Thymosin β_4 is released from human blood platelets and attached by factor XIIIa (transglutaminase) to fibrin and collagen

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ABSTRACT **The β-thymosins constitute a family of highly conserved and extremely water-soluble 5 kDa polypeptides. Thymosin β_4 is the most abundant member; it is expressed in most cell types and is regarded as the main intracellular G-actin sequestering peptide. There is increasing evidence for extracellular functions of thymosin β_4. For example, thymosin β_4 increases the rate of attachment and spreading of endothelial cells on matrix components and stimulates the migration of human umbilical vein endothelial cells. Here we show that thymosin β_4 can be cross-linked to proteins such as fibrin and collagen by tissue transglutaminase. Thymosin β_4 is not cross-linked to many other proteins and its cross-linking to fibrin is competed by another family member, thymosin β_{10}. After activation of human platelets with thrombin, thymosin β_4 is released and cross-linked to fibrin in a time- and calcium-dependent manner. We suggest that thymosin β_4 cross-linking is mediated by factor XIIIa, a transglutaminase that is coreleased from stimulated platelets. This provides 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 inflammatory responses.—Huff, T., Otto, A. M., Müller, C. S. G., Meier, M., Hannappel, E. Thymosin β_4 is released from human blood platelets and attached by factor XIIIa (transglutaminase) to fibrin and collagen.** ***FASEB J.*** **16, 691–696 (2002)**

Key Words: β-*thymosins* · *cross-linking* · *actin*

Thymosin β_4 is regarded as the principal intracellular G-actin sequestering peptide in most mammalian cells (1–6). This 43 amino acid oligopeptide forms a 1:1 complex with G-actin and thereby inhibits salt-induced polymerization to F-actin (7–11). Additional members of the β-thymosin family have been identified, and these peptides exhibit properties similar to thymosin β_4. Members of this peptide family have also been discussed to play a role in carcinogenesis and metastasis. For example, thymosin β_{10} and thymosin β_{15} are increasingly expressed in various metastatic tumors of the prostate (12), breast (12, 13), and thyroid (14) and are proposed to be prognostic markers.

Thymosin β_4 is present in very high concentrations in white blood cells (WBCs) (15), but as the peptide does not possess a signal sequence for secretion, its concentration in plasma is low. However, if clotting occurs, thymosin β_4 levels in serum can increase substantially. Extracellular thymosin β_4 may contribute to several physiological processes, including angiogenesis, wound healing, and regulation of inflammation. This peptide increases the rate of attachment and spreading of endothelial cells on matrix components (16); it stimulates migration of human umbilical vein endothelial cells (17), induces matrix metalloproteinases (18), promotes corneal wound healing, and modulates inflammatory mediators (19). The sulfoxide of thymosin β_4 has been reported to inhibit inflammatory responses (20).

As β-thymosins are small, water-soluble peptides, they would be predicted to be diluted and rapidly distributed throughout the body when released from WBCs. Therefore, to be a good candidate to mediate the foregoing processes, there should be a mechanism to retain thymosin β_4 near its site of release. In a recent report (21), we showed that thymosin β_4 serves as a specific glutaminyl substrate of tissue transglutaminase in vitro. In this report we provide evidence that thymosin β_4 can be selectively cross-linked by tissue transglutaminase to some proteins (fibrinogen, fibrin, collagen, and actin) but not others (e.g., plasmin, lactate dehydrogenase, hexokinase, glyceral-3-phosphate dehydrogenase, triosephosphate isomerase, pyruvate kinase, and alcohol dehydrogenase). Moreover, we show that after activation of human platelets with thrombin, thymosin β_4 is released and cross-linked to fibrin in a time- and Ca^{2+}-dependent manner. Since factor XIIIa (a transglutaminase) is coreleased with thymosin β_4 from thrombocytes, we suggest that it mediates this cross-linking. This provides a potential molecular mechanism to 'fix' thymosin β_4 near the sites of platelet activation, permitting it to contribute to biological processes associated with clotting and wound repair.

