Characterization of a Membrane-Associated Protein Implicated in Visna Virus Binding and Infection

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The identity of the cellular receptor(s) for visna virus, an ovine lentivirus, is currently unknown; however, previous studies from our laboratory have identified membrane-associated proteins expressed selectively in susceptible cells which bind visna virus. Moreover, a polyclonal antibody (2-23), raised against a 45-kDa visna virus binding protein, bound specifically to the surface of susceptible cells in immunofluorescence assays and significantly reduced binding of visna virus to cells (S. E. Crane et al., 1991, J. Virol., 65, 6137–6143). In this report we extend our studies of this antibody (2-23), showing both that 2-23 significantly reduces visna virus infection of susceptible cells and that 2-23 immunoprecipitates a putative protein complex consisting of a prominent 30-kDa protein, as well as the 45-kDa immunogen, specifically from radiolabeled virus-susceptible sheep cells. Further, we demonstrate that the 30-kDa protein is a membrane-associated proteoglycan substituted with a chondroitin sulfate glycosaminoglycan (GAG) chain(s) and that treatment of susceptible cells with an inhibitor of GAG synthesis significantly reduces visna virus production. Collectively, these data support a role for a proteoglycan in visna virus cell binding and infection.

INTRODUCTION

Visna virus is a member of the lentivirus family of nononcogenic retroviruses, which includes human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), bovine immunodeficiency virus (BIV), caprine arthritis–encephalitis virus (CAEV), and visna-maedi virus (visna virus), which infects sheep. Lentiviruses cause persistent infections and chronic progressive disease after prolonged incubation times in their hosts, which culminate in multiorgan pathology with an invariably fatal outcome. Differences in cellular tropism and the development of clinically distinct diseases divide the lentiviruses into two groups: HIV, SIV, and FIV replicate in both lymphocytes and macrophages, causing acquired immunodeficiency syndrome and organ-specific diseases in the lung, central nervous system (CNS), and gastrointestinal tract. In contrast, EIAV, CAEV, and visna virus replicate predominately in macrophages, do not infect lymphocytes, and cause only organ-specific disease of the CNS, lung, and joints (Clements and Zink, 1996).

Retroviruses have a common mechanism of entry that involves binding to a specific cell surface receptor followed by fusion of the viral envelope with the cell membrane. Membrane proteins with multiple transmembrane domains serve as receptors for a number of the retroviruses. The murine leukemia viruses (MLV-E) use a multiple membrane-spanning protein transporter of basic amino acids (CAT-1) as a receptor (Kim et al., 1991; Wang et al., 1991). A sodium-dependent neutral amino acid transporter serves as the receptor for feline and baboon endogenous retroviruses and for simian type D retroviruses (Tailor et al., 1999). A protein resembling a phosphate transporter (GLVR-1) serves as a receptor for both gibbon ape leukemia virus and feline leukemia virus subgroup B (Weiss and Tailor, 1995), and the chemokine receptor CXCR4 serves as a receptor for FIV (Willett et al., 1997). Although a majority of retroviruses require a single cellular receptor protein for entry, HIV and SIV utilize both CD4 and chemokine receptors (Broder and Collman, 1997; Broder and Dimitrov, 1996). CD4 is required, but not sufficient, for entry of most strains of HIV and SIV (Maddon et al., 1986). In contrast, the chemokine receptors are always required and are, in some cases, sufficient for entry of these viruses (Edinger et al., 1997; Endres et al., 1996; Marcon et al., 1997; Reeves et al., 1997).

