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Topography and Microfilament Core Association of a Cell Surface Glycoprotein of Ascites Tumor Cell Microvilli

Zeqi Sheng, Oluseyi A. Vanderpuye, Steven R. Hull, Coralie A. Carothers Carraway, and Kermit L. Carraway

Departments of Biochemistry and Molecular Biology (Z.S., C.A.C.C.) and Anatomy and Cell Biology (O.A.V., S.R.H., K.L.C.), University of Miami School of Medicine, Miami, Florida 33101

Membrane-microfilament interactions are being investigated in microvilli isolated from 13762 rat mammary ascites tumor cells. These microvilli are covered by a sialomucin complex, composed of the sialomucin ascites sialoglycoprotein-1 (ASGP-1) and the associated concanavalin A (Con A)-binding glycoprotein ASGP-2. Limited proteolysis of the microvilli releases large, highly glycosylated fragments of ASGP-1 from the microvilli and increases the association of ASGP-2 with the Triton-insoluble microvillar microfilament core (Vanderpuye OA, Carraway CAC, Carraway, KL: *Exp Cell Res* 178:211, 1988). To analyze the topography of ASGP-2 in the membrane and its association with the microfilament core, microvilli were treated with proteinase K for timed intervals and centrifuged. The pelleted microvilli were extracted with Triton X-100 for the preparation of microfilament cores and Triton-soluble proteins or with 0.1 M carbonate, pH 11, for the preparation of microvillar membranes depleted of peripheral membrane proteins. These microvilli fractions were analyzed by dodecyl sulfate gel electrophoresis, lectin blotting with Con A and L-phytohemagglutinin, and immunoblotting with anti-ASGP-2. The earliest major proteolysis product from this procedure was a 70 kDa membrane-bound fragment. At longer times a 60 kDa released fragment, 30–40 kDa Triton-soluble fragments, and 25–30 kDa membrane- and microfilament-associated fragments were observed. Phalloidin shift analysis of microfilament-associated proteins on velocity sedimentation gradients indicated that the 25–30 kDa fragments were strongly associated with the microfilament core. From these studies we propose that ASGP-2 has a site for indirect association with the microfilament core near the membrane on a 15–20 kDa segment.

Key words: membrane-microfilament interactions, ASGP-1, ASGP-2, SDS PAGE, phalloidin shift analysis

The organization of cell surface components is believed to be regulated by interactions between the membrane and elements of the cell cytoskeleton [1]. Microfilament-membrane interactions are considered to be particularly important in the dynamics of animal cell surfaces [1–4]. Understanding the mechanisms of the control of the

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topographic distribution of glycoproteins in the plane of the plasma membrane has proven to be quite difficult because of the complexity of the components and interactions involved. As a model system for investigating this type of topographic control, we have been studying the behavior of a glycoprotein complex at the cell surface of 13762 ascites rat mammary adenocarcinoma cells [5,6]. This complex is composed of two glycoproteins, a sialomucin ascites sialoglycoprotein-1 (ASGP-1) and a concanavalin A (Con A)-binding glycoprotein ASGP-2, in equimolar amounts [5]. Together they represent about 1% of the total cell protein and thus constitute the major glycoproteins at these cell surfaces [5].

A second advantage of these cells is our ability to isolate microvilli from the cell surfaces [7,8]. These microvilli are a sealed plasma membrane fraction containing intact microfilament cores [8]. In a previous study we showed that a small fraction of ASGP-2 binds to the microfilament core after extraction with nonionic detergent [9]. Interestingly, this fraction can be substantially increased by treatment of the microvilli with protease to release highly negatively charged fragments of ASGP-1 or with sialidase to release sialic acid [9]. We proposed that the binding of ASGP-2 to the microfilaments is indirect and involves an interaction at the cell surface with another cell surface component. Thus, the topographical organization of ASGP-2 at the surface of the microvilli is of considerable interest. It must have sites at the cell surface for binding to ASGP-1 and to a linker to the cytoskeleton. Moreover, our recent studies on characterization of ASGP-2 indicate that it is heavily glycosylated (S. Hull, unpublished observations) and reacts specifically with several lectins [9]. The present study was undertaken to define more fully the topography of ASGP-2 at the cell surface, including its interaction with the microvillar microfilament core.

MATERIALS AND METHODS

Materials

Ampholytes, 4-chloronaphthol and horseradish peroxidase-goat antirabbit immunoglobulin G were obtained from BioRad (Richmond, CA). Nitrocellulose membranes were from Schleicher and Schuell (Keene, NH). Con A, horseradish peroxidase, and proteases were obtained from Sigma. L-phytohemagglutinin (L-PHA) was from E-Y Laboratories (San Mateo, CA). All other chemicals were reagent grade from Sigma (St. Louis, MO).

Purification of ASGP-1/ASGP-2 Complex

Microvilli (500 μl, 6-7 mg protein), obtained as previously described from 13762 rat mammary ascites tumor cells [8], were extracted in 3.2 ml of 0.2% Triton X-100 (TX-100), 5 mM glycine, 2 mM EDTA, pH 9.5, for 15 min at room temperature. The extract was loaded onto a two-phase gradient in which the upper phase consisted of 1.0 ml of 0.2% TX-100-30 mM phosphate-buffered saline (PBS), pH 7.4, in 4% sucrose and the lower phase consisted of 0.5 ml of 2% sodium dodecyl sulfate (SDS)-30 mM imidazole, pH 7.4, in 10% sucrose. The gradient was centrifuged at 35,000 rpm for 1 h in an SW 50.1 rotor. The upper layer after this centrifugation is enriched in detergent-soluble microvillar proteins, whereas the lower layer is enriched in microfilament-associated microvillar proteins. Thus the upper layer contains ASGP-1/ASGP-2 complex among other membrane proteins. This sialomucin complex is purified from the other membrane proteins by CsCl density gradient centrifugation in the presence of

