Plasminogen promotes influenza A virus replication through an annexin 2-dependent pathway in the absence of neuraminidase

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INTRODUCTION

Influenza A viruses (IAVs) possess two major glycoproteins, haemagglutinin (HA) and neuraminidase (NA), which protrude from the viral envelope. To infect host cells, cleavage of the precursor HA molecule (HA0) into HA1 and HA2 subunits by proteases is required (Klenk & Garten, 1994). The HA proteins of highly pathogenic H5 and H7 pantropic avian virus subtypes contain multiple basic amino acids (R-X-R/K-R) that can be cleaved by an intracellular subtilisin-type enzyme. In contrast, most IAVs have a single arginine at the HA cleavage site and the HA of these viruses can only be cleaved by extracellular trypsin-like proteases (Steinhauer, 1999). Therefore, IAV replication in humans is usually restricted to the upper respiratory tract due to its dependence on these proteases present at this anatomical site (Bottcher et al., 2006), in addition to other factors such as the presence of specific receptors (Shinya et al., 2006; van Riel et al., 2006; Yen et al., 2009). Blood-derived proteases, such as plasminogen (PLG), may contribute to the pathogenesis of IAV infections by providing an alternative source of proteases for virus replication in the respiratory tract. In addition, occasionally IAVs also replicate outside the respiratory tract (Fislova et al., 2009; Mizuguchi, 2006) and the use of PLG as an alternative protease may contribute to dissemination of IAV to extra-respiratory tissues, although the mechanism of action remains poorly understood. For the IAV strain A/WSN/33 (H1N1), it was shown that its NA determined the neurovirulence of this virus (Goto & Kawaoka, 1998; Goto et al., 2001; Li et al., 1993) and that conversion of PLG, abundantly present in plasma, into plasmin provided an alternative mechanism for the cleavage of the HA molecule, contributing to dissemination of this virus to the brain (Goto & Kawaoka, 1998; Goto et al., 2001). In particular, a carboxy-terminal lysine and the lack of an oligosaccharide chain at position 146 of NA were essential for PLG binding. However, we recently reported that other strains that lack these specific features can also use PLG for their replication (LeBouder et al., 2008). In this case, the virions can activate PLG and subsequently allow HA cleavage through the host cellular protein annexin 2 (A2), which is incorporated into IAV particles. Interestingly, IAV infection also increased PLG binding at the surface of infected cells (LeBouder et al., 2008) and this feature may contribute to PLG-dependent virus replication, although the mechanism underlying this process is not clear. The goal of our study was thus to gain more insight into the role(s) of NA and A2 in PLG-dependent IAV replication. In the present study, using NA-deficient viruses, we show that NA is not required for PLG-dependent IAV replication. We
show, however, that IAV infection increased the expression of the host cellular PLG receptor A2 at the surface of infected cells, increasing the binding of PLG to IAV-infected cells and subsequent cleavage of HA. The involvement of A2 in PLG-dependent cleavage of HA and IAV replication was demonstrated by using specific inhibitors of PLG binding to A2. Collectively, these results indicate that PLG promotes IAV replication through an A2-dependent pathway in the absence of NA. These results may be of importance, as the differential capacity of various IAV strains to upregulate A2 at the surface of infected cells may be another virulence factor of IAVs.

RESULTS

PLG supports replication of NA-deficient IAV strains

First, we wished to assess whether NA was essential for PLG-dependent replication of IAV. For this purpose, we used an influenza virus that lacks a functional NA gene (Flu-ΔNA), produced by reverse genetics, and compared its replication with that of its wild-type counterpart, strain A/Puerto Rico/8/34 (H1N1) (Flu-WT). After purification of Flu-WT and Flu-ΔNA from the supernatants of infected Madin–Darby canine kidney (MDCK) cells, their protein contents were analysed by Western blotting using antibodies specific for NA, M2 and the cellular proteins A2 and p11, which binds to A2 to form heterotetramers necessary for the translocation of A2 to the cell surface. As expected, both viruses contained the viral M2 protein, but NA was only detected in the purified Flu-WT virus preparation (Fig. 1a). Furthermore, both virus preparations contained host cellular proteins such as A2 and p11, as described previously (LeBouder et al., 2008), although it seems that less A2 and p11 are incorporated into Flu-ΔNA virus preparations than for Flu-WT.

