Factor XIIIa Incorporates Thymosin β_4 Preferentially into the Fibrin(ogen) αC-Domains†

Evgeny Makogonenko,‡ Allan L. Goldstein,§ Paul D. Bishop,|| and Leonid Medved*,‡,§

Biochemistry Department, Jerome H. Holland Laboratory for the Biomedical Sciences, American Red Cross, Rockville, Maryland 20855, Department of Biochemistry and Molecular Biology, The George Washington University School of Medicine and Health Sciences, Washington, D.C. 20037, and ZymoGenetics, Inc., Seattle, Washington 98102

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ABSTRACT: It was shown recently that tissue transglutaminase and presumably plasma transglutaminase, factor XIIIa, can covalently incorporate into fibrin(ogen) a physiologically active peptide, thymosin β_4 [(Huff et al. (2002) *FASEB J. 16*, 691−696]. To clarify the mechanism of this incorporation, we studied the interaction of thymosin β_4 with fibrinogen, fibrin, and their recombinant fragments, the γ-module (γ-chain residues 148−411), and the αC-domain (Aα-chain residues 221−610) and its truncated variants by immunoblot and ELISA. No significant noncovalent interaction between them was detected in the absence of activated factor XIII, while in its presence thymosin β_4 was effectively incorporated into fibrin and to a lesser extent into fibrinogen. The incorporation at physiological concentrations of fibrin(ogen) and factor XIII was significant with molar incorporation ratios of thymosin β_4 to fibrinogen and fibrin of 0.2 and 0.4, respectively. Further experiments revealed that although activated factor XIII incorporates thymosin β_4 into the isolated γ-module and αC-domain, in fibrin the latter serves as the major incorporation site. This site was further localized to the COOH-terminal portion of the αC-domain including residues 392−610.

Activation of the coagulation cascade upon vascular injury results in generation of thrombin, which converts fibrinogen into fibrin. Fibrin polymerizes spontaneously to form blood clots that seal damaged places, thus preventing the loss of blood. Fibrin also serves as a provisional matrix on which various cell types adhere, migrate, and proliferate replacing fibrin with normal tissues during subsequent wound healing processes. Factor XIIIa, a plasma transglutaminase, covalently cross-links the fibrin clot, reinforcing its structure. In addition, it also cross-links to fibrin a number of physiologically active proteins, which may modulate the properties of the fibrin matrix. For example, covalent incorporation of α_2-antiplasmin increases resistance of the matrix to fibrinolysis, and incorporation of fibronectin may affect its ability to support cell adhesion and migration (*1*, *2*). It was reported recently (*3*) that tissue transglutaminase can selectively incorporate into fibrin thymosin β_4, another physiologically active protein that stimulates various cell activities.

Thymosin β_4 is a member of the β-thymosin family of highly conserved polar 5-kDa polypeptides found in various tissues and cell types (*4*, *5*). Originally purified from the thymus and regarded as a thymic hormone, thymosin β_4 was then found to be involved in multiple biological processes (*6*, *7*). As the main G-actin sequestering peptide, it plays an important role in regulation of actin assembly during cell proliferation, migration, and differentiation (*7*−*9*, *10*). Numerous studies implicate thymosin β_4 in the regulation of cancerogenesis, inflammation, angiogenesis, and wound healing. It was found that thymosin β_4 expression regulated tumorigenicity and metastatic activity in malignant cell lines through actin-based cytoskeletal organization (*11*). It was also shown that thymosin β_4 may stimulate tumor metastasis by activating cell migration and angiogenesis (*12*). Thymosin β_4 was found to be elevated in tube forming endothelial cells; it increases their attachment, spreading, and migration, thus promoting angiogenesis (*13*, *14*). Thymosin β_4 was also found in ulcer extracts and wound fluids at high concentrations and was suggested to function as an antibacterial factor (*15*). The stimulating role of thymosin β_4 in wound healing was demonstrated in several studies with animal models. When added topically or administered intraperitoneally, thymosin β_4 enhanced dermal wound healing in a rat full thickness model (*16*). The ability to accelerate dermal wound healing has also been observed in db/db diabetic mice and in aged mice (*17*). Thymosin β_4 has also been shown to accelerate healing of the corneal epithelium after burn injuries and to down regulate a number of corneal cytokines and chemokines reducing the inflammatory response (*18*).

It was found that thymosin β_4 serves as a specific substrate for tissue transglutaminase (*19*) and can be selectively cross-linked by it to collagen, actin, fibrinogen, and fibrin, proteins that are also involved in the aforementioned processes (*3*). It was also shown that after activation of platelets with thrombin, thymosin β_4 is released and cross-linked to fibrin

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* To whom correspondence should be addressed. Tel: (301) 738-0719. Fax: (301) 738-0740. E-mail: medvedL@usa.redcross.org.

‡ American Red Cross.

§ The George Washington University School of Medicine and Health Sciences.

|| ZymoGenetics.

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in a time- and calcium-dependent manner (*3*). On the basis of this observation and the fact that platelet factor XIII is coreleased from stimulated platelets, Huff et al. (*3*) suggested that cross-linking of platelet-released thymosin β_4 to fibrin could be mediated by factor XIIIa and could provide a mechanism to increase the local concentration of thymosin β_4 near sites of clots and tissue damage, where it may contribute to wound healing, angiogenesis, and the inflammatory response.

Fibrinogen is a chemical dimer consisting of two identical subunits, each composed of three polypeptide chains, Aα, Bβ, and γ, held together by a number of disulfide bonds (*20*, *21*). The disulfide-linked NH_2-terminal portions of all six chains form the central E region, while the COOH-terminal portions form two terminal D regions and two αC-domains (*20*−*23*). Upon conversion of fibrinogen into fibrin, thrombin-mediated removal of the NH_2-terminal fibrinopeptides A and B from the fibrinogen Aα and Bβ chains, respectively, results in exposure of their active sequences (polymerization sites) and enables interaction between E and D regions of neighboring molecules (DD/E interaction) to form a fibrin polymer. The polymer becomes cross-linked by factor XIIIa through the COOH-terminal portions of the fibrin α- and γ-chains (*21*). The intermolecular cross-linking of the γ-chains of the adjacent D regions occurs rapidly resulting in γ−γ-dimers, while cross-linking between the α-chains (αC-domains) occurs more slowly and results in formation of α-polymers (*24*, *25*). In addition, the α-chains serve for cross-linking to fibrin of such proteins as fibronectin, α_2-antiplasmin, and PAI-2 (*26*−*29*). Thus, it is tempting to hypothesize that these chains could also be involved in the cross-linking of thymosin β_4.

To test this hypothesis and to clarify the mechanism of the incorporation of thymosin β_4 into fibrin(ogen), we studied its interaction with fibrinogen, fibrin, and their recombinant fragments (domains) in the absence and presence of factor XIIIa. The study revealed that although there is no substantial noncovalent interaction between fibrin(ogen) and thymosin β_4, factor XIIIa efficiently cross-links the latter to both fibrinogen and fibrin and that cross-linking occurs mainly through the COOH-terminal portion of their αC-domains including residues 392−610.

EXPERIMENTAL PROCEDURES

Proteins and Reagents. Human fibrinogen depleted of plasminogen, fibronectin, and von Willebrand factor was purchased from Enzyme Research Laboratories (South Bend, IN). The recombinant αC-fragment corresponding to the human fibrinogen αC-domain (residues Aα221-610) and its truncated variants corresponding to the NH_2- and COOH-terminal halves (residues Aα221-391 and Aα392-610, respectively) were produced in *Escherichia coli* using the pET20b expression vector as described earlier (*30*). The recombinant γ-module comprising residues 148−411 of the human fibrinogen γ-chain was produced in *E. coli* using the same expression vector (*31*).

