Annexin II Enhances Cytomegalovirus Binding and Fusion to Phospholipid Membranes†

Christina M. Raynor,‡ J. Fraser Wright,§ David M. Waisman,|| and Edward L. G. Pryzdial*,‡

Canadian Blood Services, R&D Department, and Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, 1800 Alta Vista Drive, Ottawa, Ontario K1G 4J5, Canada, Pasteur Mérieux Connaught, 1755 Steeles Avenue West, North York, Ontario M2R 3T4, Canada, and Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada, and Cancer Biology Research Group, Department of Biochemistry, University of Calgary, Calgary, Alberta T2N 4N1, Canada

Received August 28, 1998; Revised Manuscript Received February 17, 1999

ABSTRACT: A number of studies have suggested that the anionic phospholipid (anPL)-binding protein annexin II may play a role in cytomegalovirus (CMV) infection. Since annexin II has been shown to mediate aggregation and fusion of certain membranes, we investigated whether these properties could be exploited by CMV directly. The experiments showed that purified annexin II, but not the homologous protein annexin V (AnV), can mediate the binding of ^{35}S-CMV (strain AD169) to anPL-coated microtiter wells. This association required Ca^{2+}, could be titrated by varying either annexin II (apparent $K_d = 4 \times 10^{-8}$ M) or ^{35}S-CMV, was inhibited by unlabeled CMV, and was observed for the heterotetrameric or monomeric form of annexin II. In experiments utilizing the fluorescence dequenching of octadecyl rhodamine incorporated into the CMV envelope, annexin II was furthermore found to enhance the rate of virus−anPL vesicle fusion. The observed fusion was dependent on the concentration of annexin II, Ca^{2+}, and anPL and was mediated principally by the heterotetramer. Interestingly, AnV was observed to inhibit the effects of annexin II on CMV fusion but not binding to anPL, which indicates that annexin II enhances these processes by distinct mechanisms. The results presented here provide the first direct evidence that annexin II has the capacity to bridge CMV to a phospholipid membrane and to enhance virus−membrane fusion. These observations furthermore suggest that AnV may regulate the fusogenic function of annexin II.

Annexin II is a member of the annexin/lipocortin family of proteins [for reviews, see (*1*, *2*)]. Each annexin type contains internal homology repeats and interacts with anionic phospholipid (anPL)[1] in the presence of Ca^{2+}. Annexin II is unique since it exists both as a 36 kDa monomer (AIIm), which is typical of annexins, and as a noncovalent heterotetramer (AIIt) that consists of two AIIm subunits joined by a dimer of an 11 kDa protein (p11). AIIm and AIIt coexist within a given cell, with AIIm being found in the cytosol and AIIt being predominantly bound to the intracellular leaflet of the plasma membrane (*3*). Annexin II has also been identified on the surface of a number of cell types (*4*−*7*), with antigenic data suggesting exclusively AIIm (*7*), but functional studies indicating that at least a significant proportion must be AIIt (*8*).

The known in vitro functions of AIIm and AIIt differ. At extracellular concentrations of Ca^{2+} (approximately millimolar), both forms are able to aggregate anPL-containing membranes; however, only AIIt is believed to participate in processes that may enhance fusion of apposing membranes (*9*−*12*). An even more significant difference lies within the cell, where the lower Ca^{2+} concentration (approximately micromolar) allows only AIIt to interact with anPL. In this regard, AIIt is distinct from all other annexins, including AIIm, which require much higher Ca^{2+} levels to facilitate association with anPL. The regulation of the anPL-binding, phospholipid membrane aggregation, and fusion functions of annexin II is incompletely understood (*1*) and has been shown to involve phosphorylation (*11*, *13*, *14*), pH changes (*9*), the presence of arachidonic acid (*9*), and membrane binding, (*11*, *14*). The in vivo functions of annexin II are at present speculative; however, in vitro data strongly support a role in membrane trafficking (*15*, *16*).

Recently, annexin II has been implicated in the multistep process leading to infection of cells by the highly prevalent cytomegalovirus (CMV). Like all members of the herpesvirus family, CMV is covered by an envelope that is composed of phospholipid derived from the host cell and proteins encoded by the virus as well as the host genomes. The first step of infection is a relatively weak interaction between virus

† This work was supported by Canadian Blood Services Research and Development Fund Grants TO22.926 (J.F.W. and E.L.G.P.) and HO1O.929 (E.L.G.P. and J.F.W.). J.F.W. was the recipient of a Bayer/Canadian Blood Services/Medical Research Council of Canada Scholarship.

* Corresponding author. Telephone: (613) 739-2462. FAX: (613) 739-2426. E-mail: ed.pryzdial@bloodservices.ca.

‡ Canadian Blood Services and University of Ottawa.

§ Pasteur Mérieux Connaught and University of Toronto.

|| University of Calgary.

[1] Abbreviations: AIIm, annexin II monomer; rAIIm, recombinant AIIm; AIIt, annexin II tetramer; bAIIt, bovine annexin II; AIItm, purified mixture of AIIt and AIIm; AnI, annexin I; AnV, annexin V; anPL, anionic phospholipid; CMV, cytomegalovirus; R18, octadecyl rhodamine; HSP, heparan sulfate proteoglycan; vp, virus particles.

