The Oligomeric Structure of Vaccinia Viral Envelope Protein A27L is Essential for Binding to Heparin and Heparan Sulfates on Cell Surfaces: A Structural and Functional Approach Using Site-specific Mutagenesis

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Introduction

Vaccinia virus (VV) is a member of the Poxviridae family, the largest known animal virus. It has a double-stranded DNA genome of about 187 kb.¹

Abbreviations used: VV, vaccinia virus; IMV, intracellular mature virion; GAG, glycosaminoglycan; GBS, GAG-binding site; SPR, surface plasmon resonance.

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When VV replicates in host cells, intracellular mature virions (IMVs) form in the cytoplasm and represent the majority of infectious progenies after cell lysis. During IMV infection, the virions attach to cell-surface glycosaminoglycans (GAGs) and enter cells through plasma membrane fusion via an unknown coreceptor.²³ IMV contains at least three GAG-binding envelope proteins, two of which, H3L and A27L, bind to cell-surface heparan sulfates, while the other, D8L, binds to chondroitin sulfates.⁴ In addition to its role in virion attachment, A27L is required for the intracellular wrapping of the
IMV into an intracellular enveloped virion (IEV) during virion morphogenesis.\textsuperscript{5}

Wild-type A27L contains 110 amino acid residues that can be divided into four functional domains (Figure 1(a)). Elucidation of the function of the domains of A27L will help us understand the entry mechanism of vaccinia virus. Unfortunately, structure determination by X-ray crystallography has been hampered by intrinsic self-assembly. A27L consists of: a signal peptide for protein processing (residues 1–20),\textsuperscript{6} a lysine/arginine-rich region, STKAAKKPEAKR (residues 21–32) that is essential for binding to cell surface GAGs (denoted as GAG-binding domain or GBS);\textsuperscript{7} a coiled-coil domain (residues 43–84) that is involved in self-assembly; and a C-terminal sequence (residues 85–110) that has been shown to interact with another vaccinia viral protein, A17L.\textsuperscript{8,9} Although A27L forms an oligomeric coiled-coil structure as commonly found in type I viral fusion proteins,\textsuperscript{10,11} such as influenza virus HA\textsubscript{2}, HIV gp41\textsuperscript{13,14} SIV gp41,\textsuperscript{15,16} MoMLV p55,\textsuperscript{17,18} and ebola GP2,\textsuperscript{19} however, the role of the coiled-coil region in A27L protein-mediated virus entry has not been addressed. Although A27L is not an essential protein for the vaccinia virus, previous studies have shown that both virus penetration into cells and cell fusion induced by expression of A27L were blocked by a monoclonal antibody recognizing A27L and by soluble A27L.\textsuperscript{7,20} These results suggested that A27L may regulate cell fusion either directly or indirectly.

We proposed a molecular model consisting of two distinct structural domains: a flexible, unstructured, extended coil and a more rigid, $\alpha$-helical, coiled-coil domain.\textsuperscript{21} Notably, a hydrophobic core (Asn43-Glu55) within the $\alpha$-helical coiled-coil region was highlighted as responsible for inter-helical interactions. The unstructured coil domain contains the GBS essential for binding to heparan sulfate on the cell surface. However, as shown in this study by \textit{in vitro} bioassay, the GBS alone is insufficient for binding. It was hypothesized that a cooperative structural and functional relationship exists between the self-assembled coiled coil and the unstructured single strand domains. Although within the hydrophobic core three residues Leu47, Leu51, and Leu54, positioned at $a$ and $d$ in the heptad repeat unit with a high level hydrophobicity, are highlighted as critical for self-assembly,\textsuperscript{21} the structural contribution of these Leu residues to the biological activity of the protein remains unclear.

