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Characterization of a 100-Kilodalton Binding Protein for the Six Serotypes of Coxsackie B Viruses

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Viral infection of host cells primarily depends on binding of the virus to a specific cell surface protein. In order to characterize the binding protein for group B coxsackieviruses (CVB), detergent-solubilized membrane proteins of different cell lines were tested in virus overlay protein-binding assays. A prominent virus-binding protein with a molecular mass of 100 kDa was detected in various CVB-permissive human and monkey cell lines but was not detected in nonpermissive cell lines. The specificity of CVB binding to the 100-kDa protein on permissive human cells was substantiated by binding of all six serotypes of CVB and by competition experiments. In contrast, poliovirus and Sendai virus did not bind to the 100-kDa CVB-specific protein. A fraction of HeLa membrane proteins enriched in the range of 100 kDa showed functional activity by transforming infectious CVB (160S) into A-particles (135S). In order to purify this CVB-binding protein, solubilized membrane proteins from HeLa cells were separated by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by elution of the 100-kDa protein. Amino acid sequence analysis of tryptic fragments of the CVB-binding protein indicated that this 100-kDa CVB-specific protein is a cell surface protein related to nucleolin. These results were confirmed by immunoprecipitations of the CVB-binding protein with nucleolin-specific antibodies, suggesting that a nucleolin-related membrane protein acts as a specific binding protein for the six serotypes of CVB.

Group B coxsackieviruses (CVB), comprising six serotypes (CVB1 to CVB6), are enteroviruses of the family *Picornaviridae*. They are important human pathogens and cause a variety of clinically relevant diseases such as acute and chronic myocarditis, meningitis, and autoimmune diabetes (1, 22, 25, 26, 28). The initial event in infection of susceptible cells with these single-stranded RNA viruses is the attachment of the virus to specific cell surface molecules, followed by entry of the virus into the host cell. For nonenveloped viruses, the presence of virus-binding proteins is a major prerequisite for cell and tissue tropism. Therefore, the diversity of diseases caused by CVB may reflect the presence of specific receptors in various tissues.

Identification and characterization of picornavirus receptors have been the objects of intensive investigation in the past few years. The best-characterized picornavirus receptor proteins are the intercellular adhesion molecule-1 (ICAM-1), used by human rhinoviruses of the major group, and the poliovirus receptor (PVR), which are both members of the immunoglobulin superfamily (15, 29, 38). Binding of virus is mediated by interaction of the N-terminal domains of these receptors with structures in the viral canyon (13, 35, 44, 52).

From infected HeLa cells, a virus-receptor complex was purified that contained virus capsid proteins as well as an additional cellular protein with a molecular mass of 49.5 kDa, which was suggested to be responsible for recognition and binding of CVB (37). In the brains of newborn BALB/c mice and in cultured neurons, four receptor proteins with molecular masses of 46, 44, 36, and 33 kDa were reported (51). Furthermore, a variant of CVB adapted to growth in RD cells was shown to bind to decay-accelerating factor (DAF [CD55]) (3), the previously described receptor for several echoviruses (2, 49). Recently, Shafren et al. reported that DAF might be a cell attachment receptor for serotypes B1, B3, and B5, but not for serotypes B2, B4, and B6, of CVB (46). However, previous competition assays have revealed that all six serotypes of CVB bind to a common receptor which is different from the receptor proteins of other picornaviruses (8, 36). Although a variety of data about the CVB receptor have been published, the common receptor protein for the six CVB serotypes has not yet been purified and sequenced. Here, we report the identification of a nucleolin-like 100-kDa cell surface protein with binding capacity for all six serotypes of CVB.

MATERIALS AND METHODS

Viruses and cell lines. CVB3 (Nancy strain) used in this study was derived by transfection of HeLa cells with infectious recombinant CVB3 cDNA (24). CVB1, CVB2, CVB4, CVB5, CVB6, and poliovirus type 1 (Sabin strain), obtained from the American Type Culture Collection (ATCC VR-28, -29, -184, -185, -155, and -192, respectively), were propagated in HeLa cells and maintained in Dulbecco's modified Eagle's minimal medium supplemented with 10% fetal bovine serum. Sendai virus (Fushimi D52 strain; courtesy of C. J. Buchholz) was grown in embryonated eggs and purified as described by Homann et al. (19).

