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A Biochemical Approach Reveals Cell-Surface Molecules Utilised by Picornaviridae: Human Parechovirus 1 and Echovirus 1

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Abstract Although receptor virus interactions of several *Picornaviridae* have been studied in the past, it is becoming apparent that these interactions might be more complex than previously thought. In this study, we have chosen to identify the cell-surface molecules involved in the infectious cycle of two common human pathogens and members of the *Piconaviridae* family, Echovirus 1 (Echo1) and Human Parechovirus 1 (HPEV1) also known as Echovirus 22. In order to identify the specific cell-surface protein molecules involved in Echo1 and HPEV1 infectious cycles, we have deviced a method, by which free virions were used as an affinity surface, allowing either Echo1 or HPEV1 to bind to solubilised proteins from cells susceptible to the virus infection. The virus–cell-surface protein complexes were then analysed by SDS-PAGE and two-dimensional electrophoresis. Echo1 was shown to bind to two integrin-like proteins of 150 and 120 kDa. While HPEV1 attached to two integrin-like proteins of 120 and 100 kDa. The identity of these proteins was identified via Western blotting. Thus, overall we can conclusively report that Echo1 utilises integrin $\alpha 2\beta 1$, whereas HPEV1 utilises integrin $\alpha v\beta 3$ on the cell surface. J. Cell. Biochem. 80:373–381, 2001. © 2001 Wiley-Liss, Inc.

Key words: Echo1; HPEV1; receptor; virus-receptor complex

It has been clear for many years that viruses have adapted to infect and propagate within host cells. One of the first steps of the virus infectious cycle is the ability of the virus to utilise cell-surface molecules, as receptors for binding and cell entry [Evans and Almond, 1998]. Until recently viral inhibition studies using antibody reagents against cell-surface molecules were used to determine whether cell surface proteins were used as receptor molecules by viruses.

In this work, we have chosen to study cell surface molecules used by Echovirus 1 (Echo1) and Human Parechovirus 1 (HPEV1), also known as Echovirus 22 [Hyypia et al., 1992]. Which are non-enveloped RNA viruses belonging to one of the most well-studied family of viruses, the *Picornaviridae* family. These viruses are common human pathogens and can display many clinical symptoms including: flaccid paralysis; encephalitis; respiratory disease; and exanthema [Grist and Reid, 1988].

Previous studies have shown that Echo1 utilises integrin $\alpha 2\beta 1$ as a receptor [Bergelson et al., 1992, 1993], while HPVE1 displays an RGD motif and probably interacts with αv integrins [Pulli et al., 1997]. The present study was performed to develop a sensitive approach based on immunoprecipitation and Western blotting to identify cell-surface molecules that viruses utilise. Picornaviruses have been shown in the past to bind efficiently to solubilised cell proteins [Mapoles et al., 1985]. Therefore, Echo1 and HPEV1 were chosen as representative viruses to establish a system useful for studying cell-surface molecules and virus associations. A549 cells susceptible to viral infection by HPEV1, and HeLa cells susceptible to infection by Echo1, were cell-surface labelled with biotin and then solubilised in non-ionic and Zwiterionic detergents. Free virions

Abbreviations used: Echo1, Echovirus 1; HPEV1, Human Parechovirus 1; RGD, argine-glycine-aspartic acid; mAb, monoclonal antibody; 2D, two-dimensional electrophoresis.

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were used as an affinity surface, thus allowing the virus to bind to solubilised proteins. The virus–cell-surface protein complexes were then immunoprecipitated by a virus specific serum and Protein A sepharose beads. These complexes could then be analysed by SDS-PAGE and two-dimensional gel electrophoresis and identified by immunoblotting. Our results showed that Echo1 binds to integrin $\alpha 2\beta 1$, thus being in good agreement with previous studies [Bergelson et al., 1992, 1993].

While we conclusively report that HPEV1 interacts with integrin $\alpha v\beta 3$.

EXPERIMENTAL PROCEDURES

Labeling of Cell Surface with NHS-Biotin

A549 cells (human lung carcinoma) and HeLa (human cervical carcinoma) cells were surface labelled with biotin, by using 40 μl of 0.1 M membrane-impenetrable Biotin-NHS reagent (*N*-hydroxysuccinimide ester derivative) from Amersham, in 2 ml of PBS for every 10^8 cells. After 30 min, the reaction was stopped with 1 mM ethanolamine in PBS. Cells were washed three times with PBS and lysed in lysis buffer (1% Digitonin, 15 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM phenylmethylsulphonyl fluoride).

Viruses

Echo 1 and HPEV1 were obtained from the ATCC. Each batch of virus was aliquoted and stored at −80°C. One single aliquot of virus was used per assay in order to avoid freeze and thawing the virus particles and, therefore, reducing the number of viruses capable of binding by disrupting their capsid.

Antibodies

The 23C6 integrin $\alpha v\beta 3$ specific mAb was obtained from Serotec. The Y2/51, β3 specific mAb was obtained from Zymed Labs. While the VNR139 (αv specific) mAb, the B3B11 mAb specific for integrin β1 and the BHA2.1 specific for $\alpha 2\beta 1$ were obtained from Chemicon. The HPEV1 and Echo1 specific monkey serum were from ATCC. The rabbit polyclonal serums specific for integrin α2 (AB1944), α5 (AB1928), β4 (AB1922) and β5 (AB1926) were from Chemicon. HRP-conjugated goat anti-mouse Ig and HRP-conjugated goat anti-rabbit Ig were obtained by KPL/Kirkegaard-Perry Labs and Antibodies Incorporated, respectively. Normal monkey serum was from Antibodies Incorporated.

