

Localization of mouse hepatitis virus open reading frame 1A derived proteins

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We have investigated the intracellular localization of proteolytic cleavage products encoded in the 5' portion of mouse hepatitis virus (MHV) gene 1. Immunofluorescent labeling of cells with an antiserum which recognizes p28, the ORF1a N-terminal cleavage product, resulted in widespread somewhat granular cytoplasmic staining, indicating that this protein is widely distributed in the cytoplasm of MHV-infected, but not control uninfected cells. Immunofluorescent staining of infected cells with antisera which recognize the downstream polypeptides, p65, p240 and p290 labeled discrete vesicular perinuclear structures. Double immunofluorescent labeling of BHK cells expressing the MHV receptor (BHK_{MHVRI}) and infected with MHV-A59 with a Golgi-specific anti-mannosidase II monoclonal antibody and with antiserum recognizing each of these anti-MHV ORF1a polypeptides, showed that the p240 and p290 polypeptides were localized in discrete vesicular structures that overlapped the Golgi complex. Labeling with antibodies specific for p65 colocalized with the Golgi region, and showed staining of the perinuclear cytoplasm as well. Plasmids containing sequences contained in the first 6.75 kb of ORF1a have been expressed using the coupled vaccinia virus-T7 polymerase system. Immunofluorescent labeling of transfectants with the anti-ORF1a antisera showed patterns of antigen distribution similar to those observed in cells infected with MHV-A59. A deletion analysis with constructs containing only portions of the ORF1a sequence indicated that 303 amino acids containing the first papain-like protease domain (PLP-1) was sufficient to associate this protein with the Golgi.

Keywords: coronavirus; proteolytic protein processing; protein trafficking

Introduction

Coronaviruses are widespread in nature, infect many vertebrate hosts and are important veterinary pathogens (Siddell *et al*, 1983). The human coronaviruses are most commonly associated with respiratory infections although there have been several reports associating coronaviruses with multiple sclerosis (Tanaka *et al*, 1976; Burks *et al*, 1980; Murray *et al*, 1992; Talbot *et al*, 1993). The

murine coronavirus mouse hepatitis virus (MHV), causes a broad spectrum of diseases in mice, depending upon the virus used, the route of inoculation, and the age and strain of a mouse (Siddell *et al*, 1983). Two strains of MHV, MHV-A59 and MHV JHM, produce an encephalomyelitis which extensively involves the white matter of the brain with demyelinating lesions of the spinal cord (Weiner, 1973; Lavi *et al*, 1984). Infection with these viruses provides a useful small animal model for multiple sclerosis.

MHV contains a single-stranded positive-sense RNA genome (Spaan *et al*, 1988). One of the initial steps in MHV replication is the translation of gene 1 from the input genomic RNA. Gene 1 encompasses approximately 21.8 kb at the 5' end of the genome

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and contains two overlapping large open reading frames (Lee *et al*, 1989; Bonilla *et al*, 1994). The first open reading frame ORF1a encodes 4469 amino acids. The second open reading frame ORF1b, overlaps ORF1a for 75 nucleotides and is 2771 amino acids long. *In vitro* transcription and translation studies of gene 1 have indicated that these two ORFs are translated by a ribosomal frameshifting mechanism to yield a polyprotein of almost 800 000 kDa which is co-translationally cleaved (Breedendbeek *et al*, 1990). Analysis of the nucleotide sequence and the predicted amino acid sequence revealed that ORF1a contains several likely functional domains: two hydrophobic domains, three cysteine-rich domains, a picornavirus 3C-like protease domain, and two papain-like protease domains (Lee *et al*, 1989; Bonilla *et al*, 1994). The relative positions of these ORF1a domains are depicted schematically in Figure 1. ORF1b is predicted to encode domains required for the MHV RNA-dependent RNA polymerase activ-

ity, including the canonical SDD polymerase motif, a nucleotide binding motif, a metal binding domain, and a helicase domain (Breedendbeek *et al*, 1990).

Several lines of evidence suggest that the gene 1 polyprotein is proteolytically processed into multiple polypeptides: (1) enzymatically active protease domains are encoded by ORF1a (Lee *et al*, 1989; Baker *et al*, 1989, 1993; Bonilla *et al*, 1995; Lu *et al*, 1995); (2) the extremely large size of the predicted gene 1 primary translation product, and (3) genetic evidence that as many as eight separate transacting MHV gene products are needed for RNA synthesis (Leibowitz *et al*, 1982; Stalcup *et al*, 1998). Biochemical studies using antibodies that recognize discrete portions of the protein(s) encoded by gene 1 have been useful tools to elucidate the proteolytic cleavages which process the gene 1 primary translation product (Denison *et al*, 1991, 1992, 1995; Hughes *et al*, 1995; Lu *et al*, 1995). To date, these studies have been most successful for ORF1a. An N-terminal 28 kDa protein, p28, is cotranslationally cleaved from the primary translation product by the first papain-like protease encoded in ORF1a (Denison and Perlman, 1986, 1987; Baker *et al*, 1989; Denison *et al*, 1992). Subsequently, a 65 kDa protein (p65) is cleaved from a large precursor protein and additional discrete polypeptides approximately 290 kDa (p290), 240 kDa (p240), 50 kDa (p50), and p29 kDa (3CLpro) molecular mass are generated by proteases (Denison *et al*, 1992, 1995; Hughes *et al*, 1993; Piñón *et al*, 1997; Lu and Denison, 1997). The presumed relationships of these cleavage products to each other and their positions on the MHV genome are shown in Figure 1.

Enzymes capable of directing MHV RNA synthesis sediment with the membrane fraction of the cytosol (Brayton *et al*, 1982), suggesting that gene 1 encoded polypeptides might localize to a membrane bound cytoplasmic compartment. Therefore, we have studied the intracellular localization of the various protein products of ORF1a. Since the processing pathway for ORF1a gene products is much better understood than that for ORF1b, and we have in hand well characterized antibodies recognizing defined regions encoded by ORF1a, our initial efforts have been restricted to proteins encoded within this portion of the genome. We report that the majority of the p28 protein is not membrane associated but is contained in the soluble fraction of the cytosol. In contrast the downstream polypeptides p240 and p290 were localized in the Golgi region of the cell.