detergent. The upper layer from the discontinuous sucrose density gradient step (8–10 ml) is carefully layered onto a discontinuous CsCl gradient [5] formed by successively underlaying CsCl solutions of 1.3, 1.35, 1.40, 1.45, and 1.5 g/ml densities buffered with PBS containing 0.2% TX-100. The gradients were centrifuged in an SW 28 rotor at 26,000 rpm for at least 40 h at 4°C. After centrifugation the gradients were fractionated from the bottom into 4 ml fractions using an Isco fractionator. Appropriate aliquots were analyzed for radioactivity by mixing with 2–5 ml of Ecolume (ICN, Irvine, CA) and counting on a Beckman LS-3801 scintillation counter. Alternatively, appropriate aliquots were dialyzed against 0.1% lithium dodecyl sulfate (LDS) and analyzed on 8% SDS polylacrylamide gels (PAGE).

Preparation of Antiserum Against ASGP-2

ASGP-2 was obtained by electrophoresis of ASGP-1/ASGP-2 complex on SDS PAGE gels and electroelution of the ASGP-2 band. Antibodies were elicited as described previously for microvillar membranes [9] and transmembrane complex [10] by injecting the ASGP-2 in Freund's complete adjuvant subcutaneously into female New Zealand white rabbits with booster injections in incomplete adjuvant at 2 week intervals. The rabbits were bled weekly after the second injection. For some experiments specific antibodies to ASGP-2 were blot-purified by adsorption of antiserum by the method of Smith and Fisher [11].

Electrophoresis and Blotting Procedures

SDS PAGE on 8% minigels was performed as previously described [9]. For lectin-binding analyses on electroblots or immunoblotting with antisera, proteins were transferred from gels using the BioRad Transblot system according to the manufacturer's recommendations. Briefly, SDS gels were blotted in 5 mM ethanolamine, 25 mM glycine, 20% methanol, pH 9.5, for 16 h at 30 V (0.12 mA) and 1 h at 60 v (0.33 mA). Nitrocellulose replicas were "blocked" with 20 mM Tris, 500 mM NaCl containing 0.05% Tween 20 (TBS-Tween) for 2 h and then processed for antibody or lectin staining. For Con A blots the lectin (50 μg/ml) in TBS containing 10 mg/ml bovine serum albumin (BSA) was incubated with the replica for 2 h at room temperature and washed four times with TBS-Tween (10 min each) and twice with TBS. The replicas were incubated for 2 h with horseradish peroxidase (50 μg/ml) in TBS with 10 mg/ml BSA, washed as described above, and treated with 4-chloronaphthol to reveal the lectin-binding bands. Other lectins were used as direct conjugates with horseradish peroxidase at concentrations of 4–8 μg/ml. For all lectins binding was abolished in the presence of the appropriate inhibitory saccharide.

For immunoblots the replicas were incubated with 100–250-fold dilutions of antiserum or immunoglobulin preparations in TBS-BSA for 2 h, washed as described above, and incubated with horseradish peroxidase-conjugated antibodies to rabbit immunoglobulin for 2 h. Blots were washed and reactive bands revealed with 2-chloronaphthol as described above.

RESULTS

Purification of ASGP-2 and Preparation of Anti-ASGP-2

Sialomucin complex (ASGP-1/ASGP-2) was previously isolated from detergent extracts of intact ascites cells or isolated ascites membranes [5]. To avoid contamination

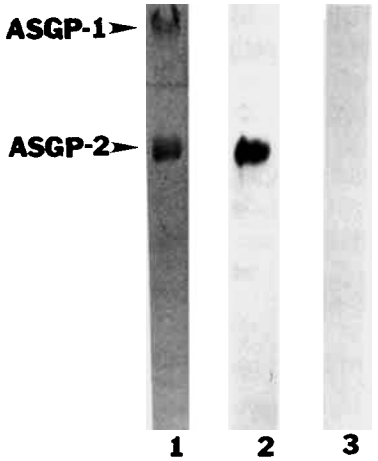


Fig. 1. SDS PAGE of purified ASGP-2/ASGP-1 complex (**lane 1**) and immunoblot staining of microvilli with anti-ASGP-2 (**lane 2**) and normal rabbit serum (**lane 3**).

from smaller proteins of whole cell extracts [5] and to minimize proteolysis, ASGP-2 was isolated from sialomucin complex extracted from ascites microvilli. The microvilli were extracted with Triton X-100 and centrifuged to remove microvillar microfilaments and associated proteins [12]. Sialomucin complex was isolated by CsCl density gradient centrifugation of the Triton extract supernatant [5]. ASGP-2 was obtained for antibody preparation by electroelution from SDS PAGE gels of the purified complex, shown in Figure 1, lane 1.

Anti-ASGP-2 antiserum produced in rabbits reacted strongly and specifically with ASGP-2 of the microvilli (Fig. 1, lane 2). No difference in specificity was observed when using antibodies immunoblot-purified from the ASGP-2 120 kDa band of SDS PAGE transfers [9]. Similar staining results were observed when immunoblot analyses were performed on the 13762 ascites cells (O. Vanderpuye, unpublished).

