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Membrane and Phospholipid Binding by Murine Coronaviral Nucleocapsid N Protein

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Evidence is presented which indicates that membrane binding of the murine coronavirus, mouse hepatitis virus (MHV) nucleocapsid (N) protein is mediated by certain lipids. Binding of N protein to membranes of mouse fibroblast L-2 cells is very specific and occurs under conditions in which no other viral or cellular proteins show detectable binding. Binding occurs with both free and nucleocapsid-associated N protein, arguing for membrane-binding sites on the N protein itself, rather than on RNA. Binding of N protein also occurs to membranes in the absence of MHV matrix (M) protein which is known to interact with the N protein. Both non-viral, single-stranded RNA and DNA inhibit membrane binding of N protein. In addition, purified phospholipid liposomes compete against N protein binding to membranes. Of various phospholipids tested, cardiolipin was the most effective in inhibiting membrane binding. The N protein was also shown to bind directly to phospholipid liposomes containing cardiolipin as well as to liposomes containing total lipids extracted from mouse L-2 cells. Because of certain structural similarities between phospholipids and nucleic acids, we speculate that membrane lipid association of the N protein may compete for RNA binding sites on the N protein. Such a mechanism may be important for processes such as nucleocapsid uncoating and nucleocapsid assembly. Most interestingly the properties of phospholipid and nucleic acid binding are markedly similar to those recently described for the bacterial DNA-binding proteins DnaA and recA.

INTRODUCTION

The nucleocapsid N protein of murine and probably other coronaviruses plays a multifunctional role in virus replication. The N protein of mouse hepatitis virus (MHV) strain A59 is a highly basic protein of 454 amino acids (Armstrong *et al.*, 1983; Skinner and Siddell, 1983). It binds RNA (Robbins *et al.*, 1986), preferentially at a site within the 5′-leader sequence of viral genomic and subgenomic RNAs (Baric *et al.*, 1988) and may play a role in viral transcription as indicated by studies of transcriptional inhibition using anti-N monoclonal antibodies (Compton *et al.*, 1987).

MHV N protein is first synthesized on soluble polysomes but rapidly becomes membrane-associated concomitant with phosphorylation (Stohlman *et al.*, 1983). The identity of the intracellular membrane(s) involved in N protein binding remains unknown. Since N protein binds with the small membrane protein M (Sturman *et al.*, 1980) and the M protein is associated with smooth intracellular membranes including transitional elements of the Golgi apparatus (Rottier and Rose, 1987), it is possible that the association of phosphorylated N protein with intracellular membranes is mediated via the M protein.

We show here that MHV N protein is able to bind cell membranes in the absence of M protein. Moreover, membrane binding of N protein is inhibited by single-stranded RNA or DNA, indicating possible relevance of the membrane-binding phenomenon in nucleocapsid assembly or disassembly. We also present evidence that membrane binding of MHV N protein may be mediated by specific host cell phospholipids and demonstrate direct binding of N protein to phospholipid liposomes. The MHV N protein thus shows properties markedly similar to certain bacterial DNA binding proteins such as DnaA (Sekimizu and Kornberg, 1988) and recA (Krishna and van de Sande, 1990) which also bind nucleic acid as well as membrane phospholipids.

MATERIALS AND METHODS

Cells and virus

The A59 strain of MHV (Manaker *et al.*, 1961) was used throughout this study. Cells used were mouse fibroblast L-2 (Rothfels *et al.*, 1959), LM (Merchant and Hellman, 1962), LMK (Kit *et al.*, 1963), rat astrocytoma C6 (Benda *et al.*, 1968), bovine kidney MDBK (Madin and Darby, 1958), human epithelial carcinoma HeLa (Gey *et al.*, 1952), human epidermoid carcinoma Hep2 (Moore *et al.*, 1955), and human diploid lung MRC5 (Jacobs *et al.*, 1970) cells. All cells were cultured as monolayers in minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS). For infection of L-2 cells, monolayer cultures were inoculated with MHV at a multiplicity of infection of 5, adsorbed 1 hr at 4°, and incubated at 37° in MEM with 5% FCS.

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Lipid extraction of L-2 cells

Monolayer cultures of L-2 cells were harvested by scraping and spun into pellets (2 min at 600 *g*). Pellets were extracted with chloroform/methanol according to Bligh and Dyer (1959) and the lipid extracts freed from non-lipid contaminants by passing through a small column of Biosil A (Bio-Rad) in chloroform/methanol (1:1, v/v).

