Annexin A2 Mediates the Localization of Measles Virus Matrix Protein at the Plasma Membrane

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ABSTRACT Annexins are a family of structurally related proteins that bind negatively charged membrane phospholipids in a Ca^{2+} -dependent manner. Annexin A2 (AnxA2), a member of this family, has been implicated in a variety of cellular functions, including the organization of membrane domains, vesicular trafficking, and cell-cell adhesion. AnxA2 generally forms a heterotetrameric complex with a small Ca^{2+} -binding protein, S100A10. Measles virus (MV), a member of the family *Paramyxoviridae*, is an enveloped virus with a nonsegmented negative-strand RNA genome. Knock-down of AnxA2 greatly reduced MV growth in cells without affecting its entry and viral RNA production. In MV-infected, AnxA2 knockdown cells, the expression level of the matrix (M) protein, but not other viral proteins, was reduced compared with that in control cells, and the distribution of the M protein at the plasma membrane was decreased. The M protein lines the inner surface of the envelope and plays an important role in virus assembly by connecting the nucleocapsid to the envelope proteins. The M protein bound to AnxA2 independently of AnxA2's phosphorylation or its association with S100A10 and was colocalized with AnxA2 within cells. Truncation of the N-terminal 10 amino acid residues, but not the N-terminal 5 residues, compromised the ability of the M protein to interact with AnxA2 and localize at the plasma membrane. These results indicate that AnxA2 mediates the localization of the MV M protein at the plasma membrane by interacting with its N-terminal region (especially residues at positions 6 to 10), thereby aiding in MV assembly.

IMPORTANCE MV is an important human pathogen, still claiming ~100,000 lives per year despite the presence of effective vaccines, and it causes occasional outbreaks even in developed countries. Replication of viruses largely relies on the functions of host cells. Our study revealed that the reduction of the host protein annexin A2 compromises the replication of MV within the cell. Further studies demonstrated that annexin A2 interacts with the MV M protein and mediates the localization of the M protein at the plasma membrane where MV particles are formed. The M protein lines the inner surface of the MV envelope membrane and plays a role in MV particle formation. Our results provide useful information for the understanding of the MV replication process and potential development of antiviral agents.

KEYWORDS measles virus, annexins, matrix protein, paramyxovirus, virus-host interactions

Measles virus (MV), a member of the genus *Morbillivirus* in the family *Paramyxoviridae*, is an enveloped virus with a nonsegmented negative-strand RNA genome (1). The virus has six genes that encode the nucleocapsid (N), phospho- (P), matrix (M), fusion (F), hemagglutinin (H), and large (L) proteins, respectively. The P gene encodes additional proteins V and C. The genomic RNA is encapsidated with the N protein, and this nucleocapsid forms a ribonucleoprotein (RNP) complex together with the RNA-dependent RNA polymerase composed of the L and P proteins. The M protein lines the

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inner surface of the envelope and is involved in virus assembly, budding, and regulation of viral RNA synthesis. The H and F proteins are envelope glycoproteins that are responsible for receptor binding and membrane fusion, respectively. Signaling lymphocyte activation molecule (SLAM; also called CD150) on immune cells and nectin-4 on epithelial cells are known to act as MV receptors (2–4).

Annexins are a family of evolutionarily conserved and structurally related proteins that bind negatively charged membrane phospholipids in a Ca^{2+} -dependent manner (5, 6). All annexins are composed of two domains: a variable N-terminal domain and a C-terminal conserved protein core. The C-terminal core consists of four highly conserved annexin repeats of 70 to ~80 amino acids, each with Ca^{2+} and phospholipid binding sites, which are involved in the translocation of annexins to the plasma membrane or other intracellular membranes upon changes of the Ca^{2+} concentration. On the other hand, the variable N-terminal domain of annexins may interact with different host proteins and be subject to posttranslational modifications, likely giving each annexin molecule unique properties.

