INVOLVEMENT OF LIPIDS IN MEMBRANE BINDING OF MOUSE HEPATITIS VIRUS NUCLEOCAPSID PROTEIN

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ABSTRACT

Evidence is presented which indicates that membrane binding of the MHV nucleocapsid (N) protein is influenced by membrane lipid composition. 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 rapidly and does not require the presence of divalent cations such as Ca^{++} or Mg^{++}. Purified phospholipid liposomes compete against N protein binding to membranes. Phospholipids consisting of cardiolipin are the most effective in inhibiting membrane binding. 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.

INTRODUCTION

Shortly after its synthesis the nucleocapsid protein becomes associated with intracellular membranes (1). The biological significance of this event is uncertain although it may serve to ensure coalescence of the various subviral components which are known to assemble at a specific cell membrane site (2,3). There is evidence that post-translational phosphorylation of the N protein is necessary for membrane association to occur (1). The nature of this association is unknown but may involve interaction between N and the integral membrane protein M which is synthesized on ribosomes present on the endoplasmic reticulum (4).

Membrane association of viral macromolecular synthesis and assembly appears to be a hallmark of coronavirus replication. Viral structural proteins are either translated on membrane-bound polysomes or become quickly associated with membranes shortly after synthesis. Virion assembly also occurs via budding through intracytoplasmic membranes, specifically those of transitional elements between the endoplasmic reticulum and the Golgi apparatus (2,3). In addition to virion assembly and synthesis of viral proteins, viral RNA is also synthesized on cytoplasmic membranes, possibly distinct with regard to the synthesis of (+) and (-) RNA species (5,6).

We report here further studies on the membrane-binding properties of the MHV N protein. We provide evidence that membrane association of N protein may involve specific phospholipids, thus raising the intriguing possibility that N protein is binding to a host cell membrane lipid "receptor" which may then be required for some step in virus replication.

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MATERIALS AND METHODS

Preparation of cell extracts

Monolayer cultures of mouse fibroblast L-2 cells (7) were mock-infected or inoculated with MHV (A59 strain) at a multiplicity of infection of five, adsorbed for 1h at 4° and then incubated at 37°. At 7h post-infection, cultures were radiolabeled for 1h with ^{35}S-methionine. The monolayers were washed with phosphate buffered saline (PBS) and extracted (15 min on ice) with 1% Triton X-100 in 10 mM tris, pH 7.4, 100 mM NaCl, 0.2 mM PMSF. Extracts were freed of detergent by overnight shaking with SM-2 Biobeads (BioRad) and clarified by centrifugation at 100,000xg. The resultant supernatants were used in binding assays.

Binding assays

Monolayer cultures of mouse fibroblast L-2, LM (8) and LM-K (9) cells were incubated for various times at 4° with radiolabeled cell extract supernatants prepared as above. Monolayers were then washed several times with PBS and the cells solubilized in dissociation buffer for analysis on SDS-PAGE and autoradiography (10).

Lipids and preparation of liposomes

Individual phospholipids, cardiolipin (CDL), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) were obtained from Sigma. Liposomes were prepared by sonication of phospholipids in PBS (11).

RESULTS

Cell membrane binding of MHV N protein

In addition to binding to intracellular membranes during its synthesis in MHV-infected cells (1), the nucleocapsid N protein was surprisingly found to bind to the outer surface of intact mouse fibroblast cells (Fig. 1). Extracts from ^{35}S-methionine-labeled mock- or MHV-infected L-2 cells were exposed to monolayer cultures of various sublines of mouse fibroblast cells for 1h at 4°. The cultures were then washed and solubilized for analysis on an SDS-polyacrylamide gel which was visualized by autoradiography. As shown in Fig. 1, all cells bound the N protein under conditions in which no other protein was observed to bind. This result suggested that the cellular component responsible for membrane binding of the N protein is present not only on intracellular membranes but also on the outer surface of the cell plasma membrane. Moreover, the results indicate that membrane-association of N protein can occur in the absence of other viral proteins, including M.

It should be mentioned that no binding of S protein was observed in the assay employed, presumably because the method of cell extract preparation disrupted higher order structure of the oligomeric form (12,13) which may be required for receptor binding.

