

VIRION-LIPOSOME INTERACTIONS IDENTIFY A CHOLESTEROL-INDEPENDENT CORONAVIRUS ENTRY STAGE

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1. INTRODUCTION

Entry of enveloped viruses depends on several cellular components, including protein or carbohydrate receptors and oftentimes co-receptors that both bind viruses to cells and catalyze the initial stages of viral surface protein refolding.¹ Endocytosis is a common prerequisite to successful entry; both the acidic pH and the proteases of the endosome can create further structural changes in the viral proteins mediating cell receptor binding and virus-cell membrane fusion.² Finally, an appropriate lipid environment, often abundant in sterols, is also frequently necessary to achieve facile fusion of viral and cellular membranes.³ In numerous seminal investigations, each of these steps has been dissected by blocking virus entry with mutant transgenes or with drugs that alter receptors or endosome or lipid environments. Although these are powerful approaches, the findings can be complicated when the transgenes or drugs create untoward pleiotropic changes in cellular functions. A complementary reductionist approach involves *in vitro* virus entry in which enveloped viruses are bound to and then fused into synthetic liposomes.⁴ In these test-tube reactions, receptors, pH, proteases and liposome bilayer compositions can be precisely defined and freely altered in ways not achievable in living cells. Such systems are useful adjuncts to understanding the biochemistry of enveloped virus entry.

In vitro virus-liposome binding assays have been recently developed for the murine coronaviruses.^{5,6} In these studies, purified MHV particles acquired hydrophobic character by incubation with soluble MHV receptors or by exposures to elevated pH, thus causing a small proportion of the virus population to associate with liposomes. We aimed to advance these studies by creating liposomes that more closely reflect authentic MHV-susceptible cells. To this end, we created synthetic liposomes containing nickel-nitriloacetic acid (NiNTA) adducts on a fraction of lipid head groups. Soluble MHV receptors containing engineered polyhistidine tags could then be attached to the liposomes, thereby creating artificial targets for virus binding. These liposomes with

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attached receptors have been useful in identifying the requirements for irreversible association of MHV to target membranes during entry.

2. RESULTS

We obtained lipids from Avanti Polar Lipids, Inc., including the novel synthetic product DOGS-NTA-Ni, which contains a chelated Ni²⁺-NTA head group that can noncovalently complex with polyhistidine tags. Lipids in various formulations [typically 69:30:1 mole ratio phosphatidylcholine (PC), cholesterol (chol), DOGS-NiNTA] were suspended from a dried state into HEPES-buffered saline and then extruded through polycarbonate filters to create 100-nm-diameter liposomes. These liposomes were then mixed with baculovirus-expressed soluble MHV receptors containing C-terminal 6x-histidine tags.⁷ These soluble receptors are designated soluble Carcino-Embryonic-Antigen, or sCEA6his. To remove unbound receptors, we adjusted the mixtures to contain 50% w/v sucrose, then overlaid stepwise with 40% – 30% – 10% w/v sucrose solutions and floated the liposomes to the 10–30% sucrose interface by ultracentrifugation. The liposome:sCEA6his complexes were collected and stored at 4°C. Estimates of the sCEA-6xhis concentrations by titration and immunoblotting indicated that our typical preparations had densities of ~50 sCEA per 100-nm liposome.

Metabolically-radiolabeled [³⁵S] MHV particles were purified by equilibrium density gradient sedimentation. Typically, we incubated ~50,000 cpm of [³⁵S] MHV (10⁵ pfu) for 1 h at 4°C with 10 mM phospholipid liposomes, in a volume of 0.2-ml of HEPES-buffered saline. Mixtures were then brought to 50% sucrose and subjected to a second round of ultracentrifugation to float the liposomes. By identifying the [³⁵S]-containing gradient fractions, we determined that MHV binding to liposomes was absolutely dependent on NiNTA lipids and on sCEA6his (Fig. 1). Notably, a control sCEAFc lacking the 6xhis tag bound to the [³⁵S] virions, but failed to link virions to liposomes (Fig. 1), even after incubation at 37°C.

To determine whether the connections between [³⁵S] MHV and liposomes were reversible, we exploited the common knowledge that noncovalent NiNTA:6his linkages are disrupted by EDTA or imidazole. To this end, we harvested [³⁵S] MHV-liposome complexes from gradient fractions and added EDTA (10 mM final concentration) for 30 min at 4°C. Reflotation in fresh sucrose gradients revealed that the EDTA exposures separated the virions, leaving the [³⁵S] near the bottom of the ultracentrifuge tubes (Fig. 2). However, MHV-liposome complexes incubated for 30 min at 37°C were insensitive to dissociation by EDTA (Fig. 2). This discovery allowed us to conclude that physiologic temperature is required to establish EDTA-resistant linkages between virus and its target lipid bilayer.

