Conformationally organized lysine isosteres in *Streptococcus pyogenes* M protein mediate direct high-affinity binding to human plasminogen

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The binding of human plasminogen (hPg) to the surface of the human pathogen group A *Streptococcus pyogenes* (GAS) and subsequent hPg activation to the protease plasmin generate a proteolytic surface that GAS employs to circumvent host innate immunity. Direct high-affinity binding of hPg/plasmin to pattern D GAS is fully recapitulated by the hPg kringle 2 domain (K2hPg) and a short internal peptide region (a1a2) of a specific subtype of bacterial surface M protein, present in all GAS pattern D strains. To better understand the nature of this binding, critical to the virulence of many GAS skin-tropic strains, we used high-resolution NMR to define the interaction of recombinant K2hPg with recombinant a1a2 (VKK38) of the M protein from GAS isolate NS5455. We found a 2:1 (m/m) binding stoichiometry of K2hPg/VKK38, with the lysine-binding sites of two K2hPg domains anchored to two regions of monomeric VKK38. The K2hPg/VKK38 binding altered the VKK38 secondary structure from a helical apo-peptide with a flexible center to an end-to-end K2hPg-bound α-helix. The K2hPg residues occupied opposite faces of this helix, an arrangement that minimized steric clashing of K2hPg. We conclude that VKK38 provides two conformational lysine isosteres that each interact with the lysine-binding sites in K2hPg. Further, the adoption of an α-helix by VKK38 upon binding to K2hPg sterically optimizes the side chains of VKK38 for maximal binding to K2hPg and minimizes steric overlap between the K2hPg domains. The mechanism for hPg/M protein binding uncovered here may facilitate targeting of GAS virulence factors for disease management.

Group A *Streptococcus pyogenes* (GAS) is a human-selective pathogen that causes both superficial self-limiting infections (e.g. pyoderma) as well as morbid and lethal maladies (e.g. toxic shock syndrome and necrotizing fasciitis). Although penicillin-type antibiotics are effective drugs against GAS diseases, more virulent pathovars have emerged because of the ability of this microbe to remodel its genome to adapt to specialized environmental niches within the host. These changes primarily occur through intra- and interspecies horizontal transfer and recombination of genetic materials, including integration of prophages and their associated virulence genes (e.g. superantigens) (1, 2).

GAS employs various virulence strategies to perpetuate in its host and cause disease. These include a variety of proteins that serve as adhesins to attach to host epithelial cell surfaces (3) as well as an outer nonimmunogenic capsule to resist antibody recognition (4) and secreted and surface-bound proteases to encourage invasion of the bacteria through natural cellular barriers (5). Further, the ability of the bacteria to form protective biofilms (6) allows colonization of the bacteria in distal tissues within the host. Among these virulence determinants, the single-chain, surface-resident multifunctional M protein, present as a single type on all GAS strains, and utilized for GAS serotyping, is of particular relevance because it is used by GAS for a variety of survival benefits (e.g. adhesion to host cells (7), evasion of phagocytosis (8), and neutralization of antimicrobial peptides (9)).

One subtype of M protein, plasminogen-binding group A streptococcal M-like protein (PAM), has an additional well-known function of coopting components of the host to evade innate immune responses. Important among these properties is the fibrinolytic system, which GAS employs to potently evade innate immunity (e.g. attenuate complement-based opsonization of GAS) (10, 11) and to disseminate into deep tissue. To accomplish this function, PAM first interacts with very high affinity to host human plasma plasminogen (hPg) (12), a step that facilitates its activation to plasmin by GAS-secreted streptokinase (13, 14). The proteolytic ability of plasmin that is localized on the GAS surface is resistant to natural plasmin inhibitors (13) and is thus utilized by the bacteria to disrupt barriers to its dissemination (e.g. fibrin that encapsulates GAS) and then resonance; ITC, isothermal titration calorimetry; AUC, analytical ultracentrifugation; BisTris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propane-1,3-diol.

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2 The abbreviations used are: GAS, group A *Streptococcus pyogenes*; DSS, disuccinimidyl suberate; hPg, human plasminogen; SPR, surface plasmon resonance; ITC, isothermal titration calorimetry; AUC, analytical ultracentrifugation; BisTris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propane-1,3-diol.
to invade into deeper distal tissues by disrupting the extracellular matrix and tight cellular junctions (15, 16). PAM is a ~42-kDa single-chain protein, assembled as a dimer in the mature protein, that has evolved in a modular fashion. The hypervariable N terminus of PAM-type M proteins contains two short A domain repeats (a1a2) that have been identified as the binding determinants for hPg (17, 18).

