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Phosphatidylserine Is Not the Cell Surface Receptor for Vesicular Stomatitis Virus

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The envelope protein from vesicular stomatitis virus (VSV) has become an important tool for gene transfer and gene therapy. It is widely used mainly because of its ability to mediate virus entry into all cell types tested to date. Consistent with the broad tropism of the virus, the receptor for VSV is thought to be a ubiquitous membrane lipid, phosphatidylserine (PS). However, the evidence for this hypothesis is indirect and incomplete. Here, we have examined the potential interaction of VSV and PS at the plasma membrane in more detail. Measurements of cell surface levels of PS show a wide range across cell types from different organisms. We demonstrate that there is no correlation between the cell surface PS levels and VSV infection or binding. We also demonstrate that an excess of annexin V, which binds specifically and tightly to PS, does not inhibit infection or binding by VSV. While the addition of PS to cells does allow increased virus entry, we show that this effect is not specific to the VSV envelope. We conclude that PS is not the cell surface receptor for VSV, although it may be involved in a postbinding step of virus entry.

Vesicular stomatitis virus (VSV) is a negative-stranded RNA virus whose envelope protein (VSV-G) has become an important tool for gene transfer and gene therapy. This envelope protein can be combined with the structural proteins from unrelated viruses in a process called pseudotyping. VSV-G pseudotyped retroviral and lentiviral vectors can be concentrated to high titers, are exceptionally stable, and have a very broad tropism (39).

Virus receptors have often been identified by the complementation of a noninfectible cell line with cDNAs from infectible cells. However, VSV has been found to infect every cell type tested. This pantropism makes it impossible to undertake this complementation approach and has made the search for the cellular receptor difficult. Here, we use the term cellular receptor to describe the specific molecule that the virus needs to contact initially in order to enter the cell, in contrast to the secondary or fusion receptor required by some viruses, such as human immunodeficiency virus.

There is evidence that the membrane lipid phosphatidylserine (PS) is important in VSV entry (24, 25). Initial experiments showed that there was a saturable binding site for VSV on Vero cells, demonstrating binding to a specific receptor (26). Subsequent studies showed that membrane extracts of Vero cells could completely inhibit VSV infection, presumably by saturating the VSV-G protein with the receptor present in the extracts (24). The factor responsible for the inhibitory activity was shown to be resistant to neuraminidase, trypsin, and heating to 100°C but was soluble in chloroform-methanol and sensitive to phospholipase C. Therefore, the possible inhibition of VSV infection by the incubation of virions with various purified phospholipids was measured, and only PS inhibited infection (24). From this indirect evidence, many people have concluded that PS is the cellular receptor for VSV.

Other experiments have demonstrated a specific affinity of the VSV-G protein for PS. For example, a particular series of heptad repeats in VSV-G bind to PS (7). Nuclear magnetic resonance studies have further shown that a different 19-amino-acid peptide from VSV-G can also bind strongly to PS liposomes (11). Finally, PS is believed to be important in the fusion step of VSV infection (6). This last study also measured the specific affinity of VSV-G protein for PS liposomes by using force spectroscopy. However, none of these studies has examined the binding of actual virions in the context of a normal cell membrane. In contrast, a study examining $CD34^+$ cells demonstrated that preincubation with cytokines increased VSV binding to cells but that PS levels were unaffected (30). This result suggests that the binding of VSV-G and PS may not be relevant in the context of the cell surface.

In most cell types, either the vast majority or all of the PS is contained on the inner leaflet of the plasma membrane (for reviews, see references 3, 36, and 41). The asymmetric distribution of this phospholipid is maintained in part by an aminophospholipid transporter, which specifically transports PS from the outer to the inner leaflet (20, 40). It is very important for most cells to minimize the amount of PS in the outer leaflet of the membrane, because PS influences apoptosis and engulfment by macrophages (for reviews, see references 27 and 37). For example, red blood cells modified by the addition of labeled PS to their surfaces and returned to mice were rapidly cleared from the circulation (28). Additionally, the molecules of PS that do appear in the outer leaflet have a very short half-life there, meaning that interactions with the virus would need to occur relatively quickly (29). The scarcity of PS on the surface of cells argues against the hypothesis that it serves as a virus receptor.

We have examined the role of PS in VSV-G-mediated virus

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entry and provide evidence that PS is not the cell surface receptor for VSV. Implications of this finding are discussed.