Kinetics and ion requirements for membrane binding of N protein

Binding of N protein to L-2 cells occured rapidly as shown by a time course study in which an extract from ^{35}S-methionine-labeled MHV-infected L-2 cells was added to L-2 cell monolayers, adsorbed for various times at 4° and the monolayers then washed to remove unbound material. SDS-PAGE analysis of the cell-bound radiolabeled proteins showed the presence of N protein as early as 5 min, with increasing amounts observed over time (Fig. 2a). Binding of N protein to cell membranes did not require the presence of cations such as Mg^{++} or Ca^{++}, although binding was slightly enhanced in response to Ca^{++} concentrations of 5 or 10 mM (Fig. 2b).

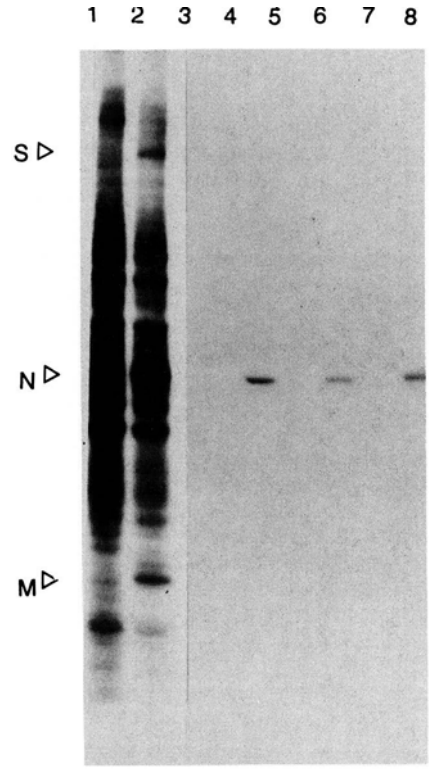
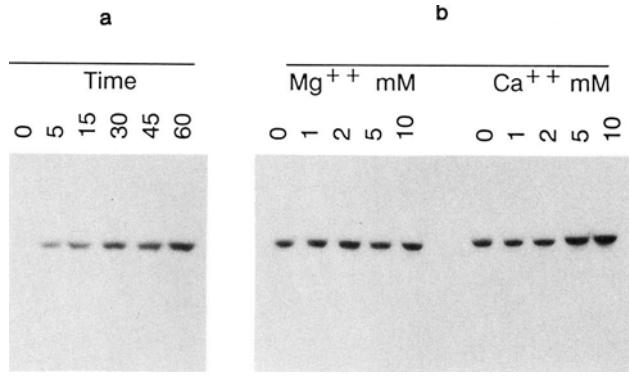


Figure 1

Autoradiographic SDS-PAGE analysis of binding of radiolabeled N protein to intact cells. Triton X-100-solubilized extracts from ^{35}S-methionine labeled, mock- or MHV-infected L-2 cells were freed of detergent by treatment with SM-2 Biobeads (BioRad) and subsequently ultracentrifuged (1h at 100,000 x g) to yield supernatant extracts shown in lanes 1 and 2, respectively. Extracts were allowed to adsorb for 1h at 4° to monolayer cultures of mouse fibroblast cells: L-2, lanes 3 and 4, LM, lanes 5 and 6, and LM-K, lanes 7 and 8. Monolayers were washed to remove unbound material and then solubilized for analysis on SDS-PAGE. Lanes 3, 5 and 7 show cell-bound radiolabeled proteins from mock-infected L-2 cells, while lanes 4, 6 and 8 show cell-bound radiolabeled proteins from MHV infected L-2 cells.

Blocking of membrane binding of N protein by phospholipid liposomes

In examining the possible nature of the cell membrane component(s) responsible for binding of the N protein, we considered the involvement of both proteins and lipids. Treatment of L-2 cells with the proteases trypsin or proteinase K did not impair the ability of these cells to bind N protein, arguing against the possibility of a membrane protein "receptor" for N. In contrast, the addition of phospholipid liposomes to L-2 cells was found to block cell-binding of N protein, specifically when cardiolipin (CDL) was the phospholipid constituent (Fig. 3). The inhibition of cell-binding was highly dependent on the phospholipid composition of the liposomes, as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) were relatively ineffective in blocking binding of N to L-2 cells.



