Involvement of annexin V in the entry of influenza viruses and role of phospholipids in infection

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Abstract Influenza viruses bind to annexin V, a widely spread non-glycosylated phospholipid-binding protein. Externally added phospholipids as well as antiserum against this protein specifically inhibit infection of these viruses in cell cultures. We conclude that annexin V plays an important role in the entry of these viruses.

Key words: Influenza virus; Binding protein; Receptor; Annexin; Phospholipid

1. Introduction

In the initial step of influenza virus infection, sialic acid (*N*-acetylneuraminic acid) on the cell surface is generally thought to be the only binding site and receptor for the virus (reviewed in [1]). However, we recently found a sialic acid-free protein in several cell lines, that clearly binds influenza A and B viruses [2]. This protein also binds phospholipids and has an apparent molecular mass of 33 kDa, suggesting that it is a member of a class of structurally homologous proteins, annexins I–V [3–7], that are known to bind phospholipids and possess very close molecular masses. We report here that a purified recombinant human annexin V, exhibiting a molecular mass of 33 kDa is equally capable of binding influenza viruses: fowl plaque virus, PR8 and Singapore. This shows that the 33 kDa virus-binding protein we found probably corresponds to annexin V. Further experiments showed that infection of influenza viruses could be inhibited by externally added phospholipids as well as by antiserum against annexin V. These results substantiated our view that annexins may serve as a second receptor for these viruses.

2. Materials and methods

2.1. Culture cell, viruses and annexin V

Continuous MDCK (Madin Darby canine kidney) cells originally obtained from the American Type Culture Collection, were grown to confluency in Dulbecco's medium containing 10% calf serum. Influenza virus strains fowl plaque virus (FPV; A/Rostock; Hav1, N1), PR 8 (A/PR/8; HO, N1) and Singapore (A/Singapore/6/86; H1, N1), were infected in these cells as described previously [2]. Recombinant annexin V was obtained from Serva, Heidelberg (rh annexin V, cat. no. BMF 306). Virus binding was detected by an overlay technique [8]. Annexin V was electrophoresed in an SDS-polyacrylamide gel and then blotted on nitrocellulose (Schleicher and Schüll: BA 85, 0.45 μm, No. 401396) and exposed to FPV. The position of virus binding was visualized by immunostaining of the viral hemagglutinin as described before [2].

2.2. Antibodies and immunostaining

Monoclonal antibodies against the hemagglutinin and the nucleocapsid protein of FPV and that against annexin 33 kDa were obtained from Drs Becht and Lim as previously described [2]. Anti-annexin V antiserum was produced in a rabbit by immunizing with 50 μg of annexin V in the presence of Freund's adjuvant at monthly intervals over a period of 2 months. The highest antibody titer obtained was about 1:500 as determined by ELISA. Immunostaining of the virus specific nucleocapsid protein (NP) in the nuclei of infected cells was performed 2 h after virus infection.

2.3. Phospholipids

All phospholipids used were Sigma grade pure compounds with the indicated catalogue numbers: phosphatidic acid (P-9511), phosphatidylcholine (P-3556), phosphadidylethanolamine (P-6386), phosphatidylinositol (P-5766), phosphatidylserine (P-7769) and sphingomyelin (S-7004). Phospholipids were added into the culture medium as clear suspensions of primarily unilamellar liposomes, using the ethanol injection method of Batzri and Korn [9]. For each phospholipid a stock solution was prepared in warm (37°C) ethanol at the concentration of 10 mM. Just before experiments, the indicated portions of this stock solution were taken up with an Eppendorf micropipette and rapidly injected into the culture medium. In all cases, the culture medium remained optically clear after the addition of phospholipids.

