Direct Interaction between Human Cytomegalovirus Glycoprotein B and Cellular Annexin II

ROBIN L. PIETROPAOLO AND TERESA COMPTON*

Department of Medical Microbiology and Immunology, University of Wisconsin—Madison Medical School, Madison, Wisconsin 53706-1532

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The betaherpesvirus human cytomegalovirus (HCMV) is a pathogen that is responsible for serious disease and death among immunocompromised populations and is the leading cause of infectious birth defects in the United States (2). As with most other herpesviruses, the mechanism whereby HCMV gains access to the interior of host cells to begin its replicative cycle has not been completely elucidated. It is apparent that HCMV entry into host cells is a complex process that likely involves sequential viral-glycoprotein–host cell receptor interactions (9). Given the fact that there is no HCMV vaccine and limited antiviral therapies, information learned about the early events in HCMV infection will lend itself toward developing effective prophylactic treatments.

HCMV binding to cell surface heparan sulfate proteoglycans (HSPGs) is the initial interaction between virus and host cell (11, 20), yet HSPGs alone are insufficient to render a cell permissive to infection (10). This initial heparin-dissociable virus binding is rapidly converted to a more stable binding state (11), possibly through engagement of a proteinaceous receptor. A number of cellular molecules have been proposed to serve as receptors for HCMV that would function downstream of the initial HSPG-virus interaction. These proteins include CD13 (23), a 92.5-kDa phosphoprotein (17, 18), and a 34-kDa protein (1, 21, 25). Recently, a 34-kDa protein from human endothelial cells, identified as annexin II, was found to specifically bind HCMV virions, suggesting a calcium-independent interaction between annexin II and an HCMV envelope component. The search for a nonphospholipid component to account for this binding led to the discovery that HCMV glycoprotein B (gpUL55) (gB) can physically interact with annexin II. We present three lines of evidence to support the conclusion that HCMV gB can bind host cell annexin II.

The binding of annexin II to the HCMV envelope occurs partially through the calcium-dependent phospholipid-binding ability of annexin II since some annexin II was dissociated from virions with chelating agents. However, a substantial proportion of virion-associated annexin II was resistant to chelation, which suggested a calcium-independent interaction between annexin II and an HCMV envelope component. The search for a nonphospholipid component to account for this binding led to the discovery that HCMV glycoprotein B (gpUL55) (gB) can physically interact with annexin II. We present three lines of evidence to support the conclusion that HCMV gB can bind host cell annexin II.

The binding of annexin II to the HCMV envelope was dependent on calcium and occurred as a result of the calcium-dependent phospholipid-binding ability characteristic of the entire family of annexin proteins, including annexin II. We confirm in this report that not only is annexin II present in HCMV virions but a majority of the virus-associated annexin II molecules are insensitive to calcium chelation. This result is not expected if annexin II is binding only viral-envelope phospholipids. The presence of EDTA- and EGTA-resistant annexin II in HCMV envelopes indicates that annexin II may be interacting with another, nonphospholipid viral component. We wished to determine if annexin II had a ligand on HCMV in addition to envelope phospholipids.

Glycoprotein B (gpUL55) (gB) of HCMV is the major constituent of the viral envelope. Monoclonal antibody studies have shown that HCMV gB plays important roles in HCMV entry, cell-cell spread of internal virions, and fusion of infected cells (19, 29, 30). In addition, gB is one envelope protein capable of heparin binding (11). Given the abundance of gB in the viral envelope and its multifunctional role in HCMV infection, we tested the ability of gB to interact with annexin II. Our results indicate that gB does form a physical association with host cell annexin II and that this interaction is not dependent on the presence of calcium.

HCMV strain AD169 was propagated, titers were determined in immortalized fibroblasts (8), and virions were purified as previously described (21). Polyclonal anti-annexin II serum was kindly provided by K. Hajjar (Cornell University Medical College) or produced in New Zealand White rabbits immunized with purified, recombinant human annexin II. To test the calcium dependence of HCMV-associated annexin II, AD169 virions were subjected to 20 mM EDTA–20 mM EGTA treatment for 1 h at 4°C. Virions were subsequently pelleted through a 20% sorbitol cushion in a Sorvall SS35C centrifuge using a SS-34 rotor at 40,000 × g for 30 min. Virion pellets were resuspended in a volume of 20% sorbitol equivalent to the supernatant volume, and fractions of each were analyzed via sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel electrophoresis (PAGE), electrotransferred to nitrocellulose, and immunoblotted with polyclonal anti-annexin II serum. Phosphorimaging (Fig. 1A) and densitometry (Fig. 1B) of immunoblots were performed with a Bio-Rad model GS-525

