gels in (b).

In view of the fact that ionic interactions play a dominant role in the heparin-neutralizing activity of vitronectin (Preissner & Müller-Berghaus, 1987; Lane *et al.*, 1987), we were interested to investigate how the ionic strength of the medium may influence the heparin-triggered phosphorylation of vitronectin. As shown in Fig. 5, NaCl (in the range 0–300 mM) essentially abolishes the heparin-dependent phosphorylation of isolated V_{65+10} at 150 mM-NaCl, whereas it only slightly inhibits the phosphorylation of V_{75} .

Identification of Ser-378 as the sole site phosphorylated by PKA

Phosphorylation of vitronectin with PKA and [γ - 32 P]ATP (under conditions that bring about stoichiometric phosphorylation of both V_{75} and V_{65+10}) and subsequent amino acid hydrolysis shows that serine is the only amino acid residue that becomes phosphorylated (Fig. 6a). Since, in the presence of heparin, only 1 mol of [32 P]phosphate is incorporated/mol of both the 75 kDa and the 65 kDa bands, and since no 32 P-labelled 10 kDa fragment is detected after reduction of V_{65+10} with thiols, we conclude that the 65 kDa polypeptide chain still contains all the 32 P label, and therefore that the PKA phosphorylation site is not within residues 380 and 459. On the other hand, when the isolated 32 P-labelled 65 kDa band is subjected to CNBr cleavage, then to SDS/PAGE(R) and autoradiography, only one phosphorylated (12 kDa) band is finally formed (Fig. 6b), suggesting that the phosphorylation site resides in one CNBr-cleavage peptide, probably the 12 kDa peptide identified by Suzuki *et al.* (1984) as the peptide containing the heparin-binding domain in vitronectin (residues 348–379; cf. Ruoslahti *et al.*, 1987).

Additional information for the identification of the PKA phosphorylation site was obtained by the fragmentation of vitronectin with α -thrombin. As shown in Fig. 7, heparin has a pronounced effect on this cleavage (cf. also Tomasini *et al.*, 1989), which yields a 58 kDa polypeptide containing the cell-

Fig. 8. Identification of Ser-378 as the sole site of PKA phosphorylation in vitronectin

The fragments of vitronectin obtained by CNBr cleavage (Fig. 6) were subjected to gel filtration on a Sephadex G-15 column equilibrated in 50 mM-Hepes buffer, pH 7.5, to remove free $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, CNBr and formic acid. The 12 kDa ^{32}P -labelled fragment was identified by counting samples from each fraction for radioactivity and subsequently detected by SDS/PAGE(R) and autoradiography. The isolated peptide was subjected to trypsin cleavage (37 °C for 22 h; 1 mg/ml) (lane A). The synthetic peptide $\text{Ser}^1\text{-Arg}^2\text{-Arg}^3\text{-Pro}^4\text{-Ser}^5\text{-Arg}^6\text{-Ala}^7$ (2.5 mM) was also subjected to phosphorylation by PKA with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (50 μM). The peptide was then subjected to trypsin cleavage for either 30 min (lane C) or 22 h (lane B). The three proteolysed samples were subjected to high-voltage electrophoresis on 0.1 mm cellulose-glass plates (20 cm $\times$ 20 cm) with a buffer composed of pyridine/acetic acid/water (1:10:189, by vol.), pH 3.5, for 1 h at 1000 V. The position of the intact peptide and the trypsin-cleaved fragments were identified by autoradiography and by staining with ninhydrin (marked by a dotted line). Note that the residual non-cleaved heptapeptide (lane C, front) and the less basic cleavage product (Arg-Pro-Ser-Arg) are clearly resolved by this procedure.

recognition site (Silnutzer & Barnes, 1984) from both the 75 kDa and the 65 kDa bands of vitronectin (Fig. 6a). However, when portions of the reaction mixture at different times were subjected to SDS/PAGE(R) and autoradiography, the phosphorylated site was found to reside not in the 58 kDa band but in a previously unidentified 17 kDa band (Fig. 7b).

The α -thrombin cleavage products were subjected to sequence analysis. As shown in Table 1, the 58 kDa polypeptide that did not contain the phosphorylation site was found to have an *N*-terminal sequence identical with that of intact vitronectin (residues 1–4 and 6–7 positively identified). On the other hand, the 17 kDa polypeptide (which contained the radioactive label) had a sequence identical with residues 305–315 (all positively identified). Two other peptides that were not labelled migrated slightly lower on SDS/PAGE than the 17 kDa band shown in Fig. 7. They were found to have a sequence identical with that of residues 380–385 in vitronectin (numbered in accordance with Jenne & Stanley, 1985). These two peptides, which migrate together, do not appear to be derived from the thrombin cleavage, and presumably represent the new *N*-terminus formed as a result

of the endogenous cleavage that yields V_{65+10} from V_{75} . The two sequences were identical apart from the appearance of either threonine or methionine at the second residue position, a single-point polymorphism that has been previously reported in sequences obtained from two different cDNA clones of human vitronectin (see Table 1).