Bovine thrombin (1000 NIH u/mg), aprotinin (4.4 TIU/mg), anti-rabbit IgG-horseradish conjugate, and fluorescein isothiocyanate (FITC)[1] were purchased from Sigma. Recombinant factor XIII was prepared as described in ref *32*. Synthetic thymosin β_4 was provided as a gift by RegeneRx Biopharmaceuticals, Inc. (Bethesda, MD). Anti-thymosin β_4 serum was prepared as described earlier (*33*).

Activation of Factor XIII. Factor XIII kept in 25 mM Tris buffer, pH 8.0, with 0.15 M NaCl (TBS), was activated either with thrombin or with $CaCl_2$; the latter was made to avoid the presence of thrombin, which could potentially activate fibrinogen. Thrombin-activated FXIII [FXIIIa(Thr)] was made by addition of bovine thrombin and $CaCl_2$ to final concentrations of 25 NIH u/mL and 2.5 mM, respectively. Ca^{2+}-activated [FXIIIa(Ca)] was made by addition of $CaCl_2$ to final concentration of 50 mM. Final concentration of FXIII in both mixtures was 1.5 mg/mL; both mixtures were incubated at room temperature for 10 min prior experiments.

Labeling of Thymosin β_4 with FITC. Fluorescence-labeled thymosin β_4 was prepared by the reaction with fluorescein isothiocyanate (FITC). Thymosin β_4 was transferred in 0.1 M $NaHCO_3$ buffer, pH 9.5, by gel-filtration on NAP5 Sephadex G-25 column (Amersham Biosciences) followed by addition of a 1.2 molar excess of FITC and incubation of the mixture at 37 °C for 2 h in the dark. Nonreacted FITC was removed on NAP5 column. The degree of labeling determined spectrophotometrically as described in ref *34* was found to be 0.9 mol of FITC per mol of thymosin β_4.

Solid-Phase Binding Assay. The interaction between thymosin β_4 and fibrin(ogen) and its fragments in the presence or absence of FXIIIa was studied by ELISA using plastic microtiter plates (Fisher Scientific). Wells of microtiter plates were coated overnight at +4 °C with fibrinogen and fibrin at 10 μg/mL or with the recombinant fragments at 20 μg/mL, all in 0.1 M $NaHCO_3$ buffer, pH 8.3. Fibrin was made by addition to the wells of a mixture containing 10 μg/mL fibrinogen, 1 NIH u/mL thrombin, and 0.044 TIU/mL aprotinin, followed by overnight incubation at +4 °C. The wells were then blocked by incubation with Super Blocker (Pierce) at 37 °C for 1 h. Following washing with TBS containing 0.05% Tween-20 (TBS-Tween), the indicated concentrations of thymosin β_4, FXIII, FXIIIa(Thr), and FXIIIa(Ca) were added to the wells and incubated for 2−2.5 h at 37 °C. Bound (incorporated) thymosin β_4 was detected by the reaction with rabbit anti-thymosin β_4 serum and peroxidase-conjugated anti-rabbit IgG. A TMB Microwell Peroxidase Substrate (Kirkengaard and Perry Laboratories Inc.) was added to the wells, and the incorporated thymosin β_4 was measured spectrophotometrically at 450 nm.

Incorporation of Thymosin β_4 into Fibrinogen and Fibrin. Reactions of incorporation of FITC-labeled and unlabeled thymosin β_4 into fibrinogen and fibrin were performed in Eppendorf tubes containing a mixture of fibrinogen at 3 mg/mL (9 μM) and thymosin β_4 or FITC-labeled thymosin β_4 at 150 μg/L (30 μM) in 100 μL of TBS with 2.5 mM $CaCl_2$. The reactions were initiated by addition of FXIIIa(Ca) or FXIIIa(Thr) to final concentration of 30 μg/mL. The final concentrations of thrombin in the FXIIIa(Thr)-containing mixtures and Ca^{2+} in the FXIII(Ca)-containing mixtures were 2.5 NIH u/mL and 2.5 mM, respectively, sufficient to rapidly form the fibrin clot, which was observed visually. The reactions with FITC-labeled thymosin β_4 lasted for 4 h at

[1] Abbreviations: FITC, fluorescein isothiocyanate; FXIIIa(Thr) and FXIIIa(Ca), factor XIII activated with thrombin and 50 mM $CaCl_2$, respectively; TBS, Tris buffered saline (25 mM Tris buffer, pH 8.0, with 0.15 M NaCl).

37 °C in the dark and were stopped by heat inactivation of the enzymes in boiling water for 5 min during which fibrinogen and fibrin denatured and precipitated. The pellets were centrifuged and washed three times in TBS and then solubilized as described in ref *35*. The amounts of fibrin(ogen) and FITC-labeled thymosin β_4 in the solubilized pellet were determined spectrophotometrically using absorption molar coefficients $E_{280,1\%} = 15.0$ (*36*) and $\epsilon_{495} = 72\ 000\ M^{-1}\ cm^{-1}$ (*34*), respectively. To prepare samples with unlabeled thymosin β_4 for analysis by SDS−PAGE and Western blot, the reaction mixtures at the indicated time were heat-inactivated as stated previously and solubilized by addition of sample buffer (Invitrogen) containing SDS and reducing agent.

Kinetic Analysis. To analyze kinetics of the incorporation of thymosin β_4 into different fibrin(ogen) fragments, the mixtures were immobilized onto the wells of microtiter plates (as described previously, except that the concentration of all fragments was 20 μg/mL) and incubated with several concentrations of thymosin β_4 in the presence of 10 μg/L thrombin-activated factor XIIIa. The incubation mixtures were inhibited every 15 min during 1 h of incubation by the addition of iodacetamide to a final concentration 10 mM, and incorporated thymosin β_4 at each time point was detected with rabbit anti-thymosin β_4 serum as described previously. The initial rates of the reaction of incorporation (V) at different concentrations of thymosin β_4 were determined from the slopes of the reaction time course plots and expressed as tangent $\alpha = A_{450}/t$ (min), where A_{450} represents absorbance at 450 nm in optical units (o.u.), which is proportional to the amount of incorporated thymosin β_4. Apparent Michaelis constants, K_m, were obtained from Lineweaver−Burk plots, $1/V$ (min/o.u.) versus 1/[S] (μM^{-1}), where [S] is concentration of thymosin β_4.

Western Blot Analysis. Detection of thymosin β_4 incorporated into fibrin(ogen) and its fragments was performed as follows. The samples prepared as described previously were electrophoresed and electrotransferred to a nitrocellulose membrane (Invitrogen) as described earlier (*37*). The membrane was blocked with Casein Blocker (Pierce) for 1 h, and thymosin β_4 was detected by the reaction with rabbit anti-thymosin β_4 serum and peroxidase-conjugated anti-rabbit IgG. Visualization of the peroxidase-labeled protein bands was performed by the procedure recommended by the manufacturer using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

RESULTS

ELISA-Detected Incorporation of Thymosin β_4 into Fibrinogen and Fibrin. To test the previous suggestion that factor XIIIa could mediate cross-linking of thymosin β_4 to fibrin(ogen) (*3*), and to clarify the mechanism of such cross-linking, we performed a direct study of the interaction of thymosin β_4 with fibrinogen and fibrin in the presence and absence of recombinant factor XIII. It should be noted that the recombinant factor consists of two a subunits (a_2), in contrast to plasma factor XIII, which consists of two a and two b subunits (a_2b_2). In this respect, recombinant factor XIII corresponds to the platelet form of factor XIII (*32*).

