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Fibrin-targeted plasminogen activation by plasminogen activator, PadA, from *Streptococcus dysgalactiae*

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Abstract: Bacterial plasminogen activators differ from each other in their mechanism of plasminogen activation besides their host specificity. Three-domain streptokinase (SK) and two-domain PauA generate nonproteolytic active site center in their cognate partner plasminogen but their binary activator complexes are resistant to α2-antiplasmin (a2AP) inhibition causing nonspecific plasminogen activation in plasma. In contrast, single-domain plasminogen activator, staphylokinase (SAK), requires proteolytic cleavage of human plasminogen into plasmin for the active site generation, and this activator complex is inhibited by a2AP. The single-domain plasminogen activator, PadA, from *Streptococcus dysgalatiae,* having close sequence and possible structure homology with SAK, was recently reported to activate bovine Pg in a nonproteolytic manner similar to SK. We report hereby that the binary activator complex of PadA with bovine plasminogen is inhibited by a2AP and PadA is recycled from this complex to catalyze the activation of plasminogen in the clot environment, where it is completely protected from a2AP inhibition. Catalytic efficiency of the activator complex formed by PadA and bovine plasminogen is amplified several folds in the presence of cyanogen bromide digested fibrinogen but not by intact fibrinogen indicating that PadA may be highly efficient at the fibrin surface. The present study, thus, demonstrates that PadA is a unique single-domain plasminogen activator that activates bovine plasminogen in a fibrin-targeted manner like SAK. The sequence optimization by PadA for acquiring the characteristics of both SK and SAK may be exploited for the development of efficient and fibrin-specific plasminogen activators for thrombolytic therapy.

Keywords: plasminogen activation; PadA; fibrin; α2-antiplasmin; enzyme mechanism

Introduction

Plasminogen (Pg) activation is a key step of physiological fibrinolysis in animals for the dissolution of blood clots.[1] The physiological Pg activators, for example, urokinase-type plasminogen activator and tissue plasminogen activator activate Pg[2] to fibrinolytic enzyme, plasmin (Pm) which degrades the blood clots. Proteolytic activity of Pm is controlled through inhibition by its principal inhibitor in plasma, that is, α2-antiplasmin (a2AP).[3] However, fibrin bound Pg/Pm is protected from inhibition by a2AP due to nonavailability of binding sites of Pm molecule occupied by fibrin.[4]

Pg–Pm system is exploited elegantly by many bacteria, for example, *Streptococcus and Staphylococcus* spp., for the infection[5] into various animal hosts

Abbreviations: a2AP, α2-antiplasmin; PadA, Pg activator form *Streptococcus dysgalacitae*; Pm/Pg, Plasmin/plasminogen; SAK, Staphylokinase; SK, Streptokinase.

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Satish Singh and Timsy Bhando contributed equally to this work.

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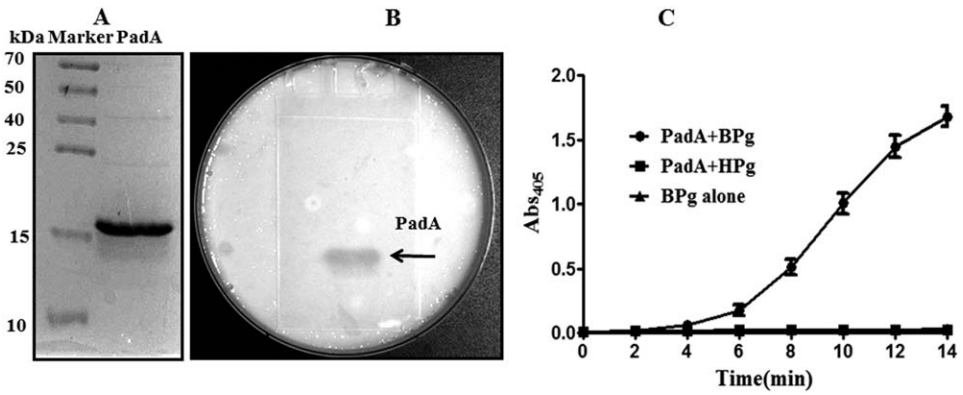

Figure 1. Functional characterization of PadA: (A) 15% SDS PAGE showing ~16 kDa purified PadA protein, (B) zymograph showing caseinolytic activity (clearance zone) of BPm generated from BPg activation by PadAin gel, and (C) PadA (75 nM) was mixed with bovine or human Pg (1.5 μ*M*) in the presence of chromozyme (1 m*M*) in HEPES buffer pH 7.4 containing 100 mM NaCl and reaction was monitored at 405 nm at 25°C to generate Pg-activation profile.