Results

Single color immunofluorescent labeling was performed to determine whether the various MHV ORF1a polypeptide products were differentially

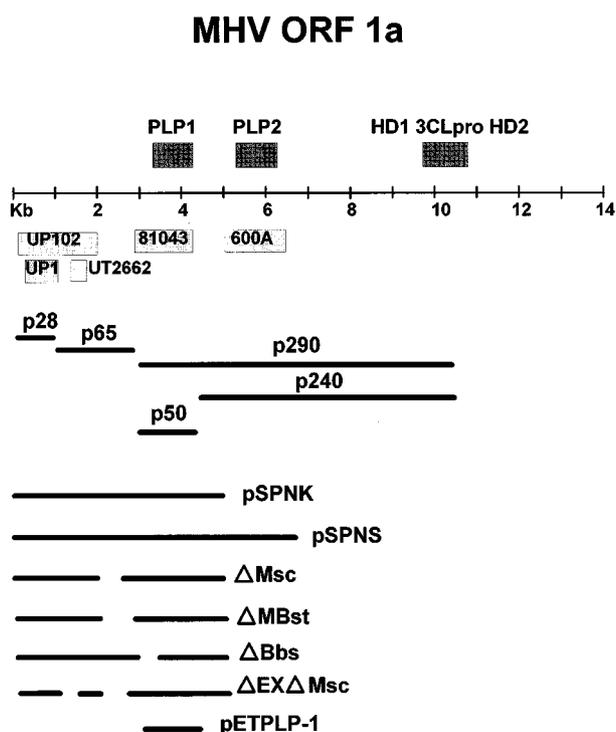


Figure 1 A schematic representation of the locations of predicted functional domains of MHV ORF1a. The positions of the two predicted papain-like protease domains, PLP1 and PLP2; the position of the predicted picornavirus 3C-like protease (3CLpro) domain and its two flanking hydrophobic domains, HD1 and HD2, are indicated by the labeled shaded boxes. The approximate locations of the ORF1a polypeptides used as immunogens and the names of the resulting antisera (UP1, UP102, 81043, 600A) are shown in boxes below the kilobase scale. The approximate lengths and map positions of the MHV ORF1a inserts in the plasmids pSPNK, pSPNS, Δ Msc, Δ MBst, Δ Bbs, Δ EX Δ Msc, and pETPLP-1 are shown.

distributed in MHV-A59-infected L2 cells. Antisera UP1 recognizes the N-terminal ORF1a cleavage product p28 (Figure 1). Immunofluorescent labeling with this antiserum resulted in a diffuse somewhat granular pattern of cytoplasmic staining with focal regions labeling more heavily, indicating that this protein is widely distributed in the cytoplasm of infected cells (Figure 2A). Immunofluorescent labeling of infected cells with antisera UP102, an antisera which recognizes the p65 cleavage product as well as p28, labeled vesicular structures in the perinuclear region in addition to staining the cytoplasm more diffusely (Figure 2B). Immunofluorescent labeling of infected cells with antisera which recognize polypeptides p50, p240 and p290, antisera 81043 (Figure 2C) and 600A (not shown), gave a different pattern, intensely labeling discrete vesicular perinuclear structures in the absence of any other cytoplasmic fluorescence, suggesting that these proteins might be localized to the Golgi.

To investigate this further, two color immunofluorescent labeling was performed using a Golgi-specific anti-mannosidase II monoclonal antibody

(mab) in addition to the various anti-ORF1a antibodies. The mouse anti-Golgi mab 53FC3 labels murine cells poorly, if at all, but clearly labels hamster cells (Burke *et al*, 1982). Therefore we utilized a BHK derived cell line which expresses recombinant MHV receptor, BHK_{MHV_R} (Dveskler *et al*, 1991), for most of the subsequent experiments. BHK_{MHV_R} cells were infected with MHV-A59 or mock-infected and labeled with either the anti-ORF1a antisera UP1, UT2662, UP102, 81043, or 600A in addition to the Golgi-specific 53FC3 mab. Figure 3A shows that most of the anti-p28 labeling (green) did not coincide with the Golgi complex which was labeled with antibody 53FC3 (red); only a modest amount of p28 antigen appeared to colocalize with the Golgi (yellow). Labeling with an anti-p65 serum gave a more complex pattern (Figure 3B). In the majority of infected cells most of the p65 labeling was not restricted to the Golgi, but was present in the peri-nuclear region with some p65 antigen colocalizing to vesicular structures which also labeled with the Golgi-specific 53FC3 mab. For approximately 25% of the cells, p65