Immunoprecipitation Protocols

A549 or HeLa cells were surface-labelled with NHS-Biotin and lysed in lysis buffer as described above. For each assay 3×10^6 cells were used. The cell lysate was pre-cleared with normal monkey serum followed by the addition of 10% Protein A Sepharose slurry (Pharmacia Biotech, Uppsala Sweden) to remove non-specific binding material. Virus receptor complexes were immunoprecipitated by the addition of 1.5×10^6 PFU of virus, that was incubated for 1 h at room temperature, followed by the addition of 2 μg of HPEV1 specific monkey serum or Echo1 specific monkey serum for 1 h at 4°C. The resulting immune complexes were isolated with 10% Protein A Sepharose slurry. All precipitates were washed five times with solubilisation buffer.

Immune complexes were eluted from protein A sepharose beads with SDS-PAGE loading buffer (125 mM Tris-HCl, 4% SDS, 20% Glycerol, 1.4 M β-mercaptoethanol, 0.1% bromophenol blue). Eluates were electrophoresed in 4–20% gradient polyacrylamide gels, or 2-D gel electrophoresis. Biotin labelled proteins were transferred to nitrocellulose membrane and for the cell-surface labelled lysates the gel was Western blotted with Streptavidin-HRP conjugate as described below.

Two Dimensional Non-Equilibrium pH Gradient Electrophoresis (NEPHGE)

The first dimensional gel mixture was prepared by adding 4.0 g urea (ultra pure grade), 1.5 ml of NEPHGE acrylamide stock solution (30% w/v acrylamide, 1.5% w/v *N*,*N*′-methylenebisacrylamide), 2.0 ml NP-40 stock solution (10% w/v NP-40), 0.5 ml Ampholine carrier ampholytes pH 3.5–10 (LKB or Pharmacia Fine Chemicals) and 2.0 ml Milli-Q water. The mixture was blended in a warm temperature (30°C) until it was homogenous and degassed for 10 min. To achieve polymerisation, 15 and 7 μl of ammonium persulfate (APS), 10% w/v and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were added, respectively. The gel solution was quickly dispensed into 2-mm glass cylindrical tubes (12 cm in length) sealed with sealing film (Nescofilm) and overlayed with 20 μl of Milli-Q-water. The tube gels were allowed to polymerise for exactly 1 h at room

temperature. In parallel, the immunoprecipitation pellets were eluted in 20 μl of NEPHGE sample buffer (700 μl NET buffer (500 mM Tris-HCl, 1.5 M NaCl, 50 mM EDTA), 100 μl 10% NP-40, 100 μl of β-mercaptoethanol, 50 μl ampholytes, 0.2 g sucrose, 1.75 g urea) for 1 h at 50°C. Prior to loading the samples the tube gels were primed with 10 μl of NEPHGE sample buffer. Once the antigen was loaded it was overlayed with 20 μl of NEPHGE overlay buffer (0.48 g urea, 10 μl ampholytes, raised to 1 ml with distilled water). The anodic (0.01 M H_3PO_4, top chamber) and cathodic (0.02 M NaOH, lower chamber) buffers were extensively degassed. The samples were electrophoresed for 2600 Vh at 200 V constant voltage, reverse polarity. The Bio-Rad Tube Gel System (model 175) was used for the first dimension. The gels were then extruded by water pressure and incubated for 1 h in 2 ml of SDS-PAGE equilibration buffer (125 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (w/v) glycerol, 1.4 M β-mercaptoethanol) at RT. The gels were continuously rotated and then stored at −20°C, awaiting SDS-PAGE analysis.

IMMUNOBLOTING

Immunoprecipitates were analysed by SDS-PAGE and transferred onto a nitrocellulose filter (Schleicher-Schuell, Germany) or immobilon P membranes (Millipore). After transfer, the membrane was blocked for 1 h in blocking solution (5% low fat dried milk dissolved in PBS with 0.1% Tween 20) and washed with 0.1% PBS-Tween 20 (two rinses, a 15 min wash, and two 10 min washes). The membrane was then incubated with Streptavidin–HRP conjugate or an appropriate dilution of antibodies, followed by 1 h incubation with an appropriate dilution of HRP conjugated goat anti-mouse Ig or HRP conjugated goat anti-rabbit Ig. The optimum antibody concentration was determined by dot blot assay (data not shown). After extensive washing with PBS with 0.1% Tween 20, the antigen was visualised using the ECL procedure (Amersham) according to the manufacturer's instructions.

