Topography of epitope-tagged F13L protein, a major membrane component of extracellular vaccinia virions

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Abstract

The protein encoded by the vaccinia virus F13L open reading frame is required for the wrapping of intracellular mature virions by cisternae derived from trans-Golgi or endosomal membranes and is an abundant, palmitylated component of the outer membrane of extracellular virions. To study the topology of the F13L protein, we constructed recombinant vaccinia viruses and plasmids that express the F13L protein with an N- or C-terminal HA epitope tag. The recombinant viruses formed normal-size plaques and the tagged proteins were incorporated into the two outer membranes of intracellular enveloped virions (IEV), indicating that the epitope-tagged proteins were functional. By selective permeabilization of the plasma membrane of infected or transfected cells, we demonstrated that the N- and C-termini of the F13L proteins in the outer IEV membrane, as well as cellular membranes, were oriented toward the cytoplasm. After fusion of the outer viral membrane with the plasma membrane, externalized virions retain the inner of the two IEV membranes. The N- and C-termini of the F13L protein were exposed on the inner surface of this extracellular viral membrane, consistent with the accepted model of biogenesis of the IEV membrane by a wrapping process. Using a coupled in vitro transcription and translation system modified by the addition of microsomes, we determined that the F13L protein associated posttranslationally with membranes. The N- and C-termini were susceptible to protease digestion and the protein could be extracted with sodium carbonate, consistent with a peripheral mode of association.

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Introduction

Poxviruses are large, enveloped DNA viruses that replicate in the cytoplasm of host cells (Moss, 2001). The assembly of vaccinia virus, the most intensively studied member of the family, begins with the formation of crescent-shaped membranes that can be visualized by electron microscopy (Dales and Siminovitch, 1961). The crescents enlarge into spheres called immature virions (IV), which then undergo internal condensation to form brick-shaped infectious intracellular mature virions (IMV). Some of the IMV are wrapped by trans-Golgi or endosomal cisternae to form intracellular enveloped virions (IEV) containing two additional membranes (Hiller and Weber, 1985; Schmelz et al., 1994; Tooze et al., 1993). The IEV are transported along microtubules to the periphery where fusion between the outer IEV membrane and the plasma membrane occurs, followed by the formation of motile actin tails that facilitate spread of cell-associated enveloped virion (CEV) to neighboring cells (Geada et al., 2001; Hollinshead et al., 2001; Rietdorf et al., 2001; Ward and Moss, 2001a,b). Some virions, known as extracellular enveloped virions (EEV), are released into the medium and mediate long-range spread (Payne, 1980).

Of the seven proteins that have been identified as constituents of the IEV membrane, the ones encoded by the F13L and B5R open reading frames (ORFs) are essential for wrapping of the IMV (Blasco and Moss, 1991; Engelstad and Smith, 1993; Wolff et al., 1993). The F13L product, called p37K for its apparent mass determined by SDS–polyacrylamide gel electrophoresis (PAGE), is a 372 amino acid nonglycosylated polypeptide that is palmitylated at cysteine residues 185 and 186 and localizes in the Golgi network (Groenbach et al., 1997; Hiller and Weber, 1985; Hirt et al., 1986; Payne, 1978; Schmelz et al., 1994). Palmitylation is required for membrane association, proper intracellular targeting, and virion wrapping (Borrego et al.,...
1999; Grosenbach and Hruby, 1998; Husain and Moss, 2001; Schmutz et al., 1995). In addition, the F13L protein contains a variant of the HKD motif that is conserved in members of the phospholipase superfamily and is required for membrane wrapping of IMV (Husain and Moss, 2001; Roper and Moss, 1999; Sung et al., 1997). Based largely on the resistance of intact EEV to protease digestion and its unreactivity to an F13L monoclonal antibody (mAb), it was suggested that the F13L protein is located on the inner side of the extracellular viral membrane (Roos et al., 1996; Schmutz et al., 1995). Here, by constructing recombinant vaccinia viruses and expression plasmids with an epitope tag on the N- or C-terminus of the F13L protein, we have determined its topology on intracellular and extracellular viral and cellular membranes. Further experiments with an in vitro system provided evidence for the posttranslational insertion of the F13L protein into membranes.