10.1021/bi982095b CCC: $18.00
Published on Web 04/01/1999

envelope constituents and cell surface heparan sulfate proteoglycan (HSP), which is believed to prime the cell for a higher affinity interaction (*17*, *18*) and subsequent CMV envelope−cell membrane fusion (*19*, *20*). Studies conducted by several investigators involving cell surface labeling and detergent solubilization of the membrane components enabled the identification of a CMV-binding protein (*6*, *21*, *22*) that was detected in appreciable amounts only on productively infected cell types (*21*) and was therefore suggested to represent the second CMV receptor (*17*, *18*). Amino acid sequence analysis later correlated this protein to annexin II (*6*). Reports that annexin II-specific antibodies inhibited both CMV infection of cells (*23*) and the fusion of cells transfected with the CMV-encoded glycoprotein B (gB) (*24*) further support a role for annexin II in CMV infection.

While considerable evidence has demonstrated that annexin II can associate separately with purified CMV (*6*, *23*, *25*) or an anPL-containing membrane (*4*, *26*−*28*), the ability for annexin II to simultaneously bind both and thereby fulfill the proposed virus receptor function has not been established. Furthermore, it is reasonable to hypothesize that the fusogenic function of annexin II may also have a role in CMV biochemistry, although this possibility has not been investigated. To address these questions directly, we have used synthetic anPL-containing membranes as a surrogate host membrane. The results obtained show that annexin II can indeed bridge the CMV surface and anPL present on a separate surface. Our studies also revealed that annexin II can participate in virus envelope−anPL membrane fusion. Thus, CMV is capable of exploiting host annexin II to mediate at least two processes that are pivotal early in the pathway leading to cell entry.

EXPERIMENTAL PROCEDURES

Virus Preparation. CMV strain AD169 was grown in human foreskin fibroblasts (HFF), purified by tartrate−glycerol ultracentrifugation, and quantified by electron microscopy as previously described (*29*). Where indicated, UV inactivation of virus was carried out for 1 h on ice at a distance of 5 cm from a germicidal lamp (30 W, G30T8, Sankyo Denki Co., Japan) and verified by plaque assay. Virus was radiolabeled by adding a total of 4 mCi of [^{35}S]-methionine (in vivo cell labeling grade, Amersham) to 8 T-175 flasks of HFF, at 1 week post-infection. ^{35}S-CMV was harvested approximately 7 days later when the cytopathic effect of infection was maximal, and was purified and quantified as described for the unlabeled preparations (*29*). Octadecyl rhodamine (R18, Molecular Probes) was incorporated into UV-inactivated CMV (R18-CMV) by adding R18 (15 μM) to virus (10^{11} particles/mL) and incubating at 22 °C for 1 h, as described (*30*). The unbound R18 was removed by chromatography over Sephadex G-75 in 20 mM HEPES, 150 mM NaCl, pH 7.4 (HBS).

Protein Preparation. The method for annexin II purification was based on a previous description (*31*). About 600 g of fresh human placenta was rinsed well in a solution of ice-cold phosphate-buffered saline (PBS) containing 5% sucrose and 1 mM benzamidine (Sigma), transferred to elution buffer (EB: 25 mM Tris, 1 mM benzamidine, 25 μg/mL pepstatin A, pH 7.7), and homogenized in 2 L of EB containing 5 mM Ca^{2+} using a Polytron (Brinkman). The homogenate was spun for 40 min at 25000*g*, and the pellet was resuspended and homogenized in 2 L of EB containing 5 mM EDTA. The second homogenate was centrifuged and the clarified supernatant applied to a 1 L column of DEAE cellulose (Pharmacia) equilibrated in 25 mM Tris, pH 7.7. The colorless flow-through was collected, concentrated by ultrafiltration (Amicon, YM 10) to 50 mL, and dialyzed against 50 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6 (MES buffer). The dialysate was applied to a 50 mL column of S−Sepharose (Pharmacia), washed, and eluted with a 0−0.6 M NaCl gradient in a total volume of 1 L of MES buffer. Phospholipase A_2 inhibitory activity was assayed as previously described (*32*). Of the two activity peaks that eluted, the first contained annexin I (AnI) and the second AIIm and p11, as determined by Western blot analysis using the respective monoclonal antibodies (Zymed). The second peak was pooled, concentrated, and subjected to MONO S chromatography (HR 5/5, Pharmacia) using a gradient of 0−0.3 M NaCl in MES buffer. Protein concentration was determined by a colorimetric assay (BCA, Pierce). To remove trace contamination of AnI, which like annexin II is known to aggregate anPL-containing membranes, the sample in HBS was passed over an immunoaffinity column specific for AnI. Evaluation of the final product by two-dimensional electrophoresis (*33*, *34*) and Western blot analysis showed that the annexin II pool contained AIIm and p11 antigen. Unlike a previous report (*27*), a range of isoelectric forms of AIIm was not observed (not shown). To approximate the cellular pool of annexin II, no attempt was made to separate the AIIm and AIIt in this mixture, which is referred to here as AIItm. The molecular mass of AIIm was used [38.5 kDa, (*12*)] to estimate the molar concentration. AIItm preparations were observed to contain a trace amount (>5%) of a nonreducible species (approximately 72 kDa) that was recognized by the AIIm mAb. The identity of this protein is currently under investigation. In some experiments, the function of AIItm was compared to human recombinant AIIm (rAIIm) and bovine AIIt (bAIIt), which were purified and characterized as previously reported (*12*). Purified recombinant annexin V (AnV), known to contain no other detectable type of annexin, was obtained as a kind gift from Dr. T. Yokoyama (Kowa Co. Ltd., Japan).

