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Identification of annexin 33 kDa in cultured cells as a binding protein of influenza viruses

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Abstract The binding of three influenza A and one influenza B virus strains to proteins of three continuously cultured cell lines was studied using protein overlay and immunostaining methods. The results obtained indicated the presence of both sialic acid-dependent and -independent binding of the virus strains; virus binding to proteins in the molecular mass range from about 40 to 103 kDa was dependent on sialic acid, whereas binding to the 33 kDa protein was independent of sialic acid. The 33 kDa binding protein was identified as annexin, a widely distributed non-glycosylated calcium-dependent phospholipid-binding protein.

Key words: Binding protein; Annexin 33 kDa; Phospholipid binding; Virus binding; Influenza virus

1. Introduction

The first step in a virus infection is the recognition of cell surface receptors by the virus. The receptor of influenza viruses is known to be sialic acid (*N*-acetylneuraminic acid), which is linked to the oligosaccharide side chains of glycolipids and glycoproteins [1,2]. Sialic acid is needed for the initial attachment of the virus to the host cell surface. This step is mediated by the hemagglutinin of the viral membrane. Sialic acid is bound to the hemagglutinin fragment HA1, while the hemagglutinin fragment HA2 is thought to mediate the fusion between viral and cellular membranes [2–5]. The fusion step, a prerequisite for virus entry, is strongly inhibited by certain phospholipids and glycolipids [6], presumably because they compete with membrane lipids involved in the fusion process. Thus, cell penetration by these viruses appears to depend on a sequence of linked reaction steps. It is possible that other, as yet unknown reactions, may also be involved in this sequence of events.

Information on virus–cell interactions can be obtained by screening cell membrane components for their ability to bind the virus. Gershoni et al. developed an overlay method to detect proteins of the erythrocyte membrane that can bind to Sendai virus [7]. We have used this method and immunostaining techniques to localize cellular proteins that can interact with influenza viruses. It was shown that three continuous cell lines, MDCK, BHK and HeLa cells, contain both sialic acid-dependent and -independent binding proteins for influenza viruses. This report concentrates on a sialic acid-independent virus-binding protein, 33 kDa annexin. This non-glycosylated protein is a member of the ubiquitous annexin family that has been shown to possess a variety of important cellular functions such as calcium and phospholipid binding [8–13], signal transduction [14], and regulation of cell proliferation [15]. That annexin can also bind viruses is a novel facet of its many biological roles.

2. Experimental

2.1. Cells and viruses

Continuous cell lines of MDCK (Madin Darby canine kidney) cells, BHK (baby hamster kidney) cells and HeLa cells were grown to confluency in Dulbecco's medium and used for protein separation and blotting. Human erythrocyte ghosts were prepared according to Fairbanks et al. [16].

2.2. Viruses and purification

Three influenza A viruses, fowl plague virus (FPV A/Rostock; Hav1N1), PR8 (A/PR/8/34; H0N1) and Singapore (A/Singapore/6/86; H1,N1) as well as one influenza B virus (B/Yamagata/16/88) were used. Viruses were propagated in the allantoic cavity of 11-day-old embryonated eggs. The allantoic fluid containing the viruses were harvested and purified on sucrose gradients according to Klenk et al. [17].

2.3. Virus labelling with [^{14}C]amino acids or fluorescein isothionate (FITC)

Viruses were grown in MDCK or BHK cells, collected from culture medium and purified on sucrose gradients according to Klenk et al. [17]. For radioactive labelling, a mixture of L-[^{14}C]amino acids were included in the medium at a concentration of 800 kBq/ml during the period of 3–10 p.i. Viruses were then harvested at 10 p.i., pelleted by centrifugation at 20,000 × *g* for 60 min and purified on sucrose gradients as above.

Purified virus concentrates containing a hemagglutinating unit of 128,000/ml were used for fluorescein labelling essentially according to Forni [18]. Fluorescein-labelled viruses were found to retain all of their hemagglutinating activity.

2.4. Polyacrylamide gel electrophoresis, blotting and overlay of viruses on protein blots

MDCK, BHK and HeLa cells grown to confluency were washed with saline and harvested by a rubber policeman and processed further for SDS-PAGE as described by Laemmli [19]. Slab gels were blotted onto nitrocellulose filters (Scheicher & Schull; BA 83, 0.2 μm, no. 401396) as described by Gershoni et al. [7], using a Mini Trans-Blot apparatus (Bio-Rad).