To further characterize the protein content of purified Flu-WT and Flu-ΔNA virus preparations, their proteins were separated by SDS-PAGE, visualized by Coomassie brilliant blue (CBB) staining (Fig. 1b) and identified by matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass-spectrometry analysis. In addition to the viral structural proteins NP and M1, A2 was also detected in both virus preparations. Furthermore, as described previously for other IAVs, actin was also present in Flu-WT and Flu-ΔNA viruses (LeBouder et al., 2008). Also, by MALDI-TOF mass spectrometry, it was found that IAV Flu-ΔNA indeed lacked NA on its particles, in contrast to Flu-WT. Finally, a band of 48 kDa corresponding to degraded NP protein was also detected in Flu-ΔNA, but not in Flu-WT. It is not clear why this virus has a degraded NP protein, but these results were also observed previously using an anti-NP antibody (Rimmelzwaan et al., 2007). Thus, both Flu-WT and Flu-ΔNA virus preparations contained more or less similar cellular and viral proteins, except for the viral NA.

To investigate whether the presence of functional NA was essential for PLG-dependent IAV replication, MDCK cells were infected with IAVs Flu-ΔNA or Flu-WT at an m.o.i. of $10^{-3}$ in the presence of various concentrations of PLG and, 24 h post-infection, infectious virus titres were determined. As shown in Fig. 1(c), PLG supported replication of IAVs Flu-WT and Flu-ΔNA in a dose-dependent manner. Importantly, none of the concentrations of PLG used displayed any signs of toxicity. Thus, these results indicate that the presence of the NA molecule at the surface of the virion or the infected cells was not required for PLG-dependent replication.

Conversion of PLG into plasmin is necessary to support IAV replication

Plasmin is the enzymically active form of its precursor PLG (Hajjar & Krishnan, 1999). Thus, next we investigated...
whether the conversion of PLG into plasmin was required to support replication of IAVs Flu-ΔNA and Flu-WT. To this end, the effect of the inhibitor 6-aminohexanoic acid (6-AHA) on IAV replication was determined. 6-AHA is a lysine analogue that binds to the high-lysine-affinity domain of PLG, inhibiting its activation (Hajjar, 1991). First, it was confirmed that IAVs Flu-WT and Flu-ΔNA replicated in a PLG-dependent fashion with similar growth kinetics (Fig. 2a) in the presence of 2 μM PLG, the usual concentration of PLG found in the serum. Interestingly, virus replication in the presence of PLG was as efficient as that in the presence of trypsin. Thus, addition of 6-AHA inhibited the replication of both viruses. 6-AHA was not toxic to cells and this inhibition was specific to PLG activation, as it did not affect replication of IAVs Flu-ΔNA and Flu-WT in the presence of trypsin, used at 1.5 μg ml⁻¹. This concentration of trypsin was used because it supported similar IAV growth kinetics as 2 μM PLG (Fig. 2a). Thus, the conversion from PLG to plasmin is necessary for the PLG-dependent replication of IAVs Flu-ΔNA and Flu-WT.

The non-structural protein NS1 is synthesized intracellularly during IAV replication. To confirm that PLG is supporting the replication of NA-deficient viruses, the synthesis of NS1 in PLG-treated MDCK cells infected with IAVs Flu-ΔNA or Flu-WT was tested by Western blot analysis. As shown in Fig. 2(b), the NS1 protein was detected in Flu-ΔNA- or Flu-WT-infected cells in the presence of PLG or trypsin, which was included as a positive control. In contrast, without PLG, NS1 was not detected, showing that PLG supported infection and the synthesis of NS1. The addition of 6-AHA to the culture blocked NS1 synthesis fully in infected cells supported by PLG, but not in those supported by trypsin (TPCK). In these experiments, the cellular protein tubulin was included as a positive control. Collectively, these results indicate that NA is not necessary for the conversion of PLG into plasmin or for PLG-dependent IAV replication and viral protein synthesis.