3. Results and discussion

Fig. 1 shows that FPV binds to annexin V at the position of 33 kDa. The results obtained with PR8 and Singapore were the same (not shown). These findings indicate that annexin V binds these viruses and that it probably corresponds to the 33 kDa virus-binding protein we found previously [2]. The crucial question is whether binding of influenza virus to annexin V (or annexin 33 kDa) is an essential step for infection. Annexins are thought to be both intracellular and plasma membrane components [10–12]. If the binding of viral phospholipids to the annexins of the plasma membrane is an essential step for infection, externally added phospholipids should compete in this reaction, resulting in the inhibition of virus infection. This rationale was tested with MDCK (Madin-Darby canine kidney) cells that were infected with the viruses FPV, PR8 and Singapore in the presence or absence of phospholipids. Fig. 2 shows typical results obtained with phosphatidylethanolamine (PE), a major phospholipid of the viral envelope [13]. At the virus input of 50 plaque forming units (pfu), the virus yield at the end of an 8 h reproduction cycle was inhibited progressively by PE at concentrations between 0.1 and 0.3 mM. When the virus input was elevated to 100 and 200 pfu, PE became less effective at these concentrations. When infection time was prolonged to 24 h, virus infection could be partially restored even in the presence of PE. These results clearly indicate that PE competitively inhibited virus infection in a dose- and time-dependent manner. All phospholipids were found to inhibit virus infection. However, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and phosphatidic acid were more effective, needing only about one half to one third the concentrations of phosphatidylchol-

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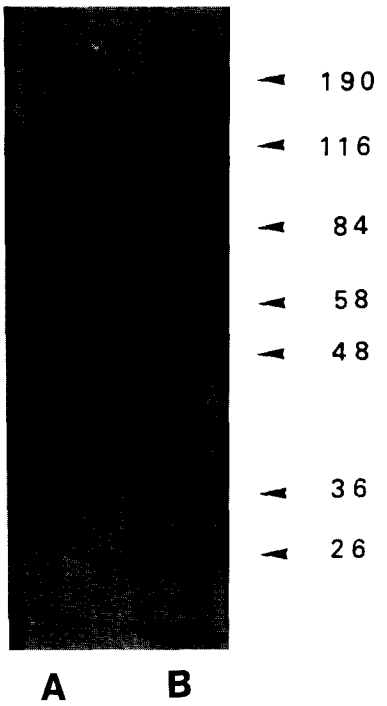

Fig. 1. Binding of influenza virus FPV to annexin V on nitrocellulose. Annexin V was separated alongside with stained molecular weight markers in an SDS-polyacrylamide gel (10%), blotted onto nitrocellulose and overlaid with the virus. (A) Annexin V; (B) molecular weight markers in kDa on the right.

ine and sphingomyelin for the complete inhibition of infection (Table 1). This is in accordance with the known binding specificity of annexins to various phospholipids [6]. The suppression of virus infection by phospholipids could also be demonstrated early by the absence of virus-specific nucleocapsid protein (NP) synthesis when phospholipids were present in the medium during the entry phase of the viruses: Fig. 3 shows that there was complete inhibition of NP synthesis 2 h after infection, when PE was present in the medium.

In further experiments anti-annexin V antiserum was used to determine whether annexin V is localized on the surface of cells and whether this antibody can block virus-binding, resulting in inhibition of virus infection. This serum was found to react with monolayers of MDCK cells and elicited plasma membrane fluorescence after treatment with FITC-labelled anti-rabbit IgG, indicating the presence of annexin V on the surface of these cells (not shown). This is in accord with reports that annexins are intracellular as well as plasma membrane components [10–12]. To test the effect of anti-annexin V antiserum on virus multiplication, it was added to cells before viruses were given and the cells were maintained in the constant presence of this antiserum. Virus infection was ascertained by immunofluorescence detection of the early virus-specific NP in the same manner as described before [14]. It was found that NP synthesis was greatly suppressed at 1:20 and 1:40 antiserum dilutions. In contrast, pre-immune serum did not show this effect (Table 1).

All the above results substantiated the view that annexin V is needed for influenza virus infection, probably as a second receptor. Influenza viruses possess spikes of hemagglutinin and neuraminidase in their envelope [15]. The hemagglutinin binds to the first receptor, sialic acid, when the virus initially encounters the cell. Reaction with annexin probably occurs after the virus is drawn to its proximity by sialic acid. Annexins have been reported to fuse membranes at neutral pH [16]. This occurs by a different mechanism than the acid-triggered fusion mediated by influenza virus hemagglutinin [17]. Influenza viruses are widely believed to fuse in the acidic environment of endosomes [18]. However, another view favours the fusion of these viruses with the plasma membrane at neutral pH [19]. In the latter case, annexins could participate in the fusion process. It is of interest to note that other enveloped viruses have also been shown to use annexins as receptors: Hepatitis B virus [20] and human cytomegalovirus [21] react respectively with annexins V and II. It remains to be estab-

