The Lack of Binding of VEK-30, an Internal Peptide from the Group A Streptococcal M-like Protein, PAM, to Murine Plasminogen is Due to Two Amino Acid Replacements in the Plasminogen Kringle-2 Domain*

Qihua Fu, Mariana Figuera-Losada, Victoria A. Ploplis, Sara Cnudde, James H. Geiger, Mary Prorok, and Francis J. Castellino

From the W.M. Keck Center for Transgene Research and the Department of Chemistry & Biochemistry, University of Notre Dame, Notre Dame, IN 46556 and Department of Chemistry, Michigan State University, East Lansing, MI 48824

Running head: Determinants of GAS dissemination

Address correspondence to: Francis J. Castellino, W. M. Keck Center for Transgene Research, 230 Raclin-Carmichael Hall, University of Notre Dame, Notre Dame, IN 46556, Tel. 574-631-9152; Fax: 574-631-8017; E-mail: fcastell@nd.edu

VEK-30, a 30-amino acid internal peptide present within a streptococcal M-like plasminogen (Pg) binding protein (PAM) from Gram-positive group-A streptococci (GAS), represents an epitope within PAM that shows high affinity for the lysine binding site (LBS) of the kringle-2 (K2) domain of human (h)Pg. VEK-30 does not interact with this same region of mouse (m)Pg, despite the high conservation of the mK2- and hK2-LBS. To identify the molecular basis for the species specificity of this interaction, hPg and mPg variants were generated, including a hPg chimera with the mK2 sequence and a mPg chimera containing the hK2 sequence. The binding of synthetic VEK-30 to these variants was studied by surface plasmon resonance. The data revealed that, in otherwise intact Pg, the species specificity of VEK-30 binding in these two cases is entirely dictated by two K2 residues that are different between hPg and mPg, viz., R220 of hPg, which is a Gly in mPg, and L222 of hPg, which is a Pro in mPg, neither of which are members of the canonical K2-LBS. Neither the activation of hPg, nor the enzymatic activity of its activated product, plasmin (hPm), are compromised by replacing these two amino acids by their murine counterparts. It is also demonstrated that hPg is more susceptible to activation to hPm after complexation with VEK-30 and that this property is greatly reduced as a result of the R220G and L222P replacements in hPg. These mechanisms for accumulation of protease activity on GAS likely contribute to the virulence of PAM+ GAS strains and identify targets for new therapeutic interventions.

From the initial early discoveries of the modular structure of plasminogen (Pg) (1), and continuing research showing the independent nature of its component domains, an appreciation of the multifunctional nature of this system, as revealed by in vivo studies of mice with an inactivated Pg gene, has continually emerged (2-4). Much of this functional diversity is revealed by the nature of the ligands that interact with Pg, especially via the constitutive kringle (K) motifs of the latent noncatalytic chain of this protein. All Pg proteins that have been studied contain 5 consecutive highly homologous kringle domains (1), which can be roughly classified by the strength of their interactions with lysine, and analogues thereof, e.g., ε-aminocaproic acid (EACA).
These small molecules mimic the manner in which Pg, and its activated product, plasmin (Pm), interact with proteins containing COOH-terminal lysine residues.

Important functional consequences accrue when Pg/Pm interacts with other proteins. For example, the binding of Pm, through its lysine-binding kringle domains, with degrading fibrin clots, wherein COOH-terminal Lys/Arg residues are liberated, further enhances the degradation of fibrin via plasminolysis (9,10). Also, the binding of Pg to COOH-terminal lysine residues of degrading fibrin stimulates its activation to Pm by fibrin-bound tissue-type plasminogen activator (tPA), and this represents a major pathophysiological pathway of activation of the fibrinolytic system by tPA (11). In addition, the lysine binding properties of Pg/Pm kringle regions results in localization of Pg/Pm on cell receptors (12), with concomitant development of cell-surface proteolytic capabilities that are important to a number of extracellular-related processes, e.g., extracellular matrix degradation, tumor cell invasion and metastasis, and wound healing (13).

The cellular binding properties of Pg/Pm are also critical for bacterial pathogenesis, and Pg receptors are present on a number of Gram-positive and Gram-negative bacteria, including Gram-positive group A streptococcus (GAS) (14), a strain that is a major cause of skin and mucosal infections in humans (15). Similar results have also been published for Pg binding and activation to Mycobacterium tuberculosis (16). In the case of GAS, human (h)Pg interacts with high affinity, through its lysine binding sites (LBS), to the Pg-binding group-A M-like protein (PAM) of GAS and is activated to Pm by streptokinase (SK), which is produced by this GAS strain. This provides Pm as a source of the cell surface protease activity of the bacteria that allows its penetration into surrounding tissue (17,18). VEK-30 is a 30-residue internal polypeptide of PAM that contains most of its N-terminal a1 and a2 repeats, but does not possess a COOH-terminal Lys residue. Binding determinants of the PAM/hPg interaction are recapitulated by VEK-30 and the K2 domain of Pg, normally a very weak lysine-binding kringle (19). We have shown that the VEK-30/hK2 interaction depends upon His, Arg, and Glu side-chains (20), and an X-ray crystallographic structure of the VEK-30/hK2 complex shows a unique binding modality of VEK-30 to the lysine binding site (LBS) of hK2. In this case, an internal "pseudo-lysine", formed from the positive VEK-30 side-chains of R17 and H18, and the negative side-chain, E20, of VEK-30, separated by one α-helical turn, is inserted into the LBS of hK2 (21).

mK2 does not interact with VEK-30 despite very high sequence identity, generally, and full conservation of all residues in the LBS. We hypothesize that 2 residues present in mPg, G220, which is an Arg in hPg, and P222, which is a Leu in hPg, may explain the species specificity. In unliganded hK2, R220 forms a salt bridge with D219, preventing the latter from completing the anionic locus with D221 (a motif that is present in all other ligand-binding kringles), thus diminishing the ability of hK2 to bind lysine analogues. Upon VEK-30 binding, R220 interacts with E7 of VEK-30, permitting D219 to reorient into the LBS whereupon the canonical anionic locus is formed (21-23). R220 is not only absent in mPg, but also in all other hPg and mPg kringles that fail to bind VEK-30. In addition, P222 of mPg is immediately proximal in sequence to the cationic binding locus (R234) of the K2-LBS. While existing structural studies
do not point to any direct role for residue 222 in ligand binding, its location within the LBS may alter the disposition of ligand-contacting residues depending upon the nature of the amino acid at this sequence position. In the absence of any structural data on mK2, we postulate that Pro at sequence position 222 causes a localized deleterious conformational change in this kringle with regard to VEK-30 binding. In order to test these hypotheses, we have constructed strategic site-directed and chimeric mouse/human Pg mutants and studied their binding affinities to VEK-30.