Liposomes

Phospholipids were obtained from Sigma: cardiolipin (C1649), phosphatidylcholine (P1013), phosphatidylethanolamine (P0510), phosphatidylserine (P6641), phosphatidylinositol (P2517). Liposomes were prepared in phosphate-buffered saline (PBS) containing bovine serum albumin (1 mg/ml) by sonication (Reeves and Dowben, 1969). Final lipid concentrations were 5 mg/ml. Mixtures of lipids were always prepared in chloroform, prior to drying down and sonication as described.

Pulse-chase radiolabeling and cell fractionation

MHV-infected cells (60-mm plates) were pulse-labeled with [^{35}S]methionine (100 μCi/ml) in methionine-free MEM for 10 min at 6.5 hr postinfection and subsequently chased for 15 min with excess unlabeled methionine. Cells were scraped and centrifuged into pellets, swollen in reticulocyte standard buffer (RSB: 10 m*M* Tris, pH 7.4, 10 m*M* NaCl, 1.5 m*M* $MgCl_2$), and dounce-homogenized. Nuclei were removed by low-speed centrifugation (2 min at 1000 *g*) and the supernatant was applied to 38-ml gradients of 15–55% (w/w) sucrose in 10 m*M* Tris, pH 7, 100 m*M* NaCl. After centrifugation for 14 hr at 20,000 rpm in a Beckman SW28 swinging bucket rotor, gradients were pierced from the bottom and drop-collected in individual fractions. Aliquots were removed from each fraction for scintillation counting of acid-insoluble radioactivity and for SDS–PAGE fluorography.

Binding studies

A high-speed supernatant fraction from Triton X-100-solubilized cells was prepared according to the method of Lee *et al.* (1981). MHV-infected L-2 cells, labeled with [^{35}S]methionine, were solubilized (15 min at 0°) with 1% Triton X-100 in RSB containing 0.2 m*M* PMSF; the mixture was shaken overnight with SM-2 Biobeads (Bio-Rad) to remove the Triton X-100, mixed with an equal volume of 2× MEM, clarified by low-speed centrifugation, and finally by ultracentrifugation (1 hr at 45k rpm in Sorvall TST 60.4 swinging bucket rotor). The 45k supernatant was added to monolayer cultures of cells. Liposomes or solutions of RNA (yeast transfer RNA, type X1, Cat. R-6750 from Sigma) or heat-denatured, snap-cooled DNA (calf thymus DNA, type 1, Cat. D-1501 from Sigma) were added as required. After incubating for 1 hr at 4°, unbound material was removed by washing with PBS, and cell monolayers were solubilized and subjected to SDS–PAGE and fluorography.

For liposome binding, mixtures of liposomes and 45k supernatant were incubated for 1 hr at 4° and overlaid onto 4 ml 5–15% (w/w) sucrose gradients in 10 m*M* Tris, pH 7, 100 m*M* NaCl. Gradients were centrifuged for 20 hr at 45k rpm in a Sorvall TST60.4 swinging bucket rotor. Gradients were fractionated from the bottom and aliquots from individual fractions analyzed by SDS–PAGE fluorography.

In vitro translation

Aliquots of 10 μg total RNA from either uninfected or MHV-infected L-2 cells were translated *in vitro* using a wheat germ extract-derived *in vitro* translation kit (Promega) according to the manufacturer's instructions and in the presence of [^{35}S]methionine. Aliquots of the translation mixture were added to L-2 cell monolayer cultures for assays of membrane binding as described above.

Sucrose gradient centrifugation of MHV nucleocapsids

MHV-infected L-2 cells, labeled with [^{35}S]methionine, were lysed hypotonically and fractionated on sucrose gradients according to the method of Mohandas and Dales (1991) with the exception that 5–25% (w/w) sucrose gradients were employed. Gradients were pierced from the bottom and the contents allowed to drain by gravity into individual fraction tubes. Aliquots of each gradient were removed for SDS–PAGE fluorography and cell-binding assays.

RESULTS

Association of newly synthesized N protein with light membranes

Newly synthesized MHV N protein becomes rapidly associated with intracellular membranes (Stohlman *et al.*, 1983). We show here that MHV N protein binds predominantly to a class of membrane distinct from that involved in the synthesis of the MHV glycoproteins S and M. Both S and M proteins are initially synthesized on endoplasmic reticular membranes (Niemann *et al.*, 1982). We performed cell fractionation studies on MHV-infected cells labeled with [^{35}S]methionine in an effort to distinguish sites of viral protein synthesis and maturation. As shown in Fig. 1A, fractionation of MHV-infected cell extracts yielded two major membrane fractions (gradient fractions 7 and 11). The heavy membrane fraction (gradient fraction 7) undoubtedly