**Results**

*Construction of recombinant vaccinia viruses that express functional epitope-tagged F13L protein*

We constructed two recombinant viruses, vF13L-HA\textsubscript{N} and vF13L-HA\textsubscript{C}, in which DNA encoding a short epitope tag derived from the influenza virus hemagglutinin (HA) was appended to either the N- or the C-terminus of the F13L ORF, respectively. An F13L deletion mutant of vaccinia virus strain Western Reserve (WR) that makes tiny plaques (Blasco and Moss, 1991) was used as the parental virus and the epitope-tagged F13L ORF was introduced into the deletion site by homologous recombination. The recombinant viruses were recognized by their large plaque size and the presence of the F13L ORF was confirmed by polymerase chain reaction (PCR). The plaques formed by vF13L-HA\textsubscript{N} and vF13L-HA\textsubscript{C} recombinant viruses were similar to those of wild-type strain WR virus (Fig. 1A), indicating that the epitope tags did not interfere with virus formation or spread.

Expression of the HA-tagged F13L proteins was demonstrated by SDS-PAGE followed by Western blotting using an anti-HA monoclonal antibody. Bands of 37 kDa, corresponding to the known mobility of the F13L protein, were detected by analysis of lysates from cells infected with vF13L-HA\textsubscript{N} or vF13L-HA\textsubscript{C} virus (Fig. 1B).

Laser scanning confocal microscopy was used to locate the epitope-tagged F13L protein in infected cells. Cells infected with vF13L-HA\textsubscript{N} or vF13L-HA\textsubscript{C} were fixed, permeabilized with Triton X-100, and then stained with a mouse mAb to the HA tag and with Alexa 568 conjugated phalloidin to visualize actin polymers. In addition to intense staining of the juxtanuclear Golgi region by the HA mAb,
punctate fluorescence was discerned in the periphery (Fig. 2). Many of these fluorescent spots were at the tips of actin tails, identifying them as CEV. Those spots that did not localize with actin tails may represent IEV or vesicles containing viral proteins. As will be shown later, IEV were distinguished from vesicles by DNA staining.

Topography of the N- and C-termini of the F13L protein in viral membranes

Having shown that the epitope-tagged F13L proteins were expressed, incorporated into viral membranes, and functioning normally as determined by the large plaque size of the recombinant viruses, more detailed studies were carried out. The epitope tags were used to investigate the membrane orientation of the N- and C-termini of the F13L protein. Cells infected with vF13L-HAN or vF13L-HAC were fixed and either the plasma membrane was selectively permeabilized with digitonin (Plutner et al., 1992) or membranes were nonselectively permeabilized with Triton X-100. The cells were then stained with a mouse mAb to HA and as a control with a rat mAb to the luminal domain of the B5R protein. The HA juxtanuclear, punctate, and plasma membrane staining of digitonin-treated cells that had been infected with vF13L-HAN or vF13L-HAC (Fig. 3) indicated that both ends of the F13L protein faced the cytoplasm. The intense staining of the vertices of the cells was consistent with the collection of IEV following microtubular transport (Ward and Moss, 2001b). Very similar staining occurred when the cells were permeabilized with Triton X-100 (Fig. 3). In contrast, the mAb to the luminal domain of B5R only stained the plasma membrane of digitonin-treated cells, confirming the selectivity of the permeabilization procedure (Fig. 3). Treatment with Triton X-100 was required to stain intracellular structures with the B5R mAb (Fig. 3).

As mentioned, both vesicles and IEV could appear as punctate structures when stained with F13L antibody. To distinguish IEV, the DNA in the digitonin-permeabilized cells was stained with 4’6-diamidino-2-phenylindole (DAPI). IEV in the periphery of the cell were identified by punctate DAPI fluorescence that colocalized with F13L (Fig. 4). F13L-staining and DAPI-non-staining punctate structures may represent endosomal or other vesicles; F13L-non-staining and DAPI-staining punctate structures may represent IMV. The cells were also stained with the mAb to B5R to distinguish the cell-surface CEV from the intracellular IEV (not shown).

The IEV membrane is composed of two layers that cannot be resolved by confocal microscopy. Therefore additional studies were carried out in which cells infected with vF13L-HAN or vF13L-HAC were analyzed by immunoelectron microscopy. Gold grains, representing the HA mAb, were detected on both IEV membranes (Fig. 5A and B), but not on IMV or in cells infected with vaccinia virus lacking the epitope tag on the F13L protein (not shown).