The following cell lines were obtained from ATCC: HeLa (human cervix carcinoma; CCL 2), A549 (human lung carcinoma; CCL 185), 293 (human embryonal kidney; CCL 1573), human liver cells (CCL 13), HepG2 (human hepatocellular carcinoma; HB 8065), Vero (green monkey kidney; CCL 81), CV-1 (monkey kidney; CCL 70), LTK$^-$ (mouse fibroblasts; CCL 1.3), and RD (human rhabdomyosarcoma; CCL 136). Rat skeletal muscle myoblasts (L6) and KS cells, a Kaposi's sarcoma-derived cell line, were provided courtesy of S. Werner and M. Stürzl, Martinsried, Germany. Cells were cultivated as monolayers in Dulbecco's modified Eagle's minimal medium supplemented with 10% fetal bovine serum.

Virus purification and radioactive labeling. Confluent monolayers of HeLa cells in 175-cm^2 flasks were washed twice with phosphate-buffered saline (PBS) and infected for 1 h at 37°C with purified virus at a multiplicity of infection of 10. Cells were washed twice with PBS and incubated in methionine-free minimal essential medium at 37°C. Two hours postinfection, 1 mCi of [^{35}S]methionine was added, and the incubation was continued for 18 h. Cells were frozen and

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quickly thawed to release virus particles. After centrifugation at 700 × *g* for 15 min to remove cell debris, the virus was pelleted at 45,000 rpm for 90 min in an SW56 rotor at 4°C. For further purification, the pellet was resuspended in 4 ml of PBS, loaded onto a 30% sucrose cushion, and centrifuged at 45,000 rpm for 12 h at 4°C. The pellet containing radiolabeled virus was resuspended in TNE buffer (10 mM Tris-HCl [pH 8.0], 10 mM NaCl, 1 mM EDTA), and aliquots were stored at −20°C.

Plaque assay. Different cell lines were infected with CVB3 as outlined above, and virus was recovered from the cell suspension by freeze-thawing. Virus titers were determined by plaque assay on HeLa monolayer cells as previously described (23).

Membrane preparation. Membrane proteins were prepared as described by Krah (30). Cell monolayers were treated with 10 mM EDTA in PBS for 10 min at 37°C to detach cells, washed twice with PBS, resuspended (6×10^7 cells per ml) in lysis buffer (1.5% octyl glucoside, 2 mM phenylmethylsulfonyl fluoride in PBS), and incubated on ice for 1 h. The lysates were centrifuged at 100,000 × *g* for 1 h at 4°C. Supernatants containing the solubilized membrane proteins were immediately used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

VOPBA. The virus overlay protein-binding assay (VOPBA) was performed as follows. Detergent-solubilized membrane proteins (25 to 100 μg per lane) were separated on SDS–7.5 to 10% polyacrylamide gels. The proteins were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore) according to the method of Kyhse-Andersen (32), followed by incubation in a renaturing buffer (RNB; 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4], 10 mM $MgCl_2$, 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol [DTT], 10% glycerol) for 12 h at 4°C. PVDF membranes were incubated for 3 h in RNB–5% bovine serum albumin (BSA) to saturate free binding sites and were exposed to [^{35}S]methionine-labeled virus (50,000 cpm/ml of RNB–5% BSA) for 24 h at 4°C. Subsequently, membranes were washed three or four times for 30 min in RNB–0.25% BSA, dried, and exposed to Kodak X-Omat AR films for 2 to 4 days.

Preparation of 135S particles. Enriched 100-kDa HeLa membrane proteins were incubated with purified [^{35}S]methionine-labeled CVB3 (20,000 cpm). The solution was incubated for 30 min at 4°C. After further incubation at 37°C for 45 min, the solution was layered onto a 15 to 30% sucrose gradient in PBS containing 0.1% BSA. Gradients were centrifuged for 3 h at 39,000 rpm in a Sorvall TH 641 rotor, fractionated from the bottom in 8-drop aliquots, and measured in a liquid scintillation counter. Heat-inactivated virions were prepared by heating of radiolabeled virus for 12 min at 56°C in the presence of 0.05% Nonidet P-40. The solution was cooled and layered onto a 15 to 30% sucrose gradient as described above.

Purification of CVB-binding proteins. Detergent-solubilized membrane proteins (about 1 mg) were separated on preparative SDS–7.5% polyacrylamide gels. The protein band corresponding to the binding activity on autoradiographs was localized with 0.3 M $CuCl_2$ as described by Lee et al. (33). The protein was eluted from the excised gel fragment by diffusion in 10 mM Tris-HCl (pH 7.5) containing 0.1% SDS at 4°C. After 24 h, the eluted protein was precipitated with 60% trichloroacetic acid containing 0.1% Triton X-100. The resulting protein pellet was washed three times with 100% acetone and resuspended in protein loading buffer. The isolated protein was separated on an SDS-polyacrylamide gel and stained with Coomassie brilliant blue to assess the purity and amount of the purified CVB-binding protein.

