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Localization of a fibrin polymerization site complementary to Gly-His-Arg sequence

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Dansyl-labeled tetrapeptide Gly-His-Arg-Pro which mimics the central fibrin polymerization site was used to investigate its binding to a number of fibrinogen fragments containing different numbers of domains. The tetrapeptide was found to bind to fragments D_H(95 kDa), D_L(82 kDa) and D_Y(63 kDa) but not to the TSD(28 kDa) fragment. The D_Y fragment differs from the TSD by the presence of β and βC domains. Therefore these domains, which are formed by the C-terminal part of the β chain, possess a polymerization site complementary to the Gly-His-Arg containing counterpart.

Fibrin; Fibrinogen fragment; Synthetic peptide; Polymerization site

1. INTRODUCTION

Polymerization of fibrin occurs due to the interaction between complementary polymerization sites. The fibrinogen molecule has two sets of these sites; one situated in the central part, another one in each terminal part of the molecule [1]. The cleavage of fibrinopeptides A and B (FPA and FPB) by thrombin from the N-terminal parts of the fibrinogen Aα and Bβ polypeptide chains leads to an exposure of the central set of the polymerization sites and spontaneous fibrin polymerization due to the interaction of the latter with the complementary sites located in the terminal regions of the neighboring fibrin molecules. It was shown that the sequences Gly-Pro-Arg and Gly-His-Arg which are exposed on α and β chains after removal of the FPA and FPB, respectively, play an important role in the formation of the central polymerization sites. Synthetic peptides Gly-Pro-Arg-Pro and Gly-His-Arg-Pro were shown to bind to fibrinogen [2]. They also bind to the D_1-fragment [3] which derives from the terminal region of fibrinogen and contains intact C-terminal regions of both β and γ chains. The D_3-fragment binds Gly-His-Arg-Pro but does not bind Gly-Pro-Arg-Pro, indicating that the 13 kDa C-terminal region of the γ-chain, missing in the D_3-fragment, is involved in the formation of a polymerization site complementary to the latter sequence [4]. A number of attempts have been made to further localize this site on the fibrinogen molecule [5–10].

Less is known about the other polymerization site complementary to the Gly-His-Arg sequence. Based on the high homology between fibrinogen β and γ chains it was hypothesized that this binding site should be situated in the C-terminal part of the β-chain, [11] although no direct experimental evidence exists. To test this hypothesis and to further localize this site we have prepared a Gly-His-Arg-Pro peptide labeled with the fluorescent Dansyl group and analyzed its binding to different fragments derived from the C-terminal part of fibrinogen.

2. MATERIALS AND METHODS

2.1. *Preparation of proteins and fragments*

Bovine fibrinogen (clottability 98%) was prepared from oxalate bovine plasma by salting out with sodium sulphate [12]. The D_H(95 kDa) fragment was obtained from a 2-h plasmin digest of fibrinogen and purified by the method described in ref. [13]. The D_L(82 kDa) fragment was obtained from an early pepsin digest of the D_H fragment as described earlier [13]. The D_Y(63 kDa) fragment was prepared by chymotrypsin digestion of the D_L fragment as described in ref. [14]. The TSD(28 kDa) fragment was prepared from prolonged pepsin digest of the D_H fragment as was reported earlier [15]. The fibrin monomer was prepared by dissolving the non-crosslinked fibrin clot at 4°C with 0.02M acetic acid as described in ref. [16].

2.2. *Peptide synthesis*

Gly-His-Arg-Pro peptide (GHRP) was synthesized by condensation of benzyl-oxycarbonyl-glycyl-histidine and *N*-(arginyl-prolyl)-*N'*-tert-butyloxycarbonyl-he xamethylene diamide in the presence of diphenylphosphorylazide. The tert-butyloxycarbonyl group was removed by treating the peptide with acetic acid saturated with HCl. A Dansyl group was introduced at the C-terminus of the peptide by reaction

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with Dansylchloride. The final product was deblocked by catalytic hydrogenolysis and purified by chromatography on silicagel. The peptide obtained (GHRP-Dansyl) was homogeneous according to thin-layer chromatography and HPLC.

