The phosphorylation of the two-chain form of vitronectin by protein kinase A is heparin dependent*

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In circulating blood, vitronectin occurs in two forms: a single-chain (75 kDa) and an endogenously clipped two-chain form (65 kDa and 10 kDa) held together by a disulfide bridge. The 75 kDa form was previously shown to be phosphorylated at Ser^{378} by protein kinase A, released by physiologically stimulated platelets. By contrast, at pH 7.5 the two-chain form is not phosphorylated at all. Heparin or heparan sulfate are shown here to modulate the conformation of clipped vitronectin at physiological pH, exposing Ser^{378} and allowing its stoichiometric phosphorylation by the kinase. At this pH the two-chain form of vitronectin in plasma exhibits a higher affinity for heparin, and behaves as a flexible molecule, which can conformationally respond to heparin and heparan sulfate, effectors involved in vitronectin function.

Conformational change; Hemostasis; Heparin; Platelet; Protein kinase A; Vitronectin

1. INTRODUCTION

Vitronectin is a multifunctional adhesive protein which plays an important role in blood coagulation and in the immuno-complement system [1–5]. It has been cloned and sequenced [6,7]: At its N-terminus (positions 1–44) it has a sequence identical to somatomedin B, followed by an Arg-Gly-Asp epitope (positions 45–47) involved in cell adhesion and spreading [1–3]. It also has a heparin-binding domain (positions 348–379) which seems to be involved in the inhibition of the heparin-catalyzed formation of the thrombin-antithrombin III complex and in other functions of vitronectin [4,5].

In circulating blood, vitronectin occurs in two molecular forms: a single-chain 75 kDa form (V_{75}), and a clipped form composed of two chains (65 kDa and 10 kDa) which are held together by a disulfide bridge (V_{65+10}) [2,8,9]. The endogenous proteinase responsible for this cleavage has not been identified, but the cleavage was shown to occur at the Arg^{379}-Ala^{380} bond [8,10]. It is currently assumed that there are 'no major functional differences between these forms' [4].

We have recently shown that V_{75} is specifically phosphorylated at Ser^{378} by PKA and that this kinase is specifically released (together with its co-substrate, ATP) upon physiological stimulation of blood platelets [11,12]. By contrast to V_{75}, V_{65+10} was not phosphorylated at all at physiological pH [12,13], in spite of the fact that Ser^{378} and the complete recognition sequence of protein kinase A (Arg-Arg-Pro-Ser-Arg) are still present in the 65 kDa chain of V_{65+10} [10]. This paper shows that, at physiological pH, V_{65+10} occurs in two distinct conformations: one in which Ser^{378} is inaccessible to phosphorylation by PKA, and another in which Ser^{378} is exposed and accessible to stoichiometric phosphorylation by this kinase. Heparin and heparan-sulfate induce and stabilize the conformation which exposes Ser^{378}

2. MATERIALS AND METHODS

2.1. *Materials*

Freshly-frozen human plasma was obtained from the blood bank of the Tel Hashomer Medical Center, Ramat Gan, Israel. 2-(*N*-Morpholino) ethanesulfonic acid, (*N*-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid), ATP, heparin, heparan sulfate, chondroitin sulfate C, dextran sulfate, polyethylene glycol (M_r = 3350) phenyl-methyl sulfonyl fluoride, benzamidine hydrochloride, reduced glutathione and affinity purified goat anti-rabbit IgG were purchased from Sigma. Rabbit anti-human vitronectin polyclonal antibodies were from Calbiochem. DEAE Sephacel and Blue Sepharose Cl-6B were purchased from Pharmacia. Hydroxyapatite and molecular weight markers for SDS-PAGE were from Bio-Rad. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham. Nitrocellulose paper (0.45 μm) was from Schleicher and Schuell.

2.2. *Procedures carried out by methods described in the literature*

Protein concentration was determined by the method of Bradford

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Abbreviations: GAG, glycosaminoglycan; Hepes, *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; PKA, protein kinase A; R, reducing conditions; NR, non-reducing conditions; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate; V_{75}, the one chain form of vitronectin; V_{65+10}, the two-chain form of vitronectin

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[14]. SDS-PAGE was carried out according to the procedure described by Laemmli [15], either under reducing conditions (R, with 2-mercaptoethanol), or under non-reducing conditions (NR, without 2-mercaptoethanol). The gels were fixed and stained with a Coomassie blue solution (0.25%) in 50% methanol and 7% acetic acid. Destaining was carried out with a 20% isopropanol solution containing acetic acid (7%). Gels assayed for radioactively labeled 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 using a toluene-based scintillant. Immunoblotting was carried out as described by Towbin et al. [16].