To explore the structural and functional relationship, we have constructed four mutants by site-directed mutagenesis: three single mutants L47A, L51A, and L54A, and one triple mutant, L47,51,54A (Figure 1(a)). The physical properties of these mutants were carefully analyzed by CD and NMR spectroscopy, and gel-filtration chromatography. The biological activity of heparan sulfate binding was examined by an \textit{in vitro} surface plasmon resonance (SPR) assay.\textsuperscript{22} Our data showed that mutation of these Leu residues effectively disrupts self-assembly such that the degree of oligomerization and the structural integrity of these mutants are affected. In contrast to the single mutants, for the triple mutant, the self-assembly hydrophobic core structure is uncoiled and the mutant has lost all biological activity. Our data provide direct evidence that the hydrophobic core structure is critical for the heparin-binding affinity. Thus, it was concluded that this set of Leu residues (Leu47, Leu51 and Leu54) sustains the self-assembly hydrophobic core structure and is essential for biological function in A27L.

**Results**

**GBS alone is insufficient for heparin binding**

To identify whether the GAG-binding site (GBS) is sufficient for heparin binding, we used solid-phase peptide synthesis to generate a 12-mer
oligopeptide corresponding to its sequence. In addition, a truncated A27L-aa in which the GBS was deleted (sDA27L) was expressed and purified from *Escherichia coli* (Figure 1(a) and (b)). An *in vitro* SPR binding assay was used to examine the heparin-binding affinity of these two samples, with sA27L-aa, serving as a positive control (Figure 2(a)). sA27L-aa displayed a typical association/dissociation binding curve fitted with a binding affinity $K_D$ of 7.7 nM (Table 1); however, neither sDA27L nor GBS was able to interact with heparin. The SPR result for sDA27L is consistent with that observed from isothermal titration calorimetry.21 Our data clearly indicate that GBS alone fails to interact with heparin. Hence, we postulate that the self-assembly coiled-coil structure acts as an indispensable scaffold that stabilizes the overall structure of sA27L, and is critical for the heparin binding interaction. To further examine this issue, we carried out site-directed mutagenesis to generate sA27L-aa mutants aimed at disrupting the coiled-coil oligomeric structure and the binding affinity.

**Table 1.** The effect of mutations in sA27L-aa on the binding to immobilized heparin

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{on}$ (mM$^{-1}$ s$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$ × 10$^{-5}$)</th>
<th>$K_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sA27L-aa</td>
<td>2.40 ± 0.04</td>
<td>1.80 ± 0.03</td>
<td>7.7 ± 0.2</td>
</tr>
<tr>
<td>L47A</td>
<td>0.58 ± 0.03</td>
<td>15.0 ± 0.9</td>
<td>260 ± 15</td>
</tr>
<tr>
<td>L51A</td>
<td>0.51 ± 0.03</td>
<td>15.0 ± 0.9</td>
<td>290 ± 17</td>
</tr>
<tr>
<td>L54A</td>
<td>0.33 ± 0.02</td>
<td>23.0 ± 1.4</td>
<td>700 ± 42</td>
</tr>
<tr>
<td>L47,51,54A</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
</tr>
<tr>
<td>sDA27L</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
</tr>
<tr>
<td>GBS</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
</tr>
</tbody>
</table>

Affinity constants ($K_D$) were calculated from the ratio $k_{on}/k_{off}$. Data represent the means ± S.D. of at least four independent experiments at five different protein concentrations. No binding (NB) was observed with triple mutant L47,51,54A or the truncated protein sDA27L.

**Spr binding assay**

We constructed four mutants via site-directed mutagenesis: three single mutants, L47A, L51A, and L54A, and one triple mutant, L47,51,54A.
The heparin-binding ability of each of these sA27L-aa mutants was assessed by SPR (Figure 2(b)). Binding affinity constant, $K_D$, values of 260 nM, 290 nM and 700 nM were derived for single mutants L47A, L51A and L54A, respectively (Table 1). The binding ability dropped by 50-fold for L47A and L51A, and 100-fold for L54A compared to sA27L-aa, while the triple mutation at Leu47, Leu51, and Leu54 lost all heparin-binding activity.