Experimental Procedures

Construction of Pg expression plasmids - Wild-type (WT)-hPg and WT-mPg expression plasmids, hPg-Puro-pMT and mPg-Puro-pMT, respectively, were generated by insertion of the cDNAs encoding these proteins into the multiple cloning site of the Drosophila S2 parent expression plasmid, puro-pMT. This latter plasmid was generated by inserting the pCoPuro cassette (24) into the commercial plasmid, pMT/BiP/V5-His(A) (Invitrogen, Carlsbad, CA). To accomplish this, a linker consisting of two oligo DNAs, 5’-cgccggtaccatcgatatcactagtgctag and 5’-cgctagcactagtgatatcgatggtaccgc, which generated NheI, SpeI, EcoRV, and KpnI sites, was inserted at NarI site located upstream of the metallothionein promoter (pMT) of pMT/BiP/V5-His. The resulting plasmid was digested by NheI and KpnI in order to insert the XbaI/KpnI fragment from pCoPuro (24), which contains the copia promoter, puromycin N-acetyltransferase (pac), and the SV40 poly(A) sequence.

The mRNA for mPg was obtained from C57Bl/6 mouse liver using TRizol reagent (Invitrogen). The first strand of cDNA was generated by SuperScript-III reverse transcriptase (Invitrogen) and the entire cDNA was obtained by the polymerase chain reaction (PCR) with ExTaq polymerase (Takara Bio USA, Madison, WI). The sequence of the cDNA was confirmed bi-directionally. After obtaining the entire cDNA, the expression cassette for mPg was amplified using the following primers;

mPg-Forward-BglII: 5’-gaagatctgactcgtgaggtgctcataagc (the BglII site is underlined)

mPg-Reverse-XbaI: 5’-cttcagatggattcctcatctctcattcc (the XbaI site is underlined).

The PCR amplicon (2398 bp) was purified by Microcon YM-100 (Millipore, Billerica, MA) and digested by BglII and XbaI, followed by its ligation into pMT-Puro through these same sites, thus providing the final expression plasmid, mPg-Puro-pMT.

The hPg expression cassette was excised using the BglII/XbaI fragment of pMT-BiP-hPg (25) and inserted into puro-pMT, through the same BglII and XbaI sites, yielding hPg-Puro-pMT, which was used for expression of hPg.

The mPg and hPg chimeric expression plasmids with K2 domain interchanges (mPg[hK2] and hPg[mK2], respectively) were generated using restriction endonuclease-free (RF) cloning (26) with the PCR primer pairs (synthesized by MWG Biotech, High Point, NC) and templates listed in Supplemental Table 1S. The PCR products were purified using the Qiaquick Gel Extraction kit (Qiagen, Valencia, CA). The total mutagenesis reaction volume was 25 µl, including 50 ng of purified hK2.
or mK2/20 ng of hPg-Puro-pMT or mPg-Puro-pMT/200 μM dNTP/1x reaction buffer (Stratagene)/1.5 μl QuikSolution/1.25 units of pfu Ultra high-fidelity DNA polymerase (Stratagene). The mixture was heated for 60 sec at 95°C, followed by 30 cycles, consisting of denaturation at 95°C for 50 sec, annealing at 60°C for 50 sec, and elongation at 68°C for 12 min. The product was then treated with 5 units of DpnI at 37°C for 2 hr to digest the methylated parental plasmid. The reaction mixture was then transformed into XL10-Gold ultracompetent cells (Stratagene). Positive clones were sequenced by the W. M. Keck Biotechnology Center (University of Illinois, Urbana-Champaign, IL).

Point mutated hPg and mPg plasmids were created with the QuikChange XL site-directed mutagenesis kit (Stratagene, LaJolla, Ca), using the primers and templates listed in Supplemental Table 1S. All final sequences were verified by DNA sequence analyses.

Construction of the WT-uPA expression plasmid - The plasmid (PDB1519) containing mouse uPA (muPA) full-length cDNA was purchased from ATCC (Rockville, MD). A primer pair, consisting of uPA-F (5'-GTAGATCTGGCAGTGTACTTGAGGTC TGAGC TC) and uPA-R (5'-CGACTCGAGTCCAGAAGGCCAGACCTTTTCT), as the PCR forward and reverse primers, respectively, was used to amplify the cDNA coding for muPA, lacking the signal peptide coding region (the first 60 bp). The muPA amplicon, digested with BglII and XhoI, was then subcloned into the puro-pMT expression plasmid, which also provides the BiP signal peptide. A stop codon was inserted just prior to the V5-His6 tags, since we desired to use a simple conventional purification protocol for the muPA protein. PCR was carried out using pfuDNA polymerase as above.

Protein expression and purification - The WT and altered Pg plasmids were purified by use of a Qiagen Plasmid Midi kit (Qiagen) and then transfected into Drosophila Schneider S2 cells using a Calcium Phosphate Transfection kit (Invitrogen). Stably transfected S2 cells were then placed under selection pressure with 10 μg/ml of puromycin. Surviving S2 cells were seeded into 1 l of fresh serum-free medium (EX-CELL 420 Serum-free medium for insect cells with L-glutamine; SAFC Biosciences, Lenexa, KS) in a 1 l spinner flask, at a cell density of 3–6 × 10^6 cells/ml. After reaching a density of 6–8 × 10^6 cells/ml, the cells were induced for 2 days by 500 μM copper sulfate/5 mg/l aprotinin (Sigma). The conditioned culture medium was immediately concentrated at 4°C in an Amicon concentration cell using a 10,000 NMWL ultrafiltration membrane (Millipore, Billerica, MA). Following dialysis against 100 mM sodium phosphate buffer, pH 7.3, at 4°C overnight, recombinant (r) Pg was purified by affinity chromatography on lysine-Sepharose as previously reported. The r-Pg was eluted from the column by 100 mM EACA in dialysis buffer. Finally, the purified protein was concentrated by Centricon Plus-20 Filter Unit with Ultracel-10 membrane (Millipore).

