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Predominant Binding of Theiler's Viruses to a 34-Kilodalton Receptor Protein on Susceptible Cell Lines

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Western immunoblots of BHK-21 cell lysates probed with the highly virulent GDVII and the less virulent BeAn strains of Theiler's murine encephalomyelitis virus (TMEV) revealed predominant binding to a 34-kDa membrane protein and much lower levels of binding to 100- and 18-kDa membrane proteins. Complete inhibition of virus binding to both the 34- and 18-kDa membrane species by excess unlabeled TMEV demonstrated specificity of binding. Virus binding was also blocked by wheat germ agglutinin, which specifically binds to sialic acid residues and blocks TMEV binding to whole BHK-21 cells. Radiolabeled TMEV also bound to 100-, 34-, and 18-kDa membrane proteins expressed on other TMEV permissive cell lines but not on the nonpermissive cell lines tested. These data suggest that a 34-kDa cellular protein may be the primary determinant of susceptibility to TMEV infection by mediating the binding of GDVII and BeAn viruses to susceptible cells.

Theiler's murine encephalomyelitis viruses (TMEVs) are naturally occurring enteric pathogens which constitute a separate serological group within the picornavirus family. More than 20 TMEVs that are divisible into two groups on the basis of neurovirulence have been described since Theiler's original isolations in 1937 (16, 32). The highly virulent TMEVs include GDVII and FA, and the much less virulent strains include all remaining isolates which cause central nervous system disease in the form of acute poliomyelitis (early onset) followed by chronic inflammation and demyelination (late onset). Demyelinating disease is associated with persistent infection that leads to immune-mediated myelin damage wherein host immune responses to virus and not self-antigens have been implicated in the demyelinating process (21, 22, 24).

TMEV neurovirulence has been mapped in part to the P1 genomic region, which encodes the four virion coat proteins (6, 11, 17), by the use of recombinants between cDNA clones of parental viruses representing the two neurovirulence groups. Recently, the P1 sequences resulting in attenuation of neurovirulence have been further localized to sequences encoding VP1 (20, 30). The dramatic effect of exchanging the virion coat proteins may rest in virion receptor determinants since the 32 amino acids composing the putative virion receptor attachment site of the closely related mengo virus reside in VP3 and VP1 (19).

Therefore, it is possible that neurovirulence depends on initial TMEV central nervous system cell interactions. The highly virulent TMEVs, unlike the less virulent strains, replicate preferentially in neocortical neurons and more extensively in pyramidal neurons in the hippocampus (18, 28). Members of both neurovirulence groups appear to replicate equally well in neurons of the thalamus, brainstem, and spinal cord. However, the identification and isolation of a specific cellular receptor has not as yet been accomplished. Using a sensitive and specific virus overlay protein binding assay to detect TMEV binding to host cell proteins, we find that representative strains of both TMEV neurovirulence groups bind predominantly to a 34-kDa protein species on the plasma membrane of cells permissive for TMEV infection; this protein is not detected in nonpermissive cell lines.

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MATERIALS AND METHODS

Cells and viruses. BHK-21 cells were grown in 100-mm plates in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mM L-glutamine, 0.15% tryptose phosphate broth (Sigma), 100 μg of streptomycin per ml, 100 U of penicillin per ml, 50 U of anti-PPLO (GIBCO) per ml, and 10% iron-supplemented bovine calf serum (Hyclone). BSC-1 cells were grown in DMEM containing 10% bovine calf serum. Madin-Darby bovine kidney and TK$^-$143 human epithelial cells were cultivated as previously described (14), as were BW 5147 mouse thymoma cells (10). Neuroblastoma NB41A3 cells were grown in Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum, while RAW 264.7 transformed macrophage cells were grown in DMEM supplemented with 10% fetal bovine serum.

After plaque purification, BeAn and GDVII virus stocks were prepared and purified by isopycnic centrifugation in Cs_2SO_4 as previously described (26).

Radiolabeling of TMEV. BHK-21 cell monolayers in 100-mm plates were infected with either BeAn or GDVII virus at a multiplicity of infection of 10 to 20. After virus adsorption for 40 min at 24°C, monolayers were washed with complete DMEM without serum and tryptose phosphate broth and incubated in the same medium for 30 min at 37°C. Infected cells were washed twice with medium deficient in methionine or phosphates and incubated in the appropriate deficient medium for 1 h at 37°C. Medium was removed and deficient medium containing 83 μCi [^{35}S]methionine (Translabel; ICN, Costa Mesa, Calif.) or $^{32}P_i$ (Amersham, Arlington Heights, Ill.) per ml was added and infected cells were harvested after 24 h. Radiolabeled virus was purified by isopycnic centrifugation in Cs_2SO_4.

