JOURNAL OF VIROLOGY, Aug. 1995, p. 4784–4791 Vol. 69, No. 8
0022-538X/95/$04.00+0
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Host Cellular Annexin II Is Associated with Cytomegalovirus Particles Isolated from Cultured Human Fibroblasts

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Received 12 January 1995/Accepted 10 May 1995

A significant amount of host cellular annexin II was found to be associated with human cytomegalovirus isolated from cultured human fibroblasts (~1,160 molecules per virion). This composition was established by four different analytical approaches that included (i) Western blot (immunoblot) analysis of gradient-purified virions with a monoclonal antibody specific for annexin II, (ii) peptide mapping and sequence analysis of virus-associated proteins and proteins dissociated from virus following EDTA treatment, (iii) electron microscopic immunocytochemistry of gradient-purified virions, and (iv) labeling of virus-associated proteins by lactoperoxidase-catalyzed radioiodination. These results indicated that annexin II was primarily localized to the viral surface, where it bound in a divalent cation-dependent manner. In functional experiments, a rabbit antiserum raised against annexin II inhibited cytomegalovirus plaque formation in human foreskin fibroblast monolayers in a concentration-dependent manner. Cumulatively, these studies demonstrate an association of host annexin II with cytomegalovirus particles and provide evidence for the involvement of this cellular protein in virus infectivity.

Human cytomegalovirus (HCMV) is a clinically significant pathogen in immunocompromised individuals. Both HCMV surface glycoproteins and specific cellular receptor molecules have been shown to contribute to virus binding and fusion to susceptible cells. For HCMV (10, 25) and other herpesviruses (22, 35, 40), initial cellular attachment was shown to be mediated by cell surface heparan sulfate proteoglycans. The HCMV surface glycoprotein complex C-II (gC-II) and the product of HCMV open reading frame UL55 (gB) were demonstrated to be heparin-binding components of the viral envelope (10, 25). Human aminopeptidase N (CD13) was shown to participate in virus binding to blood cells (43). Other studies have identified a 28- to 34-kDa protein that is widely expressed in various cell types as an HCMV receptor (1, 39, 47). Following binding, HCMV can fuse directly with the plasma membrane in a pH-independent manner (9). Two HCMV surface glycoproteins have been implicated in virus-cell fusion. One of these is the glycoprotein encoded by HCMV open reading frame UL75 (gH), which was shown to bind a 92.5-kDa phosphorylated glycoprotein on human fibroblasts (27, 28); anti-idiotypic antibodies mimicking gH could block fusion but not attachment of virions to cells (26). HCMV gB was reported to promote virion penetration into cells, transmission of infection from cell to cell, and fusion of infected cells (37). Despite these advances, the mechanism whereby HCMV binds to and fuses with the cell membrane remains incompletely understood.

A recent study demonstrated binding of HCMV to human umbilical vein endothelial cells and identified a cellular 36-kDa virus-binding protein as annexin II (52). On the basis of apparent molecular weight, proteolytic fragmentation pattern, and partial sequence data, annexin II likely corresponds to the previously described 28- to 34-kDa cellular receptor for HCMV (8a). Annexin II is a member of the annexin-lipocortin family of proteins (12) for which various functions related to calcium-dependent interaction with phospholipid membranes have been proposed. In particular, annexin II has been implicated in the bridging and fusion of biological membranes (2, 11, 13, 14). Although annexin II has been primarily described as an intracellular protein, cell surface adhesion (50, 52, 54) and receptor (7, 8, 21, 29) activities have recently been reported. Another member of the annexin family, annexin V, has been implicated as a receptor for hepatitis B virus on human liver plasma membranes (23, 38).

Host cellular proteins have been shown to associate with enveloped viruses and may contribute to virus infectivity (4, 16, 20, 48). Cell-derived class I and II major histocompatibility complex antigens were reported to be present on the surface of human immunodeficiency viruses (HIV), and antibodies to these cellular proteins were shown to inhibit HIV infectivity (4). Other studies reported that cellular cyclophilin A is incorporated into HIV type 1 (HIV-1) virions (16) and plays a functional role in infectivity (48). Here, we demonstrate that cellular annexin II is endogenously associated with the envelope of HCMV virions isolated from infected human foreskin fibroblasts and provide evidence that this virus-associated cellular protein plays a role in HCMV infectivity. A preliminary report of these findings was presented earlier (51).

MATERIALS AND METHODS

Chemicals and reagents. *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), L-glutamine, and gentamicin were purchased from Gibco BRL Canada Ltd. (Burlington, Canada). Sodium potassium tartrate, phosphotungstic acid, bovine serum albumin (BSA), cold water fish gelatin, lactoperoxidase, EDTA, polyoxyethlyene-sorbitan monolaurate (Tween 20), and other common reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.). Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and

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4-chloro-1-naphthol were purchased from Bio-Rad Laboratories Ltd. (Mississauga, Canada). Guinea pig complement was purchased from Cedarlane Laboratories (Hornby, Canada).

Cell culture. Human foreskin fibroblasts, used as target cells for infection by HCMV AD169, were grown to confluent monolayers and maintained in basal Eagle medium supplemented with 2% bovine calf serum–2 mM L-glutamine–20 μg of gentamicin per ml. Cells at passages 5 to 15 were used.