^{35}S-CMV Binding Assay. Break-away microtiter wells (Immulon 3, Dynatech) were coated with 0.3 μg per well of a mixture of 25% phosphatidylserine (PS) and 75% phosphatidylcholine (Sigma) or no phospholipid, and blocked with 10 mg/mL bovine serum albumin (BSA, Sigma) overnight at 4 °C, as previously described (*35*). Samples containing ^{35}S-CMV with or without purified annexin II, AnV, or Ca^{2+} were added in 100 μL volumes in HBS, and incubated for 1 h at 37 °C. Wells were washed 3 times in the appropriate buffer; then bound ^{35}S-CMV was quantified.

R18-CMV Fusion Assay. Fusion of R18-CMV (10^8−10^9 vp/mL) to small unilamellar vesicles, containing 75% phosphatidylcholine and 25% phosphatidylserine (PCPS), was followed at 37 °C using an SLM 8000c spectrofluorometer with excitation at 560 ± 8 nm and emission at 586 ± 8 nm in a 1 cm path length quartz cuvette, as described (*30*, *36*). Fluorescence data were accumulated in the ratio mode. Rates of fusion were derived by linear regression of the initial linear phase of the kinetic profiles. To compare different preparations of R18-CMV, the data were normalized with the

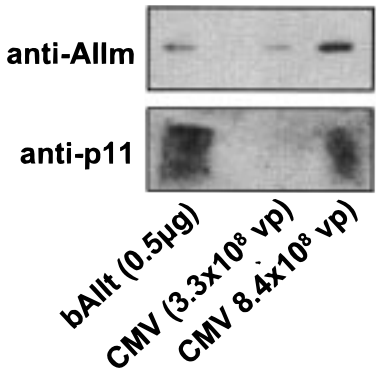

FIGURE 1: Demonstration of AIIm and AIIt associated with CMV. The indicated amounts of bAIIt and purified CMV were subjected to SDS−PAGE, electrotransferred to nitrocellulose, and probed separately with antibodies specific for AIIm or p11. Bands were detected by chemiluminescence (ECL, BioRad).

maximal rate of fluorescence change observed being 100%. The PCPS was prepared and quantified as previously reported (*37*).

RESULTS

Type of Annexin II on CMV. AIIm antigen has been previously reported as an endogenous constituent of purified CMV (*23*). To determine whether this is AIIm or AIIt, we conducted Western blot analyses, which are shown in Figure 1. The relative intensity of bands recognized by antibodies specific for human and bovine AIIm or p11 that is expected for 100% AIIt was determined by using highly purified bAIIt as a control. Both AIIm and p11 antigen were detected in association with CMV. However, in comparison to the bAIIt, the relative intensity of AIIm:P11 bands derived from CMV was significantly greater. This indicates that both AIIm and AIIt are associated with CMV and therefore both AIIm and AIIt are introduced into our experiments upon addition of CMV. For this reason and because it has not yet been reported which form(s) of annexin II may exist on the cell surface, a purified mixture of AIIm and AIIt (AIItm) was primarily investigated in our model systems to mimic the cellular pool.

Effect of Annexin II on CMV Binding to anPL-Containing Membranes. To determine whether annexin II can simultaneously interact with CMV and anPL-containing synthetic membranes, microtiter wells were coated with a source of anPL (i.e., PS) and incubated with ^{35}S-CMV in the presence or absence of annexin II. The inset to Figure 2 shows that AIItm enhanced ^{35}S-CMV binding to the PS-coated microtiter wells by at least 3-fold over the controls under these conditions. This effect was dependent on Ca^{2+} and phospholipid. Specificity was demonstrated by observing that the homologous protein AnV did not increase the binding of ^{35}S-CMV to anPL-coated microtiter wells compared to no added AIItm in the presence or absence of Ca^{2+} or anPL. When the amount of ^{35}S-CMV was held constant and the concentration of AIItm was varied, saturation binding was observed. These data are presented in Figure 2 and are corrected for the amount of ^{35}S-CMV binding in the absence of AIItm (<15% of the CPM observed at saturation). An apparent dissociation constant of 42 (±30%) nM was derived by fitting the data to a model that implicitly assumed a single class of binding sites. Thus, annexin II specifically mediates an interaction between CMV and anPL.

