Localization of viral-envelope-glycoprotein-binding sites in fibronectin

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(Received 30 August 1983/Accepted 29 December 1983)

Purified viral-envelope glycoproteins from influenza A virus were found to bind to two fragments of the fibronectin molecule. Human plasma fibronectin was digested by leucocyte cathepsin G, and three different fragments, of M_r 30000, 40000 and 120000–140000, with specific binding functions were isolated. Micelles of radio-labelled influenza A glycoprotein were allowed to bind to these fragments immobilized on polystyrene micro-titre wells. The C-terminal 120000–140000- M_r fragments that carry the cell-binding activity bound viral proteins most efficiently, whereas the 40000- M_r gelatin-binding fragment bound considerably less. The N-terminal 30000- M_r *Staphylococcus aureus*-binding fragment was negative in the assays. Laminin, a basement-membrane protein, also bound viral proteins, though less effectively than fibronectin. The binding was abolished if laminin or fibronectin fragments were pretreated with neuraminidase. This suggests that the sialic acids in the sugar moieties of these glycoproteins are involved in the binding. The affinity of viral-envelope glycoproteins for certain domains of fibronectin and for laminin may play a role in virus–cell interactions.

Fibronectin is a large- M_r protein found in plasma and connective tissues and in a number of different cultured cells. It has affinity for collagens, heparin, actin, fibrin, fibrinogen, DNA and several bacteria (see, e.g., Mosher, 1980; Ruoslahti *et al.*, 1981; Vaheri *et al.*, 1980), and it has been considered to be a non-specific opsonin in blood (Blumenstock *et al.*, 1978). Fibronectin can be digested by proteolytic enzymes into fragments that carry distinct functional domains (see, e.g., Mosher, 1980; Ruoslahti *et al.*, 1981; Vaheri *et al.*, 1980).

The glycoproteins of enveloped viruses play a central role in the pathogenicity of the viruses. The haemagglutinin of influenza A virus can bind to cellular glycoproteins at the cell surface, and the receptor structure in the glycoproteins is a terminal sialic acid (Burness, 1981). Viral-envelope glycoproteins, which are integral membrane proteins, can be purified, and water-soluble detergent-free micelles can be made (Simons *et al.*, 1978). These micelles can be used as models for viral-envelope glycoproteins.

In a previous study we found that plasma fibronectin has affinity for the envelope glycoproteins of myxoviruses and paramyxoviruses (Julkunen *et al.*, 1983). In the present work we have used purified radiolabelled viral-glycoprotein mi-

celles and isolated fragments of fibronectin to localize the interactive sites in the fibronectin molecule. Affinity of viral proteins for laminin was also studied.

Materials and methods

Viral proteins

Influenza A (H3N2, Bangkok strain) virus was grown in embryonated chicken eggs and purified in sucrose gradients, and protein micelles composed of viral glycoproteins were prepared as described previously (Julkunen *et al.*, 1983). Glycoprotein micelles were labelled with NaB^3H_4 (The Radiochemical Centre, Amersham, Bucks., U.K.) with a modification (Julkunen *et al.*, 1983) of the method of Luukkonen *et al.* (1977). The specific radioactivity was 140000 c.p.m./ μg of protein.

Cathepsin G, fibronectin and laminin

Cathepsin G (EC 3.4.21.20), a gift from Dr. J. Saklatvala (Strangeways Research Laboratory, Cambridge, U.K.), had been purified from human neutrophil leucocytes as described previously (Baugh & Travis, 1976) and had an activity of 350 units/mg in the azocasein-hydrolysis assay described by Starkey (1977).

Fibronectin was purified from human plasma by

Fig. 1. Polyacrylamide-gel electrophoresis of the cathepsin G-cleaved fragments of fibronectin

Fibronectin was digested by cathepsin G, and the digestion products and the purified fragments were analysed by 10%-polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The gel was then stained with Coomassie Brilliant Blue. Track 1, intact human plasma fibronectin (8 µg); track 2, cathepsin G digest of fibronectin; track 3, 120000–140000- M_r gelatin-non-binding fragments; track 4, 40000- M_r gelatin-binding fragment; track 5, N-terminal 30000- M_r fragment.

using gelatin–Sephacrose (Engvall & Ruoslahti, 1977) as described previously (Vuento & Vaheri, 1979). Laminin was a gift from Dr. R. Timpl (Max-Planck Institut, Martinsried bei München, Germany) and had been purified from EHS sarcoma cells as described in detail (Timpl *et al.*, 1979).

