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CYCLIC AMP-DEPENDENT PROTEIN KINASE PHOSPHORYLATES SERINE^{378} IN VITRONECTIN

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We previously observed that Ser^{378} in the heparin-binding domain of vitronectin becomes phosphorylated by a protein kinase in plasma upon addition of ATP and divalent cations. We now report that purified plasma vitronectin contains ~2.5 mol of phosphate per mol of protein and that vitronectin becomes phosphorylated during biosynthesis in human hepatoma (HepG2) cells. *In vitro*, rabbit muscle cAMP-dependent protein kinase specifically phosphorylates Ser^{378} in single-chain (75 kDa) vitronectin but does not phosphorylate the two-chain (65/10 kDa) form cleaved at Arg^{379}. Heparin affects neither the time course nor the extent of phosphorylation of Ser^{378} at neutral pH. The extent of phosphorylation of Ser^{378} achieved with cAMP-dependent protein kinase ($\geq$ 0.3 mol phosphate per mol vitronectin) is greater than that obtainable in plasma and should enable comparisons to be made of the activities of the native and phosphorylated forms.

Vitronectin (also termed serum spreading factor, complement S protein, and epibolin) is a glycoprotein that is present in human plasma at a concentration of ~4 μM (1). It has also been detected in amniotic fluid, urine, platelets, and extracellular matrix. Vitronectin binds to a specific membrane receptor on fibroblasts and endothelial cells to mediate attachment and spreading of these cells (2, 3). It also binds to soluble C5b-7 complexes of complement and prevents C9 polymerization, thereby inhibiting formation of the membrane attack complex (4, 5). Vitronectin promotes coagulation by decreasing the rate of inhibition of thrombin by antithrombin catalyzed by low concentrations of heparin (6). Conversely, vitronectin may inhibit fibrinolysis by binding to and stabilizing plasminogen activator inhibitor-1 (7). Plasma vitronectin occurs as a single-chain form (75 kDa) and a cleaved form consisting of two chains (65 kDa and 10 kDa) linked by a disulfide bond. Cleavage to the two-chain form occurs between Arg^{379} and Ala^{380} (8). The extent of cleavage is variable among individuals and correlates with a genetic polymorphism in the codon for the amino acid at position 381 (9, 10).

We have reported that vitronectin is the major protein phosphorylated in plasma upon addition of [γ-^{32}P]ATP and divalent cations (11). Sequence analysis of a ^{32}P-labeled chymotryptic

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Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

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peptide revealed that phosphorylation of vitronectin occurs on a single serine residue at position 378 adjacent to the heparin-binding domain. Phosphorylation of Ser^{378} by the protein kinase in plasma occurs preferentially on the single chain (75 kDa) form of vitronectin. Shaltiel and coworkers have suggested that a cAMP-dependent protein kinase released from thrombin-stimulated platelets phosphorylates vitronectin (12). In addition, they presented evidence that this enzyme phosphorylates a site near the heparin-binding domain, although the phosphorylation site was not identified unambiguously (13).

In this report, we demonstrate that vitronectin circulates in plasma as a phosphoprotein and that additional phosphate groups are specifically incorporated into Ser^{378} by a commercial preparation of rabbit muscle cAMP-dependent protein kinase. The characteristics of phosphorylation by this enzyme are similar to the phosphorylation observed in plasma.

Materials and Methods

Purification of vitronectin -- Fresh-frozen human plasma collected in citrate anticoagulant was purchased from the Red Cross. One microliter of each unit of plasma was subjected to immunoblot analysis as previously described to quantify the single-chain and two-chain forms of vitronectin (9). Vitronectin was purified from selected units of plasma by the method of Dahlbäck and Podack (8). Contaminating albumin was removed by absorption to rabbit anti-human albumin IgG covalently linked to Sepharose. The concentration of vitronectin was determined by absorbance at 280 nm using an extinction coefficient of 1.22 $ml \cdot mg^{-1} \cdot cm^{-1}$ (determined by quantitative amino acid analysis of a protein solution of known absorbance).