**CD and HPLC analyses**

The physical properties of sA27L mutants were carefully analyzed by CD, NMR, and gel-filtration HPLC. Studies of secondary structure were carried out by far-UV CD spectroscopy. The CD spectra exhibited distinct structured patterns in which the prominent CD ellipticity at 208 nm and 222 nm is characteristic of an $\alpha$-helix (Figure 3). The helical content, as measured from the residual ellipticity at 222 nm, amounts to 91% for sA27L-aa, 85% for L47A, 49% for L51A, 46% for L54A and 41% for triple mutant L47,51,54A (Table 2). The helicity of the single mutant L47A was reduced by only 6% compared to that of sA27L-aa, in contrast, the single mutants L51A and L54A, and the triple mutant L47,51,54A showed 40~50% less helicity. The $\alpha$-helical content in the triple mutant was similar to that of the single mutants, L51A and L54A, suggesting that Leu51 and Leu54 both contributed to the protein secondary structure.

To determine the degree of oligomerization, we carried out gel-filtration HPLC for each of these mutants (Figure 4). Using standard molecular mass markers and the molecular mass of sA27L-aa (10.3 kDa), sA27L-aa and L47A were eluted essentially as a hexamer, whereas single mutants L51A, and triple mutant L47,51,54A eluted as a tetramer, and L54A as a mixture of hexamer and tetramer.

**Table 2. Summary of data from circular dichroism, gel-filtration HPLC and nuclear magnetic resonance spectroscopy**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Helical content$^a$ (%)</th>
<th>Degree of oligomerization</th>
<th>Acid-induced dissociation (pH)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>sA27L-aa</td>
<td>91</td>
<td>Hexamer</td>
<td>3.0</td>
</tr>
<tr>
<td>L47A</td>
<td>85</td>
<td>Hexamer</td>
<td>3.0</td>
</tr>
<tr>
<td>L54A</td>
<td>49</td>
<td>Hexamer/Tetramer</td>
<td>5.0</td>
</tr>
<tr>
<td>L51A</td>
<td>46</td>
<td>Tetramer</td>
<td>6.0</td>
</tr>
<tr>
<td>L47,51,54A</td>
<td>41</td>
<td>Tetramer</td>
<td>6.7</td>
</tr>
</tbody>
</table>

$^a$ The $\alpha$-helical content of each protein was calculated with standard deviation of 2% using the equation, $[\theta]_{222} = -30,300f_\alpha - 2340$, where $[\theta]_{222}$ is the [\theta] at 222 nm and $f_\alpha$ is the fraction of $\alpha$-helical content expressed as a percentage.$^{28}$

$^b$ The degree of oligomerization was estimated by gel-filtration at neutral pH.

$^c$ The pH sensitivity measurements were determined by 2D HSQC NMR in an acid-induced dissociation approach in which full dissociation was detected (Figure 6(a)–(h)); see the text for details.
It is interesting to note that both L51A and the triple mutant appeared as tetramers but, while L51A retains a moderate binding affinity, the triple mutant has lost all heparin-binding ability.

**pH titration by 2D NMR**

We have demonstrated that 2D $^1$H/$^{15}$N heteronuclear single quantum coherence (HSQC) spectroscopy provides a reliable measure of self-assembly for the sA27L-aa protein. Typically, for sA27L-aa in its native state (pH 4), the HSQC NMR spectrum showed only one-third of the total expected number of resonances, the observed cross-peaks arising from the flexible extended coil residues Ser21-Asp42. Cross-peaks from the remaining residues involved in self-assembly coiled-coil formation were absent (Figure 5(a)). In contrast, at pH 3.0, conditions under which the sA27L-aa hexamer dissociates, nearly all amide resonances could be observed in the 2D HSQC spectrum with better resolution (Figure 5(b)).