RESULTS

In order to test whether it was possible for the virus to bind to protein molecules solubilised using mild non-ionic and Zwiterionic detergents and also optimise the concentration of antibodies used, dot immunoblots were performed using HeLa and A549 cells solubilized in 0.5% NP-40 (Fig. 1E and K), 1% Digitonin (Fig. 1F and L) or 1% CHAPS (Fig. 1D and J). The results showed that the viruses could bind to solubilised protein mole-

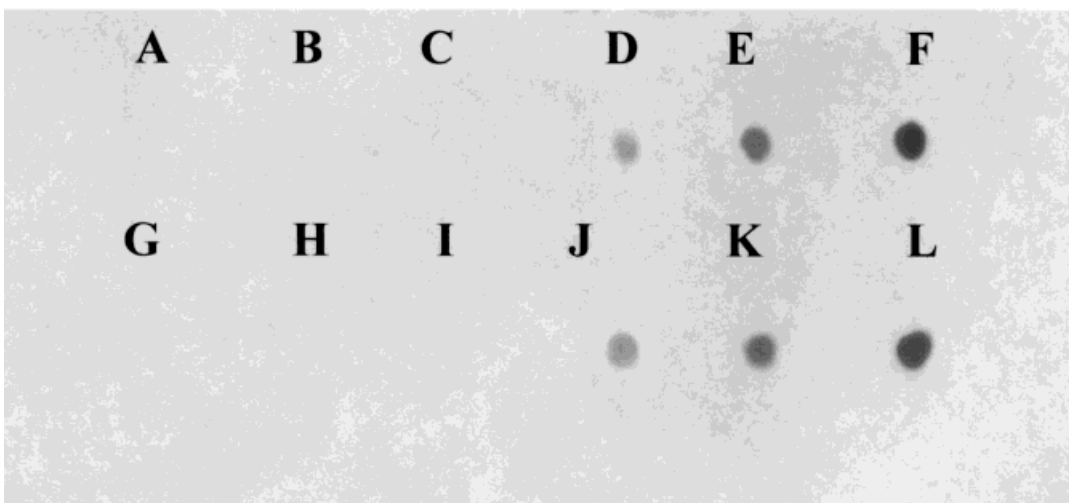

Fig. 1. Results of dot immunoblots on HeLa and A549 cells in non-ionic and zwiterionic detergents. Echo1 binding to HeLa cells solubilised in 1% CHAPS (D), 1% Digitonin (F), or 0.5% NP-40 (E) and also HPEV1 binding to A549 cells solubilised in 1% CHAPS (J), 1% Digitonin (L), or 0.5% NP-40 (K) is shown. No Echo1 binding was observed in the absence of solubilised HeLa (A) nor HPEV1 binding in the absence of solubilised A549 cells (G). Controls were also performed in the absence of Echo1 (B) or HPEV1 (H) particles, or in the absence of both Echo1 particles and specific Echo1 serum (C) or absence of HPEV1 particles and specific HPEV1 serum (I). The blots were probed with HRP-conjugated goat anti-monkey Ig. The film exposure to chemiluminescence was 2 min.

cules. No virus binding was observed in the absence of solubilised HeLa or A549 cells (Fig. 1A and G), in the absence of Echo1 or HPEV1 particles, (Fig. 1B and H) or in the absence of both virus particles and virus antiserum (Fig. 1C and I). Thus, this experiment showed that these viruses could specifically bind to solubilised cells and that 1% Digitonin was the most suitable detergent for solubilisation. This detergent was therefore used in all further immunoprecipitation experiments.

In order to immunoprecipitate the virus–cell-surface protein complexes from HeLa or A549 cells, the cell-surface molecules were biotinylated using the membrane-impenetrable Biotin-NHS reagent. Following solubilisation, free Echo1 or HPEV1 particles were used as an affinity surface thus allowing the virus to bind to solubilised receptors. Then, virus antiserum followed by Protein A sepharose beads was used to immunoprecipitate the virus–cell-surface protein complexes.

The immunoprecipitated complexes were then analysed by gradient SDS-PAGE under reducing conditions. Echo1 particles immunoprecipitated two integrin-like proteins from HeLa cell lysate, an 150 kDa protein and an 120 kDa protein (Fig. 2E). None of these proteins appeared in the absence of Echo1 virions, but in the presence of monkey Echo1 specific serum (Fig. 2A), or when an irrelevant antiserum was used (Fig. 2B). Normal monkey serum alone was also used as a control but no proteins appeared (Fig. 2C).

HPEV1 virions immunoprecipitated from A549 cell lysate, two integrin-like proteins of 120, 100 kDa and a less prominent protein of 20 kDa (Fig. 3E).

None of these proteins appeared in the absence of HPEV1 virions (Fig. 3A). No proteins appeared when an irrelevant antiserum was used as a control (Fig. 3B) or with normal monkey serum alone (Fig. 3C). When Monomac6, human monocytic cells, which are not susceptible to Echo1 or HPEV1 infection were used as a control, none of these proteins were immunoprecipitated (Figs. 2D and 3D).

