Isolation and Characterization of a 58-kDa Membrane- and Microfilament-associated Protein from Ascites Tumor Cell Microvilli*

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A 58-kDa protein is found in microvilli and in actin-containing transmembrane complexes of 13762 ascites tumor cells with immobile surface receptors; it is absent from sublines with mobile receptors. 58-kDa protein has been proposed to stabilize microvilli and restrict receptor mobility by stabilizing membrane-microfilament interactions. Antibodies against 58-kDa protein were blot-purified from antisera of rabbits injected with crude transmembrane complex and were used to monitor purification of the protein. 58-kDa protein was extracted from EDTA/EGTA-stripped microvillar microfilament cores with 1 M NaCl. A single depolymerization-polymerization cycle of the microfilaments, followed by solubilization of 58-kDa protein in 1 M NaCl and chromatography on hydroxyapatite-Sephadex G-150, purified the protein to >95% homogeneity. The native molecular weight and frictional coefficient indicated a monomeric, asymmetric structure. 58-kDa protein bound F-actin in pelleting assays and inhibited polymerization of pyrenyl-actin. It also bound phosphatidylinerine, phosphatidylinositol, and phosphatidylcholine vesicles in pelleting studies. Immunoblot analyses of endogenously and exogenously proteolyzed microvilli and their membranes and microfilament cores showed specific membrane and microfilament binding fragments of 28–30 kDa. The microfilament- and phospholipid-binding properties of 58-kDa protein and the localization of its proteolysis products are consistent with its proposed role in stabilizing membrane-microfilament interactions in the ascites cell microvilli.

Membrane-microfilament interactions are believed to play important roles in determining cell morphology and regulating the organization of components of the cell surface (Geiger, 1983, 1985; Carraway and Carraway, 1985, 1988). A number of mechanisms have been proposed for microfilament association with membranes. In the erythrocyte actin protofilaments are associated in a network with spectrin beneath the cytoplasmic surface of the membrane (Bennett, 1985). The spectrin-actin interaction is stabilized by band 4.1 (Cohen et al., 1980), and the network is linked to the membrane via spectrin-ankyrin-band 3 (Bennett and Stenbuck, 1979) and spectrin-band 4.1-glycoporphin associations (Anderson and Lovrien, 1984). In intestinal brush-border microvilli lateral attachments of the microfilament core to the membrane are formed by a complex of a 110-kDa protein (Matsudaira and Burgess, 1979) and calmodulin (Mooseker, 1985). The 110-kDa protein has myosin-like ATPase activity (Collins and Borsyenko, 1984). In resting platelets one linkage between the membranes and microfilaments is actin-binding protein (Fox, 1985).

All of these membrane-microfilament linkages are indirect. At least four plasma membrane proteins have been proposed to link directly to microfilaments. 1) Ascites tumor cell microvilli contain a glycoprotein CAG† (Carraway et al., 1983c) which can be isolated as a transmembrane complex with actin (Carraway et al., 1983a), and appears to be associated with the microvillar microfilament core (Carraway et al., 1985). 2) Purified laminin receptor (called connectin) binds to actin and causes filament bundling (Brown et al., 1983). 3) In platelets, a fraction of glycoprotein IIb-III is associated with microfilaments (Painter et al., 1985b); the filament-associated fraction is greatly increased on activation (Painter et al., 1983a). 4) A small glycoprotein (ponticulin) from Dicyostelium plasma membranes binds F-actin and is proposed to act in the assembly of microfilaments (Wuestehube and Luna, 1987).

The ascites microvillar transmembrane complex is of particular interest because of its implication in regulating cell surface receptor mobility (Jung et al., 1984). Transmembrane complex isolated from cells with mobile cell surface receptors (MAT-B1) contains actin and CAG, but the complex from cells with immobile receptors (MAT-C1) contains in addition to actin and CAG a third, cytoplasmically oriented, protein of M, 58,000 (Carraway et al., 1983a). Since the MAT-C1 cells have more stable microvilli, we have proposed that the function of this 58-kDa protein is to stabilize the microfilament-membrane interaction at the membrane (Carraway et al., 1983a, 1983b). In the present study we have begun testing this hypothesis by isolating the 58-kDa protein and examining its interaction with actin and membrane components.