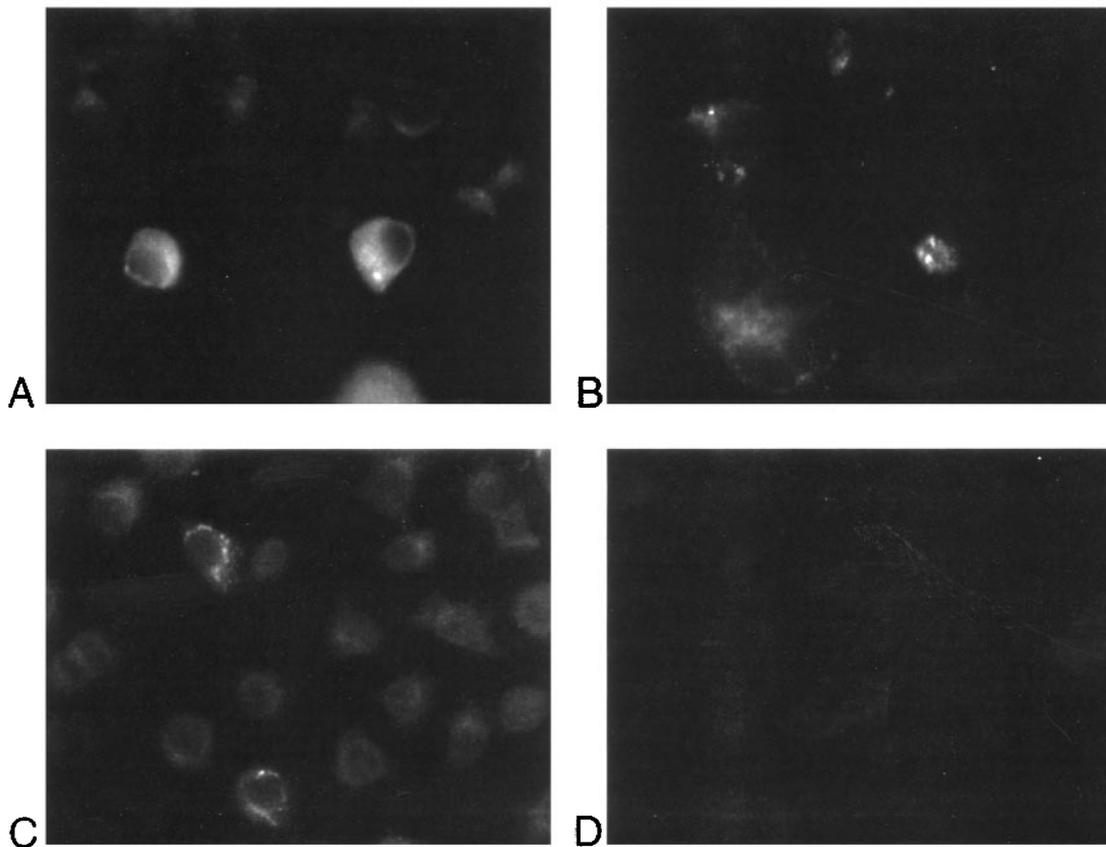


Figure 2 Immunofluorescent labeling of ORF1a products during MHV infection of mouse L2 cells. L-2 cells were infected with MHV-A59 or mock-infected, incubated for 7 h and then fixed with acetone. Infected cells were labeled with either UP1 (A), UP102 (B), or 81043 (C) and FITC-conjugated goat anti-rabbit IgG as described in Materials and methods. Mock infected cells (D) were similarly labeled using UP1 as the primary antibody.

antigen was confined to the Golgi region. Antiserum UP102, which recognizes both p28 and p65, labeled polypeptides which colocalized with the Golgi-

specific anti-mannosidase II antibody, as well as polypeptides which appeared to have an extra-Golgi location (Figure 3C). Virtually all the labeling

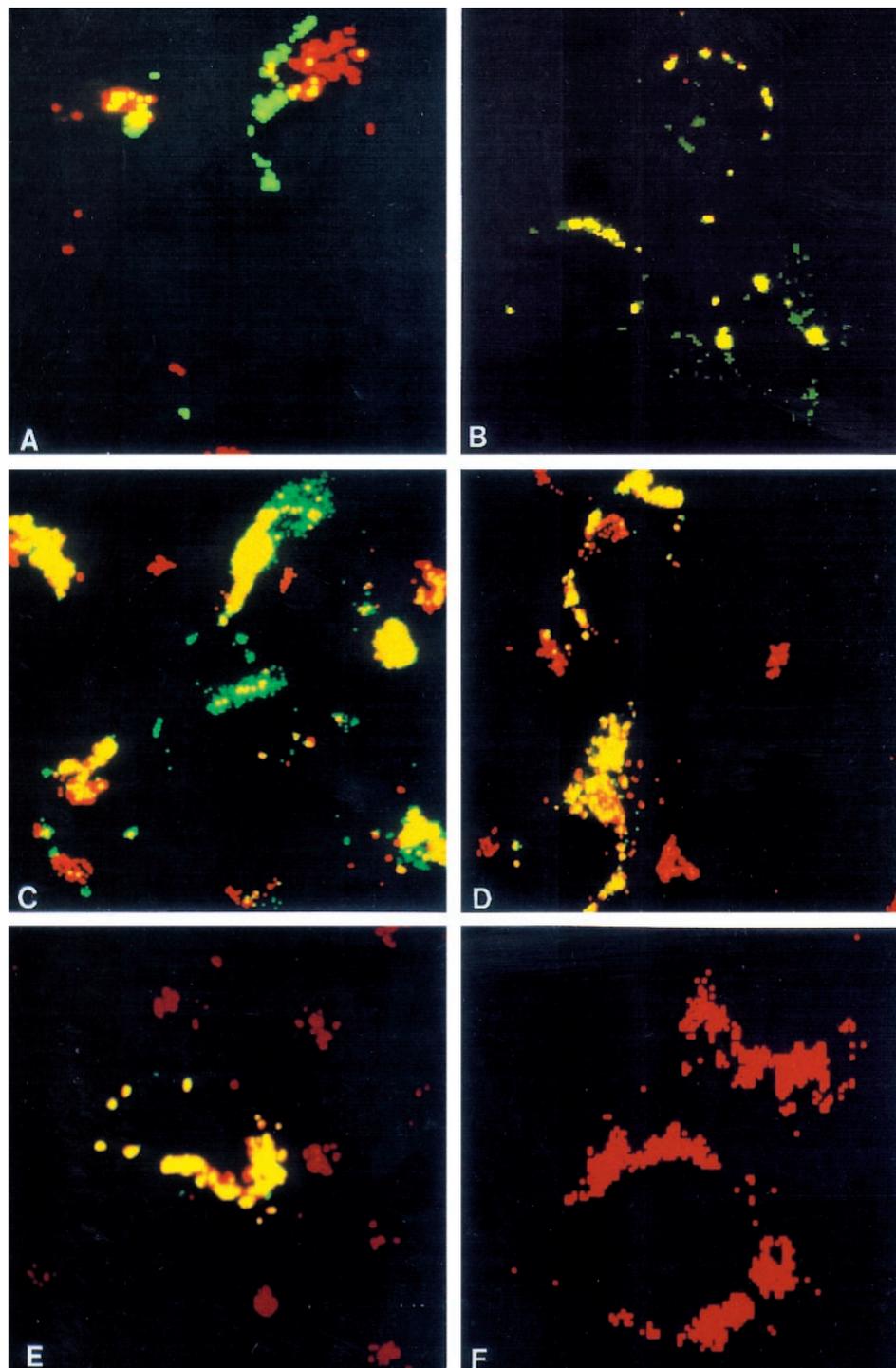


Figure 3 Immunofluorescent localization of ORF1a products in MHV-A59 infected cells. BHK cells expressing recombinant MHV receptor (BHK_{MHVR1}) were infected with MHV-A59 or mock-infected, incubated for 8 h and then fixed with acetone. Infected cells were labeled with the anti-Golgi mab 53FC3 (red) and either UP1 (A), UT2662 (B), UP102 (C), 81043 (D), or 600A (E) as described in Materials and methods. Mock infected cells (F) were stained with 53FC3 and 81043. The coverslips were viewed by confocal microscopy as described in Materials and methods. Labeling with rabbit anti-ORF1a sera (UP1, UT2662, UP102, 600A and 81043) only appears green; coincident labeling with the anti-Golgi mab and rabbit anti-ORF1a antibodies appears yellow.

of p50 and p290 polypeptides by antiserum 81043 (Figure 3D) coincided with the labeling of the Golgi complex by mab 53FC3. Virtually no green labeling was observed, indicating that the vast majority of the polypeptide(s) recognized by 81043 is localized in the Golgi region of the cell. A similar result was obtained with antiserum 600A (Figure 3E), a serum which recognizes p50 and p290. None of the anti-ORF1a sera used in these experiments stained uninfected cells (Figure 3F and data not shown).