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MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: LiChroprep RP18 (40–63 μm) and trifluoroacetic acid (Uvasol) from Merck (Darmstadt, Germany); guinea pig transglutaminase, fibrinogen, collagen, and plasminogen from Sigma (Germany); lactate dehydrogenase, hexokinase, glyceral-3-phosphate dehydrogenase/triosephosphate isomerase, alcohol dehydrogenase, and pyruvate kinase from Boehringer Mannheim (Mannheim, Germany).

Purification of actin and thymosin β_4

Actin was prepared from bovine heart muscle by the method of Pardee and Spudich (22) and stored as G-actin in G buffer (2 mM Tris, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM mercaptoethanol, 0.05% NaN_3, pH 8.0) at 0°C. Thymosin β_4 was isolated from pig spleen as described (23). The purity of the preparation was demonstrated by reverse-phase HPLC. The concentrations of thymosin β_4 and actin were determined by amino acid analysis after acid hydrolysis (6 M HCl, 155°C, 1 h) and precolumn derivatization with *o*-phthaldialdehyde/3-mercaptopropionic acid (24).

HPLC

Chromatographic conditions were controlled by a Merck-Hitachi L-6200 system supplemented with a diode array UV detector (L-7450A), a reaction pump for postcolumn derivatization (655A-13, Merck-Hitachi), and with a fluorometer (F-1050, Merck-Hitachi). The diode array detector signal was recorded on a computer using D-7000 HSM software (Merck) and the fluorescence signal on an integrator (D-2500, Merck-Hitachi).

Flow rate: 0.75 ml/min; buffer: 0.1% TFA (trifluoro acetic acid); gradient: 0 to 40% acetonitrile in 60 min; column: Beckman ODS Ultrasphere (5 μm, 4.6×250 mm); detection: UV at 205 nm and fluorescence after postcolumn derivatization with fluorescamine (25).

Generation of polyclonal antibody

A synthetic decapeptide representing the nine carboxyl-terminal amino acids of thymosin β_4 with an additional cysteine residue at the NH_2 terminus was conjugated to keyhole limpet hemocyanin (KLH, Sigma, Germany). New Zealand White rabbits were immunized with this KLH conjugate, corresponding to ~63 μg of the synthetic peptide, emulsified with complete Freund's adjuvant (Sigma, Germany). After a second immunization, the antibody was partially purified from the serum by precipitation with 50% ammonium sulfate. The precipitate was dissolved in 5 mM phosphate buffer, pH 6.5, dialyzed against PBS, and adsorbed with a 1% suspension of acetone powder from bovine heart. The resulting antiserum showed no cross-reactivity with other β-thymosins, actin, or any cellular protein in the range of 10–150 kDa, as judged by Western analysis and enzyme-linked immunosorbent assay (ELISA).

ELISA assay

Microtiter wells (PRO-BIND™, Becton Dickinson, Heidelberg, Germany) were coated with 200 μl (0.1 mg/ml) of either thymosin β_4 (positive control) or proteins to be tested by incubation for 2 h at room temperature or overnight at 4°C. Protein solutions were then removed and the wells were washed several times with 200 μl of 50 mM Tris-HCl, pH 7.4. Reagents were added as described in the figure legends; volume was adjusted to 200 μl with incubation buffer (50 mM Tris-HCl, 50 mM $CaCl_2$, 150 mM NaCl, 4 mM DTT, pH 7.3) and incubated for 2 h at room temperature. Thereafter, reaction mixtures were discarded and binding of thymosin β_4 was determined by application of ELISA using polyclonal antibody against thymosin β_4.

