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Subunit Interactions of Vesicular Stomatitis Virus Envelope Glycoprotein Stabilized by Binding to Viral Matrix Protein

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The mechanism by which viral glycoproteins are incorporated into virus envelopes during budding from host membranes is a major question of virus assembly. Evidence is presented here that the envelope glycoprotein (G protein) of vesicular stomatitis virus binds to the viral matrix protein (M protein) in vitro with the specificity, reversibility, and affinity necessary to account for virus assembly in vivo. The assay for the interaction is based on the ability of M protein to stabilize the interaction of G protein subunits, which exist as trimers of identical subunits in the virus envelope. The interaction with M protein was shown by using G proteins labeled with fluorescent probes capable of detecting subunit dissociation and reassociation in vitro. The results show that the M protein isolated from virions either as purified soluble protein or as nucleocapsid-M protein complexes interacts with the G protein in vitro and that the reaction is reversible. The interaction between the G and M proteins was not serotype specific, but no interaction between the vesicular stomatitis virus M protein and the influenza virus hemagglutinin could be detected. These results support the conclusion that the interactions described here are the ones that govern assembly of G protein into virus envelopes in vivo.

Enveloped viruses share a common structural organization in which a membranous envelope encloses a nucleoprotein core, or nucleocapsid. The envelope is acquired during virus assembly by budding from host membranes, usually the plasma membrane (recently reviewed in reference 19). Viral surface glycoproteins are usually typical of a large class of membrane proteins in having a large, glycosylated external domain, a short hydrophobic sequence that spans the virus envelope lipid bilayer, and a short sequence that is exposed on the internal surface of the virus envelope. Viral glycoproteins are inserted into host membranes after biosynthesis in the secretory pathway. During the budding process, viral proteins are incorporated into the envelope and host proteins are largely excluded. The mechanism of glycoprotein incorporation into virus envelopes is a central question of virus assembly that has remained unanswered since the molecular components of virus envelopes were first described a number of years ago (compare reviews in references 11 and 19).

Many enveloped viruses contain an internal matrix protein as a part of the viral envelope. Matrix proteins play a central role in virus assembly by binding the nucleocapsid to the cytoplasmic surface of the plasma membrane during the budding process. It is commonly believed that matrix proteins may also play a role in the assembly of viral glycoproteins into the envelope. However, there is relatively little direct evidence for such an interaction. Vesicular stomatitis virus (VSV), the prototype rhabdovirus, which has been widely used in the study of enveloped virus assembly, contains a single species of envelope glycoprotein (G protein), matrix protein (M protein), and nucleocapsid protein (N protein), as well as two minor proteins (NS and L) responsible for RNA polymerase activity. Perhaps the only direct evidence that the VSV G protein interacts directly with the M protein is provided by chemical cross-linking experiments with whole virions, which showed the existence of a cross-linked species containing the G and M proteins (6). However, this experiment could not determine whether this interaction is responsible for incorporation of the G protein into the virus envelope or what specificity the interaction possesses. Alphaviruses such as Semliki Forest virus contain only a single species of envelope glycoprotein, a single species of capsid protein, and no matrix protein. A binding site for the Semliki Forest virus glycoprotein on the capsid has been demonstrated by using anti-idiotypic (internal image) antibodies (20). This raises the possibility that the glycoproteins of other enveloped viruses may bind directly to the nucleocapsid without the participation of matrix proteins.

The VSV G protein exists in the virion as a trimer of identical subunits, as shown by chemical cross-linking experiments and sedimentation velocity analysis of detergent-solubilized G protein (4, 6, 10, 13). Using G proteins modified with fluorescein and rhodamine labels, we have shown that G protein subunits can undergo a reversible dissociation and reassociation reaction in the presence of the detergent octyl glucoside to form mixed trimers (13). A similar subunit exchange process has been described for genetically distinct G proteins in vivo (23). In results presented here, we have been able to analyze the dissociation of G protein subunits directly rather than through subunit exchange. We have found that the G protein subunit interaction is stabilized in the presence of M protein, either in the form of purified, soluble M protein or in the form of nucleocapsid-M protein complexes. This interaction between the VSV G and M proteins had the properties necessary to account for virus assembly in vivo, including specificity, reversibility, and affinity. This is the first demonstration of the interaction between a viral envelope glycoprotein and matrix protein in vitro that has these properties.