Currently, little is known about the receptors for the ungulate lentiviruses, visna virus and CAEV; however, two candidate receptors have been proposed for the visna virus receptor. Major histocompatibility complex class II antigen was previously implicated as a candidate receptor in that purified soluble ovine MHC Class II prevented virus binding to a 30-kDa protein on susceptible cells (Dalziel et al., 1991). However, the identity of this 30-kDa protein was never confirmed to be MHCII antigen...
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To extend the observation that 2-23 inhibited visna virus binding to susceptible cells, we first evaluated the ability of 2-23 to inhibit visna virus infection of SCP, a primary cell susceptible to visna virus infection. SCP cells were preincubated with IgG purified from the 2-23 serum, IgG purified from normal rabbit serum, commercial normal rabbit IgG, or no antibody (virus control) at 4°C for 1 h. Incubations were done at 4°C to prevent receptor internalization. Virus (100 TCID<sub>50</sub>) was added to the cells at 4°C for 4 h, the cells were washed, and de novo virus production was measured as described under Materials and Methods. The 2-23 IgG reduced virus production 100- to 550-fold compared to virus control and normal rabbit IgG in three independent experiments (Table 1). In contrast, the IgG from the normal rabbit serum reduced the virus production only 10-fold, compared to the virus control and rabbit IgG (Table 1). This latter result is not surprising as it is well established in our laboratory that serum components interfere with visna virus infection of cells (J. E. Clements, personal communication). Appropriate control experiments were performed to ensure that the 2-23 antiserum recognized a cellular protein and not a viral protein. A standard neutralization assay was modified such that 2-23 IgG, as well as IgG from normal rabbit serum and visna-neutralizing antiserum (NN at a 1 to 50 dilution), was incubated with visna virus (100 TCID<sub>50</sub>) for 1 h at 37°C. The virus was separated from free antibody by centrifugation prior to being used for infection of SCP cells. The NN antiserum reduced the infectivity of the virus while the IgG from 2-23 and normal rabbit serum had no effect on virus titer. Thus, the 2-23 antiserum does not reduce infectivity by binding to the virus. In any event, it is clear that the 2-23 IgG significantly reduced virus infection when allowed to bind to SCP cells but not when incubated with the virus.

### Immunoprecipitation of visna virus binding proteins from visna virus-susceptible cells

In order to study the membrane proteins recognized by 2-23, cell lysis conditions were optimized to solubilize integral membrane proteins. Nonionic and zwitterionic detergents have been shown repeatedly to be effective for membrane protein extraction (Helenius and Simons,

### RESULTS

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The 2-23 antiserum recognized specific cellular proteins when cells were solubilized in zwitterionic or nonionic detergents, such as CHAPS and n-octylglucoside, but not by ionic detergents such as SDS (data not shown). Based on these observations, we lysed 14C-labeled (most amino acids radiolabeled) SCP cells with a CHAPS-based lysis buffer and used 2-23 for standard immunoprecipitation. Normal rabbit serum was used as the negative control in immunoprecipitations. SDS–PAGE separation of 2-23 immunoprecipitates from SCP cells identified two specific protein bands of ~30 and 45 kDa (Fig. 1, lanes 11 and 12, asterisks). Of note, only these two proteins were detected consistently in 2-23 immunoprecipitations, and the 30-kDa protein was always the most prominent labeled band, in both 14C-labeled (Fig. 1) and 35S-labeled lysates (Fig. 3).

Consistent with classical receptor theory, we hypothesized that if the 30- and 45-kDa proteins were integral to virus binding/infection they would be expressed by most, if not all, visna virus-susceptible cells. Hence we examined 2-23 immunoprecipitates from 14C-labeled cell lysates of a number of visna virus-susceptible and cell lines. In addition to SCP cells (Fig. 1, lane 11, asterisks), FLK-BLV (ovine embryonic kidney), MDOK (ovine kidney), and GSM (goat synovial membrane) cells expressed both the 30- and the 45-kDa proteins (Fig. 1, lanes 1, 3, and 5, asterisks). Immunoprecipitations with 2-23 from 14C-labeled nonsusceptible cell types, QT6 (quail fibrosarcoma cells) and NIH-3T3 (mouse embryo fibroblast), did not contain either the 30-kDa protein or the 45-kDa protein (Fig. 1, lanes 7 and 9).

The 30-kDa protein is a membrane-associated protein

Classically, virus receptors are cellular membrane proteins that bind the virus to the cell surface. Therefore, we next examined 2-23 and normal rabbit serum (NRS) immunoprecipitations of proteins extracted from cellular membranes of 14C/35S-labeled SCP cells. Our results indicate that the 30-kDa protein was present in the membrane (M) fraction (Fig. 2, lane 2). Compared to nonmembrane fractions of cellular lysates (Fig. 2, lanes 4 and 6) we find that pelleted membranes contain amounts of the 30-kDa protein similar to those of the fractions of cytoskeletal aggregates (C) and that the 30-kDa protein is not detectable in the homogenate fraction (H). These results are consistent with a high turnover rate suggested by the extensive incorporation of 35S or 14C in the 30-kDa protein.