Proteolysis of Microvilli

Limited protease treatments of microvilli cleave the glycosylated portion of ASGP-1 from the membrane without cleavage of ASGP-2 and result in enhanced association of ASGP-2 with the microvillar microfilament core [9]. To determine which proteases were most effective in cleaving ASGP-2, we performed several preliminary experiments examining the effects of a number of proteases under various proteolysis conditions on intact microvilli. Analysis by SDS PAGE and immunoblotting with anti-ASGP-2, as described previously [9], gave three general observations. The results were typified by the effects observed in Figure 2. Relatively high concentrations of clostripain gave no detectable fragments of ASGP-2 associated with the microvilli (Fig. 2, lane 2). Trypsin treatment, however, produced a major fragment of about 70 kDa associated with the microvilli (Fig. 2, lane 4). Under similar proteolysis conditions proteinase K produced fragments of less than 30 kDa associated with the microvilli (Fig. 2, lane 3). Such smaller fragments can also be observed after treatments with Pronase (data not shown), but the Pronase major fragments are larger (about 40 kDa).

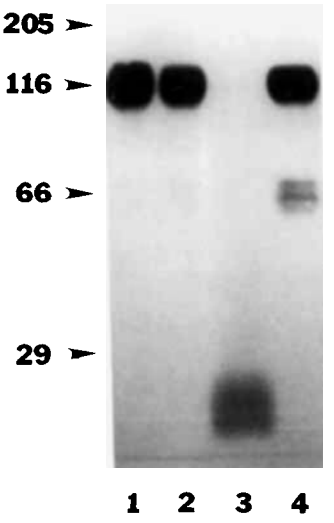


Fig. 2. Proteolysis of ASGP-2 of MAT-C1 microvilli. Microvilli (5 mg protein per ml) were incubated without protease or with 0.1 mg/ml clostripain or proteinase K in PBS (30 mM phosphate-buffered saline, pH 7.4) or 0.1 mg/ml trypsin in TBS-Ca (20 mM Tris-buffered saline containing 1 mM calcium, pH 7.4) at 25°C for 16 h. Digestion was stopped with a cocktail of protease inhibitors containing phenylmethylsulfonyl fluoride (PMSF) (0.3 mM), leupeptin (10 μg/ml), antipain (10 μg/ml), benzamidine (10 μg/ml), and aprotinin (10 μg/ml). Microvilli were washed three times in PBS containing the inhibitors, solubilized in Triton-containing buffer, and centrifuged at 15 kg for 30 min. The supernates were prepared for SDS PAGE and immunoblotting as previously described [9]. **Lane 1:** Untreated. **Lane 2:** Clostripain. **Lane 3:** Proteinase K. **Lane 4:** Trypsin. Positions of molecular weight standards are shown to left of gel.

Under appropriate conditions every protease we have examined which cleaves ASGP-2 will generate the 70 kDa fragment. Thus it appears to represent a relatively stable domain.

Identification of Released Fragments of ASGP-2

Since proteinase K appeared to be the most effective protease for cleaving ASGP-2 on microvilli to smaller analyzable products, we examined its effects in more detail. Microvilli were treated with proteinase K for timed intervals and centrifuged. Examination of the proteolysis supernates by SDS PAGE and Coomassie blue staining showed primarily proteinase K at 29 kDa (Fig. 3A). However, when the supernates were analyzed by blotting with anti-ASGP-2 (Fig. 3B), a major product of about 60 kDa was observed to appear at early times of proteolysis and to decrease after longer times.

ASGP-2 proteolysis products were also analyzed by lectin blots. ASGP-2 has been previously shown to be the major Con A-binding protein in these 13762 ascites tumor cells [5]. However, Con A is a relatively nonspecific lectin, which binds to high mannose and biantennary N-linked oligosaccharides. In contrast, *Phaseolus vulgaris* lectin (L-PHA) is specific for β1-6-linked lactosamine antennae in tri- and tetra-antennary N-linked oligosaccharides. These linkages have been implicated as markers for malignant transformation [13–15]. Moreover, a correlation has been shown between expression of β1-6-linked branching on a 130 kDa tumor cell surface glycoprotein and acquisition of metastatic potential [16]. Since the 13762 ascites cells are highly meta-