2.3. *Inhibition experiments*

The polymerization process was initiated by 10-fold dilution of the concentrated fibrin monomer solution (2.5 mg/ml) with 0.02 M veronal buffer, pH 7.4, containing 0.13 M NaCl, 1 mM $CaCl_2$, 2 mM ε-aminocaproic acid and different amounts of the D_H fragment. The appearance of the fibrin clot was registered visually. The inhibitory effect of the D_H fragment was expressed by the value $(t-t_0)/t_0$, where t_0 and t represent clotting times without and with inhibitor, respectively [17]. In the experiments where the eliminating effect of the GHRP-Dansyl peptide on anticlotting activity of the D_H fragment was studied the same amount of the D_H (0.4 mg/ml) was incubated with various concentrations of the peptide and the inhibitory effect of the mixture was tested.

2.4. *Binding experiments*

The equilibrium dialysis was performed in test tubes using Spectra/Por molecularporous membrane tubing (6.4 mm diameter). Each fragment was dissolved in 1 ml of 0.05 M Tris, pH 7.4, 0.1 M NaCl, 1 mM $CaCl_2$ and 0.02% NaN_3 buffer (TBS) to a final concentration of 1×10^{-5} M and dialyzed at room temperature while stirring against 20 ml of the same buffer containing various concentrations of GHRP-Dansyl peptide. After 48 h the fluorescence spectra of the fragment solution and dialysis buffer were recorded on Hitachi 650-10S spectrofluorimeter in the spectral range between 460 and 620 nm with excitation at λ=335 nm. The concentration of fragments in solution was determined spectrophotometrically using the following values of E_{280} for 1% solution: 20.0 for the D_H and D_L fragments [13], 16.0 for the D_Y fragment [14] and 8.0 for the TSD fragment [15].

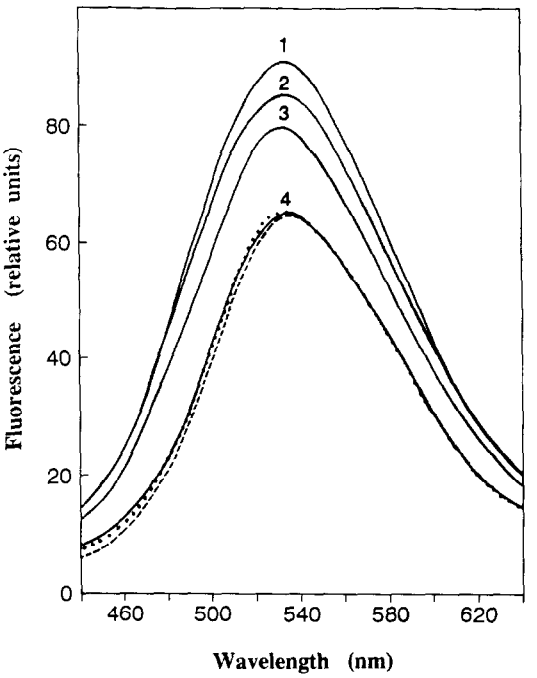

Fig. 2 Binding of the GHRP-Dansyl peptide to different fibrinogen fragments. Fluorescence spectra of the E (dotted line) and D_H, D_L, D_Y and TSD fragment solutions (solid lines 1 through 4) were recorded after dialysis against TBS containing 2×10^{-5} M GHRP-Dansyl peptide whose fluorescence spectrum is presented by the dashed line. The fragment concentration was 1×10^{-5} M.

3. RESULTS AND DISCUSSION

In order to monitor the binding of the synthetic tetrapeptide GHRP to different fibrinogen fragments it was labeled with the fluorescent Dansyl group. The latter has a fluorescence spectrum which does not overlap with that of the protein and allows quantitation of the bound peptide by fluorescence. It was shown that the binding and/or inhibition activities of the synthetic peptides GPR or GHR are very sensitive to simple structural alteration [3]. For example, acetylation of the amino group of the Gly residue in both tetrapeptides leads to elimination of their binding properties. To preserve the activity of the Gly-His-Arg-Pro peptide the Dansyl was coupled to its C-terminal proline residue as described above.