To examine the purity, the p*I* and the molecular subunit composition of the protein components immunoprecipitated by the Echo1 and HPEV1 particles, high resolution two-dimensional electrophoresis was performed using a pH gradient of 3.5–10 in the NEPGHE focussing step followed by a gradient SDS-PAGE in the second dimension. This revealed the presence of two major spots in Echo1 immuno-

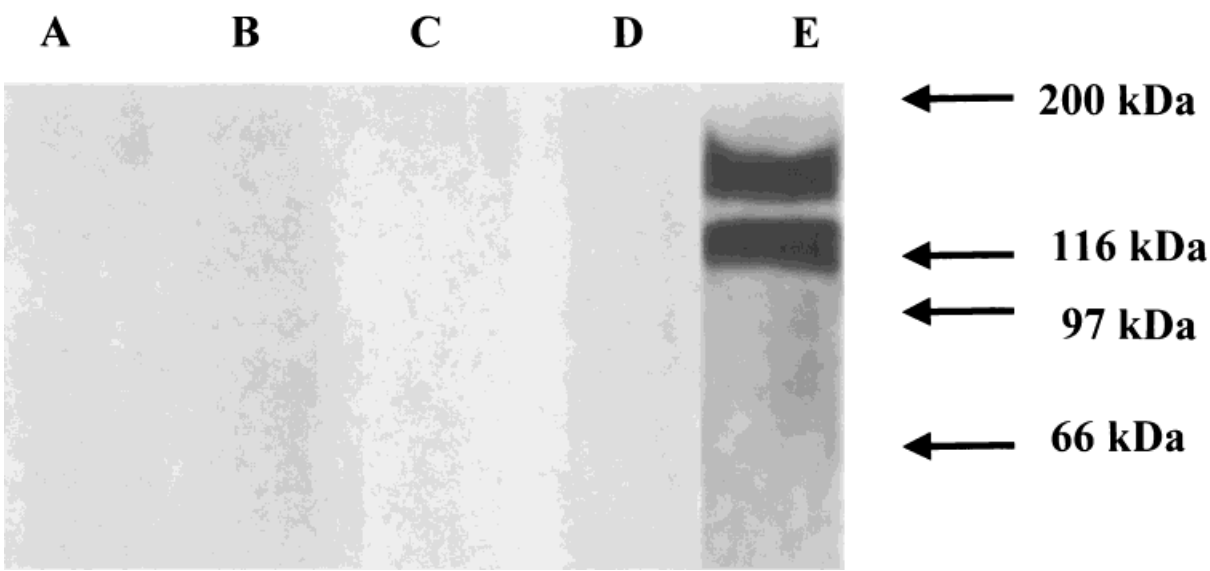

Fig. 2. SDS-PAGE analysis of immunoprecipitated Echo1 receptor complexes. Cell-surface biotinylated HeLa cells (E) were solubilised in 1% Digitonin and immunoprecipitated with Echo1 virions and Echo1 specific monkey serum. The experiment was also performed with the specific Echo1 serum alone, without the presence of Echo1 virions (A), and also using an irrelevant antiserum (B). As a control normal monkey serum alone was used (C). Biotinylated Monomac6 cells, (a human monocytic cell line not susceptible to Echo1 infection) was solubilized in 1% Digitonin and immunoprecipitated with Echo1 virions and Echo1 specific monkey serum (D). The blots were probed with Streptavidin-HRP. The positions of the molecular weight markers are indicated on the right. The film exposure to chemiluminescence was 2 min.

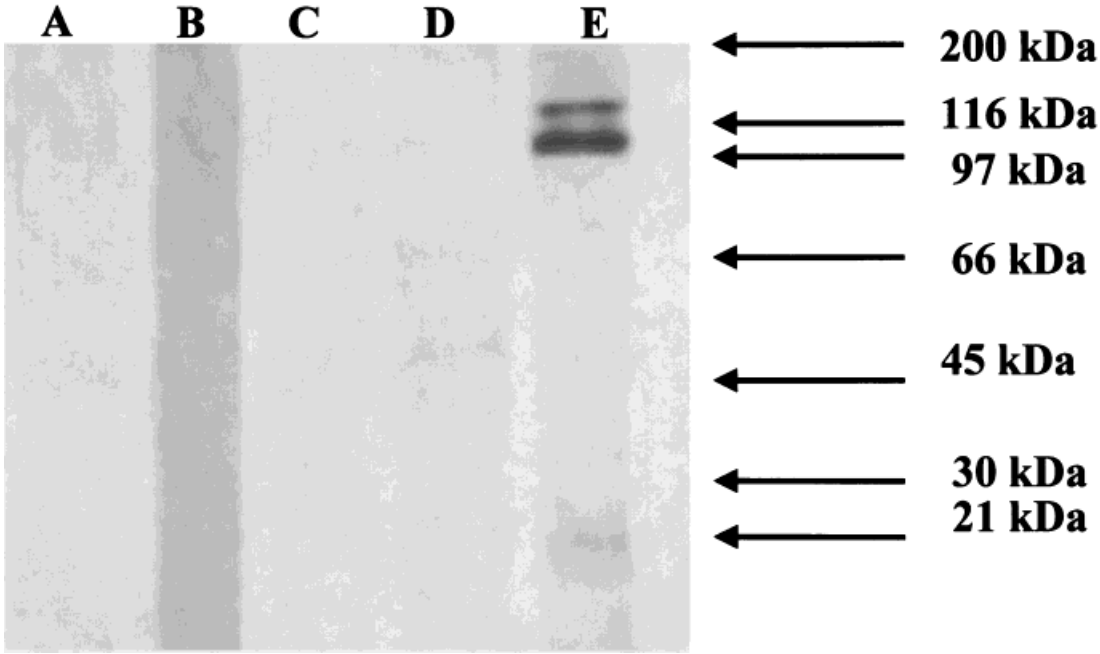

Fig. 3. SDS-PAGE analysis of immunoprecipitated HPEV1 receptor complexes. Cell-surface biotinylated A549 cells (E) were solubilised in 1% Digitonin and immunoprecipitated with HPEV1 virions and HPEV1 specific monkey serum. The experiment was also performed with the specific HPEV1 serum alone, without the presence of HPEV1 virions (A), and also using an irrelevant antiserum (B). As a control normal monkey serum alone was used (C). Biotinylated Monomac6 cells, (a human monocytic cell line not susceptible to HPEV1 infection) was solubilised in 1% Digitonin and immunoprecipitated with HPEV1 virions and HPEV1 specific monkey serum (D). The blots were probed with Streptavidin-HRP. The positions of the molecular weight markers are indicated on the right. The film exposure to chemiluminescence was 2 min.