Virus purification. HCMV AD169 was purified from infected fibroblast cultures with potassium tartrate-glycerol gradients as previously described (46) with modifications. Briefly, confluent fibroblasts were infected with HCMV at a multiplicity of infection of 0.001. At 10 to 14 days postinfection, when the full cytopathic effect was apparent, the media were harvested and cleared of cellular debris (1,000 × *g*, 10 min) and the virus particles were pelleted (30,000 × *g*, 60 min). The pellet was resuspended in 50 mM Tris–100 mM NaCl, pH 7.2, and layered onto a 15 to 35% potassium sodium tartrate gradient containing a reverse glycerol gradient (20 to 0%) made up in the same buffer. Gradients were centrifuged at 120,000 × *g* for 20 min in a swinging-bucket rotor (SW28, Beckman), and bands corresponding to viral dense bodies and infectious virions were recovered by aspiration of the visible bands (46) or by collecting fractions from the bottom of the gradient tube. With the latter procedure, determination of optical density at 550 nm (OD_{550}) was used to identify fractions containing virus particles. As a control, confluent monolayers of noninfected fibroblasts harvested with a rubber scraper were resuspended in HEPES-buffered saline (HBS; 20 mM HEPES, 150 mM sodium chloride, 1 mM calcium chloride, 1 mM magnesium chloride [pH 7.2]) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, and 1 μM leupeptin), disrupted by sonication (30 s; Fisher Scientific model FS-9), and similarly fractionated on potassium tartrate-glycerol gradients. Virus particles were resuspended in HBS, and were counted by electron microscopy with polystyrene latex spheres (365-nm diameter; Colab Laboratories, Chicago, Ill.) of known concentration as an internal standard. Briefly, mixtures of virus particles and latex spheres were adsorbed to carbon-Formvar-coated copper grids (400 mesh; Cedarlane Laboratories), negatively stained with 2% phosphotungstic acid (pH 6.5) containing 0.05% BSA, and counted directly with a Philips model EM300 transmission electron microscope. As determined by electron microscopic examination, the HCMV virion preparations were essentially free of visible cellular debris (see Fig. 3); in a representative experiment, the upper band was composed of ~90% virions and ~10% dense bodies and the lower band was composed of ~80% dense bodies and ~20% virions.

Western immunoblotting. Aspirated bands or fractions collected from potassium tartrate-glycerol gradients were washed into HBS, and aliquots were solubilized in Laemmli sample buffer and subjected to SDS-PAGE (30). The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P; Millipore, Bedford, Mass.) (49). PVDF strips were blocked with Tris-buffered saline (20 mM Tris–150 mM sodium chloride [pH 8.0]) containing 1% BSA (30 min, 20°C) and subsequently were incubated with 0.1 μg of anti-annexin II monoclonal antibody (p36-calpactin I; Oncogene Science, Inc., Uniondale, N.Y.) per ml in Tris-buffered saline containing 0.05% Tween 20. Incubation with normal mouse serum (1/50 dilution) was performed as a control for Fc-dependent interactions. Following the washing, the PVDF strips were incubated with alkaline phosphatase-conjugated goat $F(ab')_2$ anti-mouse immunoglobulin G (IgG; Serotec Canada Ltd., Toronto, Canada) (0.2 μg/ml) and bound peroxidase was detected by addition of a chromogenic substrate (5-bromo-4-chloro-3-indolyl phosphate-Nitro Blue Tetrazolium) (Sigma Chemical Co.) according to the manufacturer's instructions. In order to estimate the stoichiometry of virus-associated annexin II, various amounts of purified HCMV (10^{10}, 5×10^9, and 2.5×10^9 virus particles) and annexin II purified from human placenta (2, 1, 0.5, 0.25, and 0.125 μg) as previously described (41) were subjected to Western blotting (immunoblotting), and densitometric scanning of the bands obtained was performed. A standard curve plotting integrated band density against annexin II concentration was generated and used to estimate the amount of annexin II associated per virion in a sample containing a known number of virions. In order to determine if annexin II was present at significant levels in bovine serum, synthetic phospholipid vesicles composed of 80% phosphatidylcholine (PC)–20% phosphatidylserine (PS) or 100% PC were prepared as previously described (15). One milligram of the PC-PS or PC vesicle preparation was added to 1.5 ml of bovine calf serum alone or to serum supplemented with 1.5 μg of purified human annexin. Following incubation with gentle agitation (for 12 h at room temperature), the vesicles were pelleted and subjected to SDS-PAGE and Western blot analysis with the monoclonal annexin II antibody.

CNBr hydrolysis. SDS-PAGE-fractionated proteins were hydrolyzed with cyanogen bromide (CNBr) as described previously (53), and the resulting peptides were resolved by SDS-PAGE (20% acrylamide). Following transfer to PVDF membranes, peptides were visualized by Coomassie blue staining, excised, and subjected to amino-terminal microsequencing (33).