Biochemical characterization of the 30-kDa protein

Although the 30-kDa protein was readily detectable in both 14C- (Fig. 1) and 35S-labeled lysates, it consistently failed to stain with standard Coomassie blue, Ponceau red, colloidal Coomassie, or silver stain. Given this property of the protein, we considered the possibilities that the concentration was below the level of detection for these stains or that the protein is glycosylated. Anionic glycoproteins may not stain with these standard methods; in fact, some glycoproteins are seen as negative bands in a silver stain (Ausubel et al., 1998). Among such glycoproteins are proteoglycans which have long linear GAG chains comprising repeating disaccharides; most are membrane associated or bound to the extracellular matrix, have variable degrees of sulfation, and have a high density of neg-
ative charge. Chondroitin sulfate/dermatan sulfate, heparan sulfate/heparin, and keratan sulfate are the three classes of GAGs that are found attached to proteoglycans. Because glycoproteins such as proteoglycans resist staining by standard methods, alternate methods are often used, such as the carbohydrate-specific periodic acid Schiff (PAS) staining (Fukuda and Kobata, 1993). If used before silver staining, PAS augments the silver reaction for glycoproteins. In light of the possibility that the 30-kDa protein is glycosylated, proteins immunoprecipitated by 2-23 were separated by SDS–PAGE and stained with PAS followed by silver staining. Because this method enabled visualization of the 30-kDa protein in the gel (not shown), we concluded that the protein was likely glycosylated.

To further examine the 30-kDa protein, experiments were designed to examine the presence of sugar substituents. 35S-labeled samples immunoprecipitated with 2-23 were treated with enzymes that cleave specific sugars from glycoproteins and proteoglycans. Peptide N-glycosidase F (PNGaseF) cleaves N-linked sugars from their core proteins (Elder and Alexander, 1982). Polysaccharide lyases recognize and cleave the GAG chains from proteoglycans in a specific manner (Linhardt et al., 1986). Heparin lyase I (heparinase I) cleaves heparin, heparin lyase III (heparinase III) cleaves heparan sulfate, chondroitin ABC lyase (chondroitinase ABC) cleaves both chondroitin sulfate and dermatan sulfate, and chondroitin AC lyase (chondroitinase AC) cleaves only chondroitin sulfate chains from core proteins (Linhardt et al., 1986). Immune complexes of 2-23 from 35S-labeled FLK-BLV lysates were subjected to overnight digestion by each of the enzymes prior to separation by SDS–PAGE on a 12% gel (Fig. 3). Lanes 1 and 2 of Fig. 3 represent "untreated" immunoprecipitations with 2-23 and NRS performed exactly as in Figs. 1 and 2. As a control for the digestion conditions, "Mock"-digested samples of immunoprecipitates with 2-23 or NRS were incubated with digestion buffer only, under the same conditions as those reactions containing enzyme. Lanes 4 through 8 are immunoprecipitations treated with specific glycosidases. PNGaseF, 1.2 U (lane 4); heparinase I (HI), 50 U (lane 5); heparinase III (HIII), 50 U (lane 6); chondroitinase ABC (C-ABC), 40 U (lane 7); and chondroitinase AC (C-AC), 40 U (lane 8). Proteins were analyzed on a 12% SDS gel and detected by autoradiography.