Isolation of human thrombocytes

Twenty milliliters of ACD blood from human donors was centrifuged for 30 min at 100 *g*. The pale yellow platelet-rich plasma was transferred to a plastic tube and centrifuged again for 15 min at 150 *g*. The platelets were then pelleted by centrifugation for 15 min at 600 *g* and resuspended in a buffer consisting of 126 mM NaCl, 5 mM KCl, 0.3 mM EDTA, 10 mM Na_2HPO_4, 5 mM glucose, pH 7.3. Cell number was determined using a CASY 1 (Schärfe System, Germany) cell counter.

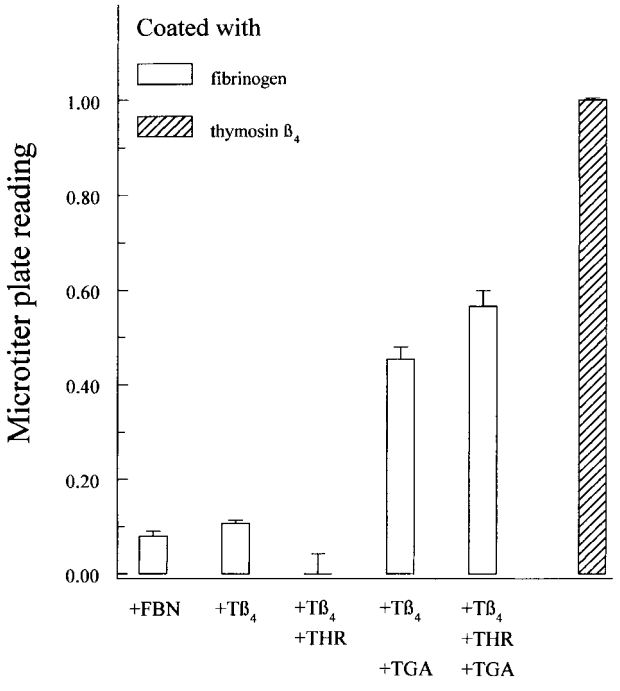


Figure 1. Cross-link of thymosin β_4 with fibrin and fibrinogen. Microtiter wells were coated with either fibrinogen or thymosin β_4 (positive control) as described in Materials and Methods. The reagents were added to the assays in the following concentrations: fibrinogen (FBN), 5 mg/ml; thymosin β_4 ($T\beta_4$), 0.04 mg/ml; thrombin (THR), 0.2 U; guinea pig transglutaminase (TGA), 0.1 U. After a 2 h incubation, reaction mixtures were removed and thymosin β_4 binding was determined using a polyclonal antibody against the peptide and peroxidase-coupled secondary anti-rabbit-IgG antibody. Data are expressed as microtiter plate reading at 490 nm after peroxidase reaction using phenylenediamine as a substrate. Each data point represents the mean of absorbance values from 6 wells ± SD. All figures are from representative experiments. Each study was repeated at least three times with similar results.