Amino acid sequencing. For internal sequencing, the purified 100-kDa protein was cleaved with endoprotease LysC (Boehringer) in the SDS-polyacrylamide gel. The peptides were eluted from the gel and separated by reversed-phase high-performance liquid chromatography (HPLC) on a Superspher 60 RP select B column (125 × 2 mm), according to Eckerskorn and Lottspeich (12). Selected peptides were subjected to Edman N-terminal sequencing on a Beckman Proton 3600 sequencer. Identification of the amino acids was performed with Beckman Microbe-HPLC System Gold.

2-D gel electrophoresis. The purified CVB-binding protein was separated by two-dimensional (2-D) electrophoresis with immobilized pH gradients as described by Görg et al. (14), with minor modifications. Briefly, the immobilized pH gradient gels with linear gradient from pH 4 to 10 were cast, dried, and stored at −20°C. For isoelectric focusing (IEF), dried immobilized pH gradient gels were cut into strips and rehydrated overnight in a solution containing 8 M urea, 0.5% octyl glucoside, and 10 mM DTT. Immobilized pH gradient gel strips were placed on a Multiphore II electrophoresis apparatus, and 2 μg of protein (dissolved in a lysis solution containing 8 M urea, 2% octyl glucoside, 5% β-mercaptoethanol, 0.4% Ampholines [pH 3.5 to 10], and 0.4% Pharmalytes [pH 3 to 10]) was applied to each rehydrated strip. IEF was performed at 15°C with an initial setting of 300 V up to a maximal constant setting of 2,000 V overnight. The focused gel strips were equilibrated twice in 10 ml of equilibration solution (0.05 M Tris-HCl [pH 6.8], 6 M urea, 30% glycerol, 2% SDS, 1% DTT, and a trace concentration of bromophenol blue) for 15 min each and directly placed onto SDS–10% polyacrylamide gels. After electrophoresis, gels were silver stained according to the method of Heukeshoven and Dernick (17) or electroblotted for VOPBAs.

Labeling of cell surface proteins with NHS-biotin. Biotinylation of HeLa cell surface proteins was performed as described by Cole et al. (7), with minor modifications. In brief, 50 μl of freshly prepared 0.1 M NHS-biotin (*N*-hydroxysuccinimide ester derivative) was added to 1 ml of HeLa cell suspension (10^8 cells) in PBS to give a final concentration of 5 mM biotin. After 30 min at 4°C, the reaction was stopped with 1 mM ethanolamine in PBS. Cells were washed three times with PBS and resuspended in PBS (2×10^7 cells per ml). Biotin-labeled cells were solubilized with 1.5% octyl glucoside as described above.

Western blots (immunoblots) and immunoprecipitations. Samples containing membrane proteins or purified nucleolin were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto PVDF membranes. Binding sites were saturated by incubation in PBS–3% BSA, followed by incubation with specific antibodies for 1 h. Filters were washed in blocking buffer, exposed to peroxidase-labeled secondary antibodies, and visualized by incubation with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate toluidinium. Human nucleolin, purified by chromatography on a heparin-agarose column, was provided by E. Genersch, Martinsried, Germany. Polyclonal anti-human pp105 antiserum was a gift of F. A. Anderer, Tübingen, Germany. For immunoprecipitations, 1 ml of octyl glucoside-solubilized, biotin-labeled HeLa membrane proteins was incubated with 2 μl of a chicken nucleolin-specific antiserum (I-8 [34], courtesy of E. A. Nigg, Epalinges, Switzerland) for 4 h at 4°C. Preswollen protein G-Sepharose was added for 2 h with vigorous shaking. Washing was carried out six times in Emerson-Schubert buffer (25 mM NaCl, 10 mM Tris-HCl [pH 8.0], 10% glycerol, 0.2% Triton X-100). The protein components of the precipitate were separated by SDS-PAGE and electroblotted onto a PVDF membrane. The membrane was blocked with 3% BSA in PBS, washed four times with PBS–0.1% Tween 20 and once in PBS, followed by a 30-min incubation with streptavidin-biotin-horseradish peroxidase complex (Amersham) (diluted 1:1,000 in PBS–1% BSA). Excess streptavidin complex was removed by five washings of the membrane as described above. For visualization of the protein band, the membrane was incubated for 30 min at 37°C with 15 ml of PBS containing 7.5 mg of diaminobenzidine and 9 μl of 30% hydrogen peroxide.