The expression of r-mouse uPA (rmuPA) was performed as for Pg. After concentrating the conditioned medium in the same manner as above, the supernatant was loaded onto a CM-Sephadex column. The column was washed with 25 mM sodium phosphate buffer, pH 7.3, and the r-muPA was eluted using a linear gradient of 25 mM sodium phosphate, pH 7.3, as the start solution, to 25 mM sodium phosphate/0.5 M NaCl, pH 7.3, as the limit solution. The associated fractions were
concentrated to about 1 ml, and loaded onto a 150 ml column of Sephadex-G75. The column was eluted with a solution of 25 mM sodium phosphate/150mM NaCl, pH 7.3. Then, the purified protein was concentrated using a Centricon Plus-20 Filter Unit with Ultracel-10 membrane (Millipore).

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) - SDS-PAGE was performed in a Mini Protean II electrophoresis apparatus (BioRad, Hercules, CA). The proteins were separated in 8% (w/v) polyacrylamide gels under reducing conditions and stained with Seeband staining solution (Gerard Biotech, Oxford, OH).

Western blots - The expressed proteins were detected by Western blotting using as the primary antibody rabbit-anti-hPg that was produced in our center, which reacts with both hPg and mPg. Goat-anti-rabbit IgG-conjugated to alkaline phosphatase (AP; Sigma) was employed as the secondary antibody. The chromogen, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, was used for detection.

Activation of Pg variants by SK - In one set of amidolytic assays, the molar ratio of SK and the Pg to be examined was set at 1:1 to monitor generation of the SK-Pm activator complex, and, in another set of assays, this molar ratio was 1:20 in order that Pm activity, from catalytic activation of Pg to Pm, by the SK-Pm complex, could be measured. A last set of assays was designed to assess the activation of mPg variants by a catalytic amount of the SK-hPm activator complex at a 1:20 molar ratio of SK-hPm:mPg. For this latter assay, SK and hPg (1:1 molar ratio) were pre-incubated for 1 hr at 25°C, after which a 20-fold molar excess of mPg variants were added.

In each assay, the reaction components were present in a volume of 200 μl, and contained 0.5 mM of the chromogenic substrate, S-2251 (Glu-Leu-Lys-p-nitroanilide; synthesized in our laboratory), and Pg (100 nM for the 1:1 molar ratio with SK, and 200 nM for the 20:1 molar ratio of Pg:SK or mPg:SK-hPm). The reaction was accelerated by addition of the required amount of SK (100 nM or 10 nM for the 1:1 and 1:20 ratios of Pg, respectively). The reaction buffer was 10 mM Heps/150 mM NaOAc, pH 7.4. The absorbance at 405 nm was measured over 30 min with a Spectramax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA, USA). Kinetic parameters were derived from plots of the absorbance at 405 nm versus time.

The assays were performed in triplicate, and the values are expressed as the mean ± S.E.

Activation of Pg variants by uPA - The assay conditions were as for SK, except that the activation of Pg (200 nM), in the presence of 0.5 mM S2251, was accelerated by addition of r-muPA at a final concentration of 0.5 μg/ml. The hydrolysis of S-2251 was monitored continually at 405 nm for 30 min and the absorbance/min was calculated. These values for hPg and mPg were taken as 100% for comparison of activations of Pg variants within the same species of Pg.

Activation of hPg and hPg variants in the presence of VEK-30 - Activation of WT and variant hPgs by an equimolar amount of streptokinase (SK) was monitored continually at 405 nm at 25°C. The reaction buffer utilized for the assays was 10 mM Heps, 100 mM NaCl, pH 7.4. Each reaction was performed in duplicate and consisted of 0.25 mM S-2251, 150 nM hPg or hPg variant, 150 nM SK, and varying concentrations of VEK-30, in a total volume of 200 μl. The effect of VEK-30 on the uPA-catalyzed activation
of hPg was also examined under the same reaction and buffer conditions. In these experiments, 25 nM hPg, 10 IU of uPA (Abbokinase; Abbott Laboratories, N. Chicago, IL), and varying concentrations of VEK-30 were employed. These sets of experiments were repeated with a randomized version of VEK-30 (AENKVDEQREYHLKEAERKLETELSELY-NH₂) that was synthesized as described for VEK-30 (20).

Surface Plasmon Resonance (SPR) - For preparation of the sensor chip, VEK-30, was synthesized as described earlier (20) and coupled to sensor chip-CM 5 to a level of, ca., 75 response units (RU) using the amine-coupling kit (BIAcore AB). In this case, the CM-5 chip was activated by 0.2 M N-ethyl-N-dimethylaminopropylcarbodiimide and 50 mM N-hydroxysuccinimide for 7 min at a flow rate of 5 µl/min. VEK-30 was diluted to a final concentration of 100 µg/ml, using 0.1 M sodium acetate buffer, pH 4.5, as the buffer. The VEK-30 solution was injected for 7 min (5 µl/min) to allow binding to the chip, after which nonbound sites on the sensor chip surface were blocked by injection of 1.0 M ethanolamine, pH 8.5, for 7 min at the same flow rate.