including humans,[6] by producing cofactors like streptokinase (SK), staphylokinase (SAK), and PauA.[7–9] These bacterial cofactors make 1:1 stoichiometric complex with Pg/Pm that acquires the ability to recognize activation loop of Pg as a substrate for the specific cleavage of Pg into Pm. SAK and SK activate human Pg (HPg) specifically while PauA is a bovine Pg (BPg) activator.[9–11] Besides host specificity, bacterial Pg activators differ from each other in their mechanism of Pg activation and their differential interactions with components of fibrinolytic system, for example, fibrin, a2AP and so forth. SAK, PauA, and SK are one-, two-, and three-domain bacterial Pg activators, respectively.[12,13] The mechanism of Pg activation by SAK differs from SK and PauA in distinct ways; (1) SK and PauA can generate a binary activator complex with their respective Pgs nonproteolytically while SAK requires human Pm (HPm) for making binary activator complex[14] and is unable to induce active site generation in HPg in a nonproteolytic mode. (2) SAK:Pm, binary activator complex is neutralized by a2AP in the absence of fibrin[15,16] while SK and PauA protect their partner Pm from a2AP inhibition[8] and catalyze nonspecific Pg/Pm conversion under physiological conditions. Understanding of these evolutionary strategies of bacteria to hijack Pg system has helped us to design and develop new thrombolytic molecules, for example, SK that can be used for treatment of human cardiovascular diseases[17,18] such as heart attack and stroke.

Considering the bacterial cofactors as potential thrombolytic molecules, detailed knowledge of their Pg-activation mechanism is an urgent need that has not been fully understood. For example, SK being a three-domain protein is a more efficient HPg activator but not fibrin specific while SAK, a single domain, fibrin-specific molecule requires Pm to initiate Pg activation.[14] The latest entry to the list of the Pg activators is a recently discovered single-domain BPg activator from *Streptococcus dysgalactiae*.[19] The most interesting and unusual feature of PadA is that being a single domain and showing 44% sequence homology to SAK, it can generate an active site in bovine plasminogen nonproteolytically like three-domain SK.[19] Functional properties of PadA under physiological conditions are unknown. The present study is aimed at delineating the Pg-activation potential of PadA in the presence of various physiological effecter molecules that regulate the fibrinolytic process. These findings demonstrate that PadA activates BPg in a fibrin-targeted manner and efficiency of its Pg activation increases several folds in the presence of fibrin. Thus, PadA represents a unique sequence-function hybrid molecule that combines the functional characteristics of SK as well as SAK. These characteristics of PadA together with its restricted substrate specificity and close sequence similarity with HPg activator, SAK, make it an interesting model for delineating the molecular mechanism and species specificity of bacterial Pg activators and design and development of new and more efficient thrombolytic drugs.

Results

Functional characterization of PadA

SDS-PAGE analysis of purified PadA protein showed approximately 16-kDa band as expected [Fig. 1(A)], and zymographic analysis exhibited clearance zone corresponding to the PadA band for the caseinolytic activity due to Pm generation [Fig. 1(B)]. Further testing of PadA indicated that it is able to activate BPg but not HPg, thus, validating its specificity for BPg [Fig. 1(C)] and supporting earlier observations.[19] There was no measurable amidolytic activity in the control reaction lacking PadA, indicating that there is no Pm contamination in the reaction.

Chloride (Cl^-) ions inhibit BPg activation by PadA

Pg activation in plasma is quite different from *in vitro* Pg-activation assays with purified protein

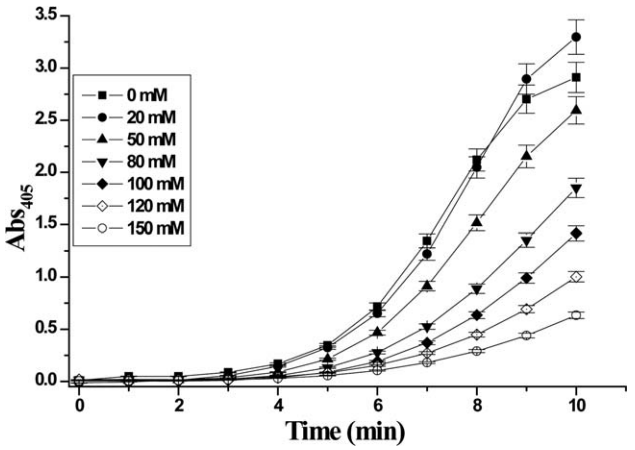

Figure 2. Effect of Cl^-ions on direct Pg activation by PadA: 75 n*M* PadA was directly added to 1.5 μ*M* BPg in HEPES buffer with increasing concentration of NaCl (0–150 m*M*) and Pm generation was monitored with chromozyme (1 m*M*) at 405 nm at 25°C. Dose dependent inhibition of Pg activation was observed. The graph shows the mean of three independent experiments.

components. As efficiency of Pg activation depends on conformational state of the Pg, we checked the ability of PadA for the BPg activation in the presence of Cl^-ions that is known to induce Pg into a closed state in plasma.[20–23] The activation of BPg by PadA was significantly delayed in the presence of Cl^- ions with prolonged initial lag phase (Fig. 2). Increasing the concentration of Cl^- ions from 20 to 150 m*M* (Fig. 2) displayed dose dependent inhibition of Pg activation. These results indicated that closed conformation of Pg is less accessible to PadA for the activation.