Annexin A2 (AnxA2; also called calpactin 1H or lipocortin 2; encoded by the gene ANXA2), a member of the annexin family, is predominantly expressed at the plasma membrane and on intracellular vesicles but is also present in the nucleus (7, 8). It has a 30-amino-acid-long unique N-terminal domain. This domain contains the binding site for S100A10, a member of the S100 protein family that is a group of small Ca^{2+} -binding proteins (9, 10). AnxA2 generally forms the heterotetrameric complex consisting of an S100A10 dimer and two AnxA2 proteins. The tyrosine residue at position 23 can be phosphorylated by Src family tyrosine kinases, whereas serine kinases may phosphorylate serine residues at positions 11 and 25 (11–13). AnxA2 has been implicated in a variety of intracellular functions, including the organization of membrane domains, vesicular trafficking, and membrane-cytoskeleton contacts. Furthermore, it plays important roles in cell-cell adhesion and extracellular matrix degradation at the outer surface of the cell as well as in transport of transcription factors and mRNA and protection against DNA damage in the nucleus (where AnxA2 functions as the monomer form rather than the heterotetramer form) (7, 8).

Given the diverse functions of AnxA2, it is not surprising that AnxA2 is involved in different replication processes of many viruses, including hepatitis C virus (HCV) (14, 15), human immunodeficiency virus (HIV) (16, 17), human papillomavirus (HPV) (18, 19), enterovirus 71 (20), bluetongue virus (21), and classical swine fever virus (22). In this study, we examined whether AnxA2 plays any role in MV replication. Our results show that AnxA2 mediates the localization of MV M protein at the plasma membrane by interacting with its N-terminal region.

RESULTS

Effects of AnxA2 knockdown on MV replication. To examine a possible role of AnxA2 in MV replication, we generated three clones (shANX 18, 22, and 29) of HeLa cells expressing human SLAM (HeLa/hSLAM cells) in which expression of AnxA2 was knocked down using short hairpin RNA (shRNA), and knockdown of AnxA2 in these clones was confirmed at mRNA (Fig. 1A) and protein levels (Fig. 1B). These clones and luciferase-knockdown HeLa/hSLAM cells (shLuci), used as a control, were infected with MV. At 24 h postinfection (p.i.), virus titers obtained in all three clones of AnxA2-knockdown cells were ~40-fold lower than that in control cells (Fig. 1C). The differences in virus titers between control and knockdown cells became less (~10-fold) at 48 h p.i. and nonsignificant at 72 h p.i. Stronger cytopathic effects in control cells (due to higher levels of virus growth) likely account for the findings at later time points (at 48 and 72 h p.i.). The results indicate that AnxA2 deficiency reduces MV replication.

In contrast to virus titers, there were no significant differences in the efficiency of virus entry, as determined with the enhanced green fluorescent protein (EGFP)-expressing recombinant MV IC323-EGFP (23) in the presence of the fusion inhibitor peptide (FIP; Z-D-Phe-Phe-Gly) (24) (Fig. 1D), and the expression levels of the N and M mRNAs, as determined by reverse transcription-quantitative PCR (RT-qPCR)

FIG 1 Effects of AnxA2 knockdown on MV replication. (A and B) AnxA2 mRNA (A) and protein (B) levels were examined by RT-qPCR (GAPDH mRNA as control) and Western blot analysis (tubulin as loading control), respectively, in AnxA2-knockdown HeLa/hSLAM (shANX 18, 22, and 29) and control (shLuci) cells. Molecular sizes (in kDa) of proteins are shown. Protein band signals were quantified, the signal intensities were normalized by the intensity of the corresponding tubulin, and relative values are shown below the respective bands. The value in shLuci is set to 1. (C) MV production in shANX 18, 22, and 29 and shLuci cells. Cells were infected with MV at an MOI of 0.02, and supernatants and cells were harvested at 3, 24, 48, and 72 h p.i. Virus titer (combined titer of cell-associated and cell-free viruses) at each time point was determined by plaque assay. Data are shown as means $\pm$ standard deviations (SD) from triplicate samples for shLuci and shANX 22 and means from triplicate samples for shANX 18 and 29. (D) MV entry in shANX 18, 22, and 29 and shLuci. Cells were infected with IC323-EGFP and incubated in the presence of FIP. The number of EGFP-expressing cells in each field was counted at 24 h p.i. under a fluorescence microscope. Data are shown as means $\pm$ SD from triplicate samples. (E) MV mRNA synthesis in shANX 18, 22, and 29 and shLuci. Cells were infected with MV, and the amounts of MV N and M mRNA were determined by RT-qPCR at 3, 24, 48, and 72 h p.i. GAPDH mRNA was used as a control. Data are shown as means $\pm$ SD from triplicate samples for shLuci and shANX 22 and means from triplicate samples for shANX 18 and 29. (F) Syncytium formation in IC323-EGFP-infected shANX 18, 22, and 29 and shLuci cells at 24 h p.i. The images observed under a light and a fluorescence microscope are shown. Scale bar, 10 μ m. *, $P < 0.05$ (Student's *t* test). Data are representative of the results from three independent experiments.