Immunoprecipitation studies were done with cytoplasmic extracts which had been fractionated into soluble and membrane-containing fractions

(Figure 4). The efficiency of fractionation of the cytoplasm into soluble and membrane portions was monitored by following the partition of the MHV N (lanes 1 and 2) and M (lanes 3 and 4) proteins, which are known to be free in the cytoplasm (N) or localized to the intermediate compartment and the Golgi (M) (Holmes *et al*, 1981; Collins *et al*, 1982). The p28 protein was precipitated predominantly from the soluble cytoplasmic fraction (lane 5) rather than the membrane pellet (lane 6). Immunoprecipitation experiments with antibodies recognizing the p240 and p290 polypeptides were difficult to interpret,

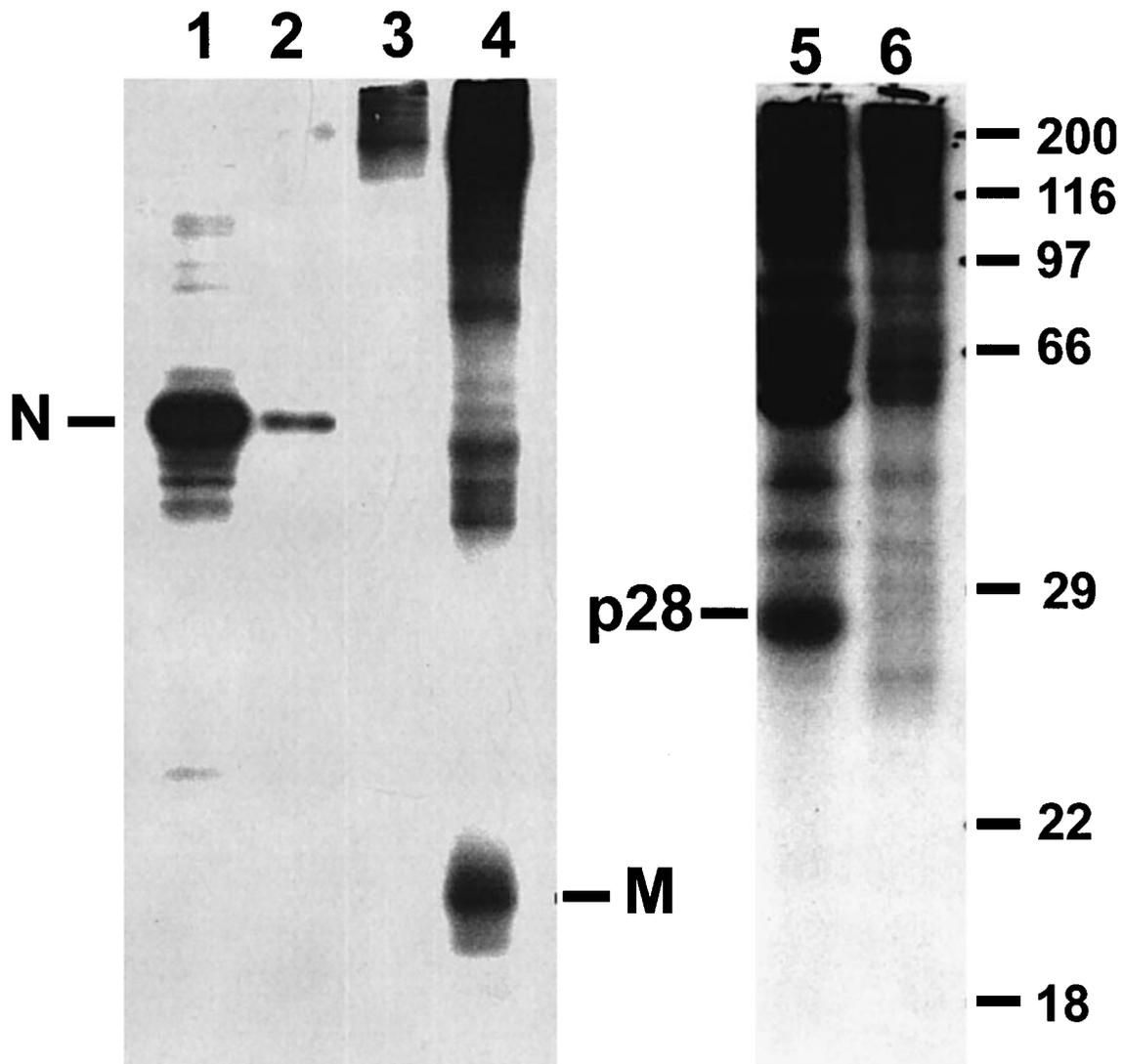


Figure 4 Subcellular fractionation of p28. MHV-infected BHK_{MHV1} cells were metabolically labeled with radioactive methionine and cysteine and cytoplasm prepared and separated into soluble and membrane-containing fractions as described in Materials and methods. Aliquots of the soluble fraction (lanes 1, 3 and 5) and the solubilized membrane-containing pellet fraction (lanes 2, 4 and 6) were immunoprecipitated with either the anti-N monoclonal antibody 1.16.1 (lanes 1 and 2), the anti-M monoclonal antibody J2.7 (lanes 3 and 4), or with the anti-p28 antibody UP1 (lanes 5 and 6). Immunoprecipitated proteins were resolved by electrophoresis in 12% polyacrylamide gels.

due to the precipitation of a smear of high molecular weight proteins from all samples (data not shown).