**PLG binding to IAV-infected cells occurs in the absence of NA**

As we demonstrated previously that IAV infection increased PLG binding to cells (LeBouder et al., 2008), we wished to investigate whether this was dependent on NA expression. To this end, the binding of PLG to cells

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**Fig. 2.** Plasmin production is necessary for IAV replication. (a) MDCK cells were infected with Flu-ΔNA or Flu-WT at an m.o.i. of 10⁻³, in the presence of 2 μM PLG and in the absence or presence of 6-AHA. At the indicated times post-infection, infectious virus titres in the culture supernatants were determined by plaque assay. Specificity of the 6-AHA inhibitor was determined on MDCK cells infected with Flu-ΔNA or Flu-WT in the presence of trypsin (lower panel). 6-AHA inhibited IAV replication in the presence of PLG (upper panel), but had no effect when IAVs were produced in the presence of trypsin (lower panel). Results show the mean values±SD from three independent experiments. (b) Western blot analysis of lysates from MDCK cells infected with Flu-ΔNA or Flu-WT in the absence (Medium) or presence of PLG or trypsin (TPCK) and in the presence or absence of 6-AHA. Viral protein synthesis was assessed by Western blot analysis using anti-NS1 antibodies and anti-tubulin as control. Numbers to the left of the figure refer to molecular masses in kDa.
infected with IAVs Flu-ΔNA and Flu-WT was analysed and compared with PLG binding to uninfected cells. PLG binding was increased after infection with IAVs Flu-WT and Flu-ΔNA compared with uninfected cells (Fig. 3a). Interestingly, the level of PLG binding correlated with the level of infection, reflected by the expression of M2 on infected cells. Of note, staining of this protein was more efficient for Flu-WT in our experiments, suggesting that Flu-ΔNA replicated less efficiently than Flu-WT. Without PLG, no signal was observed, confirming the specificity of detection of PLG binding to IAV-infected cells. To highlight the increase in PLG binding after IAV infection, we quantified the mean fluorescence intensity (MFI) of PLG binding (Fig. 3b). Results showed an increase in the MFI of PLG binding in Flu-WT- and Flu-ΔNA-infected cells compared with uninfected cells. Thus, increased PLG binding after IAV infection can occur in the absence of NA expression at the surface of infected cells.

A2 expression in Flu-ΔNA- or Flu-WT-infected cells
As it is unlikely that NA is responsible for increased PLG binding to IAV-infected cells, we wished to investigate whether A2, a cellular receptor for PLG (Hajjar & Krishnan, 1999), is involved. As shown in Fig. 4(a), cell-surface expression of A2 protein was increased after infection of MDCK cells with IAVs Flu-ΔNA or Flu-WT compared with uninfected cells. Detection of the viral protein M2 was included as a positive control for virus infection and was only observed after infection with either of the two viruses. Interestingly, the level of A2 expression correlated with the level of infection, reflected by the expression of M2 on infected cells. Also, similar results were obtained after infection of MDCK cells with A/Udorn/72 (H3N2) and A/WSN/33 (H1N1). To highlight the increase in A2 cell-surface expression after IAV infection, we quantified the MFI of A2 labelling (Fig. 4b). Results showed an increase in the MFI of A2 labelling in infected cells compared with uninfected cells. Increased cell-surface expression of A2 after IAV infection was not cell-type-specific, as similar results were also observed after IAV infection of A549 epithelial cells (Fig. 4c). Thus, infection of MDCK and A549 cells with IAVs increased the cell-surface expression of A2.

PLG binding to IAV-infected cells mediated by A2
As A2 expression on the cell surface is increased after infection with Flu-ΔNA or Flu-WT, we tested whether it mediated PLG binding to IAV-infected cells. To this end, PLG binding to Flu-ΔNA- or Flu-WT-infected cells was assessed by flow cytometry in the absence or presence of blocking anti-A2 antibodies. PLG binding to IAV-infected cells was inhibited by an A2-specific antibody, but not by an irrelevant antibody of the same isotype (Fig. 5a). To highlight these results, we quantified the MFI of PLG binding in the presence or absence of the anti-A2 antibody (Fig. 3b). Results showed an increase in the MFI of PLG binding in the presence of the isotype control versus the anti-A2 antibody. Again, the level of PLG binding to infected cells correlated with the extent of IAV infection, reflected by the expression of M2. Similar results were obtained after infection of MDCK cells with A/Udorn/72 (H3N2) but, interestingly, pre-incubation of infected cells with an anti-A2 antibody did not prevent PLG binding to cells infected with A/WSN/33 (H1N1). Collectively, these results showed that PLG binds to the surface of infected cells through A2 in the absence of NA expression.