EXPERIMENTAL PROCEDURES

Isolation of 58-kDa Protein—Cells and microvilli were obtained as previously described (Carraway et al., 1980, 1982). Microvilli (about 20 mg of protein) were extracted with 0.2% Triton X-100, 20 mM PIPES, pH 6.8, 100 mM KCl, 0.2 mM ATP, 0.5 mM EGTA, and 0.5 mM phenylmethylsulfonyl fluoride (buffer A) at room temperature for 15 min and centrifuged for 25 min at 27,000 × g to obtain the microfilament core (Carraway et al., 1985). Weakly associated proteins were removed from the core by extracting with 5 mM KCl, 5

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† The abbreviations used are: CAG, cytoskeleton-associated glycoprotein; EGTA, [ethylenbis(oxyethyleneminitrilo)]tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HEPES, N-2-hydroxyethylpiperazine-N' 2-ethanesulfonic acid; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); TMIC, transmembrane complex.
mm EDTA, 2 mM EGTA, 0.01% NaN₃, 5 mM Tris-HCl, pH 7.5, and 0.1% Triton X-100 (buffer B) overnight at 4 °C. After centrifugation at 27,000 × g for 30 min, this "stripped" core was extracted in 1 M NaCl, 7 mM phosphate, pH 7.4, 0.5 mM dithiothreitol, 0.5 mM EDTA, 0.01% NaN₃, and 0.1% Triton X-100 (buffer C) at 4 °C for 2.5 h to release 58-kDa protein along with actin and some of the peripheral membrane-associated proteins. After centrifugation at 100,000 × g the supernatant was dialyzed against 100 mM KCl, 20 mM Tris-HCl, pH 7.6, 2 mM MgCl₂, 0.5 mM ATP, 1 mM CaCl₂, 0.01% NaN₃, and 0.1% Triton X-100 (buffer D) overnight at 4 °C to repolymerize actin. The 58-kDa protein was recovered in the pellet with the microfilaments after centrifugation at 27,000 × g for 30 min. The pellet was resuspended in buffer C, incubated for 2.5 h at 4 °C, and centrifuged at 100,000 × g for 1.5 h. The resulting supernatant was applied to a Sephadex G-150 column (112 cm) overlayered with 2.5 ml of hydroxyapatite and equilibrated in buffer C. The column was washed with the same buffer until the absorbance at 280 nm was zero and eluted with 18 mM phosphate buffer, pH 7.5. Column fractions were analyzed by SDS-PAGE, and those containing 58-kDa protein were pooled and concentrated using a Centricon ultrafiltration tube (Amicon).

**Binding of 58-kDa Protein to F-Actin**—Actin (1–2 μM) was polymerized in 100 mM KCl, 50 mM NaCl, 0.5 mM ATP, 0.02% Triton X-100, 10 mM Tris-HCl, pH 7.5, for 3 h at room temperature in the absence or presence of 58-kDa protein (0.0–5 μM) and centrifuged in the Airfuge at 18 psi for 1 h. The resulting pellets were analyzed by SDS-PAGE. The effects of 58-kDa protein on polymerization of pyrenyl-actin were measured using pyrenyl-actin preparations (Carraway et al., 1986). Actin (0.4 μM, 5% pyrenyl-actin) was polymerized in 100 mM KCl, 2 mM MgCl₂, 0.5 mM ATP, 0.01% NaN₃, 0.02% Triton X-100, 5 mM Tris-HCl, pH 7.5, at 25 °C without or with 58-kDa protein at molar ratios of actin to 58-kDa protein of 476:1 and 235:1.