Within hPg, the kringle 2 (K2hPg) domain has been identified as the receptor for PAM binding (19). Kringle modules in hPg generally interact with their receptors via C-terminal lysine residues (20–24). However, PAM does not possess a C-terminal lysine. Therefore, the X-ray crystal structure (25) and NMR solution structure (26, 27) of the complex of a truncated form of the a1a2 domain of PAM53 from GAS isolate AP53 (VEK30; Table 1) was solved to propose a mechanism for this tight binding interaction. The resulting structural model that was developed involved the formation of an internal conformational isostere of lysine from side chains of VEK30 (His17, Arg18, and Glu20). Their proper orientation depended on the adoption of a VEK30 α-helix upon binding. However, the $K_d$ of this interaction was ~30–50-fold weaker than that of the PAM53–K2hPg complex. A redesign of VEK30 resulting from an addition of two C-terminal amino acids (30RH) from PAM53 or five C-terminal amino acids (30RHDDH) from PAM53 (Table 1) strengthened the binding affinity of the resulting peptides (VEK32 and VEK35, respectively) to that of full-length PAM53 (12). We then decided to assess the role of these additional amino acids in a1a2 binding to K2hPg via functional and high-resolution structural analyses.

The results presented herein demonstrate for the first time that a peptide, VKK38, from a PAM-containing isolate, NS455, harbors two binding sites for K2hPg. Upon binding to K2hPg, VKK38 undergoes a conformational transition from a predominantly random coil to an end-to-end α-helix. Overall, the data obtained reveal a mechanism for binding of PAM to hPg that will facilitate the targeting of these virulence factors of GAS in disease management.

Results

Amino acid sequence alignment for PAM53 and PAM455

The PAM-subtype M protein is present in all pattern D GAS serotypes, and its sequence variability in different GAS isolates provides natural mutations in which to assess the nature of its various functions. The most well-studied PAM was originally extracted from GAS isolate AP53 (18). GAS NS455 is another pattern D direct hPg-binding isolate (28), and we aligned PAMs from AP53 (PAM53) and NS455 (PAM455) to determine the extent to which these two proteins from different pattern D strains were similar (Fig. 1).

At the N termini, the signal peptides (residues 1–41) of the two PAMs exhibit very high homology; however, low homology was observed in the immediate downstream hypervariable domains, thus accounting for their different serotypes (emm53 for AP53 and emm52 for NS455). Following the hypervariable region are the a1 (residues Val97–Glu115) and a2 (residues Glu116–Asp130) repeats in PAM53 and, correspondingly, Val103–Glu124 and Glu125–Asp139 in PAM455. Both proteins have two RH dipeptides, one in the a1 region (113RH114 in PAM53 and, correspondingly, Val119–Glu130 in PAM455) and another in the a2 segment (126RH127 in PAM53 and 133RH134 in PAM455). These RH residues, supported by Glu125 in PAM53, are most relevant for formation of the lysine isostere needed for hPg binding. hPg binding occurs at RH1 and/or RH2. It is noteworthy that at the end of the a1 repeat in PAM455, a tripeptide insertion, 121VHD123, was found that interrupted the relative orientation of Glu125 with RH1. One central issue in this study is whether this VHD natural insert would make a significant difference in the hPg-binding pattern and structure of PAM455 compared with PAM53.

Other important domains are present in all M proteins. In PAM-type M proteins, downstream of the a2 region, somewhat higher homology is seen in the B-like repeats. The C1–C3 repeats, the D-repeats, and the Pro-Gly (PG) region, used for pattern mapping, have high homology between the PAM proteins. Both PAMs have been identified as pattern D subtypes with high tropism for skin infections (29). In addition, the downstream sortase A motifs were identical, thus ensuring that both mature PAMs are covalently anchored to the cell wall.

Binding affinities of PAMs to hPg and VEK/VKK peptides to K2hPg

The binding affinities of full-length PAMs to hPg or of VEK/VKK peptides (listed in Table 1) to K2hPg were assessed by surface plasmon resonance (SPR). The prototype PAM53 has strong binding ability to hPg, with a $K_d$ of ~1 nM, consistent with previous results (12). Despite PAM455 containing a naturally occurring, 121VHD123 motif immediately following the RH dipeptide in the a1 repeat (RH1), no effects on the binding affinity of PAM455 to hPg were found (Table 2). This is also true for the mutation D117A in PAM53 as well as the insertion (V) of 115VHD117 or 115VH117 into PAM53 at the end of the a1 domain. In addition, mutation of PAM455 to a form without the VHD tripeptide (PAM455/D121VHD123) also did not show

### Table 1

| Peptides       | Amino acid sequence |
|----------------|---------------------|
| VEK30          | GS (V'EKLTAELQRLKNEDELELARKSEY) |
| VEK30/19VHD21  | GS (V'EKLTAELQRLKNEDELELARKSEY) |
| VEK32          | GS (V'EKLTAELQRLKNEDELELARKSEY) |
| VEK32/19VHD21  | GS (V'EKLTAELQRLKNEDELELARKSEY) |
| VEK35          | GS (V'EKLTAELQRLKNEDELELARKSEY) |
| VEK35/19VHD21  | GS (V'EKLTAELQRLKNEDELELARKSEY) |

*a The GS dipeptide outside the parentheses is an exogenous product of the expression cassette and is not used in the numbering system. The C-terminal Tyr residue is included in all peptides to determine concentrations by absorption at 280 nm.

*b The residues of the binding sites are italicized, and the VHD insertions are shown in bold.