4-Methylumbelliferyl-β-D-xyloside, an inhibitor of proteoglycan synthesis, also reduces visna virus replication

To investigate the role of GAG chains in visna virus binding and infection of target cells, addition of GAG chains to proteoglycans in SCP cells was inhibited with a β-o-xyloside prior to infection, a technique used in many previous reports (Gibson and Segen, 1977; Gibson et al., 1977; Okayama et al., 1973). At low concentrations (in the mM range) these hydrophobic xylosides penetrate the cell membrane and target the Golgi apparatus, where they are added to the carbohydrate core. The xyloside 4-methylumbelliferyl-β-o-xyloside (Xylb4MU) competes with endogenous proteoglycan core proteins and acts as a GAG acceptor (Freeze et al., 1993). This competition results in the increased secretion of protein-free GAG chains and decreased production of intact proteoglycan monomers. Proteoglycans synthesized in the presence of xylosides usually display decreased GAG chain length, fewer GAG chains, and variable degrees of sulfation (Gibson and Segen, 1977; Murray et al., 1983). The xyloside primes chondroitin sulfate efficiently and heparan sulfate only weakly. Treatment of SCP cells with 1 mM Xylb4MU prior to infection resulted in a marked reduction in virus-induced cell fusion and virus production, as measured by a standard reverse transcriptase assay (Fig. 4).

The 30-kDa protein can be immunoprecipitated from primary sheep cells

Because the cumulative data presented above support a role for a 30-kDa membrane-associated chondroitin
sulfate proteoglycan, we next examined the ability of 2-23 to immunoprecipitate the 30-kDa protein from $^{35}$S-labeled primary sheep brain microvascular endothelial cells (SBEC) and sheep adipose endothelial cells (SAEC). We have previously reported the ability of both primary cell types to support visna virus replication (Craig et al., 1997). The results indicate the presence of the 30-kDa protein in 2-23 immunoprecipitates from both brain and adipose endothelial cells (Fig. 5, lanes 2 and 4). Relevantly, the 30-kDa protein was also immunoprecipitated by 2-23 from $^{35}$S-labeled primary sheep macrophages, which represent the major target cells for visna virus replication in vivo (not shown).

The cellular receptors for visna virus and the other ovine and caprine lentiviruses are currently unknown, although two previous reports have implicated, independently, the involvement of a 30-kDa membrane protein in the binding of visna virus to susceptible cells (Crane et al., 1991; Dalziel et al., 1991). In addition to the 30-kDa protein, our laboratory identified two other membrane proteins (45 and 15 kDa) from visna virus-susceptible cells that bound the virus in a VOPBA (Crane et al., 1991). Polyclonal antiserum was raised to each protein, but only polyclonal antiserum (2-23) to the 45-kDa protein specifically labeled the surfaces of SCP cells and blocked visna virus binding to SCP cells. Surprisingly, 2-23 recognized not only the immunogen (45-kDa protein) but also the 30-kDa protein in Western analysis of SCP membranes. In the present study, 2-23 significantly reduced the infection of susceptible cells and immunoprecipitated a putative complex of two proteins (30 and 45 kDa). We found the 30-kDa protein to be the most prominent in the 2-23 immunoprecipitations from both $^{14}$C and $^{35}$S-labeled cell lysates, possibly indicating that the 30-kDa protein is more abundant in cells or that this protein had a higher turnover rate in the cell. This disproportionate incorporation of both radiolabels into the 30- and 45-kDa proteins suggests that it is unlikely that the 30-kDa protein represents either a proteolytically processed form of the 45-kDa protein or a precursor for the 45-kDa protein, rather it reflects the stoichiometry of the complex. Biochemical analyses suggested that the 30-kDa protein is modified with a chondroitin sulfate GAG chain. Further, we found that the GAG chains are important for optimal efficient visna virus infection.
Analysis of the 30-kDa protein using various polysaccharide lyases demonstrated sensitivities to chondroitinase ABC (C-ABC) and chondroitinase AC (C-AC) as evidenced by the emergence of a faster migrating protein, consistent with the decrease in molecular weight expected as polysaccharides are cleaved. Although a slightly shifted band is present in the PNGaseF, heparinase I, and heparinase III digestions, the same smaller band is also present in the mock 2-23 immunoprecipitation and likely results from protein degradation due to digestion conditions and overnight incubation at 37°C. Since C-AC cleaves only chondroitin sulfate, the substantial shift in molecular weight provides evidence that the 30-kDa band is substituted with a chondroitin sulfate. The concomitant shift in molecular weight observed with C-ABC, which cleaves both chondroitin and dermatan sulfates, supports the chondroitin sulfate composition of the 30-kDa protein, as an even lower species would be expected to emerge if two sugars were cleaved. However, since a dermatan sulfate-specific lyase was not utilized, it cannot be ruled out that the single lower band emerging after C-ABC treatment may contain a heterogeneous population of singly and/or doubly cleaved 30-kDa proteins that share a common migration position in SDS–PAGE. Indeed, the shift in molecular weight is relatively small, which may suggest that there are only a few GAG chains on the core protein or that the sugar chain is very small. In any case, it is quite clear that the 30-kDa protein is membrane associated and is a chondroitin sulfate proteoglycan that may serve as a visna virus receptor. Future studies will address the role and identity of this proteoglycan in relation to other proteins comprising the complex involved in visna virus cell binding and infection.