2.3. *Vitronectin*

Vitronectin was prepared from freshly-frozen human plasma using a purification procedure described elsewhere [13]. Our preparations usually contained 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 disulfide bridge [2,8,9,17].

2.4. *The catalytic subunit of PKA*

Pure catalytic subunit of PKA was prepared according to Beavo et al. [18] and assayed as described by Kupfer et al. [19]. 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 which catalyzes the transfer of 1 μmol of [^{32}P] from [γ-^{32}P]ATP onto histone H2b per min at pH 6.5 and 30°C.

2.5 *Identification and assay of vitronectin by phosphorylation with the catalytic subunit of PKA [13]*

Aliquots of the samples to be assayed (platelet-free plasma, fractionated or purified vitronectin) were added to a reaction mixture (final volume 50 μl) which contained the following constituents at the indicated final concentrations: pure catalytic subunit of PKA (2.5 μg/ml), $Mg(CH_3COO)_2$ (10 mM), [γ^{32}P]ATP (10 μM, 50 Ci/mmol) Hepes (50 mM, pH 7.5). Phosphorylation was allowed to proceed for 10 min at 30°C and arrested by addition of 12 μl of the sample buffer to yield the following final composition: SDS (2%), 2-mercaptoethanol (0.75 M), glycerol (10%), Tris HCl buffer (25 mM, pH 6.7) bromophenol blue (0.1 mg/ml). The samples were subjected to SDS-PAGE under R or NR conditions and autoradiography or counting. Where specified, the vitronectin samples were incubated with heparin (at the indicated concentration) prior to their phosphorylation with PKA.

3. RESULTS AND DISCUSSION

At physiological pH, the catalytic subunit of PKA differentiates between the single-chain (V_{75}) form of vitronectin and its endogenously clipped two-chain (V_{65+10}) form. It phosphorylates V_{75} but not V_{65+10} (Fig. 1C). This distinction is intriguing since PKA was shown to phosphorylate Ser^{378} in V_{75}, and since the complete PKA recognition site (in this case Arg-Arg-Pro-Ser-Arg) is still present at the C terminal edge of