RESULTS

Identification of a 100-kDa CVB-binding protein. One of the first steps in virus infection is specific binding of the virus to the cell surface. To characterize this attachment protein for CVB, detergent-solubilized membrane proteins of permissive HeLa cells were tested for virus-binding activity. Membrane proteins were extracted with octyl glucoside, separated on SDS-polyacrylamide gels, and blotted onto PVDF membranes. Filters carrying the immobilized cellular proteins were renatured in a buffer containing BSA and [^{35}S]methionine-labeled CVB3. As shown in Fig. 1, the autoradiography of a VOPBA revealed a prominent virus-binding protein with an apparent molecular mass of 100 kDa. With increasing amounts of HeLa membrane proteins (25, 50, and 100 μg), dose-dependent binding of radiolabeled CVB3 to the 100-kDa protein was observed. High protein concentrations revealed binding of radiolabeled virus to additional proteins with molecular masses of 75, 50, and 30 kDa, although to a lesser extent than binding to the 100-kDa protein.

All CVB serotypes bind to the 100-kDa protein. All six serotypes of CVB were tested in the VOPBA. HeLa plasma membrane proteins were separated in SDS-polyacrylamide gels and blotted onto PVDF membranes. Blots were cut longitudinally, and each strip was probed with one of the six serotypes of CVB labeled with [^{35}S]methionine. As a control for the specificity of the interaction, two identical strips were incubated with either [^{35}S]methionine-labeled poliovirus (type Sabin 1) or Sendai virus. As shown in the corresponding autoradiograph (Fig. 2), all six serotypes of CVB (lanes 1 to 6) bind to the 100-kDa membrane protein, while poliovirus and Sendai virus (lanes 7 and 8) do not recognize this CVB-specific binding protein. Furthermore, binding of CVB to proteins in the range of 50 kDa was occasionally observed, which correlated with the strength of binding to the 100-kDa protein. These data support the assumption that the 100-kDa membrane protein of HeLa cells is a group-specific binding protein for CVB.

CVB-permissive cell lines express the 100-kDa protein. Several cell lines were tested by VOPBA for expression of the virus-binding protein and tested by plaque assay on HeLa cells (with a CVB3 multiplicity of infection of 10) for productive

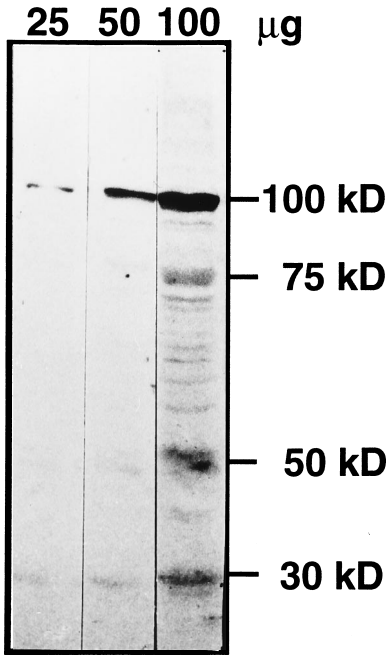

FIG. 1. Binding of ^{35}S-labeled CVB3 to solubilized HeLa membrane proteins. After solubilization with 1.5% octyl glucoside, HeLa membrane proteins were separated by SDS-PAGE (10% polyacrylamide) and blotted onto a PVDF membrane. Immobilized proteins were renatured in binding buffer with 5% BSA and incubated with [^{35}S]methionine-labeled CVB3 (50,000 cpm/ml of binding buffer) for 24 h at 4°C.

infection with CVB3. The data from these experiments are summarized as follows. Productive infection (increase in titer of >2 log units) was strictly correlated with the expression of the 100-kDa CVB attachment protein in CVB-permissive cell lines (HeLa, A549, 293, CCL13, HepG2, Vero, and CV-1). In contrast, four nonpermissive cell lines (RD, LTK$^-$, L6, and KS) did not express the specific CVB attachment protein with a molecular mass of 100 kDa or show productive infection with CVB3.

Formation of A-particles by enriched 100-kDa HeLa membrane proteins. The uptake of enteroviruses into the cell is accompanied by a conformational change in the viral capsid, mediated by the interaction of the virus and its specific binding protein. This alteration can be traced by the appearance of virus particles which have been deprived of capsid protein VP4 (A-particles). Aliquots of [^{35}S]methionine-labeled CVB3, incubated with an enriched fraction of HeLa membrane proteins in the range of 100 kDa under conditions similar to those in the

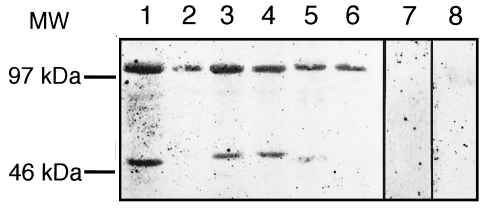

FIG. 2. Autoradiograph of a VOPBA with [^{35}S]methionine-labeled CVB1 to CVB6. HeLa proteins (50 μg of protein per lane) were prepared as described in the legend to Fig. 1. The protein blot was cut into strips, and each strip was incubated with either one of the six radiolabeled serotypes of CVB (lane 1, CVB1; lane 2, CVB2; lane 3, CVB3; lane 4, CVB4; lane 5, CVB5; lane 6, CVB6) or a radiolabeled control virus (lane 7, poliovirus; lane 8, Sendai virus). MW, molecular size.