Virus particle number. Virus particle number was estimated on the basis of virus RNA content, where an optical density at 260 nm of 1 = 5 mg of virus, and 1 mg of virus = 7.2×10^{13} particles (27). Virus yield from each purification

starting with 12 to 15 100-mm plates of infected cells was 1 to 2 mg.

Cell membrane preparation. Crude cell membranes were prepared without using detergents, as follows. Cell monolayers were washed with divalent cation-deficient phosphate-buffered saline (PBS) followed by the addition of divalent cation-deficient PBS medium containing 50 mM EDTA for 10 min at 24°C. Cells were pelleted at 800 × *g* for 5 min at 4°C, washed with PBS, swollen on ice for 15 min (2×10^7 cell per ml), and disrupted with a glass Dounce homogenizer after addition of phenylmethylsulfonyl fluoride (PMSF) to 1 mM, aprotinin to 10 μg/ml, and EDTA to 5 mM. Nuclei and cell debris were pelleted at 1,000 × *g* for 5 min and microcentrifuged for 15 s at 10,000 × *g* to further clarify the crude membrane preparation. Protein concentrations were determined by Bradford assay (Bio-Rad, Richmond, Calif.), and crude membrane extracts (8 to 10 mg/ml) were stored at −70°C.

SDS-PAGE and virus overlay protein binding assay. Either 2× nonreducing sample buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.625 M Tris-HCl [pH 6.8]) or 2× reducing sample buffer (same buffer as nonreducing, but contains 5% 2-mercaptoethanol and boiled for 3 min) was added to crude cell membrane preparations and run on 12 or 14% polyacrylamide gel electrophoresis (PAGE) in a miniprotein gel apparatus (Bio-Rad) under either reducing or nonreducing conditions as previously described (13). After electrophoresis at 180 V for 40 min, proteins were electrophoretically transferred at 350 mA for 1 h at 24°C (Minitransblot; Bio-Rad) to nitrocellulose membranes in buffer containing 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 20% methanol. Nonspecific binding was blocked by incubating the nitrocellulose membranes for 1 h at 24°C in blot buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.25% gelatin, 0.05% Tween-20) containing 5% bovine serum albumin (BSA; Sigma). After removal of blocking buffer, purified ^{32}P-labeled virions (10^5 cpm/μg of virus) were added to fresh blot buffer and membranes were incubated at 24°C for 1 h. Membranes were then washed three times for 20 min (each) in buffer containing 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.3% Tween-20, air-dried, and exposed to X-ray film (X-OMAT-AR5; Kodak) at −70°C.

Dot-blot assay for whole virus binding. Cell extracts transferred to nitrocellulose filters were screened with a 96-well dot-blot apparatus (Schleicher and Schuell, Keene, N.H.). One to 50 μg of protein was adsorbed onto nitrocellulose filters which were then air-dried, nonspecific binding sites were blocked as described above, and membranes were incubated with 3 μg of [^{35}S]methionine-labeled virus (7×10^4 cpm/μg of virus) in blot buffer for 1 h. Nitrocellulose membranes were washed, dried, and exposed to X-ray film.

Specificity of virus binding. Proteins in BHK-21 cell extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes, blocked with 5% BSA, and assembled on a slot-blot apparatus (Immunetics Corp, Cambridge, Mass.). Increasing amounts of unlabeled TMEV or wheat germ agglutinin (WGA; Boehringer-Mannheim, Indianapolis, Ind.) in 100 μl were added to each lane and incubated for 1 h. Nitrocellulose filters were washed with blot buffer prior to the addition of ^{32}P-labeled virus (10^5 cpm/μg of virus) to avoid nonspecific aggregation of excess unlabeled virions with radiolabeled virus particles. Radiolabeled virus was added to blot buffer and the nitrocellulose membranes were incubated for 1 h, washed, dried, and autoradiographed.

