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Protein blot analysis of virus receptors: identification and characterization of the Sendai virus receptor

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Receptors for Sendai virions in human erythrocyte ghost membranes were identified by virus overlay of protein blots. Among the various erythrocyte polypeptides, only glycophorin was able to bind Sendai virions effectively. The detection of Sendai virions bound to glycophorin was accomplished either by employing anti-Sendai virus antibodies or by autoradiography, when ^{125}I-labeled Sendai virions were used. The binding activity was associated with the viral hemagglutinin/neuraminidase (HN) glycoprotein, as inferred from the observation that the binding pattern of purified HN glycoprotein to human erythrocyte membranes was identical to that of intact Sendai virions. No binding was observed when blots, containing either human erythrocyte membranes or purified glycophorin, were probed with the viral fusion factor (F glycoprotein). Active virions competed effectively with the binding of ^{125}I-labeled Sendai virions (or purified HN glycoprotein), whereas no competition was observed with inactivated Sendai virus. The results of the present work clearly show that protein blotting can be used to identify virus receptors in cell membrane preparations.

Introduction

The selective binding of a particular enveloped virus to a specific cell type or tissue is an essential step in the process of infection, and is thought to be mediated via discrete viral receptors [1]. When present on the plasma membrane, these receptors render the cell amenable to viral invasion. Therefore, in studying the pathogenesis of a virus and the detailed mechanism of virus-cell interaction, it is desirable to identify its corresponding receptor and to characterize the molecular forces involved in viral binding.

As yet, only a few virus receptors have actually been identified. For example, phosphatidylserine molecules have been suggested to serve as receptors for vesicular stomatitis virus [2]. Recently, it has been postulated that the nicotinic acetylcholine receptor may serve as the rabies virus receptor [3]. Over the years, mechanisms concerning the binding of viruses belonging to the myxovirus and paramyxovirus groups have been extensively studied [1,4–6]. It has been clearly demonstrated that Sendai virus, a paramyxovirus, binds to membrane components containing sialic acid, such as sialoglycoproteins and sialoglycolipids [1,6].

A convenient method to correlate the structure of certain well characterized membrane components with their ability to serve as functional virus receptors is to insert these components into virus-

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Abbreviations: HN glycoprotein, hemagglutinin/neuraminidase glycoprotein; F glycoprotein, fusion factor.

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receptor-depleted cells [6–8]. Such cells should become susceptible to infection by viruses, the receptor for which has been inserted into their membrane. Indeed, neuraminidase-treated Madin-Darby bovine kidney cells became susceptible to infection by Sendai virions if sialoglycolipids of a certain composition were inserted into their membranes [6]. Similarly, incorporation of an isolated sialoglycoprotein (GP-2) into neuraminidase-treated bovine erythrocytes restored their ability to interact and to be lysed by Sendai virions [7].

Evidently, the above approach, i.e., insertion of isolated and characterized membrane components into cell plasma membranes in order to study their ability to serve as functional virus receptors, necessitates a preliminary knowledge of their ability to interact and bind virus particles. This was indeed the case regarding the interaction between Sendai virions and sialoglycolipids or sialoglycoproteins [6,7]. This kind of information could be obtained if a direct and quick method for following virus-ligand binding were available. For the development of such a direct scanning method, the interaction between Sendai virions and their known membrane receptors can serve as an excellent model system.

In the present work, a novel methodology which allows the screening of membrane polypeptides as putative virus receptors has been developed, namely, virus overlays of protein blots. Protein blotting has become extremely useful, not only for the detection of antigens with antibodies, but also for general analysis of protein-protein and protein-ligand interactions [9,10]. In this study, using protein blots, we demonstrate the specific binding of Sendai virus to its protein receptor on human erythrocyte ghosts. Moreover, the procedure used allows the verification of the viral proteins responsible for this binding activity.