MATERIALS AND METHODS

Cell culture. Mos-55 mosquito (*Anopheles gambiae*) cells (15), ZF4 zebrafish (*Brachydanio rerio*) cells (ATCC CRL-2050), and FHM minnow (*Pimephales promelas*) cells (ATCC CCL-42) (kind gifts from Jane Burns, University of California at San Diego, La Jolla, Calif.) were maintained at 26°C in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum (FBS) (GIBCO), penicillin, and streptomycin. XPK2 frog (*Xenopus laevis*) cells derived from adult tissue (a kind gift from Ron Reeder, Fred Hutchinson Cancer Research Center, Seattle, Wash.) were maintained at 26°C in 42% L-15 medium–43% water–15% FBS with L-glutamine, penicillin, and streptomycin. D17 dog (*Canis familiaris*) cells (ATCC CCL-183), BHK hamster (*Mesocricetus auratus*) cells (ATCC CRL-1632) (a kind gift from Adam Geballe, Fred Hutchinson Cancer Research Center), HTX human cells (a near-diploid subclone [unpublished results] of HT-1080 cells [ATCC CCL-121]), and DF-1 chicken (*Gallus gallus*) cells (12) (a kind gift from Paul Neiman, Fred Hutchinson Cancer Research Center) were maintained at 37°C and 5% CO_2 in Dulbecco's minimal essential medium with a high concentration of glucose (4.5 g per liter) and 10% FBS. QT35 quail (*Coturnix coturnix japonica*) cells (21) (a kind gift from Maxine Linial, Fred Hutchinson Cancer Research Center) were maintained at 37°C and 5% CO_2 in Ham's F-10 medium containing 10% tryptose phosphate broth, 5% newborn calf serum, 2% sodium bicarbonate, 1% dimethyl sulfoxide, and 1% heat-inactivated chick serum.

Virus production. LNCG is a Moloney murine leukemia virus-based retroviral vector made by cloning eGFP (Clontech, Palo Alto, Calif.) into the LNCX vector (19) downstream of the CMV promoter. The LNCG(RD114) virus (LNCG virus made with RD114 retrovirus envelope) was produced from the FlyRD/LNCG packaging line described previously (9). LNCG(GALV) virus (LNCG made with the gibbon ape leukemia virus [GALV] envelope) was produced from the PG13/LNCG packaging line (18). The LNCG(VSV-G) virus was generated by transient cotransfection of the pL-VSVG, pCMVtat, and pJK3 plasmids (2) with the LNCG plasmid. VSV-G/GFP was a kind gift from John Rose (Yale University, New Haven, Conn.). This virus consists of VSV with green fluorescent protein (GFP) linked to the cytoplasmic domain of the VSV-G protein (8). The addition of GFP to VSV-G does not cause a reduction in titer, is stable through multiple passages, and is incorporated into virions with almost the same efficiency as wild-type VSV-G (8). To generate stocks of this replication-competent virus, BHK cells were plated at 2.5×10^6 cells in a 75-cm^2 tissue culture flask. The following day, the medium was changed and 4 μg of Polybrene/ml was added to the flask. Virions (5×10^4) were then added, and new virus was collected 18 to 24 h later. VSV-GFP, a kind gift from John Rose, expresses soluble GFP in addition to the normal VSV proteins (4). This virus was propagated as described above for VSV-G/GFP.

Virus purification. All of the retroviral vectors used in these studies were harvested in medium exposed to producer cells or to transfected cells and were centrifuged at $1{,}000 \times g$ for 5 min to remove cells and debris. Both VSV-GFP and VSV-G/GFP were purified and concentrated before use as follows. After virus collection, the virus-containing medium was centrifuged ($1{,}000 \times g$ for 5 min) to remove cells and debris and was then frozen. After thawing, 31 ml of virus was overlaid onto 4 ml of 20% (wt/vol) sucrose in an ultracentrifuge tube. The virus was centrifuged at $90{,}000 \times g$ (26,000 rpm) for 2.5 h in an SW28 rotor (Beckman, Fullerton, Calif.). The supernatant and sucrose were removed, and the pellet was overlaid with 5 ml of cold phosphate-buffered saline (PBS) per tube. The tubes were kept on ice and in the dark for 2 h, and the pellets were resuspended, aliquoted, and frozen at −80°C.