Immunocytochemistry. Immunogold labeling was used to determine the subviral localization of annexin II in gradient-purified HCMV virions and dense bodies. Virions were adsorbed to carbon-Formvar grids in HBS. Antibody incubations were carried out with HBS containing 0.1% fish gelatin. Immobilized virions were incubated with either the annexin II-specific monoclonal antibody (final concentration, 20 μg/ml) or a 1/50 dilution of annexin II rabbit antiserum (a gift from B. Pepinsky) for 30 min, washed five times in HBS-gelatin, and then incubated with 10 nm of gold-conjugated secondary antibody (goat anti-mouse IgG or goat anti-rabbit IgG [Zymed Laboratories]) for 30 min. Following the washing, virus particles were negatively stained and examined by electron microscopy. As a positive control, an HCMV-neutralizing, gB-specific monoclonal antibody, CMVB1 (45) (a gift from B. Brodeur), was used as the primary antibody. For negative controls, the annexin II antibodies were replaced with nonimmune rabbit or mouse serum.

Radiolabeling of HCMV. To surface label HCMV, lactoperoxidase was used as described previously (32) with modifications. Briefly, to approximately 10^9 virus particles in 100 μl of HBS we added 1 μg of lactoperoxidase, 100 μCi of $Na^{125}I$, and H_2O_2 (final concentration, 2×10^{-5} M) and placed the mixture on ice for 10 min. Labeled virus particles were pelleted (10,000 × *g*, 10 min) and washed three times with HBS. To dissociate divalent cation-dependent peripheral envelope proteins, surface-radiolabeled virions were incubated in HBS containing 5 mM EDTA. After pelleting of the virions, a 50-μl sample of the supernatant was solubilized in Laemmli sample buffer before analysis by SDS-PAGE and autoradiography.

Plaque assays. Viral plaque assays were performed as described previously (42). Briefly, human foreskin fibroblasts were grown to confluency in 24-well plates. To each well was added 150 μl of a 1/5,000 dilution of a stock HCMV-containing medium derived from infected fibroblasts; this dilution was determined to yield an average of 64 viral plaques in the assay. Following infection (1 h, 37°C), the inoculum was removed and replaced with 1 ml of prewarmed culture medium containing 0.5% agar and the plates were allowed to cool. The infected monolayers were incubated in a CO_2 incubator for 10 days at 37°C, and plaques were stained with neutral red dye and counted. Annexin II-specific antibodies were assessed for their ability to inhibit plaque formation by preincubating the viruses with various dilutions (1/20, 1/200, and 1/2,000) of the anti-annexin II rabbit antiserum or various concentrations (0.1, 1, and 10 μg/ml) of the annexin II-specific monoclonal antibody (1 h, 37°C). Parallel experiments were performed without the preincubation step. Both antibodies were tested in the presence and absence of 10% guinea pig complement. CMVB1 was used as a positive control, and negative controls consisted of HCMV preincubated with equivalent dilutions of normal rabbit serum with or without added guinea pig complement.

RESULTS

Immunoblotting of HCMV with annexin II-specific monoclonal antibody. As shown in Fig. 1A, Western blot analysis of gradient-purified virions revealed a protein of ~36 kDa (lanes 1 to 3) which comigrated with purified annexin II (lanes 4 to 8). Additional bands of ~32 to 34 kDa that likely correspond to proteolytic degradation products of annexin II were also observed (52). Since receptors for the Fc region of human IgG have been reported to be expressed within the tegument of HCMV (44), normal mouse IgG was used as the primary antibody control to rule out binding of anti-annexin II antibodies to virus components via an Fc-mediated interaction. In these control experiments, the 32- to 36-kDa bands were not observed (data not shown). Virions subjected to a second gradient ultracentrifugation step were similarly reactive with the annexin II monoclonal antibody (data not shown). On the basis of a standard curve generated by densitometric scanning of the blot shown in Fig. 1A, 0.74 μg of annexin II (M_r, 38,472) was associated with 10^{10} gradient-purified virions, corresponding to 1,160 molecules per virion (Fig. 1B). In order to investigate the possibility that annexin II was associated with cellular debris distributed throughout the gradient or overlapping with the HCMV virion and dense body bands during ultracentrifugation, densitometric scanning of the annexin II antibody-reactive 36-kDa Western blot band in each gradient fraction was performed. The results are shown in Fig. 1C, indicating that two major peaks of annexin II corresponded exactly with the HCMV virion and dense body bands identified by OD_{550} and confirmed by electron microscopic examination. Annexin II antibody-reactive material was also detected in the low-density fraction that remained at the top of the gradient. When cellular lysates obtained by sonication of noninfected fibroblasts were similarly analyzed, significant amounts of annexin II were detected only at the top of the gradient.