MATERIALS AND METHODS

Isolation and fluorescent labeling of G proteins. VSV (Indiana [IND] or New Jersey [NJ] serotype) was grown in

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BHK cells. The purification of virus and the isolation and labeling of G protein with fluorescent probes has been described previously (13). Briefly, G protein was solubilized from purified virions at pH 8.5 with octyl glucoside and reacted with either fluorescein isothiocyanate or tetramethyl rhodamine isothiocyanate. G protein was purified from unreacted label and other viral components by gel filtration and sedimentation in sucrose gradients containing octyl glucoside. The reaction conditions were adjusted to give approximately one label per G protein subunit. The concentration of the purified, labeled G protein was usually around 1 μM (total G protein subunits, i.e., about 60 μg/ml).

Isolation of M protein and nucleocapsids from VSV and HA from influenza virus. Purified VSV virions were solubilized with Triton X-100 in the presence of 0.25 M NaCl, and the M protein was purified by ion-exchange chromatography in the absence of detergent as described previously (14). Nucleocapsid-M protein complexes or nucleocapsids stripped of M protein were prepared by solubilizing virions with Triton X-100 in the presence of either 10 mM NaCl or 250 mM NaCl, respectively, and then were centrifuged in sucrose gradients in the absence of detergent as described previously (9). Hemagglutinin (HA) was solubilized from the X47 strain of influenza virus and purified by ion-exchange chromatography in the presence of octyl glucoside as described previously (12). The purity of the preparations was checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and was routinely similar to the published results.

Sedimentation analysis of labeled G proteins. The sedimentation velocity of labeled G proteins was determined by centrifugation on a 10 to 17% (wt/wt) sucrose gradient containing 50 mM octyl glucoside, and the gradients were fractionated and analyzed as described previously (13). The sedimentation of G protein monomers and trimers was determined in parallel gradients containing Triton X-100 at pH 7.5 or 5.8 (4).

Fluorescence assays. Unless otherwise noted, all experiments were performed in 10 mM sodium phosphate–65 mM NaCl–50 mM octyl glucoside–1% glycerol, pH 7.0, at room temperature. Fluorescence measurements were made as described previously (12), with either a Spex Fluorolog or SLM/Aminco 8000C fluorometer.

RESULTS

Stability of monomer and trimer forms of the G proteins of VSV-IND and VSV-NJ. The stability of G protein trimers depends on the serotype of VSV from which the G protein is isolated, as shown in Fig. 1. Two serotypes of VSV, VSV-IND and VSV-NJ, have G proteins that share about 50% amino acid identity (7). G protein from either VSV-IND or VSV-NJ was solubilized with octyl glucoside and labeled with fluorescein isothiocyanate for use in the fluorescence experiments described below. Labeled G proteins were subjected to rate zonal centrifugation in sucrose gradients containing octyl glucoside (Fig. 1). The VSV-IND G protein sedimented primarily as a trimer, with a peak about two-thirds the length of the gradient, as shown previously (13). In contrast, the VSV-NJ G protein sedimented as a monomer in the presence of octyl glucoside, indicating that the VSV-NJ G protein trimers are much less stable than those of VSV-IND. These results were not influenced by the labeling procedure, since similar results were obtained with unlabeled G proteins (data not shown). Since most of the remaining experiments examined the stability of G protein trimers, the G protein from VSV-IND was used unless

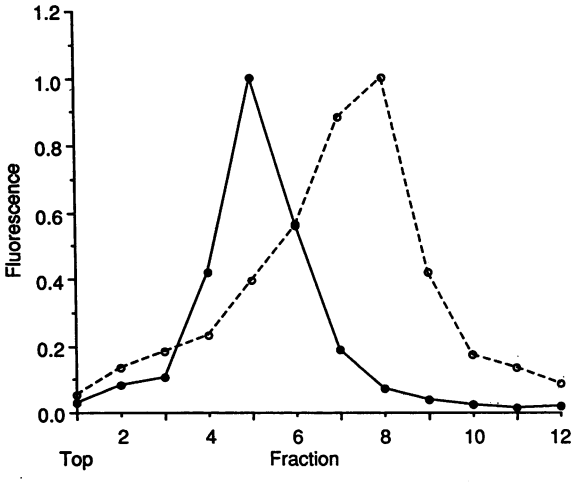

FIG. 1. Sedimentation of G proteins from VSV-IND and VSV-NJ in sucrose gradients. G proteins were isolated from VSV-IND (○) and VSV-NJ (●) and labeled with fluorescein isothiocyanate. The labeled G proteins were sedimented in a 10 to 17% sucrose gradient containing 50 mM octyl glucoside at 300,000 × *g* for 16 h. The gradients were fractionated, and the fluorescein fluorescence intensity was measured. The fluorescence of each sample was normalized to that of the peak fraction in the gradient.

otherwise noted. The VSV-IND G protein also includes some dimers and monomers as well as trimers, indicated by the asymmetric shape of the sedimentation peak (Fig. 1). In contrast, the VSV-NJ G protein peak was symmetric and, thus, reflected only the monomer population. This result is consistent with the previous finding that the VSV-IND G protein exists in a dynamic equilibrium between trimer, dimer, and monomer forms in the presence of octyl glucoside (13).