Virus assays. The VSV-G-, RD114-, and GALV-pseudotyped LNCG viruses were all assayed for infection of ZF4 cells by flow cytometry. On day 0, cells were seeded at 3×10^5 cells/well in six-well tissue culture dishes. On day 1, the medium was replaced with medium containing 4 μg of Polybrene per ml and virus was added at various dilutions. On day 4, the cells were trypsinized and washed twice in PBS. The cells were suspended in PBS at 10^6 cells/ml and examined for GFP expression by a fluorescence-activated cell sorter. Relative levels of infection were determined by counting the number of GFP-positive cells per 100,000 total cells.

The titers of VSV-G/GFP were determined by using a plaque assay in D17 cells. Cells were seeded at 4×10^6 cells per 6-cm-diameter dish. The following day, the medium was replaced with medium containing 4 μg of Polybrene per ml and virus was added at various dilutions. The plates were kept at 37°C for 2 h, and then the medium was removed. The cells were overlaid with a 1:1 mix of normal medium and 1.5% carboxymethyl cellulose. Forty-eight hours later, the overlay was removed, the cells were stained with crystal violet, and the plaques were counted.

The titers of the VSV-GFP virus were determined for all of the cell lines on the basis of GFP activity. The assays were performed with 24-well plates seeded the previous day with 5×10^4 cells/well for Mos-55 cells, 2×10^4 cells/well for ZF4 cells, and 10^4 cells/well for all other cell types. Sixty wells were seeded for each cell type per experiment. Six different serial dilutions of the virus were made, and 10 μl of each dilution was added to 10 wells per cell type. After 3 days, the cells were washed, fixed, and examined for GFP expression under a microscope. The dilution where more than 1 and less than 10 wells had some GFP-positive cells was found. The titer was calculated from this number, including the dilution factor. This method of determining titers was required because VSV does not replicate in all of these cell types, preventing the use of the plaque assay (data not shown).

Measurement of cell size. To determine the average cell size for each cell type, the cells were removed with trypsin and washed once with PBS plus 2% FBS. Photographs of cells in a hemacytometer were taken under a microscope. The average diameter of cells was measured by comparison with the calibrated grid of the hemacytometer. This number was used to calculate the relative surface area of different cell types and to estimate the amount of PS per unit surface area from flow cytometry measurements of PS per cell.

Annexin V staining. Alexa Fluor 488-conjugated annexin V, propidium iodide (PI), and annexin binding buffer were obtained from the Vybrant Apoptosis Assay Kit #2 (Molecular Probes, Eugene, Oreg.). Annexin V staining was performed by using a slight variation of the manufacturer's protocol. Cells were trypsinized from dishes, centrifuged ($1{,}000 \times g$ for 5 min), and washed once in cold PBS. Cells were counted, centrifuged ($1{,}000 \times g$ for 5 min), and resuspended to a concentration of 10^6 cells/ml in 1× annexin binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$ [pH 7.4]). One hundred microliters of cells in suspension was incubated with 5 μl of annexin V solution and 1 μl of PI (100 μg/ml) for 15 min at room temperature. Next, 400 μl of annexin binding buffer was added and the tubes were placed on ice and analyzed by flow cytometry with a FACSCaliber flow cytometer (BD Biosciences, San Jose, Calif.). Unstained cells were used as the negative control. Cells were gated on forward scatter and side scatter to eliminate cell debris and clumps. Cells that stained positive for PI (dead cells) were also excluded from analysis. Analysis was done with CellQuest software (BD Biosciences). Cell fluorescence was determined relative to a fluorescent bead standard so that results from different experiments could be compared. The geometric mean fluorescence of 10,000 cells was obtained for the unstained and stained cell populations, and the mean result for the unstained cells was subtracted from the mean result for the stained cells to determine the relative amount of cell surface PS for each cell type.

VSV-G/GFP binding assay. Cells were trypsinized and were washed twice in PBS plus 2% FBS. Cells (10^5 per binding reaction or control) were added to a 1.5-ml Eppendorf tube. All further steps were performed at 4°C to prevent virus fusion. Cells were pelleted at $960 \times g$ for 5 min and resuspended in either 1 ml of purified VSV-G/GFP in PBS or 1 ml of PBS. The cells were kept in the dark for 2 h and were shaken every 15 min to prevent settling. Next, the cells were pelleted as described above, washed once in PBS with 2% FBS, and resuspended in 400 μl of PBS with 2% FBS. Two microliters of PI (100 μg/ml) was added, and the cells were kept on ice for analysis. Flow cytometry and analysis was performed as described in the section above on annexin V staining. For the binding assays to examine the effects of annexin V treatment on VSV-G/GFP binding to ZF4 cells, this protocol was modified as follows. After cells were pelleted in Eppendorf tubes, the pellets were resuspended in 100 μl of 2× annexin binding buffer (see the section above on annexin V staining) and 100 μl of water with or without 43 μg of unlabeled annexin V. Cells were incubated for 15 min and then virus was added as described above.