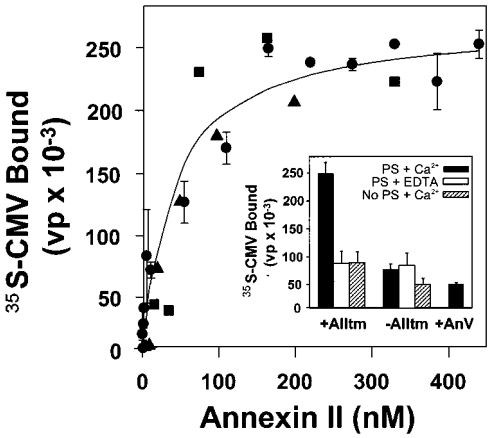

FIGURE 2: Annexin II-mediated enhancement, specificity, and Ca^{2+} dependence of ^{35}S-CMV binding to phospholipid. Constant concentrations of ^{35}S-CMV (7.3×10^{10} virions/mL) and Ca^{2+} (2 mM) were incubated in PS-coated or uncoated microtiter wells at various concentrations of AIItm (●, $n = 4 \pm$ SD), bAIIt (■, $n = 1$), or rAIIm (▲, $n = 1$), and the counts remaining bound after washing were quantified. The PS-dependent data for AIItm were iteratively fit to a binding model assuming a single class of sites after being corrected for nonspecific binding in the absence of annexin II. Inset: Microtiter wells were incubated at 37 °C with purified ^{35}S-CMV (850 CPM/10^6 virions) for 1 h with or without PS-coating, annexin II (1.3 μM), AnV (1.3 μM), or Ca^{2+} (2 mM). Each well was washed, and the remaining counts bound were quantified ($n = 4 \pm$ SD).

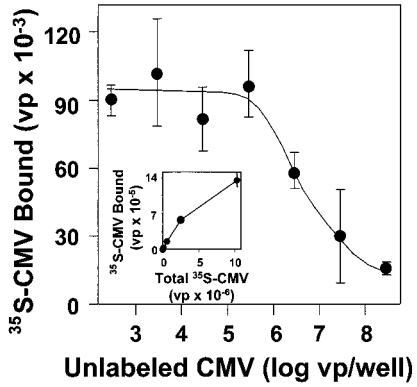

FIGURE 3: Inhibition of annexin II-mediated binding of ^{35}S-CMV to anPL by unlabeled CMV. ^{35}S-CMV (5.8×10^6 virions/well) binding to PS-coated wells in the presence of AIItm (0.5 μM), Ca^{2+} (2 mM), and the indicated amount of unlabeled virus. Inset: Constant concentrations of AIItm (1.3 μM) and Ca^{2+} (2 mM) were incubated in PS-coated microtiter wells and titrated with ^{35}S-CMV. All data were corrected for the CPM remaining bound in the absence of AIItm ($n = 4 \pm$ SD).

To determine whether AIIt or AIIm can participate in the CMV−anPL interaction, purified bAIIt and rAIIm were titrated. Their effect on ^{35}S-CMV binding to anPL, also shown in Figure 2, was indistinguishable from that of AIItm. This indicates that both AIIt and AIIm comparably bridge CMV to anPL.

The reversibility of the virus−AIItm−anPL interaction was established by showing that the anPL-bound ^{35}S-CMV was quantitatively displaced by excess unlabeled CMV (Figure 3). The inflection point of inhibition was observed to be at an amount of unlabeled CMV approximately equivalent to the amount of ^{35}S-CMV added (6×10^6 vp). To further establish the ability of AIItm to bridge CMV and anPL-containing membranes, an additional binding experiment was conducted where the AIItm was held in excess and radio-

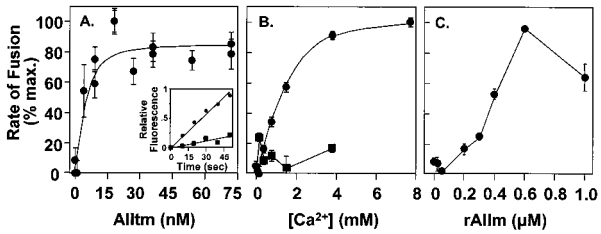

FIGURE 4: Effect of annexin II on R18-CMV fusion to anPL vesicles. Panel A: R18-CMV was added to various concentrations of AIItm and allowed to equilibrate to 37 °C. A small volume of PCPS was added to give a final concentration of 15 μM. Initial rates of fluorescence change were determined from kinetic data and relativized to the maximum rate observed. Panel A, inset: Raw data from which the rate of fluorescence signal change was derived in the presence of 18 nM (●) or no (■) AIItm. Panel B: Ca^{2+} was titrated in the presence (●) or absence (■) of AIItm (18 nM) at constant PCPS (15 μM). Panel C: As in panel A except that rAIIm was titrated (±coefficient of variation from linear regression analysis).

labeled virus was titrated (Figure 3, inset). The amount of ^{35}S-CMV bound in the absence of AIItm to PS-coated wells at each input level of ^{35}S-CMV was subtracted (<30% of the CPM observed at maximum concentration of virus) to follow AIItm-dependent binding of ^{35}S-CMV to phospholipid. The binding of ^{35}S-CMV was observed to be enhanced in the presence of AIItm and was dependent on the amount of radiolabeled virus added. Under these conditions, saturation was not achieved, which may be due to ^{35}S-CMV aggregation as a consequence of the excess AIItm concentration used.

Effect of Annexin II on the Fusion of CMV with anPL-Containing Vesicles. Having established that annexin II can facilitate virus−anPL interactions, we next investigated whether AIItm can contribute to the fusion of CMV with anPL-containing membranes. To do so, the fluorescent amphiphile R18 was incorporated into the CMV envelope at the minimal amount that induces significant autoquenching (>70%), and probe dilution due to fusion with unlabeled vesicles was followed by fluorescence dequenching (*30*, *38*−*40*). To avoid the possibility that the observed rates of fusion were dependent on the rate of binding, as previously described by Nir et al. (*41*), we used right-angle light scattering at 320 nm (data not shown) to establish concentrations of CMV and PCPS that ensured particle association was instantaneous compared to the rate of R18-CMV−vesicle fusion even at the lowest amount of AIItm (2 nM). Furthermore, to establish that the PCPS concentration was in excess and consequently did not limit the observed rates of fusion over the range of annexin II used, a PCPS titration was conducted (data not shown).