HSQC spectroscopy was used to monitor the sensitivity to pH of the sA27L-aa mutants as the spectroscopic results are correlated directly with structural stability. HSQC spectra were acquired as a function of pH starting at pH 7, in decrements of 0.5 until a dissociation state was reached. For simplicity and better contrast, we report here only two HSQC spectra for each mutant, corresponding to the associated and dissociated states, before and after the acid-induced dissociation, respectively. As for sA27L-aa, the coiled-coil domain of the L47A mutant did not uncoil until pH 3 (Figure 6(a) and (b)), suggesting that Leu47 does not contribute significantly to the stabilizing protein–protein hydrophobic interactions. In these pH-sensitivity experiments, L54A and L51A revealed an acid-induced dissociation at pH 5.0 and 6.0, respectively (Figure 6(d) and (f)), suggesting that both Leu54 and Leu51 play an important role in stabilizing the hydrophobic core structure. The triple mutant, L47,51,54A that dissociated at pH 6.5 was the least stable and most pH-sensitive oligomeric structure (Figure 6(h); Table 2).

It has been pointed out that the molecular dissociation and protein unfolding, though two different mechanisms, are highly cooperative, such that the individual molecular events are rather difficult to be dissected one from another. In all sA27L-aa mutants, the acid-induced dissociation process revealed a two-state (association/dissociation) mechanism, similar to that of urea-induced dissociation. Although more experiments need to be performed to clearly define the molecular events associated with the transition between the two states and the parameters regulating the dissociation processes, the behavior of A27L coiled-coils in response to treatment with acid was reminiscent of that of other viral fusion proteins in which the coiled coils are critical for stabilization of protein oligomers after conformational alteration triggered by treatment with acid.

While the HSQC spectra of the three single mutants L47A, L51A, and L54A, contained only one-third of the expected number of cross-peaks (Figure 6(a), (c) and (e)) as was the case for sA27L-aa at neutral pH, at least 15 additional resonances were evident in the 2D HSQC spectrum of the triple mutant (Figure 6(g)). Resonance assignments of these residues were achieved by 2D mapping analysis referenced to the spectra acquired under urea-induced dissociation conditions of pH 5.0 and 2.5 M urea. Four out of the 15 resonances were assigned specifically to Thr46, Lys48, Gln49, and Thr52. Since the appearance of cross-peaks suggests that these residues are no longer involved in the coiled coil, we propose that the self-assembly hydrophobic core domain (Asn43–Glu55) might be disentangled in the triple mutant. This hypothesis is discussed further below.

![Figure 4](image_url)
In vivo bioassay

To explore the biological activity of the four mutants, we performed three bioassays: cell binding, blocking of virus infection, and blocking of cell fusion. In the cell binding bioassay, sA27L-aa and sDA27L were included as positive and negative controls, respectively. As expected, sA27L-aa, which mediates host cell targeting, bound to cells well and sDA27L did not bind (Figure 7(a)). All three single mutants bound to cells, although the binding ability was reduced when compared to sA27L-aa, whereas the triple mutant bound poorly, if at all. We also tested the ability of the proteins to block VV entry into host cells. sA27L-aa and all single mutants blocked viral infection, whereas the triple mutant and sDA27L, which were unable to bind to cells, were also ineffective in blocking viral infection (Figure 7(b)).

We have shown previously that A27L is required for cell fusion when cells were infected with vaccinia virus. Cell fusion induced by IPTG addition in cells infected with IA27L virus was blocked by sA27L-aa, but not by sDA27L, indicating that the GAG-binding is important for A27L-mediated cell fusion. We therefore tested whether the mutants could block cell fusion (Figure 7(c)). In the presence of IPTG, IA27L-infected HeLa cells developed into large, flat, fused cells with multiple nuclei at 24 hours post-infection (Figure 7(c)b). No fusion was detected in the absence of IPTG (Figure 7(c)a). The addition of sA27L-aa into cell cultures effectively blocked cell fusion (Figure 7(c)d). sDA27L, on the other hand, did not bind to cells and, consequently, had no effect on cell fusion as described previously (Figure 7(c)c). As expected, cell fusion was blocked by the addition of any of the three single mutant proteins (Figure 7(c)e–g) but not by the triple mutant (Figure 7(c)h).