The binding of Pg variants to VEK-30 was analyzed by SPR using a BIAcoreTM 3000 (BIAcore AB, Uppsala, Sweden). All experiments were performed at 25°C employing HBS-EP (10 mM Hepes, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20, pH 7.4) as running buffer at a flow rate of 100 µl/min. The binding reactions were conducted by injecting various concentrations (1 µM - 10 µM) of Pg (diluted with running buffer) over the VEK-30-containing chip surface. Binding levels were determined by monitoring the resulting signal, which is expressed as RU. At the end of each cycle, the chip surfaces were regenerated by washing with 60 µl of glycine, pH 2.5, which did not alter the binding properties of immobilized VEK-30 bound to the chip. The data from these sensorgrams were subtracted from those of a reference flow cell prepared by the same method, with the exception that VEK-30 was not immobilized on the chip. The binding kinetic and equilibrium constants were calculated from the association and dissociation rate curves generated by applying different concentrations of the Pg analytes onto the VEK-30-containing chip, using a 1:1 binding model. All individual sensorgrams were analyzed utilizing the BioEvaluation software package version 4.1 (BIAcore AB). Specifically, determinations of \( k_{\text{obs}} \) and \( k_{\text{off}} \) were obtained through nonlinear regression fitting of the equations for monophasic association and dissociation, i.e., \( RU = RU_{\text{max}}(1-e^{-k_{\text{obs}}t}) \) and \( RU = \Delta RU(e^{-k_{\text{off}}t}) \), respectively. Values for the association rate constant \( (k_{\text{on}}) \) were determined from the equation \( k_{\text{on}} = (k_{\text{obs}}-k_{\text{off}})/[\text{Pg}] \).

Homology modeling of mK2 from hK2 - The X-ray crystal structure of hK2 has been determined and was used to model the 3-dimensional structure of mK2. The homology model of mK2 was made followed by 200 cycles of energy minimization in the software suite, Crystallography and NMR System (CNS) (27).

RESULTS

For each r-Pg listed in Supplemental Table 1S, and for r-muPA, 5 clones were randomly selected from the hundreds of E. coli transformants obtained from the individual mutated cDNAs, and the plasmids purified. After the results of restriction enzyme analysis showed that all cDNAs of the selected clones were...
properly altered, each purified cDNA insert was subjected to complete nucleotide sequencing and 2 clones with correct sequences were chosen for plasmid purification and expression in *Drosophila* S2 cells. Final yields of the proteins after purification ranged from 5-60 mg/l, and each was readily obtained at high purity (Supplemental Fig. 1S). This provided ample materials for the experiments that were conducted herein.

Each Pg was then examined for its ability to be activated by mouse r-uPA employing an amidolytic assay for the amount of Pm generated. The relative activities of each Pg variant are not significantly different from the respective parent hPg or mPg (Fig. 1A,B). muPA activated WT-mPg approximately 30% more efficiently than WT-hPg.

Additionally, activations by SK were performed for each Pg generated in this study. An equimolar ratio of SK:Pg was first employed as a reaction mixture to monitor generation of the SK-Pm activator complex (Fig. 2A). In each case of hPg variants, including the chimera in which hK2 was replaced with mK2, in the hPg background, the ability to form the active SK-hPm complex was not compromised (Fig. 2A). In the case of mPg, or any K2 variant of mPg, no measurable SK-mPm was generated. This shows that SK has a greatly reduced capacity to activate mPg and that replacement of mK2 with hK2 in mPg did not alter that fact.

Next, catalytic levels of SK were employed to activate Pg to test the ability of the SK-Pm activator complex to activate the Pg variants. The data show (Fig. 2B) that in the case of hPg variants, their activatibilities by catalytic SK-Pm are not significantly different from the parent hPg. In the case of mPg variants, since it was shown that the SK-mPm activator complex could not be kinetically demonstrated (Fig. 2A), it is not surprising that no activity was found for any mPg variant (Fig. 2B). On the other hand, mPg, and each of its variants described herein, were activated equally well by the Pg activator, SK-hPm, which was pre-formed in a separate incubation, followed by its addition to mPg (Fig. 2C).

The binding of VEK-30 to the Pg variants was assessed quantitatively by SPR. The traces obtained are illustrated in Fig. 3 and the corresponding kinetic binding values obtained from these curves are listed in Table 1. The *K_D* for interaction of Pg variants with VEK-30 was assessed by deconvolution of binding rate data of the type shown by the example provided in Fig. 3A for the WT-hPg/SK interaction at various concentrations of Pg. The on (*k_on*) and off (*k_off*) rate constants obtained from the data were used to calculate the *K_D* values, as was a global fitting of the entire curve. In each case, the differences in these two modes of calculation (Table 1) did not differ by an amount that affects the conclusions of the work. For the binding of hPg to VEK, the *K_D* value of 2.94 µM is similar to the value obtained earlier by isothermal titration calorimetry for binding of VEK-30 to isolated r-K2 of hPg (8).

While VEK-30 possessed no measurable binding to mPg (Table 1), VEK-30 was bound to a chimeric mPg, containing the hK2 for mK2 substitution, with virtually the same *K_D* as for hPg. This, coupled with the finding that VEK-30 did not measurably interact with a hPg variant with its K2 domain replaced by the mK2 module (Table 1), demonstrates that all VEK-30 binding activity to hPg is located in the K2 domain of Pg. In order to further define the binding determinants of VEK-30, and to particularly understand the structural basis of the differences between its binding to hPg...
and mPg, we made additional amino acid substitutions in these two proteins, focusing completely on the K2 region of the molecules.

The hK2 and mK2 domains are highly homologous in their amino acid sequences. However, upon examination of the VEK-30/hK2 X-ray crystal structure (21), two important amino acid substitutions in mK2 have occurred that may substantially influence its binding to VEK-30, viz., an Arg at position 220 in hPg to a Gly in mPg and a Leu at position 222 in hPg to a Pro in mPg. Both of these residues are in close proximity to K2 residues at the hK2 cationic binding loop for VEK-30. Thus, both of these specific mutations were made in hPg and mPg, and the binding of VEK-30 assessed. The results of Table 1 showed that the R220G mutation in hPg (hPg[R220G]) did not appreciably affect the Kd value for VEK-30 binding, but the L220P (in hPg[L220P]) mutation increased the Kd for VEK-30 binding by approximately 10-fold, through a decrease in the kon and an increase in the koff values for binding. Mutating both of these residues, in hPg[R220G/L222P], synergistically eliminated measurable binding of VEK-30 to this minimally mutated hPg.

