Prothrombinase Assembly on an Enveloped Virus: Evidence That the Cytomegalovirus Surface Contains Procoagulant Phospholipid

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In contrast to quiescent cells, we currently report that purified cytomegalovirus (CMV), strain AD169, constitutively expresses phosphatidylserine (PS)-like procoagulant activity. Initial evidence for this came from the observation of a CMV-dependent decrease in factor Xa clotting times. In a purified system, the assembly of a functional complex between factor Xa and the cofactor Va to form prothrombinase was found to be dependent on the addition of CMV. The corresponding dense bodies (DB) and noninfectious enveloped particles had similar activity. Quantification of the total virion and DB phospholipid, and comparison of prothrombin conversion rates to experiments conducted using known concentrations of PS-containing vesicles showed that 8.5% and 7.2% of the CMV and DB phospholipid, respectively, is procoagulant. Direct binding studies of 125I-labeled factor Xa, active site-blocked factor Xa, or the zymogen factor X, with virions or DB showed a single class of Ca2+-dependent sites with dissociation constants in the order of 10^-7 mol/L. Immunogold electron microscopy confirmed the specificity of the CMV/factor Xa association. Cumulatively, these data suggest that the CMV surface contains the necessary procoagulant phospholipid for coagulation enzyme complex assembly. This may enable CMV (and possibly other enveloped viruses) to bypass an important physiologic regulatory mechanism for the production of thrombin.

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Bovine coagulation factor X and prothrombin were purified as previously described. Factor Xa was generated from factor X by treatment with the purified activator from Russell's viper venom (RVV) (Haematologic Technologies) followed by affinity chromatography using benzamidine-Sepharose (Pharmacia, Uppsala, Sweden) to remove the factor X activation peptide, RVV, and residual unactivated factor X. The irreversible inhibition of factor Xa was accomplished by treatment with a fourfold molar excess of EGRxk (to produce EGR-factor Xa). Complete inhibition of factor Xa (1.5 μmol/L) was confirmed by a lack of detectable conversion of the chromogenic substrate S-2222 (200 μmol/L) over a 15-minute period using a kinetic microplate reader (Molecular Devices, Menlo Park, CA; Vmax) with full scale set to 0.03 optical density units (OD). Excess EGRxk was removed by Sephadex G-25 gel filtration chromatography (Pharmacia). Factor X, factor Xa, and EGR-factor Xa were radioiodinated using iodogen (Pierce, Rockford, IL) and chromatographically desalted (Excellulose 5) (Pierce) to remove unincorporated 125I. Bovine factor X was commercially prepared (Haematological Technologies) according to established protocols. The homogeneity of all proteins was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and where applicable by autoradiography using benzamidine-Sepharose (Pharmacia, Uppsala, Sweden) to remove the factor X activation peptide, RVV, and residual unactivated factor X.

To determine the concentration of CMV and DB in units of molar concentration of proteins was determined spectrophotometrically using E_{280} values and molecular weights of 12.4 and 55,000 for factor X, 12.4 and 45,000 for factor Xa, and 14.2 and 72,000 for prothrombin, and 17.4 and 170,000 for factor Va. The optical density at 280 nm was corrected for Rayleigh light scatter by subtracting 1.7 times the absorbance at 320 nm. For chemically modified proteins, the spectrophotometric determinations were measured colorimetrically using a cupric bicinchoninic acid detection system (BCA, Pierce) and known quantities of the same protein that had not been modified.