Discussion

A27L is a VV envelope protein that mediates virion attachment during viral infection. Structurally, it contains an extended random coil at the N terminus and a self-assembly coiled-coil rigid segment at the C terminus. The random coil region was shown to contain a GAG-binding domain that is essential for A27L binding to cell-surface heparan sulfate; however, the biological role
of the coiled-coil region has not been addressed. In this study, aimed at the hydrophobic core of the coiled-coil region, we specifically tackled the structural and functional relationship between these two domains by site-specific mutagenesis. The L47A mutant formed a hexamer, remained biologically active and closely resembled the sA27L-aa protein. On the other hand, the single mutants L51A and L54A formed tetramers or a mixture of hexamer and tetramer, and showed a lower level of activity. Finally, the triple mutant clearly showed no binding activity either in vitro or in vivo, indicating that a stable self-assembly hydrophobic core structure in the coiled-coil region was indeed critical for cell surface binding in sA27L.

Figure 6. The 2D $^1$H/$^1$H HSQC NMR spectra were acquired as a function of pH for the sA27L-aa mutants. For simplicity, only the spectra before and after acid-induced dissociation are presented. The spectra recorded before acid-induced dissociation are: (a) L47A at pH 4.0, (c) L54A at pH 6.0, (e) L51A at pH 6.5, and (g) L47,51,54A at pH 7.0. The spectra recorded after acid-induced dissociation are: (b) L47A at pH 3.0, (d) L54A at pH 5.0, (f) L51A at pH 6.0, and (h) L47,51,54A at pH 6.5.
The A27L protein binding to heparin or heparan sulfate via charged interactions is considered to be a multivalent effect.\textsuperscript{22,26–28} We believe that the multivalent interaction depends on the self-assembled oligomeric structure. Indeed, the interaction between isolated GBS and heparin is too weak to be detected. In addition, as shown in the SPR data, the heparin-binding affinity of the single mutants was about two orders of magnitude less than that of the control sA27L-aa, suggesting that heparin binding to sA27L \textit{in vitro} was indeed dependent on the degree of oligomerization. Note that, although the single mutants L51A and L54A, and the triple mutant L47,51,54A all formed tetramers, only the triple mutant lost its heparin-binding activity. This implies that the change in the degree of oligomerization from hexamers into tetramers does not fully account for the difference in binding.

The 2D NMR spectrum of the triple mutant at neutral pH displayed more resonances than those of other mutants, suggesting that the structure of the triple mutant differs from those of the single mutants. The appearance of cross-peaks from more than 15 amino acid residues in the HSQC spectrum of the triple mutant suggests that the hydrophobic core (Asn43–Glu55) might dissociate from the rest of the coiled-coil region. To evaluate this hypothesis, we attempted to create a triple mutant construct (Ser21–Lys64) with deletion of the C terminus of the coiled-coil region (Phe65–Asn84). Unfortunately, this construct could not be expressed in \textit{Escherichia coli} but a deletion mutant of L51A lacking the same C-terminal region, sA27L-aaC_L51A, was successfully constructed and expressed. If indeed the above-mentioned 15 amino acid residues arise exclusively from the N terminus of the hydrophobic core region, one would expect sA27L-aaC_L51A, as a mimic of the uncoiled domain, to form a monomer accordingly. Interestingly, as revealed by HPLC, it appeared as a tetramer at pH 7.0 and a monomer at pH 6.0, respectively (Supplementary Data Figure S1), consistent with the behavior of the single mutant L51A that was observed by 2D NMR (Figure 6(e) and (f)). In contrast, the controlled sA27L-aaC remained a tetramer at these pH values (Supplementary Data Figure S1). In addition, we synthesized a peptide of 28 amino acid residues corresponding to Phe65–Ala92 with a total of four heptad repeating units, termed HR-C, covering most of the coiled domain deduced from the triple mutant (Figure 6(g)). HR-C forms an oligomer rather than a monomer, as shown by the HPLC data (Supplementary Data Figure S2). Both the truncated mutant protein (sA27L-aaC_L51A) and the synthetic peptide (HR-C) showed a certain degree of helical structure by CD (Supplementary Data Figure S3 (B) and (C)).