In considering the interaction of VEK-30 with mPg, binding was restored to that of hPg with a single mutation of G220R, with a 3-fold increase in the kon value as compared to hPg (Table 1). Similarly, an individual mutation of P222L in mPg also restored VEK-30 binding to nearly the same level as hPg. Generation of the double mutant, mPg[G220R/P222L] provided a mPg that fully interacted with VEK-30. Thus, only two mutations in the K2 domain of mPg are capable of fully restoring the VEK-30 binding site.

The functional impact of VEK-30 binding to hPg was examined by evaluating the SK- and uPA-catalyzed activation of hPg in the presence of increasing concentrations of VEK-30. These experiments were performed in chloride-containing buffer, which induces Pg to assume a closed, activation-resistant conformation (28). As illustrated in Fig. 4A, VEK-30 increases the activatability of hPg by SK, in the equimolar SK/hPg complex, in a concentration-dependent manner. A randomized version of VEK-30 was without major effect in this regard, indicating that the amino acid sequence, not its composition, is responsible for this acceleration. These results suggest that occupancy of the LBS of hK2 is sufficient to enhance the activation rate of hPg. This general property of small LBS ligands, e.g., EACA, is known to occur with occupancy of K1 and K4 (29), but, to now, was not known to be a property of K2. The specificity of VEK-30 for the LBS of K2, and the increased activation of hPg consequent to VEK-30 binding, has allowed the relevance of K2 to stimulation of hPg activation to be firmly reached.

A similar activation rate enhancement was exerted by VEK-30 on the uPA-catalyzed activation of hPg (data not shown), indicating that VEK-30 promotes a form of hPg that increases its general susceptibility to activation. This feature of VEK-30 is compromised when K2-mutant forms of hPg are employed in these SK-mediated activation experiments in approximately the same order as found in the direct binding experiments. For example, formation of plasminogen activator activity with equimolar SK and hPg[R220G/L222P] is very poorly stimulated by VEK-30 (Fig. 4B), in accord with the diminished binding of VEK-30 to this form of hPg. The single K2 hPg variants, viz., hPg[R220G] and hPg[L222P], are intermediate in the
activatability between WT hPg on the higher end and hPg[R220G/L222P] as the lower limit, again in accord with the reduction in binding of these mutant plasminogens to VEK-30. For more quantitative estimates, the absorbance at 405 nm, which measures both the activation rate of the tested hPg and also the cleavage of the substrate, S2251, was plotted against time$^2$ (Fig. 4C, D) (30), and the slopes compared to each other under identical initial concentrations of all components. The data show that the activity generated in the wt-hPg complex with SK is accelerated 14-, 305-, and 360-fold by 1.5 µM, 30 µM, and 75 µM VEK-30, respectively. In addition, [R220G] and hPg[L222P] are reduced in their ability to be stimulated by VEK-30 by 28-fold and 14-fold, respectively, as compared with WT-hPg. For the double variant, hPg[R220G/L222P], the relative reduction in stimulatory capacity is 39-fold.

**DISCUSSION**

A number of receptors for Pg exist on pathogenic bacteria, with the M-proteins of Gram-positive GAS being examples of this group (31). These receptors allow tight binding of Pg to the bacterium, which in-turn secretes Pg activators, e.g., SK and staphylokinase, that activate Pg to the serine protease, Pm. These mechanisms provide proteolytic activity on the surface of the bacteria, which is used to degrade proteins of the ECM and also to activate pro-MMPs, which perform this same function. This allows bacterial dissemination through protective barriers, thus providing the capability metastasis of certain strains of GAS from mucosa to deep tissues. This process is highly species specific and, in general, bacterial SKs only activate species of Pg in which that particular bacterium is infective (32). In the case of GAS, one of the high affinity M-like protein Pg/Pm receptors is the 43 kDa surface protein PAM, originally isolated from M53 serotype GAS (33), and this protein is a major virulence factor of GAS. One binding epitope of PAM for Pg/Pm is present in the amino-terminal a1/a2 domains of PAM, an interaction that is at least in part recapitulated by a peptide from this region of the protein, viz., VEK-30 (18). Only PAM$^+$ GAS isolates bind plasminogen (34) and only GAS strains that contain the VEK-30 region of PAM, and that also express SK, acquire surface-bound plasmin (35).

From the amino- to carboxy-termini, VEK-30 consists of 6 amino acids that precede the a1 region, the 13 amino acids that constitute a1, the first 10 amino acids of a2, and a carboxy-terminal Tyr, which provides a radioiodination handle. Use of this peptide, along with fragments of hPg, showed that the Pg binding region was isolated to its K2 domain (19). With this knowledge, we have determined the X-ray crystal structure of the VEK-30/hK2 complex (21), in which we identified a unique binding mode to the LBS of Pg wherein a pseudo-lysine arrangement of functional groups, comprised of the side chains of R17, H18, and E20, are brought into proximity through one turn of an $\alpha$-helix. This structural work confirms our earlier observations in solution that these same residues were critical for the binding of VEK-30 to hK2 (20).

An elegant mouse transgenic study has demonstrated that GAS virulence is dependent upon both hPg and PAM. Here, mice with the mPg gene replaced by hPg showed enhanced virulence toward PAM$^+$-GAS and reduced virulence toward PAM$^-$-GAS (36), thereby supporting another study wherein it was shown that the PAM$^+$ genotype in GAS isolates allows hPg to
bind to the bacterial surface (34). In the current communication, we show, by direct binding measurements, that hPg interacts with the VEK-30 region of PAM. Taking advantage of the fact that mPg does not interact with VEK-30, we show that binding of VEK-30 to hPg is eliminated by replacing hK2 with mK2, and is recapitulated in mPg by replacement of mK2 with hK2. Thus, the binding of VEK-30 to hPg has been completely reduced its interaction with hPg-K2.

mK2 and hK2 are highly homologous in amino acid sequence, with only 8/78 amino acid differences. In the vicinity of the LBS of K2, there are two amino acid differences; R220 in hPg to Gly in mPg, and L222 in hPg to Pro in mPg. Of additional interest is the observation that Rhesus (rh) Pg does not interact with PAM$^{+}$-GAS (19). In this case, there are only 2 amino acid differences between hK2 and rhK2, viz., the same R220G and L220P substitutions. Thus, we sought direct proof that either, or both, of these residues conferred the PAM$^{+}$-GAS binding site to hPg.