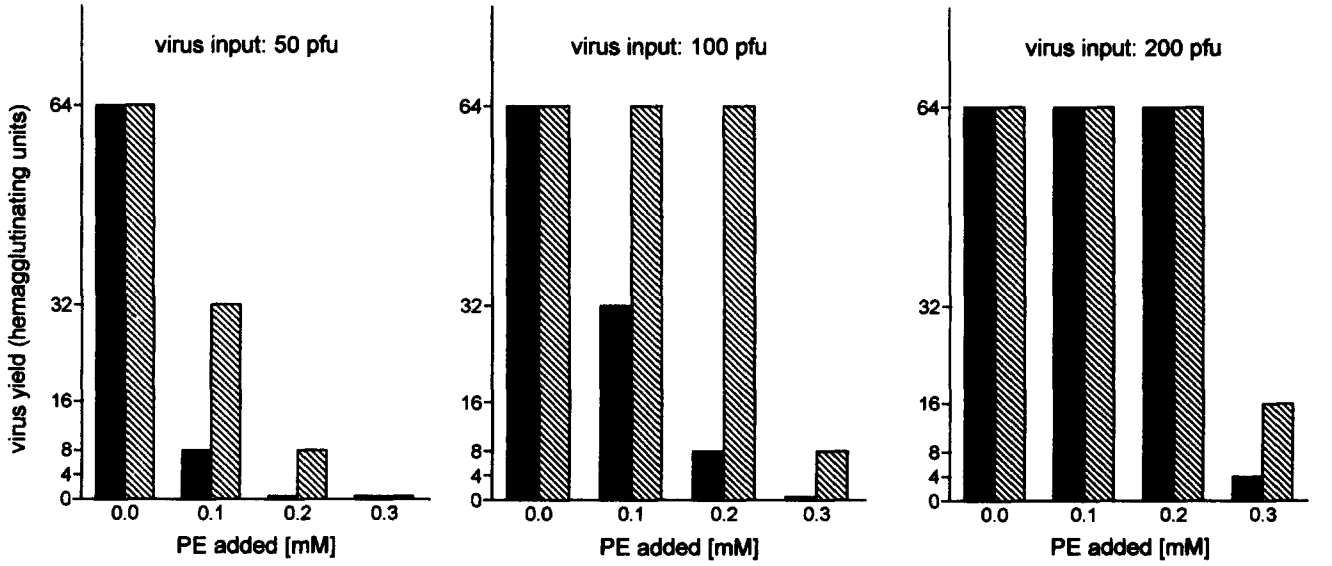

Fig. 2. Competitive inhibition of influenza virus (FPV) infection by phosphatidylethanolamine (PE) in MDCK cells. (Filled bars) Virus yield obtained 8 h after infection and (hatched bars) virus yield obtained 24 h after infection. Virus yield is shown as cell-associated hemagglutinating units measured after three cycles of freeze-thawing. PE was added as very fine unilamellar liposomes.