Generation and addition of liposomes. L-α-phosphatidyl-L-serine was obtained as a 10 mg/ml solution in chloroform-methanol (95:5) (Sigma, St. Louis, Mo.). L-α-phosphatidylcholine was obtained as a 10 mg/ml solution in chloroform (Sigma). To generate liposomes, 500 μl of phospholipid was dried in a glass tube under nitrogen and then resuspended in 1.26 ml of PBS (5 mM final concentration). This solution was sonicated on ice three times for 5 min each, by using a W-385 sonicator (Heat Systems Ultrasonics) with a microtip on output level 3. The liposomes were filtered through a 0.2-μm-pore-size syringe filter and were used immediately. ZF4 cells were plated on day 0 at 3×10^5 cells/well in six-well dishes. Liposomes were generated and added on day 1 to a final concentration of 400 μM. On day 2, the medium was replaced with medium containing 4 μg of Polybrene per ml and virus was added to the wells. On day 5, the cells were trypsinized and the virus titer for each well was determined by flow cytometry as described above.

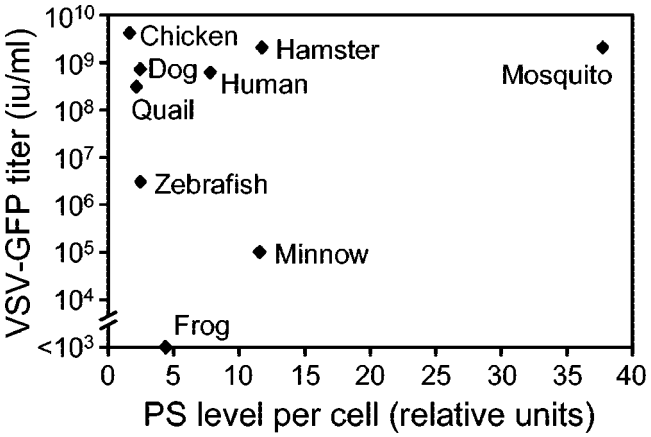

FIG. 1. VSV-GFP titer versus surface PS levels for multiple cell types. Virus titer and PS levels were measured as described in Materials and Methods. X-axis data are the geometric means of the fluorescence of 10,000 live cells measured by flow cytometry, and the units are arbitrary fluorescence units (linear scale) generated by the cytometer. The PS levels shown are representative of at least two experiments for each cell line with different batches of annexin V. Vector titer data are for one complete experiment that was repeated with very similar results.

Annexin interference assays. ZF4 cells were plated on day 0 at 5×10^4 cells/well in 24-well plates. On day 1, the medium was removed and replaced with 100 μl of 2× annexin binding buffer. Unlabeled annexin V was added to a final concentration of 12 μM, and water was added to a final volume of 200 μl. The cells were incubated at room temperature for 15 min, and the virus was added. Cells were kept at 26°C for 2 h, washed twice with PBS, trypsinized, and replated into 24-well plates in the presence of fresh annexin V (12 μM). On day 4, the wells were washed with PBS, and GFP-positive foci were counted by fluorescence microscopy.

RESULTS

Lack of correlation between surface PS levels and VSV titers in different cell types. Since VSV has such a broad tropism, we chose to use cells from a wide variety of organisms to examine the potential role of PS in virus entry. We measured PS levels on the surfaces of cells from quail, chicken, hamster, zebrafish, minnow, frog, mosquito, dog, and human by using fluorescently labeled annexin V, which binds to PS with high specificity (1, 14, 31, 32, 34). Cells were removed from their dishes, incubated with labeled annexin V, and analyzed by flow cytometry. Figure 1 shows that the different cell types exhibit a wide range of cell surface PS levels. These levels were relatively constant and did not change with factors such as cell density, passage number, or method of cell removal (data not shown). Dead cells were excluded from the analysis, because much more PS is exposed once the membrane asymmetry is disrupted during apoptosis. When annexin V staining was undertaken as described above, we consistently obtained a single, relatively tight peak for cell-bound annexin V, showing that we were measuring PS on live, healthy cells only. Once we had a reliable measure of PS levels, we examined the relationship between virus titers and PS levels on these cell surfaces. It is clear from the results shown in Fig. 1 that there is no close correlation between virus titers and PS levels for these cell lines.