Consideration of the X-ray crystal structure of the VEK-30/hK2 complex (21) offers support for the potential importance of the two amino acid substitutions on the selective ability of hK2 to interact with VEK-30. The two amino acid substitutions are in a loop in the kringle structure bounded by two Pro residues at sequence positions 218 and 224. These residues are rigorously conserved in all kringle structures that contain LBS and appear to optimally position the two critical Asp/Glu residues in lysine binding kringles (D219/E221 in hK2) that interact with the cationic region of the LBS substrates. In addition, in hK2, R220 makes a direct interaction with D7 of VEK-30. This residue, altered to a Gly in mK2, is responsible for loss of some of the binding energy in mK2 for VEK-30, and perhaps explaining the specificity of VEK-30 for hK2 over mK2. Additionally, no other hPg kringle has Arg at this position, and no other hPg kringle interacts with VEK-30.

To examine the possible effects on this binding loop of a substitution of L222 to P222, Fig. 5 shows an overlay of these same loops from X-ray crystal structures of hK2 (22) and the modeled structure of mK2. The structure of mK2, when overlayed onto the hK2/VEK-30 structure, shows that the Leu (hK2) to Pro (mK2) is forced further into the binding pocket (Fig. 5A), such that it clashes with the H18 of VEK-30, and is no longer able to effectively interact with this important ligand residue. In addition, other interactions of hK2 with VEK-30 are affected when L222 of hPg is altered to P222 in mK2 (Fig. 5B). The R17 (VEK-30)-E221 (K2) interaction in hK2 is now shifted to >3.7Å in mK2, and this interaction is also lost. Further the D219O (mainchain O) of mK2 can no longer make a contact with K14 of VEK-30, thus further disrupting binding of the ligand to mK2. Thus, both mutations in mK2 can potentially seriously disrupt binding of the VEK-30 region of PAM to K2 of mPg.

To directly test the importance of each of the two amino acid replacements in mK2 in this loop, we studied the binding of VEK-30 variant forms of mK2 and hK2 in hPg and mPg. The R220G replacement in hK2 did not significantly affect the overall $K_D$ for binding of VEK-30 to hPg[R220G] and the L222P mutation, in hPg[L222P], reduced this overall binding by 10-fold through alterations in both the $k_{on}$ and $k_{off}$ rates.
However, when both mutations were made in hPg, providing hPg[R220G/L222P], no binding was observed, showing that these two replacements acted synergistically in both mPg and rhPg to greatly attenuate binding to VEK-30.

While this downregulation of VEK-30 binding in hPg as a result of these minimal mutations in the K2 region is an important finding, even more critical is to show upregulation of binding when the reverse mutations are constructed in mPg. And, indeed, this is the case. The interaction of VEK-30 with mPg[G220R] or mPg[P222L] fully restores binding of VEK-30 and, of course, generation of a mPg variant with the double mutation, viz., mPg[G220R/P222L], produces the same effect.

The requirement of both hPg and PAM for GAS infectivity, coupled with the ability of PAM, as embodied in VEK-30, to bind K2 of hPg, necessarily invites speculation as to the mechanism underlying hPg activation by GAS. The accumulation of hPg on the bacterial surface clearly serves to raise the effective concentration of the SK-hPg complex and represents the most obvious explanation for the increased plasminolysis associated with GAS dissemination. An additional mechanistic possibility was suggested through a comparison of the X-ray crystal structures of nonliganded and VEK-30-bound angiotatin (K1-K3 of hPg) (23). In this study, an overlay of the K2 regions of both structures reveals that K1 undergoes a large rotation (48.1°) upon complexation with VEK-30. This observation implies a mechanism for SK-mediated activation of hPg on the bacterial surface, wherein hPg binding to PAM induces a conformation of Pg in which the kringle domains reorient in a manner that renders Pg more susceptible to activation. This hypothesis finds strong support in our demonstration that increasing concentrations of VEK-30 accelerate both the SK- and uPA-mediated conversion of the tight activation-resistant form of hPg to hPm. Hence, it seems likely that the ability of PAM to bind hPg not only functions to recruit hPg to the streptococcal surface, but also induces a hPg conformation that is highly amenable to activation.

In conclusion, we show herein that binding of the VEK-30 region of PAM to hPg occurs strictly through the hK2 domain, and the lack of binding of VEK-30 to mPg is due to 2 amino acid replacements between hK2 and mK2. The binding of VEK-30 to the K2 domain of hPg also results in a greatly increased rate of activation of hPg by SK. Thus, Pg binding to a cell surface GAS receptor region, represented by VEK-30, through the hPg-K2 domain, leads to enhanced activation of Pg by a bacterial-secreted Pg activator, SK. This overall mechanism of accumulation of protease activity on the GAS surface is likely responsible for the invasiveness of this bacterium. This reductionist approach to understanding the binding of PAM+GAS to hPg, and the resultant activation of hPg, will serve to identify molecular targets for new drugs that can possibly aid in attenuating the virulence of these pathogens.
REFERENCES

1. Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E., and Magnusson, S. (1978) Prog. Chem. Fibrinolysis and Thrombolysis 3, 191-209
2. Ploplis, V. A. (2001) Frontiers in Bioscience 6, D555-D569
3. Ploplis, V. A., and Castellino, F. J. (2000) Methods 21(2), 103-110
4. Ploplis, V. A., and Castellino, F. J. (2002) Thromb. Haemost. 87(1), 22-31
5. Menhart, N., Sehl, L. C., Kelley, R. F., and Castellino, F. J. (1991) Biochemistry 30, 1948-1957
6. Hoover, G. J., Menhart, N., Martin, A., Warder, S., and Castellino, F. J. (1993) Biochemistry 32(41), 10936-10943
7. McCance, S. G., Menhart, N., and Castellino, F. J. (1994) J. Biol. Chem. 269(51), 32405-32410
8. Nilsen, S. L., Prorok, M., and Castellino, F. J. (1999) J. Biol. Chem. 274(32), 22380-22386
9. Fleury, V., Gurewich, V., and Angles-Cano, E. (1993) Fibrinolysis 7(2), 87-96
10. Fleury, V., Loyau, S., Lijnen, H. R., Nieuwenhuizen, W., and Angles-Cano, E. (1993) Eur. J. Biochem. 216(2), 549-556
11. de Vries, C., Veerman, H., Koornneef, E., and Pannekoek, H. (1990) J. Biol. Chem. 265, 13547-13552
12. Miles, L. A., Dahlberg, C. M., and Plow, E. F. (1988) J. Biol. Chem. 263(24), 11928-11934
13. Miles, L. A., Hawley, S. B., Baik, N., Andronicos, N. M., Castellino, F. J., and Parmer, R. J. (2005) Frontiers in Bioscience 10, 1754-1762
14. Lottenberg, R., Broder, C. C., and Boyle, M. D. (1987) Infect. Immun. 55(8), 1914-1918
15. Carapetis, J. R., Steer, A. C., Mulholland, E. K., and Weber, M. (2005) Lancet Infect Dis. 5, 685-694
16. Monroy, V., Amador, A., Ruiz, B., Espinoza-Cueto, P., Xolalpa, W., Mancilla, R., and Espitia, C. (2000) Infec. Immun. 68, 4327-4330
17. Sodeinde, O. A., Subrahmanyam, Y. V., Stark, K., T Quan, Bao, Y., and Goguen, J. D. (1992) Science (258), 1004-1007
18. Wistedt, A. C., Ringdahl, U., Mulleresterl, W., and Sjobring, U. (1995) Molecular Microbiology 18(3), 569-578
19. Wistedt, A. C., Kotarsky, H., Marti, D., Ringdahl, U., Castellino, F. J., Schaller, J., and Sjobring, U. (1998) J. Biol. Chem. 273(18), 24420-24424
20. Schenone, M. M., Warder, S. E., Martin, J. A., Prorok, M., and Castellino, F. J. (2000) J. Pept. Res. 56, 438-445
21. Rios-Steiner, J. L., Schenone, M., Mochalkin, I., Tulinsky, A., and Castellino, F. J. (2001) J. Mol. Biol. 308(4), 705-719
22. Mathews, I. I., Vanderhoff-Hanaver, P., Castellino, F. J., and Tulinsky, A. (1996) Biochemistry 35(8), 2567-2576
23. Cnudde, S. E., Prorok, M., Castellino, F. J., and Geiger, J. H. (2006) Biochemistry 45, 11052-11060
24. Iwaki, T., Figuera, M., Ploplis, V. A., and Castellino, F. J. (2003) BioTechniques 35, 482-486
25. Nilsen, S. L., and Castellino, F. J. (1999) *Prot. Express. Purif.* **16**(1), 136-143
26. van den Ent, F., and Lowe, J. (2006) *J. Biochem. Biophys. Meth.* **67**, 67-74
27. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) *Acta Crystallogr. D Biol. Crystallogr.* **154**(5), 905-921
28. Urano, T., Chibber, B. A. K., and Castellino, F. J. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4031-4034
29. Menhart, N., Hoover, G. J., McCance, S. G., and Castellino, F. J. (1995) *Biochemistry* **34**, 1482-1488
30. Chibber, B. A. K., Radek, J. T., Morris, J. P., and Castellino, F. J. (1986) *Proc. Natl. Acad. Sci., U.S.A* **83**, 1237-1241
31. Lahteenmaki, K., Kuusela, P., and Korhonen, T. K. (2001) *FEMS Microbiol. Rev.* **25**, 531-552
32. Schroeder, B., Boyle, M. D. P., Sheerin, B. R., Asbury, A. C., and Lottenberg, R. (1999) *Infec. Immun.* **67**, 6487-6495
33. Berge, A., and Sjobring, U. (1993) *J. Biol. Chem.* **268**(34), 25417-25424
34. McKay, F. C., McArthur, J. D., Sanderson-Smith, M. L., Gardam, S., Currie, B. J., Sriprakash, K. S., Fagan, P. K., Towers, R. J., Batzloff, M. R., Chhatwal, G. S., Ranson, M., and Walker, M. J. (2004) *Infec. Immun.* **72**, 364-370
35. Ringdahl, U., Svensson, M., Wistedt, A. C., Renn, T., Kellner, R., Muller-Esterl, W., and Sjobring, U. (1998) *J. Biol. Chem.* **272**(11), 6424-6230
36. Sun, H., Ringdahl, U., Homeister, J. W., Fay, W. P., Engelberg, N. C., Yang, A. Y., Rozek, L. S., Wang, X., Sjobring, U., and Ginsburg, D. (2004) *Science* **305**, 1283-1286

**FOOTNOTES**

This study was supported by Grants HL013423 (to F.J.C.), GM0638947 (to J.H.G.), and HL073750 (to V.A.P.) from the NIH.
FIGURE LEGENDS

**Fig 1.** The uPA-catalyzed activation of hPg and mPg variants. Pg activation was monitored by the appearance of amidase activity after addition of r-muPA using a chromogenic assay. The relative level of activity produced by activation of WT-hPg and WT-mPg was considered to be 100% for proteins of same species. The activities of the hPg and mPg variants were expressed as a percentage of WT-hPg or WT-mPg activity. Assays were performed in triplicates, and values are expressed as the means ± S.E.

**Fig 2.** The SK-mediated activation of hPg and mPg variants. Pg activation was monitored by the appearance of amidase activity after SK addition using a chromogenic assay. The relative level of activity produced by activation of WT-hPg was considered to be 100%. The activities of the hPg and mPg variants were expressed as a percentage of WT-hPg activity. Assays were performed in triplicate, and values are expressed as the mean ± S.E. A. The molar ratio of SK/Pg was 1:1 and, therefore, the activity measured is primarily the SK-hPm activator complex. B. The molar ratio of SK:Pg is 1:20 and the activity measured is mainly Pm arising from catalysis of Pg by the SK-Pm equimolar complex. For A and B, the protein labels are the same and are labeled on the ordinate axis of B. C. Activation of mPg variants by catalytic levels of the preformed SK-hPm complex. SK and hPg were preincubated for 1 hr and the mixture was added to mPg at a molar ratio of 20:1 mPg:SK-hPm. The activity measured is the mPm that is generated via activation of mPg.