Figure 4, panel A, shows that AIItm enhances the observed rate of R18 probe dilution (i.e., dequenching) due to fusion with unlabeled PCPS vesicles. Under these conditions, an apparent plateau region in the titration curve was reached by 18 nM AIItm. To demonstrate the specific enhancing effect due to added AIItm, the data were corrected for the base line rate of CMV−anPL vesicle fusion. The inset to Figure 4 shows representative examples of the raw R18 fluorescence signal over time, which were collected in the presence of 18 nM or no AIItm. Linear regression of these data and derivation of slopes showed that added AIItm enhances the rate of fusion by approximately 5-fold.

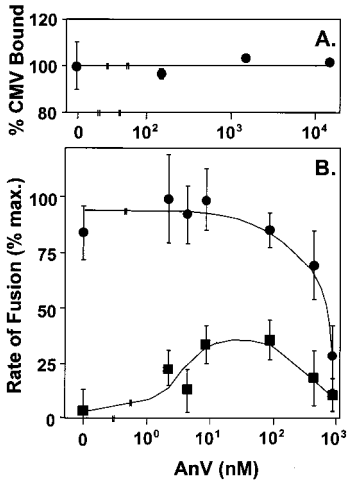

FIGURE 5: Effect of AnV on annexin II-mediated binding and fusion to anPL. Panel A: Binding of ^{35}S-CMV to anPL-coated wells was followed as described in Figure 2 with AIItm held constant (180 nM) and AnV varied. Panel B: Fusion experiments were conducted as described in Figure 4 in the presence (●) or absence (■) of constant AIItm (18 nM) with the indicated amounts of AnV.

The dependence of AIItm-mediated CMV−anPL vesicle fusion on Ca^{2+} is shown in Figure 4, panel B. The half-maximal rate of fusion was observed at approximately 1 mM Ca^{2+} in the presence of AIItm (18 nM). Conversely, the observed basal level of fusion in the absence of added AIItm was independent of the concentration of Ca^{2+}.

Since AIIm has been shown to be incapable of enhancing membrane fusion in several systems, we tested whether this is also true in our CMV model. The data shown in Figure 4, panel C, demonstrate that over the concentration range where AIItm is effective (panel A), rAIIm does not enhance R18-CMV−anPL fusion. The most likely explanation for this result is that the AIIt present in our AIItm mixture is responsible for the observed enhancement of fusion. While no significant differences were reported previously when established functions of native compared to recombinant AIIm (including AIIt formation) were evaluated (*12*), we cannot yet exclude the possibility that the relative lack of effect lies in the use of recombinant protein. We furthermore cannot eliminate a possible contribution arising from the 72 kDa species in our AIItm preparations, which we hypothesize is an AIIm dimer based on antigenicity. Interestingly, at very high concentrations of rAIIm, ranging from 400 to1000 nM, a significant enhancement of fusion was observed. This may be initiated by high avidity binding of CMV to anPL mediated by AIIm, followed by the participation of endogenous viral AIIt in the fusion process.

Effect of AnV on Annexin II-Dependent Binding and Fusion of CMV to anPL. Since AnV was not observed to enhance binding of CMV to anPL on microtiter wells, we hypothesized that competition for anPL binding sites by AnV would attenuate the effects of AIItm on CMV−anPL binding and fusion. To test this possibility, we followed ^{35}S-CMV binding to anPL-coated microtiter wells at a concentration of 180 nM AIItm, which we found saturated the available binding sites (see Figure 1). Under these conditions, the addition of up to a 100-fold molar excess of AnV reproducibly had no effect (Figure 5, panel A). Conversely, in R18-CMV−anPL fusion experiments (Figure 5, panel B), the effect of 18 nM AIItm, which we observed to give a 5-fold

enhancement of the rate of fusion (see Figure 4), was completely inhibited by a 50-fold excess of AnV. To control for possible effects of AnV alone on the fusion processes, AnV was titrated in the absence of added annexin II. Although purified AnV has been concluded to have no effect on anPL fusion processes (*42*), we observed a moderate, but significant, 2-fold increase in the rate of fusion (Figure 5, panel B). How this effect is being mediated by AnV is at present unclear, since we did not observe an enhancement in CMV−anPL binding. We are currently investigating the simultaneous contribution of other viral constituents.

DISCUSSION

Since 1993 it has been known that CMV first weakly interacts with the cell surface, which primes the cell and/or virus for a second higher affinity interaction (*18*). Previous work from our laboratories has strongly implicated the anPL-binding cellular protein annexin II as the high-affinity receptor. Notably, the purified virus was shown to selectively bind annexin II derived from the surface of solubilized host membranes (*6*), to have endogenous surface annexin II (*23*) currently shown to consist of AIIt and AIIm, and to have accessible anPL available for annexin II binding (*29*, *43*). We have further shown that a polyclonal AIIm antibody significantly decreased CMV infection (*23*). Despite considerable data implying a role for annexin II in CMV−cell interactions, there is no evidence to date that shows annexin II is able to simultaneously bind CMV and a biological membrane. Part of the goal of the current study, therefore, was to determine whether annexin II independently has this capacity. In a cellular experimental system a clear answer to this question is difficult to obtain due to the fact that CMV interacts with at least three integral cell surface constituents, including HSP (*45*), CD13 (*46*), and a 92.5 kDa phosphoprotein (*47*), and annexin II is present on the surface of cells (*4*−*7*). A model system was consequently used here in which the host cell surface was represented by synthetic phospholipid membranes containing anPL as a binding site for annexin II and a mixture of purified AIIt and AIIm (termed AIItm) represented the cellular pool. In this way, any effects could be attributed to added annexin II and not to other cellular constituents.