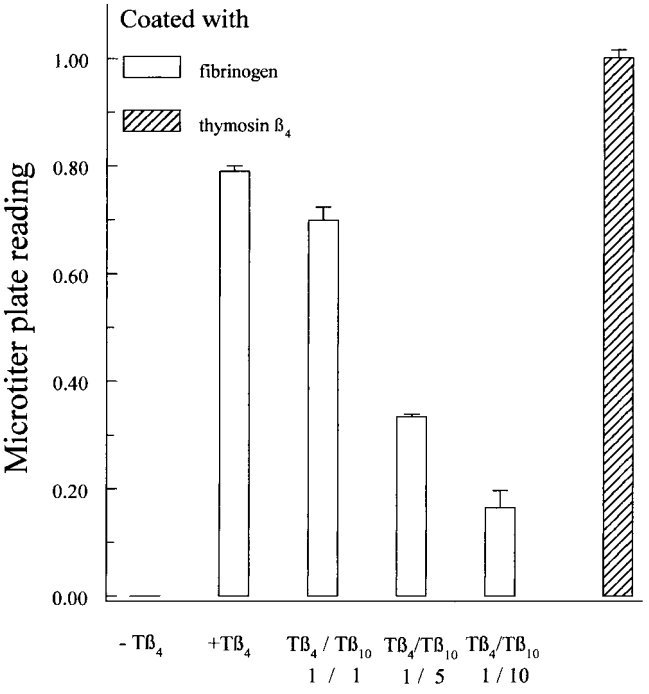


Figure 2. Thymosin β_{10} competes with thymosin β_4 in cross-linking to fibrin. Cross-linking of thymosin β_4 to fibrin by guinea pig transglutaminase decreased in the presence of increasing amounts of thymosin β_{10}. Molar ratio of thymosin β_{10} to thymosin β_4 as indicated. All other conditions are as described in legend to Fig. 1.

Determination of thymosin β_4 content in human blood platelets

2×10^8 platelets in 500 μl incubation medium were disrupted by adding 50 μl 4 M perchloric acid and incubated for 30 min at 4°C. A defined amount of the internal standard phenylalanyl phenylalanine was added. After centrifugation for 5 min at 20,000 *g*, the supernatant was carefully removed and the pH adjusted to a value between 4 and 6 by adding 10 M KOH. After a second 30 min incubation at 4°C, precipitated $KClO_4$ was removed by centrifugation and the supernatant was analyzed by HPLC as described above.

RESULTS

Selective cross-linking of thymosin β_4 to proteins by tissue transglutaminase

A microtiter plate assay was used to elucidate whether thymosin β_4 could be specifically cross-linked to other proteins by transglutaminase. First, we tested whether thymosin β_4 could be cross-linked to either fibrinogen or fibrin by tissue transglutaminase. **Figure 1** shows that thymosin β_4 is cross-linked to both of these proteins. To investigate the specificity of the reaction, we determined whether the transglutaminase catalyzed cross-linking of thymosin β_4 was competed by thymosin β_{10}. Increasing amounts of thymosin β_{10} decreased the amount of thymosin β_4 cross-linked to fibrin (**Fig. 2**), suggesting that it, too, was a substrate for transglutaminase. Besides fibrin, we observed transglutaminase-mediated cross-linking of thymosin β_4 to collagen and actin but not to a range of other proteins, including plasmin, lactate dehydrogenase, hexokinase, glyceral-3-phosphate dehydrogenase, triosephosphate isomerase, pyruvate kinase, and alcohol dehydrogenase (data not shown).

Release and cross-linking of thymosin β_4 from human platelets

We isolated human thrombocytes to determine whether thymosin β_4 is released from human platelets and can be cross-linked to fibrin. Without activation by thrombin, no cross-linked product was detected (**Fig. 3**) by the ELISA assay. In contrast, after activation with thrombin, thymosin β_4 from thrombocytes was cross-linked to fibrin, presumably by factor XIIIa. Addition of extracellular thymosin β_4 led to only a small increase in reaction product, suggesting that the reaction was saturated by endogenously generated thymosin β_4. As with purified transglutaminase, thymosin β_4 cross-linking was attenuated by addition of thymosin β_{10} (Fig. 3).

To investigate the release and cross-linking of thymosin β_4 from human platelets in more detail, we examined the time and Ca^{2+} dependence of this process.