Cleavage of HA by PLG is mediated by A2 in the absence of NA
As A2 not only binds PLG, but also converts it into plasmin (Hajjar & Krishnan, 1999), we hypothesized that A2 was
responsible for PLG-mediated HA0 cleavage in IAV-infected cells. To test this hypothesis, cells were infected with IAVs Flu-ΔNA or Flu-WT at low m.o.i. in the absence of proteases in order to detect uncleaved HA (HA0) by Western blot analysis. Sixteen hours post-infection, cells were incubated for 3 h in the absence or presence of PLG and in the presence or absence of lipoprotein (a) [Lp(a)], a competitive inhibitor of PLG binding to A2. Cells were then lysed and HA was detected by Western blot analysis using an HA-specific antibody, able to recognize both HA0 and HA2. After incubation with PLG [PLG, Lp(a)−], in addition to HA0, a band was detected at 25 kDa, corresponding to HA2. This cleavage of HA0 by PLG in infected cells was blocked fully by the addition of Lp(a), the inhibitor of PLG binding to A2. This inhibition was specific for the conversion of PLG by A2, as Lp(a) did not inhibit cleavage of HA0 in the presence of trypsin (TPCK). In uninfected cells (NI), HA0 was not detectable, confirming the specificity of the HA-specific antibody that was used. These data show that PLG-mediated cleavage of HA takes place in an A2-dependent fashion in the absence of NA.

**PLG promotes IAV replication in an A2-dependent fashion**

To further investigate the role of PLG in IAV replication, cells were infected with IAVs Flu-ΔNA or Flu-WT in the presence of PLG and in the presence or absence of the inhibitor Lp(a). At different time points post-infection, infectious virus titres were then determined by plaque assays. The PLG-dependent replication of IAVs Flu-ΔNA and Flu-WT was abrogated by the addition of Lp(a), resulting in replication kinetics similar to those seen under conditions without a protease (Fig. 7a). Again, inhibition was specific for PLG binding to A2, as Lp(a) did not affect replication of IAVs Flu-ΔNA and Flu-WT in the presence of trypsin (Fig. 7b). Thus, our results show that PLG-mediated IAV replication is dependent on A2 and independent of expression of NA.
DISCUSSION

In the present study, we have demonstrated that PLG supports the replication of IAV A/Puerto Rico/8/34 (H1N1) and that, for PLG-dependent replication, expression of the viral NA is not required. Furthermore, we have shown that PLG can bind to infected cells through the cellular protein A2, expression of which is increased upon infection. Once converted into plasmin, PLG provides an alternative protease activity responsible for the cleavage of the HA precursor molecule HA0, which is a prerequisite for influenza virus replication.

In cells, A2 is found as a monomer (A2m) and as a heterotetramer (A2t) consisting of two A2 molecules and two molecules of the protein S100A10 (p11), which is a member of the S100 protein family (Kwon et al., 2005). In the presence of micromolar cytoplasmic Ca\(^{2+}\) concentrations, A2 molecules heterotetramerize and bind reversibly to negatively charged membrane phospholipids (Balasubramanian et al., 2001; Rescher & Gerke, 2004). Thus, cytoplasmic A2 is probably translocated to the plasma membrane upon virus infection, due to dysregulation of Ca\(^{2+}\) homeostasis observed after IAV infection (Hartshorn et al., 1988). We showed here that the upregulation of cell-surface A2 allows the recruitment of PLG, a precursor of the protease plasmin, to the surface of IAV-infected cells. This phenomenon was described previously for the IAV A/WSN/33 (H1N1); it was reported that this virus used PLG efficiently through binding to its NA, which was defined by unique features of its amino acid sequence (Goto & Kawaoka, 1998; Goto et al., 2001). Of interest, it has been demonstrated that expression of the 1918 HA also facilitated robust PLG sequestration (Chaipan et al., 2009), suggesting that PLG capture is not an exclusive function of NA expression. Thus, there are different ways to achieve PLG binding to infected cells, including the...
expression of specific NA, the expression of specific HA and the expression of A2, as shown in the present study. However, the binding of PLG through A2 may be a more universal mode to acquire protease activity for the cleavage of HA, as A2 is a highly conserved cellular protein.