When fluorescein-labeled G protein was diluted to concentrations in the range of the trimer dissociation constant, the G protein trimers dissociated into dimer and monomer forms. This dissociation resulted in a decrease in the intensity of fluorescein fluorescence, as shown in Fig. 2. Labeled G protein was diluted to 4 to 120 nM in buffer containing octyl glucoside, and the fluorescence intensity of the fluorescein label was monitored as a function of time. The initial fluorescence was proportional to the concentration of labeled G protein, as expected. The data in Fig. 2A were normalized to this initial value, so that the behavior of the labeled G protein at different concentrations could be compared. At low G protein concentrations, the fluorescence intensity rapidly decreased below the initial level, while at higher G protein concentrations, there was little change in fluorescence intensity (Fig. 2A). The dependence of the fluorescence decrease on the extent of G protein dilution suggests that the fluorescence change was due to dissociation of G protein subunits.

The subunit dissociation at the two lowest G protein concentrations was biphasic, as shown in semilog plots (Fig. 2B). From the slopes of the semilog plots, the half time of the rapid phase was found to be approximately 3 min and that of the slow phase was found to be about 30 min. As shown below, the rapid phase corresponds to dissociation of G protein trimers to the new equilibrium distribution of monomers, dimers, and trimers. The rate of this dissociation ($t_{1/2}$ = 3 min) agrees with the trimer dissociation rate determined previously by analyzing the kinetics of G protein subunit

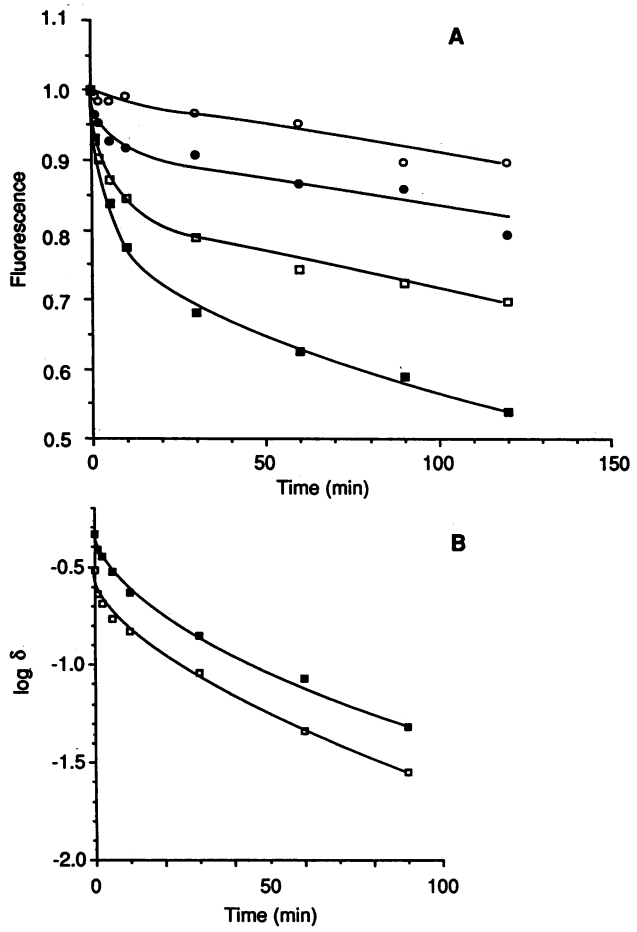

FIG. 2. Effect of dilution of labeled G protein on fluorescein fluorescence. Fluorescein-labeled G protein was diluted to a G protein subunit concentration of 4 (■), 12 (□), 40 (●), or 120 (○) nM in buffer containing 50 mM octyl glucoside, and the fluorescein fluorescence was determined at the indicated times after dilution. The fluorescence intensities of each sample were normalized to the initial fluorescence immediately after dilution (time = 0). (A) Normalized fluorescence as a function of time; (B) semilog plot of the data in panel A for 4 and 12 nM G protein, where δ is fluorescence at the indicated time minus final fluorescence at 120 min.

exchange (13). The slow phase corresponds to further dissociation of trimers due to changes in the subunits that prevent reassociation. While the rate of the rapid phase was highly reproducible, the half time of the slow phase was found to vary from about 10 min to longer than 2 h, although a half time of about 30 min was more typical.