Given these results, we suggest that the hydrophobic core structure in the triple mutant L47,51,54A is fully uncoiled as a consequence of the mutations.

Taken together, we propose a molecular model to illustrate the structural and functional relationship that closely relates the hydrophobic coiled-coil region to the GBS in the sA27L-aa mutants (Figure 8(a)). For the single mutants, the hydrophobic interaction was disturbed, leading to a reduction of the oligomerization state; however, the self-assembly core remained essentially intact. For the triple mutant, the hydrophobic interaction is substantially disrupted, leading to a distinct
oligomeric structure and resulting in the absence of multivalent integrity for heparin binding at the N terminus. In terms of heparin binding, the triple mutant is equivalent to isolated GBS, which showed no binding affinity (Figure 2(a)). We therefore conclude that a stable hydrophobic core structure responsible for oligomeric structure formation is essential for biological function in the VV envelope protein A27L.

A helical wheel representation of the coiled-coil region of A27L that is common to other type I viral fusion proteins reveals multiple inter-helical interactions (Figure 8(b)). Within the heptad repeat unit, both hydrophobic and polar residues at positions $a$ and $d$ are the major contributions to the coiled-coil structure, as revealed by studies of GCN4,29 HA$_2$ of influenza virus,30 MoMLV p55,18 and HIV gp41.31 In HIV gp41, the charged residues at positions $e$ and $g$ that potentially form salt-bridges stabilize oligomer formation.32 By protein engineering, successful inhibition of the HIV gp41 fusion protein by a designed peptide could be achieved.33 In this work, we show that the hydrophobic residues Leu47, Leu51 and Leu54 are critical for the formation of oligomers. Furthermore, the change in helical content of sA27L at different pH values is consistent with a change in the ionization state of the charged residues (Arg, Glu, and Lys) presented at positions $e$ and $g$.10 We found, using CD spectroscopy, that coiled-coil formation was not sensitive to salt concentration in the range of 20–150 mM (Supplementary Data Figure S4). Although heparin binding was shown to be sensitive to salt concentration (Supplementary Data Table S1), optimal binding affinity was found at physiological conditions with a salt concentration of 150 mM, which we used for all in vivo bioassays in this study.

Previously, several studies implied that A27L is the type I fusion protein of VV; however, recent data suggested that VV contains additional fusion proteins.34,35 Our mutagenesis study of A27L revealed that the coiled-coil region contributes significantly to the cell attachment step. Although the GBS of A27L could initiate monovalent binding to cell-surface heparan sulfate through charge–charge interactions, such interactions are of low affinity and could be stabilized only through multivalence, mediated via the coiled-coil region of A27L. Accordingly, we here conclude that a stable oligomeric structure of the C-terminal coiled-coil region is essential for the biological function of
the sA27L-aa viral protein. Given that VV contains two other GAG-binding proteins, H3L to heparan sulfate and D8L to chondroitin sulfates, it will be interesting to determine whether stable GAG-binding through protein oligomerization occurs for other viral envelope proteins.

Materials and Methods

Site-directed mutagenesis

The preparation of sA27L-aa and sDAA27L has been described. Three single mutants, L47A, L51A, and L54A, and one triple mutant, L47,51,54A, were derived from sA27L-aa using a QuickChange XL site-directed mutagenesis kit (Stratagene Inc.). To construct the single mutants, the following primer sets were used in the mutagenesis procedure described by the manufacturer: for the L47A mutant, forward primer A27L-L47A-5' (5'-gac/gac/aat/gag/gaa/act/GCC/aaa/cca/ccg/ctc/act/aat-3') plus the reverse primer A27L-L47A-3' (5'-att/agt/tag/ccg/tgt/ttt/GGC/act/ttc/cgc/att/agt/TTG/ccg/ttg/ttt/ggc/agt/-3'). The L47A mutant, forward primer A27L-L47A-5' (5'-gaa/act/ctc/aaa/caa/cgg/GCA/act/aat/ttg/gaa/aaa/aat-3') plus reverse primer A27L-L51A-3' (5'-cct/ttt/cca/ctt/att/agt/agt/ccg/ttt/gag/agt/ttc-3'); for the L54A mutant, forward primer A27L-L54A-5' (5'-aaa/cca/ccg/ctc/aaat/GCC/gaa/aaa/att/agt/aat/act/aat-3') plus reverse primer A27L-L54A-3' (5'-att/agt/aaat/cca/ccg/ctt/ttc/GC/att/agt/ttt/ggg/ccg/ttc/ttc-3'); for the L51A mutant, forward primer A27L-L51A-5' (5'-gaa/act/ctc/aaa/caa/cgg/GCA/act/aat/ttg/gaa/aaa/aat-3') plus reverse primer A27L-L51A-3' (5'-cct/ttt/cca/ctt/att/agt/agt/ccg/ttt/gag/agt/ttc-3'). All constructs were confirmed by DNA sequencing.