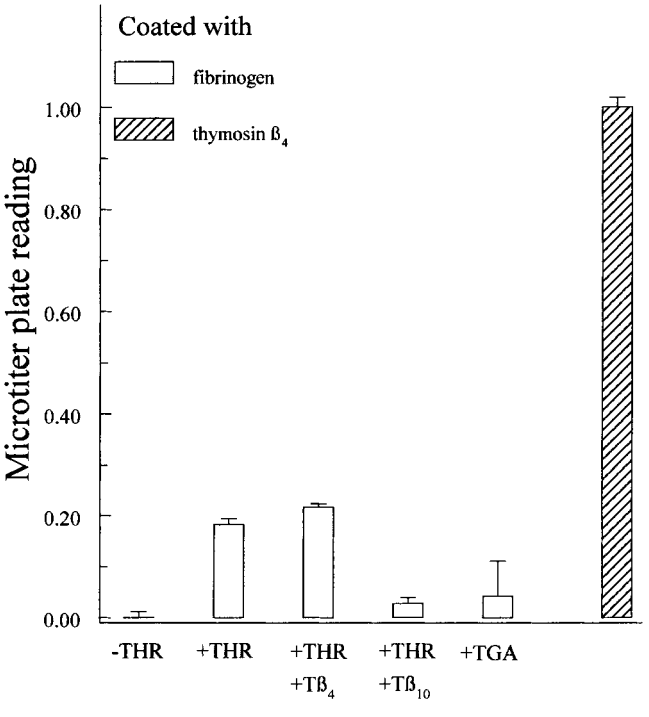


Figure 3. Thymosin β_4 is released from human platelets after activation with thrombin and cross-linked to fibrin by factor XIIIa. After activation of human platelets (2×10^8/well) with thrombin (THR, 0.2 U), thymosin β_4 was cross-linked to fibrin by the action of factor XIIIa. Addition of thymosin β_4 ($T\beta_4$) resulted in only a slight increase, whereas addition of thymosin β_{10} ($T\beta_{10}$) led to a distinct decrease in cross-linking of thymosin β_4 to fibrin. In the absence of thrombin (even in the presence of tissue transglutaminase), no cross-linking was observed.

Due to its solubility in diluted acids, thymosin β_4 content in cells can be determined in the supernatant after acid treatment of cells. If thymosin β_4 is cross-linked by transglutaminase to proteins such as fibrin, collagen, or actin, it will precipitate with these proteins, leading to a decrease in free thymosin β_4 in the supernatant. HPLC analysis showed that within 1 h after treatment of the platelets with thrombin (0.2 U), the amount of free thymosin β_4 decreased from 12 to 5.1 fg/cell; in the absence of thrombin, only a slight decrease to 10 fg/cell was observed (**Fig. 4**).

The activity of transglutaminases depends on the presence of Ca^{2+} ions. Therefore, if factor XIIIa is mediating the reaction, the omission of calcium should reduce thymosin β_4 cross-linking in the platelet assay. To study the Ca^{2+} dependence of the reaction, citrate-stabilized thrombocyte preparations were used. As expected, after a 4 h incubation in the presence of thrombin without addition of Ca^{2+}, the platelets aggregated, albeit to a lesser extent than in the presence of free Ca^{2+}, and there was almost no decrease in thymosin β_4 levels (19.4 to 19.1 fg/cell) (**Fig. 5**). Even after addition of 1 mM Ca^{2+}, only a slight decrease (18.1 fg/cell) in thymosin β_4 content was found. In contrast, when the Ca^{2+} concentration was raised to 10 and 50 mM to overcome the complex formation of Ca^{2+} with citrate, the amount of free thymosin β_4 decreased to 10.3 and 3.1 fg/cell, respectively. This is consistent with a transglutaminase-mediated thymosin β_4 cross-linking.