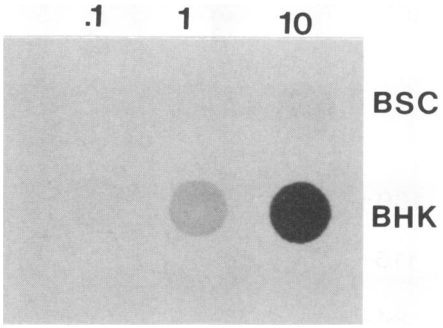

FIG. 1. Binding of [^{35}S]methionine-labeled BeAn virus to plasma membrane extracts in a virus overlay protein binding assay. Cell extracts (0.1, 1.0, and 10 μg) from TMEV-permissive BHK-21 and TMEV-nonpermissive BSC-1 cells were blotted onto a nitrocellulose filter, and the membrane was blocked in blot buffer containing 5% BSA and incubated with approximately 2.5×10^5 cpm of radiolabeled virus for 1 h.

RESULTS

Analysis of membrane extracts dot-blotted onto nitrocellulose filters. Cell extracts blotted onto nitrocellulose filters and overlaid with radiolabeled BeAn virus revealed virus binding to BHK-21 but not to BSC-1 cell extracts; increasing amounts of radiolabeled virus bound to the BHK-21 cell extract over a concentration of 1 to 10 μg of protein (Fig. 1). With longer exposure times, radiolabeled virus binding to as little as 0.01 μg of BHK-21 cell protein was detected (data not shown). Radioactive counts were detected on BSC-1 cell extracts when individual spots were cut out and counted by liquid scintillation following X-ray development, which probably represents minor nonspecific binding. TMEV does not infect BSC-1 cells; however, focal areas of nonprogressive cytopathology develop after transfection of TMEV RNA into BSC-1 cells. Infectious TMEV in the supernatants of transfected BSC-1 cultures can be passed to BHK-21 cells (data not shown). Therefore, BSC-1 cells are probably nonpermissive to TMEV because they do not have receptor molecules on their surface. Similar analyses of the nonpermissive BW 5147.G1.4, TK$^-$ 143, and MDBK cell lines also revealed only nonspecific binding (data not shown).

Radiolabeled BeAn virus binds primarily to a 34-kDa BHK-21 cell protein. To identify the protein species bound by BeAn virus, cellular proteins were separated by SDS-PAGE, transferred to nitrocellulose filters, and overlaid with ^{32}P-labeled BeAn virions (Fig. 2). Use of [^{35}S]methionine-labeled BeAn virus required long X-ray exposures and demonstrated only faint bands. The majority of TMEV binding was to a 34-kDa BHK-21 cell protein (Fig. 2, lane 1). Longer X-ray exposure times revealed BeAn binding to two other protein bands of approximately 100 and 18 kDa. Radiolabeled BeAn virus did not bind to BSC-1 cell proteins (Fig. 2, lane 2), further suggesting that the minimal virus binding to BSC-1 cells seen in Fig. 1 was indeed nonspecific. An identical 34-kDa band was observed under both reducing and nonreducing conditions; however, the 34-kDa band was more diffuse under reducing conditions (Fig. 3, lane 1), a finding expected for a glycoprotein. Previous results using neuraminidase treatment and WGA binding of whole BHK-21 cells suggest that the TMEV receptor is a sialoglycoprotein (10).

Comparison of TMEV binding to different cell lines. Analysis of three other TMEV-nonpermissive cell lines by virus

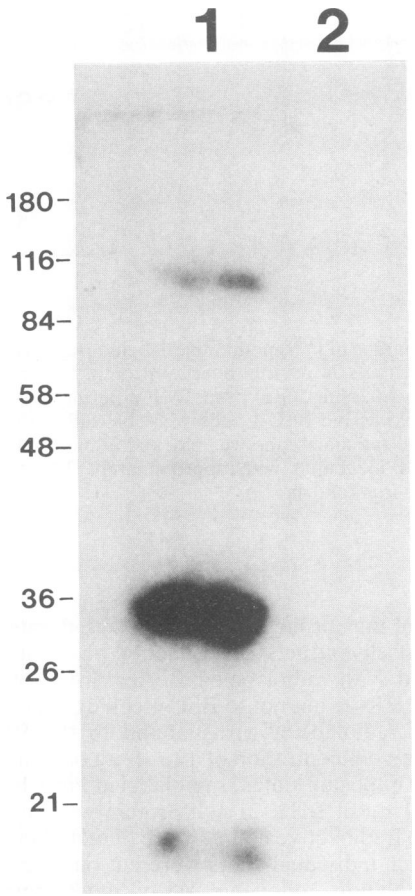

FIG. 2. Binding of ^{32}P-labeled BeAn virus to BHK-21 (lane 1) and BSC-1 (lane 2) cell extracts. One microgram of each cell extract was separated by SDS-PAGE, transferred to a nitrocellulose membrane, blocked with buffer containing 5% BSA, and probed with 10^6 cpm of virus. Molecular weight markers (10^3) are shown on the left.