To further examine the effect of Cl^- ions on substrate Pg interactions with the binary activator complex, BPg was first preincubated with 100 m*M* NaCl and was used further as a substrate for the preformed activator complex to study the Pg-activation kinetics. Cl^- ions showed 3.5-fold increase in K_m value for Pg activation by PadA while k_{cat} remains almost the same (Table I).

Cyanogen bromide digested fibrinogen but not the intact fibrinogen enhances the rate of Pg activation by PadA

Contrary to Cl^- ions effect, fibrin opens the closed conformational of Pg for its easy activation that still differs for different Pg activators.[23] The Pg-activation ability of PadA was studied further in the presence of fibrinogen and cyanogen bromide (CNBr) degraded fibrinogen fragments. The intact fibrinogen did not show any effect on the activation profile of BPg by PadA [Fig. 3(A)], whereas, increasing concentration of CNBr digested fibrinogen exhibited significant increase in the Pg activation by PadA with reduction in initial lag phase [Fig. 3(B)]. When substrate BPg was preincubated with 0.1 mg/mL CNBr digested fibrinogen, 2.5-fold decrease in K_m was observed for Pg activation by PadA:BPg binary activator complex (Table I) while k_{cat} remained the same.

Binary complex formation between PadA and BPg

The observed effects of Cl^- ions and fibrin according to kinetic constants on activation of substrate Pg were basically related to the substrate interactions with the binary activator complex. To further comprehend the effect of these molecules on the activator complex of PadA and BPg, the complex formation was studied through amidolysis of chromozyme. However, Cl^-ions (100 mM) and CNBr digested fibrinogen (0.1 mg/mL) did not affect the formation of an active complex (Fig. 4).

The PadA:BPg binary complex is strongly inhibited by a2AP

Inhibition of Pm by a2AP completes by formation of a dead complex between the two that renders the

Table I. *Steady-State Kinetic Constants of BPg Activation by Preformed PadA:BPg Binary Activator Complex*

Reaction conditions	K_m (μ*M*)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\mu M^{-1}$)
100 m*M* NaCl	0.84 ± 0.1	3.825 ± 0.25	4.55
No NaCl	0.24 ± 0.09	3.01 ± 0.17	12.54
0.1 mg/mL CNBr digested fibrinogen	0.367 ± 0.09	3.5 ± 0.2	9.54
160 n*M* a2AP	1.7 ± 0.3	1.87 ± 0.2	1.1

Substrate Pg was incubated with a fixed concentration of ions or molecules as indicated and was used for activation by 40 n*M* preformed PadA:BPg complex in 10 m*M* HEPES buffer containing 1 m*M* Chromozyme. The reaction was monitored at 405 nm at 25°C. Five independent experiments were used to calculate the constants ± SEM by Michaelis–Menten equation in Graph Pad Prism.

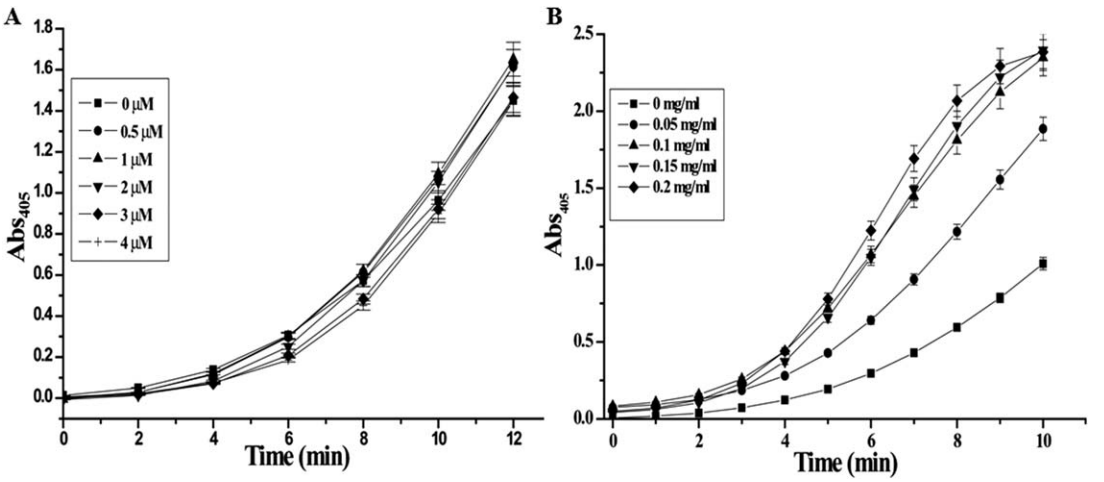

Figure 3. Effect of intact fibrinogen and CNBr digested fibrinogen on direct Pg activation by PadA: Pg activation by PadA was done in HEPES buffer with 100 m*M* NaCl as described above with increasing concentration of (A) intact fibrinogen (0.5–4 μ*M*) and (B) CNBr digested fibrinogen (0.05–0.2 mg/mL). The graph shows the mean of three independent experiments with standard error.

active site of Pm molecule incapable of carrying out its proteolytic function.[24] a2AP inhibits HPm or SAK:Pg/Pm complex with a second-order rate constant of $>10^6$ $M^{-1}s^{-1}$ [25] but SK protects the inhibition of HPm from a2AP.[26] Inhibition of PadA:BPg complex by human a2AP was studied through amidolysis by preformed binary activator complex in the presence or absence of a2AP. When preformed PadA:BPg binary complex was incubated with a2AP for 3–4 min before adding chromozyme, twofold molar excess of a2AP completely abolished the amidolytic activity (Fig. 5) indicating strong inhibition of PadA:BPg activator complex in the presence of a2AP.