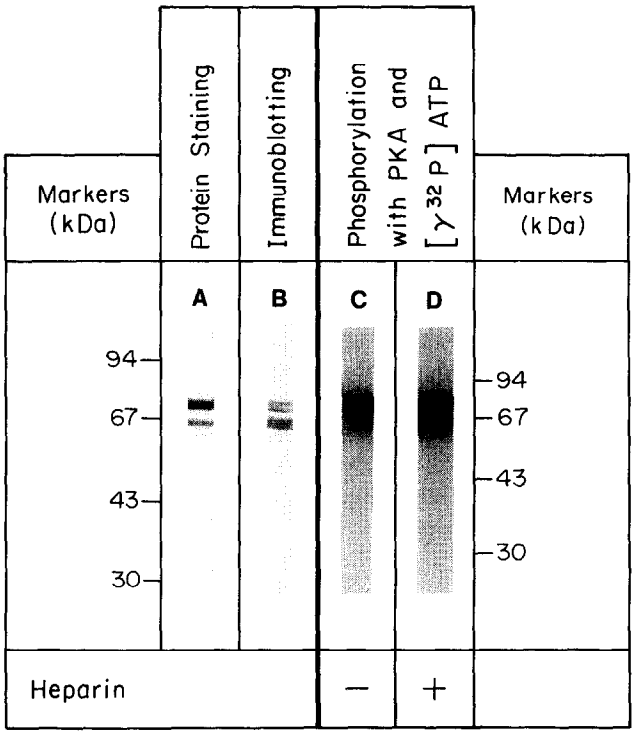


Fig. 1. Protein staining and immunoblotting of purified vitronectin and its enzymatic labeling with the catalytic subunit of PKA. Samples (10 μg) of vitronectin [13] were subjected to SDS-PAGE-R. (A) (7% gel) The gel was stained with Coomassie blue. (B) (7% gel) Immunoblotting was carried out with rabbit anti-human vitronectin and goat anti-rabbit IgG conjugated with peroxidase. (C,D) (7-20% gels) A vitronectin preparation (final concentration 2.7 μM) was phosphorylated with the catalytic subunit of PKA (25 nM) and [γ^{32}P]ATP (50 μM, 0.5 Ci/mmol) either at pH 7.5 in the absence (lane C) or in the presence (lane D) of a three-molar excess of heparin. In both lanes C and D, the reaction was allowed to proceed for 30 min at 30°C, then arrested by addition of sample buffer and boiling (3 min at 95°C). After SDS-PAGE-R the gels were subjected to autoradiography.

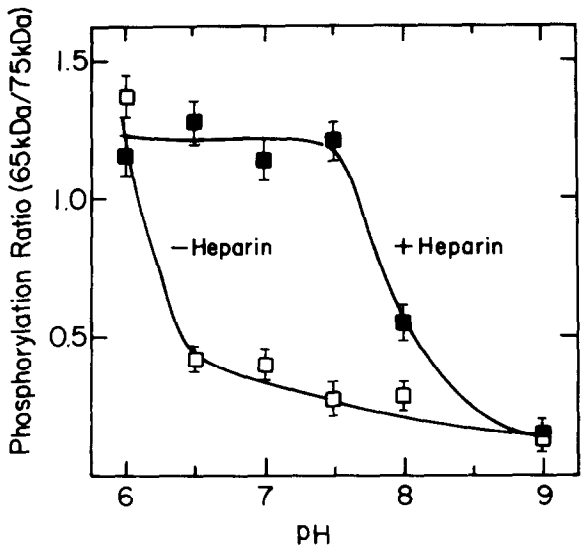


Fig. 2. Effect of heparin on the relative PKA phosphorylation of V_{65+10} and V_{75} at different pH values. Vitronectin (final concentration 1.5 μM) containing a V_{65+10} to V_{75} ratio of ~1.2 was preincubated in the absence or in the presence of heparin (a 3-fold molar excess, where indicated) for 2 h at 22°C in the following buffers: Mes 50 mM, pH 6.0 or 6.5; Hepes 50 mM, pH 7.0 or 7.5, Tris 50 mM, pH 8.0 and 9.0. Phosphorylation of vitronectin was allowed to proceed (15 min at 30°C) with the catalytic subunit of PKA (25 nM), [γ-^{32}P]ATP (10 μM, 0.5 Ci/mmol) and $Mg(CH_3COO)_2$ (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). The Coomassie blue stained bands were excised and their radioactivity was counted.