overlay protein binding to cell extracts revealed only minimal binding. Electrophoretic separation of the cell extracts, followed by incubation with BeAn virus, revealed no binding to proteins from any of the nonpermissive cell extracts but specific binding to the 100-, 34-, and 18-kDa proteins in the BHK-21 extracts (Fig. 4). These results suggest that the TMEV nonpermissiveness in the cells tested rest in the lack of a TMEV plasma membrane receptor. Proteins from two additional permissive cell lines were also analyzed by using ^{32}P-labeled GDVII virus as probe (Fig. 5). GDVII virus bound to the same BHK-21 cell proteins as BeAn virus (Fig. 5, lane 1) and also bound to the 34-kDa protein in NB 41A3 neuroblastoma cells (Fig. 5, lane 3) and RAW 264.7 transformed macrophages (Fig. 5, lane 4). The observation that BeAn and GDVII bind predominantly the same 34-kDa protein species suggests that viruses from both TMEV neurovirulence groups share a common receptor.

Specificity of virus binding to the 34-kDa BHK-21 protein. The specificity of TMEV binding to the BHK-21 cell 34-kDa protein species was analyzed in competitive inhibition experiments. Since binding of radiolabeled virus was not eliminated by using unlabeled virus suspended in a 1- to 2-ml volume (data not shown), a minislot blot apparatus in which samples were concentrated in as little as 120 μl per lane to saturate the 34-kDa protein with unlabeled virus was used.

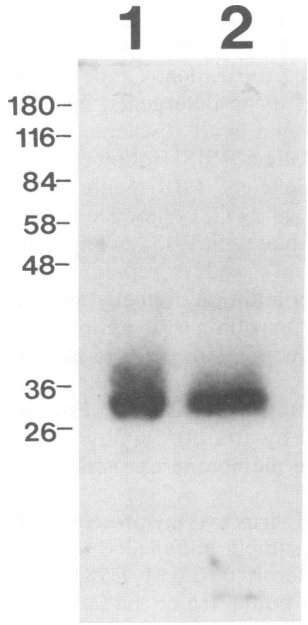

FIG. 3. Binding of ^{32}P-labeled BeAn virus to electrophoretically separated BHK-21 proteins on nitrocellulose membranes under either reducing conditions (100°C for 3 min in the presence of β-mercaptoethanol) (lane 1) or nonreducing conditions (lane 2). A filter containing separated cell proteins was probed with 10^6 cpm of radiolabeled virus, and the X-ray was developed after 1 day. Molecular weight markers (10^3) are shown on the left.

Figure 6 demonstrates that a large excess of unlabeled BeAn virus (lane 2) completely inhibited radiolabeled BeAn binding but that the addition of unlabeled, unrelated VSV (lane 3) did not affect BeAn binding. This result was confirmed in several additional competition experiments. In addition, Fig. 7A shows the effects of increasing amounts of unlabeled BeAn (to a 300-fold excess) on the binding of ^{32}P-labeled

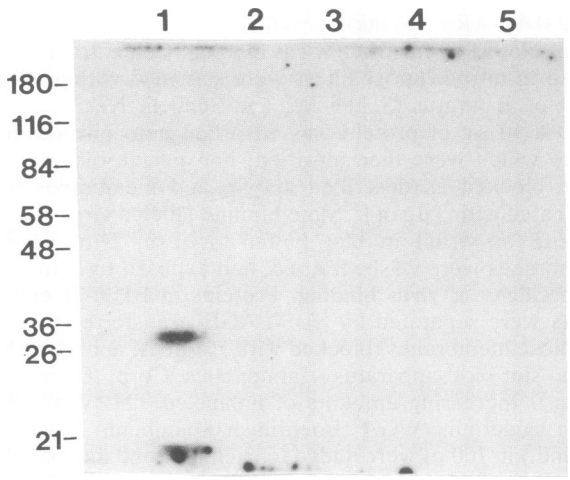

FIG. 4. Binding of ^{32}P-labeled BeAn virus to proteins of permissive and nonpermissive cell lines BHK-21 (lane 1), BSC-1 (lane 2), BW 5147 mouse thymoma (lane 3), TK$^-$143 (lane 4), and MDBK (lane 5). Cell extracts were separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with 10^6 cpm of radiolabeled virus. Molecular weight markers (10^3) are shown on the left.