MATERIALS AND METHODS

Reagents

C-ABC from Proteus vulgaris and C-AC, heparinase I, and heparinase III, all from Flavobacterium heparinum; Xylβ4MU; and minimum essential medium (Eagle's) without methionine (EMEM-met) were obtained from Sigma (St. Louis, MO). Minimum essential medium with Earle's salts (EMEM), Dulbecco's modified eagle medium (DMEM), Hanks' balanced salt solution (HBSS), and MEM Select-Amine kit (14C labeling medium and 14C/35S labeling medium) were obtained from Gibco BRL (Gaithersburg, MD). 14C labeling medium was prepared following the manufacturer's instructions for the MEM Select-Amine kit supplemented with only L-tryptophan, L-glutamine, L-methionine, and L-cystine. 14C/35S labeling medium was prepared from the MEM Select-Amine kit supplemented with only L-tryptophan and L-glutamine. Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Norcross, GA).

Cells and viruses

SCP cells were obtained as previously described (Narayan et al., 1977, 1978, 1980). GSM cells were isolated in our lab as previously described (Narayan et al., 1980). FLK-BLV cells were obtained from Dr. David Derse (NCI, Frederick, MD); MDOK cells, NIH-3T3 cells, and QT6 cells were obtained from the ATCC (Manassas, VA). SBEC and SAEC were isolated in our laboratory as described (Craig et al., 1998). All cells were cultured at 37°C with 5% CO2. SCP, FLK-BLV, and GSM cells were cultured in EMEM supplemented with 10% FBS, MDOK cells were cultured in EMEM supplemented with 10% lamb serum, and NIH-3T3 cells and QT6 cells were cultured in DMEM supplemented with 10% FBS. The SBEC and SAEC were grown in complete endothelial cell...
medium as described (Craig et al., 1998). Visna virus strain 1514 was prepared as previously described (Cork et al., 1974; Narayan et al., 1978; Pyper et al., 1984).

**Antisera**

Rabbit polyclonal antiserum 2-23 was raised against a cellular membrane protein of ~45 kDa previously shown to bind visna virus (Crane et al., 1991). Serum controls included preimmune serum collected from the 2-23 rabbit (Pre 2-23) and NRS obtained from Jackson ImmunoResearch Laboratories, Inc. (Westgrove, PA). Goat polyclonal antiserum (NN) was raised against whole visna virus. This antiserum neutralizes visna virus in the standard neutralization assay below. ChromPure Rabbit IgG (commercially pure normal rabbit IgG) was obtained from Jackson ImmunoResearch Laboratories, Inc. IgG was purified from rabbit polyclonal antiserum 2-23, normal rabbit serum, and NN neutralizing serum using the MAbTrapGII affinity purification kit (Pharmacia, Piscataway, NJ). The protein concentration of the purified IgG was measured using the Bradford-based Bio-Rad protein assay, microassay procedure (Bio-Rad, Hercules, CA).

**Virus infectivity assay**

Confluent monolayers of SCP cells cultured in 48-well dishes were incubated in EMEM + 0.5% FBS, with 300 μg/ml IgG purified from 2-23 serum, with IgG purified from NRS (described above), or with commercially purified normal rabbit IgG, for 4 h at 4°C. Visna virus (100 TCID50/well) was added to the cells in medium containing the various IgGs and incubated at 4°C. After 4 h, the cells were washed three times with EMEM + 0.5% FBS and the appropriate IgG treatment was added in EMEM + 0.5% FBS. The infected cells were incubated at 37°C for 7 days, after which the supernatant was removed, virus was quantitated by limiting dilution, and TCID50 was calculated by the method of Karber (1931).