*c Truncated peptides published previously (12) are listed for comparison.
a significant difference in hPg binding. Therefore, the presence of VHD at the end of the a1 region does not affect the binding of PAM to hPg.

The truncated peptides, including a1a2 repeats from AP53 and NS455, have been constructed and expressed in Escherichia coli BL21 cells. The expressed peptides are numbered from the Val1 at the beginning of the a1 domains; VEK peptides are from PAM53, and VKK peptides are from PAM455. Insertion of 19VHD21 into VEK peptides, VEK30 (VEK30/c14119VHD21), VEK32 (VEK32/c14119VHD21), and VEK35 (VEK35/c14119VHD21), does not change their binding affinities to hPg (Table 3). VEK30/c14119VHD21 exhibits a high dissociation rate; thus, we confirmed its $K_D$ to K2hPg using the SPR affinity method. A value of 59 ± 3 nM was determined for the $K_D$ of this interaction, which is comparable with that obtained from kinetic analysis of the binding (Fig. 2). It is noted that the binding affinity observed from VEK30 and VEK30/c14119VHD21 is about 50-fold weaker compared with that of VEK32 and VEK35, because VEK30 only harbors a single RH motif. Furthermore, VKK38, as the counterpart of VEK35, but with the 19VHD21 motif, binds to K2hPg with a $K_D$ of ~1 nm, a value very similar to that of VEK35 (1.9 nm). Thus, both sets of binding data, from full-length PAMs to hPg and from VEK/VKK peptides to K2hPg, indicate that the VHD tripeptide that naturally occurs in some PAMs does not significantly affect the binding of these ligands to hPg and K2hPg.

**VKK38 forms a complex with K2hPg with a molar ratio of 1:2**

Because of the two RH groups in VKK38, it is important to demonstrate whether each RH motif binds to K2hPg separately. The binding properties of VKK38 to K2hPg were measured by combining isothermal titration calorimetry (ITC), analytical ultracentrifugation (AUC), and NMR titrations.

ITC measurements were carried out by titrating a constant level of K2hPg in the cell with variable amounts of VKK38 and measuring the incremental liberation of heat (Fig. 3A). The exo-
thermic reaction leveled after ~40 μM of VKK38, implying saturation of all binding sites on VKK38 by K2hPg. At this stage, the concentration of K2hPg was ~95 μM, and the molar ratio of

Table 2
Binding isotherms of full-length PAMs to hPg

| Protein     | $k_{on}$ × 10^4 s^-1 | $k_{off}$ × 10^-4 s^-1 | $K_D$ nM |
|-------------|-----------------------|-------------------------|----------|
| PAM53       | 5.6 ± 0.2             | 3.1 ± 0.1               | 6.0 ± 0.02 |
| PAM53/VEK32 | 12.4 ± 1.9            | 5.5 ± 0.6               | 0.5 ± 0.06 |
| PAM53/VKK38 | 9.1 ± 0.1             | 6.2 ± 2.0               | 0.7 ± 0.2  |
| PAM53      | 10.8 ± 3.4            | 1.6 ± 0.08              | 0.2 ± 0.07 |
| PAM53/Δ231VHD | 7.0 ± 0.3           | 3.1 ± 0.002             | 0.46 ± 0.02 |

*The $K_D$ values for VEK30/K2hPg (56 nM), VEK32/K2hPg (1.1 nM), and VEK35/K2hPg (1.9 nM) are taken from a previous publication (12).*

Table 3
Binding constants of VEK/VKK peptides to K2hPg

| Peptide     | $k_{on}$ × 10^5 s^-1 | $k_{off}$ × 10^-4 s^-1 | $K_D$ nM |
|-------------|-----------------------|-------------------------|----------|
| VEK30/V19VHD | 8.0 ± 0.7             | 336 ± 2                 | 42 ± 2   |
| VEK32/V19VHD | 67 ± 5.7              | 14 ± 1.0                | 0.2 ± 0.002 |
| VKK38       | 27 ± 1.1              | 8.9 ± 0.2               | 0.3 ± 0.1 |

$K_D$ values for VEK30/K2hPg (56 nM), VEK32/K2hPg (1.1 nM), and VEK35/K2hPg (1.9 nM) are taken from a previous publication (12).

**Figure 2. Binding of VEK/VKK peptides to K2hPg**

A, kinetic analysis for VEK30/V19VHD$^{21}$. B, affinity analysis for VEK30/V19VHD$^{21}$. C, kinetic analysis for VEK32/V19VHD$^{21}$. D, kinetic analysis for VKK38. All binding experiments were conducted by SPR at 25 °C in a solution of 10 mM HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20, pH 7.4, as the running buffer at a flow rate of 10 μl/min. The concentrations of the VEK and VKK peptides were progressively increased and are indicated in each panel. The original data are shown in black lines, and the experimental curves are shown in red lines. The experimental data were fitted using a 1:1 Langmuir binding model. The binding isotherms of full-length PAMs to hPg were assayed under similar conditions. The corresponding binding constants for these curves are provided in Table 2.