**Binding of 58-kDa Protein to Phospholipid Vesicles**—Binding of 58-kDa protein to phosphatidylserine, -inositol, and -choline vesicles was assayed as described by Liu et al. (1987). Purified 58-kDa protein was dialyzed against 200 mM KCl, 1 mM EGTA, 10 mM Tris-HCl, pH 7.5, and 0.02% Triton X-100 overnight at 4 °C and centrifuged at 100,000 × g for 90 min. 58-kDa protein (20 μg/ml) without and with phospholipid liposomes (150 μg/ml) were incubated in 100 mM KCl, 10 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 0.5 mM EGTA, and 0.01% Triton X-100 at room temperature for 30 min and centrifuged in an Airfuge at 18 psi for 30 min. The resulting pellets were analyzed by SDS-PAGE.

**Preparation and Use of Antibody against 58-kDa Protein**—Crude transmembrane complex (TMC) was prepared from MAT-C1 microvilli by extraction with Triton-glycine-EDTA, pH 9.5, and centrifugation (Jung et al., 1985). Antibodies to this material were obtained by adsorption of anti-TMC antiserum or anti-TMC immunoglobulin preparations against partially purified 58-kDa protein (>80% pure, with actin as the only detectable contaminant) shown a major reacting component in the 58-kDa region of the gel (data not shown). This antibody was purified by elution from the immunoblot (Smith and Fisher, 1984) and used for immunoblot analyses of microvilli, microfilament cores, and microvillar membranes prepared by two methods. The first method was that used for preparation of MAT-B1 and MAT-C1 microvillar membranes (Carraway et al., 1982) and from which transmembrane complexes were subsequently isolated (Carraway et al., 1983a). In this procedure, microvilli were homogenized in a low ionic strength buffer at pH 9.5 to depolymerize microfilaments. The second membrane preparation was made under harsher conditions (pH 11) to solubilize peripheral membrane proteins.

**RESULTS**

Identification of 58-kDa Protein and Isolation of Anti-58-kDa Antibody—58-kDa protein was originally identified as the major protein difference between microvilli of MAT-B1 (mobile receptors) and MAT-C1 (immobile receptors) cells. It was shown to be associated both with membranes (Carraway et al., 1982) and microfilaments (Carraway et al., 1982, 1985) from the MAT-C1 microvilli. Moreover, it was identified as a component of an actin-containing transmembrane complex from the MAT-C1 microvillar membranes (Carraway et al., 1983a).

To aid in identifying the 58-kDa protein during isolation from microvilli, we prepared antibodies by injecting a crude transmembrane complex fraction greatly enriched in 58-kDa protein (Fig. 1A) (Jung et al., 1985) into rabbits. Immunoblotting of this antiserum against partially purified 58-kDa protein (>80% pure, with actin as the only detectable contaminant) showed a major reacting component in the 58-kDa region of the gel (data not shown). This antibody was purified by elution from the immunoblot (Smith and Fisher, 1984) and used for immunoblot analyses of microvilli, microfilament cores, and microvillar membranes prepared by two methods. The first method was that used for preparation of MAT-B1 and MAT-C1 microvillar membranes (Carraway et al., 1982) and from which transmembrane complexes were subsequently isolated (Carraway et al., 1983a). In this procedure, microvilli were homogenized in a low ionic strength buffer at pH 9.5 to depolymerize microfilaments. The second membrane preparation was made under harsher conditions (pH 11) to solubilize peripheral membrane proteins.

Fig. 1C shows that the immunoblot-purified antibody reacts with the 58-kDa protein from microvilli, microvillar membranes, and microfilament cores. In the microvilli the antibody also reacts with two lower molecular size polypeptides (30–32 kDa). These smaller polypeptides appear to mem-

![Fig. 1](image-url)
brane-bound, since preparation of microvillar membranes by extraction with 0.1 M carbonate to remove peripheral membrane proteins results in their retention (Fig. 1C, lane 4). Moreover, they are not detectable in microfilament cores (Fig. 1C, lane 2). A small amount of 58-kDa protein is present in membranes obtained by carbonate extraction (Fig. 1C, lane 4). However, most of it is extracted, indicating that it does not behave as an integral membrane protein.