FIG 2 Effects of AnxA2 overexpression on MV replication. (A) Expression of AnxA2 was examined by Western blotting in HEK293/hSLAM cells stably expressing Flag-tagged AnxA2. Flag-tagged AnxA2 has a higher molecular weight than endogenous AnxA2. (B) MV production in AnxA2-overexpressing HEK293/hSLAM (ANX) and control cells after MV infection. Virus titers were determined and are shown as described for Fig. 1C. (C) Efficiencies of MV entry in ANX and control cells were determined and are shown as described for Fig. 1D. (D) MV N and M mRNA levels in ANX and control cells were examined and are shown as described for Fig. 1E. (E) Syncytium formation in IC323-EGFP-infected ANX and control cells at 24 h p.i. The images observed under a light and a fluorescence microscope are shown. Scale bar, 10 μ m. *, $P < 0.05$ (Student's t test). Data are representative of the results from three independent experiments.

(Fig. 1E) between control and AnxA2-knockdown cells. Furthermore, control and ANX-knockdown cells similarly developed syncytia (reflecting the cell surface expression of the H and F proteins) after infection with IC323-EGFP (Fig. 1F). These results suggest that AnxA2 deficiency affects MV replication at a step(s) after viral protein production.

HEK293 cells are reported to express only a low level of endogenous AnxA2 (15). HEK293 cells expressing human SLAM (HEK293/hSLAM cells) were transfected with the expression vector encoding the Flag-tagged AnxA2, and a stable cell line was obtained in which the expression level of the Flag-tagged AnxA2 was 18 times higher than that of the endogenous AnxA2 (Fig. 2A). At 24, 48, and 72 h after MV infection, cells overexpressing AnxA2 produced 3 to ~5 times higher virus titers than mock-transfected control cells (Fig. 2B). There were no significant differences in the efficiency

FIG 3 Effects of AnxA2 knockdown on MV protein production and distribution. (A) Expression levels of MV proteins in shANX 22 and shLuci were examined at 24 h after MV infection using Western blot analysis. Expression levels of AnxA2 and tubulin (control) were also examined. Protein band signals were quantified, and relative values are shown below the respective bands. The value in shLuci is set to 1. (B) MV-infected shANX 22 and shLuci were stained for MV H, M, and N proteins at 24 h p.i. and examined under a confocal microscope. The nuclei were counterstained with DAPI. Scale bar, 10 μ m.

of virus entry (Fig. 2C), expression levels of the N and M mRNAs (Fig. 2D), and levels of syncytium formation after IC323-EGFP infection (Fig. 2E) between cells overexpressing AnxA2 and control cells. These results also support a role of AnxA2 in MV replication.

Expression of MV proteins in AnxA2 knockdown cells. To examine the effect of AnxA2 deficiency on viral protein production, we examined expression levels of MV proteins in infected cells at 24 h p.i. by Western blotting (Fig. 3A). The expression of the M protein was apparently reduced in shANX 22 compared with that in control cells (shLuci), whereas the other MV proteins examined (the L protein was not examined) were expressed comparably in shLuci and shANX 22. We then examined the distribution of the N, M, and H proteins in MV-infected cells by confocal microscopy (Fig. 3B). The H protein was similarly expressed and distributed in shLuci and shANX 22. In shLuci, the M protein was located at the plasma membrane as well as in the cytoplasm. In contrast, it was almost exclusively localized at the perinuclear area in shANX 22. The N

protein was distributed in the entire cytoplasm but more concentrated at the perinuclear area in shANX 22, whereas in shLuci it was evenly distributed in the cytoplasm.

