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BRIEF REPORT

Dual effect of histone H4 on prothrombin activation

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Essentials

- Prothrombin converts slowly to thrombin upon interaction with histone H4.
- Histone H4 may also affect the reactivity of prothrombin toward factor Xa.
- Histone H4 enhances or inhibits activation by factor Xa depending on cofactor Va.
- The results reveal an unanticipated dual effect of histone H4 on prothrombin activation by factor Xa.

Summary. *Background:* Recent studies have documented the ability of prothrombin to convert to the mature protease thrombin upon interaction with histone H4. The effect is abrogated by mutation of the catalytic Ser and requires the Gla domain. *Objectives:* To explore the effect of histone H4 on the reactivity of prothrombin to its physiological activator factor (F) Xa, free or assembled in the prothrombinase complex. *Methods:* The effect of histone H4 on prothrombin activation by FXa and prothrombinase is studied with kinetic assays. The potential epitope of prothrombin recognizing histone H4 is explored with electrostatic calculations using recent crystal structures. *Results and Conclusions:* Binding of histone H4 has a dual effect on prothrombin activation by FXa that is of mechanistic significance: it enhances the reaction > 10-fold in the absence of cofactor Va, but produces complete inhibition in the presence of cofactor. Histone H4 binding to prothrombin produces very slow autoactivation independent of the coagulation cascade and promotes slow thrombin generation by FXa in the absence of phospholipids. In addition, histone H4 has a rapid and drastic inhibitory effect on prothrombin activation by prothrombinase that is likely to dominate pathophysiology.

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Introduction

Prothrombin, or coagulation factor II, is a vitamin K-dependent zymogen, 579 residues long, that circulates in the blood at a high concentration (0.1 mg mL^{-1}) and long half-life (60 h) [1]. The protein has a modular assembly comprising a Gla domain, two kringles and the protease domain arranged spatially as two rigid ends (the N-terminal Gla domain/kringle-1 pair and C-terminal kringle-2/protease domain pair) connected by a flexible linker between the two kringles [2,3]. In the penultimate step of the coagulation cascade, prothrombin is efficiently converted to the mature protease thrombin by the prothrombinase complex composed of factor (F) Xa and cofactor Va assembled on a membrane surface in the presence of Ca^{2+} [4]. The conversion involves sequential cleavage at R271 and R320 along two alternative pathways that lead to generation of the inactive intermediate prethrombin-2 (cleavage at R271 first) or the active intermediate meizothrombin (cleavage at R320 first). Because neither intermediate accumulates, knowledge of which pathway of activation predominates under specific conditions may be irrelevant to physiology but carries mechanistic significance. The recent observation that prothrombin can convert to thrombin by autoactivation upon mutations around the site of cleavage at R320 [5] has mechanistic and translational relevance [6]. Autoactivation is initiated by prothrombin itself and is abrogated by mutation of the catalytic Ser. In addition, wild-type prothrombin autoactivates to thrombin upon binding of histone H4 to the Gla domain [7], revealing unexpected new properties of the zymogen worthy of further investigation.

The observation of a crosstalk between prothrombin and histone H4 should be considered in the context of the emerging field of immunothrombosis [8], an innate immune response that links coagulation to the recognition, containment and destruction of microbial pathogens. Indeed, histone H4 is one of numerous modulators released into the extracellular media during response to traumatic injury, sepsis, inflammation and cell necrosis

[9–14], where they contribute to coagulation imbalance and multiple organ dysfunction [15]. The autoactivation of prothrombin induced by binding of histone H4 occurs well within the half-life of the zymogen in the blood but is too slow (up to 8 h) to be a potential catalyst of microvascular thrombosis [16]. The interaction of histone H4 with prothrombin probably produces other effects, perhaps related to the mechanism of prothrombin activation by FXa. In order to promote autoactivation, binding of histone H4 to the Gla domain of prothrombin [7] must change the reactivity of the sites of cleavage at R271 and R320 located nearly 80 Å away [3,17]. This long-range communication is consistent with the conformational plasticity of prothrombin that emerged from recent crystallographic and spectroscopic analysis [2,3,17] and may also influence how the zymogen interacts with its physiological activator FXa, free or assembled in the prothrombinase complex. We therefore studied the kinetics of prothrombin activation by FXa in the presence of histone H4 and uncovered an unexpected dual effect of potential pathophysiological significance.