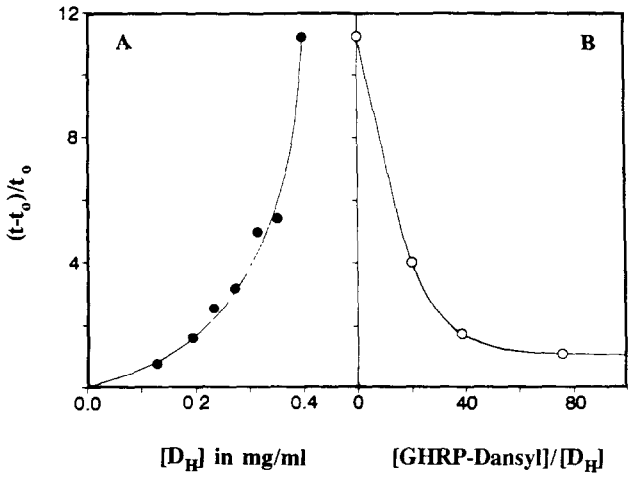

Fig. 1. Influence of the GHRP-Dansyl peptide on the anticlotting effect of the D_H fragment. Panel A represents the inhibition of the polymerization of fibrin by increasing amounts (up to 0.4 mg/ml) of the D_H fragment. Panel B demonstrates progressive elimination of the anticlotting effect of D_H fragment (0.4 mg/ml) when first incubated with various amount of the GHRP-Dansyl peptide, the X axis represents molar ratio.

Although synthetic peptide GHRP does not inhibit the fibrin polymerization process it binds to fibrinogen and/or fragment D [2–4] and reduces their anticlotting effect by blocking the polymerization sites [18]. To check the activity of the Dansyl-labeled tetrapeptide we investigated the influence of the latter on the anticlotting activity of the D_H fragment. The results are presented in Fig. 1. The addition of increasing amounts of the D_H fragment to the monomeric fibrin caused progressive retardation of the clotting time (Fig. 1A). When the D_H fragment was first incubated with increasing amounts of the GHRP-Dansyl the clotting time was substantially reduced indicating inactivation of the D_H fragment by the peptide (Fig. 1B). The degree of the inactivation was approximately the same as for unlabeled GHRP peptide tested earlier [18]. These results

indicate that the labeled peptide is an effective inhibitor of the anticlotting activity of the D_H fragment, i.e. functional activity of the Gly-His-Arg-Pro sequence was not affected substantially by the attachment of the Dansyl group to its C-terminal proline residue.

The fluorescence spectrum of a solution of 1×10^{-5} M GHRP-Dansyl peptide is presented in Fig. 2 (dashed line). Fragment E, which presumably does not bind GHRP [2] and was selected as a control, did not exhibit any fluorescence in this spectral region. After dialysis against the peptide solution, the E fragment solution acquired fluorescence whose intensity coincided with that of free peptide due to reaching an equilibrium (dotted line). At the same time, the fluorescence intensity of the D_H fragment dialyzed against the peptide solution was substantially higher (Fig. 2, solid line 1) due to binding of the peptide to the D_H fragment. Additional experiments performed with the D_H fragment dialyzed against different concentrations of the tetrapeptide allowed us to obtain a saturation curve and calculate the binding constant (k_a) and number of binding sites (n). The value of the k_a and n obtained from four repeat experiments was found to be $3.4 \pm 0.5 \times10^3$ M^{-1} and 0.92 ± 0.1, respectively (Fig. 3). These values are close to those reported earlier for human fibrinogen and D_1 fragment [3,4]. After dialysis the intensity of the fluorescence of the D_L and D_Y fragment solutions was higher than that of the GHRP-Dansyl alone while the intensity of the TSD fragment was the same as that of the tetrapeptide. This indicates that the GHRP-Dansyl binds to the D_L and D_Y fragments and does not bind to the TSD fragment.