MHV entry requires cholesterol in target lipid bilayers.⁸ To determine whether the irreversible virus-liposome linkages required cholesterol, we prepared liposomes lacking and containing 30mol% cholesterol. These two liposome formulations were indistinguishable in their irreversible, 37°C temperature-dependent capture of [³⁵S] MHV particles (Fig. 2).

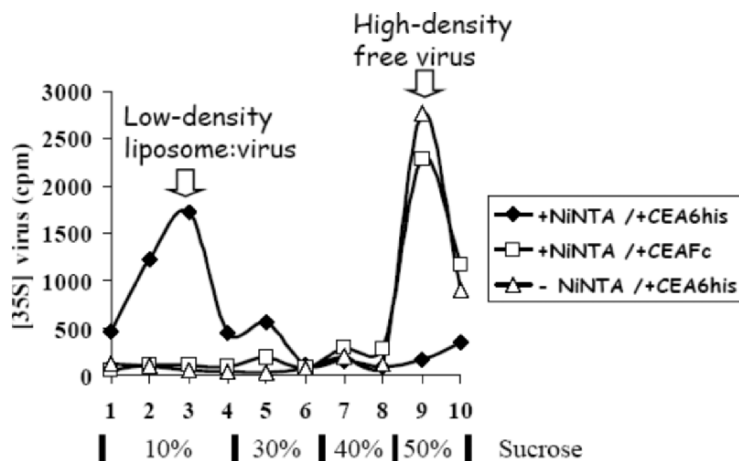


Figure 1. Virus-liposome interactions require both sCEA6his and NiNTA lipids. Liposomes with or without incorporated NiNTA lipids were incubated with either sCEA6his or sCEAFc, then with purified [³⁵S] MHV strain A59 for 1 h at 4°C before floating liposomes in sucrose gradients. The abundance of [³⁵S] in 1/10 of each of the gradient fractions were determined by scintillation counting.

3. DISCUSSION

Coronavirus particles are distinguished by their prominent spike protein trimers. These glycoproteins bind cell receptors and mediate virus-cell membrane fusions. The fusion reaction pathway is of the “class I” type⁹, which involves a temperature-dependent refolding into helical bundles. In a productive process, this refolding is preceded by

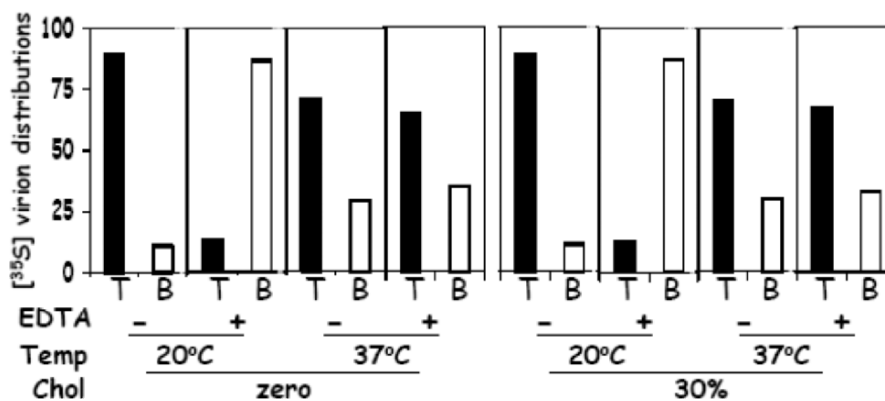


Figure 2. Stability of virus-liposome complexes against dissociation by EDTA. [³⁵S] MHV-liposome complexes, purified by floatation in sucrose gradients, were held at 20°C or 37°C prior to EDTA exposure. Samples were then subjected to a second round of floatation, and the proportion of [³⁵S] in the top (T) and bottom (B) of each gradient were determined by scintillation counting. The [³⁵S] in T and B represent virus-liposome complexes and free virus, respectively. EDTA released viruses from liposomes at 20°C, but 37°C temperatures created EDTA-stable virus-liposome complexes.

insertion of hydrophobic spike peptides, so-called fusion peptides, into target membranes. We are currently hypothesizing that the stabilized association of MHV with synthetic liposomes results from the insertion of their fusion peptides into the liposome membranes. If this hypothesis is borne out, then we can suggest that the intercalation of fusion peptides is strongly temperature-dependent but does not require cholesterol in the target membranes.

In assessing whether bona-fide fusion of virus and liposome membranes occurs, our principal strategy has been to monitor fluorescence changes as pyrene lipid probes redistribute from limiting membranes into the larger area of fused vesicles.⁴ Despite numerous attempts, we have not yet documented MHV:liposome coalescence by these methods. It is entirely possible that components required to complete the MHV membrane fusion process are missing from our *in vitro* assays. Fusion may require exposure to acids or proteases, may require proteinaceous co-factors in addition to the principal CEA receptors, and likely also requires specific sterol-containing lipid environments. We are continuing our investigations to identify the conditions that may allow completion of the spike-mediated class I fusion pathway.

4. ACKNOWLEDGMENTS

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