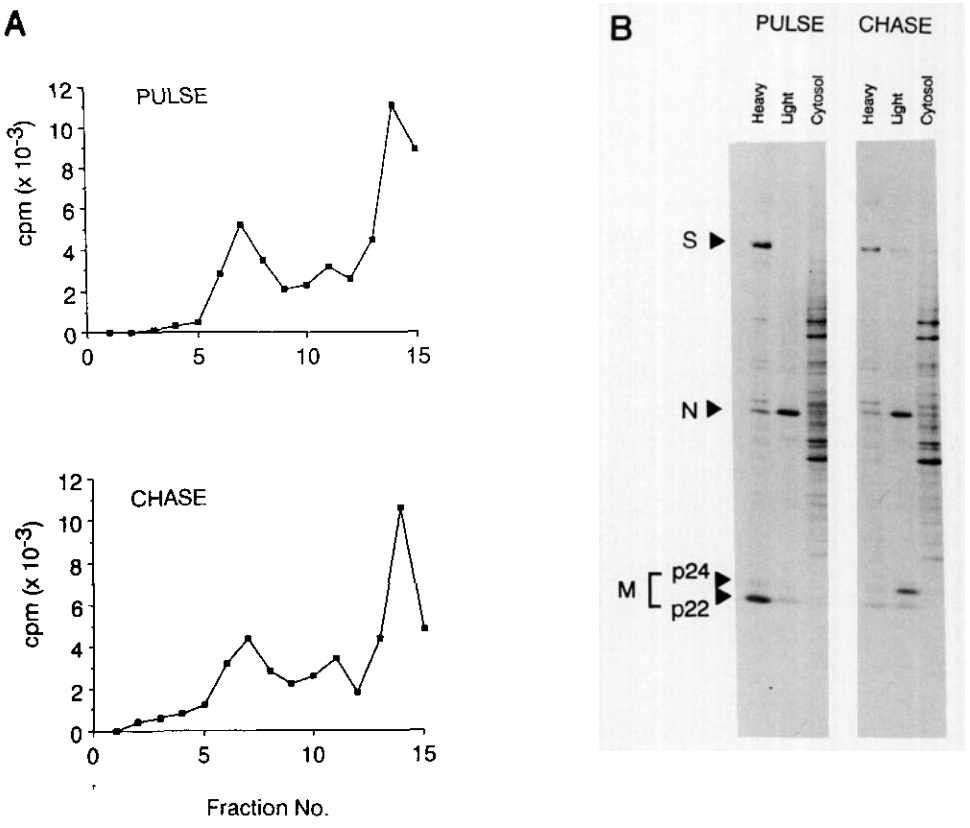

FIG. 1. Association of newly synthesized intracellular N protein with a light membrane fraction. MHV-infected L-2 cells were pulse-labeled for 10 min with [^{35}S]methionine and subsequently chased for 15 min with an excess of unlabeled methionine. Cell extracts were resolved on 15–55% (w/w) sucrose gradients which were fraction-collected and aliquots assayed for acid-insoluble radioactivity (A). Aliquots taken from fractions designated in (A), i.e., heavy (fraction 7; banding at ca 40% sucrose), light (fraction 11; banding at ca 20% sucrose) and a cytosoluble fraction were analyzed by SDS–PAGE followed by fluorography (B). The positions of the viral structural proteins S, N, and M are shown.

contains elements of the rough endoplasmic reticulum, as indicated by its greater density and the fact that newly synthesized, pulse-labeled membrane glycoproteins S and M are associated exclusively with this fraction (Fig. 1B). In contrast the light membrane fraction (gradient fraction 11) contains the bulk of newly synthesized, pulse-labeled N protein (Fig. 1B). With subsequent chase of the [^{35}S]methionine label, some of the S protein and most of the M protein are found in the light membrane fraction. Note that the M protein is now found in its glycosylated (p24) form in contrast to its unglycosylated precursor (p22). Glycosylation of the M protein occurs in smooth endoplasmic reticular membranes (Niemann, *et al.*, 1982) indicating the presence of such membranes within the light membrane fraction designated in Fig. 1. These results show that viral N protein binds to a class of intracellular membranes which is distinct from the rough endoplasmic reticulum on which the viral glycoproteins are first synthesized.

Cell binding of MHV N protein

The above finding of N protein association with a light membrane fraction prompted an investigation of binding to specific membrane surfaces. Surprisingly, we found that intact cells of a variety of sources bound MHV N protein to their outer surface. Triton X-100-solubilized extracts from [^{35}S]methionine-labeled, mock- and MHV-infected L-2 cells were freed of detergent by treatment with Biobeads and added to various cell monolayer cultures. After allowing binding for 1 hr at 4°, cultures were washed, solubilized, and analyzed for bound radiolabeled proteins by fluorographic SDS-PAGE. As shown in Fig. 2, all cell lines tested bound N protein under conditions in which no binding of any other protein of either cellular or viral origin was observed.