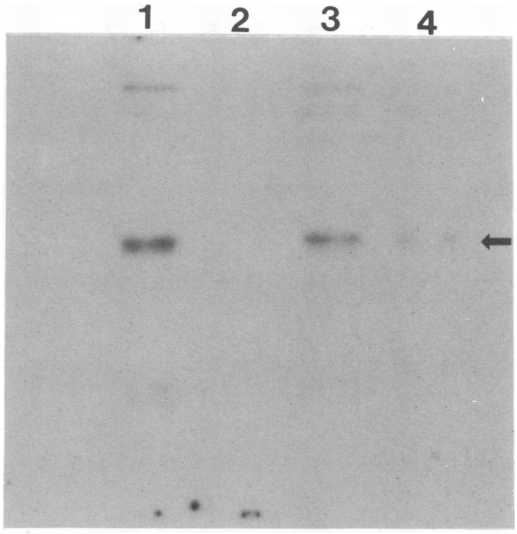

FIG. 5. Binding of ^{32}P-labeled GDVII virus to proteins of permissive and nonpermissive cell lines BHK-21 (lane 1), BSC-1 (lane 2), mouse neuroblastoma NB41A3 (lane 3), and RAW 264.7 mouse macrophage (lane 4). Cell extracts were separated by SDS-PAGE, transferred to a nitrocellulose filter, blocked with buffer containing 5% BSA, and probed with 10^6 cpm of radiolabeled virus. The arrow on the right indicates the position of the 34-kDa protein.

BeAn virus to the 34-kDa protein. The binding of radioactive virus was reduced with a 100-fold excess of unlabeled virus (Fig. 7A, lane 4) and completely inhibited with a 300-fold excess of unlabeled virus (Fig. 7A, lane 6). Virus binding to the 18- and the 100-kDa proteins was also reduced by addition of increasing amounts of unlabeled BeAn virus, although reduced binding to the 100-kDa protein is more apparent in a longer gel exposure (data not shown). Fig. 7B shows the competitive inhibition of ^{32}P-labeled BeAn virus by increasing amounts of unlabeled GDVII virus. A 200-fold excess of unlabeled GDVII reduced the binding of radiolabeled BeAn (Fig. 7B, lane 5), while a 500-fold excess was required to completely eliminate binding (Fig. 7B, lane 7). The requirement for greater concentration of unlabeled GDVII virus to block radiolabeled BeAn binding is consistent with our previous results with whole BHK-21 cells (10). These results indicate that TMEV binding to the BHK-21 cell 34-kDa protein is specific, since competitive inhibition of binding with unlabeled TMEV was concentration dependent and TMEV binding was not blocked by unrelated VSV.

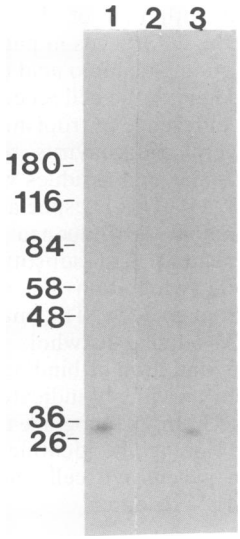

FIG. 6. Binding of ^{32}P-labeled BeAn virus to electrophoretically separated BHK-21 cell proteins. Individual lanes were first incubated with buffer (lane 1), 300 μg of unlabeled BeAn virus (lane 2), or 300 μg of unlabeled vesicular stomatitis virus (lane 3) and then probed with 1 μg of ^{32}P-labeled BeAn virus (10^5 cpm). Molecular weight markers (10^3) are shown on the left.

To further test the specificity of TMEV binding to the 34-kDa protein, electrophoretically separated BHK-21 cell proteins transferred to nitrocellulose filters were incubated with increasing amounts of WGA, washed, and incubated with radiolabeled GDVII virus. GDVII virus binding to the 34-kDa protein and to the 18-kDa protein was inhibited by increasing amounts of WGA; no effect on attachment to the 100-kDa protein was observed (Fig. 8). As shown in Fig. 8, partial inhibition of binding of 1 μg of GDVII virus was observed with 500 μg of WGA and complete inhibition with 800 μg of WGA. Therefore, WGA, which blocks TMEV binding to whole BHK-21 cells, also blocked TMEV binding to the 34-kDa membrane protein.