Interaction of the M protein with AnxA2. From the above-described results, we reasoned that the localization of the M protein at the plasma membrane is mediated by AnxA2. To test this idea, we first examined whether the M protein interacts with AnxA2. Coimmunoprecipitation experiments indeed revealed that the histidine (His)-tagged M protein coprecipitates with the Flag-tagged wild-type (WT) AnxA2 (Fig. 4A, upper). We further determined whether this interaction depends on the phosphorylation of AnxA2 and the heterotetramer formation of AnxA2 with S100A10. To this end, we produced two types of AnxA2 mutants: one [P(–)] lacking three phosphorylation sites (serine at position 11, tyrosine at 23, and serine at 25) by alanine substitutions (11–13) and another (ΔN10) missing the N-terminal 10 amino acid residues required for the interaction with S100A10 (9, 10). These AnxA2 mutants could not coprecipitate with S100A10 (Fig. 4A, lower), but the M protein was still capable of interacting with these two mutants as effectively as the WT AnxA2 (Fig. 4A, upper).

We next transfected HeLa/hSLAM cells with expression vectors encoding the M protein and Flag-tagged AnxA2 and examined the localization of the M protein and AnxA2 by microscopy (Fig. 4B). Although WT AnxA2 was mainly localized at the plasma membrane and in the cytoplasm, the two mutants did not reach the plasma membrane as efficiently as WT AnxA2 and were largely localized in the cytoplasm and nucleus. Regardless of the localization and tetramer formation of AnxA2, the M protein was colocalized with AnxA2, and it reached the plasma membrane efficiently when expressed together with WT AnxA2 but hardly reached it when expressed with P(–) or ΔN10. The localization of endogenous AnxA2 was the same as that of Flag-tagged AnxA2 in HeLa/hSLAM cells (Fig. 4B and C) and unaltered in MV-infected cells (Fig. 4C).

The above-described results indicate that the M protein interacts with AnxA2 independently of the latter's association with S100A10. To further confirm this, we prepared the His-tagged M protein and the glutathione S-transferase (GST)-tagged AnxA2 and performed an *in vitro* binding assay using these purified proteins (Fig. 4D). Although a small amount of the M protein was recovered from the GST-bound Sepharose (control), a much larger amount was recovered from AnxA2-bound Sepharose. The results indicate that the M protein directly binds to AnxA2.

The region on the M protein critical for the interaction with AnxA2. To determine the region on the M protein that is critical for the interaction with AnxA2, we generated M protein mutants missing the N-terminal 5, 10, and 20 amino acid residues (Δ5, Δ10, and Δ20). The mutant with the shortest truncation (Δ5) still interacted with AnxA2, but Δ10 and Δ20 barely did so (Fig. 5A). When Δ5 was expressed in HeLa/hSLAM cells, it was localized at the plasma membrane, although at a lower level than the WT M protein (Fig. 5B). Δ10 and Δ20 were localized in the cytoplasm and nucleus but not at the plasma membrane. Thus, the N-terminal residues at positions 6 to 10 are critical for the interaction of the M protein with AnxA2.

DISCUSSION

The MV M protein is known to associate with the inner surface of the plasma membrane (25, 26) as well as the cytoplasmic tails of the H and F proteins (27, 28). We previously reported that the MV M protein also interacts with the N protein, thereby bringing the RNP complex to the plasma membrane (29). The present study indicates that the host protein AnxA2 interacts with the N-terminal region of the MV M protein, and that this interaction is critical for the localization of the M protein at the plasma membrane. Considering its function in vesicular trafficking, AnxA2 may mediate the transport of the M protein to the plasma membrane. Alternatively, AnxA2 may be important for the retention of the M protein at the plasma membrane. Either way, through its interaction with AnxA2, the M protein bound to the RNP complex is localized at the plasma membrane, where the M protein also interacts with the envelope proteins, leading to virus assembly.

AnxA2 has been implicated as a host factor regulating the growth of many different

FIG 4 Interaction of the M protein with AnxA2. (A) His-tagged MV M protein (MV-M-His) and Flag-tagged AnxA2 (ANX-Flag) [WT, P(-), and NΔ10] were coexpressed in HeLa/hSLAM cells, and the complexes were immunoprecipitated (IP) with anti-Flag Ab and analyzed by Western blotting (WB) using anti-His (upper) or anti-S100A10 (lower) Ab. Whole-cell lysates (WCL) were used as the loading control. (-), MV-M-His but no ANX-Flag expressed. (B) Flag-tagged AnxA2 [WT, P(-), and NΔ10] and untagged M protein were coexpressed in HeLa/hSLAM cells, and their localizations were examined under a Celldiscoverer 7 microscope (Carl Zeiss) at 24 h after transfection. Scale bar, 10 μm. (C) The localization of endogenous AnxA2 in uninfected and MV-infected (24 h p.i.) HeLa/hSLAM cells as examined under a confocal microscope. (D) GST alone or GST-AnxA2 (5 μg) was immobilized on glutathione Sepharose 4B and incubated with His-tagged MV-M (0, 0.5, and 5 μg) at 4°C overnight. After washing three times, the bound proteins were eluted and subjected to Western blot analysis using anti-His Ab.