Using this model, we present the first direct evidence that annexin II can function as a bridge between ^{35}S-CMV and anPL-containing membranes. AIIm and AIIt equally mediate this interaction. The apparent K_d of 42 nM, describing the simultaneous binding of annexin II to anPL and virus, corresponds closely to previous measurements of the binary equilibrium for annexin II binding to CMV [K_d = 60 nM (*6*)] and human umbilical vein endothelial cells [K_d = 50 nM (*7*)]. Reports that CMV binds to cells with higher affinity [$K_d = 10^{-9}-10^{-11}$ M (*6*, *48*)] suggest that additional constituents and their effects of avidity are involved. The equilibrium followed here was dependent on Ca^{2+}, which is typical of the phospholipid-binding requirements of annexin II. Consistent with reports that the CMV surface has endogenous annexin II (*23*, *25*), we observed residual binding in the absence of added annexin II. As a demonstration that the CMV−membrane bridging function of annexin II is specific, AnV, which is 64% homologous in primary sequence to annexin II (*49*), did not enhance binding of the virus to the model membrane.

Having established that annexin II can bridge CMV to anPL membranes and fulfill the proposed virus receptor function, we next investigated the possibility that fusion of CMV to anPL-containing vesicles may also be facilitated by annexin II. To follow fusion, the well-documented (*30*, *38*−*40*) amphipathic fluorescent probe R18 was incorporated into the CMV envelope. We observed that AIItm (18 nM) enhanced the apparent rate of fusion by at least 5-fold under the conditions employed and in accord with the anPL binding of annexin II was dependent on Ca^{2+}. Equivalent concentrations of rAIIm did not facilitate fusion, indicating that the AIIt component of the AIItm was responsible for the observed effects in this experiment. These data provide the first evidence that annexin II can function after the CMV−membrane attachment step as a mediator of fusion between the virus and membrane, which is a process essential for host cell entry.

To understand the function and regulation of annexin II in membrane trafficking, adrenal medulla chromaffin granules have previously been well studied as a biological model. Work from several laboratories is elucidating a sequential regulatory mechanism involving phosphorylation of annexin II. The combined data suggest that chromaffin granule aggregation is mediated only by unphosphorylated annexin II (*13*). After aggregation, the annexin II must then be phosphorylated by a tyrosine kinase to facilitate membrane fusion, and only AIIt can participate in this process (*11*). The AIItm used in our studies had undetectable phosphotyrosine (not shown) when probed with a specific monoclonal antibody (Sigma). Therefore, our observation that AIItm can mediate both CMV binding and fusion to membranes without the experimental manipulation of the phosphorylation state suggests that annexin II−CMV interactions differ from annexin II−chromaffin granule interactions. It is compelling to speculate that the virus may have evolved to utilize annexin II without relying on the availability of a specific host kinase.

An important question that arises from the work presented here concerns the identity of the species present on the virus surface that could be involved in the annexin II-mediated interactions between CMV and model membranes. Our experimental system has eliminated the HSP component from the target membrane, and therefore the known involvement of viral glycoprotein C-II and gB (*45*) in attachment to HSP is not a factor here. However, our earlier studies that demonstrated the CMV envelope contains accessible anPL (*29*, *43*) are consistent with the idea that interactions of annexin II with anPL on the virus surface are involved in the CMV−membrane bridging and fusion we now report. It is unknown whether anPL-bound annexin II is required on the surface of the virus, on the surface of the target membrane, or both to participate in these functions. Experiments examining the sequence of addition of polyclonal antibody raised against annexin II that inhibited CMV infection of cells (*23*), and fluorescence resonance energy transfer between other annexins (*51*), suggest that annexin II molecules on apposing surfaces may interact. Nevertheless, the endogenous AIIt reported here to be associated with the CMV surface alone may play a role in the slow but significant rate of CMV−anPL vesicle fusion we observed in the absence of added AIItm. Reports that the endogenous annexin II binds to the virus in both Ca^{2+}-dependent (*23*)

and -independent (*25*) mechanisms suggest that anPL as well as other components on the virus surface may be involved in the interactions followed here. As a probable candidate for the latter, gB was reported previously to associate with annexin II on the CMV surface (*25*).

Fusion of CMV to the host cell membrane has been shown to involve at least two viral glycoproteins, gB (*44*) and gH (*30*, *50*). The cognate cellular ligand known for the latter, a 92.5 kDa protein (*47*), is not present in our experimental system, and consequently it is unlikely that gH contributes independently to the rates of fusion observed here. However, the previous identification of a gB−annexin II association (*25*) lead these authors to hypothesize that the complex may constitute a fusogenic unit. In this regard, a role for annexin II was suggested from earlier work involving gB-dependent cell−cell fusion, which was inhibited by an antibody to annexin II (*24*). The work presented here provides direct functional evidence that annexin II can participate in this process, which may involve annexin II interactions with both viral anPL and gB, or other as yet undocumented constituents on the CMV surface.