**PAM binds plasminogen via two lysine isosteres**

VKK38–K2hPg was calculated as 0.42, suggesting a binding stoichiometry of 2:1 (m/m) for K2hPg–VKK38.

These results were confirmed by determining the molecular weight of the VKK38–K2hPg complex utilizing AUC (Fig. 3B). To obtain the needed sample, VKK38 was mixed with excess K2hPg and the tightly bound complex of VKK38 and K2hPg was separated from free components by passing the solution over a gel filtration column. The sequence-based calculated molecular masses of VKK38–K2hPg at a 1:1 (m/m) ratio and 1:2 (m/m) ratio were 14,451 and 25,170 Da, respectively. From AUC analysis, the apparent molecular mass of the complex was 22,890 ± 230 Da, suggesting that VKK38 possesses two binding sites for K2hPg.

$^1$H–$^{15}$N HSQC experiments were recorded on [15N]VKK38 samples titrated with increasing concentrations of unlabeled K2hPg. A tight VKK38–kringle 2 complex is suggested by the observation of two cross-peaks for a subset of the residues during the titration. Amide resonances of the residues at N and C termini of VKK38 were continually shifted until a plateau was reached at an ~1:2 (m/m) ratio of VKK38 to K2hPg (Fig. 4A), again suggesting that two binding sites for K2hPg are present on VKK38 at the 1:2 VKK38/kringle 2 ratio.

Two mutant peptides, VKK38/R17A/H18A and VKK38/R34A/H35A, were separately designed by replacing RH1 and RH2 with c-cap.
RH2 with alanines to confirm the nature of the binding sites for K2hpge on VKK38. AUC analysis was then performed on the complex of VKK38/R17A/H18A–K2hpge and VKK38/R33A/H34A–K2hpge. Molecular masses of 12,500–15,500 Da were obtained for these complexes (supplemental Fig. 1S). These results further confirm that VKK38, comprising the α1α2 domain of PAM, contains two binding sites for K2hpge, and the binding is mediated by RH motifs in each repeat of the α1α2 domain.

Solution structures of VKK38 in the nonbound and K2hpge-bound forms

The structure prediction for VKK38 in its nonbound form was performed by use of the TALOS-N program on the basis of chemical shift data (Fig. 5A). NOESY spectra were analyzed to define the secondary and tertiary structure of apo-VKK38. However, most residues of apo-VKK38 only have sequential connectivities, suggesting a lack of secondary structural elements. The N- and C-terminal regions, residues Val1–Asp6 and Leu29–Tyr38, exist as random coils in VKK38. However, VKK38 has helical character formed by residues Glu7–Lys14 and Glu22–Arg28. These two α-helical segments are connected by a loop from residue Asn15 to Glu22, which contains RH1.

The conformation of VKK38 changes significantly when it forms a complex with K2hpge, as has been demonstrated from chemical shift changes (Fig. 4B), secondary structure predictions using TALOS-N, and the solution structures generated by Xplor-NIH along with NOE restraints (Fig. 5C). Bound VKK38 shows an α-helix from Asn5 to Asp37, which contains RH1.

As shown in Fig. 5C, the two RH groups are positioned on opposite faces of the VKK38 helix. However, RH2 is close to the C terminus, which leaves sufficient space for VKK38 to bind two K2hpge molecules.

Figure 3. Characterization of the binding of VKK38 to K2hpge. A, isothermal titration calorimetric analysis of VKK38 binding to K2hpge. The release of heat accompanying the interaction was measured against the molar ratio of VKK38 to K2hpge. B, sedimentation equilibrium ultracentrifugation analysis of the VKK38–K2hpge complex. In this example, a rotor speed of 36,000 rpm was used.
hydrogen bond interactions with Asp54, Asp56, and Arg69 of the second K2hPg molecule.

**Backbone dynamics of VKK38**

$^{15}$N longitudinal (spin–lattice) and transverse (spin–spin) relaxation rates ($R_1 = 1/T_1; R_2 = 1/T_2$) of the VKK38 in its nonbound and K2hPg-bound forms show a bell-like profile with highly mobile N and C termini. The $R_2/R_1$ ratio along the sequence falls in the region of 2.6 – 4.0 for the nonbound form of VKK38 (Fig. 7A). This indicates that $^{15}$N relaxation is mostly dominated by higher-frequency motions. However, the $R_2/R_1$ ratio is dramatically changed to a range of 50 – 80 for most of the residues of VKK38 bound to K2hPg due to the changes in $^{15}$N backbone dynamics as well as the increase of molecular weight from the addition of two K2hPg domains to VKK38. On the basis of the $R_2/R_1$ ratio, global correlation

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**Figure 4. Chemical shift mapping of the complex of VKK38 with K2hPg.**

A, overlay of the $^1$H-$^{15}$N HSQC spectra of [^{15}N]VKK38 (1.0 mM) to show the backbone shifts in the absence (red) and presence (blue) of 2.5 molar eq of unlabeled K2hPg. Peaks are labeled using the one-letter amino acid code and sequence position of the corresponding residue. B, combined chemical shift changes along the sequence. The positions of the two helices (H) of VKK38 are indicated above the graph.
time ($\tau_m$) estimates of 3.8 and 10.7 ns were obtained for nonbound VKK38 and VKK38 in complex with K2hPg, respectively.