Methods

Recombinant full-length prothrombin (residues 1–579) was expressed and purified as previously described [2,3,7,17]. Homogeneity and chemical identity of final preparations were verified by SDS–PAGE and by RP-HPLC mass spectrometry analysis, giving a purity of > 98%. Factor Xa and cofactor Va were purchased from Hematologic Technologies (Essex Junction, VT, USA). Recombinant histone H4 was purchased from New England BioLabs (Ipswich, MA, USA). Activation of prothrombin (0.1 mg mL^{-1}, 1.4 μM) was carried out at room temperature for 5 min as reported elsewhere [3,17] in the presence of different concentrations (0–10 μM) of histone H4 under experimental conditions of 145 mM NaCl, 5 mM $CaCl_2$, 10 mM Tris, pH 7.4. The proteolytic reaction was studied under three different conditions and started by addition of: (i) FXa (25 nM); (ii) FXa (5 nM) and phospholipids (25 μM); and (iii) FXa (10 pM), phospholipids (25 μM) and cofactor Va (10 nM). Aliquots were collected after 2 min and activation was followed at 405 nm using chromogenic substrate H-D-Phe-Pro-Arg-p-nitroanilide hydrolysis. Small unilamellar vesicles composed of phosphatidylcholine and phosphatidylserine in a 3 : 1 molar ratio were prepared by extrusion using 100 nm polycarbonate membranes and their size was confirmed by dynamic light scattering. Alternatively, activation of prothrombin was followed by SDS-electrophoresis under reducing conditions and started by: (i) FXa (40 nM); (ii) FXa (20 nM) and phospholipids (25 μM); and (iii) FXa (0.6 nM), phospholipids (25 μM) and cofactor Va (10 nM). Prothrombin (0.1 mg mL^{-1}, 1.4 μM) was buffer exchanged in 145 mM NaCl, 5 mM $CaCl_2$, 10 mM Tris, pH 7.4, and incubated at 25 °C for 5 min in the absence or presence of 3 μM histone H4, dansylarginine-N-(3-ethyl-1,5-pentanediyl) amide (60 μM), phospholipids and cofactor Va as indicated. The reaction was started by addition of FXa and quenched at different time intervals with 10 μL of NuPAGE LDS buffer containing 10% β-mercaptoethanol as reducing agent.

Results and discussion

Histone H4 significantly promotes activation of prothrombin by FXa, especially in the absence of phospholipids (Fig. 1A,D), but has a drastic inhibitory effect on the same reaction when FXa is assembled in the prothrombinase complex with cofactor Va and phospholipids (Fig. 1G). This indicates that binding of histone H4 makes prothrombin a better substrate for FXa, especially in the absence of phospholipids, but a very poor substrate for the prothrombinase complex. Histone H4 also affects the pathway of prothrombin activation. The effect is particularly evident in the absence of phospholipids (Fig. 1B, C), where the consumption of prothrombin is accelerated and a new band appears in SDS-PAGE to document an additional cleavage at R155 to generate prethrombin-1 before activation proceeds along the prethrombin-2 pathway. The reaction is minimally perturbed in the presence of phospholipids (Fig. 1E,F) but progressively inhibited in the presence of prothrombinase, where activation proceeds along the meizothrombin pathway (Fig. 1H,I).

The multifaceted effect of histone H4 on prothrombin activation and autoactivation [7] strengthens the emerging role of histones as double-edged swords in immunothrombosis [8,16]. During response to traumatic injury, sepsis, inflammation and cell necrosis the levels of histones, RNA and polyamines become elevated in the blood [9–14] and contribute to coagulation imbalance, inflammation and multiple organ dysfunction [15]. The concentration of histones in the blood reaches 20 μM under pathological conditions [18], which is a level sufficient to promote autoactivation [7] and all the effects on activation by FXa reported in this study (Fig. 1). The prothrombotic effect due to autoactivation [7] is unlikely to be of physiological significance because it is too slow and easily surpassed by thrombin activation along the intrinsic pathway of the coagulation cascade triggered by necrotic tissue [11]. The procoagulant effect mediated by enhanced thrombin generation by FXa in the absence of phospholipids (Fig. 1A) is more rapid than autoactivation, but again its physiological significance diminishes when membrane surfaces are readily available for assembly of the prothrombinase complex. In this case, the effect of histone H4 turns into rapid and complete inhibition of thrombin generation (Fig. 1G) and is likely to dominate the pathophysiology. Indeed, addition of histone H4 to human plasma significantly and progressively prolongs both PT and APTT (data not shown). The recent suggestion of a prothrombotic effect of histones mediated by