Materials and Methods

Preparation of Sendai virus and membrane vesicles containing either the viral HN or the F glycoproteins

Sendai virus was isolated from the allantoic fluid of fertilized chicken eggs, and its hemagglutination titer was determined [11]. Membrane vesicles, containing either the purified viral HN or F glycoproteins, were prepared as previously described [12]. Iodination of Sendai virions or of the viral HN and F glycoproteins was performed by the use of $Na^{125}I$ (Amersham, U.K.) and Iodogen™ (Pierce, U.S.A.) [13].

Anti-virus antibodies

Rabbit anti-Sendai virus antibodies were obtained after injection of reconstituted viral envelopes [14]. The IgG fractions of the rabbit antiserum were obtained after precipitation in ammonium sulfate [15]. The anti-Sendai virus antibodies reacted with the viral F and HN polypeptides, as analyzed by immuno-overlays of viral protein blots (data not shown).

Human erythrocyte ghosts

Human erythrocyte ghosts were prepared from recently outdated human blood, type O, Rh^+, according to the method of Fairbanks et al. [16].

Preparation of glycophorin from human erythrocytes

The major human erythrocyte membrane glycoprotein, glycophorin, was purified from freshly drawn blood, as described previously [17]. When indicated, the purified glycophorin (600 μg) was treated for 1 h at 37°C with 50 μl of neuraminidase (*Vibrio cholera*, 1 U/ml, Behringwerke, F.R.G.) in 1 ml of acetate buffer (50 mM sodium acetate-acetic acid (pH 5.5), 154 mM NaCl and 6 mM $CaCl_2$).

Gel electrophoresis and protein blotting

Human erythrocyte ghosts (70–100 μg) or purified glycophorin (5 μg) were separated by SDS-polyacrylamide gel electrophoresis on 10% acrylamide slab gels, using the Laemmli buffer system [18]. Protein blotting onto nitrocellulose membrane filters was performed as described earlier [19], for 3–6 h at 70–90 V, using an apparatus equipped with an electrode array designed to generate a gradient electric field so as to compensate for the effect of molecular weight on the transfer efficiency. It has been well documented that, using this apparatus and the present experimental conditions, the electrical field generated throughout the different tracks of the gel is homogeneous [20]. Therefore, the efficiency of transfer for the same polypeptides in the various tracks

is identical [20]. A Bio-Rad 250 V/2.5 A power supply was used.

Probing of the blots

A. Immuno-detection of bound Sendai virus antigens. Blots were probed with Sendai virions or with Sendai virus isolated components (HN or F polypeptides), as indicated. Prior to probing, the blots were quenched in 3% bovine serum albumin complemented with 10% newborn calf serum in phosphate-buffered saline (140 mM NaCl, 10 mM sodium phosphate, pH 7.4) for 1–2 h at 25°C. Then the probes (Sendai virus: 150 μg/blot; HN polypeptide: 60 μg/blot; F polypeptide: 60 μg/blot) were added to the blots in a solution of 15 ml 3% bovine serum albumin in phosphate-buffered saline, and incubated for 2 h at 25°C with constant shaking. The blots were subsequently washed at least five times for 15 min in phosphate-buffered saline (in some instances the filters were allowed to wash overnight with no apparent deleterious or advantageous effects). After the wash, the blots were incubated with anti-Sendai virus antibodies (150 μg) in 15 ml of 50 mM Tris-HCl (pH 7.6), 0.05% Tween 20 and 3% bovine serum albumin for 1 h, washed four times and incubated with horseradish peroxidase conjugated to goat anti-rabbit-IgG (Bio-Yeda, Rehovot, Israel), diluted 1 : 1000 in 15 ml of phosphate-buffered saline–3% bovine serum albumin. After washing, the presence of horseradish peroxidase was demonstrated by reacting the blots with 25 mg diaminobenzidene and 0.003% H_2O_2 (v/v) in 50 ml of 50 mM Tris-HCl (pH 8.6) [21].