These results indicate that intact 58-kDa protein, but not the lower M, polypeptides, is associated with the microfilament core (Fig. 1C, lane 2). Moreover, they suggest that two cross-reacting polypeptides of about 30 kDa associate specifically with the membrane. These lower M, proteins increase in preparations of microvilli which have been stored, with a concomitant loss of 58-kDa protein. They are most likely proteolytic degradation products resulting from cleavage of 58-kDa protein by an endogenous protease (see subsequent section on proteolysis studies).

The results of the extraction and immunoblotting experiments indicate that the protein recognized by the antibody is associated with transmembrane complex, based on the method of antibody preparation. Moreover, it is strongly associated with microfilaments and with microvillar membranes, as suggested previously (Carraway et al., 1982, 1983a, 1985).

Purification of 58-kDa Protein—Since most of the 58-kDa protein is associated with microfilament cores when microvilli are extracted under microfilament-stabilizing conditions (Carraway et al., 1985; Carraway and Weiss, 1985), microfilament cores served as the starting material for purification of the 58-kDa protein (Fig. 2). After extraction of microvilli under filament-stabilizing conditions, the resulting microfilament cores (Fig. 3, lane 3) were “stripped” with a low ionic strength EDTA/EGTA buffer to remove loosely associated proteins, and the 58-kDa protein was solubilized in 1 M NaCl (Fig. 3, lane 6). After centrifugation the supernatant from the NaCl extraction was dialyzed against 100 mM KCl to repolymerize the microfilaments and permit reassociation of the 58-kDa protein (Fig. 3, lane 9). This pellet was resolubilized in 1 M NaCl and applied to a column of Sephadex G-150 overlaid with hydroxyapatite (Fig. 4). In preliminary purification attempts we had noted that 58-kDa protein was unusual among microvillar proteins in not binding significantly to hydroxyapatite. The hydroxyapatite layer removed proteins which would have co-migrated with 58-kDa protein on the gel filtration column. A summary of the purification results is shown in Table I.

The primary contaminants of the 58-kDa protein preparation are polypeptides of 45-55 kDa which are variable in amount. Since 58-kDa protein is susceptible to proteolysis,2 we examined the preparation by immunoblot analysis of SDS-PAGE gels using antibody against 58-kDa protein. As shown in Fig. 5, the lower M, polypeptides were reactive with the immunoaffinity-purified antibody, suggesting that they are indeed degradation products of the 58-kDa protein. Although intact microvilli contain immunologically cross-reactive low M, polypeptides (approximately 30 kDa) they contain little if
TABLE I

Purification of 58-kDa protein from MAT-C1 microvilli

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein</th>
<th>Total 58-kDa protein</th>
<th>Percent 58-kDa protein</th>
<th>Purification</th>
<th>Yield</th>
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<tbody>
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<td>-fold</td>
<td>%</td>
<td>Y’all</td>
<td>%</td>
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<td>49</td>
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<tr>
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<td>1.2</td>
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<td>41</td>
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<tr>
<td>NaCl supernatant 2</td>
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<td>0.24</td>
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<td>29</td>
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<tr>
<td>Hydroxypatite/Sephadex G-150</td>
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<td>0.052</td>
<td>1.2</td>
<td>1.0</td>
<td>15</td>
</tr>
</tbody>
</table>

FIG. 5. Immunoblot analysis of purified 58-kDa protein. Antibody directed against 58-kDa protein was immunoblot-purified from antiserum prepared as described under “Experimental Procedures” and used to stain immunoblots of preparation of 58-kDa protein (lanes 1) and microvilli (lanes 2). A, silver-stained SDS-PAGE gel; B, immunoblot with 58-kDa protein; C, immunoblot with preimmune serum.