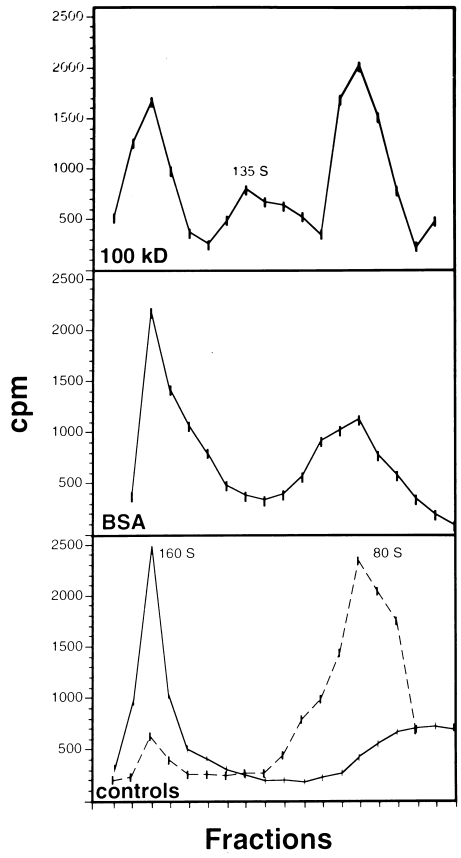

FIG. 3. A-particle formation by HeLa membrane proteins enriched in the range of 100 kDa. A fraction containing the CVB-binding protein (top panel) and an equal amount of BSA (middle panel) were exposed to purified [^{35}S]methionine-labeled CVB3. Solutions were incubated for 30 min at 4°C. After further incubation at 37°C for 45 min, reaction mixtures were layered onto a 15 to 30% sucrose gradient in PBS containing 0.1% BSA. After centrifugation, the gradients were fractioned from the bottom in 8-drop aliquots. Virus particles deprived of capsid protein VP4 (A-particles) sediment at 135S. Purified CVB3 resulted in a 160S peak, and heat-inactivated virions resulted in an 80S peak (bottom panel).

binding experiments, were analyzed by sedimentation through sucrose gradients. As depicted in the top panel of Fig. 3, interaction of the HeLa protein fraction with radiolabeled CVB3 separated the virus into three peaks of 160S (cosedimenting with native virus), 135S (A-particles), and 80S (empty capsids). Incubation of ^{35}S-labeled virus with 400 μg of BSA did not result in the formation of 135S particles (Fig. 3, middle panel). Profiles of purified virus (160S peak) and heat-inactivated virions (80S peak) are shown in the bottom panel of Fig. 3.

Characterization of the purified CVB-binding protein. In order to identify the CVB-binding protein by amino acid sequencing, it was necessary to obtain large amounts of purified protein. The purification of the 100-kDa protein by column chromatography turned out to be unsuitable because of the strong degradation of the virus-binding protein during this procedure. Therefore, the protein was purified to homogeneity from HeLa cells by preparative SDS-PAGE. Detergent-solubilized membrane proteins from about 3×10^9 cells were separated, and bands of the appropriate size were eluted from the gel. The eluted 100-kDa proteins were pooled and examined by SDS-PAGE. Coomassie staining of the purified protein revealed a 100-kDa band with a minor contaminant (Fig. 4A).

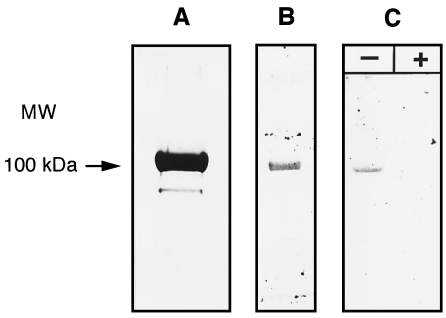

FIG. 4. Analysis of the purified 100-kDa CVB-binding protein. (A) Five micrograms of purified CVB-binding protein was separated on an SDS–7.5% polyacrylamide gel and visualized by Coomassie staining. The position of the binding protein is indicated by an arrow. (B) Autoradiograph of a binding assay with [^{35}S]methionine-labeled CVB3. An aliquot of 50 ng of the purified CVB-specific protein was treated as described for panel A and blotted onto a PVDF membrane. The VOPBA was carried out as described in the legend to Fig. 1. (C) Binding of ^{35}S-labeled CVB3 in the absence (−) or presence (+) of unlabeled CVB (250-fold excess of unlabeled competitor virus). MW, molecular size.