viruses. By virtue of its functions in the organization of membrane domains and vesicular trafficking, AnxA2 supports virus assembly by interacting with HCV nonstructural protein 5A (NS5A) (14), HIV Gag (16), and classical swine fever virus NS5A (22). Our results show that AnxA2 is also essential for MV assembly. AnxA2 forms the heterote-

FIG 5 Region on the M protein critical for the interaction with AnxA2. (A) His-tagged MV M protein (WT, $\Delta 5$, $\Delta 10$, and $\Delta 20$) and Flag-tagged AnxA2 were coexpressed in HeLa/hSLAM cells, and the complexes were immunoprecipitated (IP) with anti-Flag Ab and analyzed by Western blotting using anti-His Ab. Whole-cell lysates (WCL) were used as the loading control. Flag-tagged AnxA2 was not expressed in the sample at the far left. (B) Untagged M proteins (WT, $\Delta 5$, $\Delta 10$, and $\Delta 20$) were expressed in HeLa/hSLAM cells, and their localizations were examined under a confocal microscope at 24 h after transfection. Scale bar, 10 μm .

tramer with S100A10. In the case of bluetongue virus, S100A10, rather than AnxA2, within the tetramer interacts with NS3, thereby allowing intracellularly assembled virions to engage in the cellular exocytic machinery for nonlytic virus release (21). AnxA2 is also known to promote viral genome replication, as it recruits HCV NS proteins on the lipid raft to form the HCV replication complex (15). Furthermore, AnxA2 expressed at the outer surface of the plasma membrane can enhance entry of HPV16 and enterovirus 71 by interacting with their capsid proteins (18, 20). AnxA2 may even affect virus growth in the nucleus, as exemplified by its binding to infectious bronchitis virus RNA and altering the frameshift efficiency (30).

In AnxA2-knockdown cells, both the M and N proteins were more concentrated at the perinuclear area where the RNP complex is first formed after MV infection (Fig. 3B). This likely occurred because the localization of the M protein (and that of the RNP complex as well) at the plasma membrane was compromised. It is notable that the expression of the M protein, but not other MV proteins, was reduced in AnxA2-knockdown cells (Fig. 3A). The M protein is a basic protein with several hydrophobic regions. It is possible that the M protein is stabilized within the cell by interacting with other host and/or viral proteins, and that in the absence of AnxA2 the M protein may be destabilized and then degraded.

The two AnxA2 mutants examined [P(−) and N Δ 10] were predominantly expressed in the nucleus but not at the plasma membrane (Fig. 4B). Their inability to form a complex with S100A10 and/or the lack of the nuclear export signal (mapped to AnxA2 residues 3 to 13) (31) may have caused the changes in their localization. Tyr23 phosphorylation-deficient AnxA2 was reported to exhibit a dominant-negative phenotype (32), also contributing to the dysfunction of the nuclear-cytoplasmic cycling of the mutant AnxA2.

The mutant M proteins ($\Delta 10$ and $\Delta 20$), which do not bind to AnxA2, were also largely localized in the nucleus (Fig. 5B). Since there is no known nuclear localization signal in the MV M protein, the results suggest the presence of a host factor(s) which recruits the MV M protein to the nucleus in the absence of AnxA2. Generally, viruses in the family *Paramyxoviridae* replicate in the cytoplasm. However, a certain fraction of M proteins of Sendai virus (33), Newcastle disease virus (NDV) (34), respiratory syncytial virus (35), and Nipah virus (36) were observed in the nuclei of infected cells. In fact, the nuclear-cytoplasmic trafficking of the M protein is important for the replication process of Nipah virus (36) and NDV (37). Recently, it has been shown that the MV M protein can

be located in the nucleus and inhibit host cell transcription (38). All these observations suggest that the MV M protein receives posttranslational modification in the nucleus for its proper functioning.

In summary, our study demonstrates that AnxA2 mediates the localization of the MV M protein at the plasma membrane, thereby facilitating MV assembly. This adds one more example to a growing list of various roles the host protein AnxA2 plays in virus growth.