Partial amino acid sequencing of the HCMV-associated protein. As a consequence of the Western blot results, the possi-

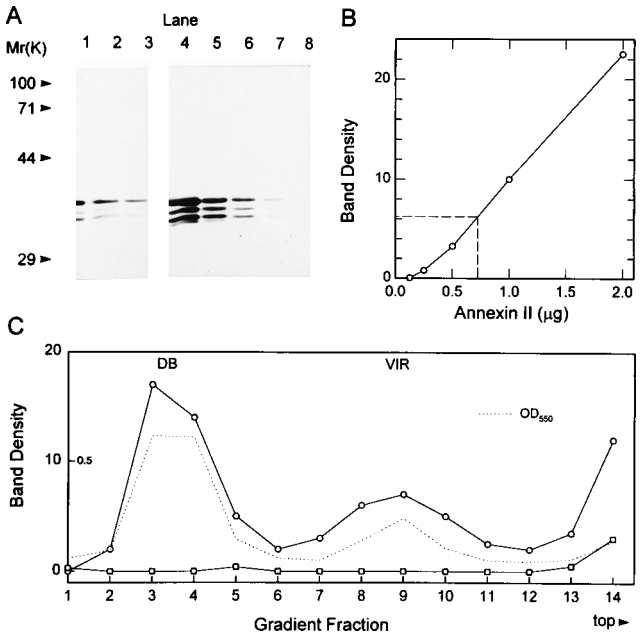

FIG. 1. Western blot analysis of HCMV. (A) HCMV virions isolated by potassium tartrate-glycerol gradient ultracentrifugation subjected to SDS-PAGE (12% acrylamide) and transferred to PVDF membranes. Western blotting was performed with a monoclonal annexin II primary antibody and an alkaline phosphatase-conjugated secondary antibody (see Materials and Methods). Lanes 1 to 3, dilutions containing 10^{10}, 5×10^9, and 2.5×10^9 HCMV particles, respectively; lanes 4 to 8, dilutions containing 2, 1, 0.5, 0.25, and 0.125 μg, respectively, of purified human annexin II. Molecular weight standards (in thousands [K]) are indicated by arrowheads. (B) Quantification of bands visualized in panel A by scanning densitometry with a Xerox model 830 scanner and IPLab Gel software (Signal Analytics Corp.). Integrated band densities were plotted against the known amounts of purified annexin II, and the resulting standard curve was used to determine the quantity of annexin II associated with 10^{10} virus particles (dashed line). (C) Quantification by densitometric scanning of bands resulting from fractions collected following potassium tartrate-glycerol gradient ultracentrifugation of HCMV-infected fibroblast cultures (○) or sonicated, non-infected fibroblasts (□) subjected to Western blot analysis as described above. The localization of HCMV virions (VIR) and dense bodies (DB) in gradient fractions was determined by OD_{550} and confirmed by electron microscopic analysis (see Materials and Methods). The value of 0.5 indicated on the vertical axis corresponds to OD_{550}.

bility was raised that the virus-associated 36-kDa protein could correspond to fibroblast-derived annexin II or to bovine annexin II derived from the cell culture medium. Alternatively, an ~36-kDa viral protein that cross-reacted with the annexin II monoclonal antibody was detected. Amino-terminal sequence analysis of a CNBr fragment derived from the virus-associated 36-kDa protein resulted in a sequence identical to amino acids (Met-170)Val-171 to Ile-185 of human annexin II (Table 1). Since the human and bovine sequences differ in only 6 of 338 residues (98.2% identity), the sequence data obtained could not distinguish between them.

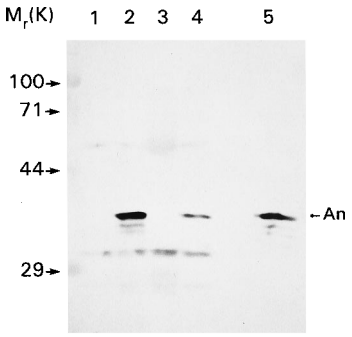

FIG. 2. Assay for annexin II in bovine calf serum. To 1.5 ml of bovine calf serum was added 1.5 μg of purified human annexin II (lanes 2 and 4), prior to addition of 1 mg of PC-PS (lane 1 and 2) or PC (lane 3 and 4) vesicles (see Materials and Methods). After incubation, vesicles were pelleted, washed five times, and subjected to SDS-PAGE and Western blot analysis with monoclonal annexin II antibody. In lane 5, 1 μg of purified annexin II was loaded directly. The annexin II band (An) and molecular weight standards (in thousands [K]) are indicated by arrows.

Lack of detection of annexin II in bovine serum. To investigate the possibility that virus-associated annexin II was derived from the bovine serum used for culturing cells, two approaches were used. In direct Western blotting of 10 μl of bovine serum with the monoclonal annexin II antibody, no bands were detected (data not shown). The possibility that low levels of annexin II present in bovine serum were concentrated on the surface of virions was investigated by using synthetic phospholipid vesicles to adsorb annexin II from serum. PC-PS or PC vesicles that were incubated with 1.5 ml of bovine serum, washed, and subjected to SDS-PAGE and Western blotting had no detectable annexin II bound to them (Fig. 2, lanes 1 and 3). In positive controls, PC-PS vesicles incubated with serum supplemented with 1.5 μg of purified annexin II (the amount of annexin II estimated to be associated with HCMV isolated from infected cell culture medium containing 1.5 ml of bovine serum) and subsequently analyzed by Western blotting reacted strongly with the monoclonal annexin II antibody (lane 2). PC vesicles adsorbed smaller amounts of annexin II (lane 4), consistent with the known anionic phospholipid-binding properties of annexin II. These data indicated that the level of endogenous annexin II in bovine serum is insufficient to account for the amount of annexin II associated with HCMV.