B. Autoradiographic demonstration of bound ^{125}I-labeled Sendai virus proteins. In this case, ^{125}I-labeled Sendai virions or ^{125}I-labeled Sendai virus envelope glycoproteins were used to probe the blots (10–20 μg/blot, equivalent to $(5–10)\cdot 10^5$ cpm). Prior to probing, the blots were quenched with 2% bovine serum albumin in phosphate-buffered saline for 1 h at 25°C. Then the probes were added to the blots in 5 ml of the above quenching buffer, and incubated with continuous shaking for 2 h at 4°C. After five washes, each for 10 minutes, the blots were autoradiographed at −70°C, using Kodak X-AR-5 film and a Lightning-Plus (DuPont) intensifying screen. Exposures were carried out for 12–96 h.

Staining of blots

The transferred protein pattern could be demonstrated, without modifying the proteins, by a method of negative staining in which the nitrocellulose membrane filter is stained, while the protein bands remain unstained (to be published elsewhere).

Results

Immunodetection of Sendai virions bound to their membrane ligands

In order to determine whether virus binding to immobilized erythrocyte membranes could be detected, dot-blot experiments were performed. Our results demonstrate that by applying this approach, viral binding can be detected using as little as 125 ng protein of erythrocyte membranes (Fig. 1A) or 8 ng of glycophorin (Fig. 1B) as ligands (see arrows). This corresponds well with data showing that glycophorin represents 4% of the erythrocyte membrane proteins [22]. This binding could be prevented by pretreatment of the Sendai virions with dithiothreitol or preincubation with fetuin, a sialoglycoprotein (Fig. 1A). Moreover, no binding could be observed with neuraminidase-treated glycophorin (Fig. 1B).

Fig. 2 depicts the binding of Sendai virions to blotted human erythrocytes and purified glycophorin. As shown, all the erythrocyte polypeptides are represented on the nitrocellulose membrane filters (compare lanes a and c). The same applies to the purified glycophorin polypeptide (lanes b and d). Among the various erythrocyte membrane polypeptides, only glycophorin A binds Sendai virions (lane e). Binding of Sendai virions to the purified erythrocyte glycophorin polypeptide can also be seen (lane f). A strong signal is visible at the location of the glycophorin dimer, while a faint signal is detectable in some experiments for the residual amounts of the monomeric form of this protein (see Fig. 3a, c) [22].

Reduction of Sendai virions with dithiothreitol has been shown to specifically inactivate its binding protein, i.e., the viral envelope HN glycoprotein [23]. Dithiothreitol-treated virions are unable