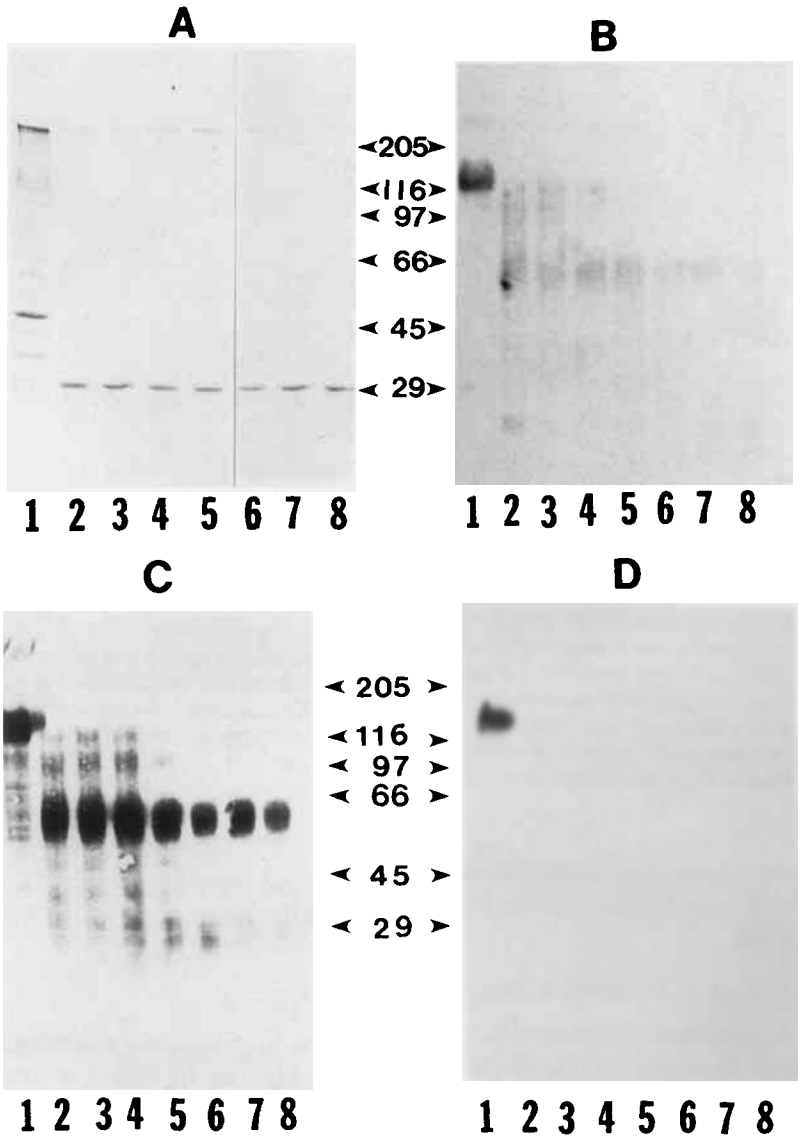


Fig. 3. Proteinase K release of soluble fragments of ASGP-2. Microvilli (3.5 mg protein per ml) were incubated for timed intervals with 0.1 mg/ml proteinase K in PBS at 37°C. The mixture was treated with 10 mM PMSF and centrifuged at 12,000*g* for 30 min. The supernates were prepared for SDS PAGE and blotting with anti-ASGP-2, Con A, and L-PHA. **A:** Coomassie blue. **B:** Anti-ASGP-2. **C:** Con A. **D:** L-PHA. **Lane 1:** 0 min (PMSF added immediately before proteinase K). **Lane 2:** 30 min incubation with proteinase K. **Lane 3:** 1 h. **Lane 4:** 2 h. **Lane 5:** 4 h. **Lane 6:** 8 h. **Lane 7:** 12 h. **Lane 8:** 16 h.

static, the presence and location of the β1,6-linkages on ASGP-2 is of considerable interest. Con A blotting, like anti-ASGP-2 blotting, showed a 60 kDa fragment which was released from the microvilli. In contrast, no product staining with L-PHA could be detected in the microvillar supernates after proteolysis (Fig. 3D).

Microvillar supernates from control samples contained small amounts of ASGP-2. High-speed centrifugation (100,000*g*) removed this material, suggesting that it results

from unsedimented vesicles. The fact that such material was not observed in any of the proteolyzed samples suggests that the proteolysis and removal of negatively charged ASGP-1 fragments allows sedimentation of the vesicles at lower speeds, probably as a result of their aggregation.

Identification of Membrane-Associated Fragments of ASGP-2

ASGP-2 has been previously reported to be an integral membrane protein [5]. To identify a proteolytic fragment(s) which contains the transmembrane domain of ASGP-2, microvilli treated with proteinase K were sedimented, extracted with Triton X-100, and centrifuged to separate soluble membrane proteins from those associated with the microfilament core. Analysis of the Triton-soluble proteins by SDS PAGE and Coomassie blue staining showed few changes other than the loss of ASGP-2 (Fig. 4A). In contrast, blotting of gel transfers with anti-ASGP-2 showed the rapid appearance of a major band at about 70 kDa and a minor band at about 40 kDa and the delayed appearance of a minor band in the 25–30 kDa range (Fig. 4B).

Con A blots were more complex because of the presence of other Con A-staining glycoproteins. However, the 70 kDa ASGP-1 fragment was readily detected as a Con A-binding species (Fig. 4C). No major L-PHA binding fragments were found in the Triton supernates (Fig. 4D). The fact that L-PHA-binding fragments are not seen in any of the fractions after proteolysis indicates that they must be too small to be detected by SDS PAGE analysis, since proteolysis is unlikely to reduce the lectin binding.

To determine whether the Triton-soluble fragments of ASGP-2 are integral membrane proteins, we extracted the microvilli with carbonate buffer at pH 11 [10], which is reported to remove peripheral proteins from membranes [17]. The carbonate supernate showed primarily a 60 kDa fragment on Con A and anti-ASGP-2 blots (data not shown). We believe that it is the same as the fragment released from the microvilli after proteolytic digestion, and that its presence on the microvilli is due to weak binding to the microvillar surface. The membrane pellet from the carbonate extraction was examined by SDS PAGE and Coomassie blue staining (Fig. 5A) and by blotting with anti-ASGP-2 (Fig. 5B), Con A (Fig. 5C), and L-PHA (Fig. 5D). Both anti-ASGP-2 and Con A blots indicate the presence of a 70 kDa ASGP-2 fragment, which appears rapidly with proteolysis and then becomes degraded, with the appearance of 25–30 kDa fragments. These results suggest that the 70 and 25–30 kDa fragments, as well as ASGP-2, are integral membrane proteins. No major L-PHA-binding fragments were found associated with the membranes (Fig. 5D), again suggesting that the L-PHA binding site is released as a small fragment.

Identification of Microfilament-Associated Fragments of ASGP-2

To evaluate the effects of proteolysis on the microfilament association of ASGP-2, microfilament cores were isolated from Triton extracts of proteolyzed microvilli. The core fractions were analyzed by SDS PAGE and Coomassie blue staining (Fig. 6A) and by anti-ASGP-2 (Fig. 6B) and Con A (Fig. 6C) blots. Treatment with proteinase K demonstrates binding to the microfilament core of both intact ASGP-2 and the 70 kDa fragment at the earliest times of proteolysis. At longer treatment times core-binding fragments of <30 kDa were observed (Fig. 6B, lanes 6–8). No 30–40 kDa species was found associated with the cores.