Lack of correlation between VSV binding and the amounts of PS in different cell types. Even though VSV titers and PS levels were not correlated, we reasoned that since there are multiple steps to virus entry, PS could still play a role in virus binding and the differences in titers could be due to postbinding factors—especially when cells from different tissues and organisms are used. However, if PS is the cell surface receptor for VSV, then cells that express more PS on the surface should bind more virus. To quantify virus binding, we used a GFP-labeled VSV, VSV-G/GFP. In this virus, GFP is attached to the cytoplasmic tail of the VSV-G protein (8), allowing virus binding to be observed under a microscope or detected in a flow cytometer. Binding was undertaken at 4°C to prevent fusion. Figure 2 demonstrates that there is no correlation between the amount of PS on the surface of a cell and the ability of that cell to bind VSV. For example, mosquito cells have a very high level of PS yet they bind virus poorly, while hamster cells show the opposite pattern. The inset to Fig. 2 is an enlargement of the part of the graph containing cells that have lower levels of virus binding and lower levels of PS. It further highlights the lack of close correlation between VSV binding and PS levels.

One potential concern with these results is the lack of a strong correlation between VSV binding and titers (data not shown). However, there are several possible reasons for this result. One possibility is that a postbinding step is rate limiting for infection between cell types. If this were the case, a low level of binding might be sufficient to mediate virus entry and a subsequent step (such as the rate of endocytosis) would play a larger role in determining virus titers for different cell types. Since the cell lines utilized in our study are from a variety of organisms and tissue types, there are a large number of variables that may affect virus titers.

Increasing the PS levels on cell surfaces causes a nonspecific increase in vector titer. To examine the relationship be-

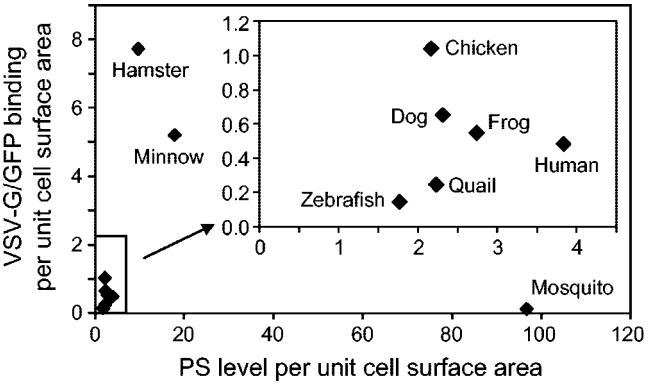

FIG. 2. VSV-G/GFP binding versus surface PS levels for multiple cell types. The PS level determinations are the same as in Fig. 1 before scaling for cell size. VSV-G/GFP binding was measured at 4°C as described in Materials and Methods. To account for significant differences in cell size between cell types, both PS levels and binding levels are expressed as fluorescence per unit cell surface. Data shown are the geometric means of 10,000 cells for both PS and binding. The experiment was repeated with a different stock of VSV-G/GFP with very similar results. PS levels versus VSV-G/GFP binding for all cell types are shown. Inset, enlargement of the part of the graph containing the six cell types with the lowest levels of PS among those tested.

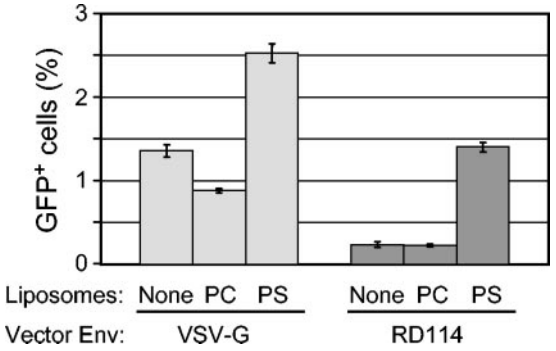

FIG. 3. Effects of liposome addition on virus infection. PC and PS liposomes were generated as described and added to cells. Twenty-four hours later, the retrovirus vector LNCG, pseudotyped with either RD114 or VSV-G envelope proteins, was added to cells in the presence of 4 μg of Polybrene per ml. After 3 days, the cells were removed from the dishes and GFP-positive cells were counted by flow cytometry. Cells not incubated with the virus showed fewer than five GFP-positive cells in the 10^5 cells analyzed. Data shown are the means ± standard deviations of results from three replicates from one experiment. The experiment was repeated twice with very similar results.

