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Effects on human plasminogen conformation and activation rate caused by interaction with VEK-30, a peptide derived from the group A streptococcal M-like protein (PAM)

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ABSTRACT

In vertebrates, fibrinolysis is primarily carried out by the serine protease plasmin (Pm), which is derived from activation of the zymogen precursor, plasminogen (Pg). One of the most distinctive features of Pg/Pm is the presence of five homologous kringle (K) domains. These structural elements possess conserved Lys-binding sites (LBS) that facilitate interactions with substrates, activators, inhibitors and receptors. In human Pg (hPg), K2 displays weak Lys affinity, however the LBS of this domain has been implicated in an atypical interaction with the N-terminal region of a bacterial surface protein known as PAM (Pg-binding group A streptococcal M-like protein). A direct correlation has been established between invasiveness of group A streptococci and their ability to bind Pg. It has been previously demonstrated that a 30-residue internal peptide (VEK-30) from the N-terminal region of PAM competitively inhibits binding of the full-length parent protein to Pg. We have attempted to determine the effects of this ligand–protein interaction on the regulation of Pg zymogen activation and conformation. Our results show minimal effects on the sedimentation velocity coefficients ($S^{\circ}_{20,w}$) of Pg when associated to VEK-30 and a direct relationship between the concentration of VEK-30 or PAM and the activation rate of Pg. These results are in contrast with the major conformational changes elicited by small-molecule activators of Pg, and point towards a novel mechanism of Pg activation that may underlie group A streptococcal (GAS) virulence.

1. Introduction

In order for invasive pathogens to migrate beyond the site of infection, host physiological barriers such as the extracellular matrix, the basement membrane, and encapsulating fibrin networks must be overcome. To circumvent these impediments, proteolytic enzymes can facilitate dissemination of the microorganism. Along these lines, recruitment of a host protease to the bacterial surface represents a particularly effective mechanism for enhancing invasiveness [1–3].

Group A streptococcus (GAS is highly adapted to human hosts and affects over 700 million people worldwide each year. While the majority of these cases are classified as mild, a host of severe and aggressive invasive GAS infections befall approximately 18 million individuals per year [4]. Two of the more distinctive features of GAS are the production of cell wall-attached M proteins and the secretion of streptokinase (SK). The former minimize phagocytosis and mediate GAS binding to a number of host proteins, while the latter is a highly efficient activator of human plasminogen [5]. Plasminogen (Pg) is the zymogen of the serine protease plasmin (Pm) and circulates in blood and in most extravascular fluids. Fully active Pm is generated as a result of cleavage of R^{561}–V^{562} peptide bond and cleavage of the N-terminal 77-residues of the Pg precursor. Pm is the primary fibrinolytic agent in mammalian systems, but has also been implicated in processes such as cell migration, wound healing, angiogenesis, and the growth and metastasis of tumors [6]. The critical nature of activated host Pg in bacterial infectivity in humans has been reinforced through work demonstrating that mice expressing the transgene for hPg exhibit a striking increase in mortality when infected with human GAS isolates in comparison to littermate controls [7,8].

M proteins belong to a family of well-characterized surface proteins that form α-helical coiled-coils on the bacterial surface. One member of the M protein family, Pg-binding group A streptococcal M-like protein

Abbreviations: 6-AHA, 6-aminohexanoic acid; GAS, group A streptococcus; PAM, plasminogen binding group A streptococcal M-like protein; Pg, plasminogen; Pm, plasmin; SK, streptokinase; uPA, urokinase-type Pg activator

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(PAM), is a 43 kDa single polypeptide chain that binds Pg or Pm with high affinity. Its role as a virulence factor in concert with human Pg/Pm has been confirmed in a study in which a GAS strain expressing a mutated version of PAM deficient in Pg-binding was found to be markedly less virulent than the strain expressing wt-PAM in mice carrying the hPg transgene [9]. Contained within the N-terminal region of PAM are two tandem repeats that direct the binding of PAM to Pg. Despite the obvious absence of a C-terminal lysine residue within this span, the PAM docking site on Pg has been localized to the lysine-binding site (LBS) of kringle 2 (K2), the weakest of the lysine-binding kringles [10]. Binding studies have established that a 30-residue peptide (VEK-30) that overlaps with this internal Pg-binding sequence, corresponding to PAM residues 85–113 with a non-native Tyr appended to the C-terminus as a spectrophotometric handle, is a convenient, readily synthesized surrogate ligand for full-length PAM [10,11]. Crystal structures of K2 and K1–K3 in complex with VEK-30 have revealed that the through-space orientations of the Arg^{17}, His^{18}, and Glu^{20} side-chains of VEK-30 are roughly isosteric with the charged moieties of a C-terminal lysine [12,13]. Additionally, the finding that the binding of VEK-30 to K1–K3 results in the disappearance of K3 electron density [13] suggests that a large conformational change may be operative in Pg-PAM functionality, akin to that observed for Pg in the presence of small-molecule activators such as 6-aminohexanoic acid (6-AHA) [14,15]. Preliminary data from a recent study, in which VEK-30 was found to augment SK-mediated activation of Pg, is likewise supportive of this premise [16]. The present study was thus undertaken to determine if structural alterations attend the binding of VEK-30 to Pg, as evaluated by the effects of the bacterial peptide on the hydrodynamic volume and activation rate of Pg. The effects of full-length wt-PAM on Pg activation properties were also examined. The results of this study point towards a physiological scenario in which VEK-30 and PAM effect subtle structural changes in Pg that render it more susceptible to the action of the urokinase-type Pg activator (uPA) and SK, potentially facilitating the invasive character of PAM-expressing GAS strains.