The relationship of the microfilament core-associated fraction to the released and Triton-soluble fractions is shown in Figure 7, in which all the products from the

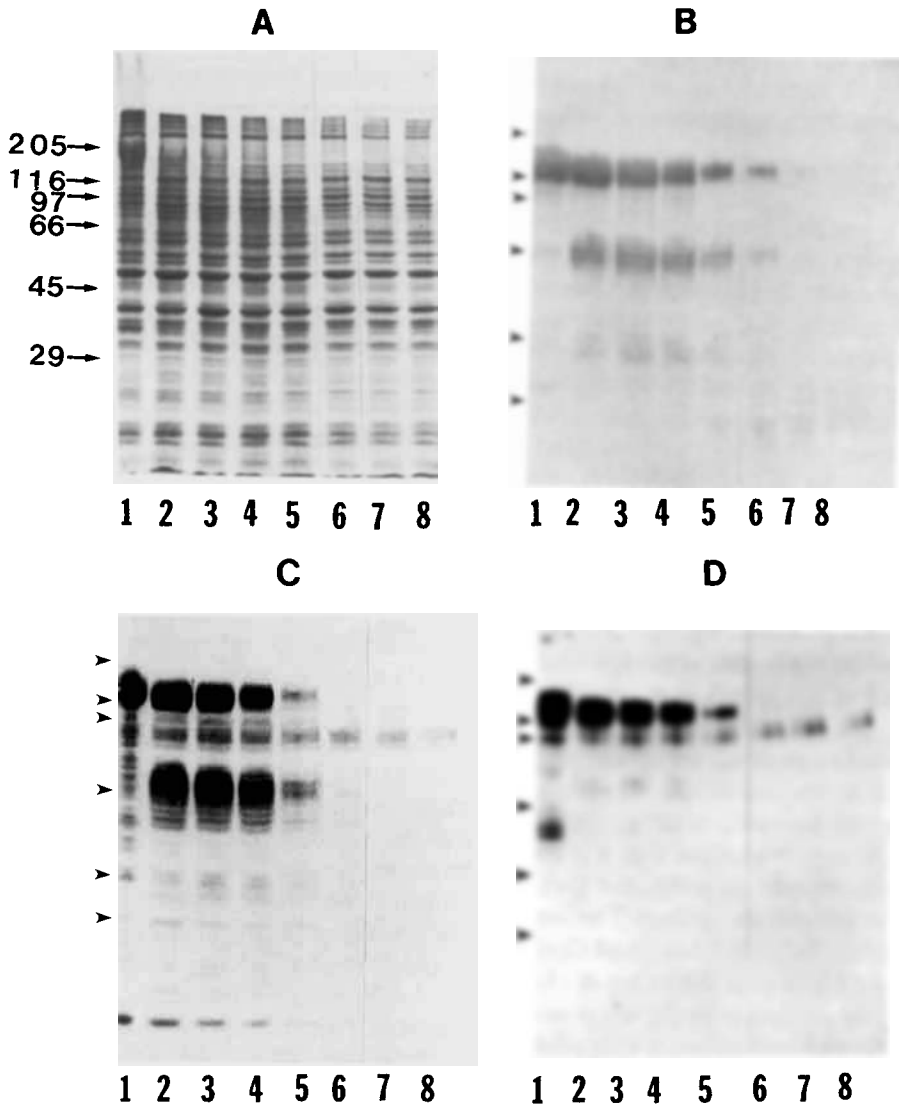


Fig. 4. Triton-soluble fragments of ASGP-2 from proteinase K-treated microvilli. Pellets of microvilli, treated with proteinase K and centrifuged as described for Figure 3, were extracted with Triton-PIPES-KCl (TPK) buffer [12] and centrifuged to generate Triton-soluble microvillar proteins, which were prepared for SDS PAGE and blotting. **A:** Coomassie blue staining. **B:** Anti-ASGP-2 blot. **C:** Con A blot. **D:** L-PHA blot. Lane designations are as in Figure 3.

proteolyzed microvilli were analyzed by immunoblotting with anti-ASGP-2 under identical conditions on the same gel. These results show clearly that the first product to be detected (30 min) is the 70 kDa membrane-associated fragment. Since only minor fragments are detected at 30 min in any of the other fractions, the remainder of the ASGP-2 must have been cleaved to products unreactive toward anti-ASGP-2 or too small to be detected by our electrophoresis procedure. These products must contain the