CEV, which contain only the inner of the two IEV membranes, were examined by immunoelectron microscopy to determine the orientation of the N- and C-terminal ends of the F13L protein. The clearest results were obtained by immunostaining with mAb and Protein A gold prior to fixation and sectioning. Under these conditions, only CEV with defects in their membrane were stained and the gold grains clearly lined the inner side of the CEV membrane (Fig. 5C and D).

Thus, by confocal microscopy, we demonstrated that the F13L protein on the outer IEV membrane has N- and C-termini that face the cytoplasm and by immunoelectron microscopy we showed that the ends of the F13L protein of the inner membrane face the enclosed virion.

Topography of the N- and C-termini of the F13L protein in cellular membranes

The above results were consistent with the formation of the IEV membrane by wrapping of IMV with cisternal vesicles that have the F13L protein on their cytoplasmic surface. Our previous studies indicated that the F13L protein associates primarily with the Golgi complex and endosomes when expressed in uninfected cells and suggested that these cisternae are precursors of the wrapping membranes in infected cells (Husain and Moss, 2001). To determine the topology of the F13L protein in these structures, we constructed plasmids in which a cytomegalovirus promoter regulated F13L-HAN and F13L-HAC expression. Following transfection, unpermeabilized cells or cells permeabilized with digitonin or Triton X-100 were stained with anti-HA mAb. No staining of unpermeabilized cells was detected (Fig. 6). Staining was similar, however, when the cells were permeabilized by either method (Fig. 6), indicating that the F13L protein was on the cytoplasmic face of membranes when synthesized in the absence of other viral proteins.

In vitro synthesis and membrane association of F13L-HAN and F13L-HAC polypeptides

Although palmitylation of the F13L protein is required for its association with membranes (Borrego et al., 1999; Groesenbach and Hruby, 1998; Husain and Moss, 2001; Schmutz et al., 1995), it had been suggested that two small hydrophobic regions near the center of the protein might be membrane anchors (Hirt et al., 1986). To further study the association of F13L with membranes, we constructed plasmids containing the F13L-HAN or F13L-HAC ORF regulated by a bacteriophage T7 promoter for use as templates in a coupled transcription and translation system containing canine pancreatic microsomes. Sedimentation through a sucrose cushion and SDS–PAGE were used to assess membrane-association. Identical results were obtained with the N- and C-tagged protein so only results with the former are shown here. In the absence of microsomes, a band with an
estimated molecular mass of approximately 37 kDa was detected exclusively in the supernatant fraction when the template was either F13L-HAN (Fig. 7A) or F13L-HAC (not shown). Upon inclusion of microsomes in the reaction, the 37-kDa band was also detected in the membrane pellet fraction (Fig. 7A). To investigate the topology of the F13L protein, the supernatant and pellets were treated with protease under conditions in which only cytoplasmically oriented proteins are susceptible (Betakova et al., 1999; da Fonseca et al., 2000). Both ends of the F13L protein were digested, as no protein bands were detected in either the supernatant or the pellet fraction (Fig. 7A and data not shown).

Treatment with sodium carbonate was carried out under...
Fig. 3. Localization of epitope-tagged F13L and B5R proteins in infected cells permeabilized with digitonin or Triton X-100. HeLa cells were infected with vF13L-HA\textsubscript{N} or vF13L-HA\textsubscript{C} for 20 h at 37°C, fixed, permeabilized for 5 min with 0.2% Triton X-100 at room temperature or 25 μg of digitonin per milliliter at 0°C, and then stained with anti-HA mAb followed by rhodamine red conjugated anti-mouse Ig (red). The cells were then stained with an anti-B5R rat mAb followed by FITC-conjugated anti-rat Ig (green) and analyzed by confocal microscopy. Bars, 10 μm.
conditions in which proteins containing a transmembrane domain remain bound (Betakova et al., 1999; da Fonseca et al., 2000; Howell and Palade, 1982). The complete extraction of the F13L protein with sodium carbonate indicated that it does not traverse the membrane (Fig. 7B). This result differed from that previously described for extraction of EEV with sodium carbonate (Schmutz et al., 1995), possibly because in that study the released F13L protein was trapped between the EEV and IMV membranes or the F13L protein was associated with other viral proteins. Previous studies indicated that the F13L protein partitions in Triton X-114, only when palymitylated (Schmutz et al., 1995). A similar approach was used to analyze the hydrophobicity of the F13L polypeptide synthesized in the presence and absence of microsomes. After temperature-induced phase separation, proteins from the aqueous and detergent phases were resolved by SDS–PAGE. In the absence of microsomes, the F13L protein was mostly in the aqueous phase (Fig. 7C). A larger fraction was in the detergent phase when microsomes were present (Fig. 7C), suggesting that palymitylation of F13L occurred under these conditions, albeit incompletely. The ratios of F13L in the aqueous and detergent phases were 3:1 and 10:1 in the presence and absence of microsomes, respectively.