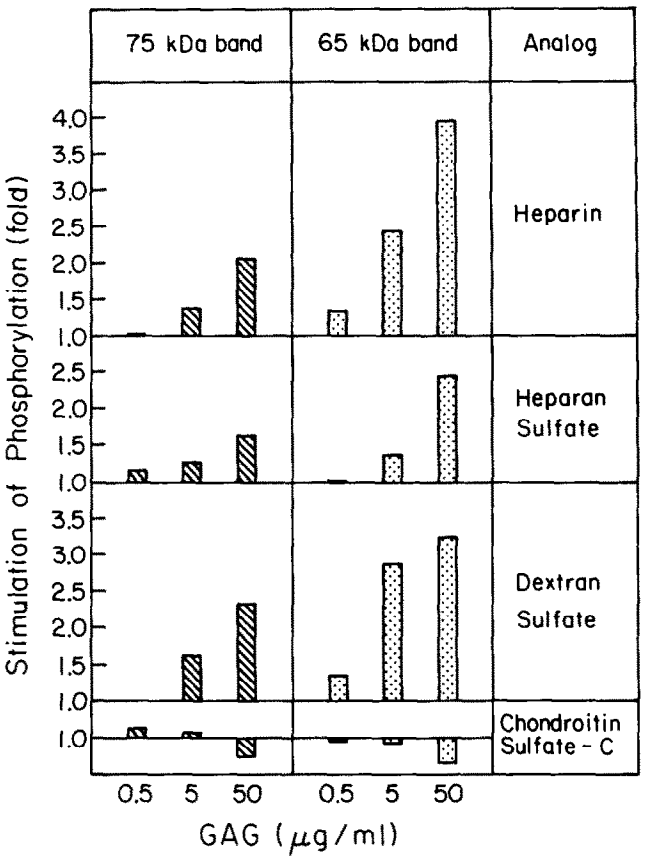


Fig. 3. Effect of heparin and other glycosaminoglycans (GAG) on the phosphorylation of the 75 and 65 kDa polypeptide bands of vitronectin. Duplicate samples (50 μl) containing vitronectin (2 μM) in Hepes (50 mM), pH 7.5 were incubated (120 min at 23°C) with heparin, heparan sulfate, dextran sulfate and chondroitin sulfate C. The phosphorylation reaction was initiated by the addition of the catalytic subunit of PKA (4 μl of 1 mg/ml) and 6 μl of a solution containing $Mg(CH_3COO)_2$ (100 mM) and [γ-^{32}P]ATP (0.5 mM, 0.5 Ci/mmol). The reaction was arrested after different times (0–15 min) by mixing the samples with 14 μl of a solution containing SDS (10%) and 2-mercaptoethanol (3.75 M) and boiling for 3 min. The incorporation of [^{32}P] was determined quantitatively by counting the 75 kDa and 65 kDa Coomassie blue stained bands of vitronectin excised from a dry 10% SDS-PAGE-R.

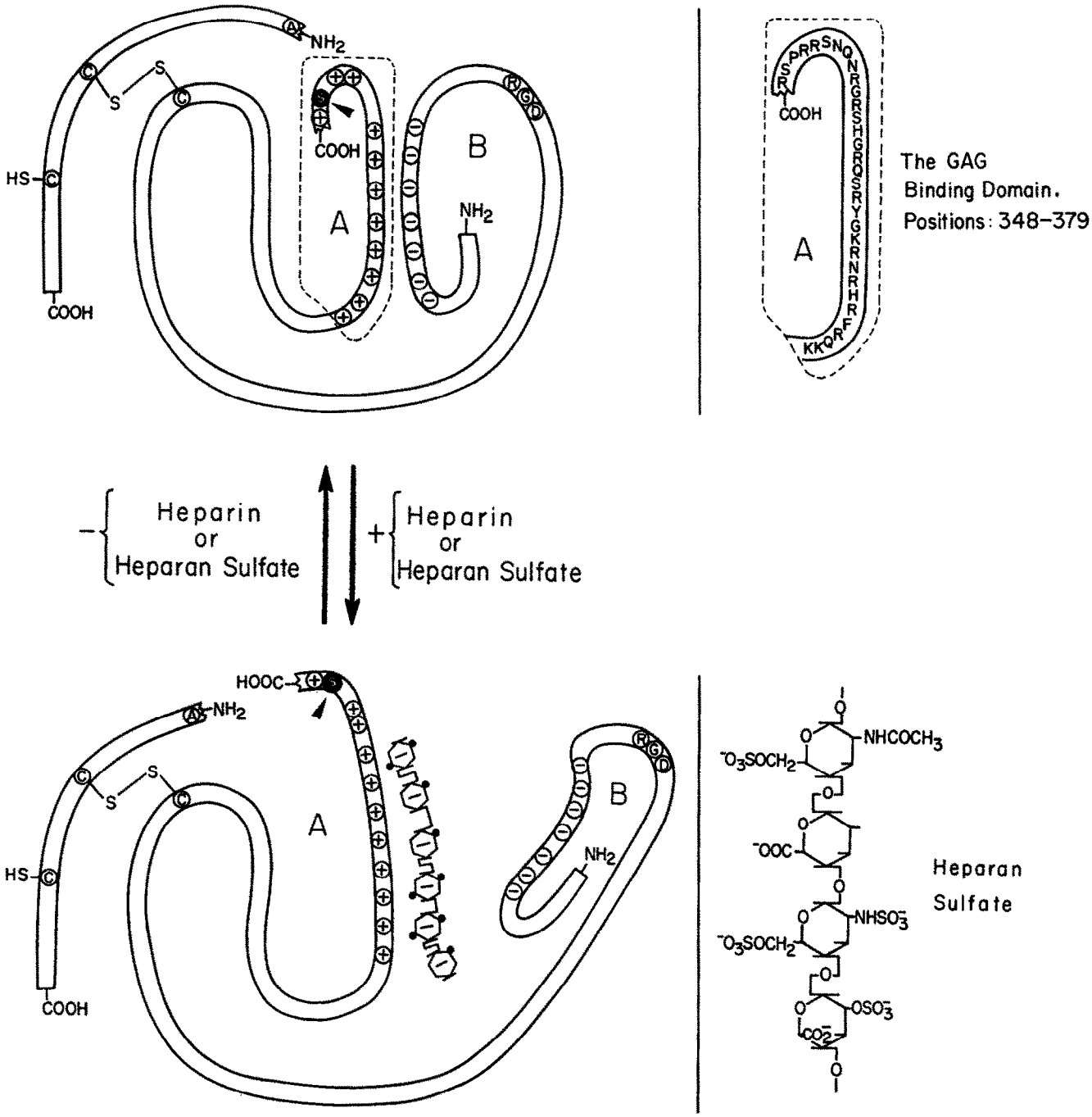
the 65 kDa chain in V_{65+10} [10,12]. We assumed therefore that the endogenous cleavage which converts V_{75} into V_{65+10} may bring about a conformational change whereby Ser^{378} becomes 'buried' and inaccessible to the kinase. Indeed, it is possible to achieve a phosphorylation of Ser^{378} in V_{65+10} if the reaction is carried out in the presence of heparin (Fig. 1, compare lanes C and D). Furthermore, if the relative stoichiometry of phosphorylation of V_{65+10} and V_{75} is determined as a function of pH, it can be seen that in the presence of heparin, not only V_{75} but also V_{65+10} undergoes a quantitative phosphorylation at physiological pH (Fig. 2). In the absence of heparin such a V_{65+10} / V_{75} phosphorylation ratio can be achieved only at pH 6.0, where the structure of V_{65+10} is presumably loosened to make Ser^{378} accessible to phosphorylation. At pH 7.5, the stoichiometry of phosphorylation of V_{65+10} was found to be 0.99 ± 0.02 mol ^{32}P/mol in the presence of heparin but only 0.15 ± 0.03 mol in the absence of this GAG.