PadA is recycled after inhibition of PadA:BPg complex by a2AP

As a2APstrongly inhibited the amidolytic activity of the PadA: BPg complex, we further examined whether this binary complex remains functionally dead or gets disrupted. Therefore, we checked Pg-activation kinetics after incubating the PadA:BPg activator complex with fourfold molar excess of a2AP for 3–4 min. Surprisingly, the mixture of

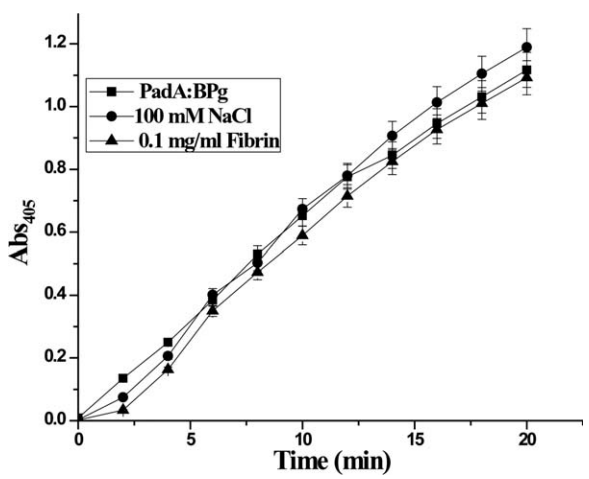

Figure 4. Effect of Cl^- ions, CNBr digested fibrinogen on PadA:BPg binary complex: BPg was preincubated with respective effector molecules and formation of active binary complex with PadA (150 n*M*, PadA:BPg) was studied through amidolysis of 1 m*M* chromozyme. 100 micromolar and 0.1 mg/mL CNBr digested fibrinogen did not affect the formation of binary activator complex. Three independent experiments were done to show the representative one.

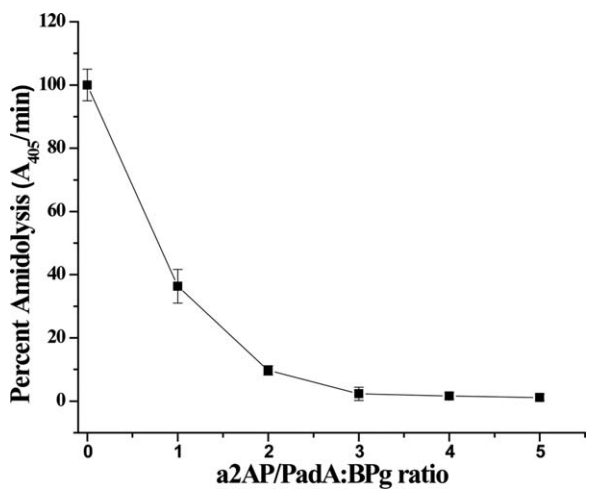

Figure 5. Inhibition of PadA: BPg binary activator complex with a2AP: Equimolar PadA:BPg binary activator complex was preincubated for 3–4 min. a2APin increasing concentration was allowed to react with preformed PadA:BPg (150 n*M*) complex and then amidolytic activity of this solution was measured at 405 nm after adding chromozyme. Percent amidolytic activity was plotted against inhibitor (a2AP) versus substrate (PadA:BPg) ratio. Fifty percentage inhibition of amidolytic activity was observed at 150 n*M* a2AP concentration.