Inspection of the hydrophobicity plot of the entire predicted ORF1a polypeptide reveals that the only clearly predicted transmembrane domains are far downstream, flanking the picornavirus 3C-like protease domain. The sequences contained within p290 and p240 extend into this region of ORF1a (Figure 1). To investigate if these putative transmembrane domains are required for targeting p290 and p240 for localization in the Golgi region, we have utilized the plasmids pSPNK and pSPNS which contain the first 4482 nucleotides and 6574 nucleotides, respectively, of ORF1a under the control of the T7 promoter. These constructs do not include the two transmembrane domains surrounding the 3C-like protease domain. The polypeptides encoded by both of these constructs contain a functional papain-like protease and cleave p28 from the primary *in vitro* translation products (Bonilla *et al*, 1995; Hughes *et al*, 1995). These two constructs were expressed in BHK_{MHV} cells using the coupled vaccinia virus T7 polymerase system. Cells transfected with pSPNK and infected with vTF7.3 were fixed at 8.5 h post transfection and labeled with antisera 81043 or UP1 in combination with the anti-Golgi mab 53FC3. Since the amino acid sequences recognized by antiserum 600A are not encompassed by this plasmid, this antiserum was not used for this experiment. The labeling patterns observed were very similar to those observed during infection with MHV-A59. Labeling of p28 antigen with the UP1 antiserum demonstrated that this polypeptide did not specifically localize to the Golgi region (Figure 5A) but is more widespread in the cytosol. As shown in Figure 5B, all the staining of pSPNK-expressed p240/p290-related proteins by antisera 81043 coincided with the staining of the Golgi complex by antibody 53FC3. Labeling of p65 with antisera UT2662 yielded a predominantly perinuclear pattern, with most, but not all, of the labeling coincidental with labeling by the Golgi anti-mannosidase II antibody (data not shown). Additional experiments with the plasmid pSPNS produced similar results (not shown). These *in vivo* results showing that spacial dissociation of p28, and presumably proteolytic cleavage accompanying this spacial dissociation, from translation products which contain the first papain-like protease domain but not the second protease domain are consistent with *in vitro* studies showing that p28 cleavage only requires the first papain protease domain (Baker *et al*, 1989; Hughes *et al*, 1995).

The data present above indicated that the predicted transmembrane domains flanking the 3C-like protease domains were not necessary for localization of p290/p240 in the Golgi region of the cell. In an effort to determine the sequences

targeting the polypeptide containing the epitopes recognized by antibody 81043 to the Golgi region of the cell, a series of plasmids containing internal deletions relative to pSPNK were expressed. Trans-

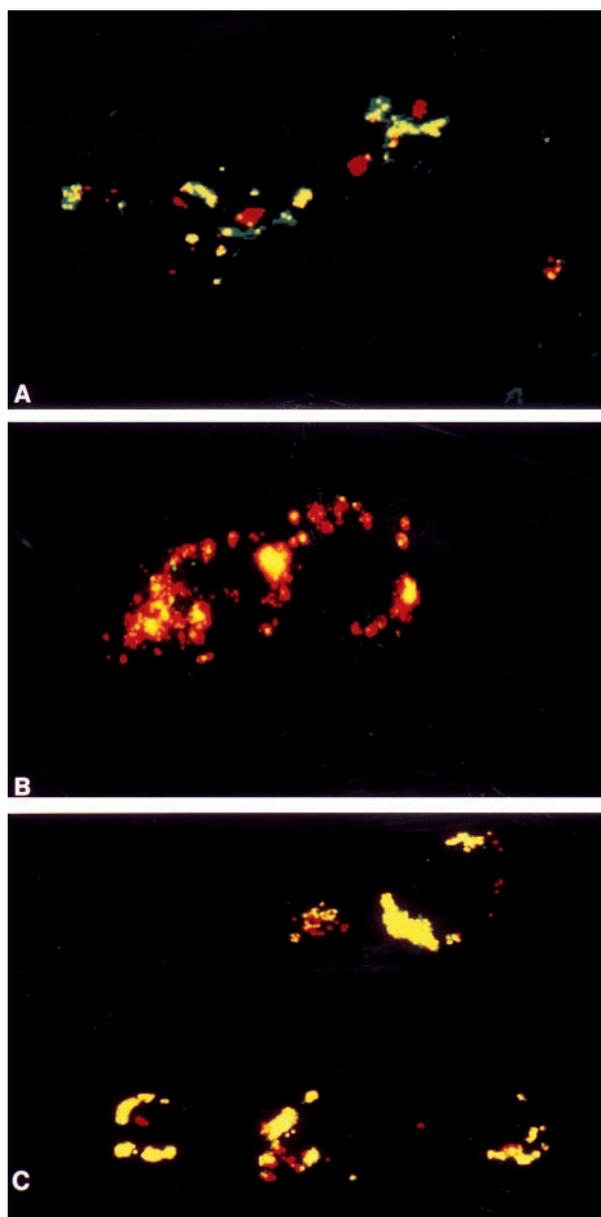


Figure 5 Immunofluorescent localization of ORF1a products in transfected cells. BHK_{MHV} cells were infected with vTF7.3 and transfected with either pSPNK (A and B) or pETPLP-1 (C) as described in Materials and methods. Eight and one half hours after the addition of DNA the coverslips were fixed and subsequently labeled with the anti-Golgi mab 53FC3 (red) and with either the anti-p28 antiserum UP1 (A) or antiserum 81043 (B and C). The coverslips were visualized by confocal microscopy. Labeling with rabbit anti-ORF1a sera (UP1 and 81043) only appears green; coincident labeling with the anti-Golgi mab and rabbit anti-ORF1a antibodies appears yellow.

fection of the plasmids Δ Msc, Δ MBst, Δ Bbs, and Δ EX Δ Msc (Bonilla *et al*, 1995), into vTF7.3 infected cells followed by immunofluorescent labeling with antiserum 81043 and the anti-Golgi mab 53FC3 indicated that the polypeptide segment recognized by 81043 localized to the Golgi region of the cell, in spite of the absence of various upstream polypeptide segments (data not shown). The 81043 antiserum was raised against a prokaryotic fusion protein encompassing the first papain-like domain in ORF1a. To determine if the first papain-like protease domain contained the sequences directing association with the Golgi region, the plasmid pETPLP-1 was expressed in BHK_{MHVRI} cells and the location of this domain determined by double immunofluorescent labeling with antiserum 81043 and mab 53FC3. As shown in Figure 5C, the labeling pattern obtained with antiserum 81043 coincides with that obtained with 53FC3, indicating that the first papain-like protease domain encoded by pPLP-1 contains sequences directing its association with the Golgi region of the cell.