tween PS levels and virus entry in a single cell type, we attempted to increase the PS levels on the cell surface by the addition of PS to cells. We choose to use ZF4 zebrafish cells for these experiments, because they exhibited an intermediate level of cell surface PS and showed an intermediate infection rate. As a negative control, we added phosphatidylcholine (PC), a phospholipid similar to PS, which is normally found in the outer leaflet of the plasma membrane. We generated phospholipid liposomes as described in Materials and Methods. Cells were incubated with liposomes for 24 h and analyzed for cell surface PS levels. The PS liposomes caused a reproducible doubling in the surface PS levels on ZF4 cells, while PC liposomes had no effect on surface PS levels (data not shown). After incubation with liposomes, the ZF4 cells were exposed to VSV-G- or RD114-pseudotyped viruses containing GFP, and titers were measured by flow cytometry. While a reproducible increase in titer was found, it was not specific for VSV-G, as shown in Fig. 3. The enhancement of infection was, however, specific for PS when compared to results with PC. Similar results were obtained with a GALV-pseudotyped virus (data not shown).

Annexin V shows saturable binding to cell surface PS. While there is no correlation between VSV binding to cells and their PS levels, we wanted to examine the interaction between the virus and PS in a more direct manner. It has been demonstrated that high concentrations of annexin V can prevent both macrophage recognition of apoptotic cells and platelet coagulation by virtue of binding to all available PS (5, 13). The binding of annexin V to PS is both specific and strong, with a K_d of 9 to 15 nM on cells (23, 35) and 40 pM on phospholipid vesicles (33). Importantly, annexin V can also detect and bind to very low levels of PS (16, 17).

To determine whether the labeled annexin V from Molecular Probes could saturate the PS in ZF4 cells, we used a slight modification of the annexin V staining described above. Cells were prepared and analyzed as normal, but instead of adding 5 μl of annexin V we added from 1 to 75 μl of annexin. The exact concentration of annexin V in the solution from Molecular Probes is proprietary, but by spectrometry we determined the protein concentration to be about 1 mg/ml. Because of the high cost of labeled annexin V, we also obtained some unlabeled, purified annexin V (0.86 mg/ml) from Jonathan Tait (University of Washington, Seattle). To make sure the annexin V could also saturate the PS on ZF4 cells, we performed a competition experiment. This experiment was undertaken as a normal annexin V staining except that cells were incubated with 0.1 to 40 μl of unlabeled annexin for 15 min prior to the incubation with labeled annexin V and PI.

Figure 4 (top) shows the binding curve of labeled annexin V to ZF4 cells, and demonstrates saturable binding of annexin V as well as some nonspecific binding at higher concentrations. Figure 4 (bottom) shows the results of the competition experiment between unlabeled annexin V and the labeled annexin V used in flow cytometry. At relatively low concentrations of unlabeled annexin, the binding of labeled annexin to cells was almost completely blocked. From these experiments, we concluded that we could successfully saturate the PS on the surface of cells and examine the effects on infection.

Saturating concentrations of annexin V do not interfere with virus entry into cells. Cells were incubated with 86 μg of annexin V/ml, which is more than sufficient to mask the available PS (Fig. 4, bottom), or with buffer only. LNCG(VSV-G) was then added to these cells (in the absence of Polybrene) and allowed to incubate for 2 h. After this time, the cells were trypsinized to remove any virus remaining outside the cells, and the cells were replated in the presence of fresh annexin V.