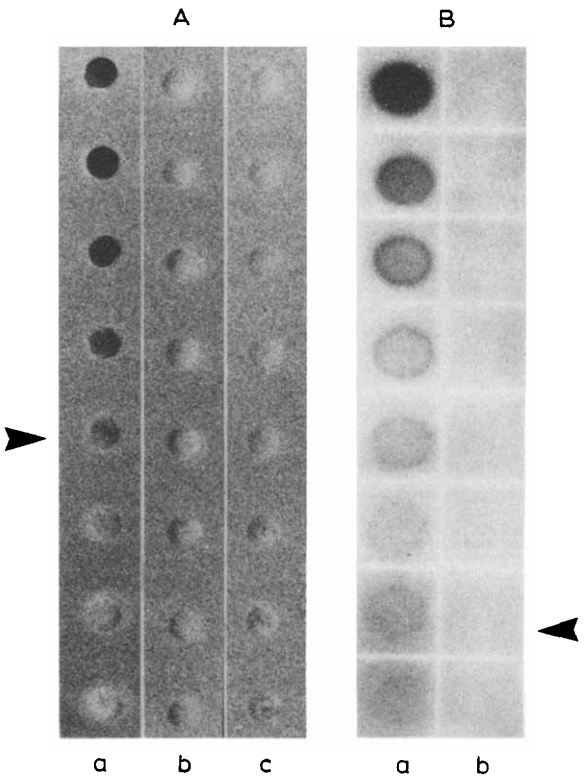

Fig. 1. Binding of Sendai virions to erythrocyte ghost membranes as revealed by dot-blots. (A) Decreasing amounts of human erythrocyte ghost membranes (2-fold dilutions starting with 2 μg protein or (B) decreasing amounts of glycophorin (2-fold dilution starting with 0.5 μg protein) were applied to nitrocellulose membrane filters, (A) using a Bio-Dot apparatus (Bio-Rad) (B) or by dotting manually. The filters were quenched and then probed with Sendai virions (15 μg/ml), after which the filters were incubated with anti-Sendai virus antibodies followed by (A) goat anti-rabbit-IgG conjugated to horseradish peroxidase or (B) ^{125}I-protein A ($3 \cdot 10^5$ cpm/3 ml phosphate-buffered saline + 3% bovine serum albumin), as described in Materials and Methods. In the case of ^{125}I-protein A, the dots were autoradiographed as described for detection of ^{125}I-Sendai virions in Materials and Methods. The filters were reacted with the following probes. A(a), Sendai virions; (b), dithiothreitol-treated Sendai virions: Sendai virions were reduced with dithiothreitol (5 mM) for 30 min at 37°C; (c), Sendai virions (45 μg) which were first incubated with 60 mg (in 3 ml phosphate-buffered saline–bovine serum albumin) of fetuin for 30 min at room temp. and then used for probing. B(a), Sendai virions; (b), as for (a), but neuraminidase-treated glycophorin was applied to the nitrocellulose membrane filters. Arrows indicate practical limit of detection, i.e., 125 ng erythrocyte protein or 8 ng of glycophorin.

to bind to their respective membrane receptors and are incapable of inducing hemolysis (which reflects virus-cell fusion) [23]. Indeed, the results in

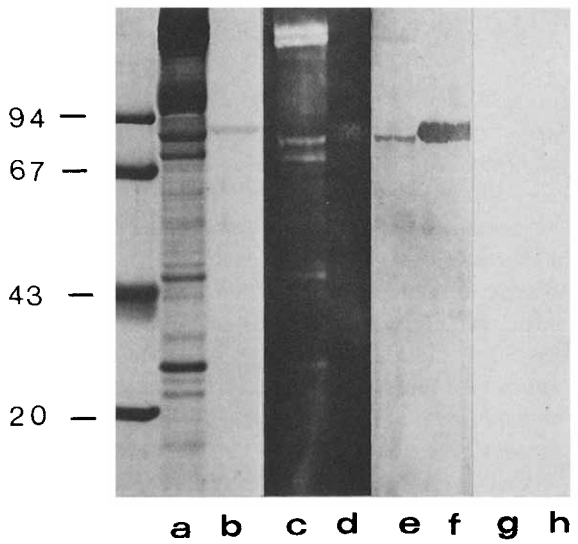

Fig. 2. Binding of Sendai virions to human erythrocyte membranes. Human erythrocyte ghost membranes were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane filters and probed with Sendai virions, as described in Materials and Methods. The virus was immunodetected, as described in Materials and Methods. Numbers indicate molecular mass in kDa. (a) and (b), Coomassie blue staining: (a), human erythrocyte ghost membranes; (b), purified glycophorin. (c)–(h), nitrocellulose membrane filters with either transferred human erythrocyte ghost membranes or purified glycophorin: (c), protein blot negatively stained demonstrating that all the various erythrocyte polypeptides are transferred and represented on the filter; (d), as for (c), but with purified glycophorin; (e) and (f), human erythrocyte ghost membranes and purified glycophorin probed with Sendai virions, respectively; (g) and (h), same as (e) and (f), but the blots were probed with dithiothreitol-treated virions. Sendai virus particles were treated with 5 mM dithiothreitol, as described in Fig. 1.

lanes g and h (Fig. 2) show that no signal could be detected when dithiothreitol-treated virions were incubated with blots containing either human erythrocyte ghost polypeptides or purified glycophorin, respectively.