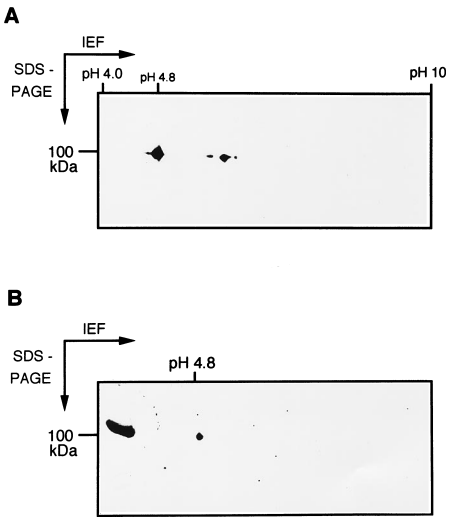

FIG. 5. Analysis of the purified CVB-specific binding protein by 2-D gel electrophoresis. An aliquot of the purified CVB-binding protein (1 μg) was separated by IEF in an immobilized pH gradient (pH 4 to 10) followed by SDS-PAGE (10% polyacrylamide). (A) Silver staining of the 2-D gel. (B) Proteins separated by 1-D and 2-D gel electrophoresis were blotted onto a PVDF membrane, renatured, and incubated with [^{35}S]methionine-labeled CVB3. Binding of radiolabeled CVB3 to the 1-D-separated protein is shown on the left side of the autoradiograph. A single CVB-binding protein with a molecular mass of 100 kDa and a pI at pH 4.8 was detected by 2-D gel electrophoresis.

A small aliquot of the purified protein was tested in a VOPBA, demonstrating that the eluted protein retained its binding properties towards CVB, as shown in the corresponding autoradiograph (Fig. 4B). In addition, the specificity of CVB binding to the purified protein was determined by competition assays. The interaction of radiolabeled CVB3 with the purified 100-kDa protein was completely inhibited by a 250-fold excess of unlabeled virus, as shown in Fig. 4C. Other RNA viruses, such as poliovirus and Sendai virus, did not compete for CVB binding (data not shown).

To examine the purity and the monomeric character of the eluted 100-kDa protein, high-resolution 2-D electrophoresis was applied. IEF was carried out in immobilized pH gradients (pH 4 to 10), followed by SDS-PAGE. Silver staining analysis of the purified protein revealed one dominant and three minor protein spots with pIs of 4.8 to 6.5 (Fig. 5A). However, as shown in the autoradiograph of the 2-D VOPBA (Fig. 5B), only binding of radiolabeled CVB3 to the major protein spot at pI 4.8 was observed. The same results were obtained with unpurified extracts of HeLa cell membrane proteins (data not shown). In contrast to the complex pattern of 2-D electrophoresis-separated proteins after silver staining, binding of [^{35}S]methionine-labeled CVB3 resulted in a single protein spot with a molecular mass of 100 kDa. In summary, these results demonstrate that the CVB-binding protein was eluted from preparative SDS gels in a homogeneous and functionally active form suitable for amino acid sequence analysis.

For internal amino acid sequence analysis, the purified protein was digested in the gel with endoproteinase LysC. After elution of the peptides, the cleavage mixture was fractionated by reversed-phase HPLC, and five peptides were subjected to amino acid analysis. A comparison of the peptide sequences with sequences in a protein database (Swiss Prot) revealed that all five peptides showed consensus sequences with the C-terminal part of human nucleolin (Table 1).

Cell surface localization of the CVB-binding protein. The nature of the CVB-binding protein was further evaluated by immunological assays. The purified CVB attachment protein and a nuclear extract of HeLa cells were separated by SDS-PAGE, transferred to a PVDF membrane, and analyzed by Western blotting with a human nucleolin-specific antiserum (anti-pp105). The anti-pp105 antiserum, raised against the cytosolic form of nucleolin (40), detected a 50-kDa protein and a 100-kDa protein in the nuclear extract (Fig. 6A, lane 1) as well as the 100-kDa CVB-binding protein purified from plasma membranes (Fig. 6A, lane 2), demonstrating the close relationship between these two proteins. Furthermore, cell surface biotinylation was used to demonstrate membrane localization of the CVB attachment protein (Fig. 6B). Intact human HeLa and RD cells were labeled with NHS-biotin before solubilization of membrane proteins and subsequent immunoprecipitation. Precipitated biotinylated proteins were visualized by means of a streptavidin-peroxidase complex. An antiserum (I-8) with specificity to chicken nucleolin was able to precipitate a 100-kDa biotinylated protein from HeLa cells (Fig. 6B, lane 3). As expected, antiserum I-8 did not precipitate a specific protein from RD cells (Fig. 6B, lane 2), since these cells do not express the 100-kDa CVB-binding protein on the cell surface (described above). It is important to note that in contrast to anti-pp105 antiserum, the chicken nucleolin-specific antiserum I-8 does not recognize the nuclear form of human nucleolin (4, 34). However, a biotinylated membrane protein from HeLa cells was immunoprecipitated by use of this antiserum, indicating that the CVB attachment protein is not completely identical to human nucleolin of the nucleolus but rather was a 100-kDa cell surface membrane protein with homology to nucleolin. The autoradiograph of a corresponding VOPBA in Fig. 6C demonstrates that the biotinylated 100-kDa cell