2. Experimental

2.1. Proteins and peptides

Wild type (wt) human Glu^1-Pg was a gift from Enzyme Research (South Bend, IN). SK from *Streptococcus equisimilis* was expressed and purified as described [17]. Wt-PAM (GAS strain NS13) and the mutants $K^{98}A/K^{111}A$ (KK-PAM), $R^{101}A/H^{102}A$ (RH-PAM) and $K^{98}A/R^{101}A/H^{102}A/E^{104}A/K^{111}A$ (KRHEK-PAM) were expressed and purified as described in an earlier study [18]. VEK-30 and a scrambled version of VEK-30 (sVEK-30), consisting of identical amino acid composition but randomized primary structure, were synthesized as previously published [12].

2.2. Analytical ultracentrifugation

All experiments were conducted in a Beckman XL-I Optima™ analytical ultracentrifuge (Beckman-Coulter, Fullerton, CA) operated at 20 °C. Sedimentation coefficients of wt Pg in the presence and absence of VEK-30 were determined under previously specified conditions [19]. The concentration of Pg was 0.5 mg/mL. Runs conducted in the presence of VEK-30 employed Pg:VEK-30 ratios of 1:5 and 1:20 (mol:mol). Analysis of the sedimentation velocity curves using Beckman XL-A/XL-I software (version 4.0) generated the S_{app} values. For the determination of the apparent molecular weight ($M_{w,app}$) of PAM by sedimentation equilibrium, the protein was dissolved to a final concentration of 22 μM in 10 mM HEPES/100 mM NaCl pH 7.4, and rotated at speeds of 10,000 and 15,000 rpm. The data were analyzed using aforementioned software provided by Beckman, as was the simulation of the radius vs. absorbance curve for purely monomeric PAM. The experimentally derived apparent molecular weight was determined from global fitting of the data from three separate scans at the two indicated speeds.

2.3. Pg activation assays

Activation of full-length Glu^1-Pg was followed at 25 °C in a Spectramax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA) at 405 nm. The zymogen (27–270 nM) was incubated with 250 μM of the chromogenic substrate, S-2251 (Chromogenix, Milan, Italy) and varying concentrations of VEK-30 or PAM proteins, as indicated. After 10 min, Pg activation was initiated by the addition of Abbokinase (human uPA for injection, Abbott Laboratories, Chicago, IL) or by either an equimolar amount of SK (with respect to the molar concentration of Pg) or a catalytic amount of pre-formed SK–Pm. The latter was made immediately prior to use by mixing 3 μM each of Glu-Pg and SK (molar ratio) in 10 mM HEPES/100 mM sodium acetate, pH 7.4, and incubating for 30 min at room temperature. These conditions were sufficient for complete conversion of Pg to Pm, as confirmed by performing SDS-PAGE on the samples under reducing conditions in which the light and heavy chains of Pm, but not single-chain of the Pg zymogen, were observed. All activation assay reactions consisted of a total volume of 200 μL in either 10 mM HEPES/100 mM NaCl, pH 7.4 or 10 mM HEPES/100 mM sodium acetate, pH 7.4. All the data presented correspond to the average of duplicate assays. Each experiment was repeated at least twice.

2.4. Surface plasmon resonance (SPR)

All the experiments were carried out in a BIAcore X instrument (BIAcore AB, Uppsala, Sweden). Pg or wt-PAM proteins were coupled to CM-5 sensor chips by the amine coupling method, to a level of 200–1000 resonance units (RU). All assays were carried out at 25 °C in HBS-EP buffer (BIAcore). Progress curves for binding were obtained by injecting PAM proteins (0.5 nM–50 nM) over immobilized Pg. Regeneration of the chip surface was accomplished by injecting 100 μL of 10 mM glycine pH 1.5. The entire set of sensorgrams for each experiment was X- and Y-transformed and the binding data were fitted to a 1:1 Langmuir binding model using the BIAevaluation 4.1 software (BIAcore AB). Association and dissociation rate constants (k_{on} and k_{off}) were fitted globally and separately. From these data, the equilibrium dissociation constants (K_d) were calculated. Validation of the K_d values derived from the kinetic data was achieved through evaluation of steady-state binding data by plotting response at equilibrium versus analyte concentration.

3. Results

3.1. Effects of the bacterial PAM peptide on the sedimentation properties of Pg

The present study was undertaken to determine if the interaction between the PAM peptide and full-length Pg had discernible consequences on the tertiary structure of the latter. For this purpose, sedimentation velocity analyses were performed on full-length Pg in the presence and absence of VEK-30 in Cl^-- and acetate-containing buffers. Cl^- is considered to be a negative effector of Pg activation that promotes the closed, tight conformation of the zymogen, while acetate ion is much less effective in this regard [20]. In contrast to Cl^-, C-terminal lysine analogs (such as 6-AHA) are considered to be positive effectors of activation, due to their ability to promote the open, relaxed conformation of Pg [14]. This is dramatically illustrated by the approximately 1 unit decrease in sedimentation coefficient ($S°_{20,w}$) of Pg when saturating amounts of 6-AHA are added to Pg in Cl^--containing buffer [14,19,20]. As can be concluded from the data summarized in Table 1, the effect of a 5-fold molar excess of VEK-30 on Pg conformation is negligible in Cl^- buffer and small, but significant, in acetate buffer. At a 20:1 molar ratio of