Heteronuclear $^{15}$N-NOE values for nonbound VKK38 decrease toward both the N and C termini (Fig. 7B), suggestive of fast motions and high flexibility at each end. Comparably higher $^{15}$N NOE enhancements have been observed when VKK38 is bound to K2hPg, especially in the areas of RH1 and RH2. The average $^{15}$N NOE value is 0.78 for the region between Asp6 and Asp35, indicating that a rigid structure is formed in VKK38 upon binding to K2hPg.

Discussion

Structural changes in VKK38 upon its binding to K2hPg

In the current study, we provide a high-resolution characterization of the hPg-binding a1a2 domain (VKK38) of PAM455 in complex with its binding partner in hPg, K2hPg, using NMR and molecular dynamics approaches. Two RH motifs (RH1 and RH2) in VKK38, which are indispensable for interacting with hPg, were found in flexible peptide regions in the absence of K2hPg, suggesting that the binding sites for K2hPg are exposed and readily available to interact with K2hPg.

For comparison, the structural model of VEK30 in the nonbound form was predicted on the basis of the chemical shift data of this peptide using TALOS-N and CS-Rosetta programs (31). It is noted that in the nonbound form, the RH1 motif in VEK30 is also located in a flexible region of this peptide (Fig. 5B). In VKK38, the random secondary structures between Ala15 and Asp21 and between Leu29 and Tyr38 include the critical RH1 and RH2 dipeptides. However, upon binding to K2hPg, both RH1 and RH2 are structured as part of an end-to-end helix in the bound conformation of VKK38. These data imply that a1a2 repeats may adapt better to more flexible and exposed conformations that better fit the binding sites for K2hPg. Subsequently, the binding of VEK and VKK peptides to their receptor, K2hPg, induces a conformational transition allowing both VEK30 and VKK38 to adopt a fully helical form.

The role of VHD motif

On the basis of the structure of VEK30/K2hPg determined previously (25–27), a model was proposed in which the lysine-binding site of K2hPg interacts with residues Arg17, His18, and Glu20 of VEK30 (Arg113, His114, and Glu116 in PAM numbering). The charged side chains of these residues, contained...
within a single turn of the VEK30 helix, assume a spatial arrangement that is isosteric with the carboxylate and \(-\text{H9280} -\text{amino}\) groups of a C-terminal lysine residue that is necessary for binding of hPg to its known receptors. Thus, because neither VEK30 nor PAM contain a C-terminal lysine, these three residues could be critical for VEK30 binding to K2hPg. Our present study shows that the insertion of the naturally occurring VH3 motif between 17RH18 and Glu20 does not significantly affect the binding of PAM to hPg, as seen from the measured binding constants for full-length PAM, the mutant PAMs, and the truncated peptide variants.

Comparisons of the solution structures of VKK38 and VEK30 (Fig. 5D) suggest that the VHD tripeptide insertion in VKK38 (and in PAM) might not significantly change the orientation of the RH dipeptide (i.e. \(3^\text{R} \text{H}^{136}\) in VKK38 and \(3^\text{R} \text{H}^{136}\) in PAM455), which is needed to maintain strong binding to hPg. Meanwhile, hydrogen bond interactions between RH1, at residues Arg17, His18, and Asp21 in VKK38, and K2hPg have been observed from the binding model (Fig. 6B).

The residues 17RH18 and Glu20 in VEK30 (113RH114 and Glu116 in wild-type PAM53) are spaced by two amino acid residues and juxtaposed to one another, an arrangement that provides the bipolar ligand required of the lysine-binding site in K2hPg. Instead of Glu20 in VEK30 (or Glu116 in PAM53), residue Asp21 in VKK38 (Asp123 in PAM455) provides the negatively charged environment for interacting with K2hPg. The corresponding bipolar ligand could also be composed by 17RH18 and Asp21 in VKK38. Thus, we propose that in PAM455 and other strains of GAS containing PAM with the VHD inclusion, 119RH120 and Asp123, which occupy the positive and negative ends of the lysine-binding site of K2hPg, provide the pseudolysine for the binding to K2hPg.

Based on the same mechanism, and in confirmation of this conclusion, the insertion of the VHD tripeptide in VKK30, VEK32, or VEK35 does not significantly affect the binding affinities of the peptides or their full-length counterpart PAMs with hPg or K2hPg (Table 1).

**The role of the RH motifs in the a1a2 domain of PAM**

PAM is a virulence factor expressed by pattern D strains of GAS. However, no structure for this protein is available despite its pathological significance. PAM was previously shown to be a dimer (12, 32). Two RH motifs, located in flexible region of the a1a2 domain of PAM, play important roles in its binding to hPg. Residues Arg113 and His114 of the a1 repeat of PAM have been shown to make numerous salt bridges and hydrophobic-type electrostatic interactions with recombinant K2hPg, forming a pseudoligand similar to the lysine analogue, \(-\text{H9280} -\text{aminocaproic acid}\) (25). It is thus likely that the corresponding residues Arg126 and His127 in the a2 repeat of PAM interact with hPg in a similar fashion. Our current study for a truncated PAM clearly shows that each VKK38 peptide contains two binding sites for K2hPg which raises the question as to whether each PAM molecule could bind two hPg molecules. In the dimeric forms of PAM and these peptides, it is possible that one of the RH sites is masked. Thus, further structural studies of dimeric forms of extended a1a2 domains are required, and these are best studied by X-ray crystallography.