DISCUSSION

We have identified a 34-kDa plasma membrane protein on TMEV-permissive cells to which the majority of virus binding occurs by using a virus overlay protein binding assay that has been used successfully to identify receptor proteins for reovirus (9), Sendai virus (12), mouse hepatitis virus (4), and cytomegalovirus (1, 31). This assay can detect virus binding to less than 100 ng of protein in a crude membrane preparation (12).

BeAn and GDVII, representing the two TMEV neurovirulence groups, bound predominantly to the 34-kDa protein present on BHK-21 cells and to the same protein on two TMEV-susceptible mouse cell lines, NB 41A3 neuroblastoma and RAW 264.7 transformed macrophage cells. The binding was specific, since it was blocked by unlabeled TMEV and by WGA, which inhibits TMEV binding to whole BHK-21 cells by greater than 90% (10). Thus, TMEV of both neurovirulence groups bind to a common receptor molecule present on permissive cells. In addition, TMEV appeared to bind specifically to 100- and 18-kDa proteins on permissive cells, but to a much lesser extent than to the 34-kDa protein. By contrast, no binding of radiolabeled TMEV was observed with cellular extracts on dot-blots or the separated proteins of the four TMEV-nonpermissive cell lines tested. Our preliminary data indicating that BSC-1 cells can be transfected with TMEV RNA (unpublished data) suggest that nonpermissiveness in this instance reflects the absence of a membrane TMEV receptor.

The finding that TMEV binds predominantly to the 34-kDa protein separated under denaturing conditions suggests that these viruses recognize a specific epitope on the 34-kDa protein rather than an overall conformation of the 34-kDa protein together with another protein(s) in a complex. However, the possible role of other proteins, such as the 18-kDa species, in TMEV attachment to permissive cell lines cannot be excluded at present. The diffuse appearance of the 34-kDa protein under reducing conditions suggests the presence of intramolecular disulfide bonds or some posttranslational