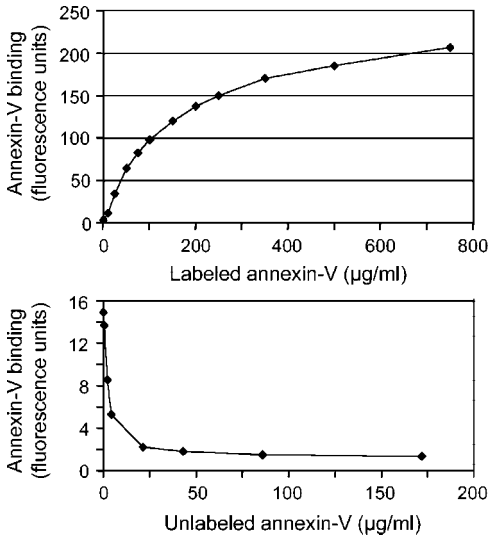

FIG. 4. Saturable annexin V binding to ZF4 cells. (Top) Alexa Fluor 488-labeled annexin V binding to ZF4 cells. Annexin V staining was performed as described in Materials and Methods, with variable amounts of annexin V. Data shown are the geometric means for 10,000 live cells minus the mean fluorescence of cells not exposed to annexin V. (Bottom) Competition of unlabeled annexin V with labeled annexin V on ZF4 cells. Staining with labeled annexin V was performed as described in Materials and Methods (5 μl of labeled annexin V per 100 μl of incubation mixture; ~50 μl/ml) except that cells were exposed to various amounts of unlabeled annexin V for 15 min prior to the addition of labeled annexin. Each data point is the geometric mean fluorescence of 10,000 live cells minus the mean fluorescence of cells not exposed to annexin V.

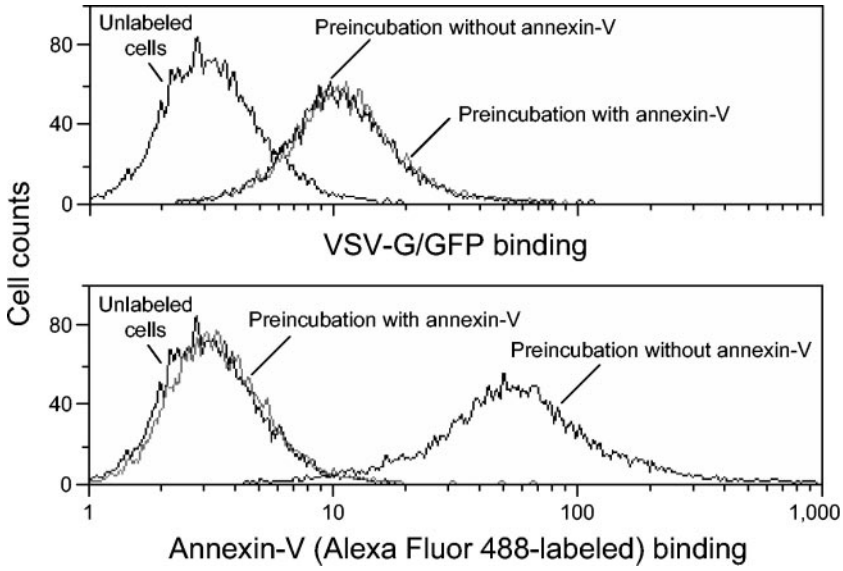

FIG. 5. Annexin V binding to ZF4 cells and effect on VSV-G/GFP binding. Cells were harvested for virus binding, treated with unlabeled annexin V (86 μg/ml) or buffer, and then measured for virus binding or labeled annexin V binding as described in Materials and Methods. The unlabeled cells are the same in both panels, and the grey curves in both panels represent the cells pretreated with 86 μg of unlabeled annexin V/ml. (Top) VSV-G/GFP binding to ZF4 cells with or without treatment of unlabeled annexin V (86 μg/ml). (Bottom) Labeled annexin V binding to cells with or without pretreatment of unlabeled annexin V (86 μg/ml). This experiment was repeated with virtually identical results.

Not only did a saturating amount of annexin V not interfere with virus infection, it increased the LNCG(VSV-G) infection rate by 27 ± 7% (mean ± standard deviation of results from three independent experiments).

Saturating concentrations of annexin V do not affect virus binding to cells. To examine the effects of annexin V saturation more directly, we also measured the effect on virus binding. ZF4 cells were treated as described above for the VSV-G/GFP binding assays. Annexin V (86 μg/ml) was added to cells, and the cells were incubated for 15 min prior to virus addition and the subsequent flow cytometry as normal. A subset of these treated cells was also stained with labeled annexin V. Figure 5 shows that there is no effect of annexin V treatment on VSV binding to ZF4 cells. In contrast, the binding of labeled annexin V is completely eliminated in the cells treated with unlabeled annexin V, demonstrating that the saturation of cell surface PS was complete and remained so throughout the assay.