Protein blots were treated with block solution (PBS containing 1% bovine serum albumin and 1% polyvinyl pyrrolidone) for 2 h at room temperature. The blots were washed with PBS and incubated with virus suspensions containing 1024 hemagglutinating units/ml at 4°C overnight. The blots were washed and air dried. In the case of ^{14}C-labelled viruses, virus binding was detected by autoradiography using Kodak X-OMAT AR film. In the case of fluorescein-labelled viruses, bound viruses were detected directly in a chamber equipped with a multi-wavelength illuminator.

2.5. Monoclonal antibodies and immunostaining

Monoclonal antibody against the hemagglutinin fragment HA1 of FPV was kindly supplied by Dr. H. Becht of the Institute of Virology, Justus-Liebig Universität, Giessen. This antibody was produced and characterized as previously described [20]. Monoclonal antibody against annexin 33 kDa was supplied by Dr. Lim of our Institute [21].

Protein blots overlayed with FPV as described above were immersed

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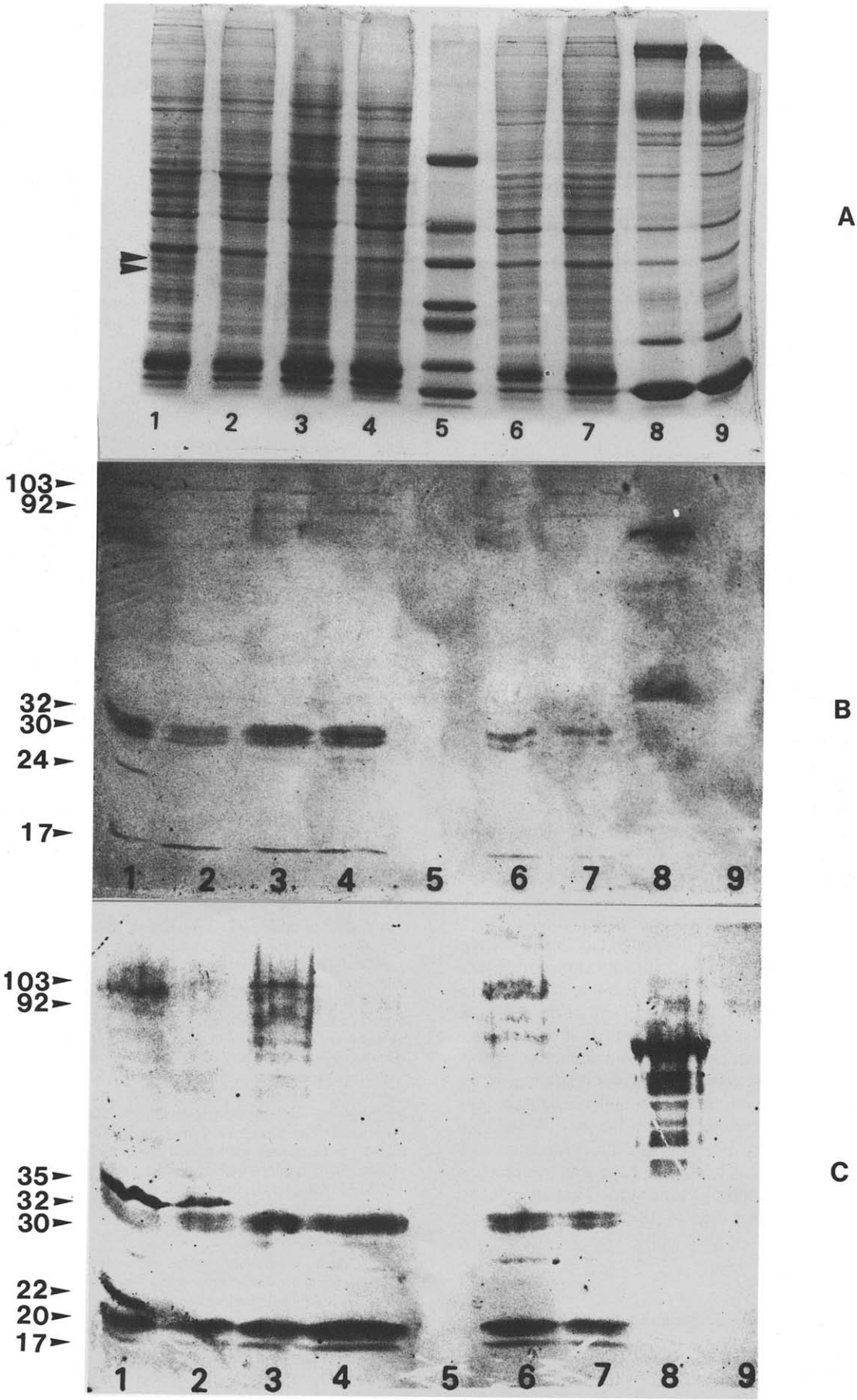
A
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in a 1:400 diluted monoclonal anti-FPV antibody solution for 2 h at 4°C. The sheets were washed five times with PBS and treated once with block solution for 30 min, then incubated with a 1:1000 diluted peroxidase-coupled anti-mouse IgG (Sigma cat. no. A9044) for 2 h at room temperature. The general procedure was basically as described by Gershoni et al. [7]. Immunodetection of annexin 33 kDa was performed similarly as above.