The fluorescence decrease following dilution of the labeled G protein was presumably due to an increase in nonspecific quenching mechanisms in dimers or monomers compared with those in trimers. The fluorescence decrease was not accompanied by changes in the excitation or emission maxima, the fluorescence depolarization, or the pK_a of the fluorescein, which were virtually identical to published values (13). The relative fluorescence intensity of G protein dimers versus monomers could not be measured. Thus, the contributions of trimer versus dimer dissociation to the fluorescence changes could not be assessed. Nonetheless, the changes in fluorescein fluorescence provided a sensitive, rapid spectroscopic assay for the association state of G protein subunits.

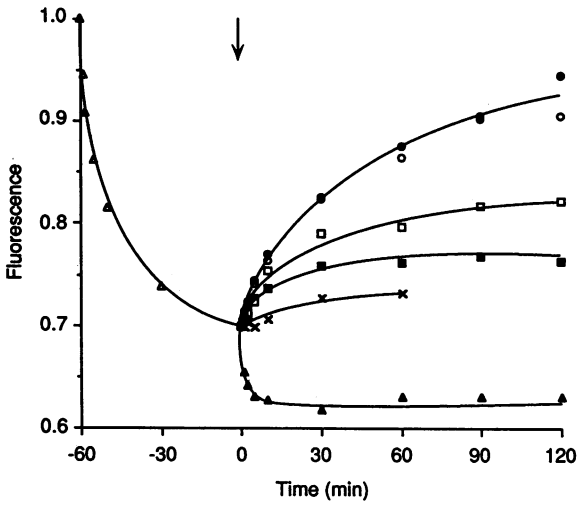

FIG. 3. Reversibility of fluorescence changes in labeled G protein. Fluorescein-labeled G protein isolated from VSV-IND was diluted to 4 nM (total G protein subunits) and incubated for 60 min (△). Unlabeled G protein from VSV-IND was added so that the total G protein concentration was 120 (○), 40 (●), 16 (□), or 12 (■) nM. Alternatively, unlabeled G protein from VSV-NJ (×) or rhodamine-labeled G protein from VSV-IND (▲) was added (40 nM). Fluorescein fluorescence was determined as a function of time of addition of excess G protein (arrow). Fluorescence intensities shown were normalized to the initial fluorescence immediately after dilution of the labeled G protein (time = −60 min).

Reversibility of dissociation of G protein subunits. The dissociation of labeled G protein was reversible when the G protein concentration was increased. Two different fluorescence assays were used to demonstrate the reassociation of labeled G protein subunits upon raising the G protein concentration. In the first, unlabeled G protein was added, which would be expected to increase the fluorescein fluorescence upon reassociation with labeled subunits. In the second assay, rhodamine-labeled G protein, which would undergo resonance energy transfer with fluorescein-labeled subunits upon reassociation, was added (13). This would result in a decrease in fluorescein fluorescence. Since resonance energy transfer between fluorescein and rhodamine requires that the labels be within about 5 nm, G protein subunits have to be in contact in order for energy transfer to occur. As shown in Fig. 3, labeled G protein was diluted to 4 nM, and the fluorescence was allowed to decay for 1 h. Upon addition of unlabeled G protein to a final concentration of 40 or 120 nM, the fluorescence recovered to near the initial level, and 50% recovery was obtained at a concentration of 12 to 16 nM (Fig. 3). Addition of unlabeled G protein from VSV-NJ did not reverse the fluorescence decrease, as expected, since it is incapable of forming trimers in the presence of octyl glucoside. No increase in fluorescence was observed when rhodamine-labeled G protein was added. In fact, the fluorescein fluorescence decreased further in the presence of rhodamine-labeled G protein because of resonance energy transfer to the rhodamine label. The results of the experiments in Fig. 3 show that dilution of the labeled G protein leads to dissociation of G protein subunits, which is reversible upon increasing the G protein concentration.