Purification of recombinant proteins

The recombinant proteins were expressed in bacteria and purified as described. All recombinant proteins had the third L51A mutation was introduced into the double wild-type A27L.2 For NMR studies, the transformed bacteria were grown at 37 °C with 1 mM IPTG and harvested; the C terminus, which did not interfere with the function of the L51A mutant, forward primer A27L-L51A-5' (5'-gag/gac/aat/gag/gaa/act/GCC/aaa/cca/ccg/ctc/act/aat-3') plus reverse primer A27L-L51A-3' (5'-att/agt/tag/ccg/tgt/ttt/GGC/act/ttc/cgc/att/agt/TTG/ccg/ttg/ttt/ggc/agt/-3') for the L51A mutant, forward primer A27L-L54A-5' (5'-aaa/cca/ccg/ctc/aaat/GCC/gaa/aaa/att/agt/aat/act/aat-3') plus reverse primer A27L-L54A-3' (5'-att/agt/aaat/cca/ccg/ctt/ttc/GC/att/agt/ttt/ggg/ccg/ttc/ttc-3'); for the L54A mutant, forward primer A27L-L54A-5' (5'-aaa/cca/ccg/ctc/aaat/GCC/gaa/aaa/att/agt/aat/act/aat-3') plus reverse primer A27L-L54A-3' (5'-att/agt/aaat/cca/ccg/ctt/ttc/GC/att/agt/ttt/ggg/ccg/ttc/ttc-3'). In the primer sequence, the upper case letters indicate the mutated sequences. To construct the triple mutant, a double mutant (L47,54A) construct was generated by introducing the L54A mutation into the single L47A mutant construct using the A27L-L54A-5' and A27L-L54A-3' primers, then the third L51A mutation was introduced into the double mutant using the forward primers A27L-L47,51,54A-5' (5'-gaa/act/ctc/aaa/caa/cgg/GCA/act/aat/ttg/gaa/aaa/aat-3') and the reverse primer A27L-L47,51,54A-3' (5'-cct/ttt/cca/ctt/att/agt/agt/ccg/ttt/gag/agt/ttc-3'). All constructs were confirmed by DNA sequencing.

Measurement of circular dichroism (CD) spectra

The CD spectra of sA27L-aa and the four mutant proteins were recorded on a Jasco J-7200 spectrometer using a quartz cuvette with a 1 mm path-length. The spectrometer was equipped with a water-bath and the experiments were carried out at ambient temperature (25 °C). The CD spectra were recorded at 200–260 nm in the far-UV region with a bandwidth of 1 nm at a scan speed of 50 nm/minute and a resolution of 0.2 nm. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard. Five scans were averaged, then the base-lines were subtracted; no numerical smoothing was applied. The mean residue ellipticity [θ]MRE, in deg cm² dmol⁻¹ for the spectra was calculated using the equation:

\[
[\theta]_{MRE} = \frac{[\theta]}{C} \times l 
\]

where [θ] is the measured ellipticity (in mdeg), C is the mean residue molar concentration, and l is the path-length (in cm). The α-helical content of each protein was calculated using the equation:

\[
[\theta]_{222} = -30300 f_{\alpha} - 2340 \times 10^6 
\]

where [θ]_{222} is the [θ] at 222 nm and f_{\alpha} is the fraction of α-helical content expressed as a percentage. CD spectra are presented as the mean residue ellipticity.