Kinetics and effects of divalent cations on membrane binding of N protein. A total extract of ^{35}S-methionine-labeled MHV-infected cells, prepared as described in the Materials and Methods, was added to monolayer cultures of L-2 cells and incubated for various times at 4° (A) or for 1h at 4° in the presence of various concentrations of $MgCl_2$ or $CaCl_2$ (B). Monolayers were washed and the cells solubilized for autoradiographic SDS-PAGE.

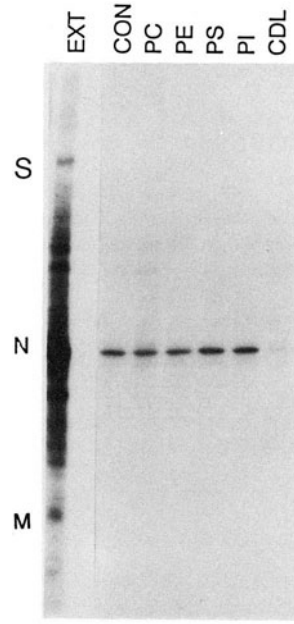


Figure 3

Blocking of membrane binding of N by phospholipid liposomes. An extract (EXT) of ^{35}S-methionine-labeled MHV-infected cells, prepared as described in the Materials and Methods, was added to monolayer cultures of L-2 cells in the absence (CON) or presence of phospholipid liposomes (PC, PE, PS, PI, CDL) at a final concentration of 250 μg/ml. After incubation for 1h at 4° the monolayers were washed and the cells solubilized for SDS-PAGE and autoradiography.

DISCUSSION

The nucleocapsid N protein of coronaviruses, including MHV, is a protein of approx MW 43-50k. Basic amino acids are localized in the amino-terminal two-thirds of the protein; in contrast, the carboxy terminal one-third is acidic. The N protein sequence contains a considerable proportion of serines (7-10%) some of which are phosphorylated. Phosphorylation occurs soon after biosynthesis of the N protein at which time N becomes associated with internal cell membranes (1). This observation has prompted speculation that phosphorylation is the trigger for membrane association of N. The biological significance of this event is uncertain although it may serve to ensure coalescence of the various subviral components which are known to assemble at a specific cell membrane site (2,3). Nucleocapsid N protein incorporated into virions is also phosphorylated; the recent finding of a cellular N protein-reactive phosphatase localized in the endosomal compartment may suggest a role for N protein dephosphorylation during the entry process (14). A potentially crucial observation is that cultured glial cells, induced to differentiate in vitro become resistant to MHV (strain JHM) and less capable of dephosphorylating the N protein (15).

MHV N protein binds RNA in a manner (depending on *in vitro* conditions) which suggests sequence specificity (16,17). In particular a short sequence present within the 5'-terminal leader region of both genomic and messenger RNAs has been reported to bind N protein (18). The finding of N protein bound to mRNAs is somewhat surprising, since mRNAs are not encapsidated during assembly. It has been speculated that binding of N may act as a translational regulator of viral mRNAs (19). The N protein is apparently required for viral RNA transcription, since anti-N monoclonal antibody has been shown to inhibit MHV RNA synthesis *in vitro* (20). Evidence that discreet regions of the N protein participate in RNA binding comes from varied sources. For example, a 40k processing product of the N protein has been reported which no longer binds RNA (21). Furthermore, carboxy-truncated forms of the N protein show much reduced RNA-binding ability (22).

The findings from the present study suggest that, in addition to RNA, the MHV nucleocapsid protein has the capacity to bind membrane phospholipids. Such dual affinity might conceivably have a role in facilitating either assembly or disassembly, should RNA and membrane phospholipid compete for the same binding sites on N. Such considerations are perhaps similar to those described for the bacterial dnaA (23) and recA (24) proteins which also bind both nucleic acid and phospholipid. Interestingly, recA and dnaA proteins show strong affinity for cardiolipin (23,24), the phospholipid which was found to be the most effective in blocking membrane association of the MHV N protein.

Although cardiolipin is a major component of bacteria, it is a relatively minor component of animal cells in which it is mainly associated with mitochondria (25). We suggest that the MHV nucleocapsid protein may associate either with non-mitochondrial cardiolipin or a phospholipid with similar structural characteristics. A possible candidate is bis(monoacylglycero)phosphate a lysosomal phospholipid, the synthesis of which is enhanced in certain virus infections, eg. vaccinia (26,27) and mengo (28). Studies are in progress to identify possible alterations in cellular lipid metabolism upon infection with MHV.

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