During infection with MHV the bulk of the MHV M protein is retained in the pre-Golgi intermediate compartment (Tooze *et al*, 1984; Krijnse-Locker *et al*, 1994) where virus budding takes place. To investigate the likely possibility that the site of budding and the location of ORF1a proteins are different, double immunofluorescent labeling was performed using the anti-M protein monoclonal antibody (mab) J.2.7 (Fleming *et al*, 1989), generously provided by Dr John Fleming, and the anti-ORF1a antibody 81043. As shown in Figure 6, these two

antibodies produce patterns of staining which only overlap to a limited extent. This result is consistent with our localization of the papain-like protease domain to the Golgi in Figures 3 and 5, and the accumulation of the majority of the M protein in the intermediate compartment.

Discussion

To better understand the functions of the MHV ORF1a encoded proteins we have initiated a series of experiments to determine their intracellular location. As reported here, p28 is more widely distributed than p290/240, as judged by immunofluorescent labeling with the UP1 antibody, and p28 partitions into the soluble fraction of the cytosol, suggesting that it is not membrane bound like the other ORF1a proteins. This was true in MHV-A59 infected L-2 and BHK_{MHVRI} cells and in BHK_{MHVRI} cells transfected with pSPNS, pSPNK, and pETPLP-1, plasmids which each contain a single papain-like protease domain (PLP1 in Figure 1). This is the first *in vivo* experiment which indicates that the first papain-like protease, rather than the second papain-like protease domain or the downstream 3C-like protease, cleaves p28 from the ORF1a primary translation product. Cell-free translation studies utilizing many of the same constructs employed in this work previously demonstrated that the first papain-like protease domain cleaves p28 from the ORF1a primary translation product *in vitro* (Bonilla *et al*, 1995). Thus there is good agreement between the results obtained *in vivo* and *in vitro*.

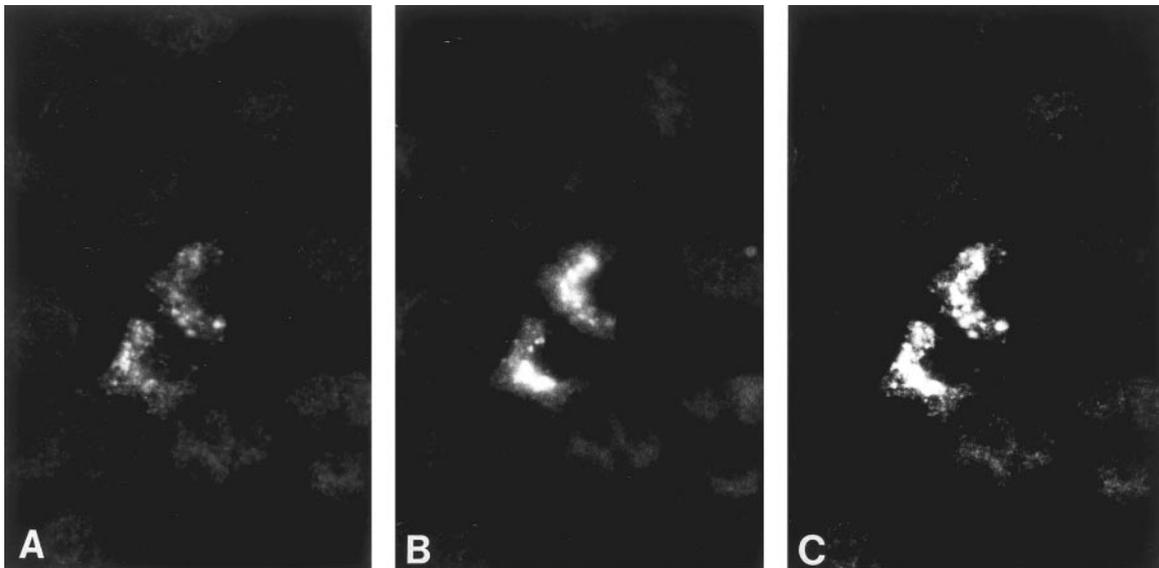


Figure 6 Double immunofluorescent labeling of ORF1a products and M protein in MHV-A59 infected cells. L2 cells were infected with MHV-A59 and fixed with acetone at 6 h post infection. Cells were doubly labeled with a mixture of rabbit anti-ORF1a antibody 81043 and anti-M protein monoclonal antibody J.2.7 as primary labels, and a mixture of FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG as secondary labels. Antibody 81043 labeling is shown in (A). Antibody J.2.7 labeling is shown in (B). Simultaneous labeling with both antibodies is shown in (C).

We have demonstrated by double immunofluorescent labeling that several ORF1a polypeptides colocalize to the Golgi region of the cell. Both the 600A and 81043 antisera recognize the p290 and p240 ORF1a cleavage products and label the Golgi region of infected cells. The downstream putative hydrophobic transmembrane domains M1 and M2 (Figure 1) cannot be responsible for the association of p240/p290 with the Golgi, since plasmids lacking the coding sequences for these domains, pSPNK and pSPNS, directed the polypeptide product recognized by the 81043 antibody to the Golgi region of the cell. Further expression studies with the plasmid pETPLP-1 indicated that the first papain-like protease domain by itself contains sequences capable of directing the association of p240/p290 with the Golgi. Since no clear hydrophobic domain is contained within this region, it is likely that the protein is bound to Golgi membranes by protein-protein interactions. Further experiments are needed to more precisely determine the mechanism targeting these proteins to the Golgi.

The distribution of labeling for the epitopes recognized by our anti-p65 antibody was more complex. About 25% of MHV-A59 infected cells showed a substantial degree of colocalization with the Golgi marker anti-mannosidase II. The remainder of the cells gave a less discrete perinuclear pattern of staining. We do not have a firm explanation for this cell to cell variation in the distribution of p65, although part of this variation could be due to the slower liberation of p65 from its precursor as compared to p28 (Denison *et al*, 1995). This precursor is thought to contain the first papain-like protease domain which we have shown associates with the Golgi when expressed in isolation, and could account for the staining pattern we observed. If this is so, than the labeling of the Golgi with UT2662 could reflect the localization of the p65 precursor polypeptide.