All members of the annexin/lipocortin family of proteins interact with anPL in the presence of Ca^{2+}. We therefore postulated that AnV, which on its own did not affect binding or fusion of CMV to anPL, would competitively inhibit the enhancing effects of annexin II on binding and fusion of CMV to anPL. Interestingly, AnV was found to attenuate the observed AIItm-mediated enhancement of CMV−anPL fusion with no effect on the role of AIItm in CMV−anPL binding. These data therefore suggest that annexin II affects the binding and fusion of CMV to anPL-containing membranes by discrete mechanisms. Since more than one type of annexin is invariably found within a given cell, the observations presented here furthermore suggest that interactions between different annexins may have a regulatory function.

There is evidence in the literature that in addition to CMV, influenza A and B (*52*) and hepatitis B (*53*) use annexin molecules during infection. Furthermore, a role for anionic phospholipid in hemorrhagic septicemia virus, sendai virus, vesicular stomatitis virus, rubella virus, sindbus virus, and vaccinia virus infection has been suggested (*54*−*59*). Cytomegalovirus, herpes simplex virus types 1 and 2, measles virus, murine hepatitis virus, and avian hemangioma retrovirus cause host cells to participate in the activation of blood coagulation (*57*, *60*, *61*), which usually involves the expression of anPL (*62*). Thus, the presence of accessible anPL on cells or viruses is a common theme that could indicate a relatively general role for anPL during infection.

ACKNOWLEDGMENT

We express our appreciation to M. Sutherland for quantification and evaluation of purified CMV by electron microscopy and to N. Brooks for conducting the Western blot analyses. We are also grateful to Dr. R. Lemieux for insightful discussion.

REFERENCES

1. Waisman, D. M. (1995) *Mol. Cell. Biochem. 149/150*, 301−322.
2. Siever, D. A., and Erickson, H. P. (1997) *Int. J. Biochem. Cell Biol. 29*, 1219−1223.
3. Thiel, C., Osborn, M., and Gerke, V. (1992) *J. Cell Sci. 103*, 733−742.
4. Chung, C. Y., and Erickson, H. P. (1994) *J. Cell Biol. 126*, 539−548.
5. Yeatman, T. J., Updyke, T. V., Kaetzel, M. A., Dedman, J. R., and Nicolson, G. L. (1993) *Clin. Exp. Metastasis 11*, 37−44.
6. Wright, J. F., Kurosky, A., and Wasi, S. (1994) *Biochem. Biophys. Res. Commun. 198*, 983−989.
7. Hajjar, K. A., Jacovina, A. T., and Chacko, J. (1994) *J. Biol. Chem. 269*, 21191−21197.
8. Kassam, G., Choi, K.-S., Ghuman, J., Kang, H.-M., Fitzpatrick, S. L., Zackson, T., Zackson, S., Toba, M., Shinomiya, A., and Waisman, D. (1998) *J. Biol. Chem. 273*, 4790−4799.
9. Drust, D. S., and Creutz, C. E. (1988) *Nature 331*, 88−91.
10. Ali, S. M., Geisow, M. J., and Burgoyne, R. D. (1989) *Nature 340*, 313−315.
11. Regnouf, F., Sagot, I., Delouche, B., Devilliers, G., Cartaud, J., Henry, J.-P., and Pradel, L.-A. (1995) *J. Biol. Chem. 270*, 27143−27150.
12. Kang, H.-M., Kassam, G., Jarvis, S. E., Fitzpatrick, S. L., and Waisman, D. M. (1997) *Biochemistry 36*, 2041−2050.
13. Hubaishy, I., Jones, P. G., Bjorhe, J., Bellagambe, C., Fitzpatrick, S., Fujita, D. J., and Waisman, D. M. (1995) *Biochemistry 34*, 14527−14534.
14. Mayorga, L. S., Beron, W., Sarrouf, M. N., Colombo, M. I., Creutz, C., and Stahl, P. D. (1994) *J. Biol. Chem. 269*, 30927−30934.
15. Raynal, P., and Pollard, H. B. (1994) *Biochim. Biophys. Acta 1197*, 63−93.
16. Gerke, V., and Moss, S. E. (1997) *Biochim. Biophys. Acta 1357*, 129−154.
17. Compton, T., Nepomuceno, R. R., and Nowlin, D. M. (1992) *Virology 191*, 387−395.
18. Compton, T., Nowlin, D. M., and Cooper, N. R. (1993) *Virology 193*, 834−841.
19. Keay, S., and Baldwin, B. (1992) *J. Virol. 66*, 4834−4838.
20. Keay, S., Merigan, T. C., and Rasmussen, L. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 10100−10103.
21. Nowlin, D. M., Cooper, N. R., and Compton, T. (1991) *J. Virol. 65*, 3114−3121.
22. Adlish, J. D., Lahijani, R. S., and St. Jeor, S. C. (1990) *Virology 176*, 337−345.
23. Wright, J. F., Kurosky, A., Pryzdial, E. L. G., and Wasi, S. (1995) *J. Virol. 69*, 4784−4791.
24. Bold, S., Ohlin, M., Garten, W., and Radsak, K. (1996) *J. Gen. Virol. 77*, 2297−2302.
25. Pietropaolo, R. L., and Compton, T. (1997) *J. Virol. 71*, 9803−9807.
26. Hajjar, K. A., Guevara, C. A., Lev, E., Dowling, K., and Chacko, J. (1996) *J. Biol. Chem. 271*, 21652−21659.
27. Ma, A. S. P., Bell, D. J., Mittal, A. A., and Harrison, H. (1994) *J. Cell. Sci. 107*, 1973−1984.
28. Evans, T. C., and Nelsestuen, G. L. (1994) *Biochemistry 33*, 13231−13238.
29. Pryzdial, E. L. G., and Wright, J. F. (1994) *Blood 84*, 3749−3757.
30. Keay, S., and Baldwin, B. (1991) *J. Virol. 65*, 5124−5128.
31. Huang, K. S., Wallner, B. P., Mattaliano, R. J., Tizard, R., Burne, C., Frey, A., Hession, C., McGray, P., Sinclair, L. K., Chow, E. P., Browning, J. L., Ramachandran, K. L., Tang, J., Smart, J. E., and Pepinsky, R. B. (1986) *Cell 46*, 191−199.
32. Comëra, C., Rothhut, B., and Russo-Marie, F. (1990) *Eur. J. Biochem. 188*, 139−146.
33. O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007−4021.
34. Glenney, J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4258−4262.
35. Pryzdial, E. L. G., Bajzar, L., and Nesheim, M. E. (1995) *J. Biol. Chem. 270*, 17871−17877.
36. Hoekstra, D., de Boer, T., and Wischut, J. (1984) *Biochemistry 23*, 5675−5681.
37. Pryzdial, E. L. G., and Kessler, G. E. (1996) *J. Biol. Chem. 271*, 16621−16626.