The ability of a given cell to bind N protein did not correlate with susceptibility to MHV infection. For example, MHV replicates in L-2, LM and LMK cells but not in C6, MDBK, Hep2, HeLa or MRC5 cells. However, all these cells bound N protein (Fig. 2), indicating the presence of a common surface membrane component able to specifically bind the N protein.

Parenthetically, no binding of S protein was observed to any cell line examined under the conditions of this assay. The S protein has been identified as the cell receptor binding protein (Collins *et al.*, 1982) and is

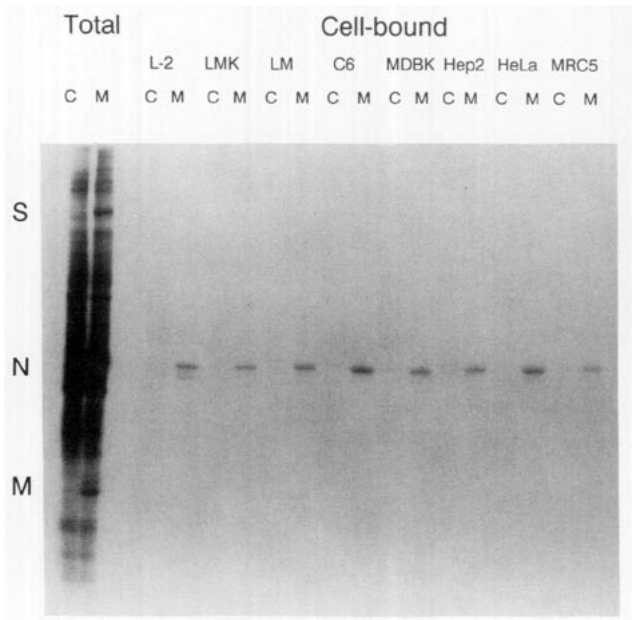

FIG. 2. Binding of MHV N protein to whole cells. Solubilized cell extracts (100,000 *g* supernatant) from [^{35}S]methionine-labeled, mock-control (C), or MHV-infected (M) L-2 cells were applied to monolayer cultures of L-2, LMK, LM, C6, MDBK, Hep2, HeLa, or MRC5 cells and incubated for 1 hr at 4°. Monolayers were washed with PBS, solubilized in SDS dissociation buffer, and aliquots examined by SDS–PAGE fluorography.

probably an oligomer (Delmas and Laude, 1990; Vennema *et al.*, 1990). It is likely that the method of Triton X-100 solubilization used to prepare the cell extracts used in our assay (Fig. 2) disrupted higher order structure required for receptor binding.

Membrane-binding of free and nucleocapsid-associated N protein

Extracts prepared from [^{35}S]methionine-labeled, MHV-infected cells showed the presence of nucleocapsid-associated and free N protein (Fig. 3). As shown by sucrose gradient sedimentation, MHV nucleocapsids were found predominantly in the heaviest gradient fraction (fraction 1 of Fig. 3A) although considerable amounts of N protein were also found in lighter fractions. Radiolabeling of actinomycin D-treated, MHV-infected L-2 cells with ^{3}H-uridine showed the bulk of trichloroacetic acid-precipitable radiolabel to be present in fraction 1, although lesser amounts were also found in fractions 2–6 (data not shown). The MHV nucleocapsid is known to be quite labile (Masters and Sturman, 1990), thus giving rise to less dense forms of the N protein which likely include oligomers and monomers (see also Robbins *et al.*, 1986). In some gradient fractions, particularly fraction 2, considerable M protein was observed, consistent with previous evidence of M:N protein interactions (Sturman *et al.*, 1980).

Assays of individual gradient fractions for cell-binding activity showed N binding in virtually all fractions where N was present (Fig. 3B). Thus, the N protein retains its membrane-binding properties whether in free or nucleocapsid-associated form.