In ELISA experiments, when thymosin β_4 at 150 μg/mL (30 μM) was incubated with immobilized fibrinogen, only a low signal was observed in the absence of factor XIII as well as in the presence of nonactivated factor XIII, suggesting that the interaction between them is very weak, if any (Figure 1). When thymosin β_4 was incubated with immobilized fibrin in the absence or presence of nonactivated factor XIII, the signal was higher but still low. At the same time, in the presence of factor XIIIa, which was activated by the addition of $CaCl_2$ to avoid conversion of fibrinogen into fibrin in the wells, the signal substantially increased, suggesting that factor XIIIa mediates binding (incorporation) of thymosin β_4 into fibrinogen. A similar situation was observed with immobilized fibrin except that the level of the incorporation was higher than that into fibrinogen. The incorporation in both cases was dose-dependent (Figure 1, inset). The incorporation into fibrin was further increased when factor XIII was activated with thrombin instead of Ca^{2+}. Such differences could be due to different specific activities of these two factor XIIIa species. These results indicate that activated factor XIII, in a manner similar to tissue transglutaminase, mediates the incorporation of thymosin β_4 into both fibrinogen and fibrin. They also suggest that there is no significant noncovalent interaction between thymosin β_4 and both fibrinogen and fibrin.

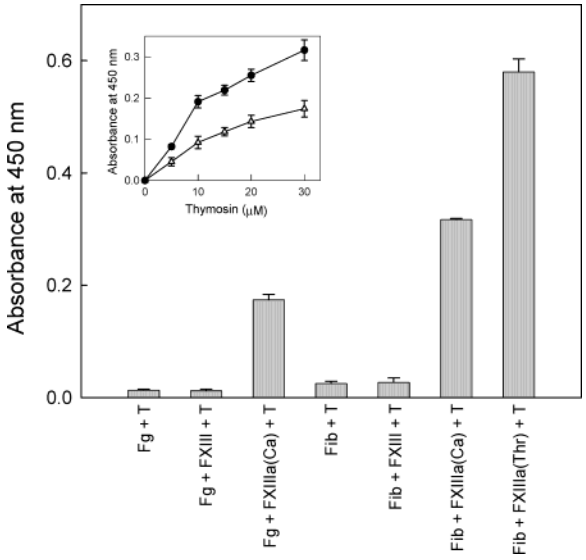

FIGURE 1: ELISA-detected interaction of thymosin β_4 with immobilized fibrinogen and fibrin in the presence and absence of factor XIII. Immobilization of fibrinogen (Fg) and fibrin (Fib), their incubation with thymosin β_4 (T) without and with nonactivated factor XIII (FXIII), thrombin-activated factor XIII [FXIIIa(Thr)] and Ca^{2+}-activated factor XIII [FXIIIa(Ca)], and subsequent detection of bound thymosin β_4 with anti-thymosin β_4 serum were performed as described in Experimental Procedures. Thymosin β_4 was added at 150 μg/mL, all factor XIII species were at 30 μg/mL, and thrombin was at 2.5 NIH u/mL. The inset shows concentration dependence of the incorporation of thymosin β_4 into immobilized fibrinogen (triangles) and fibrin (circles) in the presence of 30 μg/mL FXIIIa(Ca). Error bars represent the standard deviation of three independent determinations.

Further Analysis of the Incorporation of Thymosin β_4 into Fibrinogen and Fibrin. To further characterize factor XIIIa-mediated incorporation of thymosin β_4 into fibrin(ogen), we analyzed a mixture of thrombin, factor XIII, thymosin β_4, and fibrin at different time points by immunoblotting. The mixture and the samples for analysis were prepared as described in Experimental Procedures. The samples were electrophoresed in reduced conditions to separate individual

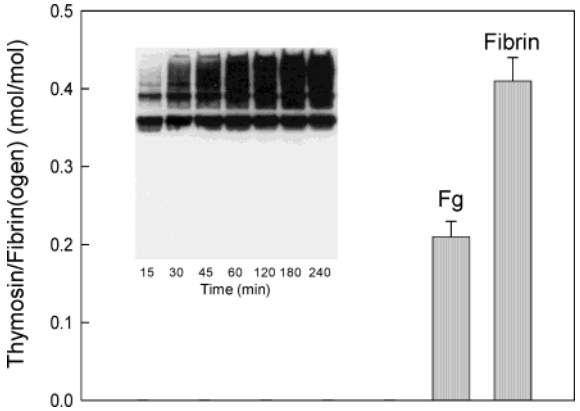

FIGURE 2: Quantitative analysis of the factor XIIIa-mediated incorporation of thymosin β_4 into fibrinogen and fibrin. The reactions of incorporation and determination of the amounts of FITC-labeled thymosin β_4 incorporated into fibrinogen (Fg) and fibrin were performed as described in Experimental Procedures. Error bars represent the standard deviation of three independent experiments. The inset shows the time course of the incorporation of thymosin β_4 into fibrin detected by Western blot analysis. The samples were prepared as described in Experimental Procedures, electrophoresed in 4−12% polyacrylamide gel in reduced conditions, and then electrotransferred to nitrocellulose membrane and probed with anti-thymosin β_4 serum.

chains, α, β, and γ, electrotransferred to a nitrocellulose membrane, and probed with anti-thymosin β_4 serum. The results of immunoblotting presented in Figure 2 indicate that factor XIIIa incorporates thymosin β_4 into fibrin covalently, like tissue transglutaminase, and that the amount of the incorporated (cross-linked) thymosin β_4 seems to reach saturation after 4 h. This time was selected to evaluate the degree of the incorporation. For this purpose, thymosin β_4 was labeled with a FITC chromophore group, which enabled its direct measurement in fibrinogen/thymosin β_4 and fibrin/thymosin β_4 mixtures. Such modification did not influence its incorporation into either fibrinogen or fibrin since the pattern of incorporation revealed by Western blot analysis was essentially the same as that presented in Figure 2. A similar mixture as above but with FITC-labeled thymosin β_4 was incubated for 4 h, after which the degree of incorporation was estimated based on the spectrophotometrically determined amounts of fibrin(ogen) and incorporated FITC-thymosin β_4 in each sample. The results revealed that at the selected conditions, which include physiological concentration of fibrinogen (about 9 μM), factor XIIIa incorporated a substantial amount of FITC-thymosin β_4, about 0.2 and 0.4 mol per mol of fibrinogen and fibrin, respectively (Figure 2).

Incorporation of Thymosin β_4 into Individual Fibrin(ogen) Chains. To establish which of the three fibrin(ogen) chains are involved in cross-linking with thymosin β_4, we analyzed the time course of factor XIIIa-mediated cross-linking of fibrinogen and fibrin in the presence and absence of thymosin β_4 by SDS−PAGE and Western blot. It is well-known that in fibrin factor XIIIa cross-links rapidly the COOH-terminal portions of the γ-chains to produce $\gamma-\gamma$ dimers followed by cross-linking of the α-chains to form α−α dimers, trimers, and α-polymers; fibrinogen is cross-linked in a similar way but at slower rate (*21*). When analyzed by SDS−PAGE in reducing conditions, the bands corresponding to the individual polypeptide chains of fibrinogen and fibrin, Aα, Bβ, γ, and α, β, γ, respectively, were well-resolved