2.6. Miscellaneous

Cells harvested from monolayers with rubber policeman and suspended in physiological saline were treated with 0.1 unit of neuraminidase from *Vibrio cholerae* (Behringwerke, Germany) for 2 h at 37°C in a total volume of 1 ml prior to electrophoresis. Protein blots were also treated with neuraminidase in the same manner.

Periodate oxidation was performed on protein blots in a 0.1 M sodium periodate solution for 2 h at room temperature in a similar way as described by Suttajit and Winzler [22].

Dansyl phosphatidylethanolamine was prepared by reacting a commercially available phosphatidylethanolamine (Sigma cat. no. P5149) with dansyl chloride in the same manner as that described for synthesis of dansyl cerebroside [23]. Dansyl phosphatidylethanolamine was dissolved in PBS at a concentration of 1 μg/ml by a brief ultrasonic treatment.

3. Results and discussion

SDS-PAGE of the proteins (Fig. 1A) of MDCK (lanes 1, 2), BHK (lanes 3,4), HeLa (lanes 6,7) cells and erythrocytes (lanes 8, 9) produces complicated patterns. As can be seen from this figure, protein patterns of the same cells are not different when the cells have been pretreated with the neuraminidase of *Vibrio cholerae* before electrophoresis (Fig. 1A, lanes 2, 4, 7 and 9). However, when these protein patterns were blotted onto nitrocellulose, then overlayed with ^{14}C-labelled influenza virus strains FPV and PR 8, it was found that the viruses were bound to different proteins. Fig. 1B, C, lanes 1, 3, 6 and 8 show the patterns of virus-binding when cells have not been treated with the neuraminidase: generally, FPV and PR 8 were bound differently to proteins of 35, 92 and 103 kDa and to fast moving compounds near the gel front. However, both viruses were equally bound to the 30 and 33 kDa proteins in all cultured cell lines. When erythrocyte proteins were used, both viruses bound only to the higher molecular mass proteins (Fig. 1B, C, lane 8).

Binding patterns were altered by treatment of cells with the neuraminidase (Fig. 1B, C, lanes 2, 4 and 7); the high molecular mass proteins in the 90 kDa range were no longer able to bind the viruses, whereas binding to the 30 and 33 kDa proteins was retained by both FPV and PR 8. The same results were observed after destruction of sialic acid on the blot with periodate (not shown). These results showed that the binding to the 30 and 33 kDa proteins is independent of sialic acid. In similar studies with proteins from erythrocytes, the removal of sialic acid with neuraminidase resulted in the abolition of all virus binding (Fig. 1B, C, lane 9). Sialic acid is therefore an essential constituent of all virus-binding proteins of erythrocytes.