**Experimental procedures**

**Bacterial strains**

Pattern D GAS isolates AP53 and NS455 were provided by G. Lindahl (Lund, Sweden) and M.J. Walker (Queensland, Australia), respectively. Both strains were cultured on sheep blood agar plates or in Todd–Hewitt broth (BD Bacto, Franklin Lakes, NJ) supplemented with 1% (w/v) yeast extract (THY) at 37 °C in an atmosphere of 5% CO₂.

**Figure 6. Binding model of VKK38 to K2hPg.** A, the model is derived from both Xplor-NIH and HADDOCK. K2hPg molecules (1 and 2) are illustrated as white ribbons, and VKK38 is shown as a green ribbon. The defined binding sites used in HADDOCK are colored red. Hydrogen bond interactions between K2hPg and the \(1^\text{R} \text{H}^{113}\text{VHD}^{117}\) motif in VKK38 (6) and the \(1^\text{R} \text{H}^{113}\text{EE}^{116}\) motif in VEK30 (C) are compared. The binding site residues of K2hPg and VKK38 and of K2hPg and VEK30 are shown as green and red sticks, respectively.

**Figure 7. NMR-based characterization of the backbone relaxation of unbound \([15\text{N}]\text{VKK38}\) (gray) and \([15\text{N}]\text{VKK38}\) bound to K2hPg (black).** A, variations of the R2/R1 ratio; B, heteronuclear \(\text{H}^1 - \text{15N}\) NOE enhancements along the VKK38 amino acid sequence. The standard deviation is indicated by the vertical error bar.
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**Protein expression plasmids**

To construct the PAM expression plasmids, the coding sequence for PAM from AP53 (PAM53), comprising amino acid residues 42–392, which did not contain the N-terminal signal peptide and C-terminal LPXTG cell membrane anchoring domain, was amplified from GAS AP53 genomic DNA with primers pamF and pamR. The latter primer introduced a coding sequence for a His$_6$ tag at the 3’-end of the pam gene to aid purification. After double digestion with NcoI and EcoRI, the PCR product was ligated into NcoI/EcoRI-digested pET28a.

The same cloning, digestion, and ligation strategy was used to clone the coding sequence for PAM from NS455 (PAM455), containing amino acid residues 42–401. A similar strategy was used to generate the PAM mutants, PAM53/▼$^{115}$VHA$^{117}$, PAM53/▼$^{115}$VHD$^{117}$, and PAM455/▲$^{121}$VHD$^{123}$.

**PAM-derived peptide expression plasmids**

The construction of peptide expression plasmids was performed as described previously (27). PAM-derived VEK/VKK peptides from GASAP53 and NS455, respectively, were expressed in *E. coli* BL21 (DE3) employing the His$_6$-tagged streptococcal protein GB1 domain fusion expression system. The final constructs contained, sequentially from the 5’-end, an ATG initiation codon, a His$_6$ tag for purification purposes, the GB1 domain for enhanced solubility, a 9-residue linker, and a thrombin cleavage site, LVPR ↓ GS. DNA fragments containing the VEK/VKK coding sequences were inserted downstream of this cassette into the bacterial expression vector, pET-15b (Novagen, Gibbstown, NJ). A translation stop codon was placed immediately downstream of the open reading frame. Thus, all peptides cleaved with thrombin possessed a GS dipeptide at immediately downstream of the open reading frame. Then, all peptides cleaved with thrombin possessed a GS dipeptide at their N termini. In addition, a Tyr residue was intentionally placed at the C termini of each peptide for 280-nm absorption properties (12).

*E. coli* Top10 (Invitrogen) cells were transformed by electroporation. Clones that harbor plasmids inserted with correct genes were screened by DNA sequencing.

**Full-length PAM gene expression and protein purification**

To express PAM53 and PAM455, *E. coli* BL21 (DE3) cells (New England Biolabs) were transformed with the corresponding expression plasmids. The overnight cultures of the transformed cells were inoculated at 1% (v/v) in 1 liter of LB broth plus 40 μg/ml kanamycin and incubated at 37 °C until an A$_{680}$ nm of 0.6–0.8 was reached. Protein expression was induced by the addition of 0.8 mM isopropyl-1-thio-β-D-galactopyranoside. The resulting cultures were grown for 5 h at 37 °C and then centrifuged. The resulting cell pellets were resuspended in 40 ml of binding buffer with 1 mM PMSF, the cells were then disrupted by sonication, and the supernates were collected after centrifugation at 10,000 rpm for 30 min at 4 °C (14).