Given the compelling evidence that PS does not play a role in VSV binding to cells, we reexamined the original result demonstrating PS inhibition of VSV infection (24). There are no published reports replicating the finding that PS inhibits VSV plaque formation. We generated PS liposomes by using the original published protocol (24) or by using the protocol described in Materials and Methods. In several experiments, we never observed a decrease in VSV plaque formation of more than 10%. Because the original published protocol was insufficiently detailed to repeat exactly, it is possible that the results obtained in the previous studies were dependent on the exact method used to generate PS liposomes. In our hands, the exposure of VSV to the PS liposomes that were made with two different procedures had no effect on VSV infection.

DISCUSSION

Discovering the identity of the cell surface receptor for VSV has been greatly hindered by the broad tropism of the virus. The original finding that PS could inhibit virus infection when incubated with virions has been accepted as evidence that PS is the receptor for VSV. We have been unable to duplicate this result; furthermore, we demonstrate through more direct assays that PS is not the cell surface receptor for VSV. We have shown that the levels of PS on cell surfaces do not correlate with either VSV titers or levels of VSV binding to those cells. Furthermore, when all available PS molecules are bound to annexin V, the infection of VSV is unaffected. Most importantly, when enough annexin V is added to cells to completely eliminate binding by additional annexin V, the binding of VSV to these cells is totally unaffected.

We are confident that VSV and annexin V are not able to simultaneously bind PS based on both affinity and steric arguments. As previously discussed, annexin V binds to PS with a very low K_d and is unlikely to be displaced by the virus. Furthermore, the area of the phospholipid head of PS is 0.7 nm^2 compared to an annexin V molecule at 25.5 nm^2, so one annexin V molecule is thought to cover an area of the plasma membrane containing 35 lipid molecules (22). Therefore, we feel confident that when cells are treated with a saturating concentration of annexin V, no other large compound, especially a bulky VSV virion, can access the PS in the cell membrane.

That the surface PS levels on the cell lines studied here varied by 25-fold is a novel finding. Most previous work has focused on the amount of PS that is externalized during apoptosis or the total amount of PS present in the cell and has not focused on comparing cell surface levels of PS between cell

types. Furthermore, most studies using annexin V to examine PS levels have focused on mammalian cells. Here, we have shown that cells from mosquito express far higher levels of cell surface PS than most other cell types. Since the mosquito cells were the only insect cell line utilized, this finding could potentially have implications for the role of PS in different organisms.

The discovery that virus infection can be enhanced after PS is added to cells is somewhat surprising. It is not clear what is causing the nonspecific enhancement of infection with these viruses. The effect must be general, since RD114- and GALV-pseudotyped viruses enter cells by fusion at the plasma membrane and VSV enters cells through endocytosis. One possibility is that changing the phospholipid composition of the membrane nonspecifically enhances virus fusion. We plan to further investigate this phenomenon to see if this enhancement is receptor specific and if it would be a useful tool for increasing virus transduction in some refractory systems.

While the VSV-G protein clearly binds to PS, as demonstrated by others, this interaction does not appear to be relevant for VSV binding to cells, suggesting the presence of an undetermined cell surface receptor. Previous studies have examined the VSV-G and PS interaction by using liposomes containing PS or PS bound to an artificial substrate. They have shown that VSV-G does not bind or fuse to PC liposomes and will bind or fuse increasingly well as more and more PS is added to these liposomes (6, 10, 38). Based on these data for PS binding in vitro, we predicted that cell lines with a higher level of PS would bind more virus. However, we did not observe any strong correlation between PS levels and virus binding. This finding suggests that the in vitro interaction demonstrated between VSV-G and PS is either coincidental or relates to events that take place somewhere other than the cell surface. Previous studies have also demonstrated that VSV-G has some affinity for other negatively charged phospholipids, suggesting that even the interaction with PS may not be specific.

A recent study has shown that the pH-dependent conformational change of VSV-G depends on the presence of PS within the target membrane (6). Furthermore, the rate of the fusion reaction depends on the amount of PS present in the endosome. It is possible that PS functions as a fusion receptor within the endosome. This possible function could help explain some of the documented interactions between VSV-G and PS.

Therefore, we propose a model in which the VSV-G protein interacts with an unknown cellular receptor, followed by receptor-mediated endocytosis. During the pH-induced conformational change, the VSV-G protein could then interact with the PS within the endosome, allowing fusion to occur. Because of the broad tropism of VSV, the cellular receptor must also be something ubiquitously found on animal cells.

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