Immunogold electron microscopy with annexin II antibodies. Immunocytochemical localization of annexin II on purified virions is shown in Fig. 3. When virus particles were incubated with either annexin II-specific rabbit antiserum (Fig. 3A) or monoclonal antibody (Fig. 3B) and developed with a gold-conjugated secondary antibody, binding of gold particles to HCMV was observed. The distribution patterns of gold particles were similar for both treatments; however, at the concentrations used the rabbit antiserum produced stronger labeling.

TABLE 1. Amino acid sequence analysis of CNBr peptide CNBr10 from HCMV-associated protein p36[a]

Protein origin	Amino acid identified in cycle no.:														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
HCMV-p36 CNBr 10	V	A	L	A	K	G	R	R	A	E	D	G	S	V	I
Hu-annexin II[b] (predicted) V-171–I-185	V	A	L	A	K	G	R	R	A	E	D	G	S	V	I

[a] Results of analysis from PVDF, using an Applied Biosystems, Inc., 475 microsequencer.
[b] Hu-annexin II, human annexin II.

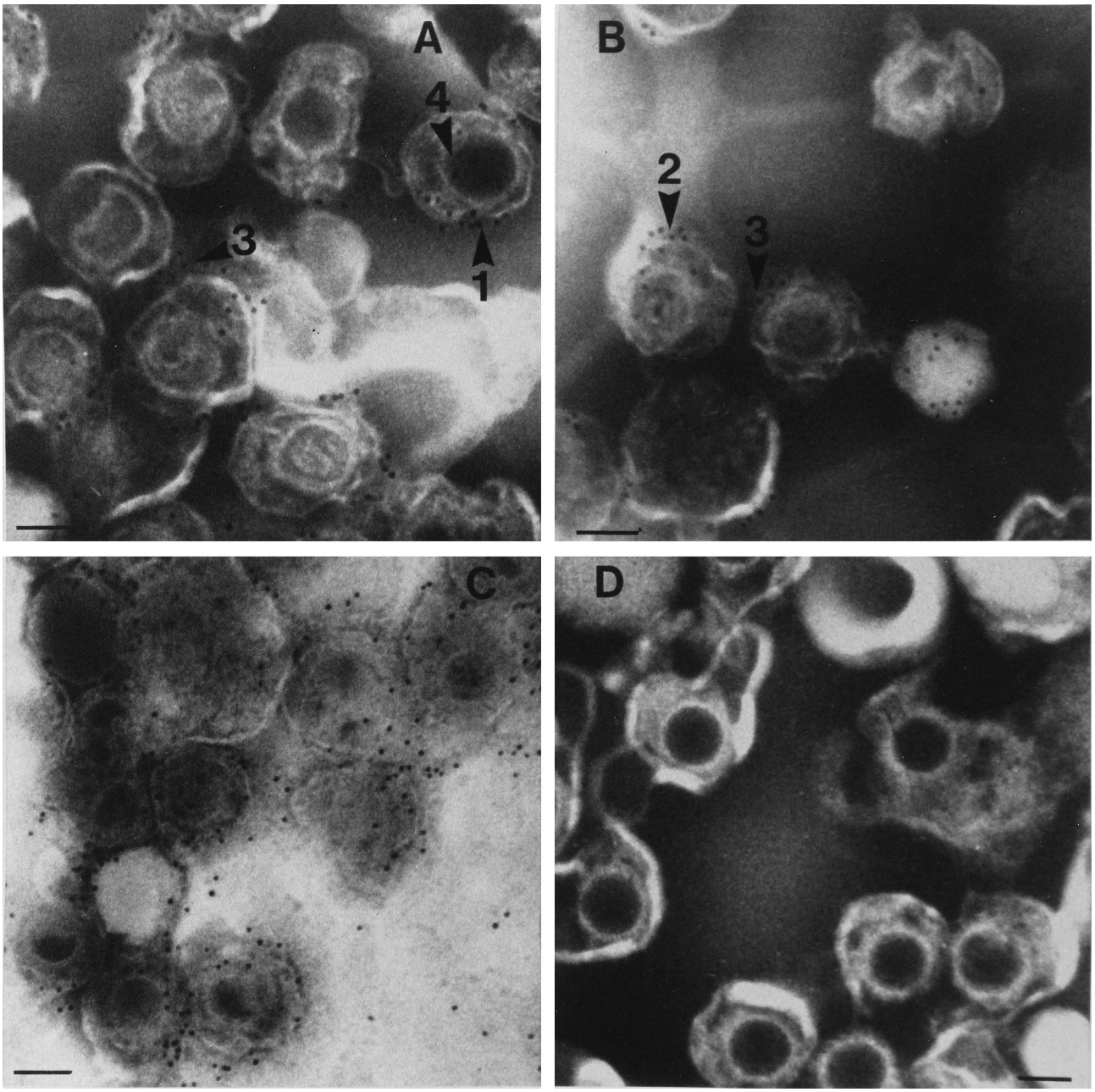

FIG. 3. Electron microscopic immunocytochemistry. HCMV virions were purified by gradient ultracentrifugation; adsorbed to Formvar grids; incubated with rabbit annexin II antiserum (A), annexin II monoclonal antibody (B), monoclonal antibody CMVB1 (C), or normal rabbit serum (D); and washed. Following incubation with 10 nm of gold particle-conjugated secondary antibodies, virions were negatively stained and examined by transmission electron microscopy. Annexin II was found associated with the envelopes of stain-penetrated (arrowhead 1) and non-stain-penetrated (arrowhead 2) virions, localized at the interface of neighboring virus particles (arrowhead 3), and bound to the nucleocapsids of virions that had lost their envelopes (arrowhead 4). Bar, 100 nm.