Membrane-binding of *in vitro* translated N protein

The ability of MHV N protein to bind to membranes in the absence of M protein was further demonstrated by a membrane-binding assay of proteins synthesized in an *in vitro* translation system. Total RNA isolated from both uninfected and MHV-infected L-2 cells was translated in a wheat germ-derived *in vitro* system in the presence of [^{35}S]methionine. The translation mixtures were added to L-2 cell monolayers, incubated for 1 hr at 4°, and washed. Monolayers were then solubilized and examined by SDS–PAGE and fluorography. *In vitro* translation of RNA from MHV-infected L-2 cells gave rise to clear radiolabeled bands corresponding to the viral M and N proteins. The S protein could not be clearly identified. The N protein appeared as two bands, possibly reflecting different phosphorylation or alternative processing stages. Both N bands were found to bind to L-2 cell membranes, whereas negligible binding of M was observed (Fig. 4, compare lanes B and D). The possible role of additional proteins (for example the two membrane-binding proteins observed in lane D below the region of the M protein) was not in-

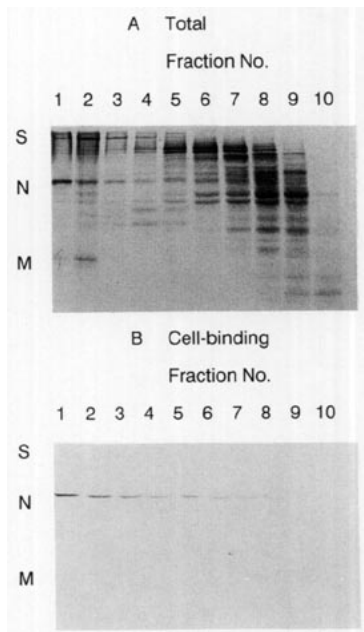

FIG. 3. Cell binding of sucrose gradient-fractionated MHV N protein. A detergent-solubilized extract from [^{35}S]methionine-labeled MHV-infected cells was ultracentrifuged in a 15–25% (w/w) sucrose gradient and subsequently fraction-collected. Aliquots of individual fractions were analyzed by fluorographic SDS–PAGE (A) or applied to L-2 cell monolayers in a cell-binding assay (B). Fractions 1 and 10 represent the bottom and top of the gradient, respectively.

vestigated further. We note however that similar-sized proteins were not observed in other membrane-binding assays (e.g., Figs. 2 and 3) and we therefore think it unlikely that such proteins may act as accessory binding factors for N.

Competition for membrane binding of N protein by RNA and DNA

Although sequence-specific binding between the MHV N protein and MHV leader RNA is known (Baric *et al.*, 1988; Stohlman *et al.*, 1988), the N protein is also able to bind RNA in a sequence-independent manner (Robbins *et al.*, 1986). This latter property is likely crucial for encapsidation of viral RNA in which the initiation event of N protein binding to the leader sequence has already occurred. We reasoned that membrane binding of N protein might occur at sites also involved in RNA binding. The ability of N protein to bind membranes was examined in the presence of RNA and DNA to compete for common binding sites. As shown in Fig. 5, both RNA and DNA were effective inhibitors of N protein binding to L-2 cell membranes.

At first glance the results of Figs. 3 and 5 are difficult to reconcile. Although nucleocapsid-associated N protein retains membrane binding properties (Fig. 3), N protein binding can be inhibited by an excess of RNA (Fig. 5). A likely explanation is that an equilibrium exists between membranes and RNA for N protein binding. Either RNA or membrane may compete for the other in binding the N protein. This interesting phenomenon is explored below under Discussion.

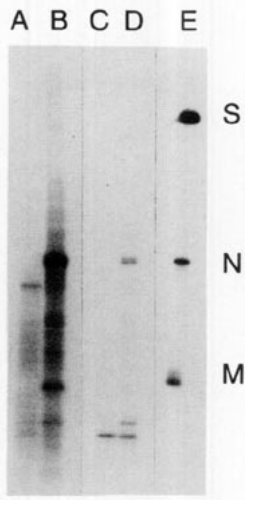

FIG. 4. Binding of *in vitro* translated N protein to L-2 cells. Aliquots of RNA from uninfected or MHV-infected L-2 cells were translated in a wheat germ system in the presence of [^{35}S]methionine. Translation mixtures were added to monolayer cultures of L-2 cells, incubated 1 hr at 4°, washed, and the monolayers were solubilized for analysis by fluorographic SDS–PAGE. Lanes A and B are [^{35}S]methionine-labeled proteins translated *in vitro* using uninfected and MHV-infected L-2 cell RNA, respectively. Lanes C and D are the L-2-cell-bound proteins from A and B. Lane E is a standard mixture of MHV proteins, prepared by radiolabeling MHV-infected L-2 cells with [^{35}S]-methionine for 15 min at 7 hr postinoculation.