The supernates containing the tagged proteins were loaded onto a Ni$^{2+}$-Sepharose affinity chromatography column (HisTrap HP, GE Healthcare) at 4 °C. The column was washed with binding/wash buffer (50 mM Tris, 300 mM NaCl, 40 mM imidazole, pH 8.0), the proteins were eluted with a solution containing 50 mM Tris, 300 mM NaCl, 250 mM imidazole, pH 8.0. The eluates contained purified PAM53 or PAM455, and their molecular weights were confirmed by MALDI-TOF mass spectrometry on an Autoflex III system (Bruker Daltonics, Bremen, Germany).

**PAM-derived peptide expression and purification**

Unlabeled truncated VEK and VKK peptides (Table 1) were expressed in a similar fashion as full-length PAMs. To prepare $^{15}$N/13C]VKK38 for NMR experiments, expression was accomplished in M9 minimal medium containing $^{15}$N/[NH$_4$]Cl (99%; Cambridge Isotope Laboratories, Andover, MA) as the sole nitrogen source and/or $^{13}$C]glucose (99%; Isotec, Champaign, IL) as the sole carbon source. A single *E. coli* BL21 colony expressing $^{15}$N/13C]VKK38 was selected and grown in 2 ml of LB broth for ~4 h. The cells were collected by centrifugation, resuspended in 100 ml of prefiltred M9 medium, and grown at 30 °C overnight. The overnight culture was then inoculated into 1 liter of prefiltred M9 medium with 100 μg/ml ampicillin and grown at 37 °C to an A$_{680}$ nm of ~2.0. After induction with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside for 5 h at 37 °C, cell pellets were collected and frozen at −80 °C. After resuspension in 40 ml of binding buffer with 1 mM PMSF, the cells were then disrupted by sonication, and the supernates were collected after centrifugation at 10,000 rpm for 30 min at 4 °C (26).

The VEK and VKK peptides were purified using a Ni$^{2+}$-Sepharose affinity chromatography column, as above, and concentrated by ultrafiltration. Thrombin (1,000 units; ERL, South Bend, IN) was then added. The cleaved fragments were further separated using a HiTrap HP affinity column (GE Healthcare). The flow-through fractions contained the desired peptides, which were then applied to a p-aminobenzamidine-agarose affinity column (Sigma) to remove thrombin. The integrity of all proteins and peptides was determined by MALDI-TOF mass spectrometry (supplemental Table 1S). For all peptides, single mass peaks were obtained at the correct molecular weights, indicating, for the labeled peptides, that complete incorporation of the heavy isopes occurred.

**SPR**

The binding kinetics of full-length PAMs to hPg and VEK/VKK peptides to K$_2$hPg were measured in real time by SPR using a BIACore X100 Biosensor system (GE Healthcare). All binding experiments were conducted at 25 °C employing HBS-EP (10 mM HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20, pH 7.4) as the running buffer at a flow rate of 10 μl/min. Both hPg and K$_2$hPg, diluted to 40 μg/ml in 10 mM NaOAc, pH 4.5, were injected into flow cell 2 for immobilization on the CM5 sensor chip surface using the amine-coupling kit, to a level of ~900 response units. Nonbound sites on the sensor chip surface were blocked afterward by injection of 1 M ethanolamine, pH 8.5.

All binding experiments were conducted by injecting various concentrations of analytes in HBS-EP buffer over the hPg or K$_2$hPg-coupled CM5 chip surface. Each concentration was injected for an association time of 2 min followed by a 6-min dissociation time. The chip surface was regenerated between
cycles using 10 mM glycine, pH 1.5 (for hPg) or pH 2.0 (for K2hPg), which did not change the properties of ligands bound to the CM5 chip. The binding data from these sensorgrams were subtracted from those obtained using a reference flow cell prepared by the same method, but without immobilizing ligands on the chip. Sensorgrams were analyzed using BIA evaluation software version 2.0.1 (GE Healthcare). The apparent equilibrium $K_d$ values were calculated from the instrument software by the ratio of the dissociation ($k_{off}$) and association rates ($k_{on}$). Nonlinear fitting of the association and dissociation curves with a 1:1 binding model was employed (12, 33).