precipitates (Fig. 4A). The 150 spot had an apparent p*I* of 5.0, whereas the 120 kDa spot had an apparent p*I* of 5.1. In the absence of Echo1 virus particles, no proteins appeared (Fig. 4B).

In the HPEV1 immunoprecipitates two major spots appeared. An 100 and an 120 kDa spot with an apparent p*I* of 5.1. Upon extended exposure a 20 kDa spot appered with an apparent p*I* of 5.1 (Fig. 5A). No proteins appeared in the absence of HPEV1 particles (Fig. 5B).

To identify these receptor proteins used by the viruses, we proceeded to Western blotting. Since it is known that Echo1 [Bergelson et al., 1992, 1993] and HPEV1 [Pulli et al., 1997] interact with integrins, and these isolated proteins had an apparent p*I* and molecular weight similar to integrins, we used a panel of α and β integrin chain specific mAbs, to blot the membranes and identify these proteins. Antibodies specific for integrins α2, α5, αv, β1, β3, β4, β5 were used, followed by HRP-conjugated goat anti-mouse Ig or by HRP-conjugated goat anti-rabbit Ig, depending on the species of the integrin antibody that had been used. Our results showed that the identity of the proteins precipitated by Echo1 virus particles were the α and β chains of integrin α2β1, (Fig. 6) while HPEV1 virus particles precipitated integrin αvβ3 (Fig. 7).

DISCUSSION

Specific receptors have been identified for only a few of the viruses that cause human disease [Fields, 1996]. Approaches that have been used in the past to identify cell-surface receptors involved in virus attachment to host cells, include binding of detergent solubilised cell extracts to specific ligands immobilised on inert surfaces, as in affinity chromatography [Jacobs and Cuatrecasas, 1981; Linsley et al., 1981], or as in solid phase assay [Krah and Crowell, 1982]. One of the main drawbacks of these approaches is that most receptors constitute only a small fraction of the total protein in the cell membrane, thus purification can be difficult. Another drawback is that the conformational state of the specific ligand used in such methods might be altered once immobilised, thus making the interaction with the virus in some cases almost impossible.