In vitro heparin binding analysis by surface plasmon resonance (SPR)

Real-time SPR22 experiments were performed on a BIAcore biosensor 3000 system (BIAcore, Uppsala, Sweden) with 10 mM Heps (pH 7.0), 0.15 M NaCl, 0.005% (w/v) Polysorbate 20 as the running buffer at a flow-rate of 10 μl/minute. Biotinylated heparin was immobilized on the sensor chip surface coated with streptavidin (Sensor Chip SA). SA sensor chips were purchased from BIAcore and biotinylated heparin (CalBioChem) was used without further purification. To immobilize the heparin on an SA sensor chip, 10 μg/ml of biotinylated heparin in running buffer was injected into a flow-cell of a SA sensor chip at a flow-rate of 10 μl/minute until the desired amount of the biotinylated heparin was captured. The control surface was also generated, using the same procedure, by replacing the biotinylated heparin with running buffer. Recombinant proteins at five different concentrations (10 μM, 5 μM, 2.5 μM, 1.25 μM and 0.625 μM) were injected over the heparin-coated surface for 300 s for association, respectively, followed by a 600 s running buffer injection for dissociation. Parallel injections of analytes over a control surface were performed for background measurements. The heparin-coated surface was regenerated for subsequent injections by two successive injections of 120 μl of 1 M NaCl.

NMR spectrometry

All NMR spectra were recorded at 23 °C on a Bruker DRX 500 MHz spectrometer equipped with a 5 mm inverse triple resonance (1H/13C/BB) z-axis gradient probe; the 13N-labeled proteins (0.8–1.0 mM) were examined in Shigemi NMR tubes (5 mm outer diameter). The detailed procedures for NMR measurement, data acquisition, and analysis have been described.

Peptide synthesis

A peptide sequence corresponding to amino acid residues 21–32 (STKAKKPEAKR) was synthesized in
an automated, solid-phase peptide synthesizer PS3 (Rainin), using Fmoc chemistry and PyBOP activation. The peptide product was purified by HPLC using a reverse phase C-18 column (SUPELCO, 250 mm x 10 mm, 5 μm) on an Agilent 1100 system. Purity and homogeneity were confirmed by electrospray ionization and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

HPLC gel-filtration chromatography

The degree of oligomerization of sA27L-aa and its mutants was determined by gel-filtration chromatography. An aliquot of 20 μl of protein was applied to a KW-802.5 8 mm x 300 mm column (Shodex) equilibrated at room temperature with PBS (pH 7.0). Samples were eluted at a flow-rate of 0.5 ml/minute and monitored by measuring UV absorbance at 220 nm. The column was calibrated with protein standards (Amersham Biosciences) by running a set of gel-filtration markers that included albumin (67 kDa), ovalbumin (25 kDa), and ribonuclease A (13.7 kDa).

Cell binding, cell infection and cell fusion assays

Cell binding assays were performed using HeLa cells, as described. The amount of bound protein was quantified using a luminescent image analyzer LAS-1000plus (Fujifilm Inc) and Image Gauge v.4.0 software using standard curves of purified sA27L. Virus infection blocking assays were performed using HeLa cells essentially as described, with slight modifications. In brief, HeLa cells were pretreated with different sA27L-aa mutants (50 μg/ml) at 4 °C for 30 minutes and infected with VV expressing green fluorescence protein (GFP) driven by an early promoter at a multiplicity of infection (MOI) of 10 at 4°C for another 30 minutes in the presence of the mutant protein. Cells were washed, incubated for two hours at 37°C and subjected to GFP analysis on a fluorescence-activated cell sorter (excitation 488 nm, emission 520 nm). Cell fusion assays were performed using HeLa cells, as described. The fused cells were photographed using a Nikon inverted microscope: magnification, 200 x.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2005.04.024

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