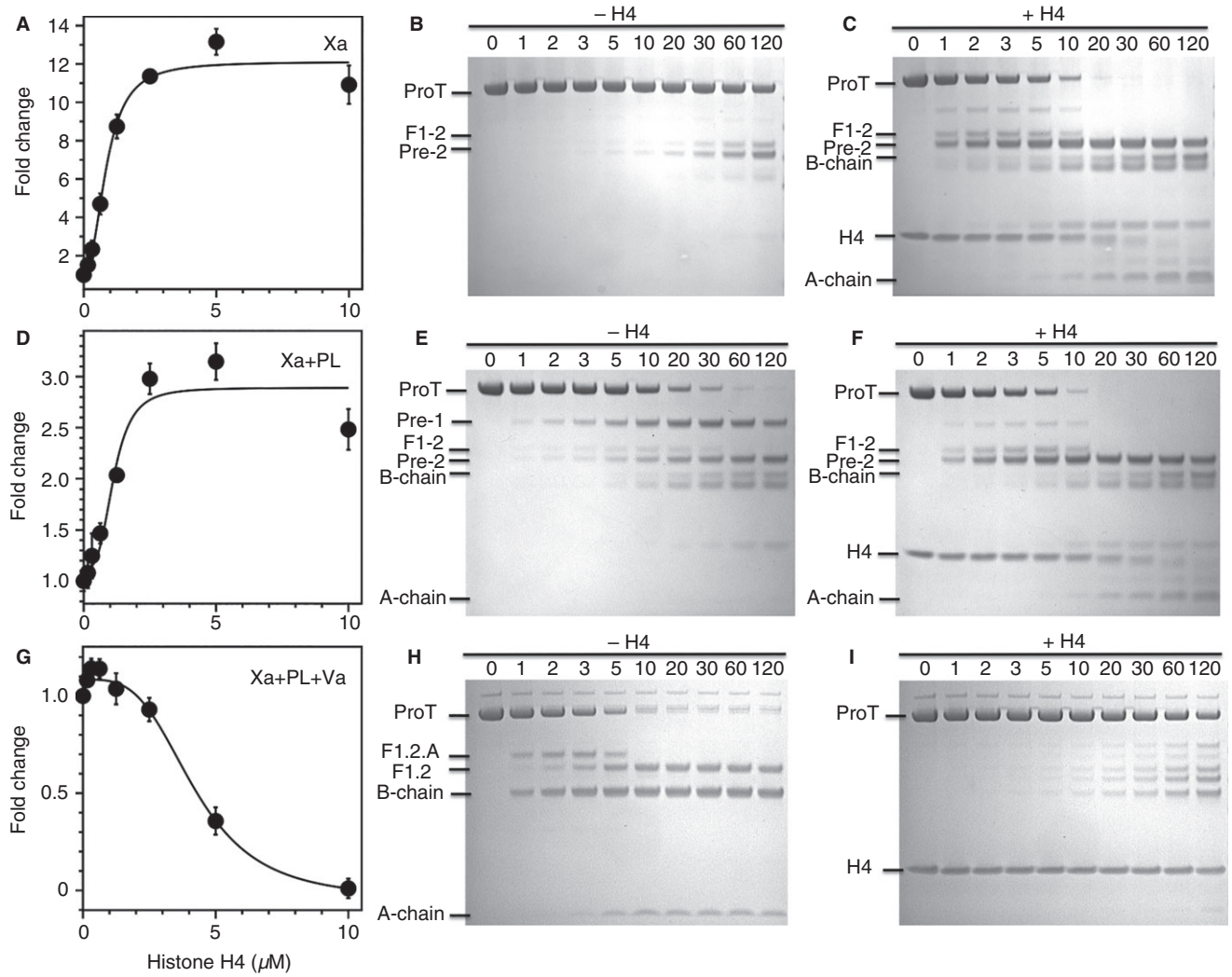

Fig. 1. Effect of histone H4 on prothrombin activation by factor Xa. Effect of histone H4 on the initial velocities of thrombin generation in the absence (A) or presence (D) of phospholipids, and in the presence of cofactor Va and phospholipids (G). The fold change measures the ratio between the initial velocity in the presence (v_i) and the absence (v_0) of histone H4. The concentration of histone H4 producing half of the maximal effect is (A) 0.9 ± 0.2 μM, (D) 1.0 ± 0.2 μM and (G) 3.5 ± 0.5 μM. The effect of histone H4 was also monitored by following the disappearance of prothrombin over time using SDS-PAGE under reducing conditions. In all assays, dansylarginine-N-(3-ethyl-1,5-pentanediyl) amide was added to inhibit thrombin and meizothrombin activity. Factor Xa cleaves prothrombin at R155, R271 and R320. Cleavage at R155 generates prethrombin-1 (Pre-1) and fragment-1 (F1) composed of the Gla domain and kringle-1. Cleavage at R271 produces prethrombin-2 (Pre-2) and fragment 1.2 (F1.2) composed of the Gla domain and the two kringles, and additional cleavage of prethrombin-2 at R320 generates the A and B chains. Direct cleavage of R320 generates the B chain and fragment 1.2.A (F1.2.A), where fragment 1.2 is attached to the A chain. In the presence of 3 μM histone H4, prothrombin activation by factor Xa is enhanced with (E, F) or without (B, C) phospholipids, but is inhibited in the presence of cofactor Va and phospholipids (H, I). The inhibition completely abrogates thrombin generation at high concentrations.

inhibition of the regulatory feedback mediated by the thrombomodulin-dependent protein C activation [13] needs re-evaluation in the context of our observations. It is difficult to envision a prothrombotic effect of histones, and certainly histone H4, that is mediated by inhibition of the thrombin-thrombomodulin complex if thrombin generation by the prothrombinase complex is completely inhibited in the first place. The dominant effect of histone H4 on prothrombin activation is therefore inhibitory, at least toward the major reaction driving thrombin generation in the coagulation cascade. Further investigation is clearly needed to clarify the effect of histones on prothrombin and other coagulation factors, which is more complex than currently assumed [11,16].

The interaction of histone H4 with prothrombin also has substantial mechanistic significance, especially in the context of recent crystallographic analysis [2,3,17]. Binding of histone H4 to the Gla domain is required for autoactivation [7] and probably mediates the dual effect on FXa activation reported here because of involvement of the