TABLE 1. Amino acid sequences of tryptic peptides derived from the purified 100-kDa CVB-binding protein

Peptide	Amino acid sequence	Amino acid position in human nucleolin
1	FGYVDFESAED	349–359
2	TLVLSNLSYSATEETLQEV	487–505
3	GLSEDTTE	578–585
4	ESFDGSVRA	590–598
5	GFGFVDF	611–617

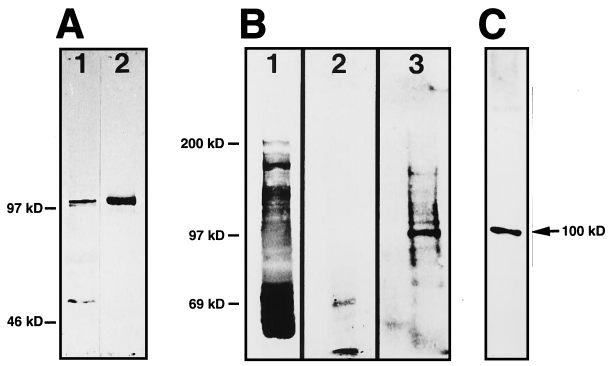

FIG. 6. Characterization of the 100-kDa CVB-binding protein by Western blotting and immunoprecipitation. (A) Western blot analysis. HeLa nuclear protein extract (lane 1) and purified CVB-binding cell surface protein (lane 2) were analyzed by Western blotting with an antiserum against human nucleolin (pp105) as described in Materials and Methods. (B) Immunoprecipitation of biotinylated membrane proteins with chicken nucleolin-specific antibodies. HeLa cells and RD cells were labeled in a 5 mM solution of NHS-biotin and lysed with 1.5% octyl glucoside. The lysates were immunoprecipitated with chicken nucleolin-specific antibodies. Total biotinylated HeLa cell lysate (lane 1) and immunoprecipitates of biotinylated protein from RD cells (lane 2) and HeLa cells (lane 3) were separated by SDS-PAGE. Proteins were blotted onto a PVDF membrane and visualized with a streptavidin-biotin-horseradish peroxidase complex. A 100-kDa nucleolin-like membrane protein was precipitated from HeLa cells but not from RD cells. (C) VOPBA with immunoprecipitated biotinylated protein from HeLa cells. Biotinylated HeLa membrane protein was treated as described in the legend to Fig. 5B. The protein was incubated with [^{35}S]methionine-labeled CVB3 and visualized by autoradiography.

surface protein precipitated from a membrane fraction of HeLa cells was still capable of binding radiolabeled CVB3. These results strongly indicate that a nucleolin-like cell surface protein is the specific binding protein for all six serotypes of CVB.

DISCUSSION

We have identified a 100-kDa plasma membrane protein on CVB-permissive cell lines as a specific binding protein for all six serotypes of CVBs. This protein was detected by use of VOPBAs, a method which has been proved to be a useful tool for studying protein interactions with virus particles (11, 39). In addition to the distinct binding of CVB to the 100-kDa protein, further bands with molecular masses of 75, 50, and 30 kDa were detected. All six serotypes of CVB, but not two other RNA viruses (poliovirus and Sendai virus), were found to bind specifically to the attachment protein, which is in accordance with the finding that all six serotypes of CVB compete for the same binding protein (36). CVBs are characterized by a broad tissue tropism and infection of a wide range of mammalian cells in culture (9). The CVB-specific binding protein was found to be expressed in all CVB-permissive cell lines tested, but could not be detected in nonpermissive RD, LTK$^-$, L6, and KS cells.

One of the early products of the interaction of enteroviruses with their specific binding proteins is the formation of virus particles which have been deprived of capsid protein VP4 (A-particles) (10). Furthermore, it has been shown that cell-free systems containing receptor proteins can alter enterovirus and rhinovirus particles into A-particles as well (16, 52). The formation of A-particles by the enriched fraction of 100-kDa membrane proteins from HeLa cells demonstrates that the interaction between virus and cellular proteins is not restricted to binding but also comprises functional alteration of the virus particles. The correlation between virus binding to the 100-kDa HeLa membrane protein and A-particle formation by HeLa membrane proteins enriched in the range of 100 kDa strongly suggests that the 100-kDa CVB-binding protein is a putative receptor for all six serotypes of CVB.