The differential distribution of the ORF1a proteins p28 and p240/290 allows several conclusions to be drawn. Although the function of p28 remains unknown, it is likely that the site of action for this protein is in the cytosol, distinct from the location of the PLP1-containing polypeptides which are confined to the Golgi region of the cell. Second, the localization of the p240 and p290 polypeptides containing the papain-like protease domains to the Golgi region of the cell suggests that the processing of the ORF1a translation products occurs in or near this cellular compartment. The observation that replication and transcriptionally active RNAs and RNA dependent RNA polymerase activity are associated with cell membranes (Brayton *et al*, 1982) raises the possibility that the polypeptides involved in MHV RNA replication and transcription could also be specifically localized to the Golgi region.

The intracellular localization of non-virion proteins thought to be required for MHV RNA synthesis to a membrane containing intracellular structure, is similar in some respects to observations made with other plus-stranded RNA viruses. Equine arteritis virus (EAV), an arterivirus, and MHV, a coronavirus, are both members of the nidovirus family, a classification based on their similar replication strategies and genetic organizations, particularly in the organization of ORF1a and ORF1b which contain genes required for viral RNA transcription and replication (den Boon *et al*, 1991). Recently Van Dinten *et al* (1996) have shown that during infection with EAV proteins containing the EAV polymerase and helicase domains are localized in the perinuclear region of the cell. They have also demonstrated an association of ORF1a derived polypeptides in a complex with these ORF1b derived proteins and have hypothesized the formation of a membrane bound replicase complex consisting of ORF1a and ORF1b derived proteins. Our data is not completely consistent with extending this concept to the coronaviruses. We have demonstrated that MHV ORF1a PLP1 containing polypeptides colocalize with the Golgi, an observation which is consistent with the underlying similarity of the arterivirus and coronavirus systems and the homology of the PLP1 domain encoded by the two virus groups. However, the MHV p28 and p65 polypeptides are not confined to the Golgi and are inconsistent with a model in which all of the ORF1a and ORF1b proteins are located in a replicase complex. There are no obvious homologs to the MHV p28 and p65 polypeptides in the arterivirus genome, thus our results for these proteins may not apply to the arteriviruses.

Cells infected with either poliovirus or Sindbis contain *de novo* synthesized vesicular structures containing polypeptides which are components of the viral RNA biosynthetic machinery (Bienz *et al*, 1992; Miller and Brown, 1993). For poliovirus it is clear that these structures are sites of RNA replication (Takeda *et al*, 1986; Bienz *et al*, 1992). In this respect our results differ somewhat from those obtained with these two viruses, in that the MHV proteins we have studied localized to a pre-existing structure, namely the Golgi complex. However, we have not yet demonstrated the intracellular location of MHV replication complexes. During infection with MHV the assembly of viral nucleocapsids and virus budding takes place primarily in smooth-walled vesicles transitional between the Golgi and ER (Tooze *et al*, 1984), and it has been suggested that RNA synthesis might be localized to this compartment (Dubois-Dalcq *et al*, 1984). Future studies with antibodies which recognize downstream ORF1b domains predicted to be components of the MHV RNA replication and transcription machinery

coupled with *in vitro* biochemical studies of MHV transcription are needed to better address these issues.

Materials and methods

Cells and virus

The origin and growth of L-2 cells and BHK cells (BHK_{MHV}) expressing recombinant MHV receptor have been described (Leibowitz *et al.*, 1981; Dveksler *et al.*, 1991). The origin and growth of MHV-A59 has been described (Leibowitz *et al.*, 1981). The recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase, vTF7.3 was kindly provided by Dr Bernard Moss (Fuerst *et al.*, 1987).

Antibodies

Monoclonal antibodies J2.7 and 1.16.1 which recognize the membrane (M) protein and the nucleocapsid (N) protein, respectively, have been described previously (Fleming *et al.*, 1989; Denison *et al.*, 1991).

Most of the polyclonal antibodies used in this study (UP1, UP102, 600A, 81043) have been described previously (Denison *et al.*, 1992, 1995; Hughes *et al.*, 1993). These antisera were raised in rabbits using prokaryotic/viral fusion proteins as immunogens. An antibody recognizing p65, the ORF1a cleavage product immediately downstream from the N-terminal p28 cleavage product, was raised by immunizing a goat with a purified polyhistidine-tagged polypeptide expressed in *E. coli*. A cDNA fragment encoding a portion of the p65 polypeptide (nucleotides 1376–1774, Bonilla *et al.*, 1994; Denison *et al.*, 1995) was amplified by PCR using an upstream primer, CCGGATCCTTGT-TGTGGTGACACCTGCG, which introduced a *Bam*HI site onto the 5' end of the fragment, and a downstream primer, AACAAAGTGGCTTCGCTGC. The amplified cDNA fragment was digested with *Xho*I, filled in with Klenow, digested with *Bam*HI and the resulting 399 nucleotide fragment cloned into the plasmid pET15b (Novagen) utilizing a *Bam*HI site and a *Bpu*1 102I site which had been filled in with Klenow (Rosenberg *et al.*, 1987; Studier *et al.*, 1990). The resulting plasmid, pET15b-p65, contained 132 amino acids of p65 fused to an

upstream polyhistidine sequence via a vector-derived synthetic sequence containing a thrombin cleavage site, all under the control of the T7 promoter. The plasmid carrying this construct was transferred to the inducible T7 polymerase expressing strain, BL21(DE3). The histidine-tagged polypeptide was induced with 1 mM IPTG and partially purified by affinity chromatography over a nickel-containing resin using the directions supplied by the vendor (Novagen). Further purification of the histidine-tagged p65 fragment was achieved by preparative SDS-PAGE. Antibodies to p65 were prepared by Bethyl Laboratories, Inc. (Montgomery, TX, USA). A goat was immunized once subcutaneously with 100 µg of purified protein emulsified in complete Freund's adjuvant and boosted six times at biweekly intervals with the same amount of antigen in incomplete Freund's adjuvant. The resulting antiserum was designated UT2662. The reactivity of UT2662 with p65 was verified by immunoprecipitation of coupled *in vitro* transcription/translation reactions programmed with pET15b-p65 as template.

The locations of the viral sequences encoding the various polypeptides used to raise these antibodies, as well as the locations of the proteins recognized by these antibodies, are summarized in Table 1 and depicted schematically in Figure 1. The Golgi-specific anti-mannosidase II monoclonal antibody 53FC3 was purchased from the Berkeley Antibody Company (Burke *et al.*, 1982). This antibody labels hamster but not mouse Golgi complex. Fluorescein-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG were purchased from Jackson Research.