Another experiment was performed with the B/Yamagata strain that had been prelabelled with fluorescein. The fluorograms of virus binding are shown on Fig. 2. As in the previous case, the virus bound to proteins in the high molecular mass range, as well as to the 33 kDa protein before neuraminidase treatment (Fig. 2, lane 2). After neuraminidase treatment, only the 33 kDa protein remained bound to the virus (Fig. 2, lane 3). Virus binding to the 35 kDa protein and other fast moving compounds were not observed in this case.

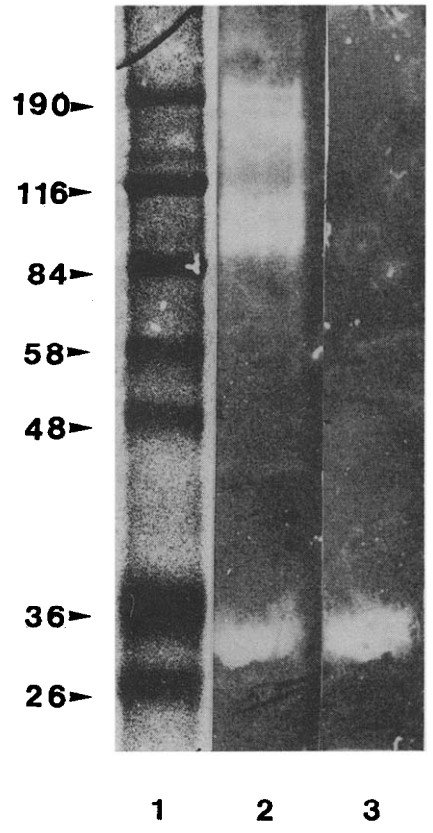

Fig. 2. Overlay of FITC-labelled virus (B/Yamagata) on protein blots of BHK cells. Lane 1, marker proteins; in lane 2 the blot was untreated and in lane 3 the blot was treated with neuraminidase prior to overlay with virus.

In another experiment using immunoblotting for the detection of virus binding, influenza virus strain FPV was found to bind to several high molecular mass protein bands as well as to the 33 kDa protein and some fast moving compounds (Fig. 3A, lane 2). After treatment of the protein blots with neuraminidase, only the 33 kDa protein remained capable of binding the virus (Fig. 3A, lane 3). Thus the 33 kDa protein was again found to be the common component of the cells that bound the viruses in a sialic acid-independent manner. To identify this sialic acid-independent virus-binding protein of 33 kDa, we compared it with other widely distributed proteins of the same molecular mass. It was found that one of such proteins, 33 kDa annexin, was stained exactly at this position in a Western blot, using a monoclonal antibody against this protein (Fig. 3A, lane 4).

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Fig. 1. Total proteins (A) of cultured cells and positions of virus binding for FPV (B) and PR8 (C) strains on nitrocellulose blots of these proteins. MDCK (lanes 1, 2), BHK (lanes 3, 4), HeLa (lanes 6, 7) cells and human erythrocyte ghosts (lanes 8, 9); lanes 1, 3, 6 and 8 are from cells that had not been treated with neuraminidase prior to SDS-PAGE; lane 5 is a molecular weight standard mixture, showing, from top to bottom, 66, 45, 36, 29, 24, 20 and 14 kDa.