Even though addition of unlabeled G protein reversed the effects of dilution of labeled G protein, initial fluorescence levels in Fig. 3 were not obtained. The extent to which the

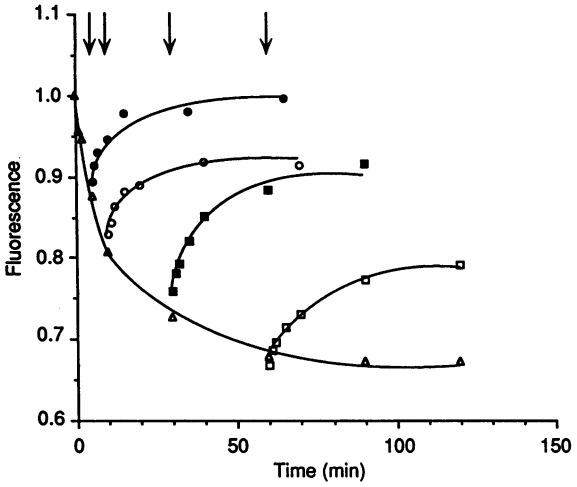

FIG. 4. Effect of time of dissociation on reversibility of G protein subunit dissociation. Fluorescein-labeled G protein was diluted to 4 nM (total G protein subunits). Unlabeled G protein (40 nM) was added (arrows) after 5 (●), 10 (○), 30 (■), or 60 (□) min, or no unlabeled G protein was added (△). Fluorescein fluorescence was determined at the indicated times after dilution. Fluorescence intensities of each sample were normalized to the intensity immediately after dilution (time = 0).

decline in fluorescence could be reversed depended on the length of time the G protein was incubated at a low concentration, as shown in Fig. 4. Fluorescein-labeled G protein was diluted to 4 nM and incubated for various times before unlabeled G protein was added to a concentration of 40 nM. The extent to which the fluorescence could be recovered upon addition of unlabeled G protein declined steadily over the course of a 1-h incubation at low concentration. This result indicates that prolonged incubation under conditions that promote dissociation of G protein subunits leads to changes in the G protein that prevent reassociation. Therefore, in subsequent experiments, dissociation of G protein subunits was allowed to proceed for only brief periods, generally 30 min or less.

Association of labeled G protein with M protein. M protein and nucleocapsids can be isolated from VSV after solubilization of the virus envelope with Triton X-100 in 0.25 M NaCl, which dissociates the M protein from the nucleocapsid. Alternatively, nucleocapsid-M protein complexes can be isolated by solubilizing the envelope at a low salt concentration. These complexes have the tightly coiled, bullet-shaped structure found in virions (17). VSV M protein, nucleocapsids, or nucleocapsid-M protein complexes were purified and tested for their ability to interact with fluorescein-labeled G protein. None of these preparations altered the fluorescence properties of labeled G protein when the G protein was at a concentration high enough to be primarily in the form of trimers. However, if labeled G protein trimers were first allowed to dissociate, an increase in fluorescence intensity was observed upon addition of M protein, either in the form of purified M protein (Fig. 5A) or nucleocapsid-M protein complexes (Fig. 5B). The purified M protein had approximately half the activity of the nucleocapsid-M protein complexes at equivalent M protein concentrations. In contrast, nucleocapsids had little effect on the fluorescence of labeled G protein unless added at an order-of-magnitude-higher concentration (Fig. 5C). This activity is presumably due to the 10 to 20% residual M protein that remained bound to the nucleocapsids. These data indicate that the M protein of VSV interacted with dissociated forms of the labeled G protein, resulting in an increase in the fluorescence intensity. As shown below, this fluorescence increase was due to reassociation of G protein subunits induced by M protein binding. In some experiments, such as that shown in Fig. 5, the M protein preparations were able to increase the fluorescence intensity above the level present immediately after dilution (i.e., above a value of 1.0 in Fig. 5). This is due to