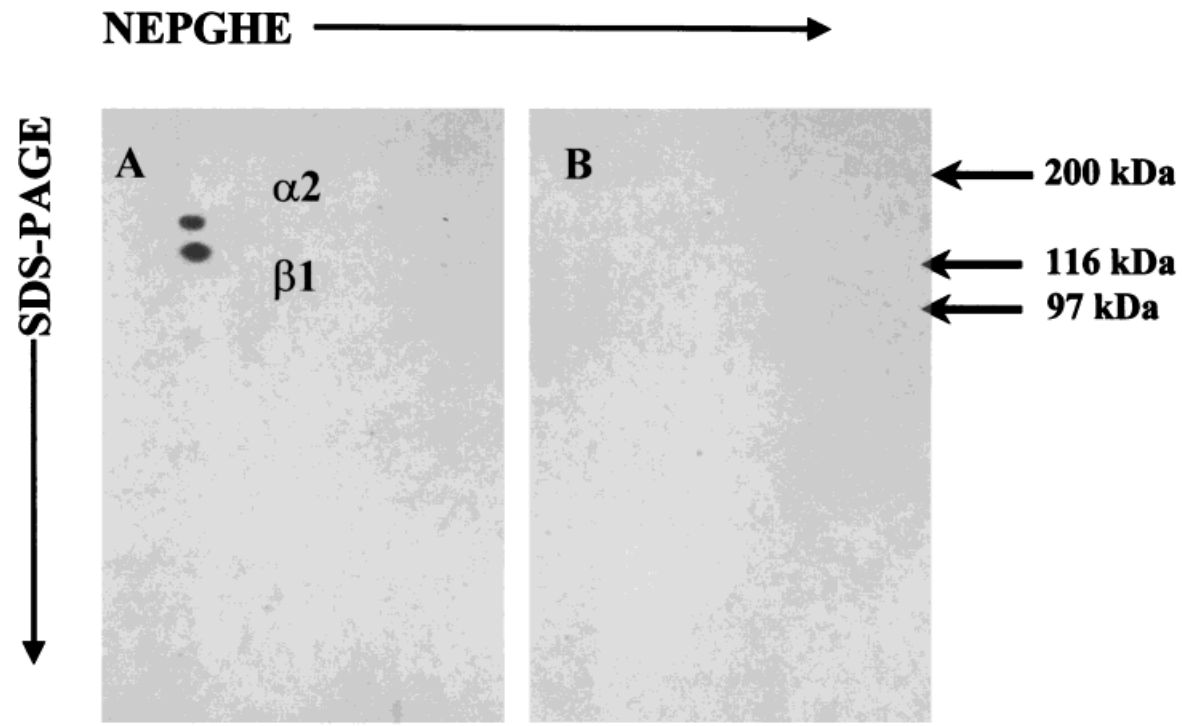

Fig. 4. Two-dimensional gel electrophoresis of immunoprecipitated Echo1 receptor complexes. Cell-surface biotinylated HeLa cells were solubilised in 1% Digitonin and immunoprecipitated with Echo1 virions and Echo1 specific iserum (A), or with Echo1 specific serum alone (B). The positions of the integrin α2 and β1 chains, are indicated. The acidic end of the gel is loaded to the left. The blots were probed with Streptavidin-HRP. The positions of the molecular weight markers are shown to the right. The film exposure to chemiluminescence was 5 min.

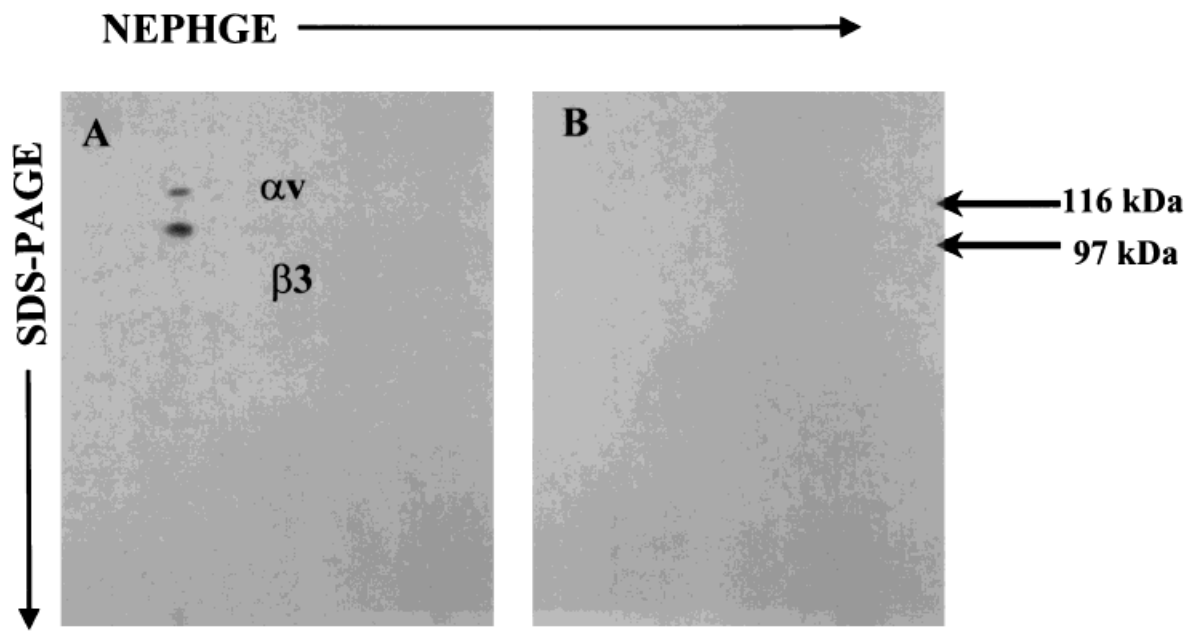

Fig. 5. Two-dimensional gel electrophoresis of immunoprecipitated HPEV1 receptor complexes. Cell surface biotinylated A549 cells were solubilised in 1% Digitonin and immunoprecipitated with HPEV1 virions and HPEV1 specific serum (A), or with HPEV1 specific iserum alone (B). The positions of the integrin αv and β3 chains, are indicated. The acidic end of the gel is loaded to the left. The blots were probed with Streptavidin-HRP. The positions of the molecular weight markers are shown to the right. The film exposure to chemiluminescence was 5 min.