**Modified neutralization assay**

In a standard neutralization assay visna virus (100 TCID50/100 μl) is preincubated with dilutions of neutralizing antibody in EMEM + 0.5% FBS for 1 h at 37°C. Virus and antibody are then added to confluent monolayers of SCP cells in 96-well plates. The negative control is virus preincubated with medium without antisera or with preimmune serum and is processed the same as above. Virus cytopathic effects (cpe) are recorded at 7 days postinoculation (p.i.) and neutralization dose is calculated by the method of Karber (1931). To determine whether the 2-23 antiserum had any neutralizing effect on the virus, this standard assay was modified as follows. Virus and antibody (2-23 IgG and normal rabbit IgG at 300 μg/ml and NN at 1 to 50 dilution) were incubated for 1 h at 37°C after which virus was pelleted through a cushion of 20% sucrose/Tris 25 mM, pH 8.0, NaCl 150 mM, EDTA 2 mM in a Sorvall 5CRC high-speed centrifuge with the SH-MT rotor at 13,500 rpm to remove free unbound antibody. The virus pellet was resuspended in 200 μl of EMEM + 0.5% FBS and used to infect SCP cells as described above. Virus cpe were evaluated 7 days p.i.

**Metabolic labeling of cells**

Cells (~1.0 × 10^6) were washed twice with HBSS, starved in 14C-labeling medium for 1 h at 37°C, and labeled overnight at 37°C in 14C-labeling medium supplemented with 1% FBS and 10 μCi/ml L-[U-14C]amino acid mixture from NEN Life Science Products, Inc. (Boston, MA). For double-labeling experiments with both Tran35S-Label (ICN Pharmaceuticals, Irvine, CA) and [14C]amino acids (referred to as 14C/35S labeled), ~2.0 × 10^5 cells were starved in 14C/35S labeling medium for 1 h and incubated overnight at 37°C in the same medium supplemented with 1% FBS, 70 μCi/ml Tran35S-Label, and 10 μCi/ml L-[U-14C]amino acids. 35S-labeled cells (~1.0 × 10^5) were starved in EMEM-met for 1 h at 37°C and labeled for 5 h at 37°C in EMEM-met supplemented with 1% FBS and 70–150 μCi/ml Tran35S-Label (referred to as 35S-labeled throughout).

**Immunoprecipitation**

Radiolabeled cells were washed three times with HBSS and lysed on ice for 1 h in lysis buffer (6.5 mM CHAPS, 50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, containing complete protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN)). Lysates were clarified by centrifugation for 10 min at 12,000 rpm, and the supernatant was assayed for total protein concentration using the Bradford-based Bio-Rad protein assay, microassay procedure (Bio-Rad). Immunoprecipitations from 0.1 to 0.5 mg of protein were performed overnight with either 2-23, Pre 2-23, or the normal rabbit serum control. Following incubation with protein G–Sepharose beads (Amersham, Arlington Heights, IL), immune complexes were pelleted (1 min at 12,000 rpm), washed three times with lysis buffer, washed once with lysis buffer without CHAPS, and finally resuspended in 2X solubilization buffer (Laemmli, 1970). Following resolution by SDS–PAGE, gels were fixed for 1.5 h (20% isopropanol, 10% glacial acetic acid), rinsed in dH2O, and soaked for 30 min in 1 M salicylic acid, sodium salt (Aldrich Chemical Co., Milwaukee, WI), prior to being dried and processed for autoradiography using either Kodak X-OMAT blue or Kodak MR film (Kodak, Rochester, NY). Films were scanned using the UMAX Powerlook II scanner into Adobe PhotoShop 5.0, and figures were assembled using Adobe Illustrator 8.0.