**AUC**

AUC experiments were performed in a Beckman XL-I analytical ultracentrifuge (Beckman-Coulter, Fullerton, CA) at 25 °C in the absorbance detection mode at 280 nm. Protein samples were diluted to a final concentration of 0.1–0.3 mg/ml in 100 mM sodium phosphate buffer, pH 7.4. A six-sector cell loaded with 110 μl of samples and 125 μl of reference buffer was used. The apparent molecular weight for the complex of VKK38–K2hPg was determined by sedimentation equilibrium at rotation speeds of 32,000–48,000 rpm. The partial specific volumes of VKK38 and K2hPg were calculated from their amino acid sequences as described elsewhere (45). All 15N relaxation parameters, including longitudinal relaxation rates ($R_1$), transverse relaxation rates ($R_2$), and steady-state heteronuclear 1H-15N NOEs, for free and complexed VKK38 were measured at 298 K using standard pulse sequences as described elsewhere (45). All 15N $R_1$/$R_2$ relaxation experiments were carried out in an interleaved manner with a 2-s recycle delay between scans. The relaxation delays used for free and complexed VKK38 were 10, 50, 110 × 2, 180, 300, 420, 570 × 2, 650, 850, 1100, and 1500 ms for the $R_1$ experiments and 16, 32 × 2, 48, 64, 80.1, 96 × 2, 112, 128, 160.1, 176, and 192 ms for the $R_2$ experiments. Duplicate spectra were used to estimate experimental errors. The relaxation rates were determined by fitting the cross-peak intensities to a single exponential function using the nonlinear least-squares method. The error in the rate constants was assessed from Monte Carlo simulations. 1H-15N NOE experiments were carried out in the absence and presence of a 3-s proton saturation period before the 15N excitation pulse, using recycle delays of 4 and 7 s. Heteronuclear NOE values were obtained from the ratios of the peak intensities measured with and without proton saturation. Peak intensity uncertainties were estimated from the noise level of the spectra.

**NMR spectroscopy**

NMR spectra for resonance assignments and NOE identification were recorded at 298 K on a Bruker AVANCE II 800 spectrometer, equipped with a 5-mm triple resonance (TCI, 1H/13C/15N) cryoprobe. All spectra were conducted on samples of uniformly 15N- or 13C/15N-labeled recombinant VKK38 in 20 mM BisTris-d19, 2 mM DSS, 0.1% NaN3, 5% D2O, 95% H2O, pH 6.7, except for 2D 1H/13C HSQC, 3D HCCH-TOCSY (34), and 13C-edited NOESY experiments, for which the sample was dissolved in 2H2O. NMR data were processed with TopSpin version 3.5 software and analyzed by using Sparky (35). 1H chemical shifts were referenced to internal DSS, 13C and 15N chemical shifts were referenced indirectly to DSS (36). The following spectra were collected: 15N HSQC (37, 38), 13C- and 15N TOCSY-HSQC (39) (80-ms mixing time), HNCO/HNCACB/CO (40, 41), HNCA (34), HNCA/CBCA(CO)NH (42), and C(CO)NH (34) for the backbone and aliphatic side chain resonance assignments, as well as 15N NOESY-HSQC (43) and 13C NOESY-HSQC (44) to collect intramolecular NOE distance constraints for use in the structure calculations.

To solve the structure of the VKK38–K2hPg complex, 15N-dispersed NOESY (200-ms mixing time) and 13C-dispersed NOESY (80-ms mixing time) spectra were collected on the mixture of [15N/13C]VKK38 and natural abundance K2hPg at a ratio of 1:2 m/m. Intermolecular distance constraints were determined by 13C-half-filtered 3D NOESY experiments (100-ms mixing time) performed on samples with [13C]VKK38 to unlabelled K2hPg. Control 13C-half-filtered 3D NOESY experiments (100-ms mixing time) were acquired as controls on free [13C]VKK38 or K2hPg samples under identical conditions.

**NMR structure calculations**

Assignments for backbone atoms are 92 and 77% complete for VKK38 in the apo and bound forms, respectively, and >80% for side-chain resonances. Using Sparky, a total of 120 and 162 proton distance constraints for VKK38 in the apo and bound forms were obtained from analyzing the 15N and 13C NOE spectral data. Backbone torsion angles ($\phi$ and $\psi$) were predicted using TALOS-N (46).

All restraint information was applied in a simulated annealing protocol using XPLOR-NIH version 3.6 (47, 48). A summary of the experimental constraints as well as pertinent structural statistics is provided in supplemental Table 2S. Approximately 200 structures were calculated, from which 20...
structures with the lowest restraint energy values were further refined with implicit water. The quality of the structures was analyzed with PROCHECK version 3.5.4 (49, 50). For the 20 final conformers of apo-VKK38 and VKK38 bound to K2hPg at 100 and 94.7% of all residues were found in the favored and allowed regions of the Ramachandran plot, respectively. Visualization of the structures was performed using PyMOL.

Molecular modeling

K2hPg was modeled to the bound structure of VKK38 at a ratio of 1:2 (m/m). A total of 39 intermolecular NOEs from the NMR solution structure of VEK30/K2hPg (BMRB entry ID 16311) were used as distance restraints for structure calculations for VKK38–K2hPg at residues 17RH18. The lowest energy conformer of the 200 calculated structures was used as the starting template to bind with the second K2hPg molecule at the binding site. This was as carried out using the HADDOCK web server combined with the chemical shift perturbation data from 15N HSQC titration experiments for VKK38–K2hPg and previous results for VEK30–K2hPg. The result generated with the lowest HADDOCK score was selected to present the binding model of VKK38–K2hPg at a ratio of 1:2.

Data deposition

Backbone shift assignments and the experimental restraints used in the structure calculation for VKK38 in the nonbound and K2hPg-bound forms have been deposited in the BioMagResBank with accession numbers 30272 and 30271, respectively. The coordinates of the calculated structure ensembles have been deposited in the Protein Data Bank with the accession code 5V4U for VKK38 in the K2hPg-bound form.

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