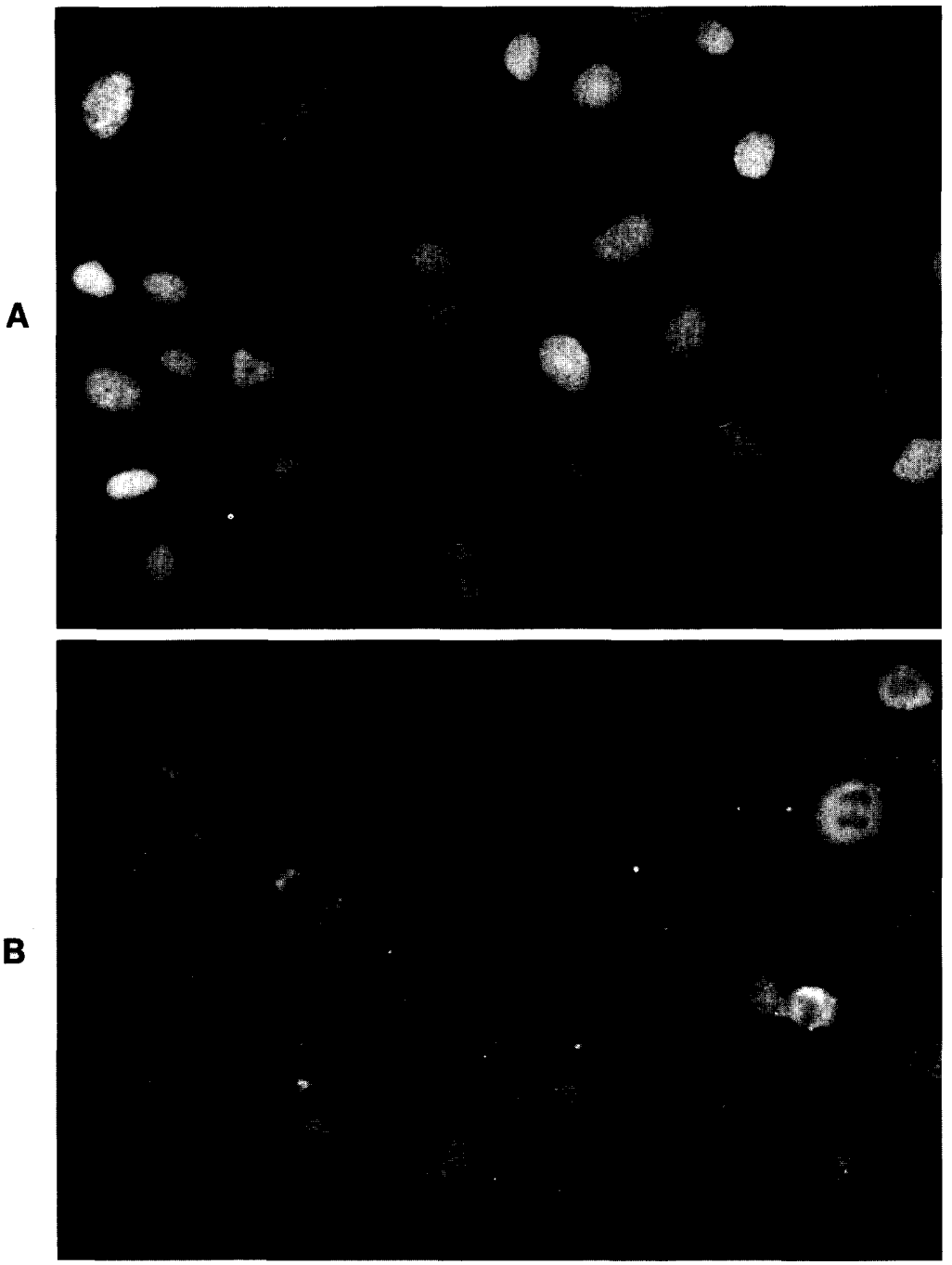

Fig. 3. Inhibition of viral nucleocapsid protein (NP) synthesis by PE. (A) Positive test for NP in the nuclei of cells in the control. (B) Negative test for NP in the nuclei of PE supplemented sample. MDCK cells were covered in Dulbecco's medium, supplemented or unsupplemented with PE (0.3 mM), and infected with FPV at an input of 50 pfu for 2 h. Dishes were fixed with methanol, treated with a monoclonal antibody against NP and stained with a fluorescein-labelled anti-mouse IgG.

Table 1
Inhibition of FPV infection in MDCK cells by various supplementations in the culture medium

Supplementation in medium	Dilution	Concentration (mM)	% infected cells[b]
PA, PS, PI, PE		0.1–0.2	0
PC, Sphm[a]		0.4–0.6	0
Pre-immune serum	1:20–1:40		100
Anti-annexin V antiserum	1:20–1:40		0–10
	1:80–1:160		10–100

[a]PA, phosphatidic acid; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Sphm, sphingomyelin.
[b]Estimated by counting the numbers of NP-positive and NP-negative cells in a microscopic field.

lished how widely annexins are used as receptors for enveloped viruses in general.

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