Incorporation of [^{32}P]phosphate into vitronectin during biosynthesis in HepG2 cells -- HepG2 cells were grown to confluence in a 150-cm^2 flask in D-MEM/F-12 medium (Gibco) containing 10% fetal bovine serum. They were then washed and cultured for 24 h in serum-free medium containing 100 µCi of [^{32}P]phosphoric acid (9000 Ci/mmol, New England Nuclear). The conditioned medium was removed and immunoprecipitated as previously described (11).

Phosphorylation of purified vitronectin by cAMP-dependent protein kinase -- Vitronectin (4-6 µM) was incubated with 0.07-0.22 µM [γ-^{32}P]ATP (4500 Ci/mmol, ICN Radiochemicals), 5 µg/ml rabbit muscle cAMP-dependent protein kinase (Sigma), and 10 mM $MgCl_2$ in 50 mM HEPES buffer, pH 7.0. Some incubations also contained cAMP, unlabeled ATP, the dodecapeptide protein kinase inhibitor (Sigma) described by Cheng *et al.* (14), and/or porcine intestinal heparin (Sigma) at the final concentrations indicated in the figure legends. The incubations were carried out at 30 °C, and the reactions were terminated by adding an equal volume of SDS sample buffer and heating at 100 °C for 4 min. Samples were subjected to SDS-PAGE under reducing conditions (15). Protein bands were detected with Coomassie Brilliant Blue and by autoradiography. The labeled bands were excised from the gel and counted for Cerenkov radiation. The radioactivity in the vitronectin band on SDS-PAGE was divided by the total radioactivity loaded onto the gel; this fraction was then multiplied by the total amount of ATP in the sample to give the amount of phosphate incorporated.

Phosphate analysis -- Phosphate was determined by the colorimetric assay of Ames (16) after dialysis of the sample against 0.1 M ammonium bicarbonate.

Identification of the phosphorylation site -- Vitronectin (5.5 µM, 68% single-chain form) was incubated with 11 nM [γ-^{32}P]ATP, 1 mM unlabeled ATP, 10 mM $MgCl_2$, 12 µM cAMP, and 5 µg/ml rabbit muscle cAMP-dependent protein kinase in 2 ml of 50 mM HEPES buffer, pH 7.0, for 2 h at 30 °C. Following dialysis to remove unincorporated [γ-^{32}P]ATP, the phosphorylated vitronectin was reduced with 10 mM dithiothreitol and alkylated with 20 mM iodoacetamide in 6 M guanidine-HCl (BRL), 0.5 M Tris-HCl, pH 8.5. The sample was dialyzed against 0.1 M ammonium bicarbonate, divided in half, and incubated with 2% by weight of either TPCK-trypsin (Sigma) or TLCK-chymotrypsin (Sigma) for 24 h at 37 °C. The digested samples were lyophilized and dissolved in 0.1% trifluoroacetic acid (Pierce) containing 2 M urea (BRL). The peptides were separated on a 4.6 × 250 mm Altex C-18 (5 µm) HPLC column with a gradient of 0-75% acetonitrile in 0.1% trifluoroacetic acid. The labeled peaks were pooled separately and

rechromatographed on the same column with a gradient of 0-65% acetonitrile in 100 mM ammonium acetate, pH 4.8. Amino acid sequencing was performed as previously described (11).

Results and Discussion

Purified plasma vitronectin contains 2.49 ± 0.44 (mean ± SD, n = 8) mol phosphate per mol protein as determined by a colorimetric phosphate assay. The phosphate cannot be removed by dialysis against 6 M guanidine-HCl and, therefore, appears to be covalently linked to vitronectin. No phosphate was detected in samples of human serum albumin and bovine γ-globulin analyzed in an identical manner. The phosphate groups in plasma vitronectin could be incorporated during biosynthesis in the liver or by modification of the circulating protein elsewhere in the body. As shown in Fig. 1 (*lane 2*), human hepatoma (HepG2) cells cultured in the presence of [^{32}P]phosphoric acid secrete a number of [^{32}P]phosphoproteins into the conditioned medium. A 75 kDa phosphoprotein is precipitated from the conditioned medium by anti-vitronectin antibodies (*lane 3*) but not by control antibodies (*lane 1*), indicating that HepG2 cells secrete single-chain vitronectin that is phosphorylated during biosynthesis. Further work will be required to determine the sites of phosphorylation.