**Cells and viruses.** Human foreskin fibroblasts (passage 5 to 15) were grown to confluent monolayers in Basal Medium Eagle supplemented with 5% bovine calf serum, 2 mmol/L glutamine and 20 μg/mL gentamicin. The confluent fibroblasts were inoculated with CMV (lab strain AD169, Advanced Biotechnologies Inc, Columbia, MD) at a multiplicity of infection of 0.01, and harvested from the supernatant when a full cytopathic effect was apparent (10 to 14 days post infection). The cell supernatant was spun at 700g to clear cellular debris. To pellet the virus, the resulting supernatant was centrifuged at 23,000g for 25 minutes with 5 x 10^8 viral particles in 50 μL of HBS/CaCl_2 at 21°C. Following equilibrium, the mixture was layered onto 200 μL of 20% sucrose, and the particle-bound and free 125I-ligand were separated by differential centrifugation at 12,000g in a Beckman microfuge E (Beckman Instruments, Irvine, CA). The number of molecules of 125I-ligand that were bound was determined and corrected for nondisplicable binding by subtracting the amount that remained associated with the virus or DB in the presence of unlabelled ligand at a 50-fold molar excess over the highest concentration of labeled ligand that was used. All points were done in triplicate. Dissociation constants (K_d's) and the number of binding sites were derived by iteratively fitting the binding isotherm to a rectangular hyperbola that described a single class of binding sites.

**ImmunoGold electron microscopy.** The direct observation of factor Xa-CMV surface complexes was facilitated by immunogold staining. In these experiments, purified virions were adsorbed onto a carbon/formvar grid (400 mesh) and subsequently incubated with factor Xa (1 μmol/L) at room temperature in HBS/CaCl_2. The viral particles were then incubated with a 1:100 dilution of rabbit polyclonal antifactor X IgG (Diagnostica Stago, Asnieres, France). Gold (10 nm)-conjugated antirabbit IgG was added as a secondary antibody to detect factor Xa bound to the virions by transmission electron microscopy. After the incubation of each reagent for 30 minutes, five cycles of washing in HBS/CaCl_2, 0.1% cold-water fish gelatin was conducted to remove unbound ligands.

**RESULTS**

**Effect of factor Xa clotting activity.** As an initial investigation to determine whether CMV can function as a
source for procoagulant phospholipid, clotting assays were performed using factor X/VII-depleted plasma as the substrate and purified factor Va (70 nmol/L) as the enzyme. In these experiments (Fig 1), approximately 10^6 virions/mL were found to decrease the factor Xa clotting times from 92 seconds in the absence of added phospholipid (Fig 1, lane B) to 40 seconds (Fig 1, lane C). One cycle of freezing and thawing of the virions did not significantly alter these values (data not shown). Clotting times are logarithmically related to enzymatic activity. Therefore, these observations demonstrate a large enhancement of factor Xa-dependent generation of functional thrombin by CMV, even in the presence of normal plasma anticoagulants. Because phospholipid was the only missing constituent of the prothrombinase complex in this assay, these data are consistent with the CMV surface functioning as the necessary procoagulant phospholipid. A similar effect was observed for DB and NIEP that were isolated from the CMV-infected cell supernatant (data not shown). To verify the dependence on factor Xa, the clotting times were found to be greater than 120 seconds in the absence of factor Xa, regardless of whether CMV or rabbit brain cephalin was added (Fig 1, lane A).

When excess rabbit brain cephalin (Sigma) was used instead of the viral particles, factor Xa (35 nmol/L) clotting times were further reduced from 40 seconds to 22 seconds (Fig 1, lane E). This correlated to approximately 10-fold higher factor Xa activity in the presence of cephalin as shown by comparison to factor Xa assayed at 3.5 mmol/L in the presence of cephalin (Fig 1, lane D). These data demonstrated that the accessible procoagulant phospholipid provided by CMV at the concentration used was rate limiting.