Physical properties of 58-kDa protein

- Stokes radius: 4.8 nm
- Sedimentation coefficient: 2.9 S
- Frictional ratio: 1.9
- Mₙ, native: 57,000
- Mₙ, SDS-PAGE: 58,000

Properties of 58-kDa Protein—The proposed membrane-microfilament stabilizing function of 58-kDa protein (Carraway et al., 1983a, b) would suggest that it should bind to the membrane (or a membrane protein) and to one or more actin monomers of the microfilaments attached to the membrane. Such a function might be expected to require a multimeric or asymmetric structure. Gel filtration of the 58-kDa protein indicated a Stokes radius of 4.8 nm (Table II), which is larger than expected for a globular protein of Mₙ, 58,000. From this Stokes radius, the amino acid analysis for calculation of the partial specific volume and a sedimentation coefficient of 2.9 S, a native Mₙ of 57,000, and a frictional coefficient of 1.9 were calculated. The close correspondence of the Mₙ determined by SDS-PAGE and gel filtration/sedimentation suggests that detergent binding does not significantly influence the size of 58-kDa protein. These results indicate that 58-kDa protein is an asymmetric monomer.

Binding of 58-kDa Protein to Actin—¹²⁵I-actin overlay and G-actin affinity chromatography studies suggested that 58-kDa protein does not bind G-actin (data not shown). Incubation of 58-kDa protein with G-actin under actin polymerization conditions resulted in the binding of the protein to the microfilaments formed (Fig. 6). However, the amount of F-actin pelletable after polymerization was substantially decreased in the presence of 58-kDa protein. Only about 50% of the 58-kDa protein was pelleted with the actin under the conditions used, as expected if part of the 58-kDa protein is bound to filaments too short to pellet.

The results of the pelleting studies suggest that 58-kDa protein is acting as either a capping or a severing agent on the filaments. Transmission electron microscopy of negatively stained preparations suggested a general decrease in the length of the filaments in the presence of 58-kDa protein (data not shown). The absence of severing activity was demonstrated by incubation of preformed microfilaments with 58-kDa protein. No decrease in pelletable actin or change in fluorescence in pyrenyl-actin-containing microfilaments was observed in the presence of 58-kDa protein (data not shown). 58-kDa protein does have an inhibitory effect on actin polymerization. Pelleting studies indicated a half-maximal effect at a 58-kDa protein:actin ratio of about 1:10 (Fig. 7).

Since some microfilament-capping proteins are also able to nucleate polymerization, we examined the effects of 58-kDa protein on the polymerization of pyrenyl-actin (Fig. 8). These results clearly show that 58-kDa protein inhibits polymerization and does not act as a nucleator.

Phospholipid Binding of 58-kDa Protein—As a membrane-
Fig. 7. Inhibition of actin polymerization by 58-kDa protein. Actin (1 \(\mu\)M) was polymerized in the presence of different concentrations of 58-kDa protein (0.023–0.5 \(\mu\)M) as described for Fig. 6. Airfuge pellets were analyzed by SDS-PAGE with quantitation of the stained gels by densitometer.

58-kDa Membrane- and Microfilament-associated Protein

Fig. 8. Effect of 58-kDa protein on polymerization of pyrenyl-actin. Actin (2.4 \(\mu\)M, 5% pyrenyl-actin) was polymerized as described under “Experimental Procedures” in the absence of 58-kDa protein (●), a 470:1 ratio of actin to 58-kDa protein (■), or a 235:1 ratio of actin to 58-kDa protein (□).

The possibility of different membrane- and microfilament-binding domains may reside in different halves of the molecule. Moreover, the weak actin binding of the smaller fragment may indicate that interactions between the domains enhance the interaction of 58-kDa with actin.
binding domains was supported by proteolysis studies with trypsin. Tryptic fragments of 28–31 kDa were found exclusively in the Triton-soluble fraction, whereas a fragment of 27 kDa was found associated with the microfilaments (Fig. 11).

The simplest interpretation of these collected results is that the proteases, both exogenous and endogenous, can cleave the 58-kDa protein into fragments of roughly equal size, one of which contains the microfilament-association site, whereas the other contains the membrane-binding site.

Immunoblot Analysis of 58-kDa Protein in MAT-B1 and MAT-C1 Cells—Our previous hypothesis that the 58-kDa protein is involved in stabilizing microvilli and the cell surface of the 13762 MAT-C1 ascites cells was based in part on its absence from microvilli of the MAT-B1 cells. The apparent sensitivity of a region of the 58-kDa protein to proteolysis indicates that one reason for this absence could be degradation of the protein by endogenous proteases. Therefore, MAT-B1 and MAT-C1 cells were examined by immunoblot analysis for 58-kDa protein or degradation products. In MAT-C1 cells only the 58-kDa protein was observed (Fig. 12). No cross-reacting product was found in the MAT-B1 cells, indicating that any 58-kDa protein or degradation product present is below the detection limit of the procedure used.