MATERIALS AND METHODS

Cells and viruses. Cells stably expressing human SLAM (Vero/hSLAM [39], HeLa/hSLAM [40], and HEK293/hSLAM [41]) were established in our laboratory and maintained in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries) supplemented with 10% fetal bovine serum and penicillin-streptomycin (Gibco). IC323 is a recombinant MV based on the pathogenic IC-B strain (42). For some experiments, IC323 expressing EGFP (IC323-EGFP) was used (23). In all experiments, MV infection was performed at a multiplicity of infection (MOI) of 0.1, except for those shown in Fig. 1C and 2B, where an MOI of 0.02 was employed. For titration, viruses were harvested by one cycle of freezing and thawing of infected cells and their supernatants, followed by sonication. The virus suspensions were clarified by low-speed centrifugation and titrated on Vero/hSLAM cells.

Plasmids. To construct expression plasmids, fragments encoding the following proteins were individually subcloned into the expression plasmid pCA7 (43): the MV M protein, His-tagged MV M protein (MV-M-His), untagged and His-tagged MV-M proteins in which the N-terminal 5, 10, and 20 amino acids were deleted ($\Delta 5$, $\Delta 10$, and $\Delta 20$ and $\Delta 5$ -His, $\Delta 10$ -His, and $\Delta 20$ -His, respectively), Flag-tagged AnxA2 (ANX-Flag), Flag-tagged AnxA2 in which the serine-to-alanine substitutions were introduced at positions 11 and 25 and the tyrosine-to-alanine substitution at position 23 [ANX-Flag-P(–)], and Flag-tagged AnxA2 in which the N-terminal 10 amino acids were deleted (ANX-Flag-N Δ 10). To produce recombinant proteins in the bacterial system, the fragments encoding ANX and MV-M-His were subcloned into pGEX-5X-1 (GE-Healthcare) and pCold III vector (TaKaRa), respectively.

Gene knockdown by shRNAs. Sequences targeting AnxA2 and luciferase mRNAs were designed using BLOCK-IT RNAi Designer (Invitrogen) and inserted into pRS-U6/puro (OriGene). To generate cells with the gene of interest constitutively knocked down, HeLa/hSLAM cells were transfected with pRS-U6-sh-AnxA2 or pRS-U6-sh-luciferase and selected in medium containing 1 μ g of puromycin/ml.

Virus entry assay. To determine the efficiency of virus entry, cells were infected with IC323-EGFP for 1 h at 37°C. The infected cells then were washed, and the fresh medium containing FIP (Peptide Institute Inc.) was added to block the second round of infection by progeny viruses. At 24 h p.i., the number of EGFP-expressing cells was counted under a fluorescence microscope.

Coimmunoprecipitation and Western blot analysis. Subconfluent monolayer HeLa/hSLAM cells on 6-well plates were transfected with 2 μ g of pCA7-MV-M-His, together with 2 μ g of pCA7-ANX-Flag, pCA7-ANX-Flag-N Δ 10, or pCA7-ANX-Flag-P(–), using polyethylenimine Max (Polysciences). At 48 h posttransfection, the cells were washed with phosphate-buffered saline (PBS) and lysed in 1 ml of the lysis buffer (50 mM Tris-HCl, pH 8.0, 280 mM NaCl, 1% Triton X-100, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol, 1 mM dithiothreitol [DTT]) containing the protease inhibitor cocktail (Sigma). After incubation for 2 h at 4°C, the lysates were centrifuged at 20,000 $\times$ g for 10 min at 4°C. A small amount (50 μ l) of each supernatant was mixed with an equal volume of the 2 $\times$ SDS loading buffer (125 mM Tris-HCl, pH 6.8, 10% 2-mercaptoethanol, 4% SDS, 0.1% bromophenol blue, 20% glycerol) and kept as the whole-cell lysate sample. The rest of the cleared supernatant was incubated with anti-Flag monoclonal antibody (MAb) (F1804; Sigma) or normal mouse IgG and with Dynabeads pan mouse IgG (Invitrogen) overnight at 4°C with gentle shaking. After intensive washing with the lysis buffer, the polypeptides in precipitated complexes were fractionated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Immobilon-P; Millipore). The membranes were incubated with rabbit anti-His polyclonal antibody (pAb) (PM032; Medical & Biological Laboratories) or anti-S100A10 MAb (ab89438; Abcam), followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Invitrogen) or anti-mouse IgG (Jackson Immuno Research). Chemiluminescent signals (Chemi-Lumi One Super; Nacalai Tesque) were detected and visualized using a VersaDoc 5000 imager (Bio-Rad). In other experiments, lysates were prepared from MV-infected cells, fractionated by SDS-PAGE, and blotted onto membranes, as described above. Abs used to detect MV proteins were previously described (44). The M protein was detected by a rabbit pAb (a gift from T. Kohama). Tubulin was examined as a loading control by using anti-tubulin Ab (sc-5286; Santa Cruz Biotechnology).