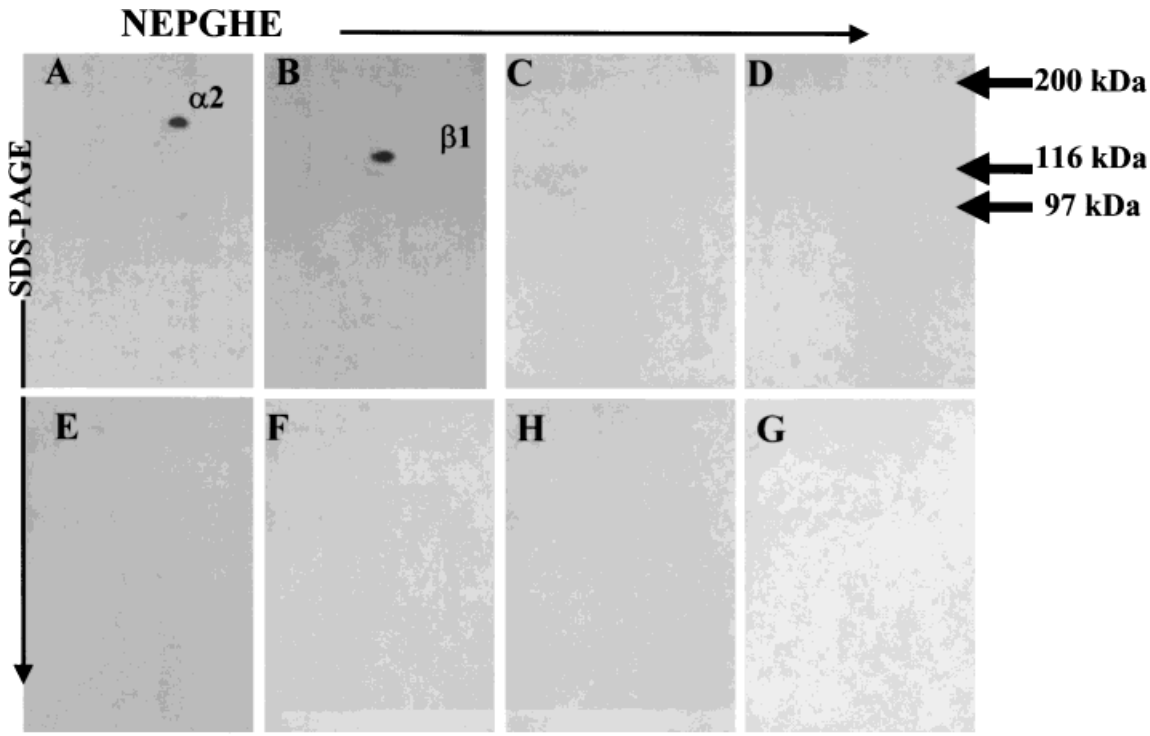

Fig. 6. Western blotting of nitrocellulose membranes containing Echo1 immunoprecipitated receptor complexes. The membrane was probed and with α2 specific rabbit serum (AB1944)(A), with β1 specific mAb (B3B11) (B), with αv chain specific mAb VNR139 (C) with α5 specific rabbit serum (AB1928) (D), with β4 specific rabbit serum (AB1922) (E), with β5 specific rabbit serum (AB1926) (F) β3 chain specific mAb Y2/51 (G). Followed by probing with HRP-conjugated goat anti-mouse Ig or by HRP-conjugated goat anti-rabbit Ig, depending on the species of the integrin antibody that had been used. The film exposure to chemiluminescence was 5 min.

In order to gain more insight into the mechanisms underlying virus attachment we established a method for immunoprecipitating receptor–cell-surface protein complexes by utilising virion particles as affinity surfaces for solubilised proteins in cell extracts followed by the addition of mAbs specific for the virions and Protein A Sepharose beads. The immunoprecipitated virus–cell-surface protein complexes could be subsequently identified by SDS-PAGE and immunoblotting.

The advantage in using this approach is that the ligand, in this case the virus, has not been modified in any way, and since it is not immobilised and is in solution it is behaving as it would when binding to host cells.

In this study, we have decided to use this approach in order to identify cell-surface molecules utilised, by Echo1 and HPEV1 which are members of the *Picornavirus* family, a family of small non-enveloped RNA-viruses which include several pathogens of man and animal and their medical and economic importance has initiated a considerable research activity [Grist and Reid, 1988]. This is a diverse family of viruses and not all members utilise the same receptor molecules. ICAM-1 was identified as a receptor for 90% of rhinoviruses [Greve et al., 1989] and several Coxsackie A viruses [Shaffren et al., 1997a,b]. Integrins are also known to function as receptors for some Picornaviruses: integrin α2β1 is a receptor molecule for Echo1 and Echo2 [Bergelson et al., 1992, 1993] whereas integrin αvβ3 is recognised by foot-and-mouth disease virus [Neff et al., 1998; Berinstein et al., 1995] and also Coxsackievirus A 9 [Triantafilou et al., 1999, 2000]. HPEV1 which displays an RGD motif in the VP1 capsid protein [Hyypia et al., 1992], which a motif recognised by αv integrins [Hynes, 1992; Ruoslahti, 1991; Ruoslahti and Pierschbacher, 1987], is also thought to utilise integrins as its receptors molecules [Pulli et al., 1997].

This family of viruses can successfully bind to solubilised cell extracts [Krah and Crowell, 1982; Mapoles et al., 1985], therefore, by using this modified immunoprecipitation method we have immunoprecipitated the candidate recep-