Fragments of fibronectin

Purified plasma fibronectin was digested with cathepsin G, and the N-terminal 30000- M_r *Staphylococcus aureus*-binding, the 40000- M_r gelatin-binding and the C-terminal 120000–140000- M_r fragments were isolated as described in detail previously (Vartio, 1982; Vartio *et al.*, 1983). The purity of the fragments was determined by using 10%-polyacrylamide-gel electrophoresis (Laemmli, 1970) in the presence of sodium dodecyl sulphate (Fig. 1).

Radioassay

Polystyrene micro-titre plates (Lindbro, Tjtertek; Flow Laboratories, Hamden, CT, U.S.A.) were coated with fibronectin, laminin, bovine serum albumin or fragments of fibronectin, all in phosphate-buffered saline (0.14M-NaCl/0.01M-

sodium phosphate buffer, pH 7.4) at 37°C for 2 h. The plates were washed twice with phosphate-buffered saline containing 0.05% Tween 20 and once with distilled water, and used for the assay.

Micelles of radiolabelled influenza A glycoproteins were diluted with unlabelled micelles at a ratio of 1:3, and were allowed to bind on to the solid-phase material at protein concentrations of 0.1–0.32 µg/ml (in phosphate-buffered saline containing Tween 20; 50 µl/well).

After 2 h incubation at 37°C, the wells were washed twice with phosphate-buffered saline containing Tween 20 and once with distilled water, and the bound viral proteins were eluted into 150 µl of hot 2% (w/v) sodium dodecyl sulphate. The radioactivity was determined in a liquid-scintillation counter. Duplicate determinations were made.

Other techniques

Fibronectin, fragments of fibronectin and laminin were treated in solution with *Vibrio cholerae* neuraminidase (Behringwerke, Marburg, Germany) in order to remove sialic acids from the sugar residues of proteins (Drzeniek, 1973), at an enzyme/substrate ratio of 1:15, at final pH 6.2 at 37°C for 2 h. After the treatment the proteins were used for coating of the micro-titre plates as described above. Protein determinations were done by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

To study the coating properties of the different fibronectin fragments before and after neuraminidase treatment, the fragments were radioiodinated by using the chloramine-T method (Krohn *et al.*, 1972).

Results

Binding of micelles of influenza A glycoprotein to fibronectin fragments

Plastic micro-titre wells were coated with purified fragments of fibronectin, and the binding of micelles of radiolabelled influenza A virus glycoprotein to various immobilized fragments of fibronectin was then studied. Efficient dose-dependent binding was observed to the wells coated with the C-terminal 120000–140000- M_r fragments of fibronectin. Considerably less binding of viral proteins to the 40000- M_r gelatin-binding fragment was observed, whereas the binding of the 30000- M_r *S. aureus*-binding fragment was comparable with that of bovine serum albumin (Fig. 2). In order to study possible differences in the coating efficiencies of the fibronectin fragments, radiolabelled fragments (5 µg/ml) were allowed to bind on to polystyrene plates. The binding took place almost at equimolar proportions (1.2–1.4:1.4:1.0 for

Fig. 2. Binding of influenza A glycoproteins to fibronectin fragments

Micelles of radiolabelled influenza A glycoprotein were allowed to bind to solid-phase fibronectin fragments, and the bound radioactivity was eluted by sodium dodecyl sulphate and quantified. Radioactivity bound to the 120000–140000- M_r fragment with cell-binding activity (120–140 kDa), the 40000- M_r gelatin-binding fragment (40 kDa) and the 30000- M_r *S. aureus*-binding fragment (30 kDa) and to bovine serum albumin (BSA).

fragments 120000–140000- M_r , 40000- M_r and 30000- M_r , respectively), and, accordingly, differences in the proportions of amounts of bound protein were observed (18.9 : 6.1 : 3.4 in terms of ng of protein/well).

Binding of viral-envelope glycoproteins to laminin

The basement-membrane glycoprotein laminin was included in the study because its sugar moieties are known to be rich in sialic acids (Cooper *et al.*, 1981). Viral-envelope glycoproteins bound to solid-phase laminin in a dose-dependent manner (Fig. 3). The binding was less efficient than the binding of viral proteins to fibronectin. There were no major differences in the coating efficiency of fibronectin and laminin (results not shown).