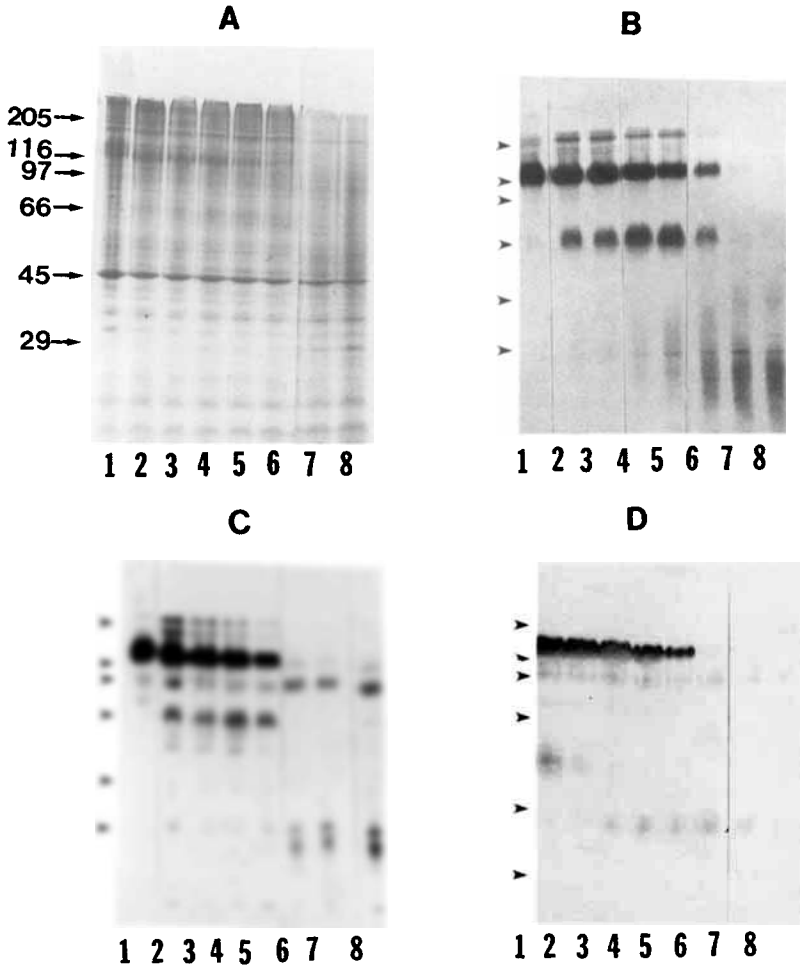
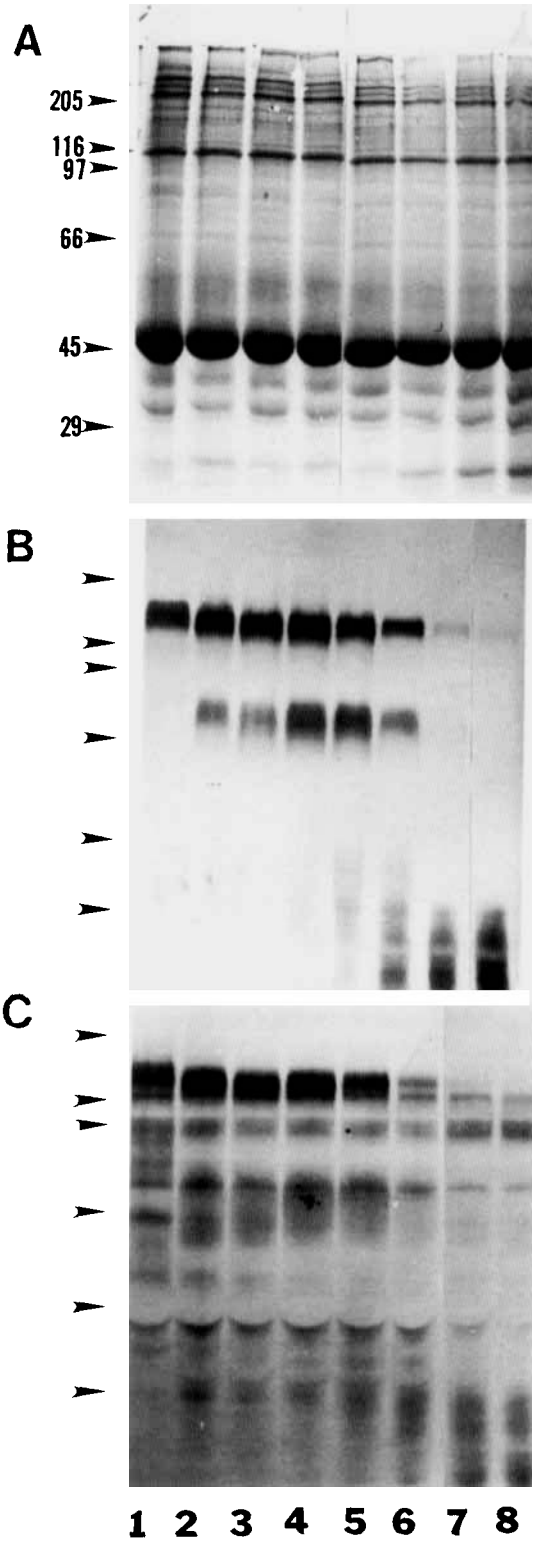


Fig. 5. Membrane-associated ASGP-2 fragments from proteinase K-treated microvilli. Pellets of microvilli, treated with proteinase K and centrifuged as described for Figure 3, were incubated with 0.1 M sodium carbonate, pH 11, for 45 min at 25°C. The mixture was centrifuged 30 min at 12,000*g*, and the membrane pellets were prepared for SDS PAGE and blotting. **A:** Coomassie blue. **B:** Anti-ASGP-2. **C:** Con A. **D:** L-PHA. Lane designations are as in Figure 3.

binding site for L-PHA, which disappears as a result of proteolysis and must be too small for detection on these gels. The detection of the 60 kDa soluble fragment at longer proteolysis times suggests that it is being derived from the 70 kDa fragment by further cleavage. If the 60 kDa fragment were derived directly from ASGP-2, it should have appeared earlier. Proteinase K treatment also produces small amounts of 30–40 kDa Triton-soluble fragments at the earliest times (Fig. 7,B). The major products detected by antiASGP-2 blots (Fig. 7,B,C) after prolonged proteolytic digestion are the 25–30 kDa fragment(s), which are associated predominantly with the microfilament core.