Additional phosphorylation of purified plasma vitronectin occurs upon incubation with rabbit muscle cAMP-dependent protein kinase in the presence of $MgCl_2$ and [γ-^{32}P]ATP (Fig. 2). Phosphorylation by this enzyme is stimulated by cAMP and inhibited by the dodecapeptide

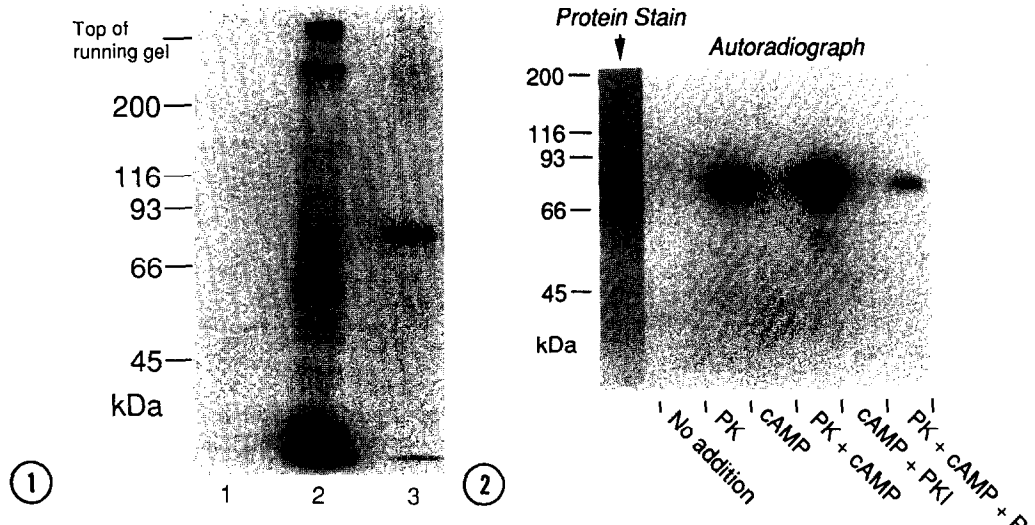

Fig. 1. *Phosphorylation of vitronectin by HepG2 cells.* HepG2 cells were cultured for 24 h in serum-free medium containing [^{32}P]phosphoric acid. The conditioned medium (*lane 2*) or immunoprecipitates of the conditioned medium with control (*lane 1*) or anti-vitronectin (*lane 3*) antibodies were analyzed by SDS-PAGE. An autoradiograph of the gel is shown.

Fig. 2. *Phosphorylation of vitronectin by cAMP-dependent protein kinase.* Purified vitronectin (4 μM) was incubated at 30 °C for 10 min with 0.22 μM [γ-^{32}P]ATP and 10 mM $MgCl_2$ in 50 mM HEPES buffer, pH 7.0. Some incubations contained 5 μg/ml rabbit muscle cAMP-dependent protein kinase (*PK*), 12 μM cAMP (*cAMP*), and 100 μg/ml protein kinase inhibitor (*PKI*) as indicated. The samples were subjected to SDS-PAGE under reducing conditions. Protein bands were detected with Coomassie Brilliant Blue and autoradiography.

inhibitor of cAMP-dependent protein kinase described by Cheng *et al.* (14). No phosphorylation of purified vitronectin occurs in the absence of the protein kinase. Although equal amounts of the single-chain (75 kDa) and two-chain (65/10 kDa) forms of vitronectin were present in this experiment, the purified protein kinase phosphorylated only the single-chain form. Phosphorylation of vitronectin by the purified protein kinase reached a maximal level of ~0.3 mol phosphate per mol vitronectin after 2-3 hours (Fig. 3) as determined from the incorporation of ^{32}P into the 75 kDa vitronectin band on SDS-PAGE. Chain *et al.* (13, 17) have reported that heparin stimulates phosphorylation of the 65 kDa polypeptide at pH 7.0 and that phosphorylation of the 65 kDa polypeptide occurs in the absence of heparin at pH 6.0 or at low ionic strength. However, we did not observe any phosphorylation of the 65 kDa polypeptide in the presence of heparin (Fig. 3).