Effect of CMV on prothrombinase activity. To confirm that CMV accelerated factor Xa clotting activity by promoting the assembly of prothrombinase, the conversion of prothrombin to thrombin was followed in a purified system. In this experiment (Fig 2), prothrombin (1.4 mmol/L), factor Va (2 mmol/L), and CMV (at 1.28 × 10^9 or 0.64 × 10^9 particles/mL) or CMV, respectively, and CMV at 1.28 × 10^9 or 0.2 × 10^9 particles/mL were combined, and the increase in fluorescence caused by binding of DAPA to newly formed thrombin was monitored. When all reactants were present in this system, considerable thrombin generation was observed when either CMV or DB were used. Rates of 68 and 57 nmol/L thrombin/minute at the higher concentration of CMV and DB, respectively, and 38 and 32 nmol/L thrombin/minute at the lower concentration, were derived by regression of the initial linear phase of the kinetic profiles with percentage standard deviation in each case of less than 5% for three separate measurements. The fact that decreasing the concentration of viral particles also decreased the rate of thrombin generation confirmed the dependence on CMV and DB (Fig 2). If either factor Xa, factor Va, or viral particles were omitted from the reaction mixture, prothrombin conversion was not detectable (data not shown). This demonstrated the requirement for each added prothrombinase constituent to
generate thrombin and shows that CMV and DB do not express endogenous factor Xa-like enzymatic or factor Va-like cofactor activity. These data further support the conclusion that CMV and DB accelerate factor Xa activity by providing the necessary procoagulant surface for assembly of prothrombinase.

An important trigger for the assembly of prothrombinase is the exposure of cellular PS, of which a major physiologic source is activated platelets. Therefore, to obtain an indication of the capacity for viral phospholipid to act in thrombin production, the proportion of phospholipid within CMV or DB that functions as a procoagulant was determined for comparison to the known value for platelets. The first step to accomplish this was to quantify the total amount of phospholipid associated with CMV or DB, which showed 0.47 × 10⁻¹⁸ moles of phospholipid per virion and 1.50 × 10⁻¹⁸ moles of phospholipid per DB. These amounts corresponded within 25% to the theoretical values derived by calculating the spherical surface area of CMV (diameter ~100 nm) or DB (diameter ~200 nm) and using the area of a phospholipid polar head group (~0.65 nm²). The difference may be accounted for by constituents in the envelope other than phospholipid.

Based on the phospholipid quantification, the virus particle-dependent DAPA experiments presented in Fig 2 involved 600 and 300 nmol/L of total viral or DB phospholipid. To determine the relative amount of procoagulant phospholipid associated with the virus or DB, the resulting initial velocities of thrombin production were compared with a standard curve constructed by using rate limiting concentrations of PCPs as the source of procoagulant phospholipid (Fig 2, inset). It was calculated that 8.5% mol and 7.2% mol of the total CMV and DB phospholipid, respectively, has accessible PS-like procoagulant activity.

¹²⁵I-ligand binding to CMV and DB. The parameters governing the equilibrium between factor Xa or factor X and PCPs have been studied extensively in the past. These values provide criteria to distinguish whether the binding of factor Xa or factor X to CMV or DB, is mediated exclusively by phospholipid on the virus surface or if an alternative mechanism is indicated. Therefore, the direct binding of ¹²⁵I-labeled-factor Xa or -factor X to CMV and DB was followed.

As an initial investigation to determine whether specific and Ca²⁺-dependent binding could be detected, one concentration of ¹²⁵I-ligand (0.1 μmol/L) was used in triplicate and incubated with the viral particles for 1 hour at 21°C. These single concentration experiments are presented in Fig 3 and support the conclusions drawn from our clotting and DAPA assays by showing specific interactions between factor X, factor Xa, or chloromethylketone-inactivated factor Xa (EGR-factor Xa) and CMV (Fig 3, bar A). In each case, the interaction was reversible by addition of an excess of unlabeled ligand (Fig 3, bar B) and was found to be inhibited by chelation of Ca²⁺ (Fig 3, bar C).