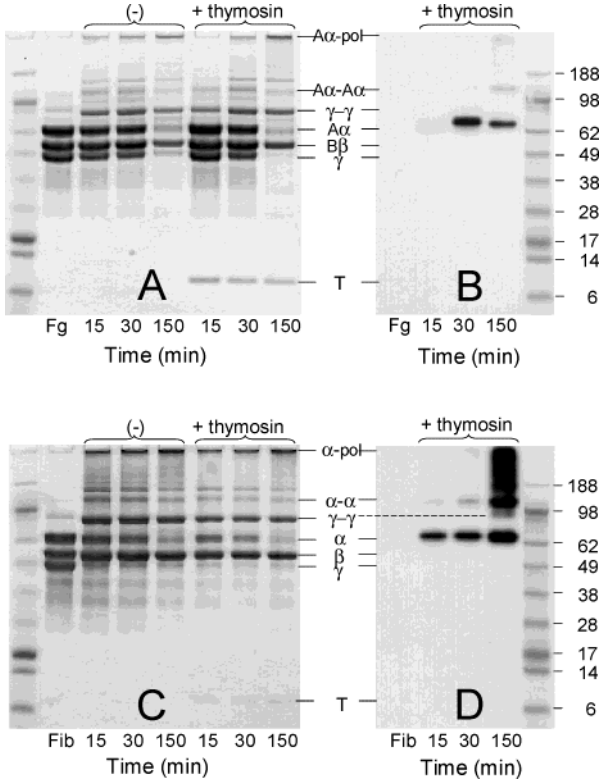

FIGURE 3: Analysis of the factor XIIIa-mediated incorporation of thymosin β_4 into fibrinogen and fibrin by Western blot. The reaction mixtures of fibrinogen and factor XIIIa(Ca) (upper panels) or fibrin and factor XIIIa(Thr) (lower panels) in the absence (−) or presence (+) of thymosin β_4 were heat-inactivated at the indicated time points and prepared for SDS−PAGE analysis as described in Experimental Procedures. The samples were electrophoresed in 4−12% polyacrylamide gels in reduced conditions and then either stained with Coomassie Blue R350 (A and C) or electrotransferred to nitrocellulose membrane and probed with anti-thymosin β_4 serum (B and D). The bars indicate the expected positions for the individual polypeptide chains of fibrinogen (Aα, Bβ, and γ) and fibrin (α, β, and γ), their dimeric (Aα−Aα, α−α, and $\gamma-\gamma$) and oligomeric (Aα−pol, α−pol) forms, and for thymosin β_4 (T). The dashed line in panel D shows the expected position for thymosin β_4 incorporated into $\gamma-\gamma$ dimer (see text). The left outer lanes in panels A and C and the right outer lines in panels B and D contain protein markers of the indicated molecular masses (SeeBlue Plus2 Prestained Standards, Invitrogen).

(Figure 3A,C). Incubation of fibrinogen with factor XIIIa resulted in the progressive depletion of the bands corresponding to the γ- and Aα-chains and simultaneous accumulation of the bands corresponding to the $\gamma-\gamma$ dimers and the Aα−Aα dimers and trimers. The appearance of some material that was observed at the start of the gel most probably corresponds to the Aα polymers. When fibrinogen was incubated with factor XIIIa in the presence of thymosin β_4, no substantial difference in the intensity of the bands corresponding to the individual chains and their cross-linked variants was found (Figure 3A). Similar results were obtained with fibrin except that the cross-linking of its α- and γ-chains occurred more rapidly, as expected, and the amount of the material at the start was higher (Figure 3C). Subsequent Western blot experiments revealed that after 30 min of incubation, substantial amounts of thymosin β_4 were incorporated into fibrinogen Aα-chain and that after 150 min of incubation some thymosin β_4 was also incorporated into the Aα−Aα dimer (Figure 3B). The incorporation of thymosin β_4 into the fibrin α-chain and the α−α dimer was much more

rapid, and after 150 min of incubation, substantial amounts of thymosin β_4 were also observed in higher molecular mass forms of the α-chain (α-polymers) (Figure 3D). It should be noted that in control experiments, we compared incorporation of thymosin β_4 into fibrin by FXIIIa(Thr) and tissue transglutaminase and found that the incorporation occurred in a similar manner, although the rates of the incorporation as well as the α−α cross-linking by the transglutaminase seemed to be slower (not shown).

These results indicate that the fibrinogen Aα- and fibrin α-chains contain the major sites for covalent incorporation of thymosin β_4. At the same time, the appearance after 150 min of incubation of a low-intensity band with the mobility between that of the γ−γ and α−α dimers (Figure 3D) suggests that thymosin β_4 could also be incorporated into the fibrin γ-chains (γ−γ dimer) Alternatively, this band may correspond to a proteolytically truncated variant of the α−α dimer.

Incorporation of Thymosin β_4 into Recombinant Fibrin(ogen) Fragments. It is well-established that the COOH-terminal portions of the fibrinogen Aα- and γ-chains forming the αC-domain and the γ-module contain reactive Gln and Lys residues that are cross-linked by factor XIIIa in fibrin (*21*) and therefore could potentially be involved in cross-linking with thymosin β_4. To test this speculation and to further localize the cross-linking sites for thymosin β_4 in fibrin(ogen), we analyzed the incorporation of thymosin β_4 into the recombinant γ-module (residues γ148−411) and the αC-domain (Aα221−610) and its truncated variants, the Aα221-391 and Aα392-610 subfragments, by SDS−PAGE and Western blotting. Incubation of the αC-domain and the γ-module with factor XIIIa in the presence of thymosin β_4 resulted in effective cross-linking and appearance of their higher molecular mass forms, dimers, trimers, and oligomers (Figure 4A). This is in agreement with the previous observations in which we found a similar cross-linking pattern for both recombinant fragments (*28*, *31*). At the same time, the cross-linking of the Aα221-391 and Aα392-610 subfragments, which contain mainly acceptor Gln and donor Lys residues, respectively (*28*), was much less effective. When the samples were electrotransferred to nitrocellulose membrane and probed with anti-thymosin β_4 serum, substantial amounts of thymosin β_4 were detected in the αC-domain, the γ-module, and their higher molecular mass variants, dimers, trimers, and oligomers (Figure 4B). The incorporation into the Aα392-610 subfragment monomer and oligomers was also substantial, while only very small amount of thymosin β_4 was detected in the Aα221-391 oligomers. These results suggest that thymosin β_4 could be cross-linked to both the αC-domain and the γ-module and that the reactive Lys residues of the Aα392-610 region of the former are involved in the cross-linking.

The above-mentioned observations were confirmed by ELISA. When thymosin β_4 was incubated with the immobilized γ-module or the αC-domain variants in the presence of factor XIIIa, it was incorporated effectively into the γ-module and into the αC-domain and the Aα392-610 subfragment, while the incorporation into Aα221-391 was very low (Figure 5). It should be noted that the incorporation of the γ-module was almost twice lower than that of the αC-domain variants at all concentrations studied (Figure 5, inset). When thymosin β_4 was incubated with the same

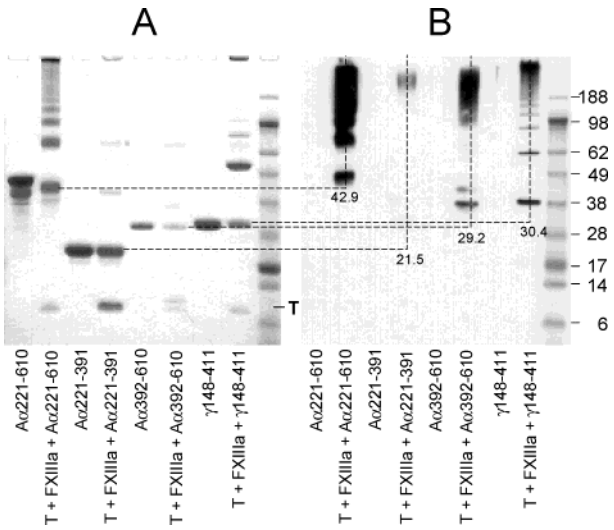

FIGURE 4: Analysis of the factor XIIIa-mediated incorporation of thymosin β_4 into the recombinant fibrinogen fragments by Western blot. The indicated fragments or the reaction mixtures of the fragments at 1 mg/mL with thymosin β_4 (T) at 150 μg/mL and thrombin-activated factor XIIIa (FXIIIa) at 30 μg/mL, all incubated for 2.5 h, were electrophoresed in 4−12% polyacrylamide gels in reduced conditions and then either stained with Coomassie Blue R350 (A) or electrotransferred to nitrocellulose membrane and probed with anti-thymosin β_4 serum (B). The broken lines show the expected positions for the individual fragments, the numbers indicate their apparent molecular masses, and position of thymosin β_4 is indicated by T. The right outer lanes in both panels contain the same protein markers as in Figure 3.

immobilized species in the presence of nonactivated factor XIII or without it, the incorporation was very low in all cases. This suggests that, as in the case with fibrinogen and fibrin, there is no significant noncovalent interaction between thymosin β_4 and the recombinant fragments.

