PRIMARY POLYMERIZATION SITES IN THE D-DOMAIN OF HUMAN FIBRINOGEN

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INTRODUCTION

The conversion of fibrinogen into a fibrin clot is initiated by the limited thrombin proteolysis resulting in the release of fibrinopeptide A and the exposure of NH₂-terminal polymerization sites "A" in the E domain of the fibrinogen molecule. Complementary polymerization sites "a" capable of interacting with the NH₂-terminal sites have been attributed to the D domain, but their exact location in the fibrinogen molecule is uncertain. There are inconsistent preliminary data indicating that sites "a" may be present in sequences either Y₁₉₅-₂₆₄ or Y₂₆₅-₄₁₁. The present studies were initiated to assess this discrepancy and identify the location of a polymerization sites "a" in the D domain.

MATERIALS AND METHODS

Peptides - A 9-amino acid fibrinogen peptide fragment (GPRVVERHK) corresponding to residues 17-24 of the Aa chain, containing Lys at the COOH terminus which is not present in the natural sequence, was synthesized by Peninsula. The peptide was dissolved in 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.3 (PBS) and radioiodinated using IODO-beads (Bio-Rad).

Inhibition of fibrin monomer polymerization by GPRVVERHK was measured spectrophotometrically at 350 nm.

Peptide cross-linking - Fibrinogen dissolved in PBS was mixed with ¹²⁵I-GPRVVERHK and binding proceeded for 30 min at room temperature. The primary cross-linking agent used in this study was disuccinimidyl suberate (DSS, Pierce Chemical Co.). The cross-linking reactions were terminated after 20 min at room temperature by addition of 1 M glycine buffer, pH 8.0. Fibrinogen cross-linked with the radioiodinated GPRVVERHK was hydrolysed by plasmin (0.5 CTA) for 2 hours at 37° C in the presence (10 mM) or absence of calcium ions. Digestion was stopped by addition of trasyrol (500 KIU), aliquots of the digest were mixed with Laemmli Sample buffer and separated by SDS PAGE.

Preparative recovery of the cross-linked GPRVVERHK - Fragment D1, the major fibrinogen cleavage product cross-linked with ¹²⁵I-GPRVVERHK, was separated from plasmic digest of fibrinogen by immunoaffinity chromatography on anti-FgD antibodies immobilized on Sepharose 4B. FgD₁-GPRVVERHK was further digested with 0.1 CTA units of plasmin in the presence of 20 mM EGTA for 24 hours at 37° C. The digest
was separated by HPLC on the reverse phase C₁₈ column. Peptide fractions containing the radioactive GPRVVERHK were rechromatographed and directly applied into a gas phase sequenator (Applied Biosystem model 475A).

RESULTS

The peptide GPRVVERHK bound to fibrin monomers and when added in a 4000-fold molar excess abolished their polymerization. However, it was approximately 10-fold less potent in inhibiting of fibrin monomer polymerization than GPRP. IC₅₀ of GPRVVERHK and GPRP were 385 μM and 39 μM, respectively.

To locate a binding site for GPRVVERHK in the fibrinogen molecule, this protein was preincubated with 22.59 μM of ¹²⁵I-GPRVVERHK for 30 minutes at room temperature and then chemical cross-reacting reagent DSS was added. After 20 minutes incubation at room temperature, the DSS was neutralized with 1 M glycine buffer, and samples of fibrinogen solution were analyzed by SDS-PAGE under reducing conditions. Autoradiograms indicated that the radioactive peptide was predominantly bound to the Y chain (Fig. 1: curve 1). Though the concentration of the DSS was very low (0.2 mM), there was a residual cross-linking of the polypeptide chains within the fibrinogen molecule. The specificity of the cross-linking of ¹²⁵I-GPRVVERHK was explored by assessing the capacity of nonlabeled GPRVVERHK to inhibit the interaction of the radiiodinated peptide with polypeptide chains of fibrinogen. To estimate the relative inhibition of the cross-linking reaction by different concentrations of

Fig. 1. Densitometric scanning of autoradiogram showing the specific binding of ¹²⁵I-GPRVVERHK to the Y chain of human fibrinogen. Cross-linking experiments performed in the absence (curve 1) and in the presence of various concentrations of the unlabeled peptide; a 40-fold (curve 2) and 100-fold (curve 3) molar excess was used.