We have previously shown that the D_H (95 kDa) fragment consists of five domains. Among them are coiled-coil thermostable TSD domains formed by the N-terminal regions of all three α, β and γ chains and two pairs

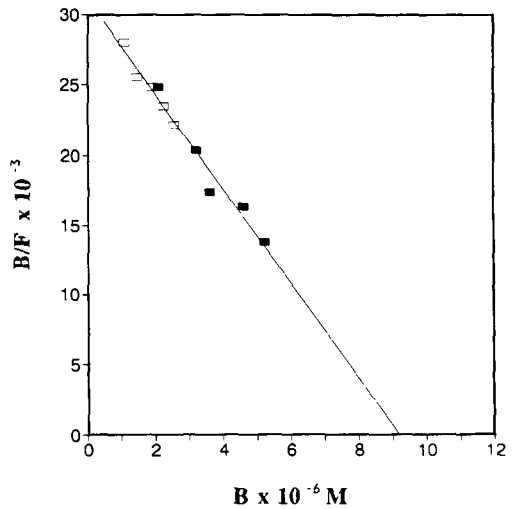

Fig. 3. Scatchard analysis of binding of the GHRP-Dansyl peptide to the fibrinogen fragment D_H. Open and closed squares represent results obtained from two different experiments. B, concentration of the peptide bound to D_H fragment; F, concentration of free peptide.

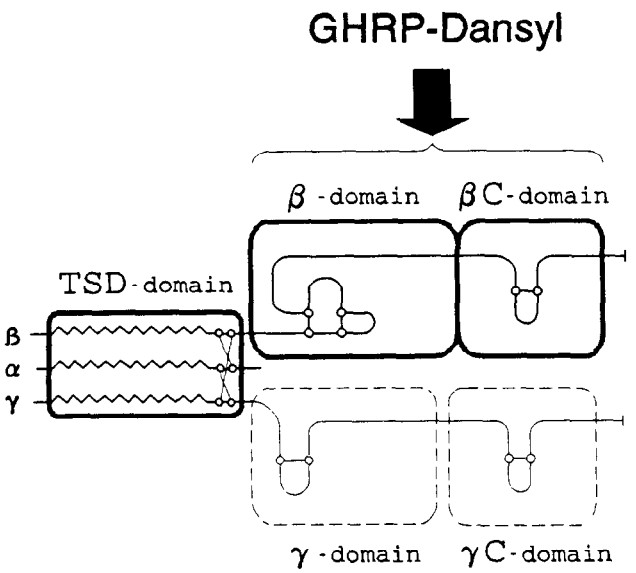

Fig. 4. Diagram of the arrangement of TSD, β, βC and γ, γC domains in the D_H fragment and binding of GHRP-Dansyl to them. Dashed lines indicate γ and γC domains which are removed upon preparation of the D_Y fragment. Individual polypeptide chains are denoted as α, β and γ.

of thermolabile domains, β, βC and γ, γC, formed by the C-terminal regions of the β and γ chains, respectively [14,19]. The domain structure of the D_H fragment is presented in Fig. 4. These domains could be proteolytically removed in a step-wise manner to produce D_L(82 kDa), D_Y(63 kDa) and TSD(28 kDa) fragments [14]. The latter is represented by the TSD domain, while the two former contain in addition to the TSD also $\beta + \beta C + \gamma$ and $\beta + \beta C$ domains, respectively. The exact amino acid sequence of the fragments will be published elsewhere (Litvinovich et al., in preparation). D_Y is the smallest fragment which still binds Dansyl labeled tetrapeptide (Fig. 2). Because it differs from the TSD fragment by the presence of the β and βC domains, the latter two contain the polymerization site complementary to the central Gly-His-Arg containing counterpart. The β and βC domains are formed by the C-terminal part of the β chain [14,19]. Thus this region of the fibrinogen molecule is involved in the formation of the polymerization site complementary to the Gly-His-Arg containing counterparts as was predicted by Doolittle and Laudano [11].

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