**Preparation of cell membrane fractions**

SCP cells (~2 × 10^6, 14C/35S labeled, as described above) were washed three times in ice-cold PBS and...
incubated (at 4°C) in PBS containing 1 mM EDTA until the adherent cells could be released by slapping the sides of the flasks. Released cells (>95%, as assessed microscopically) were pelleted (2000 rpm, 4°C, 8 min) and resuspended in 7 ml of ice-cold homogenization buffer (25 mM HEPES, pH 7.3; 0.5 mM EGTA; 0.5 mM sodium orthovanadate; 0.1 mM sodium molybdate; 1 mM sodium fluoride) containing protease inhibitors (2 Complete tablets/50 ml; Boehringer Mannheim, Mannheim, Germany). Cell lysates were prepared by dounce homogenization (100 strokes) essentially as described previously (Bhat et al., 1999). Low-speed centrifugation (1000g, 10 min, 4°C) of homogenates was performed to remove unlysed cells, nuclei, and cell fragments (Jesaitis et al., 1982). The resulting supernatant was subjected to high-speed centrifugation (10,000 rpm, 7 min, 4°C) to pellet cytoskeletal aggregates (Sambrook et al., 1971). Pellets (representing cytoskeletal aggregates) were incubated on ice while the supernatants were subjected to centrifugation (417,000g, 2 h, 4°C) in order to pellet cell membranes. Resulting supernatants were designated “homogenates” (~7 ml), decanted into tubes, and incubated on ice. Residual supernatant was removed from the ultracentrifuge tubes containing visible membrane pellets using cotton-tipped applicators. Proteins were solubilized from membrane pellets (M) and cytoskeletal aggregates (C) in 3 ml of homogenization buffer supplemented with 10 mM CHAPS (overnight, 4°C). Accordingly, the homogenate fraction designated H (representing the supernatant following ultracentrifugation) was supplemented with CHAPS (to achieve a final concentration of 10 mM) and processed as described above. Proteins from each fraction were concentrated using Centricon3 protein concentrators (above) and equal amounts of protein (derived from each fraction) were used in immunoprecipitation assays.

Glycosidase and polysaccharide lyase treatment of immunoprecipitations

35S-labeled immunoprecipitations (from 500 μg lysate) were prepared from FLK-BLV cells as described above. Following the final wash in lysis buffer without CHAPS, immune complexes were resuspended in 50 μl of the appropriate buffer containing 50 μl heparinase I, 50 μl heparinase III, 40 μl chondroitinase ABC, or 40 μl chondroitinase AC (units represent “Sigma units”). Buffers for the lyases were 50 mM sodium phosphate with 100 mM NaCl, pH 7.1 (heparinase I); 50 mM sodium phosphate, pH 7.6 (heparinase III); and 50 mM Tris, with 60 mM sodium acetate, pH 8.0 (chondroitinase ABC and chondroitinase AC). Overnight digestions with heparinase I, chondroitinase ABC, and chondroitinase AC were incubated at 37°C, while heparinase III digestions were incubated at 30°C. Digestion with PNGaseF was performed essentially according to the manufacturer’s specifications, using the N-Glycosidase F Deglycosylation Kit from Boehringer Mannheim. Briefly, immune complexes were resuspended in 5 μl of denaturation buffer and 5 μl of water and heated at 95°C for 3 min. Reaction buffer (10 μl) and N-glycosidase F (1.2 U) were then added, and the digestions were incubated overnight at 37°C. Enzyme-treated immune complexes were resolved by 12% SDS-PAGE as described above. “Mock” immunoprecipitates were incubated in the appropriate enzyme-specific buffer in the absence of enzyme and processed identically. “Untreated” immunoprecipitates were processed exactly as described under “Immunoprecipitation” above.

Inhibition of proteoglycan synthesis

SCP cells were grown to confluence in the presence of EMEM + 10% FBS or in EMEM + 10% FBS supplemented with 1 mM Xylβ4MU. Cells were washed twice with HBSS, after which the medium was changed to EMEM + 0.5% FBS with or without 1 mM Xylβ4MU, and cells were infected with 1 × 10^6 TCID₅₀/ml visna virus for 8 h at 37°C. Cells were washed twice with EMEM + 0.5% FBS, and the medium was replaced with either EMEM + 0.5% FBS or medium supplemented with 1 mM Xylβ4MU. Supernatants collected 5 days after infection were assayed for visna virus production by reverse transcriptase activity (Clabough et al., 1991).

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