RT-qPCR. For RT-qPCR, total RNA was extracted from MV-infected cells with the TRIzol reagent (Life Technologies), treated with DNase I, and reverse transcribed into cDNAs using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega) and random hexamer primer. MV N and M and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were quantified using SYBR premix Ex Taq II (TaKaRa) and a LightCycler (Roche) as described previously (43).

Immunofluorescence staining. HeLa/hSLAM cells seeded on coverslips were infected with IC323 for 1 h at 37°C, washed with PBS, and cultured with fresh medium. Transfection with expression plasmids was performed where indicated. At 24 h p.i. or posttransfection, the cells were simultaneously fixed and permeabilized with PBS containing 2.5% formaldehyde and 0.5% Triton X-100. The cells were treated with 10% normal donkey serum and then incubated with appropriate combinations of primary and secondary Abs. For detecting endogenous AnxA2, the cells were fixed with 100% methanol, treated with

PBS containing 1% bovine serum albumin, 10% normal donkey serum, 0.3 M glycine, and 0.1% Tween 20, and then incubated with Abs. The following Abs were used: mouse anti-N and anti-M MABs (provided by T. Kohama), mouse anti-H MAB (a gift from Y. Fukuda), rabbit anti-Flag pAb (F7425; Sigma), rabbit anti-AnxA2 MAB (ab178677; Abcam), Alexa Fluor 488-conjugated donkey anti-mouse IgG (H+L) (Molecular Probes), and Alexa Fluor 488- or Alexa Fluor 594-conjugated donkey anti-rabbit IgG (H+L) (Molecular Probes). The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) in some experiments. The stained cells were observed under a confocal microscope or CellDiscoverer 7 live cell imaging system (Carl Zeiss).

In vitro binding assay. The *E. coli* XL10-Gold strain containing pGEX-5X-1-AnxA2 was grown in LB medium at 37°C. At an optical density at 600 nm (OD₆₀₀) of 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM and the culture was continued for a further 4 h at 37°C. The cells were pelleted and resuspended in PBS containing the protease inhibitor cocktail and 1% Triton X-100. After incubation at 4°C overnight and sonication, the lysate was clarified by centrifugation. GST-tagged AnxA2 recombinant protein was purified with glutathione Sepharose beads (GE Healthcare). GST-tagged AnxA2 recombinant protein was eluted with 50 mM reduced glutathione and dialyzed against PBS. His-tagged MV-M protein was expressed in Rosetta2 *E. coli* cells (Novagen) by incubating them at 37°C in Overnight Express instant LB medium (Novagen) containing 10 ml glycerol and 100 mg ampicillin per liter following induction with 0.1 mM IPTG until the OD₆₀₀ reached 0.6. The incubation then was continued at 15°C overnight with shaking. The cells were harvested by centrifugation, resuspended in the lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 5 mM imidazole, 5 mM DTT, pH 8.0), and lysed by sonication. The lysate was centrifuged, and the supernatant was filtered, purified using an Ni²⁺-nitrilotriacetic acid affinity column (COSMOGEL His-Accept; Nacalai Tesque) in the lysis buffer, and then eluted with the elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 500 mM imidazole, 5 mM DTT, pH 8.0) at 4°C. The eluate was dialyzed against PBS, and insoluble debris was removed by centrifugation. Protein concentration was determined by 280-nm UV absorbance. GST-AnxA2 was immobilized on glutathione Sepharose 4B and incubated with His-tagged MV-M recombinant protein in the binding buffer (50 mM HEPES, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 2 mM MgCl₂, 0.2% Triton X-100, protease inhibitor cocktail) at 4°C overnight. After washing three times, the bound protein was eluted by boiling in the SDS loading buffer and subjected to Western blot analysis.

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