The peripheral association of the F13L protein with membranes suggested that it occurs posttranslationally. This was assessed using cycloheximide, an inhibitor of protein synthesis. When added at the start of the transcription–translation reaction, the drug entirely prevented synthesis of the F13L protein, indicating its activity. When cycloheximide was added after the transcription–translation reaction, but before addition of microsomes, membrane association was detected indicating that it can occur posttranslationally (Fig. 7D).
Discussion

To investigate the topology of the F13L protein, the major membrane component of the extracellular virion envelope, we constructed recombinant viruses that express the F13L protein with an HA epitope tag at the N- or C-terminus. The use of these constructs distinguished our study from previous ones, which depended on a mAb to an unmapped epitope on the F13L protein. Synthesis and viral membrane-association of the epitope-tagged proteins were demonstrated with an HA-specific mAb by Western blotting and by fluorescence and electron microscopy. Virus plaque size and replication were unaffected, indicating that the epitope tags did not compromise the function of the F13L protein. In contrast, deletion or certain mutations of the F13L gene cause a severe reduction in virus transmission and plaque size (Blasco and Moss, 1991; Roper and Moss, 1999).

Fig. 6. Reactivity of epitope-tagged F13L protein in transfected uninfected cells permeabilized with digitonin. HeLa cells were transfected with plasmids pF13L-HA<sub>N</sub> or pF13L-HA<sub>C</sub> for 24 h at 37°C and were either unfixed and unpermeabilized, fixed and then permeabilized for 5 min with 0.2% Triton X-100 at room temperature, or fixed and then permeabilized with 25 μg digitonin per milliliter at 0°C. The cells were stained with anti-HA mAb followed by rhodamine red conjugated anti-mouse Ig and examined by confocal microscopy. Unstained cells were visualized by differential interference contrast. Bars, 10 μm.

Fig. 7. Membrane association of in vitro synthesized F13L-HA<sub>N</sub> protein. Proteins were synthesized using a reticulocyte transcription and translation system in the presence of [35S]methionine with or without added microsomes. Supernatant (S) and pellet (P) fractions were obtained by centrifugation through a sucrose cushion and analyzed by SDS-PAGE and autoradiography. (A) Susceptibility to protease digestion. Supernatant and pellet fractions were incubated with pronase for 30 min on ice before analysis. (B) Extraction of membrane-bound F13L protein with sodium carbonate. All reactions contained the DNA template. The pelleted microsomes were resuspended in NTE buffer or 0.1 M sodium carbonate, pH 11.0 and incubated on ice for 20 min before repelleting and analysis. (C) Phase separation of proteins. At the end of the reaction, the mixture was mixed with buffer containing 1% Triton X-114 and aqueous and detergent phases were obtained by lowering the temperature. Samples of the total reaction mixture (T) and aqueous (A) and detergent (D) phases were analyzed. (D) Posttranslational association of F13L protein with membranes. Cycloheximide and microsomes were added to a reaction at 0 time (0') or cycloheximide and microsomes were added to a reaction after 60 min incubation (60'). The latter samples (right two lanes) were incubated for an additional 30 min before analysis.
The orientation of the F13L protein on vesicles and IEV was determined by selective permeabilization of the cellular plasma membrane with a low concentration of digitonin. Under these conditions, both the N- and the C-terminal epitope tags reacted with the HA mAb, indicating their cytoplasmic location. As a control, we showed that the B5R membrane protein did not react with a mAb to its luminal domain under the same conditions, but did react when the cells were permeabilized with Triton X-100. The N- and C-termini of the F13L protein were also oriented toward the cytoplasm when it was expressed by transfection in the absence of other viral proteins. Under these conditions, the F13L protein is associated primarily with the Golgi complex, endosomal vesicles, and the plasma membrane (Husain and Moss, 2001). In infected cells, the presence of the F13L protein in both the inner and the outer IEV membranes was visualized by immunoelectron microscopy. Furthermore, we could directly demonstrate the epitope-tagged proteins lining the internal side of the CEV membrane by electron microscopy, a result that was consistent with previous determinations of the resistance of the F13L protein-to-protease digestion and the inability to label intact extra-cellular virions with a mAb (Roos et al., 1996; Schmutz et al., 1995). The presence of the F13L protein on the internal side of the CEV membrane indicated that it must have the same topology on the inner side of the two IEV membranes. The locations of the F13L protein on the cytoplasmic side of the outer IEV membrane and the side of the inner IEV membrane facing the virion are consistent with the wrapping of IMV by cisternal membranes containing the F13L protein on their cytoplasmic side, as diagrammed in Fig. 8. A similar model was suggested by Schmutz and co-workers (Schmutz et al., 1995).