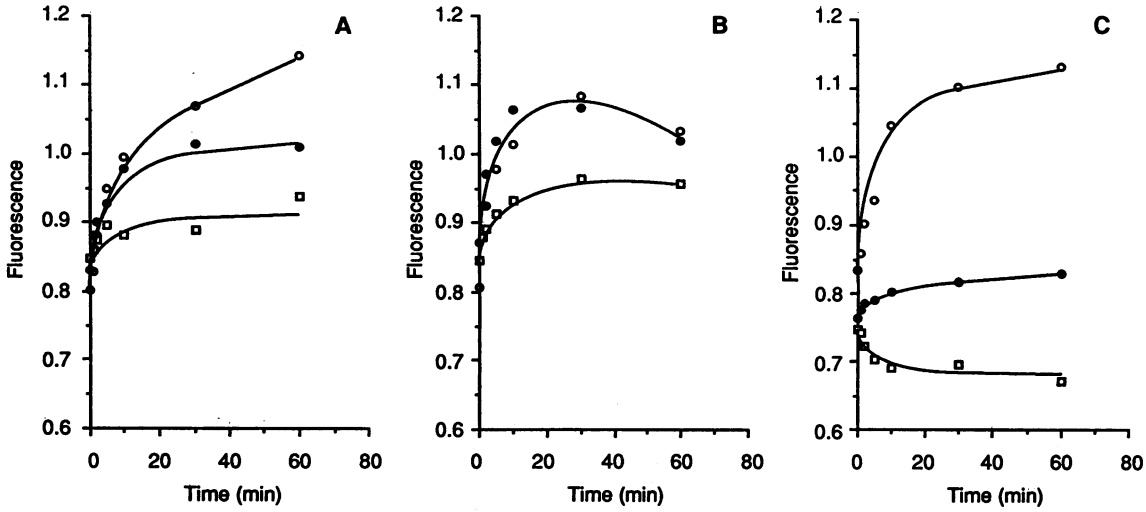

FIG. 5. Effect of M protein and nucleocapsids on fluorescence of labeled G protein. Fluorescein-labeled G protein was diluted to 6 nM (total G protein subunits) and incubated for 5 min. Either purified M protein (A) or nucleocapsid-M protein complexes (B) were added at an M protein subunit concentration of 40 (□), 120 (●), or 400 (○) nM. (C) Alternatively, nucleocapsids were added at equivalent N protein concentrations (nucleocapsid-M protein complexes are approximately 50% M protein by weight). Fluorescein fluorescence was determined at the indicated times after addition of M protein or nucleocapsids. Fluorescence intensities of each sample were normalized to the intensity immediately after dilution of the G protein.

TABLE 1. Effect of salt concentration and pH on interaction of G and M proteins

[NaCl] (mM)	pH	Normalized fluorescence[a]	
		Without M protein	With M protein
115	7.0	0.94	1.11
250	7.0	0.93	1.21
115	8.5	0.82	0.90

[a] Fluorescein-labeled G protein was diluted to a subunit concentration of 25 nM at the indicated pH and NaCl concentrations, and the fluorescence intensity was determined immediately after dilution and after a 10-min incubation. Purified M protein (120 nM) was added, and the fluorescence intensity was determined 10 min later. The fluorescence shown was normalized to the initial intensity immediately after dilution of the labeled G protein.

the fact that the G protein preparations are usually partially dissociated prior to dilution.

The experiments in Fig. 5 were performed at an ionic strength (65 mM NaCl) intermediate between the salt concentration necessary to maintain the integrity of nucleocapsid-M protein complexes (9) and that required to maintain the solubility of the purified M protein (14). At this NaCl concentration, about 50% of the purified M protein aggregates in the presence of octyl glucoside. However, the aggregated M protein does not appear to bind the labeled G protein, since centrifugation of the mixture of M and G proteins to pellet the aggregates does not remove any of the labeled G protein (data not shown). Furthermore, the purified M protein interacted with the labeled G protein in the presence of 0.25 M NaCl (Table 1), which maintains the M protein in a soluble form (14). M protein has little effect on labeled G protein at pH 8.5, the pH used to solubilize G protein from the virus envelope (Table 1). It has been shown that efficient solubilization of G protein from virions requires a pH above 8 (15). The data presented in Fig. 5 and Table 1 suggest that this pH dependence may be due to M protein binding. In further experiments (not shown), G protein-binding activity of either the purified M protein or nucleocapsid-M protein complexes declined steadily on subsequent days following isolation, presumably because of denaturation of the M protein. No binding activity was observed by 3 to 4 days following M protein isolation.

The increase in fluorescence of labeled G protein upon addition of M protein was due to reassociation of G protein subunits, as shown in resonance energy transfer experiments (Fig. 6). In the experiment shown in Fig. 6A, rhodamine- and fluorescein-labeled G proteins were first mixed at a 3:1 ratio and incubated for 30 min to allow the subunits to undergo exchange to form mixed trimers. The mixture was then diluted to 8 nM (total G protein subunit concentration) to induce trimer dissociation prior to the addition of purified M protein. As a control, unlabeled G protein was mixed with fluorescein-labeled G protein at a 3:1 ratio and was subjected to the same experimental protocol. Immediately after dilution, the fluorescence of the rhodamine-containing heterotrimers was about 20% less than that of the control mixture (time = 0 in Fig. 6A) because of fluorescence quenching via resonance energy transfer, as shown previously (13). The fluorescence of both samples declined over the course of 30 min following dilution. In the case of the rhodamine-containing mixture, this was due primarily to the dissociation of residual trimers that lack rhodamine. Little if any net change in fluorescein fluorescence should result from dissociation of rhodamine-containing heterotrimers, since the nonspecific