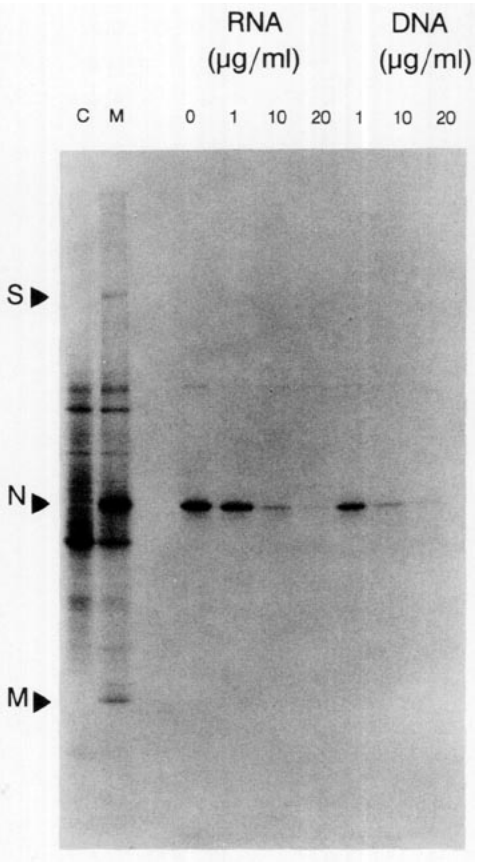

FIG. 5. Blocking of MHV N protein binding to L-2 cells by either single-stranded yeast transfer RNA or heat-denatured calf thymus DNA. A supernatant preparation of Triton X-100-solubilized, [^{35}S]-methionine-labeled, MHV-infected L-2 cells was applied to L-2 cell monolayers in the absence or presence RNA or DNA at the indicated concentrations. After 1 hr incubation at 4°, monolayers were washed, solubilized, and analyzed by fluorographic SDS–PAGE.

Competition for membrane binding of N protein by liposomes

In an effort to determine the nature of the membrane component(s) which bind N protein, we treated L-2 cell cultures with proteases, trypsin and proteinase K, using sufficient concentrations of enzyme to detach cell monolayers from their plastic substrate. Neither protease was able to decrease binding of N protein to the cell membranes (data not shown). This result raised the possibility that N protein binding to membranes was not mediated by cellular proteins. We considered the possibility that N protein might bind to lipids and assessed the ability of specific phospholipid liposomes to block binding of N protein to L-2 cells. Shown in Fig. 6 are the results of cell binding in the presence of cardiolipin or phosphatidylcholine (PC) liposomes. While PC liposomes had little effect on N protein binding to L-2 cells, cardiolipin (CDL) was a marked inhibitor of

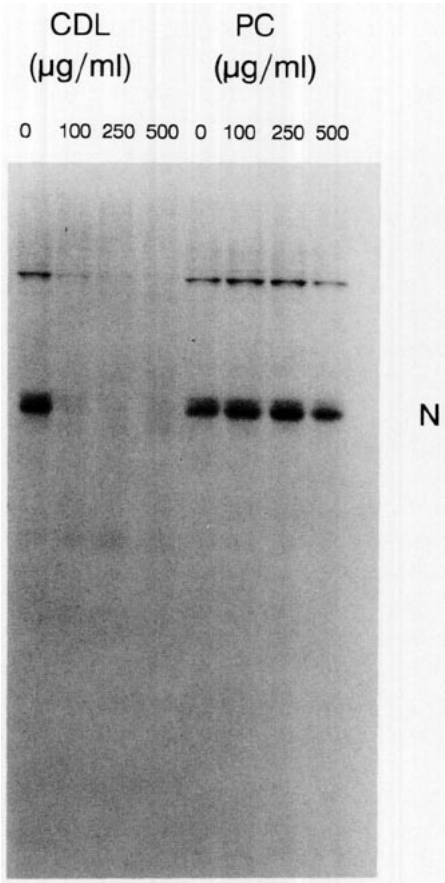

FIG. 6. Blocking of MHV N protein binding to L-2 cells by phospholipid liposomes. A supernatant preparation of Triton X-100-solubilized, [^{35}S]methionine-labeled, MHV-infected L-2 cells was applied to L-2 cell monolayers in the absence or presence of cardiolipin (CDL) or phosphatidylcholine (PC) liposomes at the indicated concentrations. After 1 hr incubation at 4°, monolayers were washed, solubilized, and analyzed by fluorographic SDS–PAGE.

binding. Other phospholipids tested (data not shown) were phosphatidylethanolamine (no effect), phosphatidylserine, and phosphatidylinositol (both with little effect).