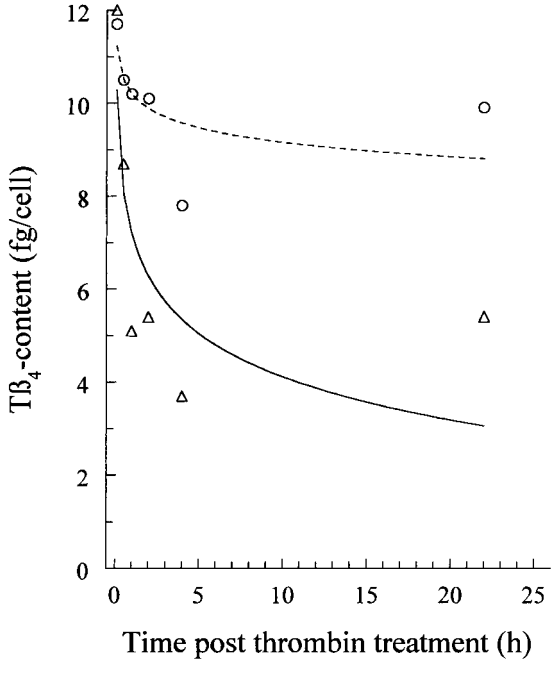


Figure 4. Time dependence of thymosin β_4 release from human platelets. Within 1 h after treatment of human platelets (2×10^8 in 500 μl buffer) with thrombin (△, 0.2 U), the concentration of free (acid-soluble) thymosin β_4 decreased to less than half the initial concentration, whereas in the absence of thrombin (○) only a slight decrease (less than 20%) was found. In neither case did longer incubation times result in remarkable changes.

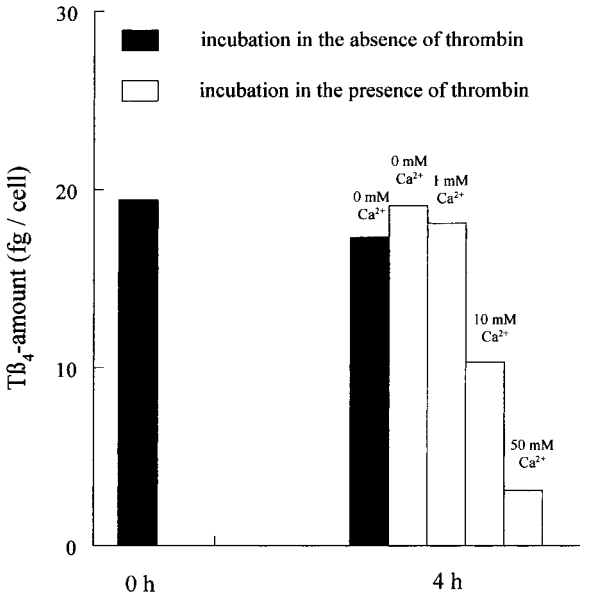
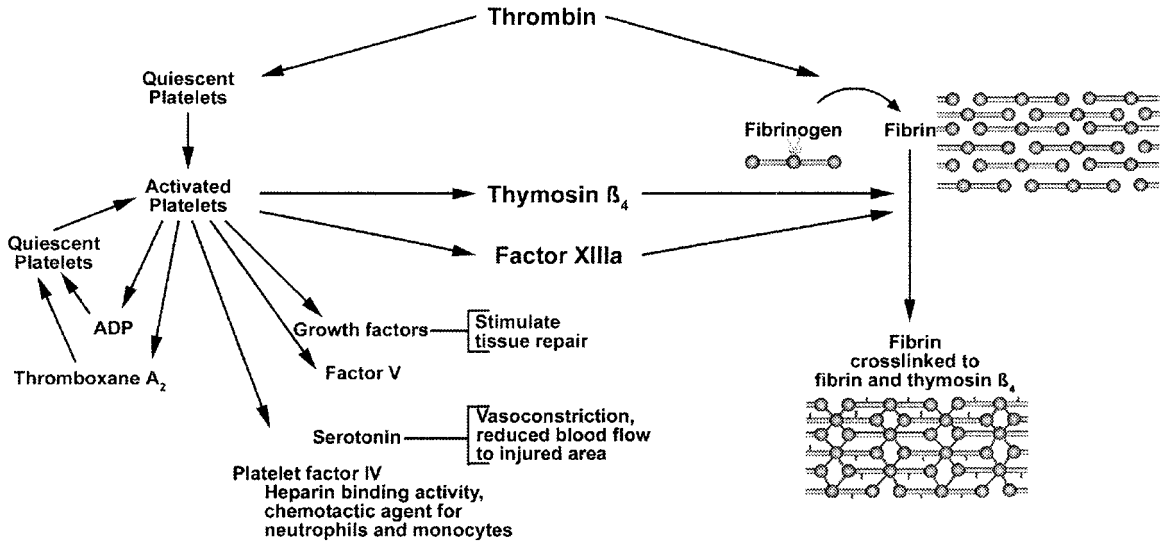