38. Doms, R. W., Blumenthal, R., and Moss, B. (1990) *J. Virol. 64*, 4884−4892.
39. Srinivasakumar, N., Ogra, P. L., and Flanagan, T. D. (1991) *J. Virol. 65*, 4063−4069.
40. Stegmann, T. (1993) *J. Biol. Chem. 268*, 26886−26892.
41. Nir, S., Stegmann, T., and Wilschut, J. (1986) *Biochemistry 25*, 257−266.
42. Ernst, J. D., Hoye, E., Blackwood, R. A., and Mok, T. L. (1991) *J. Biol. Chem. 266*, 6670−6673.
43. Sutherland, M. R., Raynor, C. M., Leenknegt, H., Wright, J. F., and Pryzdial, E. L. G. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 13510−13514.
44. Navarro, D., Paz, P., Tugizov, S., Topp, K., Lavail, J., and Pereira, L. (1993) *Virology 197*, 143−158.
45. Kari, B., and Gehrz, R. (1993) *J. Gen. Virol. 74*, 255−264.
46. Soderberg, C., Giugni, T. D., Zaia, J. A., Larsson, S., Wahlberg, J. M., and Moller, E. (1993) *J. Virol. 67*, 6576−6585.
47. Baldwin, B. R., Kleinberg, M., and Keay, S. (1996) *Biochem. Biophys. Res. Commun. 219*, 668−673.
48. Taylor, H. P., and Cooper, N. R. (1989) *J. Virol. 63*, 3991−3998.
49. Burger, A., Berendes, R., Liemann, S., Benz, J., Hofmann, A., Gottig, P., Huber, R., Gerke, V., Thiel, C., Romisch, J., and Weber, K. (1996) *J. Mol. Biol. 257*, 839−847.
50. Keay, S., and Baldwin, B. (1992) *J. Virol. 66*, 4834−4838.
51. Zaks, W. J., and Creutz, C. E. (1991) *Biochemistry 30*, 9607−9615.
52. Otto, M., Gunther, A., Fan, H., Rick, O., and Huang, R. T. C. (1994) *FEBS Lett. 356*, 125−129.
53. Neurath, A. R., and Strick, N. (1994) *Virology 204*, 475−477.
54. Luan, P., Yang, L., and Glaser, M. (1995) *Biochemistry 34*, 9874−9883.
55. Chejanovsky, N., Zakai, N., Amselem, S., Barenholz, Y., and Loyter, A. (1986) *Biochemistry 25*, 4810−4817.
56. Estepa, A., and Coll, J. M. (1996) *Virology 216*, 60−70.
57. Mastromarino, P., Cioe, L., Rieti, S., and Orsi, N. (1990) *Med. Microbiol. Immunol. 179*, 105−114.
58. Kuge, O., Akamatsu, Y., and Nishijima, M. (1989) *Biochim. Biophys. Acta 986*, 61−69.
59. Oie, M. (1985) *Virology 142*, 299−306.
60. Fung, L. S., Neil, G., Leibowitz, J., Cole, E. H., Chung, S., Crow, A., and Levy, G. A. (1991) *J. Biol. Chem. 266*, 1789−1795.
61. Resnick-Roguel, N., Eldor, A., Burstein, H., Hy-Am, E., Vlodavsky, I., Panet, A., Blajchman, M. A., and Kotler, M. (1990) *J. Virol. 64*, 4029−4032.
62. Mann, K. G., Nesheim, M. E., Church, W. R., Haley, P., and Krishnaswamy, S. (1990) *Blood 76*, 1−16.

BI982095B