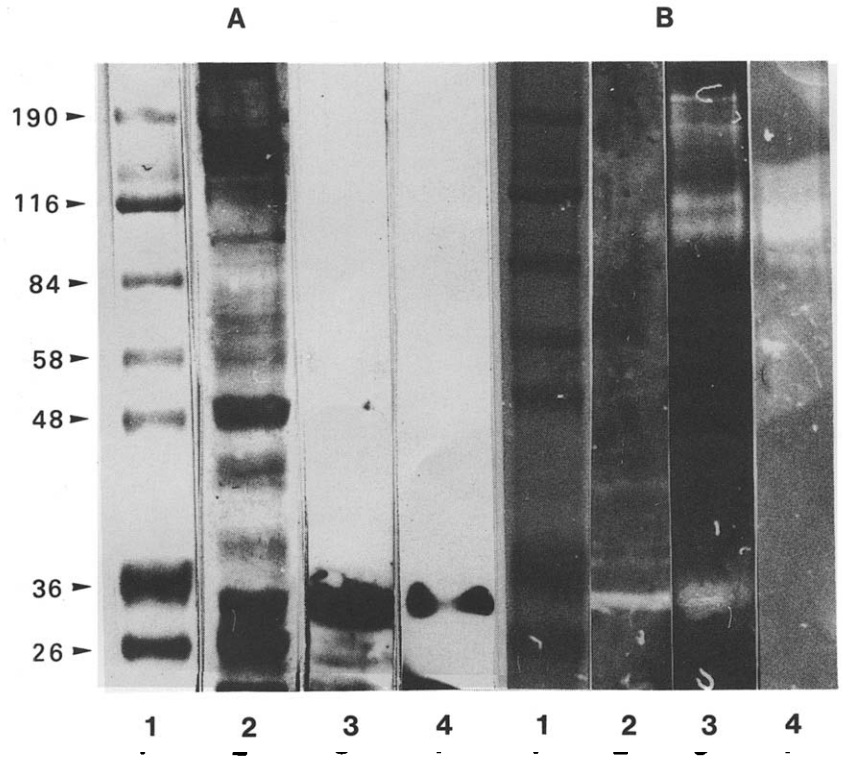

Fig. 3. Binding patterns of FPV, 33 kDa annexin, dansylphosphatidylethanolamin and A/Singapore on protein blots of BHK cells. (A) Immunostaining: lane 1, marker protein; lane 2, FPV overlaid on blot of BHK cell proteins; lane 3, FPV overlaid on blot of BHK cell proteins pretreated with neuraminidase; lane 4, overlay of 33 kDa annexin on BHK cell protein blot. (B) Fluorogram: lane 1, marker protein; lane 2, dansylphosphatidylethanolamin; lane 3, overlay of A/Singapore on blot; lane 4, overlay of A/Singapore on blot pretreated with monoclonal antibody against 33 kDa annexin.

From the following additional evidence, using fluorescein-labelled virus (A/Singapore), the 33 kDa virus-binding protein was conclusively identified as 33 kDa annexin, a family of phospholipid binding proteins. First, dansyl phosphatidylethanolamine, a fluorescence-labelled phospholipid, was shown to be bound exactly to the 33 kDa position (Fig. 3B, lane 2). Second, this position corresponds to that of the 33 kDa virus-binding protein detected by fluorescence-labelled virus (Fig. 3B, lane 3). Third, protein blots pretreated with the monoclonal antibody against 33 kDa annexin lost the ability to bind the viruses at the 33 kDa position while retaining the virus binding in the higher molecular mass range (Fig. 3B, lane 4), showing that free, unbound annexin is necessary for virus binding.

The above findings are surprising because sialic acid is generally considered to be the only cellular receptor of influenza viruses [1,2]. Influenza viruses possess densely packed glycoprotein spikes extending from the lipid bilayer which contains phospholipids. Some bending or compression of the spikes during virus attachment to the cell might make the phospholipids accessible to 33 kDa annexin. In this way, virus binding can then occur independently of sialic acid. It is possible that 33 kDa annexin of the plasma membrane can function as a 'second' receptor to enable infection of influenza viruses that are detached from the 'first' receptor, sialic acid, by the viral neuraminidase. In contrary, it is also possible that virus binding to annexin is a mechanism used by cells to fend off virus infection: virus that binds to annexin may be immobilized on the plasma membrane and thereby prevented from entering the cell. Thus, the consequence of virus binding to 33 kDa annexin can be either promotion or inhibition of infection, depending on which of the above mechanisms is operating.

33 kDa annexin may be of interest as a binding protein for enveloped viruses in general. It is noteworthy that the same technique has revealed the presence, in several cell lines, of 30–35 kDa binding proteins for a number of unrelated enveloped viruses. A recent study reported the binding of human cytomegalovirus to two proteins belonging to lymphocyte and fibroblast cell membranes. Their molecular masses were estimated to be 32 and 34 kDa [24]. Similarly, proteins with molecular masses between 30 and 33 kDa have recently been identified as putative receptors for the visna virus [25]. These binding proteins have not been characterized in detail, but because of the closeness of the reported molecular masses and their common ability to bind phospholipid-containing enveloped viruses, some of these may well be the same 33 kDa annexin described in the present study. What role annexin plays in the infection of these viruses is of great interest for further studies.

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