It was previously shown that factor XIIIa cross-linking of the γ-chains of fibrin exhibits apparent Michaelis behavior (*35*). Assuming that factor XIIIa behaves as a Michaelis enzyme when cross-linking thymosin β_4 to the immobilized γ-module and αC-domain variants, one could determine the kinetic parameters of such cross-linking. The analysis of the kinetic data performed as described in Experimental Procedures and presented in Figure 6 revealed the following values of apparent Michaelis constants (K_m) for the reaction of incorporation, 183 ± 29 μM for the incorporation of thymosin β_4 into the γ-module, and 17.6 ± 2.5 and 8.6 ± 3.7 μM for that into the αC-domain and its Aα392-610 subfragment, respectively. The much higher K_m value for the γ-module than those for the αC-domain and its subfragment indicates that the cross-linking of thymosin β_4 to the αC-domain variants is much more efficient. In this connection, the K_m for the Aα392-610 fragment is comparable to the $K_m = 6.2$ μM determined previously for the factor XIIIa-mediated γ−γ cross-linking (*35*). The 2-fold difference in the K_m values for the αC-domain and the Aα392-610 subfragment could be explained by competition between reactive Gln residues of thymosin β_4 and the Aα221-391 region of the αC-domain for reactive Lys residues of the αC-domain Aα392-610 region (i.e., between αC to αC and thymosin β_4 to αC cross-linking). In agreement, the double-reciprocal plot for the αC-domain and the Aα392-610 subfragment exhibits a pattern characteristic for competitive inhibition.

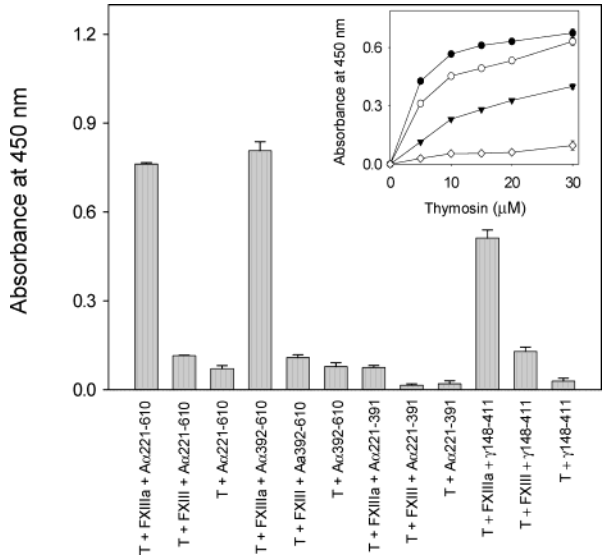

FIGURE 5: ELISA-detected incorporation of thymosin β_4 into the recombinant fibrinogen fragments. Immobilization of the indicated fragments, their incubation with thymosin β_4 (T) in the absence or presence of nonactivated factor XIII (FXIII) and thrombin-activated factor XIII (FXIIIa), and subsequent detection of bound thymosin β_4 with anti-thymosin β_4 serum were performed as described in Experimental Procedures. Thymosin β_4 was added at 150 μg/mL, and all factor XIII species were at 30 μg/mL. Error bars represent the standard deviation of three independent determinations. The inset shows concentration dependence of the incorporation detected by ELISA. Wells of the microtiter plates were coated with the Aα221-610 (open circles), Aα392-610 (closed circles), Aα221-391 (diamonds), and γ148-411 (triangles), and the indicated concentrations of thymosin β_4 were added together with thrombin-activated factor XIIIa followed incubation for 2 h at 37 °C. Bound thymosin β_4 was detected with anti-thymosin β_4 serum as described in Experimental Procedures. The error bars reflect the standard deviation of two independent determinations.

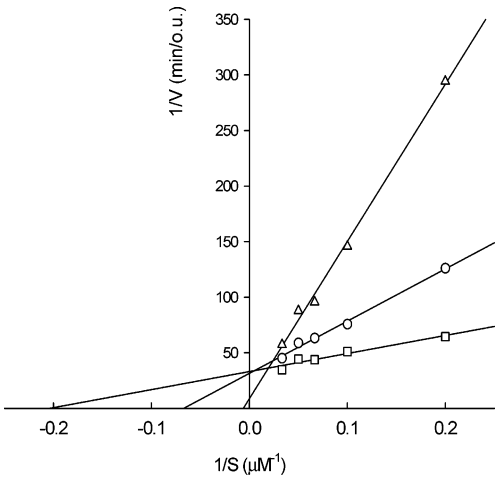

FIGURE 6: Lineweaver−Burk plots of the rate dependence of the reaction of incorporation of thymosin β_4 into the recombinant Aα221-610 (circles) and Aα392-610 (squares) fragments, and the γ-module (triangles) on thymosin β_4 concentration. The initial rates (V) were calculated as described in Experimental Procedures.

Altogether, the results presented in Figures 3−6 indicate that factor XIIIa effectively cross-links thymosin β_4 to the COOH-terminal portion of the isolated αC-domain including residues Aα392-610, that the incorporation into the isolated γ-module is less effective, and that in fibrinogen or fibrin the incorporation occurs mainly into the αC-domains.

DISCUSSION

Fibrin(ogen) plays an important role in wound healing through interactions with physiologically active proteins and cell receptors. Particularly, the fibrin matrix stimulates an inflammatory response and capillary tube formation by endothelial cells (angiogenesis), which are essential steps in the wound healing process, through interaction with the leukocyte integrin Mac-1 and endothelial cell receptor VE-cadherin, respectively (*38*, *39*). Fibrin also interacts with high affinity with basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (*40*, *41*) providing colocalization of these potent stimulators of angiogenesis at sites of fibrin deposition and their contribution to wound healing. Fibrin can also retain insulin-like growth factor-1 (IGF-1) through high affinity interaction with insulin-like growth factor binding protein-3 (IGFPB-3), which forms a complex with IGF-1 (*42*). It was demonstrated recently that another potent angiogenic and wound healing factor, thymosin β_4, can also be incorporated into fibrin by tissue transglutaminase (*3*) and can apparently further increase the wound healing potential of fibrin matrix. The major goal of this study was to clarify the mechanism of this incorporation.