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 control experiments similar to those shown in Fig. 1D, we found that heparin actually reduces the rate of phosphorylation of a protein (histone IIb) and a peptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly [20]) which are commonly used substrates of PKA [21]. In the presence of heparin the rate of phosphorylation of the protein is reduced ~1.6-fold and that of the peptide is reduced ~3.6-fold. It seems therefore that heparin exerts its major effect on the conformation of V_{65+10}, exposing its otherwise 'buried' Ser^{378} and allowing its rapid stoichiometric phosphorylation. The physiological proteolytic nicking of V_{75} to yield V_{65+10} thus yields a structurally flexible form of vitronectin which can now 'sense' and conformationally respond to heparin.

In an attempt to assess the possible physiological significance of the heparin dependency in the phosphorylation of V_{65+10} and V_{75}, we compared the ability of several GAG analogs to stimulate this phosphorylation. As seen in Fig. 3, heparan sulfate (a widely distributed GAG found in the arteries, on cell surfaces and the basal laminae) also brings about a significant stimulation of the PKA phosphorylation of both V_{75} and V_{65+10}, but chondroitin sulfate-C does not. Interestingly, the highly charged heparin analog dextran sulfate stimulates the phosphorylation of vitronectin even better than heparin (Fig. 3), suggesting that the negative charges of heparin play an important role in this stimulation, probably interfering with the ionic interactions between the cluster of basic amino acids found at the heparin-binding domain of vitronectin (A) and repelling the negatively charged domain (B) (Scheme I).

Furthermore, when recalcified plasma is phosphorylated by PKA, then chromatographed on immobilized heparin, the phosphorylated V_{75} is excluded at physiological pH and ionic strength, whereas V_{65+10} is adsorbed on the column and requires a higher ionic strength to be detached (Fig. 4), suggesting that under physiological conditions V_{65+10} has a distinctly higher affinity for heparin (see also [22]). The structural change resulting from the cleavage of V_{75} into V_{65+10} may therefore promote the binding of vitronectin to tissue surfaces that contain GAGs such as heparan sulfate or heparin. In this context it is noteworthy that the binding of heparin to vitronectin was shown to be enhanced by thrombospondin [23] and by the thrombin-antithrombin III complex [19].