* Corresponding author. Mailing address: Department of Medical Microbiology and Immunology, 1300 University Ave., MS 497, University of Wisconsin—Madison Medical School, Madison, WI 53706-1532. Phone: (608) 262-1474. Fax: (608) 262-8418. E-mail: tcompton@facstaff.wisc.edu.
Molecular Imager and Bio-Rad Molecular Analyst software as per manufacturer’s instructions. In control-treated virions, 11% of the annexin II was found in the supernatant fraction (Fig. 1B, lane 2) while 89% of the annexin II remained associated with virions (Fig. 1B, lane 1). The 11% of annexin II found in the supernatant fraction of control-treated virions likely represents spontaneous dissociation or virion degradation. In virions treated with the chelators EDTA and EGTA, 42% of HCMV-associated annexin II was dissociated from the envelope of HCMV into the supernatant fraction (Fig. 1B, lane 4) when the value (53%) was adjusted for spontaneous dissociation. Virions treated with high concentrations of EDTA and EGTA retained 47% of the virion-associated annexin II (Fig. 1B, lane 3). Either this population of chelator-resistant annexin II is binding to HCMV via a calcium-independent interaction or it is not available to the action of the chelating agents. The latter scenario is unlikely since immuno-cytochemical analysis revealed that almost all of the annexin II present on HCMV was localized to the virion envelope, not to an internal structure (32). Additionally, annexin II has been reported to be resistant to EDTA dissociation from other biological membranes, although the nature of the EDTA-resistant interaction between annexin II and membranes has not been characterized (14, 28). Furthermore, the original virus overlay experiments identifying annexin II (then known as a 30- to 34-kDa protein) as an HCMV-binding protein were performed with buffers containing EDTA (1, 21). All of these factors suggest that annexin II may be binding to HCMV through a calcium-independent, and potentially phospholipid-independent, interaction.

To test the idea that HCMV gB may be the viral ligand for annexin II, membrane proteins from annexin II-abundant, permissive human fibroblasts (IF) (8) and annexin II-poor, non-permissive human epithelial 293 cells were subjected to SDS-PAGE followed by ligand blotting. Membranes were prepared from IF and 293 cells by a hypotonic lysis protocol, and protein content was quantitated by the Bio-Rad Protein Assay. Equal amounts (75 μg) of membrane protein from IF and 293 cells were analyzed in the experiments. As previously reported (15), annexin II was found on IF and 293 cells, although IF membranes contained 11% of annexin II compared to 89% in 293 cells (Fig. 1B, lanes 1 and 2). Strikingly, the recognition pattern of tissue plasminogen activator (t-PA) (provided by B. Schwartz, University of Wisconsin—Madison), a known physiological ligand for annexin II (15), was nearly identical to that of gB-S (Fig. 2, lanes 1, 2, 5, and 6). Both gB-S and t-PA preferentially recognized the more slowly migrating component of the characteristic annexin II doublet (Fig. 2, lanes 1, 3, and 5). Whether this doublet pattern represents full-length and proteolytic forms of annexin II or phosphorylated versus non-phosphorylated forms remains to be determined. No proteins of the molecular size of annexin II were detected with anti-t-PA antibodies (provided by B. Schwartz), normal rabbit serum, or a recombinant soluble form of another viral glycoprotein, hemagglutinin (HA) from influenza virus (provided by V. Hinshaw, University of Wisconsin—Madison). The use of HA is a particularly germane control since this viral glycoprotein was also produced in a baculovirus expression system, indicating that production in insect cells was not mediating the effect of binding. Also of note is the difference in signal strength resulting from the use of membranes from two cell lines expressing different levels of annexin II. Since the levels of annexin II in 293 cells are much reduced compared to the IF cell line, the fact that in both gB-S- and t-PA-probed lanes a signal is detectable only in the IF lanes further indicates that the protein species being bound is annexin II.

Proteins associated with one another are generally able to be communoprecipitated provided appropriate lysis conditions are employed. We next conducted immune-precipitation–immunoblotting experiments to further examine interaction be-
tween gB and annexin II. At 72 to 96 h postinfection, IF cultures infected with HCMV strain AD169 at a multiplicity of infection of 1 were harvested and subjected to immune precipitation analysis employing either anti-gB or anti-annexin II antibodies. For these experiments, cells were lysed in RIPA buffer (1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 50 mM Tris [pH 7.5]) plus protease inhibitors (2 μg each of antipain, aprotinin, chymostatin, leupeptin, and pepstatin A per ml) for 30 min on ice and residual insoluble material was removed by centrifugation. Clarified cell lysates were adjusted to a volume of 1 ml with TNE (0.15 M NaCl, 5 mM EDTA, 50 mM Tris [pH 7.4]) and precleared for 1 h with a formalin-killed staphylococcal/protein G suspension (Sigma). For precipitations, either an antibody cocktail consisting of monoclonal anti-gB antibodies 27-78, 7-17, and 9-3 (4, 24) or affinity-purified anti-annexin II antibodies were added and the mixtures were incubated at 4°C. Immobilized protein A (Pierce) was added and allowed to collect immune complexes for 1 h at room temperature. Protein A pellets were washed at least five times with TNE plus 0.1% Triton X-100 and once with Tris-buffered saline (20 mM Tris [pH 7.4], 150 mM NaCl). Pellets were resuspended in SDS-PAGE sample buffer lacking β-mercaptoethanol, boiled for 3 min, resolved through SDS–10% PAGE, and electrotransferred onto nitrocellulose membranes. The composition of the immunoprecipitates was confirmed by immunoblotting with anti-annexin II antibodies as described above for HCMV-infected cells. Immune complexes were resolved by SDS–10% PAGE and the gel was dried and phosphorimaged (Fig. 3C). Similarly to the experiments employing HCMV-infected cells, immunoprecipitation of HCMV virions with anti-annexin II antibodies resulted in the coimmunoprecipitation of a high-molecular-weight complex (Fig. 3C, annex II/gB) that was resistant to treatment with 2% SDS sample buffer and boiling. These results demonstrate that gB and annexin II are interacting within the viral envelope. To further demonstrate an interaction between annexin II and HCMV gB, recombinant gB-S was utilized in affinity chromatography experiments. Recombinant gB-S lacks both transmembrane and cytoplasmic sequences to facilitate secretion and was engineered to contain a carboxy-terminal histidine tag (His6) for purification via metal-chelate chromatography (7). A