The stoichiometry of phosphorylation of vitronectin by the cAMP-dependent kinase was confirmed by the colorimetric phosphate assay. The phosphate content of a sample of vitronectin that contained >90% single-chain form increased from 2.32 to 2.64 mol phosphate per mol protein after treatment with cAMP-dependent protein kinase for 3 h under the conditions of the experiment shown in Fig. 3. The difference in phosphate content (0.32 mol per mol vitronectin) agreed with the value determined from incorporation of [^{32}P]phosphate.

A preparation of vitronectin was phosphorylated by cAMP-dependent protein kinase in the presence of [γ-^{32}P]ATP and digested with trypsin or chymotrypsin to identify the phosphorylation

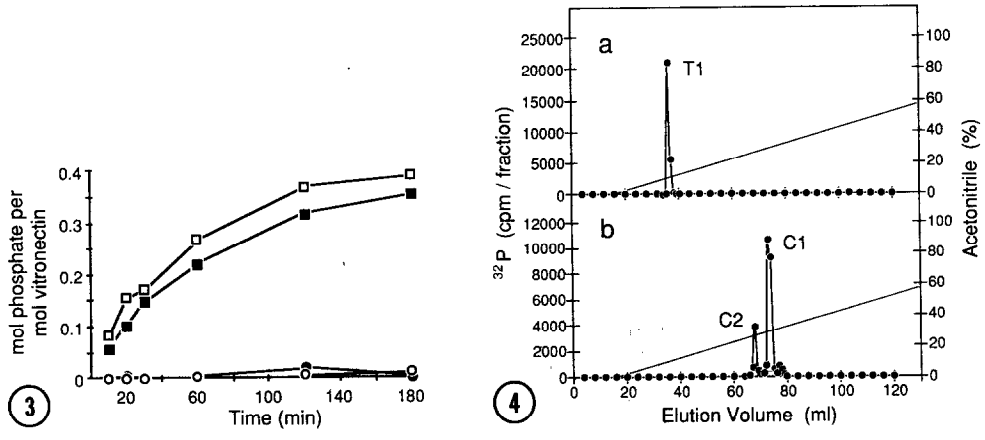

Fig. 3. *Time course of phosphorylation in the presence and absence of heparin.* Vitronectin preparations (6 μM) containing >90% of the single-chain or two-chain form were incubated at 30 °C with 5 μg/ml cAMP-dependent protein kinase, 0.07 μM [γ-^{32}P]ATP, 30 μM unlabeled ATP, 12 μM cAMP, 10 mM $MgCl_2$, and 120 mM NaCl in 50 mM HEPES buffer, pH 7.0. Aliquots were removed at each time point and immediately heated at 100 °C with SDS sample buffer. Following SDS-PAGE, the Coomassie-stained vitronectin bands were cut from the gel and counted for Cerenkov radiation. Molar phosphate incorporation was calculated as described in Materials and Methods. ■, single-chain vitronectin; □, single-chain vitronectin plus heparin (68 μg/ml); ●, two-chain vitronectin; ○, two-chain vitronectin plus heparin (68 μg/ml).