Taken together, these results would place the site of PKA phosphorylation between positions 306 and 379, which accommodates the heparin-binding domain of vitronectin (Suzuki *et al.*, 1984; Ruoslahti *et al.*, 1987). Within this region, there is one serine residue in an optimal sequence for phosphorylation by PKA, since two adjacent basic amino acid residues are usually found at positions $n-2$ and $n-3$ *N*-terminal to a serine or threonine residue at a position n for phosphorylation by PKA (Kemp *et al.*, 1977; Feramisco *et al.*, 1980). This sequence in vitronectin (Arg-Arg-Pro-Ser-Arg, residues 375–379) contains two additional features: (a) the occurrence of a proline residue at position $n-1$, and (b) the occurrence of an arginine residue at position $n+1$. To ascertain that this particular sequence does indeed represent a substrate for PKA, we synthesized the peptide $\text{Ser}^1\text{-Arg}^2\text{-Arg}^3\text{-Pro}^4\text{-Ser}^5\text{-Arg}^6\text{-Ala}^7$ (identical with positions 374–380 in vitronectin) and showed (B. Korc-Grodzicki, M. Fridkin & S. Shaltiel, unpublished work) that it constitutes a good peptide substrate for PKA with K_m and V_{max} of similar values to those reported in the literature for Kemptide (16 μM and 20 $\mu\text{mol/min per mg}$ respectively; Kemp *et al.*, 1977). To establish further that Ser-378 is the sole site phosphorylated by PKA, we subjected the 12 kDa ^{32}P -labelled fragment obtained by CNBr cleavage (Fig. 6b) to trypsin cleavage and showed that a single radioactively labelled peptide was obtained that co-migrated on thin-layer electrophoresis with the tryptic product of the Ser-5-phosphorylated synthetic heptapeptide (Fig. 8). The tryptic products in both cases (Arg-Pro-Ser-Arg) correspond to the sequence around Ser-378 in vitronectin (positions 376–379).

DISCUSSION

Previously we have shown that PKA is specifically released following thrombin stimulation of platelets (Korc-Grodzicki *et al.*, 1988a,b). In human plasma, vitronectin was shown to be the sole substrate for PKA and to undergo a phosphorylation that requires Mg^{2+} (Korc-Grodzicki *et al.*, 1988b, 1990). The phosphorylation of vitronectin by PKA is distinct from an Mn^{2+} -dependent phosphorylation of this and other plasma proteins that has been independently observed and reported to involve an unidentified protein kinase in plasma (McGuire *et al.*, 1988). In both cases, however, the site of phosphorylation was found to be at Ser-378 within the heparin-binding domain.

In this paper we have examined the phosphorylation of vitronectin by PKA using the pure catalytic subunit of the enzyme, to establish the molecular basis for the differential phosphorylation of V_{75} versus V_{65+10} . On the basis of the results reported above, we conclude that the endogenous cleavage of V_{75} brings about a distinct conformational change that 'buries' its site of phosphorylation by PKA. Furthermore, it is shown that heparin can exert a major effect on the conformation of V_{65+10} , exposing this site and allowing its stoichiometric phosphorylation (Scheme 1).

The interactions between vitronectin and heparin are thought to be primarily ionic (Suzuki *et al.*, 1985; Jenne & Stanley, 1985; Lane *et al.*, 1987). We propose (Scheme 1) that, upon binding to the protein, the heparin polyanion interferes with the electrostatic interactions between the cluster of basic amino acid residues found in the heparin-binding domain and the negatively charged somatomedin B domain, which contains the Arg-Gly-Asp cell-

recognition sequence (cf. Fig. 7 in Preissner & Müller-Berghaus, 1987), thus exposing Ser-378 and making it amenable to phosphorylation by PKA. In the absence of heparin, the phosphorylation site remains 'buried' in the molecule.

Under physiological conditions of pH and ionic strength and in the absence of some additional or alternative effector, it is evident (from the results shown in Fig. 5) that the phosphorylation of vitronectin by PKA would be limited to V_{75} . It is pertinent to note, however, that heparin binding to vitronectin, which is below maximal in the native structure (Barnes *et al.*, 1985; Preissner & Müller-Berghaus, 1987), is enhanced by thrombospondin following platelet activation (Tomasini & Mosher, 1985) and by exposure of vitronectin to the thrombin-antithrombin III complex (Tomasini & Mosher, 1988). The possible role of these molecules, as well as other plasma (or serum) proteins, in modulating the phosphorylation of vitronectin by PKA requires further investigation.

In the native state, a number of the functional domains of vitronectin that are located in separate well-defined regions of the protein (Suzuki *et al.*, 1984) appear to be 'buried'. These include the heparin-binding site (Barnes *et al.*, 1985; Preissner & Müller-Berghaus, 1987), the collagen-binding site (Gebb *et al.*, 1986) and the cell-recognition site (Preissner *et al.*, 1988). Altered conformational states of vitronectin (which have been measured by the expression of an antigenic epitope) have implicated several possible effectors (including heparin and the thrombin-antithrombin III complex) in the modulation of cell adhesion, binding of vitronectin to extracellular-matrix collagen and cell-surface constituents such as thrombomodulin (Tomasini & Mosher, 1988; Tomasini *et al.*, 1989; Hildebrand *et al.*, 1989).

The exact difference in the physiological assignment of the two circulating forms of vitronectin, V_{75} and V_{65+10} , has yet to be established. Nonetheless, the results described here demonstrate that there is a marked difference between these two forms of vitronectin, not merely in their binding to heparin (cf. Ill & Ruoslahti, 1985; Izumi *et al.*, 1988), but also in their specific conformational response to this modulator. The work presented in this paper shows that vitronectin has a single phosphorylation site for PKA and makes use of this specific phosphorylation to probe the conformation of the molecule. This novel approach enabled us to show that V_{65+10} has a unique functional flexibility, which is illustrated by the lack of phosphorylation by PKA in the absence of heparin, contrasted with a stoichiometric phosphorylation at physiological pH in the presence of heparin.

Although the role of phosphorylation by PKA remains to be established, the identification of Ser-378 as the sole site for PKA action, and the proximity of the phosphorylation site to the point of cleavage that converts V_{75} into V_{65+10} , focuses attention on a putative role for PKA in the modulation of this cleavage. Further studies are required to examine the possible relationship between the phosphorylation of vitronectin by PKA and the endogenous cleavage of the intact molecule into its two-chain form.

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