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**FIG. 3.** Coimmunoprecipitation of HCMV-infected immortalized fibroblast lysates. Infected (+) or mock-infected (−) lysates were immunoprecipitated (IP) with a mixture of anti-gB antibodies, affinity-purified anti-annexin II (annII) antibodies, or bovine immunoglobulin G (IgG). Immunoprecipitates were subjected to SDS–10% PAGE under nonreducing conditions, electrotransferred to nitrocellulose, and immunoblotted. (A) Immunoprecipitates were blotted with anti-annexin II, followed by protein A-conjugated horseradish peroxidase (HRP). This blot was stripped in 100 mM β-mercaptoethanol–2% SDS–62.5 mM Tris (pH 6.7) for 30 min at 50°C and then immunoblotted with 27-78 (anti-gB) followed by protein A-conjugated HRP (B). Monomeric annexin II and the combined annexin II-gB signal (ann II/gB) are indicated. (C)35S-labeled HCMV virions were subjected to immunoprecipitations with anti-annexin II and anti-gB antibodies and resolved through SDS–10% PAGE under nonreducing conditions. The phosphorimage of the dried gel is shown.
cellular lysate prepared from IF cells in Tris-buffered saline containing 1% n-octylglucoside (Boehringer Mannheim) was diluted to 0.1% n-octylglucoside and applied to gB-S immobilized on an agarose-nitrilotriacetic acid (NTA)-Ni²⁺ matrix. The affinity matrix was stringently washed with 40 mM imidazole-NaPO₄–300 mM NaCl–10% glycerol, pH 6.0. As shown in Fig. 4A and B, annexin II was retained on the gB-S column and was eluted under conditions that removed gB-S. In contrast, annexin II was not retained on a column lacking gB-S (Fig. 4C). These results, again, strongly imply that gB and annexin II are capable of forming an association, given that annexin II was bound by immobilized gB-S in the presence of a complete complement of detergent-solubilized cellular proteins.

This is the first report of a cellular binding protein (annexin II) for HCMV gB, a multifunctional glycoprotein that is the immunodominant and most abundant constituent of the HCMV envelope. As was previously suggested, it is likely that HCMV acquires annexin II concomitantly with the cellular lipid bilayer during virion envelopment (32). This proposal is supported by the fact that annexin II is found abundantly on endosomal membranes (14, 16), which are a proposed site for final HCMV envelopment (27).

Since annexin II is ascribed membrane trafficking and fusion functions (3, 5, 6, 12, 13, 31), and given the biological roles assigned to HCMV gB, it is attractive to hypothesize that the annexin II-gB complex may be involved in one of the many membrane fusion events required during HCMV infection and dissemination such as fusion at cell surface, egress of virions, envelopment, cell-cell spread, and release of particles from host cells. Since annexin II is also an intracellular protein, found in the cytoplasm (34), in association with the cytoskeleton (22, 26), and on endosomal membranes (14, 16), it is possible that gB and annexin II are associating intracellularly.

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Studies to address the kinetics, compartmentalization, and function of the interaction between cellular annexin II and HCMV gB are under way.

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FIG. 4. Immobilized gB-S binds annexin II from a cell lysate. Secretory gB (gB-S) was immobilized by virtue of a carboxy-terminal His₆ tag (7) on Ni²⁺-NTA affinity resin and was exposed to IF cell lysates. Recombinant gB-S and any associated cellular proteins were eluted from the matrix, and fractions were resolved in parallel by SDS–12.5% PAGE under reducing conditions. (A) Column fractions were immunoblotted with anti-gB antibody 27-78. (B) Duplicate column fractions were immunoblotted with annexin-antibody (anti-annexin II) serum. (C) Fractions from a control experiment in which a cell lysate was applied to Ni²⁺-NTA resin that lacked gB-S were immunoblotted with anti-annexin II serum gB-S collected from baculovirus-infected insect cell medium (gB-preload) was applied to a Ni²⁺ column. IF cell lysates were prepared in 1% n-octylglucoside (imid), and then proteins were eluted with 500 mM imidazole (eluates 1 to 4).