Binding of PLG at the surface of infected cells is not sufficient to support IAV replication. PLG is a pro-protease and conversion of PLG into its active form plasmin by a host factor is necessary for IAV replication. Thus, in addition to the binding of PLG at the surface of infected cells, the presence of a host PLG-activation system is also crucial for IAV replication. As A2 not only binds PLG, but also mediates the conversion of PLG into plasmin (Hajjar & Krishnan, 1999), it is thus likely that A2 is responsible for the activation of PLG at the surface of infected cells. In addition, as infection with IAV A/WSN/33 also upregulates expression of A2, it is possible that PLG binding and activation occur through both the NA of this virus and A2. A2 is probably not expressed in all cell lines, which consequently could be a limiting factor for PLG-dependent virus replication. Also, proteins that modulate the activation of PLG or plasmin, such as lipoprotein, which can block the binding of PLG to A2 or plasmin inhibitors (Favier et al., 2001), may influence the pathogenesis of IAV infections.

In addition to binding to infected cells, PLG recruitment probably also occurs at the surface of the virions, as we demonstrated previously that purified virions do contain A2 and can cleave PLG into plasmin (LeBouder et al., 2008). However, the A2 content of virus will probably depend on the A2 expression of the cells. Thus, cells that do not express A2 may not release A2-containing IAV particles, unless A2 is induced after infection, which may contribute to an increase of PLG-dependent virus replication. Among several viral determinants (Conenello et al., 2007; Garcia-Sastre, 2006; Rogers et al., 1983) and host immune responses (de Jong et al., 2006; Khoufache et al., 2009; LeBouder et al., 2009; Le Gal et al., 1999), the differential capacity of IAVs to use PLG for their replication may be an alternative viral determinant of IAV virulence.

Finally, our results also explain why several IAV strains that lack a carboxy-terminal lysine and an oligosaccharide at position 146 of the NA can replicate in a PLG-dependent fashion (LeBouder et al., 2008; Tumpey et al., 2005). Apparently, some IAVs can use PLG in the absence of NA, using A2 as an alternative way to recruit PLG and convert it into plasmin at the surface of infected cells.

The use of A2 is not restricted to IAVs, as other viruses, such as rabbit vesivirus (Gonzalez-Reyes et al., 2009), human immunodeficiency virus (Harrist et al., 2009; Ryzhova et al., 2006) and cytomegalovirus (Derry et al., 2007), also exploit this protein to increase their replication, which may be related to the fact that A2 is a highly conserved protein.

Collectively, the results presented here suggest that the PLG/A2 pathway may be an important host factor contributing to the pathogenesis of IAV infections.

**METHODS**

**Virus strains, cells, antibodies and reagents.** Wild-type (Flu-WT) and NA-deficient (Flu-ΔNA) influenza viruses A/Puerto Rico/8/34 (H1N1) were used in the present study. The latter was produced by
reverse genetics as described previously (Rimmelzwaan et al., 2007). MDCK (ATCC CCL 34) and human alveolar type II A549 cells (ATCC; CCL 185) were grown as described previously (Adrián Cabestré et al., 1999; Riteau et al., 2001a). For Western blot analyses, the following antibodies were used: monoclonal anti-HA (C102; Santa Cruz Biotechnology), monoclonal anti-M2 (Santa Cruz Biotechnology), polyclonal anti-NA (kind gift from Dr R.G. Webster, Memphis, TN, USA), polyclonal anti-A2 (Santa Cruz Biotechnology or Meridian Life Science), monoclonal anti-tubulin (Sigma-Aldrich), monoclonal anti-S100A10 (p11) (BD Biosciences), polyclonal anti-ERK (Cell Signaling Technology), polyclonal anti-PLG (Kordia), polyclonal anti-NS1 (Santa Cruz Biotechnology) and HRP-coupled antibodies directed against mouse, rabbit or goat (P.A.R.I.S.). Trypsin was purchased from Becton Dickinson, and PLG, 6-AHA and Lp(a) from Sigma-Aldrich.