Effect of neuraminidase treatment on the binding

Fibronectin, its fragments and laminin were treated with neuraminidase in solution to remove the terminal sialic acids of the proteins. Plastic micro-titre wells were then coated with the treated

Fig. 3. Binding of influenza A glycoproteins to fibronectin and laminin

Micelles of radiolabelled influenza A glycoprotein were allowed to bind to solid-phase fibronectin (FN), laminin (LAM) and bovine serum albumin (BSA).

and non-treated proteins. The binding of micelles of radiolabelled influenza A virus glycoprotein to solid-phase proteins was then determined. The binding of the micelles to fibronectin was decreased, as shown previously (Julkunen *et al.*, 1983). The treatments of laminin with neuraminidase also decreased the binding to background level. The binding to 120000–140000- M_r and 40000- M_r fragments were decreased similarly. Neuraminidase treatment of proteins did not significantly affect their binding efficiency on to plastic. The most notable decrease after neuraminidase treatment was obtained for the 30000- M_r fragment (13%), the decrease with the other fragments or fibronectin and laminin being less.

Discussion

In a previous study we have characterized the interaction of glycoproteins of several myxoviruses and paramyxoviruses with fibronectin (Julkunen *et al.*, 1983). Dose-dependent binding was observed when fibronectin was used in either the solid or the soluble phase. In the present study we have used certain fragments of fibronectin with

known biological functions to analyse further which parts of the fibronectin molecule are responsible for the binding.

The major binding activity was detected in the C-terminal 120000–140000- M_r fragment that also contains the cell-binding activity (Hahn & Yamada, 1979; Sekiguchi & Hakomori, 1980). The 40000- M_r fragment that carries the gelatin-binding activity (Vartio, 1982) also bound viral proteins, whereas the 30000- M_r *S. aureus*-binding fragment did not bind viral proteins. All three fragments bound nearly at equimolar proportions to the plastic micro-titre wells.

Treatment of fibronectin or its proteolytic fragments with *V. cholerae* neuraminidase, which removes the terminal sialic acids from the sugar moieties (Drzeniek, 1973), totally abolished the binding of viral proteins. Most of the carbohydrate residues of fibronectin are localized in the gelatin-binding region or close to it (Sekiguchi & Hakomori, 1980; Sekiguchi *et al.*, 1981). Influenza A virus can bind to glycoproteins that contain sialic acids. It has been suggested that the haemagglutinin molecule binds predominantly to sialic acids that are linked to galactose with α -(2–3)-linkages (Burness, 1981). Plasma fibronectin contains sialic acids, and according to the study by Wrann (1978) they are terminally linked to galactose with α -(2–4)- or α -(2–6)-linkages. This may explain the relatively inefficient binding to 40000- M_r gelatin-binding fragment. On the other hand, the 120000–140000- M_r fragment, which contains a minor part of the carbohydrates of the molecule (Sekiguchi *et al.*, 1981), may have suitable linkages in sugar residues for the viral glycoproteins to bind to.

Laminin is a glycoprotein mainly found in basement membranes (Rhode *et al.*, 1979; Foidart *et al.*, 1980), but more recently it has also been found at the surface of mouse peritoneal macrophages (Wicha & Huard, 1983). Influenza A-virus glycoproteins were also found to bind to laminin, and, as in the case of fibronectin, the binding was inhibited by pretreatment of laminin with neuraminidase, suggesting that the terminal sialic acids of the sugar residues are involved in the binding.

Fibronectin has affinity for several macromolecules and bacterial species, and it has been considered to be a non-specific opsonin in blood. Mosher & Proctor (1980) localized the *S. aureus*-binding site of fibronectin in a 27000- M_r tryptic fragment corresponding to the N-terminal 30000- M_r fragment of the present study. Interestingly, this fragment did not bind viral proteins, suggesting a binding mechanism different from that of bacteria. The interaction of viral proteins with fibronectin may play a role in the opsonization of viruses or in virus–cell interactions.

This was supported by the Paulo Foundation, the Finnish Cultural Foundation, the Science Foundation of Lääke Co. and the Finnish Cancer Foundation.

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