We used an in vitro transcription-translation system to explore the mechanism of association of the F13L protein with cellular membranes. In vitro synthesized F13L protein could bind to microsomal membranes with the N- and C-termini facing the exterior, as indicated by their susceptibility to protease digestion. The microsomes increased the partitioning of the F13L protein into the Triton X-114 detergent phase, suggesting that they catalyzed palmitoylation as occurs posttranslationally in vivo. The ability to extract the F13L protein with sodium carbonate indicated that the central hydrophobic domain was not membrane-spanning, in agreement with our other data. Taken together, these results indicated that the F13L protein associates peripherally with membranes via the palmitoylated regions, as suggested by others (Grosenbach and Hruby, 1998; Grosenbach et al., 1997; Schmutz et al., 1995). In accord with this idea, we found that association of the F13L protein with microsomal membranes could occur posttranslationally.

In conjunction with other studies (Baek et al., 1997; Husain and Moss, 2001, 2002; Roper and Moss, 1999; Sung et al., 1997), we believe that the F13L protein binds peripherally to the Golgi complex and through an intrinsic lipase activity or by activating a cellular lipase, induces the formation of vesicles and cisternae that include additional viral membrane proteins, and wraps the IMV to form IEV. The N- and C-terminal cytoplasmic domains of the F13L protein may have additional undetermined roles in the association of the cisternal membranes with the IMV, the trafficking of IEV through the cytoplasm or fusion of the outer IEV membrane with the plasma membrane.

**Materials and methods**

**Cells and viruses**

HeLa, RK-13, and BSC-1 cells were grown and subcultured in Dulbecco’s modified Eagle’s medium (DMEM) and Eagle’s modified medium (EMEM), respectively, supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C with 5% CO₂. For virus titrations and analysis of plaque size, infected BSC-1 monolayers were fixed and stained with 0.1% crystal violet in 20% ethanol.

**Plasmids**

F13L polypeptides with an HA epitope tag at the N- or C-terminus were expressed from plasmid pcDNA3 (Invitrogen) under bacteriophage T7 or cytomegalovirus promoters. An HA epitope sequence was attached to F13L ORF at the N- or C-terminus by PCR. The PCR fragments were cloned into plasmid pcDNA3 to form recombinant plasmids pF13L-HA_N and pF13L-HA_C. Plasmid pGF13L-HA_N and pGF13L-HA_C were generated by replacing the F13L-GFP fragment with F13L-HA_N and F13L-HA_C coding sequences in plasmid pGF13L (Husain and Moss, 2001).
Construction of recombinant vaccinia viruses

Recombinant vaccinia viruses expressing F13L-HA\(_{N}\) and F13L-HA\(_{C}\) polypeptides were constructed as described earlier (Husain and Moss, 2001). Briefly, HeLa cells were infected with a vaccinia virus F13L deletion mutant (Blasco and Moss, 1991) and then transfected with either pGF13L-HA\(_{N}\) or pGF13L-HA\(_{C}\). After 3 days of incubation at 37°C, cells were harvested and lysates were used to infect BSC-1 cells. Large recombinant plaques were picked and purified by three rounds of infection of BSC-1 cells. The presence of the correct F13L fragment in the genome of each recombinant virus was confirmed by PCR.

Transfection and infection

Plasmids used for transfection were prepared with the Qiagen plasmid preparation kit. HeLa cells were transfected using Lipofectamine (LF) 2000 (Invitrogen) according to the manufacturer. Briefly, cells were grown on glass coverslips until they were 80% confluent. LF 2000 and DNA were diluted separately in Opti-MEM I medium (Invitrogen), mixed, and incubated at room temperature for 20 min. The complex of LF 2000 and DNA was added to the cells for 5 h at 37°C and replaced with fresh medium with 10% FBS and incubations were continued until 24 h. For infection, virus stocks were diluted to the appropriate multiplicity in culture medium with 2.5% FBS and added to cell monolayers in wells or coverslips. After 2 h of incubation at 37°C, the virus inoculum was replaced with fresh culture medium (2.5% FBS) and incubated for a further 18 to 20 h.