Fig. 3 demonstrates that the viral HN glycoprotein confers upon Sendai virus its ability to bind to glycophorin. As can be seen, identical patterns were obtained when either isolated and purified HN glycoproteins (lane c) or intact Sendai virions (lane a) were incubated with blots of purified glycophorin. No signal could be detected in blots incubated with the viral fusion protein, the F glycoprotein (lane e), indicating that no poly-

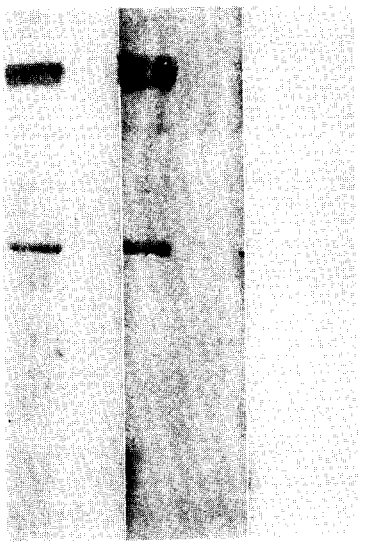

Fig. 3. Binding of the viral HN glycoprotein to purified glycophorin: Dependence on the presence of sialic acid residues. Glycophorin (a, c, e) or neuraminidase-treated glycophorin (b, d, f) were transferred to nitrocellulose membrane filters, probed with Sendai virus (a, b), NH (c, d) or F (e, f) viral glycoproteins, and then immunodetected as described in Materials and Methods.

peptide receptor exists in the human erythrocyte membrane for the F protein. Evidently, the presence of sialic acid residues is essential to allow attachment between the virus and the glycophorin polypeptides. Neither Sendai virions nor the purified HN glycoprotein were able to attach to neuraminidase-treated glycophorin (see lanes b and d).

Detection of Sendai virus membrane ligands by the use of ^{125}I-labeled Sendai virions

Sendai virions can be radiolabeled to give virus particles with a high specific activity, while still preserving their binding and fusogenic abilities [13,24]. These radiolabeled virions (or radiolabeled purified HN and F glycoproteins) were used to directly detect Sendai virus ligands in human erythrocyte membranes. In order to determine optimal working conditions, erythrocyte ghost membranes were directly applied to nitrocellulose membrane filters which were then probed under various conditions. Fig. 4 summarizes these experiments. Not surprisingly, both radiolabeled Sendai virions and membrane vesicles containing radiolabeled HN glycoproteins (Fig. 4a) provided strong signals, similar to those obtained when non-labeled probes were used in the immunodetection studies (see Fig. 1). However, the use of labeled probes provided an additional advantage, i.e., the ability to perform competition experiments. The dots of ghost membranes were preincubated with non-labeled virions which subsequently prevented the binding of radiolabeled probes (Fig. 4b). Dithiothreitol-inactivated virions, as expected, did not compete with the binding of radiolabeled virions (Fig. 4c). Furthermore, bind-