Fig. 4. *Isolation of [^{32}P]phosphopeptides from vitronectin.* A vitronectin preparation (5.5 μM) containing 68% of the single-chain form was phosphorylated as described in Materials and Methods. The phosphorylated vitronectin was reduced, alkylated, and digested with TPCK-trypsin (*panel a*) or TLCK-chymotrypsin (*panel b*). Peptides were separated on a 4.6 × 250 mm Altex C-18 (5 μm) HPLC column with a gradient of 0-75% acetonitrile in 0.1% trifluoroacetic acid.

site. The [^{32}P]phosphopeptides were fractionated by reverse phase HPLC in 0.1% trifluoroacteic acid. A single labeled peptide was obtained from the trypsin digest (Fig. 4a). This peak (*T1*) contained 94% of the the total incorporated radioactivity. Two labeled peptides were obtained from the chymotrypsin digest (Fig. 4b). The major peptide (*C1*) contained 67% of the incorporated radioactivity and the minor peptide (*C2*) contained 13%. All three of the labeled peptides were associated with peaks detected by absorbance at 210 nm (not shown). The three labeled peptides were pooled separately and rechromatographed on the same column in 100 mM ammonium acetate, pH 4.8, to ensure that they were homogeneous. A single peak of radioactivity was detected after each of the runs in ammonium acetate (not shown).

The amino acid sequences of the two major [^{32}P]phosphopeptides are illustrated in Fig. 5. The phenylthiohydantoin derivative of the third residue in tryptic peptide T1 was not identified due to interference by residual ammonium acetate in the sample. However, the sequence Arg-Pro-X-Arg occurs only once in vitronectin at positions 376-379 and includes Ser^{378}. Peptide C1, the more abundant of the two chymotryptic peptides, matched the vitronectin sequence at positions 374-381 and contained peptide T1. These data indicate that the phosphorylation site utilized by cAMP-dependent protein kinase is Ser^{378}. The minor peptide C2 had a sequence similar to that of vitronectin beginning at position 337 and apparently resulted from incomplete chymotrypsin cleavage.

The phosphorylation of vitronectin by rabbit muscle cAMP-dependent protein kinase is similar to the phosphorylation observed when ATP and divalent cations are added to plasma (11). The site of phosphorylation in both cases is Ser^{378}, and phosphorylation is specific for the single-chain form of vitronectin. These findings support the hypothesis that the enzyme responsible for phosphorylation of vitronectin in plasma is a cAMP-dependent protein kinase. It remains to be determined whether Ser^{378} becomes phosphorylated in plasma under physiological conditions. In this regard, Martin demonstrated phosphorylation of vitronectin by 0.3 μM [γ-^{32}P]ATP in EDTA- or heparin-anticoagulated whole blood or in clotting blood without addition of exogenous divalent cations (18). Potential sources of ATP and protein kinase for phosphorylation of vitronectin are damaged cells or stimulated platelets. Platelets contain substantial quantities of ATP complexed with Mg^{++} in their dense granules and secrete the nucleotide during hemostasis, producing a final concentration of ATP in serum estimated to be ~20 μM (19).

Ser^{378} is located at the C-terminal end of the heparin-binding domain of vitronectin (20). The heparin-binding domain has been implicated in several of the biological activities of

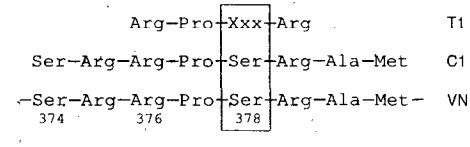

Fig. 5. *Amino acid sequences of [^{32}P]peptides.* The sequences obtained for the labeled tryptic (*T1*) and chymotryptic (*C1*) fragments were compared to the published vitronectin sequence (*VN*) (23, 24).

vitronectin: (a) inhibition of the heparin-catalyzed thrombin-antithrombin reaction (6); (b) binding to soluble C5b-7 complexes of complement and prevention of C9 polymerization which thereby inhibits the membrane attack complex (21); (c) inhibition of perforin-induced cytolysis (21); and (d) binding of plasminogen (22). Phosphorylation of vitronectin during platelet aggregation may alter one or more of these activities and thereby provide a mechanism for coordinated regulation of hemostasis, fibrinolysis, and complement activation. The use of purified cAMP-dependent protein kinase to phosphorylate Ser^{378} should allow functional comparisons to be made of the phosphorylated and non-phosphorylated forms of vitronectin.

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