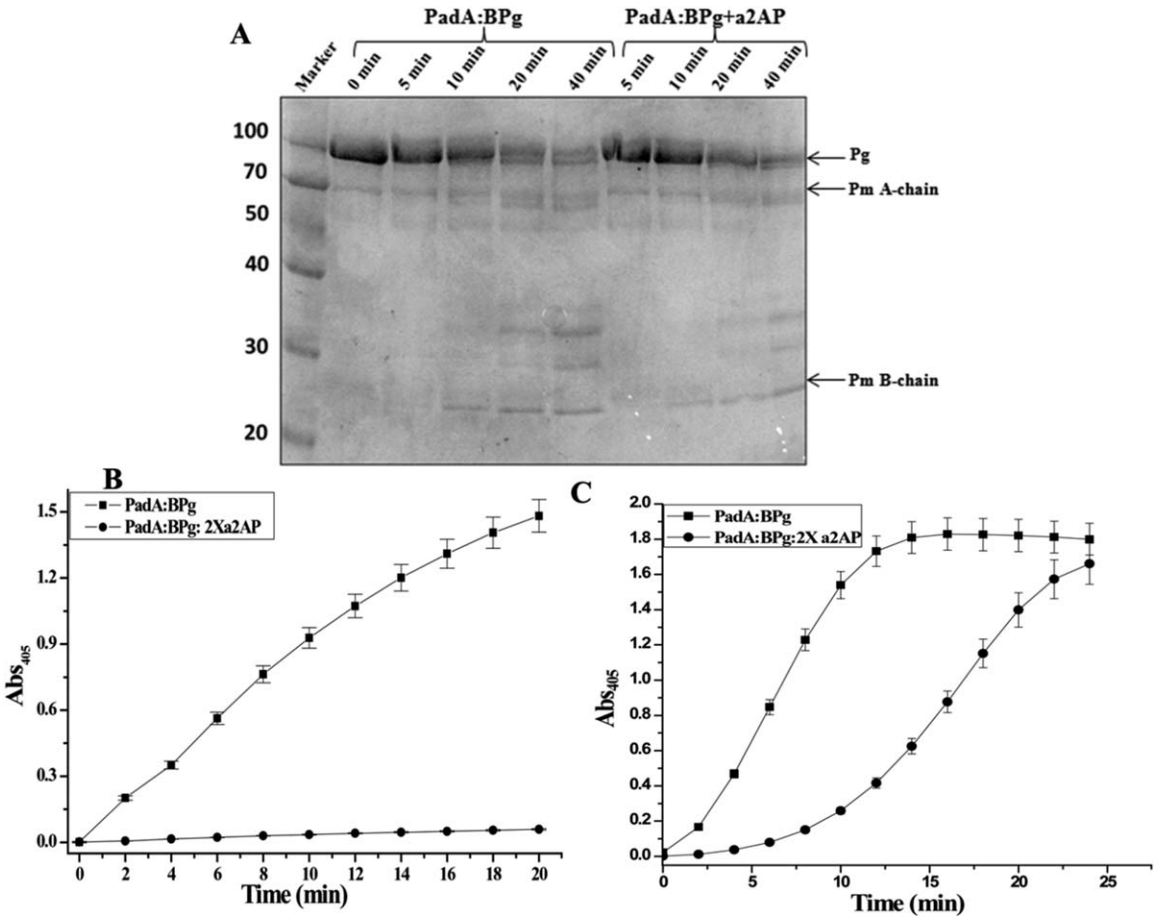

Figure 6. Effect of a2AP on Pg-activation ability of PadA: PadA: BPg complex was made to react with fourfold molar excess of a2AP, and then, this mixture was used to activate excess of BPg (1.5 μ*M*) (A). The samples were taken at different time intervals and run on 12% SDS PAGE. Simultaneously, reaction was monitored through amidolytic assay (B) amidolysis by 150 n*M* PadA:BPg complex in the absence and presence of 4× or four fold concentration of a2AP over complex showed inhibition of complex and (C) Pg-activation profile by a2AP inhibited complex of PadA:BPg also shows the retained Pg-activation ability. The results shown are representative of three experiments with standard error.

PadA:BPg and a2AP was still capable of activating the substrate Pg; however, the rate of activation was significantly reduced with a twofold increase in K_m and twofold decrease in k_{cat} (Table I). To further confirm our results of a2AP inhibition, activation of Pg was analyzed on SDS PAGE [Fig. 6(A)] and the reaction was simultaneously followed through amidolytic [Fig. 6(B)] and Pg-activation assay [Fig. 6(C)]. These experiments clearly suggested that a2AP inhibits the binary activator complex as shown by amidolysis but this complex is still capable of activating BPg as shown by SDS PAGE and Pg-activation profile which is possible only due to release or recycling of PadA from the inhibited complex.[15]

PadA does not protect bovine Pm from inhibition by a2AP

To study the effect of PadA specifically on inhibitory action of a2AP on bovine Pm (BPm), amidolytic assays of BPm were done in the absence or presence of a2AP and effect of increasing concentration of PadA in molar excess was studied. Even 20-fold molar excess of PadA over BPm could not protect the inhibitory action of a2AP (Fig. 7). Thus, PadA resembles SAK but not SK in its effect on protection of Pm from a2AP inhibition.