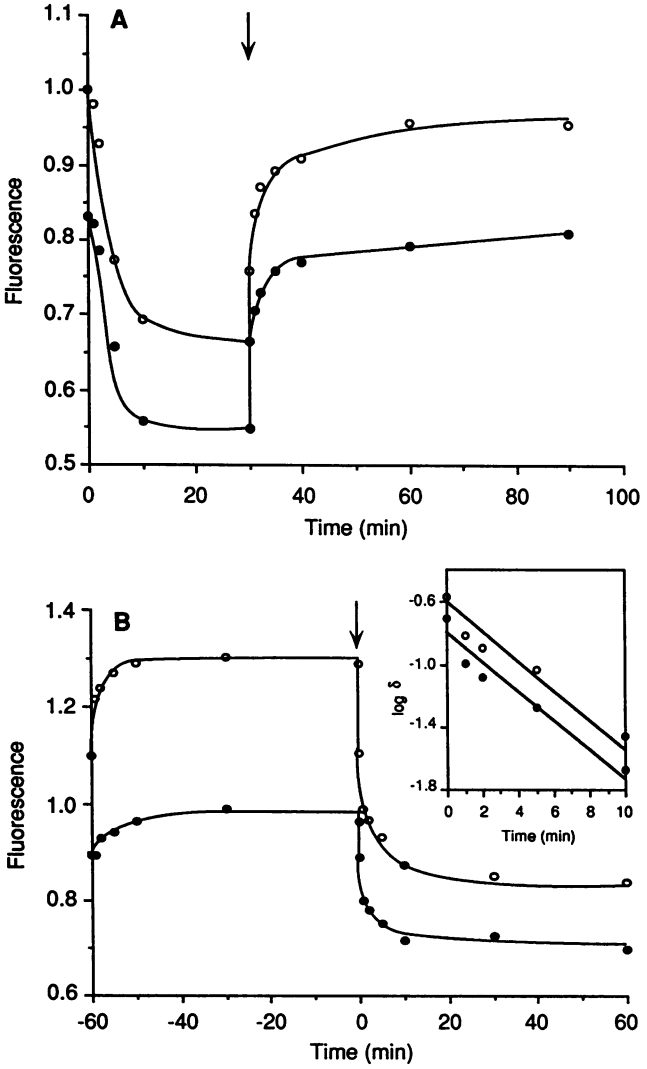

FIG. 6. Resonance energy transfer between labeled G proteins in the presence of M protein. (A) Fluorescein-labeled G protein was mixed at a 3:1 ratio with either rhodamine-labeled (●) or unlabeled (○) G protein at a total G protein subunit concentration of 1 μM for 30 min. The mixtures were then diluted to a final G protein subunit concentration of 8 nM. Fluorescein fluorescence was determined at the indicated times after dilution to 8 nM. After 30 min (arrow), purified M protein was added (final concentration = 500 nM). Fluorescence intensities of each sample were normalized to the initial fluorescence of the mixture lacking rhodamine-labeled subunits. (B) Fluorescein-labeled G protein was diluted to a G protein subunit concentration of 8 nM and incubated for 5 min. Either unlabeled G protein (8 nM) (●) or nucleocapsid-M protein complexes (200 nM M protein) (○) were added. After 60 min, rhodamine-labeled G protein (90 nM) was added to each sample. Fluorescence is shown as a function of time relative to the addition of rhodamine-labeled G protein (arrow). (Inset) Semilog plot of the data in panel B calculated as described in the legend to Fig. 2B.

quenching mechanism would compensate for the lack of energy transfer upon dissociation. Upon addition of M protein, the fluorescence intensity of both samples increased. However, in the case of the rhodamine-containing mixture, the fluorescence intensity only returned to the initial quenched level rather than increasing to the level

TABLE 2. Effect of octyl glucoside concentration on interaction of G protein with serum albumin and M protein[a]

Protein	Protein concn (μg/ml)	% Increase in fluorescence at:	
		50 mM OG	150 mM OG
BSA	1	8.6	5.5
	3	39	18
	10	34	30
NCM	0.8	21	19
	2.4	32	38
	8.0	39	63

[a] Fluorescein-labeled G protein was diluted to a subunit concentration of 4 nM at the indicated concentration of octyl glucoside (OG) and incubated for 10 min. Bovine serum albumin (BSA) or nucleocapsid-M protein complexes (NCM) were added at the indicated concentrations, and the fluorescence intensity was determined as a function of time. Data shown are the percent increase in fluorescence determined after 30 min over the intensity just prior to addition of BSA or NCM.