**DISCUSSION**

In animal cells membrane-microfilament interactions have been implicated in the determination of cell morphology, cell motility, organization of cell surface molecules, and transmembrane signalling (Geiger, 1983; Carraway and Carraway, 1985, 1988). An understanding of these interactions at the molecular level requires the characterization of the proteins involved. How does 58-kDa protein compare with other membrane- and microfilament-associated proteins? It is strongly bound to the plasma membrane, but appears not to be a transmembrane protein. Thus, it differs from integral membrane glycoproteins which can link microfilaments directly or indirectly to the membrane. A new classification for cytoplasmic proteins which can associate transiently and reversibly with the membrane has been proposed (Burn, 1988). These "amphitropic" proteins bind lipid specifically and in some cases also bind actin or other cytoskeletal proteins. The erythrocyte skeleton proteins spectrin and 4.1 have been reported to bind phosphatidylserine (Momers et al., 1979; Shiffer et al., 1988; Cohen et al., 1988), and 4.1 requires lipid (Anderson and Marchesi, 1985) for its membrane interaction. A number of proteins found in nonerythroid cells bind lipid specifically. Examples of these are a-actinin (Burn et al., 1985) and vinculin (Ito et al., 1983), proposed to anchor microfilaments to plasma membranes. The 110-kDa protein of intestinal brush-border microvilli is reported to have hydrophobic properties and to bind acidic phospholipids (Glenney et al., 1984). Calpain and related proteins, which are associated with membranes of many cell types, bind both phospholipid and microfilaments in a Ca²⁺-dependent manner (Glenney, 1986; Liu et al., 1987). Thus a number of submembrane proteins have the potential to interact with actin and with lipids of the cytoplasmic surface of the plasma membrane. How these lipid interactions may contribute to protein functions remains unclear. In the case of the 58-kDa protein, an interaction with the membrane lipid could certainly contribute to its strong association with the membrane, providing support for its proposed role in stabilizing membrane-microfilament interactions. Unlike the examples cited above, 58-kDa protein appears not to show binding specificity for phospholipid head groups. This observation might suggest that binding occurs predominantly with the hydrophobic portion of the lipid. Binding of 58-kDa protein to phospholipid does not rule out the possibility of interaction with other membrane proteins, such as CAG. Studies with purified CAG will be necessary to answer this question.

The association of 58-kDa protein with actin resembles the microfilament-capping proteins which bind only to an end of microfilaments (Korn, 1982; Weeds, 1982; Craik and Pollard, 1982). However, 58-kDa protein differs from most capping proteins in its inability to promote severing or nucleation and its lack of requirement for calcium. Moreover, the strong association of 58-kDa protein with membranes suggests that its primary function is not as a cytoplasmic regulator of actin filament length. Our previous studies led us to propose that 58-kDa protein stabilizes the interaction of microfilaments
with the cytoplasmic surface of the microvillar membrane in MAT-C1 microvilli (Carraway et al., 1983a). Its properties are consistent with that role, since it is an elongated molecule which can bind to the membrane and to microfilaments. We would suggest that the apparent capping activity results from its ability to bind to microfilaments near their ends and interfere with the addition of monomers to the filaments. This type of filament binding, coupled to an interaction with CAG and/or the bilayer, would be consistent with our hypothesis of a role in membrane-microfilament stabilization.

The proteolysis studies are also consistent with this type of functional model, suggesting that 58-kDa protein is composed of two domains of roughly equal size. The putative membrane-binding domain is observed in freshly isolated microvilli. We suggest that it may result from cleavage of 58-kDa protein by a calcium-dependent protease in the microvilli. Triton lysis of microvilli in the presence of calcium results in the rapid binding domain is observed in freshly isolated microvilli. We

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REFERENCES