Fibrin protects PadA:BPg complex from inhibition by a2AP during clot lysis

Finally, interactions of PadA with BPg, fibrin, and a2AP in the clot environment were studied by following the lysis of fibrin clots containing BPg by PadA. To check that, Pg (500 n*M*) was incorporated into the fibrin clots (as described in experimental procedures), and then, PadA (100 n*M*) was added to initiate clot lysis in the presence or absence of a2AP to study its effect on clot lysis. Clot lysis profile of PadA in the presence or absence of a2AP was same that clearly indicated that the activator complex is

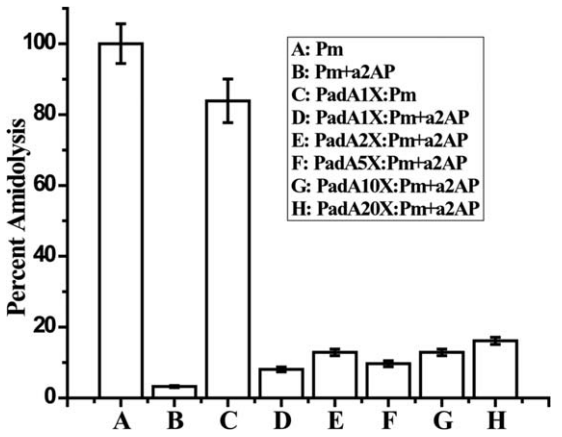

Figure 7. Influence of PadA on the inhibition of Pm by a2AP: 10 n*M* BPm was used for amidolysis of chromozyme (0.5 m*M*) in the absence or presence of a2AP and reaction was monitored spectrophotometrically at 405 nm. To study the effect of PadA on BPm inhibition by a2AP, 2–20 fold molar excess of PadA was incubated with Pm for 5 min, and then, a2AP was used to inhibit the complex for 3 min. Percent amidolytic activity was calculated as *dA/dt* and plotted against PadA concentration. PadA does not show any protective effect for Pm against inhibition by a2AP.

completely protected from inhibition by a2AP (Fig. 8) in the presence of fibrin clots.

Discussion

The bacterial Pg activators show an interesting evolutionary relationship among their sequence, structure/domain organization, and function. SK and PauA can activate their cognate Pg nonproteolytically but their Pg/Pm-activator complexes are resistant to a2AP inhibition. Conversely, SAK cannot activate HPg nonproteolytically but functions as a fibrin-specific Pg activator due to inhibition of SAK:HPm complex in plasma by a2AP. For bacterial Pg activators, capability of nonproteolytic active site generation and susceptibility to a2AP inhibition (or fibrin specificity) appear "two mutually exclusive functions." The newly discovered bacterial Pg activator, PadA, is an interesting fibrinolytic agent that has been recently reported to be capable of nonproteolytic active site generation in Pg. The present study unravels the role of various components of blood on functional activity of PadA and demonstrates for the first time that its Pg-activator activity is enhanced significantly and targeted specifically at the fibrin surface. These characteristics of PadA together with its close homology with HPg activator, SAK, may be exploited for designing and development of new therapeutic molecules having better thrombolytic potential.

Activation of Pg by bacterial Pg activators proceeds through the formation of a binary functional activator complex followed by the catalysis of substrate Pg involving multiple conformational changes. Under physiological conditions in plasma, Pg remains constantly in contact with various components of fibrinolytic system that may affect its conformational state and activation ability. For example, Cl^- ions are known to induce activation resistant closed (α) conformation of Pg (in plasma) while fibrin opens up Pg molecule to its γ-conformation.[21–23,27,28] However, activators differ in their Pg-activation ability that is dependent on kringle-mediated interaction of Pg with its activator. Both Cl^- ions and fibrin were found to influence the Pg-activation ability of PadA in a concentration dependent manner. Incubation of Pg substrate with Cl^- ions (100 m*M*) at a concentration approximately equal to physiological concentration (~140 m*M*) caused 3.5-fold increase in K_m while k_{cat} remained unchanged. In contrast, in the presence of CNBr digested fibrinogen, overall catalytic efficiency of PadA was doubled due to decrease in K_m. This is in variance with SK that does not require fibrin for the efficient Pg activation.[29] The catalytic switch that converts SK-mediated Pg activation from fibrin-independent to fibrin-dependent mode relies within the 1–59 amino-terminal residues of SK.[30] These results exemplify the mechanistic distinction of PadA from SK in having permanent fibrin specificity. In solution, Pg remains in a closed conformation that changes significantly when it binds to fibrin through its kringle domains. Enhanced Pg activation by PadA in the presence of fibrin indicates that open conformation of BPg is favored for the Pg activation by PadA. Contrary to substrate Pg activation, fibrin, and Cl^- ions did not affect the formation of binary activator complex between PadA and BPg as shown by its amidolytic profiles. These features of PadA

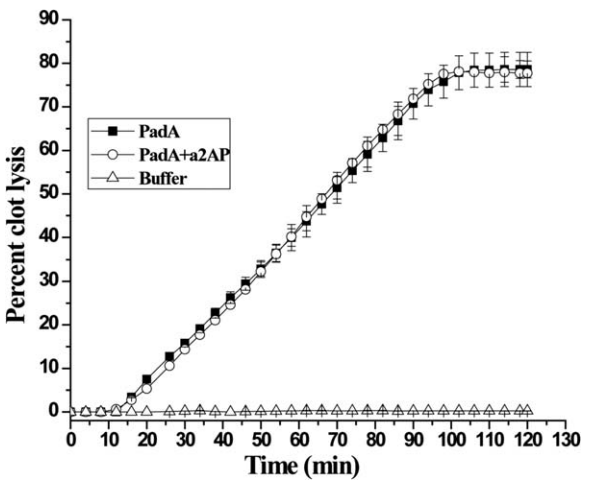

Figure 8. Clot lysis by PadA in the presence or absence of a2AP: 100 nM of PadA (with or without 500 n*M* a2AP) was used for the lysis of the fibrin clots containing 500 nM Pg and percentage of clot lysis were plotted against time. The graph shows that during clot lysis fibrin protects PadA:BPg inhibition by a2AP.