On the basis of previous findings and of the observations reported here, we propose that the conformational change triggered by the V_{75} to V_{65+10} conversion



Scheme I. Schematic representation of the conformation of V_{65+10} at physiological pH, in the absence and in the presence of heparin or heparan sulfate. Note the electrostatic interactions between the positively charged heparin binding domain (A) and the negatively charged N-terminal domain (B). Also note that in the absence of heparin, Ser^{378} (arrow) is inaccessible to phosphorylation by PKA (buried), and that upon binding of heparin (or heparan sulfate), Set^{378} becomes exposed.

and the subsequent heparin-dependent PKA phosphorylation could be a physiological regulatory process. This proposal stems from the following facts: (a) V_{65+10} is a normal constituent of human blood, present in an equal amount to V_{75} in 38–59% of the population [17,24], and in a large excess over V_{75} in up to 22% of the population [24]; (b) The PKA phosphorylation of V_{65+10} is heparan sulfate (or heparin) dependent; (c) Heparan sulfate is present in the sub-endothelium of blood vessels and in platelets [2,25]; (d) Plasma V_{65+10} adheres to immobilized heparin at physiological pH and ionic strength (see also [26]); (e) The K_m's of PKA for vitronectin, ATP and Mg^{2+} are compatible with the concentrations of these blood constituents at the locus of a hemostatic event (Korc-Grodzicki and Shaltiel, unpublished results); and

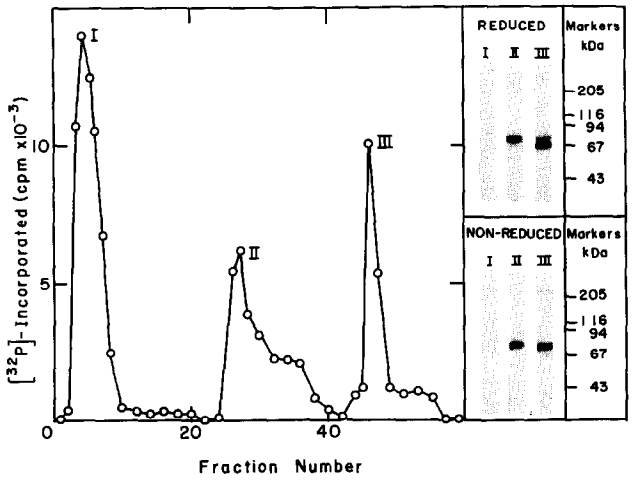


Fig. 4. Immobilized heparin separates plasma V_{75} from V_{65+10} under physiological pH and ionic strength. To human plasma (250 μl) we added $CaCl_2$ (final concentration 20 mM). After standing for 1 h at 4°C, the fibrin clot was removed by centrifugation (5 min at 12000 rpm) and the supernatant was subjected to phosphorylation with the C subunit of PKA and [γ-^{32}P]ATP at pH 6.0 (see section 2), then dialyzed against a sodium phosphate buffer (25 mM, pH 6.0) which included benzamidine (2 mM) and glutathione (1 mM), The sample was then applied on a heparin-agarose column (0.5×4.0 cm) equilibrated with the same buffer. The column was washed with 10 ml of the same buffer and then elution was attempted with 10 ml of a sodium phosphate buffer (50 mM, pH 7.2), then with 10 ml of the sodium phosphate buffer (50 mM, pH 7.2) which also contained 0.5 M NaCl. Fractions (0.5 ml) were collected, counted, and each of the peak fractions (I, II and III) was subjected to SDS-PAGE (7% gels) under R and NR conditions, then to autoradiography (right panels) to detect the presence of V_{75} and of V_{65+10}.

(f) PKA is specifically released from physiologically stimulated platelets alongside with its co-substrate ATP [11,12]. In any case, the heparin-dependent PKA phosphorylation of V_{65+10} should be kept in mind when working with heparinized blood, and in studying the effect of heparin as an anticoagulant.

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