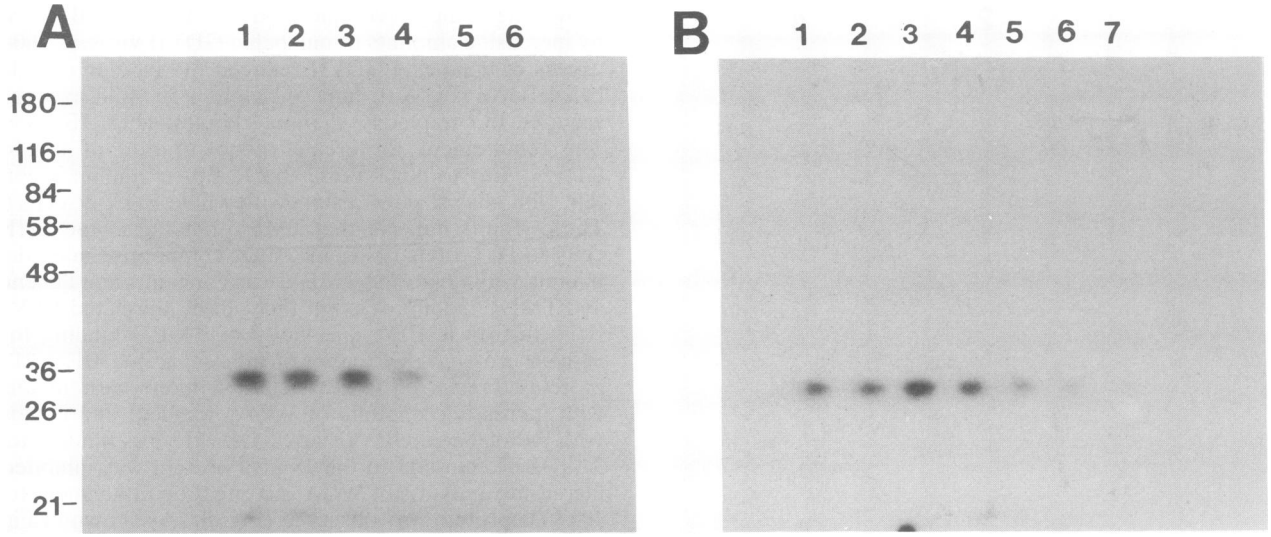

FIG. 7. Competitive binding of ^{32}P-labeled BeAn virus to BHK-21 cells. Proteins were separated by SDS-PAGE, transferred to nitrocellulose filters, and blocked with unlabeled BeAn virus (A) or unlabeled GDVII virus (B). Lane 1, blotting buffer only; lane 2, 25-fold excess of competing unlabeled virus; lane 3, 50-fold excess; lane 4, 100-fold excess; lane 5, 200-fold excess; lane 6, 300-fold excess; lane 7 (B only), 500-fold excess. Molecular weight markers (10^3) are shown on the left.

modification, such as oligosaccharide processing, since the TMEV receptor appears to be a sialoglycoprotein (10).

The ability of a virus to bind a specific cellular receptor molecule is a critical determinant of pathogenicity for several different viruses (2, 7, 8, 25, 29). Binding experiments in vitro suggest that both GDVII and BeAn recognize the same cellular receptor but bind to it differently (10). However, it is not known whether both viruses bind the same receptor on central nervous system cells. Interestingly, the lethality of GDVII virus correlates with its greater ability to infect hippocampal dentate and neocortical neurons; BeAn virus infects neither neocortical neurons nor, apparently, dentate neurons (28). Recently, attention has focused on the TMEV-receptor interaction in pathogenesis, since a major neurovirulence determinant has been mapped to the P1 genomic region which encodes the virus coat proteins (6, 11, 20, 30). It is possible that changes in the surface amino acids composing the cardiovirus receptor attachment site or "pit" alter virus receptor activity in selected neuronal populations (15, 19, 23). We are currently constructing additional recombinant viruses between GDVII and BeAn to further localize the P1 determinant.

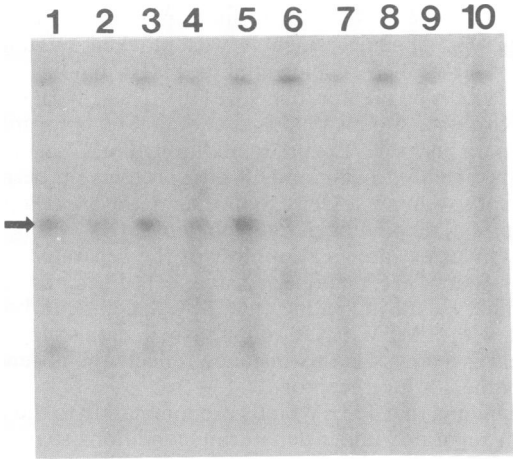

FIG. 8. Competitive inhibition of binding of ^{32}P-labeled GDVII virus to BHK-21 cell proteins by WGA. Cell extracts were electrophoretically separated, transferred to a nitrocellulose filter, incubated with WGA, and washed prior to addition of radiolabeled virus (5×10^4 cpm per lane). Lane 1, blotting buffer only; lane 2, 100 μg of WGA; lane 3, 200 μg; lane 4, 300 μg; lane 5, 400 μg; lane 6, 500 μg; lane 7, 600 μg; lane 8, 700 μg; lane 9, 800 μg; lane 10, 900 μg. The lefthand arrow indicates the position of the 34-kDa protein.

Experiments with a related picornavirus, encephalomyocarditis virus (EMCV), have suggested that differences in the receptor binding ability of the nondiabetogenic strain EMCV-B compared with that of the diabetogenic strain (EMCV-D) explain the differences in pancreatic tropisms of the two viruses (3). A single amino acid change in the virion coat protein that recognizes the cell receptor is thought to be responsible for this difference in tropism between EMCV-B and EMCV-D. Several picornavirus receptors, including EMCV (5), possess sialic acid residues which are necessary for virus binding (33, 34). This is especially interesting since studies of escape mutants of influenza virus have demonstrated that single amino acid substitutions can lead to changes in sialic acid recognition that are associated with changes in virus tropism (25). The marked difference in sensitivity of TMEV binding to whole BHK-21 cells with neuraminidase (90% inhibition of binding of BeAn virus but none of GDVII) seen previously indicates a possible difference in the way these TMEVs recognize the cellular receptor (10). The molecular cloning and identification of the 34-kDa receptor protein on permissive cell lines should serve to characterize the TMEV receptor.

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