differ from SK and display similarity with SAK-mediated activation of substrate Pg.[31] The finding that fibrin but not intact fibrinogen modulates catalytic efficiency of Pg activation by PadA implies that it may not induce fibrinogen degradation in plasma.

Fibrin-specific Pg activation is an important determinant of therapeutic applicability of a thrombolytic agent. The fibrin-specific Pg activation by SAK occurs mainly due to inhibition of SAK:HPm complex by a2AP in plasma. Amidolytic activity of PadA:BPg binary enzymatic complex was completely inhibited by a2AP at twofold molar excess. Surprisingly, this inhibited binary activator complex was still able to activate BPg although with altered kinetics. At fourfold molar excess of a2AP over PadA:BPg complex, PadA showed 4–5 fold loss of its catalytic efficiency (Table I) but still PadA was capable of activating substrate Pg. The conversion of Pg to Pm under these conditions was also observed on SDS PAGE. These results strongly corroborate the behavior of PadA similar to SAK in terms of its inhibition by a2AP and its property to get recycled subsequently for another round of Pg activation.[15] To explore the effect of PadA directly on BPm:a2AP interactions, amidolysis by Pm and PadA (excess):Pm in the presence of a2AP was studied. However, PadA could not protect BPm from inhibition by a2AP as has been observed during interactions of SAK and Pm in the presence of a2AP. Finally, clot lysis experiments directly proved that fibrin protects PadA:BPg binary activator complex from a2AP inhibition. Taken together, these results demonstrate that PadA is a fibrin-specific Pg activator under physiological conditions.

Under physiological conditions, where Cl^- ions suppress Pg-activation susceptibility due to its closed conformation, bacterial infection by *Streptococcus* or *Staphyloccous* species may be affected. *Streptococcus dysgalactiae* causes mastitis disease in ruminants and septic arthritis specifically in lambs characterized by inflammation of mammary glands and synovial joints, respectively.[32,33] Pg is an important molecule in inflammation due to its role in extracellular matrix degradation and movement of inflammatory leukocytes.[30,34] Therefore, the differential capability of different bacterial cofactors is a major determinant for severity of infection and depends on the differential behavior of bacterial Pg activators in plasma under the influence of regulators like Cl^- ions and fibrin.

The higher order structural organization empowers bacterial Pg activators (like SK or PauA) with an inherent capability to generate an activator complex with Pg nonproteolytically and the protection of activator from inhibition by a2AP. Such a mechanism may provide a leading edge to pathogenic bacteria to generate systemic Pg activation that is advantageous for its dissemination during host infection. From negative selection theory of evolution, the shedding of structures from three domains (SK) to single domain (SAK) may compromise the Pg-activation ability of a bacterial cofactor but the molecule is reassured by attaining high-fibrin specificity to exploit the fibrin induced enhancement factor of Pg activation. PadA:BPg activator complex is protected from a2AP inhibition at fibrin surface. Moreover, the single domain Pg activators (like SAK or PadA) whose complexes with Pg/Pm are inhibited by a2AP in circulation are equipped with an intrinsic capacity to leave the activator complex and get recycled for grasping other partner molecules. The present study highlights the evolutionary adaptation by bacteria to precisely design a single domain, fibrin-specific Pg activator for bovine species as SAK for humans. The thrombolytic potential of a Pg activator is measured through its catalytic efficiency in clot environment (fibrin specificity) and its neutralization in plasma for its safety. The applicability of widely used SK for thrombolytic therapy is blemished by its nonspecific systemic Pg activation in plasma. Complete understanding of the mechanisms of Pg activation by PadA will provide new strategies for development of thrombolytic agents for human thrombolytic therapy. Additionally, close sequence similarity of PadA with HPg activator, SAK, and its species specificity for the BPg makes it an attractive model to understand molecular basis of species specificity in bacterial Pg activators and mechanism of proteolytic and nonproteolytic active site generation during cofactor mediated Pg activation.

Material and Methods

Reagents and chemicals

BPg (Enzyme Research Laboratories, USA), HPg, HPm, and chromozyme PL (Tosyl-glycyl-polyl-lysine-4-nitranilide-acetate) (Roche Diagnostics, Germany) were utilized in this study. Bovine fibrinogen, 4-methylumbelli-feryl-*p*-guanidino- benzoate (4-MUGB), and human a2AP were procured from Sigma. Ni-NTA resin and bicinchoninic acid kit were purchased from Thermo Scientific Pierce. DNA modifying enzymes and *E. coli* strains JM109 and Bl21 DE3 were procured from New England Biolabs or Fermentas. All other biochemicals used were of highest purity grade available commercially.