Although all transglutaminases catalyze the same reaction, formation of covalent γ-glutamyl-ϵ-lysyl isopeptide bonds between reactive Gln and Lys residues, and their specificity toward substrates may differ. For example, while factor XIIIa, a plasma transglutaminase, specifically cross-links in fibrin the γ- and α-chains, resulting in the $\gamma-\gamma$ dimers and α-polymers, respectively, tissue transglutaminase is less specific and can also generate $\alpha-\gamma$-chain cross-links (*43*, *44*). The cross-linking patterns for the serine protease inhibitor (serpin), PAI-2, to fibrin(ogen) were also found to be different for tissue transglutaminase and factor XIIIa (*29*). It was originally shown that thymosin β_4 is incorporated into fibrin by the guinea pig liver tissue transglutaminase; its incorporation into fibrin by factor XIIIa was hypothesized based on the facts that thrombin-activated platelets corelease factor XIII and thymosin β_4 and that the latter becomes cross-linked to fibrin (*3*). In this study, we demonstrated directly that thymosin β_4 is incorporated by factor XIIIa to both fibrinogen and fibrin. Furthermore, we found that the degree of the incorporation is rather high, 0.2 and 0.4 mol of thymosin β_4 per mol of fibrinogen and fibrin, respectively. Since concentration of fibrinogen in plasma is about 9 μM, local concentration of fibrin at places of fibrin deposition should be much higher. Taking into account that thymosin β_4 exhibits its proangiogenic activity at 0.1 nM to 1 μM (*14*, *16*, *45*), such a degree of incorporation is obviously physiologically significant and should be sufficient to increase the wound healing potential of a fibrin clot.

It is known that factor XIIIa incorporates into fibrin a number of plasma proteins, α_2-antiplasmin, PAI-2, fibronectin, thrombospondin, and von Willebrand factor (*26*, *29*, *46*−*48*). The mechanism of incorporation is established only for some of them. For example, fibronectin binds to the fibrin αC-domains noncovalently with high affinity prior to covalent cross-linking with factor XIIIa; the recognition sites and the reactive Gln and Lys residues in each protein are located in different regions, providing proper orientation of the cross-linking sites (*37*). In addition, factor XIIIa interacts with the αC-domains (*49*), further increasing the specificity

of the reaction. To test whether noncovalent binding of thymosin β_4 precedes its cross-linking to fibrin, we studied its interaction with immobilized fibrinogen and fibrin in the presence and absence of nonactivated factor XIII. In contrast to other proangiogenic factors, such as bFGF and VEGF, which exhibit high affinity to fibrin (*40*, *41*), no noticeable noncovalent interaction was observe with thymosin β_4 in all cases (Figure 1). The incorporation was observed only in the presence of activated factor XIIIa, suggesting that the covalent cross-linking may be the only mechanism to retain thymosin β_4 in fibrin clot.

The results of this study clearly indicate that although thymosin β_4 could be incorporated by factor XIIIa into the isolated γ-module and the αC-domain variants, in fibrin(ogen) it is cross-linked mainly to the αC-domains, namely, to their Aα392-610 regions. The analysis of distribution of the identified reactive Lys and Gln residues in thymosin β_4 and fibrin(ogen) provides a reasonable explanation for this finding. It was reported that thymosin β_4 contains a reactive amine donor, Lys38, and two amine acceptors, Gln23 and Gln36, which could be involved in the cross-linking reaction with other proteins (*19*, *50*). There are only two reactive residues in the γ-chain involved in the intermolecular $\gamma-\gamma$ cross-linking, Gln398 (or Gln399) and Lys406, both located in the γ-module. When the isolated γ-module was treated with factor XIIIa, the cross-linking seemed to occur randomly resulting in dimers and trimers/oligomers; thymosin β_4 was incorporated in all these species (Figure 4). In fibrin, these regions are aligned by the DD:E interactions in an antiparallel manner facilitating cross-linking between Gln398/399 of one chain and Lys406 of another to form $\gamma-\gamma$-dimers. The efficiency of this cross-linking reaction is much higher than that between these residues and thymosin β_4; therefore, it is not surprising that little or no incorporation of thymosin β_4 into the fibrin γ-chains was observed in this study.

In contrast to the γ-chain, the Aα-chain contains multiple reactive glutamine and lysine residues. The following residues were found to be involved in the cross-linking between the α-chains in fibrin or the recombinant αC-domains, Gln221, 237, 328, and 366, and Lys508, 539, 556, 580, and 601 (*28*, *51*, *52*). The Aα-chain Lys303 was shown to serve as amine donor in factor XIIIa-mediated cross-linking of the serpin α_2-antiplasmin to fibrin(ogen) (*27*). This Lys is not reactive toward another serpin, PAI-2, which is cross-linked by tissue transglutaminase and factor XIIIa through other Aα-chain lysine residues, 148, 176, 183, 230, 413, and 457 (*29*). The study with a synthetic peptide mimicking the cross-linking region of α_2-antiplasmin revealed that it is incorporated into fibrin α-chain through 12 reactive lysine residues, Lys418, 448, 508, 539, 556, and 580, which accounted for 78% of the total activity, and less reactive Lys208, Lys219 and/or 224, and Lys427, 429, 601, and 606 (*53*). At least 10 lysine residues within fibrin(ogen) Aα368-610 region were implicated in cross-linking reactions with fibronectin (*54*). The above-mentioned analysis indicates that most of the identified reactive residues in fibrin are located in its αC-domains, that the 392-610 region of the αC-domain, to which thymosin β_4 is preferentially cross-linked, contains at least 11 reactive Lys residues, and that among these residues only half are utilized in the $\alpha-\alpha$ cross-linking. It also suggests that although thymosin β_4 could compete for reactive lysine residues involved in the $\alpha-\alpha$ cross-linking, its cross-linking to the αC-domains may occur independently of their intermolecular $\alpha-\alpha$ cross-linking, providing its efficient incorporation into fibrin. Thus, the reactive lysine residues of the αC-domains are designed not only to serve for the $\alpha-\alpha$ cross-linking but also to simultaneously accommodate physiologically active proteins, including thymosin β_4, which could modulate properties of fibrin matrix contributing to wound healing and other physiological and pathological processes.

Fibrinogen polymerizes in a controllable fashion to make a clot that easily adheres to different cells and is nonimmunogenic and biodegradable. These make it an ideal hemostatic and bioadhesive (fibrin sealant) that has been used increasingly in numerous surgical applications as a hemostatic agent for the arrest of bleeding and to assist tissue sealing and wound healing (*55*−*57*). The use of fibrin sealants in wound healing and other therapies can be enhanced by including bioactive agents. For example, it was shown in a number of cellular and animal models that fibrin can serve as a vehicle for localized delivery of antibiotics (*58*, *59*) and growth factors (*60*−*66*). Recent studies in animal models have demonstrated the ability of thymosin β_4 to accelerate wound healing (*16*−*18*), making it a good candidate for supplementing fibrin sealants. While antibiotics encapsulated by fibrin are released slowly due to low solubility (*59*), the retention of growth factors in fibrin sealants was achieved through their high affinity interaction with fibrin (*60*, *65*, *66*) or through their direct covalent cross-linking to it (*63*). The ability of thymosin β_4 to be incorporated into fibrin(ogen) by cross-linking with factor XIIIa could be used for its immobilization on fibrin sealants. This study demonstrates the high efficiency of such incorporation into both fibrinogen and fibrin, supporting the feasibility of such approach. The wound healing properties of thymosin β_4-loaded fibrin sealants need to be tested.

In summary, in this study we confirmed the previous suggestion (*3*) that thymosin β_4, a bioactive peptide, could be incorporated into fibrin by covalently cross-linking with factor XIIIa, demonstrated high efficiency of its incorporation into both fibrinogen and fibrin at physiological concentrations of the components, and localized the incorporation sites within the Aα392-610 region of the fibrin(ogen) αC-domains. This study supports the feasibility of incorporation of physiologically significant amounts of thymosin β_4 into fibrin sealants for delivery to places of wound healing.