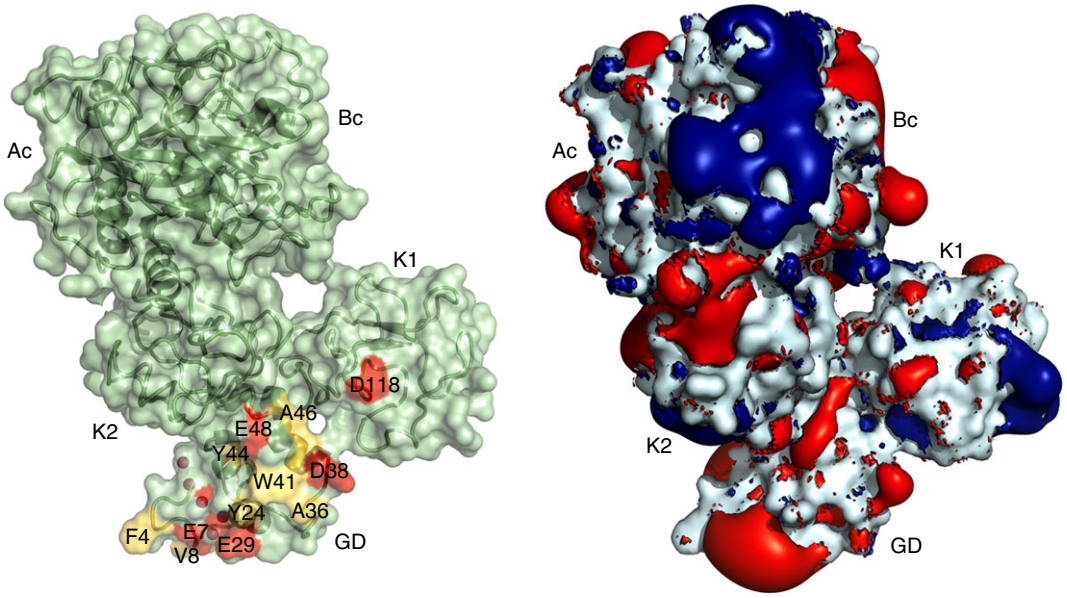

Fig. 2. Regions of prothrombin for potential interaction with histone H4. The crystal structure of prothrombin devoid of residues 146–167 in the linker connecting the two kringles [3] reveals the general assembly of the modular components of the zymogen (GD, Gla domain; K1, kringle-1; K2, kringle-2; Ac, A chain; Bc, B chain). The left panel shows acidic (red) and hydrophobic (gold) residues with solvent exposure > 70% in the Gla domain and adjacent kringle-1 that are prime candidates for mutagenesis screens aimed at identifying the epitope of prothrombin recognizing histone H4. Two of these residues (Y44 and D118) are associated with naturally occurring mutations causing severe bleeding [20–22]. The panel at right shows the electrostatic potential surface (red, negative; blue, positive), contoured at $\pm$ 2*k*T/*e* under conditions of 140 mM NaCl, pH 7.4. The surface identifies the Gla domain and neighbor kringle-1 as regions of charge complementarity to the positively charged histone H4.

sites of cleavage at R271 and R320. Furthermore, the interaction changes the conformation of prothrombin and makes R155 available for cleavage by FXa even in the absence of phospholipids (Fig. 1c). The effect underscores the molecular plasticity of prothrombin revealed by recent crystal structures and the important role of the linker connecting the two kringles, where R155 resides, in brokering long-range communication within the zymogen [2,3,17]. The inhibitory effect of histone H4 when prothrombin is bound to prothrombinase can be explained in terms of a direct antagonism of cofactor Va, whose epitope includes the Gla domain of the zymogen [19]. Histone H4 carries a net positive charge and binds to prothrombin through an electrostatically driven mechanism [7]. The crystal structure of prothrombin devoid of residues 146–167 [3] reveals a cluster of solvent-exposed acidic residues interspersed through hydrophobic residues in the Gla domain and adjacent kringle-1 (Fig. 2). The locale is associated with an area of strong and negative electrostatic potential that may help steer the positively charged histone H4 to its site and directly compete for cofactor Va binding. Interestingly, the naturally occurring mutations Y44C [20,21] and D118Y [22] affect this region of prothrombin and their bleeding phenotype may be explained by a weakened binding of cofactor Va. Alternatively, the epitope of prothrombin recognizing histone H4 may change upon binding to prothrombinase and the inhibitory effect on prothrombinase may originate from interference with FXa, rather than, or in addition to, cofactor Va. Furthermore, conformational transitions induced by histone H4 binding may have long-range effects on kringles and the protease domain, thereby disrupting interactions with prothrombinase at multiple sites beyond the Gla domain. A rigorous dissection of the molecular origin of the intriguing effect of histone H4 on prothrombin will therefore require extensive mutagenesis and functional and structural analysis using the strategy developed recently for the study of this zymogen [17]. The translational appeal of such an analysis should also be emphasized. Identification of the locale for histone H4 binding to prothrombin may point to new targets of therapeutic intervention where the activity of prothrombinase is inhibited through interference with prothrombin rather than with the enzyme FXa or cofactor Va.

Addendum

N. Pozzi and E. Di Cera designed the research and analyzed the data; N. Pozzi performed the research; E. Di Cera wrote the manuscript.

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Disclosure of Conflict of Interests

N. Pozzi is a co-founder of Hemadvance, LLC; E. Di Cera has a financial interest in the Verseon Corporation.

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