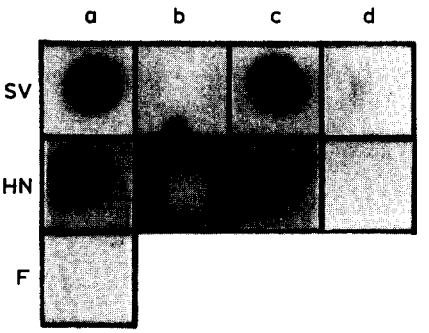

Fig. 4. Inhibition of the binding of labeled Sendai virus (SV) particles to human erythrocyte ghost membranes by native Sendai virus. Human erythrocyte ghost membranes (0.7 μg) were applied to nitrocellulose membrane filters using a Bio-Dot apparatus (Bio-Rad). The dots were probed with either ^{125}I-labeled Sendai virions or ^{125}I-labeled viral glycoproteins. The viral antigens were detected by autoradiography, as described in Materials and Methods. (a), Sendai virions (10^5 cpm/μg), HN glycoprotein ($2 \cdot 10^5$ cpm/μg) and F glycoprotein ($4 \cdot 10^4$ cpm/μg). In all experiments 10^6 cpm were used. (b) Prior to probing with ^{125}I-virus or with ^{125}I-HN glycoproteins, the nitrocellulose membrane filters were incubated for 1 h at 4°C with 120 μg unlabeled, active Sendai virus, after which labeled virions were added as described in (a). (c), As for (b), but instead of active virions, the nitrocellulose membrane filters were incubated with 120μg of dithiothreitol-treated virions (dithiothreitol treatment was as described in Fig. 1). (d), As in (a), but the nitrocellulose membrane filters were incubated prior to probing with neuraminidase for removal of sialic acid residues from the erythrocyte membranes as follows: a volume of 10 μl of neuraminidase (*Vibrio cholera* 1 U/ml, Behringwerke, F.R.G.) was added to nitrocellulose membrane filters immersed in 2 ml of acetate buffer (pH 5.5) (see Materials and Methods). After incubation for 1 h at 37°C with shaking, the filters were washed twice with phosphate-buffered saline and then probed with ^{125}I-Sendai virions or ^{125}I-HN glycoproteins as described in (a).

ing of radiolabeled probes could be completely prevented by incubating the dotted nitrocellulose membrane filters with neuraminidase prior to probing (Fig. 4d). As can be seen (Fig. 4a), no radiolabeled F-glycoproteins could be detected.

In view of these results, radiolabeled Sendai virions and membrane vesicles containing the HN glycoprotein were used to probe protein blots of either purified glycophorin (Fig. 5a) or erythrocyte ghosts (Fig. 5b) separated on SDS-polyacrylamide gels. As can be seen, only glycophorin bound the probes. Moreover, active non-labeled virions could compete effectively with the signal obtained (Fig. 5d). Competition was not achieved by preincubation of the nitrocellulose membrane filters with dithiothreitol-treated HN glycoproteins (Fig. 5e) or with heat-inactivated virions (Fig. 5f). Treatment of the blots with neuraminidase also prevented virus binding (Fig. 5c). Once again, exclusive binding only to glycophorin was observed when radiolabeled, purified HN glycoproteins were used (Fig. 5g).

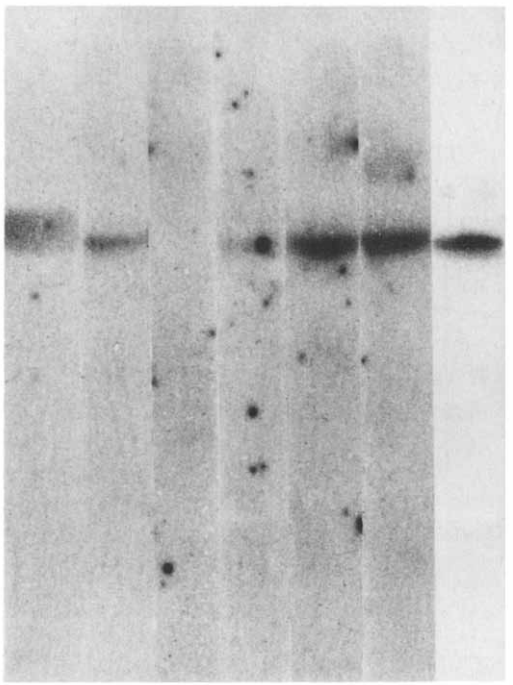

Fig. 5. Use of ^{125}I-Sendai virions to detect receptors for Sendai virus in human erythrocyte ghost membranes. Detection of radiolabeled viral components bound to the nitrocellulose membrane filters was as described in Materials and Methods. (a) and (b), ^{125}I-Sendai virions (30 μg, $1.8 \cdot 10^4$ cpm/μg) were incubated with nitrocellulose membrane filters containing purified glycophorin or human erythrocyte membranes, respectively. (c), the nitrocellulose membrane filters were treated prior to addition of ^{125}I-Sendai virions with neuraminidase, as described in Fig. 4; (d), as in (b), but the nitrocellulose membrane filters were preincubated with unlabeled, active Sendai virions (240 μg/5 ml) for 1 h at 4°C, after which ^{125}I-Sendai virions were added; (e), as in (d), but the preincubation was performed with 240 μg of inactive, dithiothreitol-treated Sendai virions; (f) as in (d), but the preincubation was carried out with 240 μg of heat-inactivated, native Sendai virions. Heat inactivation was performed by incubation of Sendai virions for 1 h at 60°C, (g), ^{125}I-HN glycoprotein (30 μg of $2.4 \cdot 10^4$ cpm/μg).