**Virus production, purification and composition analysis.** MDCK cells were seeded at a density of 2 × 10^5 cells per 150 cm^2 tissue-culture flask and infected with IAVs at an m.o.i. of 10^-2 in Eagle’s minimum essential medium (EMEM) containing 1 μg TPCK-treated trypsin ml^-1, as done previously (Khoufache et al., 2009; LeBouder et al., 2009). For the propagation of Flu-ANA, NA from *Vibrio cholerae* (Sigma, # N6514) was added at a concentration of 0.1 μM ml^-1. Two days post-infection, the supernatant was harvested and purified by using a sucrose gradient as described previously (LeBouder et al., 2008). Virion-composition analysis was performed by either Western blotting (Riteau et al., 2003a, b) or MALDI-TOF mass spectrometry (LeBouder et al., 2008), as described previously.

**Kinetics of virus replication and inhibition by 6-AHA.** MDCK cells were infected with IAV at an m.o.i. of 10^-2 in EMEM supplemented with 2 μM PLG (unless indicated otherwise) or 1.5 μg TPCK-treated trypsin ml^-1 in the presence or absence of 6-AHA at a concentration of 3 mg ml^-1. For the propagation of IAV Flu-ANA, NA from *Vibrio cholerae* was added at a concentration of 0.1 μM ml^-1. At various time points post-infection, supernatants were collected and virus titres were determined by plaque assay as described previously (Riteau et al., 2006).

**Inhibition of viral protein synthesis by 6-AHA using Western blot analysis.** MDCK cells were infected with IAVs as described above in the presence of 5 μM PLG or 1.5 μg TPCK-treated trypsin ml^-1, with or without 6-AHA (30 mg ml^-1). Forty-eight hours post-infection, cells were lysed and proteins were analysed by Western blot analysis as described previously (Bernard et al., 2006).

**Detection of PLG binding to cells by flow cytometry.** MDCK or A549 cells were infected with the indicated IAV strain at an m.o.i. of 10 for 24 h. Cells were then incubated for 45 min with PLG (2 μM) and then washed extensively with PBS. Bound PLG was detected using a PLG-specific goat antibody (Jackson ImmunoResearch) by flow-cytometry analysis as described previously (Pascale et al., 2008; Riteau et al., 2001b).

**Detection of upregulation of A2.** MDCK cells were infected with the indicated IAV strain at an m.o.i. of 10 for 24 h. Protein expression was then assessed by flow-cytometry analysis as described previously (Riteau et al., 1999, 2001c).

**Antibody blocking experiments.** MDCK cells were infected with the indicated IAV strain at an m.o.i. of 10 for 24 h. Cells were then pre-incubated for 45 min with either an isotype control or a specific anti-A2 antibody. Subsequently, the cells were incubated with 2 μM PLG for 45 min, washed extensively with PBS and tested by flow cytometry for binding of PLG as described previously (Khalil-Daher et al., 1999; Khoufache et al., 2009).

**Conversion of HA0 into HA1 and HA2.** MDCK cells were infected with the indicated IAV strain at an m.o.i. of 0.5 for 16 h in the absence of proteases. Cells were then incubated with PLG (5 μM) or TPCK-treated trypsin (1.5 μg ml^-1) in the presence or absence of the competitive inhibitor of PLG binding to A2, Lp(a), at a concentration of 15 μM for 3 h (for Flu-WT) or 7 h (Flu-ANA). Cells were then lysed and proteins from the lysate were analysed by Western blot analysis as described previously (Menier et al., 2000).

**Kinetics of virus replication and inhibition by Lp(a).** MDCK cells were infected with the indicated IAV strain at an m.o.i. of 0.5 for 16 h in the absence of proteases. Cells were then incubated with PLG (5 μM) or TPCK-treated trypsin (1.5 μg ml^-1) in the presence or absence of Lp(a) at a concentration of 15 μM for 3 h (for Flu-WT) or 7 h (Flu-ANA). Lp(a) is a competitive blocker of PLG binding to A2 (Hajar & Krishnan, 1999). Then, fresh EMEM was added to the cultures (in the case of propagation of Flu-ANA, supplemented with exogenous NA from *Vibrio cholerae* at 0.1 μM ml^-1). Virus titres were determined in the supernatants of infected cells by plaque assay at the indicated time points post-infection, as described previously (Riteau et al., 2006).

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**REFERENCES**


Annexin 2 allows plasminogen-dependent IAV replication