Annexin II was found to be associated with the envelope of both stain-penetrated and non-stain-penetrated virions; several annexin II molecules were localized at the interface of neighboring virus particles. Annexin II was occasionally observed to be bound to the nucleocapsid of a virion that had lost its envelope. Counting of gold particles associated with virions that had been labeled with the rabbit antiserum yielded an average value of 12 ± 7 (standard deviation) gold particles per virion. Importantly, there was minimal labeling of cellular debris present in the virus preparation, confirming that the annexin II present in the virus preparation was specifically associated with virus particles. The positive-control primary antibody, CMVB1, also resulted in labeling of virus envelopes (Fig. 3C); however, there were differences in the labeling patterns observed when the CMVB1 and the annexin II-specific antibodies were used. The antigen recognized by CMVB1 (gB) was homogeneously distributed on the virus envelope, while clustering of annexin II molecules on the virus envelope was frequently observed. Furthermore, while all virions were well labeled with CMVB1, annexin II was found to be present on virions at variable levels; for example, more annexin II appeared to be associated with aggregated virions. In negative

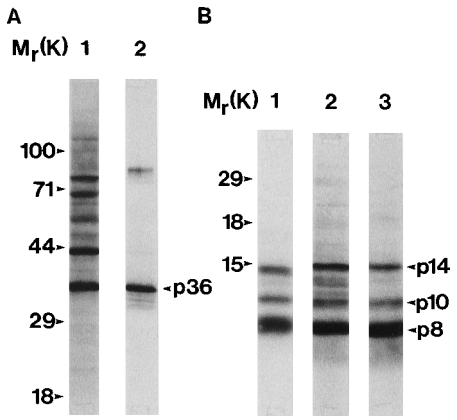

FIG. 4. Surface radioiodination of HCMV. (A) Autoradiograph of SDS-PAGE (12% acrylamide) gel of ^{125}I-surface-labeled HCMV (lane 1) and proteins (p36) eluted from labeled HCMV with 5 mM EDTA (lane 2). (B) Autoradiograph of SDS-PAGE (20% acrylamide) gel of CNBr fragments derived from endothelial cell annexin II (52) (lane 1). The HCMV-associated 36-kDa protein was excised from the panel A gel and hydrolyzed with CNBr; the resulting peptides (p14, p10, and p8) were separated by SDS-PAGE (20% acrylamide) and detected by autoradiography. Lanes 2 and 3 of panel B correspond to lanes 1 and 2, respectively, of panel A. Molecular weight standards (in thousands [K]) are indicated by arrowheads.

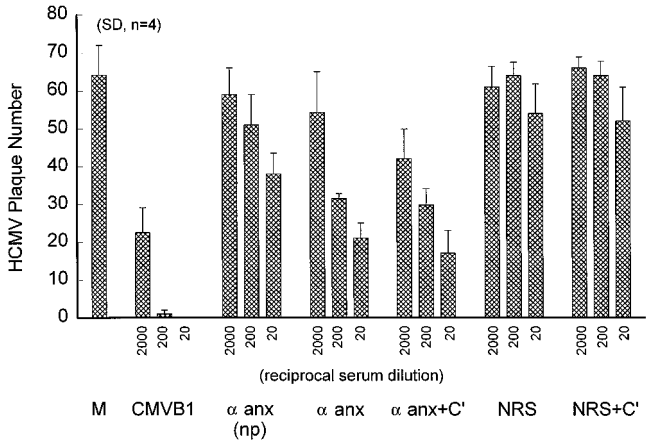

FIG. 5. HCMV plaque reduction by an annexin II antiserum. Confluent monolayers of human foreskin fibroblasts were infected with a quantity of HCMV sufficient to give 64 ± 9 viral plaques. The virus was preincubated (1 h, 37°C) in medium alone (M) and in medium containing dilutions of CMVB1 ascites, rabbit annexin II antiserum (α anx), or normal rabbit serum (NRS), with or without addition of 10% guinea pig complement (C′). Plaque formation was also assessed by use of the annexin II antiserum with no preincubation (np). Viral plaques were counted as described in Materials and Methods. The error bars indicate standard deviations (SD).

controls in which annexin II-specific antibodies were replaced with normal rabbit serum (Fig. 3D) or normal mouse serum (not shown), no association of gold particles with virus was detected.