Plasmids and transfections

Plasmids pSPNK and pSPNS are derived from cDNAs and contain MHV-A59 gene 1 sequences from the *Nar*I site at nucleotide 182 to the *Kpn*I site at nucleotide 4664 and the *Spe*I site at nucleotide 6756, respectively (Bonilla *et al.*, 1994; 1995; 1997). In both cases the MHV-A59 cDNA is oriented in the plasmid pSP72 such that the coding sequences are under the control of the T7 promoter. pSPNK contains the coding sequence for p28, p65, and the first papain-like domain of MHV-A59; pSPNS extends past the second papain-like domain (Figure

Table 1 Antisera used for immunofluorescent localization of A59 ORF1a products.

Antisera	Size of viral sequences used as immunogen (aa)	cDNA for fusion proteins spans nucleotides	Polypeptides immunoprecipitated from infected cells
UP1	228	461–1144	p28
UP102	601	182–1984	p28, p65
UT2662	132	1376–1774	p65
81043	363	2879–3968	p290, p240
600A	495	5000–6486	p290, p240

1). The plasmids Δ Msc, Δ MBst, Δ Bbs, and Δ EX Δ Msc are derivatives of pSPNK containing a series of in-frame deletions between the p28 cleavage site and the first papain-like domain (Bonilla *et al*, 1995). The plasmid pETPLP-1 contains ORF1a sequences from nucleotides 3393–4301. This sequence was amplified by PCR using primers which introduced an *Nde*I site upstream of the ORF1a sequence and an *Xba*I site downstream. The *Nde*I site provides an initiation codon for protein synthesis. The amplified DNA was then cloned into the *Nde*I and *Xba*I sites of the vector pHB40P. PHB40P is a derivative of pET3a and contains *Kpn*I, *Xba*I, and *Xho*I sites inserted between the *Bgl*II and *Bam*HI sites in the parent vector (Rosenberg *et al*, 1987; Studier *et al*, 1990).

For transfection experiments, 60 mm dishes containing sterile coverslips were seeded with 2.2×10^6 BHK_{MHV} cells and incubated overnight. BHK_{MHV} cells were used rather than murine cells to allow subsequent immuno-labeling with the Golgi-specific 53FC3 monoclonal antibody. The cells were then infected at a MOI=5 with recombinant vaccinia virus expressing T7 polymerase, vTF7.3. The cultures were incubated at 37°C for 2.5 h and then transfected with 5 μ g of plasmid using lipofectin (Life Technologies) according to the manufacturer's instructions. After further incubation for 2.5 h the transfection medium was replaced with fresh DME, and the cells were incubated for a further 6 h, at which time they were washed with PBS and fixed with cold acetone.

Indirect immunofluorescence

L-2 cells and BHK_{MHV} cells were grown for 2 days on glass coverslips or chamber slides and infected with MHV-A59 at a calculated MOI of 0.3 or mock-infected. At 6–7 h post-infection (L-2 cells), or at 8 h post-infection (BHK_{MHV} cells), cells were fixed for 5 min with cold acetone. For transfection experiments, BHK_{MHV} cells were similarly fixed 8.5 h after transfection. The fixed coverslips were washed twice with phosphate buffered saline, pH 7.4 (PBS) containing 0.2% Tween 20 and treated with 10% non-fat milk in PBS overnight at 4°C to block non-specific staining. The coverslips were then washed 2–3 times with PBS containing 0.2% Tween 20, and subsequently incubated on ice with rabbit anti-ORF1a antibody (1:100 dilution in PBS containing 3% BSA) for 90 min. Following incubation with primary antibody, the coverslips were washed extensively with PBS containing 0.2% Tween 20, incubated for 60 min with FITC-conjugated goat anti-rabbit IgG (1:100 dilution), and washed five times with PBS containing 0.2% Tween 20. Coverslips were mounted and examined by fluorescent microscopy. For double labeling experiments, a mixture of rabbit anti-ORF1a antibodies (diluted 1:100) and mouse monoclonal anti-mannosidase II anti-

body (diluted 1:600) was used as a primary label, and a mixture of FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG was used in a secondary labeling step. The cells were mounted in elvanol mounting solution for examination with a Multi-probe 2001, CLSM confocal microscope (Molecular Dynamics). The CLSM produces simultaneous dual channel images. Colocalization of the two chromophores was revealed by the yellow color resulting from their overlapping emission.

Immunoprecipitation and cell fractionation

Confluent monolayers of L2 cells in 35 mm wells were infected with A59 at an MOI of 10. The infected monolayers were incubated with DME containing one tenth of the normal amount of methionine and cysteine and supplemented with 2% FBS. At 2 h post infection actinomycin D was added to the medium to a concentration of 10 μ g/ml. At 7 h post infection, a time when approximately 50% of the cells were fused to form large syncytia, monolayers were washed with methionine-free and cysteine-free DME, and labeled with 250 μ Ci of [³⁵S]-methionine and [³⁵S]-cysteine in 0.5 ml of the same medium for 2 h. Monolayers were harvested and fractionated essentially as described previously (Zoltick *et al*, 1990). After labeling, the monolayers were washed five times with buffer A (10 mM Tris, pH 7.4, 150 mM NaCl, 80 μ g/ml PMSF), scraped into 1 ml of the same buffer, and collected by centrifugation for 5 min at 500 g. The cells were resuspended in 0.3 M sucrose, held on ice for 10 min, and disrupted with 15 strokes of a Dounce homogenizer. As judged by phase microscopy, more than 90% of the cells were broken by this procedure. Nuclei and unbroken cells were removed by centrifugation at 800 g for 8 min. The crude post-nuclear extract was fractionated into soluble and membrane-containing fractions by centrifugation at 13 000 g for 20 min. The membrane-containing pellet was solubilized in 100 μ l of Buffer B (10 mM Tris, pH 7.4; 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). An equal volume of 2 \times Buffer B was added to the soluble fraction of the post-nuclear supernate.

Soluble and membrane-containing samples representing approximately equal numbers of cells (25% of a 35 mm well) were used for each immunoprecipitation reaction. Immunoprecipitation was performed as described previously (Yu *et al*, 1994). When monoclonal antibodies were used for immunoprecipitation, Protein A beads were precoated with goat anti-mouse IgG. Immunoprecipitates were resolved by SDS-PAGE on 12% polyacrylamide gels and the immunoprecipitated proteins detected by fluorography.

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