The 100-kDa CVB-binding protein, purified to homogeneity from HeLa cells by preparative SDS-PAGE, retained its specificity towards CVB, as shown in VOPBAs and by inhibition with cold virus. After two-dimensional electrophoresis of the purified 100-kDa HeLa protein, a single protein spot (100 kDa [pI 4.8]) with virus-binding capacity could be detected. It is unlikely that the 100-kDa CVB-binding protein is a dimer of two molecules of the previously described 50-kDa CVB receptor protein (37), since it retained its molecular mass of 100 kDa even in the presence of 8 M urea during 2-D PAGE.

By sequencing of tryptic peptides of the purified CVB-binding protein, the CVB-binding protein was identified as a membrane protein with homology to nucleolin. Nucleolin is a phosphoprotein abundantly expressed in exponentially growing cells, primarily localized in the cell nucleoli (47). The polypeptide is composed of 707 amino acids with a calculated molecular mass of 77 kDa and an apparent molecular mass of 100 kDa (48). In nucleoli, this protein was shown to be involved in the control of pre-rRNA transcription (5), ribosomal assembly (6), and nucleocytoplasmic transportation of ribosomal components (4). Originally, nucleolin was described by Pfeifle et al. (42) as phosphoprotein pp105, localized on the cell surface of different cell lines after endogenous phosphorylation of intact cells. Further investigations showed that pp105 is predominantly localized in the nucleolus but is also expressed on the cell surface (40, 41). Cell surface expression of nucleolin or nucleolin-related proteins was further substantiated by the findings of other groups. A 109-kDa lipoprotein-binding protein and a 110-kDa laminin-binding protein were identified as nucleolin or nucleolin-related proteins on the cell surface, indicating that nucleolin itself or putative splicing products may function as cell surface receptors or binding proteins (27, 45). Another nucleolin-related phosphoprotein (pp100), which serves as a substrate for an ecto-protein kinase on the cell surface, was observed in HeLa cells (21). Recently, Krantz et al. (31) have purified a 100-kDa fructosyllysine-specific binding protein with sequence homology to nucleolin from cell membranes of the monocyte-like cell line U937. The 100-kDa CVB-binding protein described in this paper may be a further member of this family of nucleolin-like cell surface proteins.

Even though the sequences of the five peptides of the CVB-binding protein showed 100% identity with sequences in the C-terminal part of nucleolin, binding of CVB to partially purified nuclear nucleolin was not observed (data not shown), indicating sequential and structural differences between these two proteins. In our study, we used the method of cell surface biotinylation to demonstrate that the CVB-binding protein is a nucleolin-like protein expressed on the plasma membrane. Sequence homology of the CVB attachment protein to nucleolin was confirmed by immunological assays. Two different antisera, a chicken nucleolin-specific antiserum (I-8) and a polyclonal antiserum raised against human phosphoprotein pp105, were tested. Both nucleolin isolated from HeLa nuclei and the purified CVB-binding membrane protein were recognized by the anti-pp105 polyclonal antiserum (Fig. 6A), demonstrating that these proteins share common immunologic determinants. However, the chicken nucleolin-specific antiserum, which does not recognize human nucleolin, was yet able to immunoprecipitate a 100-kDa cell surface protein from HeLa cells (Fig. 6B). Moreover, this precipitated protein retained its binding capacity towards radiolabeled CVB3 (Fig. 6C). Thus, it appears that the CVB-binding protein is immunologically distinct

from human nucleolar nucleolin. Experiments to inhibit virus binding by nucleolin-specific antibodies, however, were not performed because of the limited amount of available antiserum, which therefore was preferentially used for the immunological characterization of the CVB-binding protein.

During the past few years, receptors for several picornaviruses have been identified (2, 3, 15, 18, 20, 37, 38, 43, 50, 51). The first receptor proteins for picornaviruses discovered were members of the immunoglobulin superfamily and the integrin family. The recently identified minor group rhinovirus receptor belongs to the low-density lipoprotein receptor family (18), and the receptor for echovirus 7 and related viruses was shown to be the DAF (CD55) (2, 49). The data presented in this paper suggest that a 100-kDa CVB-binding protein may be a putative receptor for CVBs. Ultimately, proof of the functional role of this nucleolin-like CVB-binding protein in virus entry will require identification of the receptor-encoding cDNA and transfer of CVB permissivity to nonpermissive cells by gene transfer.

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