CNBr digested fibrin was prepared following the published procedures.[35] BPm was prepared by incubating molar excess of BPg (21.7 μM) with catalytic amount of PadA (5 nM) and diluted in excess to use it for experiments with negligible concentration of PadA. Concentration of Pm was determined by MUGB titration.[36,37]

Cloning, expression, and purification of recombinant proteins

The DNA sequence encoding PadA gene was retrieved from UNIPROT. The nucleotide sequences of the

mature PadA protein (without signal sequence) were artificially synthesized by Genscript and were provided cloned in their standard vector pUC57C. The gene was subcloned in pET28C (with N-terminal His Tag) vector with NdeI-BamHI restriction sites although conventional cloning methods and expressed in Bl21 DE3 expression host. His tagged PadA protein was purified by Ni-nitrilotiacetic acid (Ni-NTA) chromatography and used for subsequent biochemical assays after extensive dialysis.

Zymography and caseinolytic activity for BPg activation by PadA. BPg (500 μg/plate) was mixed with 5% skimmed milk and 1% agarose in 10 m*M* HEPES buffer (pH 7.5, 100 m*M* NaCl) in a petriplate, and SDS-PAGE gel containing PadA (after washing in assay buffer) was over layered for 6 h at 37°C to see its caseinolytic activity as described earlier.[38]

Kinetics of Pg activation by PadA

Pg-activation profile of PadA was generated by mixing 75 n*M* of PadA with 1.5 μM BPg in HEPES buffer (10 mM HEPES pH 7.5, 0.1% BSA, and 0.01% Tween 80, with or without 100 mM NaCl) containing 1 mM chromozyme in a 96 wells microtiter plate and generation of Pm was measured as a function of time at 405 nm at 25°C in a BioTEK Power wave X microplate reader.[39,40] To examine the effect of various effectors on Pg activation, the activation profiles of PadA:BPg complex were generated with increasing concentration of Cl^- ions, fibrinogen, and CNBr digested fibrinogen. To study the kinetics of Pg activation by PadA:BPg complex, equimolar concentration of PadA (0.75 μ*M*) and BPg (0.75 μ*M*) was incubated in assay buffer at 37°C for 5 min to generate the PadA:BPg bimolecular complex. The preformed activator complex (40 n*M*) was then mixed with increasing concentration of substrate BPg and generation of Pm was measured spectrophotometrically at 25°C using chromozyme (1 m*M*) as described above. The kinetic constants for Pg activation were calculated by Michaelis–Menten plot using Graph Pad Prism software. Substrate BPg was preincubated with a fixed concentration of NaCl (100 m*M*), CNBr digested fibrinogen (0.1 mg/mL) before adding preformed PadA:BPg complex to calculate the apparent kinetic constants. To study the effect of a2AP on Pg activation, PadA:BPg complex (40 n*M*) was incubated with a2AP (160 n*M*) for 3 min and then kinetics of Pg activation was studied as described above.

Complex formation assay

To study the complex formation between PadA and BPg, 150 n*M* PadA was mixed with 150 n*M* BPg in HEPES buffer, and formation of binary activator complex was studied through amidolysis of 1 m*M* chromozyme at 405 nm in a microplate reader.[19] To study the effect of various molecules like Cl^-ions, CNBr digested fibrinogen on complex formation between BPg and PadA, BPg was preincubated with respective ions or molecules, and then, the complex formation was monitored continuously with chromozyme. To generate the amidolysis profile of preformed binary activator complex, PadA (0.75 μ*M*), and BPg (0.75 μ*M*) were preincubated in assay buffer for 5 min, and finally, 150 n*M* of this complex was used to follow the amidolytic kinetics of chromogenic substrate chromozyme (0.5 m*M*). The inhibitory effect of a2AP on PadA:BPg binary activator complex formation was studied by monitoring the amidolysis with increasing concentrations of a2AP. Effect of PadA on inhibition of BPm by a2AP was studied through amidolysis using PadA in molar excess.

Clot lysis assay

Clot lysis assay was performed following the earlier described procedure[41] with minor modifications. Briefly, fibrinogen (2 mg/mL) was mixed with 500 n*M* BPg and 1 m*M* $CaCl_2$ in HEPES buffer (pH 7.5) containing 100 m*M* NaCl. Hundred microliter of this mixture was added to wells of microtiter plate containing 1 National Institute of Health (NIH) unit/mL thrombin and clots were allowed to form for 2 h at 37°C in the wells of microtiter plate. PadA (100 nM) was layered on the clots in the presence or absence of a2AP (500 n*M*: equimolar to Pg) and clot lysis was followed continuously at 405 nm in plate reader at 37°C. Percentage of clot lysis was calculated by using the formula-

$$\text{Percentage of clot lysis} = \frac{100 - \text{A405(final)}}{\text{A405(initial)}} \times 100$$

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