Before generating complete binding curves to obtain Kₐ's and stoichiometry, a time course of the interaction between ¹²⁵I-factor X or -factor Xa and CMV was conducted to establish an appropriate incubation period to reach equilibrium (data not shown). Based on these kinetic profiles, a 25-minute incubation time was used to acquire the binding data describing the equilibrium between ¹²⁵I-factor X, -factor Xa, or -EGR-factor Xa and CMV or DB shown in Fig 4. For each of these equilibria, Kₐ's and the number of ligand molecules bound per particle at saturation were derived from the nonlinear least squares fit of the data and are presented in Table 1. These experiments show that regardless of the ligand or type of particle, the Kₐ's are similar and range between 0.12 and 0.45 μmol/L, which are comparable to the Kₐ's previously reported for the binding of factor Xa (0.11 μmol/L) or factor X (0.47 μmol/L) to PCPs. Because factor X/Xa-protein receptor interactions are typically 10 to 100-fold stronger, the binding of factor X, factor Xa, and EGR-factor Xa to viral particles is consistent with a phospholipid receptor-mediated interaction. Other characteristics of the binding to CMV or DB resembled binding to synthetic phospholipid vesicles, including: (1) a functional factor Xa active site was not required (Fig 4); (2) each interaction required Ca²⁺ (Fig 3); and (3) factor Va (10 nmol/L) enhanced the amount of ¹²⁵I-factor Xa (10 nmol/L) associated with CMV (data not shown).

The number of molecules of each ¹²⁵I-ligand bound per viral particle at saturation was also derived from the iterative fit of the binding data (Table 1). Approximately four times fewer factor X, factor Xa, or EGR-factor Xa binding sites were shown to exist per CMV (~400 molecules/particle) than on DB (~1200 molecules/particle). This value correlates with the relative surface area of the particles (1.4, CMV:DB).
**PROTHROMBINASE ASSEMBLY ON THE CMV SURFACE**

Figure 4. Binding of factor X, factor Xa, or EGR-factor Xa to CMV or DB. Various concentrations of 
$^{32}$P-ligand were combined with CMV (○) or DB (■) in HBS/Ca$^{2+}$ and equilibrated at 25°C for 30 minutes. 
Radiactive counts that were not displaced by excess cold ligand were subtracted. The binding isotherms were 
iteratively fit to an equation describing a single class of sites.

*Immunogold electron microscopy.* Further evidence for an interaction between factor Xa and CMV was obtained 
by performing immunogold electron microscopy. In these experiments, polyclonal antifactor X/Xa rabbit IgG as a pri-
mary antibody and gold-conjugated antirabbit IgG as a sec-
ondary antibody were used to detect the factor Xa remaining 
bound to CMV after extensive washing. The dense spots on 
the CMV particles shown in Fig 5A (an example is indicated 
by an arrow) identify sites of factor Xa binding that were 
labelled by a gold particle. Virions that were stain-penetrated 
(visible nucleocapsids) and not stain-penetrated are both 
shown in Fig 5A to interact with factor Xa. The envelope 
of stain-penetrated virions was frequently seen to contain 
protrusions (blebs) that bound factor Xa. An example of this 
structure is shown in the upper right corner of Fig 5A. The 
majority of virions were labeled by gold particles. Those 
that lacked detectible factor Xa binding were attributed to 
the rigorous washing procedure used to reduce the back-
ground or to aggregation of the virus. The specificity of the 
detection system was demonstrated in Fig 5B by omitting 
the primary antibody, which completely eliminated the bind-
ing of gold particles to CMV. A similar result was observed 
when the virions were preincubated with chelator EDTA, 
which reverses the interaction of factor Xa with procoagulant 
phospholipid (data not shown).

Periodically, gold label was found in association with 
small particles having less than 10% of the virus diameter. 
Examples of these are indicated by an arrow in Fig 5C and 
D. Because particles of this size would be separated from 
virions during purification, it is probable that they are formed 
during the experiment and may originate from the envelope 
protrusions. This is consistent with the observation that these 
particles were also recognized by a neutralizing monoclonal 
antibody (CMVBI, received as a gift from Dr B. Brodeur, 
Laboratory Centre for Disease Control, Ottawa, Canada) that 
is specific for a surface epitope on CMV (Fig 5C). As with 
virions and DB, the omission of the antifactor Xa antibody 
eliminated the detection of gold bound to these small particles.

In a typical viral preparation, particles much larger than 
virions corresponding to less than 2% of the particles were 
observed. Their size indicated that they were cell-derived. 
Figure 5D shows an example of these darkly staining species 
in association with a viral aggregate. Although factor Xa 
binding to the viral component in this panel was observed, 
binding to the large particle was not demonstrable.