N protein binding to cardiolipin liposomes

In order to determine whether phospholipids, such as cardiolipin, could bind the N protein, we mixed solubilized [^{35}S]methionine-labeled cell extracts with liposomes consisting of either PC alone or PC containing 15% mol% CDL (designated CDL/PC liposomes). Mixtures were subsequently fractionated on 5–15% sucrose gradients to separate unbound proteins from lipid-associated proteins (present at the top of the gradient). As shown in Fig. 7, very little protein was associated with PC liposomes (fraction 8 of Fig. 7A). In contrast, most of the N protein was bound to CDL/PC liposomes (fraction 8 of Fig. 7B). Binding was specific in that none of the S or M proteins was associated with the CDL/PC liposomes (Fig. 7B). Similar assays, using

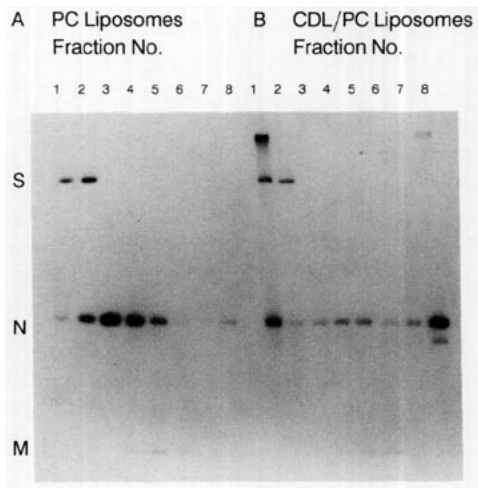

FIG. 7. Binding of MHV N protein to phospholipid liposomes. A supernatant preparation of Triton X-100 solubilized, [^{35}S]methionine-labeled, MHV-infected L-2 cells was mixed with either PC liposomes or CDL/PC (15:85 mol%) liposomes, incubated 60 min at 4°, and applied to a gradient of 5–15% (w/w) sucrose. The ultracentrifuged gradients were fraction-collected and individual fractions immunoprecipitated with anti-MHV antiserum for analysis by fluorographic SDS-PAGE. For each gradient (A or B) fractions 1 and 8 represent the bottom and top, respectively. Liposomes banded in fraction 8 of the gradients.

mixed liposomes containing PC and PE, PS, or PI, showed little binding of N protein (data not shown, but similar to PC liposomes alone [Fig. 7A]).

N protein binding to liposomes containing L-2 cell lipids

The possibility that membrane-binding of N protein might involve an L-2 cell lipid component was investigated by using a liposome binding assay similar to that described in the previous paragraph. Liposomes were prepared consisting of PC and L-2 cell lipids in a 2:1 (w/w) ratio and used in an N protein binding assay as described above. Sucrose gradient analysis (Fig. 8)

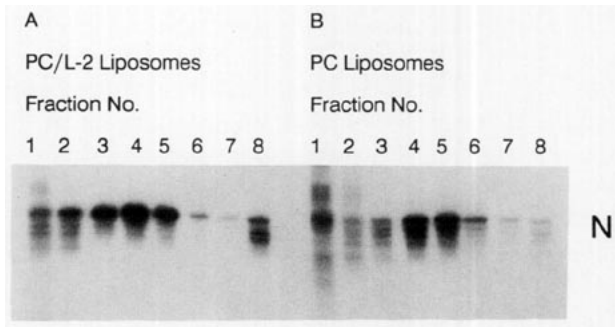

FIG. 8. Binding of MHV N protein to liposomes containing L-2 cell lipids. A supernatant preparation of Triton X-100 solubilized, [^{35}S]-methionine-labeled MHV-infected L-2 cells was mixed either with PC liposomes or liposomes containing a 2:1 (w/w) ratio of PC and lipids extracted from L-2 cells. Mixtures were incubated 60 min at 4° and applied to sucrose gradients and processed as described in the legend for Fig. 7. Only the region of the gel comprising the general area around the N protein is shown.

showed considerable binding of N protein to such liposomes (present at the top of the gradient), whereas little binding of N protein was observed with control PC liposomes (compare fraction 8 in Figs. 8A and 8B). This result strongly suggests the existence of a host cell lipid which is responsible for N protein binding. It may be noted that in this experiment, considerable heterogeneity of the N protein was observed, as indicated by the appearance of several radiolabeled protein bands. Such heterogeneity remains largely unexplained but may reflect events such as proteolytic processing (Cheley and Anderson, 1981) or enzymatic dephosphorylation (Beushausen *et al.*, 1987; Mohandas and Dales, 1991).