Western blotting

Infected cells were harvested and washed once with phosphate-buffered saline (PBS). Cells were directly resuspended in RIPA buffer (50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 1% Triton X-100; 0.2% SDS; 1% sodium deoxycholate), vortexed briefly, and incubated on ice for 15 min. The lysates were centrifuged at 16,000 g for 15 min at 4°C. The supernatant was collected, reduced, and denatured with SDS and analyzed by electrophoresis on a 12% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and incubated overnight in PBS with 5% milk at 4°C. The membrane was then washed with PBS three times and incubated with anti-HA mAb (HA.11) diluted 1 to 1000 in 5% milk–PBS for 1 h. After washing the membrane four times with PBS-T (PBS containing 0.1% Tween 20) the membrane was incubated as above with horseradish peroxidase conjugated anti-mouse secondary antibody diluted 1 to 2000. The membrane was washed as above and proteins were visualized by Super signal chemiluminescence substrate (Pierce).

Confocal and electron microscopy

Transfected or infected cells were fixed with cold 4% paraformaldehyde in PBS at room temperature for 20 min. Fixed cells were permeabilized for 5 min with PBS containing either 0.2% Triton X-100 at room temperature or 25 µg of digitonin per milliliter at 0°C. Permeabilized cells were incubated with primary antibodies diluted in 10% FBS for 1 h followed by secondary antibody diluted in 10% FBS for 30 min at room temperature. For double staining, cells were stained separately with each antibody. Stained cells were washed extensively with PBS and coverslips were mounted in 20% glycerol. Fluorescence in the cells was examined under a Leica TCS NT inverted confocal microscope and images were overlaid using Adobe Photoshop version 5.0.2.

Procedures for immunostaining and electron microscopy were similar to those previously described (da Fonseca et al., 2000) except immunostaining was carried out prior to fixation or cryosectioning where indicated.

In vitro transcription–translation

Polypeptides were synthesized in vitro using the reticulocyte TNT quick-coupled transcription–translation system (Promega) according to the manufacturer’s protocol. Briefly, 0.5 µg of plasmid and [\(^{35}\)S]methionine (final concentration of 20 µCi/ml) were added to 20 µl of TNT mixture, and the mixture was incubated at 30°C for 90 min. When indicated, 2 µl of canine pancreatic microsomal membranes (Promega) was added. At the conclusion of the reaction, the mixture was layered over 0.5 M sucrose and centrifuged at 16,000 g for 20 min at room temperature. The supernatant at the top was removed and the membrane pellet was resuspended directly in SDS–sample buffer or in NTE buffer (100 mM NaCl; 10 mM Tris–HCl, pH 7.5; 1 mM EDTA). Polypeptides were resolved on a 4 to 20% or 12% polyacrylamide SDS gel and detected by autoradiography.

To investigate posttranslational association of polypeptides with microsomes, protein synthesis was stopped at 60 min by adding 200 µg of cycloheximide per milliliter. After addition of 2 µl of microsomes, the incubation was continued for 30 min.

Pronase and sodium carbonate treatments

Proteins were synthesized in vitro in the presence or absence of microsomes and the latter were purified through a sucrose cushion and resuspended in NTE buffer. Pronase (0.05 units, Sigma) was added to the protein suspension and incubated at 0°C for 30 min. The protease action was stopped by adding 1 µl of protease inhibitor cocktail (Sigma).

For sodium carbonate treatment, proteins were synthesized in vitro in the presence of microsomes. The reaction mixture was centrifuged at 16,000 g for 20 min at room temperature. The membrane pellet was resuspended gently...
in NTE buffer or 0.1 M sodium carbonate, pH 11.0 and incubated at 0°C for 20 min. Membranes were purified through a sucrose cushion as above and the proteins were resolved by SDS–PAGE and detected by autoradiography.

Triton X-114 extraction

Protein was synthesized in vitro with or without microsomes and the reaction mixture was extracted with buffer containing 1% Triton X-114 for 5 min on ice as previously described (Bordier, 1981). Proteins in the resulting aqueous and detergent phases were resolved by SDS–PAGE and detected by autoradiography.

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References


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