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REFERENCES

1. Sakata, Y., and Aoki, N. (1982) Significance of cross-linking of α_2-antiplasmin inhibitor to fibrin in inhibition of fibrinolysis and in hemostasis, *J. Clin. Invest. 69*, 536−542.
2. Corbett, S. A., Wilson, C. L., and Schwarzbauer, J. E. (1996) Changes in cell spreading and cytoskeletal organization are induced by adhesion to a fibronectin-fibrin matrix, *Blood 88*, 158−166.
3. Huff, T., Otto, A. M., Muller, C. S. G., Meier, M., and Hannappel, E. (2002) Thymosin β_4 is released from human blood platelets and attached by factor XIIIa (transglutaminase) to fibrin and collagen, *FASEB J. 16*, 691−696.
4. Low, T. L., Hu, S. K., and Goldstein, A. L. (1981) Complete amino acid sequence of bovine thymosin β_4: a thymic hormone that

induces terminal deoxynucleotidyl transferase activity in thymocyte populations, *Proc. Natl. Acad. Sci. U.S.A. 7*, 1162−1166.
5. Hannappel, E., Xu, G. J., Morgan, J., Hempstead, J., and Horecker, B. L. (1982) Thymosin β_4: a ubiquitous peptide in rat and in mouse tissues, *Proc. Natl. Acad. Sci. U.S.A. 79*, 2172−2175.
6. Oates, K. K., and Goldstein, A. L. (1995) Thymosin, in *Biologic therapy of cancer* (DeVita, V. T., Hellman S., Rosenberg, S. A., Eds.) pp 841−852, JB Lippincott, Philadelphia.
7. Hannappel, E., and Huff, T. (2003) The thymosins. Prothymosin α, parathymosin, and β-thymosins: structure and function, *Vitam. Horm. 66*, 257−296.
8. Safer, D., Golla, R., and Nachmias, V. T. (1990) Isolation of a 5-kDa actin-sequestering peptide from human blood platelets, *Proc. Natl. Acad. Sci. U.S.A. 87*, 2536−2540.
9. Safer, D., Elzinga, M., and Nachmias, V. T. (1991) Thymosin β_4 and Fx, an actin-sequestering peptide, are indistinguishable, *J. Biol. Chem. 266*, 4029−1032.
10. Sanders M. C., Goldstein A. L., and Wang Y.-L. (1992) Thymosin β_4 (FX peptide) is a potent regulator of actin polymerization in living cell, *Proc. Natl. Acad. Sci. U.S.A. 89*, 4678−4682.
11. Kobayashi, T., Okada, F., Fujii, N., Tomita, N., Ito, S., Tazawa, H., Aoyama, T., Choi, S. K., Shibata, T., Fujita, H., and Hosokawa, M. (2002) Thymosin β_4 regulates motility and metastasis of malignant mouse fibrosarcoma cells, *Am. J. Path. 160*, 869−882.
12. Cha, H. J., Jeong, M. J., and Kleinman, H. K. (2003) Role of thymosin β_4 in tumor metastasis and angiogenesis, *J. Natl. Cancer Inst. 95*, 1674−1680.
13. Grant, D. S., Konsella, J. L., Kibey, M. C., LaFlamme, S., Burbello, P. D., Goldstein, A. L., and Kleinman, H. K. (1995) Matrigel induces thymosin β_4 gene in differentiating endothelial cells, *J. Cell Sci. 108*, 3685−3694.
14. Malinda, K. M., Golgstein, A. L., and Kleinman, H. K. (1997) Thymosin β_4 stimulates directional migration of human umbilical vein endothelial cells, *FASEB J. 11*, 474−481.
15. Frohm, M., Gunne, H., Bergman, A.-C., Agerberth, B., Bergmen, T., Boman, A., Liden, S., Jornval, H., and Boman, H. (1996) Biochemical and antibacterial analysis of human wound and blister fluids, *Eur. J. Biochem. 237*, 86−92.
16. Malinda, K. M., Sidhu, G. S., Mani, H., Banaudha, K., Maheshwari, R. K., Goldstein, A. L., and Kleinman, H. K. (1999) Thymosin β_4 accelerates wound healing, *J. Invest. Dermatol. 113*, 364−368.
17. Philp, D., Badamchian, M., Scheremeta, B., Nguyen, M., Goldstein, A. L., and Kleinman, H. K. (2003) Thymosin β_4 and a synthetic peptide containing its actin-binding domain promote dermal wound repair in db/db diabetic mice and in aged mice, *Wound Repair Regen. 11*, 19−24.
18. Sosne, G., Szliter, E. A., Barrett, R., Kernacki, K. A., Kleinman, H., and Hazlett, L. D. (2002) Thymosin β_4 promotes corneal wound healing and decreases inflammation in vivo following alkali injury, *Exp. Eye Res. 74*, 293−299.
19. Huff, T., Ballweber, E., Humeny, A., Bonk, T., Becker, C., Muller, C. S., Mannherz, H. G., and Hannappel, E. (1999) Thymosin β_4 serves as glutaminyl substrate of transglutaminase. Labeling with fluorescent dansylcadaverin does not abolish interaction with G-actin, *FEBS Lett. 464*, 14−20.
20. Doolittle, R. F. (1984) Fibrinogen and fibrin, *Annu. Rev. Biochem. 53*, 195−229.
21. Henschen, A., and McDonagh, J. (1986) Fibrinogen, fibrin, and factor XIII, in *Blood Coagulation* (Zwaal, R. F. A., Hemker, H. C., Eds.) pp 171−241, Elsievier Science Publisher, Amsterdam.
22. Erickson, H. P., and Fowler, W. E. (1983) Electron microscopy of fibrinogen, its plasmic fragments, and small polymers, *Ann. N. Y. Acad. Sci. 408*, 146−163.
23. Medved, L. V., Gorkun, O. V., and Privalov, P. L. (1983) Structural organization of C-terminal part of fibrinogen Aα-chains, *FEBS Lett. 22*, 291−295.
24. Schwartz, M. L., Pizzo, S. V., Hill, R. L., and McKee, P. A. (1973) Human Factor XIII from plasma and platelets. Molecular weights, subunit structures, proteolytic activation, and cross-linking of fibrinogen and fibrin, *J. Biol. Chem. 248*, 1395−1407.
25. Francis, C. W., and Marder, V. J. (1987) Rapid formation of large molecular weight α-polymers in cross-linked fibrin induced by high factor XIII concentrations. Role of platelet factor XIII, *J. Clin. Invest. 80*, 1459−1465.
26. Mosher, D. (1975) Cross-link of cold-insoluble globulin by fibrin-stabilized factor, *J. Biol. Chem. 250*, 6614−6621.
27. Kimura, S., and Aoki, N. (1986) Cross-linking site in fibrinogen for α_2-plasmin inhibitor, *J. Biol. Chem. 261*, 15591−15595.
28. Matsuka, Y. V., Medved, L. V., Migliorini, M. M., and Ingham, K. C. (1996) Factor XIIIa-catalyzed cross-linking of recombinant αC-fragments of human fibrinogen, *Biochemistry 35*, 5810−5816.
29. Ritchie, H., Lawrie, L. C., Crombie, P. W., Mosesson, M. W., and Booth, N. A. (2000) Cross-linking of plasminogen activator inhibitor 2 and α_2-antiplasmin to fibrin(ogen), *J. Biol. Chem. 275*, 24915−24920.
30. Tsurupa, G., and Medved, L. (2001) Identification and characterization of novel tPA- and plasminogen-binding sites within fibrin(ogen) αC-domains, *Biochemistry 40*, 801−808.
31. Medved, L., Litvinovich, S., Ugarova, T. Matsuka, Y., and Ingham, K. (1997) Domain structure and functional activity of the recombinant human fibrinogen γ-module (γ148−411), *Biochemistry 36*, 4685−4693.
32. Bishop, P. D., Teller, D. C., Smith, R. A., Lasser, G. W., Gilbert, T., and Seale, R. L. (1990) Expression, purification, and characterization of human factor XIII in *Saccharomyces cerevisiae*, *Biochemistry 29*, 1861−1869.
33. Naylor, P. H., McClure, J. E., Spangelo, B. L., Low, T. L. K., and Goldstein, A. L. (1984) Immunochemical studies of thymosin: radioimmunoassay of thymosin β_4, *Immunopharmacology 7*, 9−16.
34. Ingham, K. C., and Brew, S. A. (1981) Fluorescent labeling of the carbohydrate moieties of human chorionic gonadotropin and α_1-acid glycoprotein, *Biochim. Biophys. Acta 670*, 181−189.
35. Lewis, K. B., Teller, D. C., Fry, J., Lasser, G. W., and Bishop, P. D. (1997) Cross-linking kinetics of the human transglutaminase, factor XIII[A_2], acting on fibrin gels and γ-chain peptides, *Biochemistry 36*, 995−1002.
36. Doolittle, R. F. (1973) Structural aspects of the fibrinogen to fibrin conversion, *Adv. Protein Chem. 27*, 1−109.
37. Makogonenko, E., Tzurupa, G., Ingham, K., and Medved, L. (2002) Interaction of fibrin(ogen) with fibronectin: further characterization and localization of the fibronectin-binding site, *Biochemistry 41*, 7907−7913.
38. Ugarova, T. P., and Yakubenko, V. P. (2001) Recognition of fibrinogen by leukocyte integrins, *Ann. N. Y. Acad. Sci. 936*, 368−385.
39. Martinez, J., Ferber, A., Bach, T. L., and Yaen, C. H. (2003) Interaction of fibrin with VE-cadherin, *Ann. N. Y. Acad. Sci. 936*, 386−405.
40. Sahni, A., Odrljin, T., and Francis, C. W. (1998) Binding of basic fibroblast growth factor to fibrinogen and fibrin, *J. Biol. Chem. 273*, 7554−7559.
41. Sahni, A., and Francis, C. W. (2000) Vascular endothelial growth factor binds to fibrinogen and fibrin and stimulates endothelial cell proliferation, *Blood 96*, 3772−3778.
42. Campbell, P. G., Durham, S. K., Hayes, J. D., Suwanichkul, A., and Powell, D. R. (1999) Insulin-like growth factor-binding protein-3 binds fibrinogen and fibrin, *J. Biol. Chem. 274*, 30215−30221.
43. Murthy, S. N., and Lorand, L. (1990) Cross-linked Aα−γ chain hybrids serve as unique markers for fibrinogen polymerized by tissue transglutaminase, *Proc. Natl. Acad. Sci. U.S.A. 87*, 9679−9682.
44. Shainoff, J. R., Urbanic, D. A., and DiBello, P. M. (1991) Immunoelectrophoretic characterizations of the cross-linking of fibrinogen and fibrin by factor XIIIa and tissue transglutaminase. Identification of a rapid mode of hybrid α-/γ-chain cross-linking that is promoted by the γ-chain cross-linking, *J. Biol. Chem. 266*, 6429−6437.
45. Koutrafouri, V., Leondiadis, L., Avgoustakis, K., Livaniou, E., Czarnecki, J., Ithakissios, D. S., and Evangelatos, G. P. (2001) Effect of thymosin peptides on the chick chorioallantoic membrane angiogenesis model, *Biochim. Biophys. Acta 1568*, 60−66.
46. Sakata, Y., and Aoki, N. (1980) Cross-linking of α_2-plasmin inhibitor to fibrin by fibrin-stabilizing factor, *J. Clin. Invest. 65*, 290−297.
47. Hada, M., Kato, M., Ikematsu, S., Fujimaki, M., and Fukutake, K. (1982) Possible cross-linking of factor VIII related antigen to fibrin by factor XIII in delayed coagulation process, *Thromb Res. 25*, 163−168.
48. Bale, M. D., Westrick, L. G., and Mosher, D. F. (1985) Incorporation of thrombospondin into fibrin clot, *J. Biol. Chem. 260*, 7502−7508.
49. Procyk, R., Bishop, P. D., and Kudryk, B. (1993) Fibrin-recombinant human factor XIII a-subunit association, *Thromb. Res. 71*, 127−138.