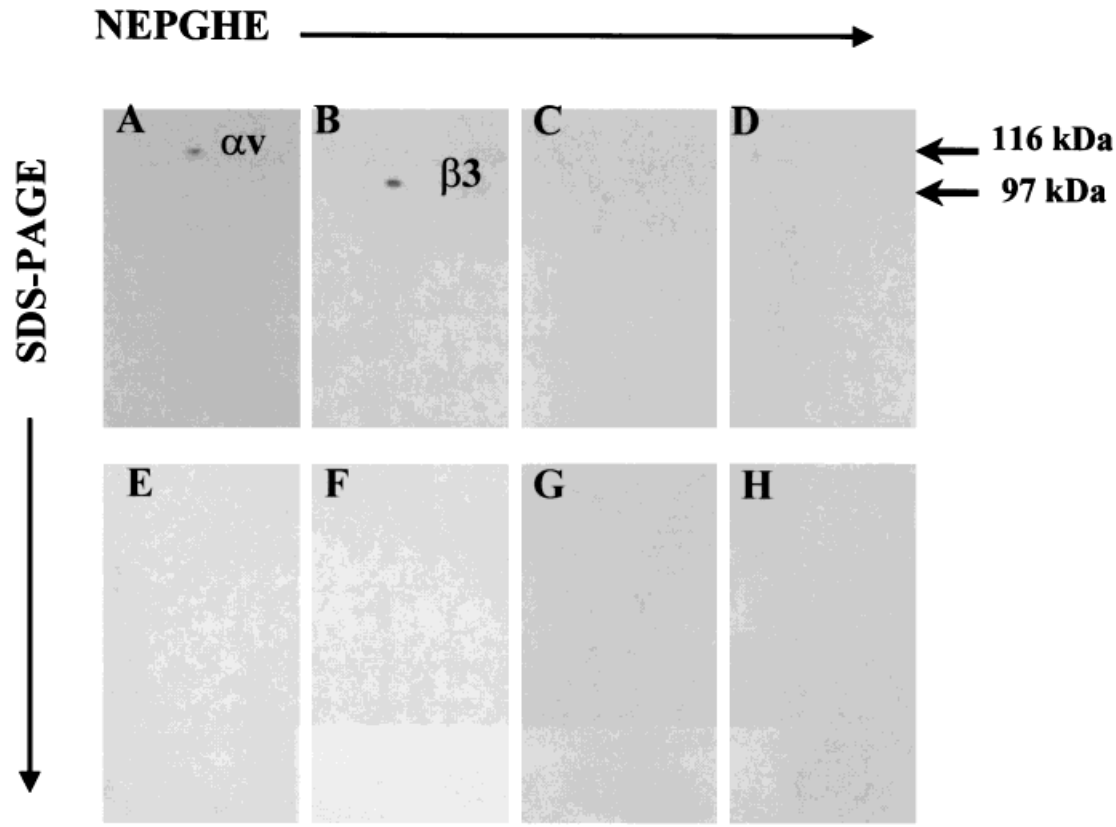

Fig. 7. Western blotting of nitrocellulose membranes containing HPEV1 immunoprecipitated receptor complexes. The membrane was probed with αv chain specific mAb VNR139 (A), with β3 chain specific mAb Y2/51 (B) with α2 specific rabbit serum ((AB1944) (C), with α5 specific rabbit serum (AB1928) (D), with β4 specific rabbit serum (AB1922) (E), with β5 specific rabbit serum (AB1926) (F), and with β1 specific mAb (G). Followed by probing with HRP-conjugated goat anti-mouse Ig or by HRP-conjugated goat anti-rabbit Ig, depending on the species of the integrin antibody that had been used. The film exposure to chemiluminescence was 5 min.

tor–virus complexes and analysed them by SDS-PAGE and two-dimensional gel electrophoresis. In the case of Echo1, two proteins having molecular weights of 120 and 150 kDa, respectively, were immunoprecipitated. By subsequent immunoblotting, we found that these two proteins were the β1 and the α2 subunits of integrin α2β1 which is in good agreement with previous work [Bergelson et al., 1992, 1993] that suggests that Echo1 utilises this integrin as its main receptor.

In the case of HPEV-1 two proteins were also immunoprecipitated with molecular weights of 120 and 100 kDa. By subsequent immunoblotting we found that these proteins were the αv and β3 subunits of integrin αvβ3. Thus, showing that HPEV1 interacts with integrin αvβ3.

The receptor binding epitopes of these viruses are not known, there could be either conformational or sequence specific, therefore it is difficult to estimate the number of cell-surface molecules that each virus particle binds to. However, by making some assumptions we have attempted to estimate the sensitivity of this assay when applied to Echo1. The number of virus particles used per assay was 1.5×10^6 PFU, by assuming that the concentration of virus particles capable of receptor binding was 10-fold greater, the total number of virus particles capable of binding in our assay was 1.5×10^7. In each assay we used 3×10^6 cells, separately we had quantified the number of integrin α2β1 per cell [Triantafilou et al., 2000]. There were ≈81,000 molecules of integrin α2β1 per cell, therefore the number of integrin α2β1 receptors in our assay is 2.4×10^{11}. If we assume that one virus particle binds one α2β1 integrin, then the amount of integrin on the blot can be calculated. Therefore, the 1.5×10^7 virus particles bind 1.5×10^7 integrin molecules, since the molecular weight of integrin α2β1 is 270,000 Da there were 2.5×10^{-17} mol

of integrin, so the amount of integrin $\alpha2\beta1$ on the gel is ≈ 6.7 pg of protein. If we assume that each protomer of the virus capsid binds one integrin $\alpha2\beta1$ molecule, then the virus binds 60 integrin $\alpha2\beta1$ molecules and the amount of integrin on the gel is 0.4 ng. Thus, the minimum amount of receptor available on the blot is in the range of picograms.

In conclusion, we have deviced an immunoprecipitation method to identify cell-surface molecules utilised by viruses during the crucial virus attachment stage. Using two Picornaviruses we have shown that we can successfully immunoprecipitate and identify by immunoblotting cell surface proteins which interact with viruses. We believe that this approach has a wide applicability and can be extended to analyse virus–cell-surface protein complexes of practically any virus with capsid.

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