DISCUSSION

Coronaviruses, such as MHV, bud from intracellular membranes, in contrast to many other viruses which acquire their lipid envelope by budding through the plasma membrane. The site of budding for coronaviruses is thought to be determined by the M protein which is retained in the Golgi region (Rottier and Rose, 1987), while the other viral glycoprotein (S) is present both on intracellular as well as surface membranes (Sturman and Holmes, 1985). The M protein also has the property of binding N protein (Sturman *et al.*, 1980), which may play an important role in virus assembly and in stabilizing virus structure. The results from the present study indicate that the phenomenon of membrane association of N can occur independently of the M protein. It is nevertheless quite possible that interactions between M and N play a role in certain replicative events such as the proper envelopment of the viral nucleocapsid during assembly.

The role of phosphorylation of the MHV N protein was not investigated in this study. While Stohlman *et al.* (1983) demonstrated post-translational phosphorylation of the MHV (JHM) N protein in DBT cells, we have not been able to uncouple phosphorylation from translation in MHV-infected L-2 cells and conclude that in these cells, phosphorylation of N protein occurs either cotranslationally or immediately post-translation (unpublished data). The extent of phosphorylation of N protein synthesized in an *in vitro* wheat germ system has not been investigated to date and so we cannot comment on whether phosphorylation is required for membrane-association of the N protein. This would be an interesting matter to investigate, since the association of N protein with intracellular membranes in MHV(JHM)-infected DBT cells was shown to occur at the same time as phosphorylation of the N protein (Stohlman *et al.*, 1983).

The ability of MHV to apparently induce membrane proliferation inside infected cells has been reported by several laboratories (David-Ferreira and Manaker, 1965; Massalski *et al.*, 1982; Dubois-Dalcq *et al.*, 1982; van Berlo *et al.*, 1986; Tooze *et al.*, 1984), although the underlying mechanisms are not understood. However, membrane proliferation in cells infected with certain picornaviruses such as mengo is connected with altered phospholipid metabolism (Penman, 1965; Amako and Dales, 1967), raising the possibility that virus-induced changes in host cell membrane metabolism may reflect a requirement of certain viral proteins for specific host cell lipids.

The property of MHV N protein to bind both membranes and nucleic acids has striking similarity to certain bacterial DNA-binding proteins such as DnaA (Sekimizu and Kornberg, 1988), which is involved in DNA replication and recA (Krishna and van de Sande, 1990), which is involved in genetic recombination and DNA repair. RecA has also been recently shown to bind acidic phospholipids which may mediate membrane binding and compete for DNA binding sites (Krishna and van de Sande, 1990). RecA also has the ability to polymerize cooperatively and nonspecifically on nucleic acid substrates to form a helical filament (Story *et al.*, 1992), a property likely shared by the MHV N protein.

Cardiolipin appears to be a preferred phospholipid in terms of its affinity for nucleic acid binding proteins, such as DnaA and recA (Sekimizu and Kornberg, 1988; Krishna and van de Sande, 1990). CDL was also found to be the most effective among common phospholipids (PC, PE, PS, PI, and CDL) in binding MHV N protein in the present study. Although CDL is a common lipid constituent of prokaryotes it is a relatively minor component of eukaryotic cells, where it is predominantly localized in mitochondria (White, 1973). We suspect that in the course of natural infection, membrane binding of MHV N protein is mediated by extramitochondrial cardiolipin or by an as yet unidentified lipid with similar structural characteristics. Cardiolipin is a member of the phosphatidylglycerol class of phospholipids of which there are derivatives in eukaryotic cells. As examples are acylated forms of bis(monoacylglycero)phosphate, normally enriched in secondary lysosomes (Wherett and Huterer, 1972) and which, intriguingly, are elevated in concentration during certain viral infections, e.g., vaccinia (Stern and Dales, 1974; Hiller *et al.*, 1981) and mengo (Schimmel and Traub, 1987). We are presently attempting to identify candidate N protein-binding lipids in L-2 cells.

The MHV N protein has been shown to bind RNA non-specifically (Robbins *et al.*, 1986) and this report (Fig. 5), although evidence for high affinity binding of the 5′-terminal leader sequence of MHV RNA has been presented (Baric *et al.*, 1988; Stohlman *et al.*, 1988). It is likely that the initiation event for encapsidation of viral RNA is mediated by N protein binding at the leader site followed by sequential addition of N protein mole-

cules along the viral RNA to form the helical nucleocapsid. As in the tobacco mosaic virus model (Butler and Klug, 1978), only the initiation event would require a specific RNA sequence for N protein binding. Since MHV mRNAs are unencapsidated but do contain N protein bound to their leader sequences (Baric *et al.*, 1988; Stohlman *et al.*, 1988), conditions inside the infected cell must favor the maintenance of viral RNA in a form which is relatively free from associated N protein. Conceivably, intracellular binding of MHV N protein by membrane-associated phospholipids could facilitate these conditions.

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