50. Safer, D., Sosnick, T. R., and Elzinga, M. (1997) Thymosin β_4 binds actin in extended conformation and contact both the barbed and pointed ends, *Biochemistry 36*, 5806−5816.
51. Cottrell, B. A., Strong, D. D., Watt, K. W. K., and Doolittle, R. F. (1979) Amino acid sequence on the α-chain of human fibrinogen. Exact localization of cross-linking acceptor site, *Biochemistry 18*, 5405−5410.
52. Corcoran, D. H., and Ferguson, E. W. (1980) Localization of a cross-link donor site in the α-chain of human fibrin, *Thrombosis Res. 19*, 883−888.
53. Sobel, J. H., and Gawinowicz, M. A. (1996) Identification of the α-chain lysine donor sites involved in factor XIIIa fibrin cross-linking, *J. Biol. Chem. 271*, 19288−19297.
54. Matsuka, Y. V., Migliorini, M. M., and Ingham, K. S. (1997) Cross-linking of fibronectin to C-terminal fragment of the fibrinogen α-chain by factor XIII, *J. Protein. Chem. 16*, 739−745.
55. Jackson, M. R. (1996) Tissue sealants: current status, future potential, *Nat. Med. 2*, 637−638.
56. Spotnitz, W. D. (2001) Commercial fibrin sealants in surgical care, *Am. J. Surg. 182, suppl. 2*, 8S−14S.
57. Albala, D. M. (2003) Fibrin sealants in clinical practice, *Cardiovasc. Surg. 11, Suppl. 1*, 5−11.
58. Woolverton, C. J., Huebert, K., Burkhart, B., and MacPhee, M. (1999) Subverting bacterial resistance using high dose, low solubility antibiotics in fibrin, *Infection. 27*, 28−33.
59. Woolverton, C. J., Fulton, J. A., Salstrom, S. J., Hayslip, J., Haller, N. A., Wildroudt, M. L., and MacPhee, M. (2001) Tetracycline delivery from fibrin controls peritoneal infection without measurable systemic antibiotic, *J. Antimicrob. Chemother. 48*, 861−867.
60. Pandit, A. S., Feldman, D. S., Caulfield, J., and Thompson, A. (1998) Stimulation of angiogenesis by FGF-1 delivered through a modified fibrin scaffold, *Growth Factors 15*, 113−23.
61. Pandit, A. S., Wilson, D. J., and Feldman, D. S. (2000) Fibrin scaffold as an effective vehicle for the delivery of acidic fibroblast growth factor (FGF-1), *J. Biomater. Appl. 14*, 229−242.
62. Sakiyama-Elbert, S. E., and Hubbell, J. A. (2000) Development of fibrin derivatives for controlled release of heparin-binding growth factors, *J. Control. Release 65*, 389−402.
63. Zisch, A. H., Schenk, U., Schense, J. C., Sakiyama-Elbert, S. E., and Hubbell, J. A. (2001) Covalently conjugated VEGF-fibrin matrixes for endothelialization, *J. Control. Release 72*, 101−113.
64. Mackenzie, D. J., Sipe, R., Buck, D., Burgess, W., and Hollinger, J. (2001) Recombinant human acidic fibroblast growth factor and fibrin carrier regenerates bone, *Plast. Reconstr. Surg. 107*, 989−996.
65. Petratos, P. B., Felsen, D., Trierweiler, G., Pratt, B., McPherson, J. M., and Poppas, D. P. (2002) Transforming growth factor-β_2 (TGF-β_2) reverses the inhibitory effects of fibrin sealant on cutaneous wound repair in the pig, *Wound Repair Regen. 10*, 252−258.
66. Wong, C., Inman, E., Spaethe, R., and Helgerson, S. (2003) Fibrin-based biomaterials to delivery human growth factors, *Thromb. Haemost. 89*, 573−582.

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