The strong interaction of the 25–30 kDa fragment with the microfilament core was verified by phalloidin shift analysis (Fig. 8), performed as described previously [18]. A substantial fraction of the 25–30 kDa fragment comigrated with the microfilament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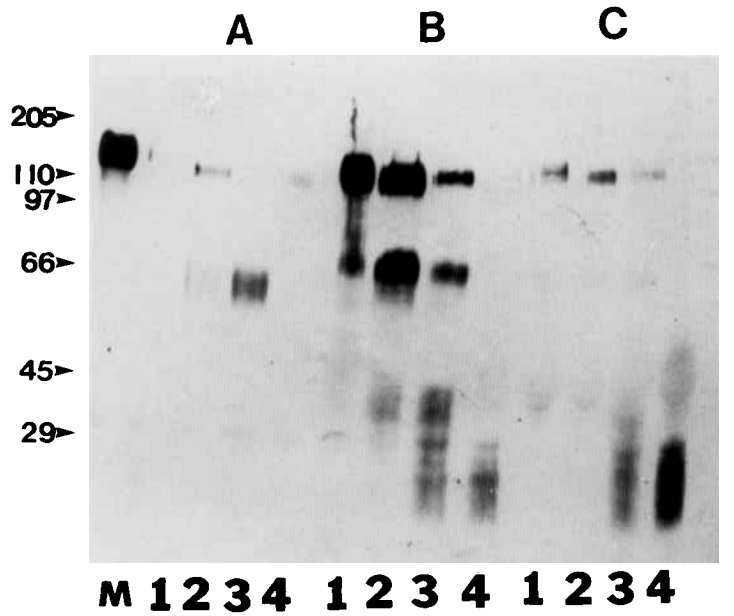

Fig. 7. Analysis of released, membrane-associated, and microfilament-associated fragments of ASGP-2. Samples of fractions from protease-treated microvilli, prepared as described in Figures 3, 4, and 6, were subjected to electrophoresis on the same gel and blotted with anti-ASGP-2 for direct comparisons. **Lane M:** Untreated microvilli. **A:** Soluble fragments, as in Figure 3. **B:** Triton-soluble fraction, as in Figure 4. **C:** Microfilament core-associated fraction, as in Figure 6. **Lanes 1–4** represent protease treatment times of 0, 0.5, 4, and 16 h, respectively.

fraction on the velocity sedimentation gradients in the presence and absence of phalloidin. More weakly associated proteins, even α-actinin of the microvilli, dissociate from the microfilaments during this procedure [18].

DISCUSSION

The results of these proteolysis studies can be described by a relatively simple working model, assuming that the ASGP-2 is an integral membrane glycoprotein with one transmembrane segment (Fig. 9). In the proposed model 120 kDa ASGP-2 is cleaved to a 70 kDa membrane-associated fragment in the first step. The 70 kDa fragment is then cleaved further at two different sites: 1) to release a 60 kDa soluble fragment, leaving a 10 kDa membrane-associated fragment (not detected), and 2) to generate a 25–30 kDa fragment(s), which can associate with protein(s) linked to the microfilament core. This model proposes that a 15–20 kDa segment of ASGP-2 near the external face of the membrane bilayer is involved in the indirect association of ASGP-2 with the microfilament core. This model accounts for the major products observed by

Fig. 6. Microfilament core-associated ASGP-2 fragments from proteinase K-treated microvilli. Microfilament cores were prepared by TPK extraction and centrifugation as described in Figure 4 and analyzed by SDS PAGE and blotting. **A:** Coomassie blue. **B:** Anti-ASGP-2. **C:** Con A. Lane designations are as in Figure 3.