**Fig 3.** Kinetic analyses of the interaction of VEK-30 with r-Pgs by SPR. VEK-30 (100 µg/ml) was injected onto a CM5 sensor chip at a flow rate of 5 µl/min for 7 min. Approximately 75 RU of VEK30 was immobilized through use of amine coupling. Various amounts of r-Pgs were then injected onto the surface of the chip at a rate of 100 µl/min, and the binding levels were detected in real time by SPR. A. Example of the binding curves of WT-hPg to the immobilized VEK-30 at 4 different hPg concentrations (that are indicated on the graph), from which the binding kinetics were determined. B. Examples of the binding curves of various Pgs (that are indicated on the graph), each at a concentration of 10 µM.

**Fig 4.** The effects of VEK-30 on the SK-catalyzed activation of hPg K2 variants. Activation of Pg (150 nM), after addition of an equimolar amount of SK, in the presence and absence of VEK-30. The activity generated was monitored by the appearance of amidase activity using a chromogenic assay with 0.25 mM S2251. A. The rate of appearance of amidase activity of the complex formed from WT-hPg and SK at the concentrations of VEK-30 labeled on the graph. B. The activity of the indicated Pg K2-variants in the presence of 30 µM VEK-30. C. Replot of the data in A according to time² for purposes of quantitation. D. Replot of the data in B according to time² for purposes of quantitation. In C and D, the actual data are shown by the red lines and the linear regression fits to the data, from which slopes have been calculated, are shown by the black dashed lines. In all cases, the assay buffer consisted of 10 mM Na-Hepes, 100 mM NaCl, pH 7.4.
Fig 5. Homology model of the mK2 structure at the lysine cation binding site loop, residues 218-224. A. Overlay hK2 (cyan) from its X-ray crystal structure with the model of mK2 (magenta), bounded by the highly conserved Pro residues at each end of each loop. The numbering of residues begins at Glu1 of hPg and are numbered according to the hPg sequence. Residues numbered in black type are identical between hK2 and mK2. The two amino acids that differ between the two K2 structures, at residues 220 and 222, are labeled and numbered in cyan type for hK2 and magenta type for mK2. B. The mK2 model (magenta), from residues 219-222, overlaid on the same residues of crystal structure of the hK2 (cyan)/VEK-30 (yellow) X-ray structure. Side-chains of VEK-30 that interact with the cation binding loop of hK2, viz., D91, K98, R101, H102, are projected from the VEK-30 ribbon. These are numbered (in green) from the amino-terminus of PAM.
**Table 1.** Kinetic binding parameters for VEK-30 to various human and mouse plasminogens

| Variant             | $k_{on}$ $10^4$ M$^{-1}$sec$^{-1}$ | $k_{off}$ sec$^{-1}$ | $K_D^b$ µM |
|---------------------|-----------------------------------|----------------------|------------|
| WT-hPg              | 5.71 ± 0.83                       | 0.165 ± 0.019        | 2.94 (2.89) ± 0.57 |
| WT-mPg              | n.b.$^a$                          | n.b.                 | n.b.       |
| hPg[mK2]            | n.b.                              | n.b.                 | n.b.       |
| mPg[hK2]            | 5.51 ± 0.54                       | 0.111 ± 0.03         | 2.07 (2.01) ± 0.78 |
| hPg[R220G]          | 29.1 ± 7.8                        | 0.546 ± 0.032        | 2.00 (1.88) ± 0.60 |
| mPg[G220R]          | 16.6 ± 8.4                        | 0.311 ± 0.013        | 2.20 (1.87) ± 1.05 |
| hPg[L222P]          | 1.30 ± 0.59                       | 0.313 ± 0.022        | 27.6 (24.1) ± 1.01 |
| mPg[P222L]          | 7.36 ± 0.46                       | 0.349 ± 0.007        | 4.75 (4.74) ± 0.19 |
| hPg[R220G/L222P]    | n.b.                              | n.b.                 | n.b.       |
| mPg[G220R/P222L]    | 9.54 ± 1.36                       | 0.287 ± 0.039        | 3.07 (2.91) ± 0.84 |

$n.b.$, no binding observed.

$^b$The value of the $K_D$ is estimated from software fits of the entire binding curve. The value in parenthesis is obtained from the ratio of the $k_{off}/k_{on}$. 
Figure 1

A

Relative activity (%)

hPg  hPg[R220G]  hPg[mK2]  hPg[L222P]  hPg[R220G/L222P]

B

mPg  mPg[G220R]  mPg[hK2]  mPg[P222L]  mPg[G220R/P222L]
Figure 3A

![Graph showing response units over time for different concentrations of a substance. The x-axis represents time in seconds, ranging from 0 to 120, while the y-axis represents response units, ranging from 0 to 60. Different concentrations (10 μM, 5 μM, 2 μM, 1 μM) are indicated by distinct lines of different colors.](http://www.jbc.org/Downloaded from)
Figure 3B

Graph showing the response units over time for different samples.

- hPg[R220G]
- mPg[hK2]
- hPg
- mPg[P222L]
- mPg[G220R/P222L]
- mPg[G220R]
- hPg[L222P]
- hPg[R220G/L222P]
- hPg[mK2]
- mPg
Figure 4
Figure 5
The lack of binding of VEK-30, an internal peptide from the group A streptococcal M-like protein, PAM, to murine plasminogen is due to two amino acid replacements in the plasminogen Kringle-2 domain

Qihua Fu, Mariana Figuera-Losada, Victoria A. Ploplis, Sara Cnudde, James H. Geiger, Mary Prorok and Francis J. Castellino

J. Biol. Chem. published online November 26, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M705063200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2007/11/28/M705063200.DC1