Phosphatidylserine Is Not the Cell Surface Receptor for Vesicular Stomatitis Virus

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Received 10 May 2004/Accepted 18 June 2004

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 titters, 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...
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. XPF2 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–normal medium and 1.5% carboxymethyl cellulose. Forty-eight hours later, the cells were washed, fixed, and 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 day before with 5 10^5 cells/well for Mos-55 cells, ZF4 cells, and 10^6 cells/well for all other cell types. Sixty wells were seeded for each cell type per experiment. Six different serial dilutions of the viruses were made, and 10 μl of each dilution was added to 10 wells per cell type. After 3 days, the cells were trypsinized, 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 dilution, 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 x g for 5 min), and washed once in cold PBS. Cells were counted, centrifuged (1,000 x g for 5 min), and resuspended to a concentration of 10^5 cells/ml in 1:4 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 FACS-Calibur 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 stained and unstained 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-GFP binding assay.** Cells were trypsinized and washed twice in PBS plus 2% FBS. Cells (10^6 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 x g for 5 min and resuspended in either 1 ml of puriﬁed VSV-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 unlabelled annexin V. Cells were incubated for 15 min and then virus was added as described above.

**Generation and addition of liposomes.** 1-α-phosphatidylycholine-serine was obtained as a 10 mg/ml solution in chloroform-methanol (95:5) (Sigma, St. Louis, Mo.). 1-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 PI solution (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl_2 [pH 7.4]). 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 liposome 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% carbomethoxy cellulose. Forty-eight hours later, the overlay was removed, the cells were stained with crystal violet, and the plaques were counted.
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-
between 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 phosphatidylethanolamine (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. Data shown are the geometric means for 10,000 live cells minus the mean fluorescence of 10,000 live cells. 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 V for 15 min prior to the incubation with labeled annexin V and PI.

**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. 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.

**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.
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 with virus infection, it increased the LNCG(VSV-G) infection rate by 27 ± 7% (mean ± standard deviation of results from three independent experiments).

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 unidentified 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.

ACKNOWLEDGMENTS

We thank John Rose, Jonathan Tait, Jane Burns, Ron Reeder, Adam Gебalle, Maxine Linial, and Paul Neiman for gifts of materials and John Rose for helpful advice.

This work was supported by NIH Viral Oncology training grant T32 CA09229 and NIH grant HL54881.

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