Figure 5. The decrease in concentration of free thymosin β_4 depends on the presence of Ca^{2+}. Human platelets (2×10^8 in 500 μl buffer) were incubated in either the absence (■) or presence of 0.2 U thrombin (□) for 4 h. Ca^{2+} was added as indicated above the bars. In the absence of thrombin, no change in the concentration of free thymosin β_4 occurred (19.4 fg/cell vs. 19.1 fg/cell). In the presence of thrombin and increasing amounts of Ca^{2+}, an appropriate decrease in the concentration of free thymosin β_4 was detected.

DISCUSSION

In this study we show selective, transglutaminase-mediated cross-linking of thymosin β_4 to fibrin, fibrinogen, collagen, and actin. This property is likely shared by other β-thymosins, as thymosin β_{10} attenuates thymosin β_4 cross-linking. We also demonstrate that after activation of human thrombocytes with thrombin, thymosin β_4 is cross-linked to fibrin, most likely by factor XIIIa.

In recent years, several reports have shown that thymosin β_4 may be involved in various cellular processes including angiogenesis, wound healing, and regulation of inflammation. For example, Kleinman and co-workers (16) have found that thymosin β_4 mRNA increases fivefold during the morphological differentiation of endothelial cells into capillary-like tubes. Transfection of these cells with thymosin β_4 caused an increased rate of attachment and spreading on matrix components and an accelerated rate of tube formation on Matrigel (16). The same group also demonstrated that thymosin β_4 stimulated the migration of human umbilical vein endothelial cells (17) and induced matrix metalloproteinase 2 in vitro and in vivo (18). Young et al. have shown that thymosin β_4-sulfoxide generated from monocytes in the presence of glucocorticoids inhibits inflammatory responses (20). As some of these responses involve extracellular thymosin β_4, this raises the question of how small, water-soluble peptides would not be rapidly diluted and distributed to distant sites in the body. Therefore, we postulated that some site-



Scheme 1. Integration of thymosin β_4 into platelet aggregation and blood coagulation. After stimulation with thrombin, quiescent platelets change shape and release several mediators of blood coagulation and wound healing. Release of ADP leads to the aggregation of thrombocytes and facilitates formation of a hemostatic plug. This plug is unstable. To ensure long-term closure of a wound, it must be stabilized by the formation of a fibrin clot. Fibrin arises from proteolytic removal of the fibrinopeptides A and B from fibrinogen by thrombin. Fibrin monomers first join to establish the so-called 'soluble clot.' Cross-linking of the monomers by the action of factor XIIIa results in the formation of covalent isopeptide bonds (insoluble clot). As our results show, thymosin β_4 (shown as vertical bars) is also released from platelets after activation and fixed to the fibrin clot by factor XIIIa.

specific mechanisms must exist to retain the peptide in the vicinity of the clot or tissue damage after its release.

We tested whether transglutaminase-mediated cross-linking of thymosin β_4 to extracellular proteins represents one possible mechanism to immobilize the peptide near its site of release. Thymosin β_4 serves as a specific substrate of tissue transglutaminase in vitro (21). Here we have extended this observation by showing that in the case of platelet aggregation and blood coagulation, thymosin β_4 can be cross-linked to extracellular proteins. We propose that this cross-linking is catalyzed by factor XIIIa, a transglutaminase coreleased with thymosin β_4 from activated thrombocytes (**Scheme 1**). The cross-linking is to relevant proteins, such as fibrin and collagen, which are known to play an important role in processes such as blood coagulation and wound healing. This may provide mechanistic insight into how thymosin β_4 and related peptides can modify a range of biological responses in the extracellular milieu. FJ

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