**DISCUSSION**

Virus envelopes are complex structures containing protein 
that is encoded by the host and virus genome, and phospho-
lipid derived exclusively from the host cell. The envelope 
functions early in the infection process by providing the 
molecular species required for host-cell binding, fusion, and/or 
penetration. In the present study, a novel role for the 
envelope phospholipid of CMV is described. The data are 
consistent with CMV functioning as a source of procoagulant 
phospholipid (eg, PS). This conclusion is based on factor Xa-
dependent clotting assays, fluorescent detection of thrombin 
generation in a purified system, direct binding of radioiodin-
ated ligands, and immunogold electron microscopy. To-
gether, these observations show that the CMV surface phos-
pholipid fundamentally differs from that of quiescent cells, 
which have a control mechanism(s) to restrict the expression 
of procoagulant phospholipid specifically to areas of vascular 
injury. Indirect evidence that HSV-1 also expresses 
procoagulant phospholipid comes from our previous identi-
fication of PS binding annexin II on its surface. Thus, 
CMV and likely HSV bypass a normal regulatory pathway 
by continuously presenting procoagulant phospholipid to 
clotting factors in plasma.

Stimulated platelets are considered to be the most abun-
dant physiologic source of procoagulant phospholipid. A 
procedure involving enzymatic digestion of the polar head 
groups revealed that approximately 11% mol of the throm-in/collagen-activated platelet surface phospholipid is PS. 
In the current study, a comparable value for PS-like procoag-
ulant phospholipid activity was estimated for CMV (8.5% 
mol) and DB (7.2% mol). Because our work is based on a 
functional assay, these percentages may be influenced by 
competition with endogenous PS binding annexin II or

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**Table 1. Dissociation Constant and Stoichiometry for the Binding 
of Factor X, Factor Xa, or EGR-Factor Xa to CMV or DB**

<table>
<thead>
<tr>
<th>Ligand Bound to CMV</th>
<th>kd (µmol/l) ± SD</th>
<th>Molecules/Particle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor X</td>
<td>0.31 ± 0.07</td>
<td>256 ± 17</td>
</tr>
<tr>
<td>Factor Xa</td>
<td>0.45 ± 0.11</td>
<td>328 ± 25</td>
</tr>
<tr>
<td>EGR-Factor Xa</td>
<td>0.15 ± 0.03</td>
<td>284 ± 15</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Ligand bound to DB</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor X</td>
<td>0.26 ± 0.06</td>
<td>1,123 ± 68</td>
</tr>
<tr>
<td>Factor Xa</td>
<td>0.24 ± 0.07</td>
<td>1,233 ± 103</td>
</tr>
<tr>
<td>EGR-Factor Xa</td>
<td>0.12 ± 0.03</td>
<td>1,264 ± 64</td>
</tr>
</tbody>
</table>

Parameters were derived from the least squares fit of the binding isotherms in Fig 4.
other constituents of the CMV surface. Additionally, procoagulant phospholipid with a specific activity differing from that of PS may exist in the viral envelope, which would consequently affect our estimate. Although the phospholipid content was not measured for NIEP, these particles were also observed to participate in thrombin generation using DAPA as a fluorescent probe (data not shown). Therefore, virus, DB, and NIEP would be expected to provide procoagulant phospholipid, especially at the sites of viral egress from host cells where the local concentration of virus particles would be maximal. Numerous host cell biochemical changes in response to CMV or HSV have been reported. We now propose that viral PS-like procoagulant activity may participate in the stimulation of these cellular changes. A pathway based on the potent cell modulatory activity of thrombin is suggested and illustrated in Fig 6. From the data presented here, it is evident that factor Xa and factor Va can associate with the viral phospholipid in the presence of Ca^{2+} to form prothrombinase. Procoagulant phospholipid is known to function as an essential prothrombinase cofactor by: (1) stabilizing the interaction with factor Va{sup 62,63}; (2) concentrating the enzyme, cofactor and substrate{sup 60,61}; (3) assembling the prothrombinase components in a productive orientation{sup 62,63}; (4) inhibiting the activated protein C-mediated inactivation of factor Va{sup 64}; and (5) inhibiting the inactivation of factor Xa by antithrombin III{sup 65}. We suggest that the same procoagulant properties are conferred to factor Xa and factor Va by the virus envelope phospholipid and account for the accelerated thrombin generation that we have observed. A novel mechanism has been reported for the production of factor Xa on the surface of HSV-infected endothelial cells{sup 68-69}. This is mediated by the virus-encoded glycoprotein C, which functions as a factor X receptor and activation cofactor. Glycoprotein C is also expressed as an envelope constituent on the HSV surface{sup 68,69} and may conceivably have similar procoagulant properties as the cell surface species. The presence of a viral tenase would fit into our proposed model by initiating prothrombinase assembly in coordination with procoagulant phospholipid on the virus surface. A similar pathway may also be identified on CMV.