Radioiodination of virus-associated annexin II with lactoperoxidase. In order to further evaluate the distribution of annexin II on virus particles, and its susceptibility to dissociation by EDTA, radioiodination experiments using lactoperoxidase were undertaken. As shown in Fig. 4A (lane 1), a number of virus-associated proteins were labeled when HCMV particles were surface iodinated. One of the major proteins detected following SDS-PAGE and autoradiography was a band of an apparent M_r similar to that of annexin II (~36,000). Evidence that the radiolabeled 36-kDa protein corresponded to annexin II was obtained by CNBr peptide mapping indicating that the peptides derived from the virus-associated protein corresponded to those derived from endothelial cell-derived annexin II (Fig. 4B, lanes 1 and 2). When surface-iodinated virions were treated with EDTA, bands of M_r of ~36,000 and ~80,000 were eluted from virus particles (Fig. 4A, lane 2). CNBr peptide mapping analysis of the 36-kDa band indicated that it also corresponded to annexin II (lane 3). The 80-kDa protein dissociated by EDTA was not further analyzed. These results support the notion of a peripheral association of annexin II on the virus envelope and together with previous results (52) indicate that annexin II interacts with virus surface phospholipids in a calcium-dependent manner.

HCMV plaque inhibition by annexin II-specific antiserum. Inhibition of HCMV infection of human fibroblasts by the annexin II-specific antiserum and monoclonal antibody was investigated by using a plaque reduction assay (Fig. 5). A partial but significant concentration-dependent reduction in the number of viral plaques was observed when HCMV particles were preincubated with various concentrations of antiserum. In a representative experiment, a 68% ± 6% (standard deviation) reduction in plaque number was observed when the virions were preincubated in the presence of a 1/20 dilution of antiserum. Coincubation with 10% guinea pig complement did not significantly increase the ability of the antiserum to inhibit HCMV plaque formation (74% ± 9% reduction in plaque number, with a 1/20 dilution of antiserum). Experiments carried out without the preincubation step resulted in a significantly reduced inhibitory effect (40% ± 8% reduction in plaques, with a 1/20 dilution of antiserum). In contrast to the result obtained with the annexin II rabbit antiserum, preincubation of viruses with various concentrations of the annexin II monoclonal antibody did not reduce the number of viral plaques (data not shown). In control experiments, preincubation of HCMV with equivalent dilutions of normal rabbit serum had a minimal effect on the number of viral plaques formed (14% ± 12% plaque reduction without added complement and 19% ± 15% with added complement).

DISCUSSION

There is a previous report of evidence that annexin II expressed on the surface of endothelial cells contributes to HCMV cell binding (52). A concurrent study by Hertogs and coworkers reported that the related protein annexin V (endonexin II) expressed on human liver plasma membranes was a binding protein for hepatitis B virus (23). Other recent studies have implicated annexin II in a number of cell surface binding phenomena, including tumor cell adhesion to endothelial cells (50), binding of plasminogen and tissue plasminogen activator to endothelial cells (7, 21), binding of the alternatively spliced segment of tenascin-C to glioma and endothelial cells (8), and placental Fc-receptor activity (29). These reports indicate that members of the annexin family of proteins can act as cell surface receptors that may contribute to the infectivity of some viruses.

In the course of a previous study (52), a 36-kDa protein that comigrated with annexin II during PAGE was observed to be endogenously associated with highly purified HCMV particles. In view of recent reports that virus-associated cellular proteins can contribute to virus infectivity (4, 16, 48), together with the finding that cellular annexin II (a known membrane-bridging and potentially fusogenic protein) (2, 11, 13, 14) can bind to the virus surface (52), the 36-kDa virus-associated protein was

further characterized. Western blot analysis of HCMV particles (Fig. 1) prepared by a protocol previously shown to yield highly purified virions (46) indicated that the 36-kDa protein was reactive with a monoclonal annexin II antibody. Western blotting of fractions from potassium tartrate-glycerol gradient-fractionated viruses indicated two peaks of annexin II-containing material that comigrated with the peaks corresponding to HCMV virions and dense bodies. CNBr peptide mapping (Fig. 4) and sequence analysis (Table 1) confirmed that the virus-associated protein was annexin II and not a virus-encoded species that was cross-reactive with the monoclonal antibody. In conjunction with our finding that annexin II was not detectable in bovine calf serum at levels sufficient to account for the virus-associated material by a sensitive affinity adsorption technique, these results indicate that HCMV-associated annexin II was likely acquired from the infected cells during viral egress.

Electron microscopic immunocytochemistry confirmed that annexin II is associated specifically with HCMV particles (Fig. 3). This conclusion is based on the observation that annexin II molecules detected by gold-conjugated secondary antibodies were closely associated with the envelopes of virions and dense bodies and in some cases were associated with the exposed nucleocapsids of broken virions. In these studies, we observed that virions that were present in aggregates had significantly more gold particles associated with them than isolated virions. This finding may be the result of a differential effect of the washing used in the preparation of samples on aggregated and isolated virions. Alternatively, annexin II may be preferentially associated with a subpopulation of virus particles that tend to form aggregates. The observation that gold particles were frequently detected between neighboring virions supports the possibility that annexin II contributes to virion aggregation. Typically, annexin II was found to be unevenly distributed on the surface of virions. On the basis of reports that annexins can self-associate on phospholipid membranes (3, 55), it is reasonable to expect that annexin II molecules present on the virus surface are clustered together. Because annexin II is known to bind to actin filaments (18, 19), an alternative possibility is that annexin II-actin complexes released following lysis of virus-infected cells bind to the virus surface.