Discussion

In order to study and search for membrane polypeptides that may serve as potential virus receptors, we have used the protein blotting technique. Two detection systems have been shown to be effective for the identification of membrane components which specifically bind Sendai virions in human erythrocytes. The demonstration of virus-ligand interaction was achieved with the use of either specific anti-Sendai virus antibodies or radiolabeled virus particles.

An alternative method to determine and study the ability of various glycolipids to serve as ligand for animal viruses, again using Sendai virions as a model system, was recently developed [25]. Glycolipids extracted from either human erythrocytes or human brain were separated on plastic-treated thin-layer plates. The chromatogram was then overlaid with Sendai virions. With this method, a particular receptor may be specifically detected in mixtures of phospholipids or glycolipids extracted from a target cell membrane [25].

Our present results clearly demonstrate that among the various human erythrocyte polypeptides, only glycophorin A binds Sendai virions effectively. The major sialoglycoprotein of the erythrocyte membrane is glycophorin A which bears about 60% of the total sialic acid residues of the erythrocyte membrane [22]. A pure preparation of glycophorin A can be obtained either by gel filtration of a detergent solution of the erythrocyte membrane glycoproteins or by a wheat germ agglutinin-affinity system from detergent-

solubilized membranes [26]. Two additional sialoglycoproteins, glycophorin B and glycophorin C, were isolated from the erythrocyte membrane [22].

Glycophorin A migrates on SDS gel electrophoresis in two positions, representing its dimer and monomer forms. Glycophorin A exists in the erythrocyte membrane predominantly as a dimer. However, the conversion of the dimer to the monomer is dependent on protein and detergent concentration, temperature, time of incubation and ionic strength of the buffer, and, moreover, it is reversible [22].

Binding of intact virions to glycophorin is mediated by the viral HN glycoprotein, as demonstrated by experiments showing that membrane vesicles containing the purified HN glycoprotein bind to the same polypeptide. The binding activity was abolished by heat inactivation or by dithiothreitol reduction of the viral particles or of the purified HN glycoprotein. The requirement for sialic acid as a first recognition site is demonstrated by the fact that erythrocyte membranes or purified glycophorin, which were treated with neuraminidase, no longer served as efficient receptors. Furthermore, binding could be prevented by competition with a soluble sialoglycoprotein such as fetuin. Hence, it seems that the binding observed in the present work indeed reflects the binding activity needed for attachment of virions to living cells. It appears that using the method developed here, other sialoglycoproteins of the erythrocyte, besides glycophorin A, do not bind Sendai virions. This could be due either to their presence at concentrations which are too low to allow their detection or to actual structural differences discerned by the virus itself.

It is noteworthy that no protein receptors could be detected for the F glycoprotein in untreated or neuraminidase-treated erythrocyte membranes (not shown). These results may support the view that the first recognition site of the F glycoprotein are the cell membrane phospholipid molecules [27]. However, the possibility that a membrane polypeptide could serve as receptor for the F glycoprotein cannot be excluded. Such a putative receptor may lose its activity by denaturation under our experimental conditions.

The present results demonstrate that particular material such as enveloped animal viruses or reconstituted virus envelopes can serve as an efficient probe for protein blotting overlays. The virus overlay of protein blots should therefore provide the means to screen further for potential virus receptors in various cells and tissues, and to test the compatibility of a variety of membrane polypeptides for the binding of virions from different groups. Once such a membrane polypeptide is identified for its ability to bind virus particles, it should be tested for its biological activity, i.e., as a functional virus receptor, by incorporation into virus receptor-depleted cells [6–8].

Acknowledgments

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