Virus-cell interactions are an important aspect of the proposed mechanism for cell modulation by thrombin generated on herpesviruses. For CMV the initial attachment with cells is believed to involve cellular heparan sulfate proteoglycan and the viral gC II glycoprotein complex{sup 67,70,71}, which is represented in Fig 6. A similar interaction with heparan sulfate proteoglycan is also required for HSV infection and involves viral gC I and gB{sup 67,70,71}. The effect of the virus-cell interactions in our model is to anchor the virus close to the host cell surface so that thrombin produced on the virus is proximal to cellular thrombin receptors. This is expected to increase the probability for thrombin to interact with cell receptors before inactivation by plasma anticoagulants can occur.
When bound to its cell receptor, thrombin proteolytically induces the production of intracellular effectors leading to cell functional changes. Interestingly, the modulation of specific intracellular biochemical pathways following CMV binding has been described and is similar to that induced by thrombin, including increased phospholipase activity resulting in the generation of inositol trisphosphate (IP3) and diacylglycerol (DAG), changes in intracellular calcium concentrations, alteration of intracellular protein phosphorylation patterns, and induction of proto-oncogenes c-fos, c-myc and c-jun. This parallel suggests that thrombin generated on the virus may be a direct trigger for the host intracellular changes attributed to infection. The probability that several agonists contribute simultaneously to cell modulation during infection cannot be overlooked. However, thrombin produced at the virus surface is a good candidate for a cell stimulus during the initial stages of infection.

One of the biologic effects of thrombin is conversion of anticoagulant cells into a procoagulant state. Therefore, the procoagulant activity associated with CMV or HSV infected cells may be stimulated by thrombin generated on the virus surface. This could account for reports that demonstrate endothelial cell expression of procoagulant activity in response to CMV or HSV occurs before the virus enters the cell. Once the host cell becomes procoagulant, thrombin production would be amplified and additional cell stimulation would result. The involvement of thrombin in herpesvirus-mediated cell modulation has been observed by the loss of HSV-induced cellular adhesion after immunodepleting prothrombin from growth media or by using thrombin-specific inhibitors. In addition, a role for thrombin has been shown in hepatitis virus pathology using an inhibitory monoclonal antibody to a unique murine hepatitis virus (MHV)-3-induced prothrombinase on macrophages.

Virus envelope phospholipid is derived from the host cell during egress. Interestingly, the envelope phospholipid composition of several virus types has been analyzed and shown to differ from that found in the host cell membrane. This indicates that the selection of phospholipid during the formation of the envelope is not random. Of particular relevance to the current study is a recent report showing the %mol of PS within the human immunodeficiency virus (HIV)-1 and HIV-2 envelope was markedly enhanced compared with that of the host cell (1.4- and 2.5-fold, respectively). Although the proportion of PS on the external leaflet of the phospholipid bilayer is unknown, this observation suggests that HIV may also exhibit a procoagulant effect similar to that observed by us for CMV. Therefore, we are investigating other virus types for procoagulant phospholipid activity, which may be a consequence of a general mechanism for envelope formation.

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