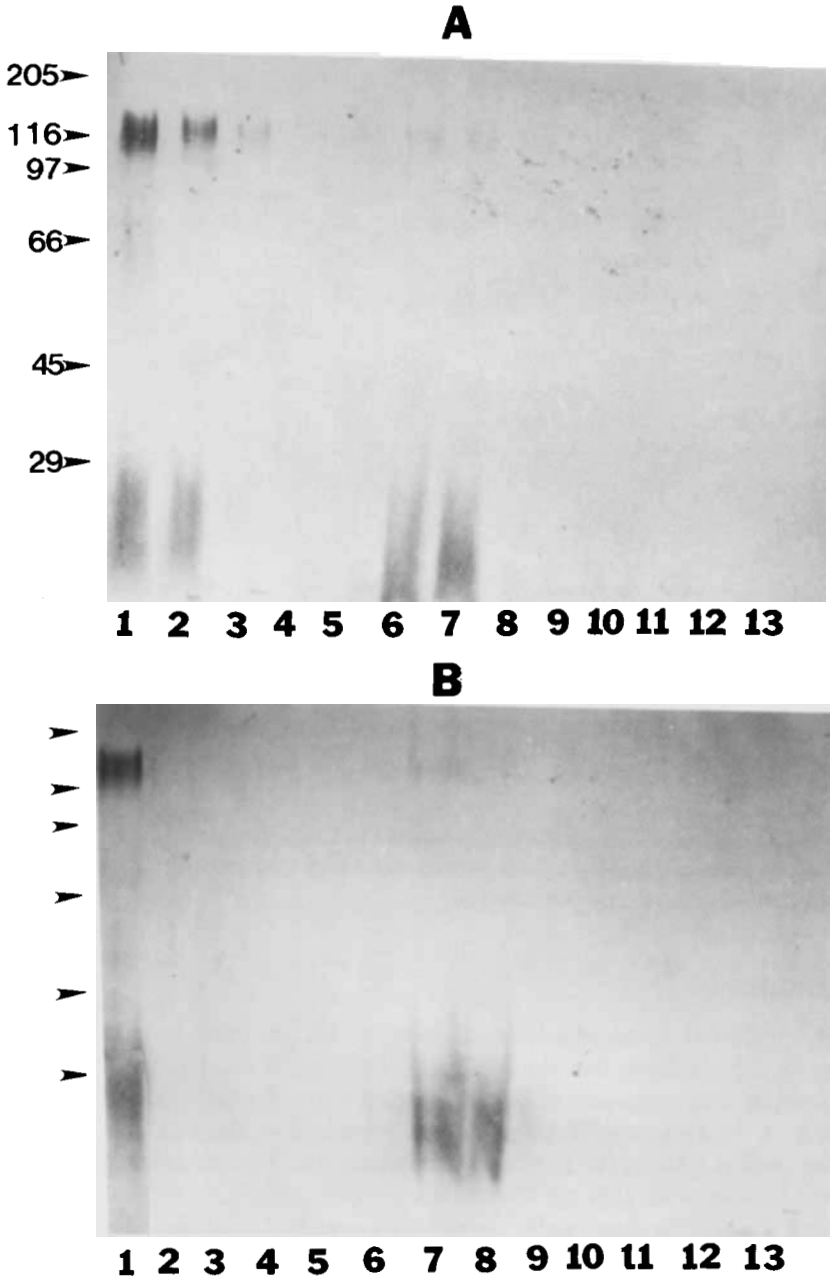

Fig. 8. Phalloidin shift analysis of 25–30 kDa fragment binding to microfilament core. Microvilli were treated with proteinase K for 16 h as described for Figures 3–7, extracted with Triton X-100 in the absence (**A**) and presence (**B**) of phalloidin as described previously [12,18], and subjected to velocity sedimentation analysis on sucrose density gradients [18]. Fractions from the gradients were analyzed by SDS PAGE and immunoblotting with anti-ASGP-2. Microfilament-containing fractions were 6 and 7 in the absence of phalloidin and 7 and 8 in the presence of phalloidin.

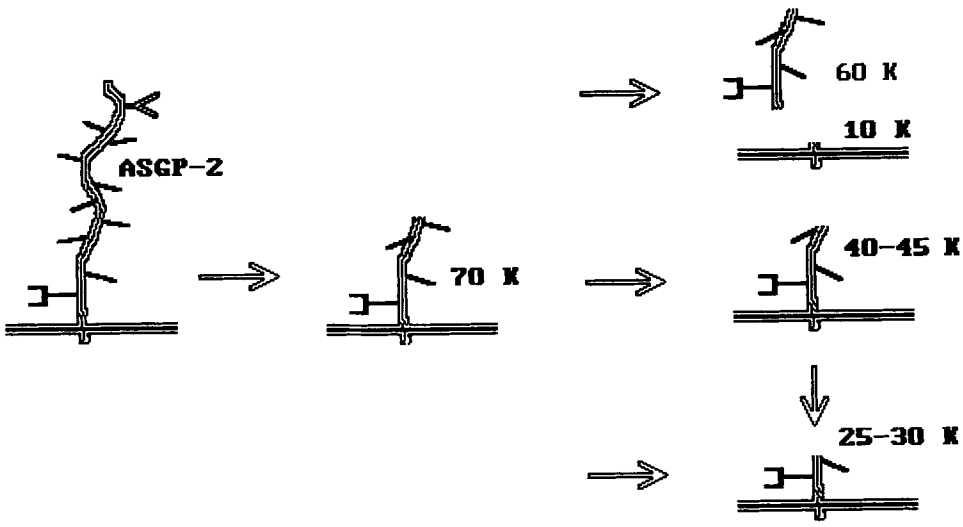

Fig. 9. Model for the topography, binding domains, and primary proteinase K cleavage sites of ASGP-2. L-PHA-binding site (⊣); Con A binding sites (⌇); association site to microfilament core (⊐).

proteinase K proteolysis, with respect to size, binding properties, and localization behavior of the fragments. Obviously, other cleavage sites may generate minor products. Moreover, interpretation is more complicated if ASGP-2 has multiple membrane-spanning segments.

The most interesting feature of these studies is their implication for transmembrane control of cell surface topography. Treatment of 13762 MAT-B1 cells with Con A leads to capping of the sialomucin complex [6]. An early step in this process is Con A linkage of the sialomucin complex to a transmembrane complex associated with the microfilament core [19]. Thus, the ability of Con A to serve as a bridge between two different cell surface glycoproteins can explain induction of topographical redistributions of cell surface components by Con A. However, this explanation does not suffice for monospecific ligands, such as antibodies. How do monospecific ligands induce linkage to the cytoskeleton? We would suggest that the proteolysis of ASGP-2 might be an example or model for the first stage in the receptor redistribution process. Our results suggest that proteolysis strengthens binding of an ASGP-2 domain to transmembrane microfilament-associated glycoproteins by reducing intramolecular interactions which inhibit this binding. We envision that antibody binding to a cell surface antigen might similarly induce a conformational change which would uncover a cryptic binding site on the antigen which is specific for binding to a transmembrane microfilament-associated glycoprotein. This hypothesis is similar to the X-protein model of Bourguignon and Singer [20], but it also provides a mechanism by which many receptors or antigens can be linked to a single X-protein or small number of X-proteins via a common set of peptide domains. This model also suggests that both intramolecular and intermolecular interactions can play a role in linkage of cell surface components to the cytoskeleton. Although this model is speculative, it can be tested if ASGP-2 peptides involved in binding to the transmembrane microfilament-associated glycoproteins can be isolated.

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