The calcium-dependent interaction of annexin family proteins with anionic phospholipids is well characterized (5, 11–13, 34). In view of our finding that >90% of the virus-associated annexin II could be dissociated following treatment with EDTA, it is likely that annexin II binds to anionic phospholipids expressed on the surface of virions in a calcium-dependent manner. Wright and coworkers previously demonstrated calcium-dependent binding of purified annexin II to HCMV particles (52). Pryzdial and Wright similarly showed calcium-dependent binding of purified coagulation factors X, Xa, and Va to virions (41). Neurath and Strick recently demonstrated that annexin V (a putative cellular receptor for hepatitis B virus [23]) binds to lipid components of the virus (38). Cumulatively, these observations support the possibility that the lipids associated with enveloped viruses, through their interaction with lipid binding proteins, contribute to virus infectivity and pathogenesis.

Annexins are known to bridge phospholipid vesicles (5, 34), and evidence indicating that annexin II plays a role in membrane binding and fusion events during exocytosis has accumulated (2, 11, 13, 14). Annexin II-mediated membrane interactions are proposed to be triggered by the increase in intracellular calcium concentration that occurs following cell activation (13). Hence, we hypothesized that annexin II molecules on the surface of HCMV that are exposed to extracellular calcium concentrations (millimolar range) are capable of participating directly in virus-cell binding and/or fusion events. To test this hypothesis, antibodies to annexin II were examined for their ability to inhibit HCMV infectivity. We found that preincubation of HCMV with serial dilutions of an annexin II rabbit antiserum resulted in a concentration-dependent reduction in virus infectivity relative to that of control sera. In view of reports that annexin II is expressed on the surface of various cell types (7, 8, 21, 31, 50, 52, 54), the observed inhibitory effect could also be due to binding of the antibody to cell surface annexin II. However, when the plaque assay was performed without preincubation of viruses with antiserum, the inhibitory effect was significantly reduced, suggesting that antibody binding to virus-associated annexin II is responsible, at least in part, for inhibition. One explanation for the weak neutralization titer of the antiserum is that the epitope(s) on annexin II that contributes to virus-cell interactions is only weakly immunogenic. Alternatively, virus-associated annexin II may contribute to a secondary pathway of infection which enhances but is not essential for virus infectivity. A significant observation was that inhibition of virus plaque formation by the annexin II antiserum was independent of the added complement. Previous studies showed that complement-independent neutralizing antibodies inhibit infection by interfering with virion penetration into cells (17, 24, 36). Hence, our results suggest that HCMV-associated annexin II contributes to cell penetration by the virus. The inability of the monoclonal annexin II antibody to inhibit HCMV plaque formation suggests that the epitope recognized by this antibody is not important for infectivity.

These results raise questions concerning how annexin II becomes associated with the viral surface. One possibility is that following virus-induced cell lysis, released cytosolic annexin II binds to the phospholipids expressed on the surface of virus particles as a result of exposure to elevated calcium concentrations. Alternatively, in view of its reported expression on the surface of several cell types (7, 8, 21, 31, 50, 52, 54), annexin II may be acquired concurrently with the phospholipid bilayer during viral budding. This process would be analogous to the mechanism proposed for the acquisition of host major histocompatibility complex molecules by HIV-1 and -2 (4). On the basis of a recent report that acidic isoforms are enriched in annexin II isolated from extracellular pools (31), further characterization of virus-associated annexin II will likely contribute to an understanding of how this cellular protein becomes associated with the virus.

The presently available evidence implicates three HCMV surface glycoprotein complexes in virus binding to cells or initiation of fusion of the virus envelope and cell plasma membrane (25, 27, 28, 37). In addition, several cellular HCMV receptors have been proposed (1, 20, 25, 27, 28, 39, 43, 52). While these reports suggest that multiple interactions are involved in HCMV binding to cells, the interrelationship between interactions is poorly understood. Here, we provide evidence that host cellular annexin II is acquired during virus egress from infected cells and is bound to anionic phospholipids expressed on the virus surface. On the basis of its ability to bind anionic phospholipids that are known to be expressed on the surface of activated cells, virus-associated annexin II, likely in association with other interactions, may contribute to membrane binding and fusion events required for virus entry. Studies are in progress to further test this model.

ACKNOWLEDGMENTS

We thank Mathias Haun, Steve Smith, Steven Doyle, Wah-kiam Chia, and Tina Raynor for skilled technical assistance; B. Brodeur for CMVB1 ascites; and B. Pepinski for annexin II antiserum. We also

thank B. Brodeur, B. Pepinski, and T. Compton for helpful scientific discussion.

This investigation was supported by The Canadian Red Cross Blood Services, by Miles Biologicals grant HO10-813 (to J.F.W. and S.W.), by The Canadian Red Cross Blood Research and Development grant HO10-936 (to J.F.W. and E.L.G.P.), and by National Institutes of Health grant NS 29261 (to A.K.). J.F.W. is a recipient of a Bayer/Canadian Red Cross Society/Medical Research Council of Canada scholarship.

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