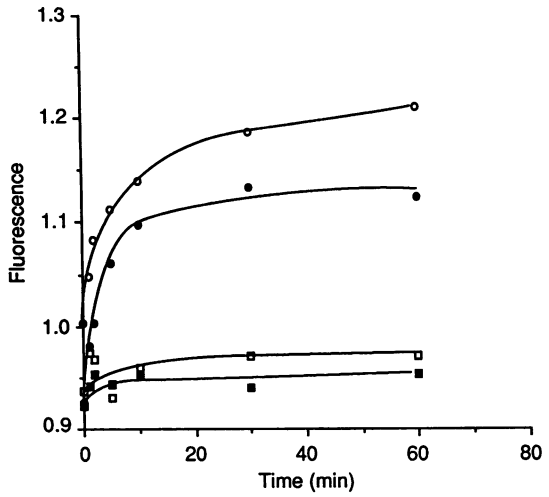

FIG. 7. Serotype specificity of the interaction between G and M proteins. Fluorescein-labeled G proteins were prepared from either VSV-IND (○, ●) or VSV-NJ (□, ■). Labeled G proteins were diluted to a subunit concentration of 5 nM and incubated for 30 min. Nucleocapsid-M protein complexes isolated from either VSV-IND (○, □) or VSV-NJ (●, ■) were added (200 nM M protein), and the fluorescence intensity was determined at the indicated times after addition of M protein. Intensities were normalized to the initial intensity after dilution of the labeled G protein.

achieved in the control mixture. This result shows that upon binding to M protein, the labeled G protein subunits could undergo resonance energy transfer and must, therefore, have reassociated.

A similar conclusion was reached in the experiment shown in Fig. 6B. In this case, fluorescein-labeled G protein was diluted to 8 nM to induce subunit dissociation, and then either unlabeled G protein or nucleocapsid-M protein complexes were added. After a 60-min incubation, rhodamine-labeled G protein was added. The fluorescence intensity of both samples declined after addition of rhodamine-labeled G protein, indicating that the G protein was able to undergo subunit exchange so that resonance energy transfer between fluorescein- and rhodamine-labeled subunits could take place. The time course of the subunit exchange was virtually identical in the presence or absence of nucleocapsid-M protein complexes, as shown by semilog plots of the data (Fig. 6B, inset), which had identical slopes corresponding to half times of 2 to 3 min. This result provides further evidence that upon binding to M protein, the G protein subunits are also bound to each other. Furthermore, the binding of G protein to M protein must be reversible, since the G protein subunits must dissociate in order to undergo exchange with rhodamine-labeled subunits.

Specificity of the interaction between G and M proteins. The experiments shown in Fig. 6 indicate that M protein binding enhanced the interaction of G protein subunits. However, they do not address the specificity of this effect, since the G protein subunit interaction is sensitive to many factors such as type of detergent and pH (4, 13). A survey of the effects of nonviral proteins on the fluorescence of labeled G protein revealed that serum albumin also bound to G protein and enhanced the subunit interaction, as shown in Table 2. The activity of serum albumin is presumably due to its ability to bind lipids or detergents associated with the labeled G protein and differs from the activity of the M protein as shown by the effect of increasing the concentration of the detergent octyl glucoside. Fluorescein-labeled G protein was diluted to 4 nM to induce subunit dissociation in the presence of either 50 or 150 mM octyl glucoside. After a 10-min incubation, either serum albumin or nucleocapsid-M protein complexes were added at various concentrations, and the increase in fluorescein fluorescence was determined (Table 2). The higher detergent concentration competitively inhibited the binding of serum albumin to the labeled G protein, as shown by the amount of albumin required to give maximal fluorescence. In contrast, the binding of the nucleocapsid-M protein complexes to labeled G protein was actually enhanced at the higher octyl glucoside concentration. These data suggest that the effect of serum albumin was mediated by binding to detergent associated with the G protein, while the effect of M protein was not due to binding to octyl glucoside.

Further evidence for the specificity of M protein binding to G protein was provided by analyzing the binding of M protein to heterologous viral glycoproteins. The serotype specificity of the interaction between the G and M proteins was tested by mixing proteins isolated from VSV-IND and VSV-NJ in homologous and heterologous combinations. Fluorescein-labeled G proteins from VSV-IND and VSV-NJ were incubated at a G protein subunit concentration of 4 nM for 30 min to allow the subunits to dissociate and then mixed with nucleocapsid-M protein complexes isolated from the two virus serotypes (Fig. 7). The nucleocapsid-M protein complexes from both serotypes induced the reassociation of subunits of the VSV-IND G protein indicated by an increase in fluorescence intensity. The nucleocapsid-M protein complexes from VSV-NJ were only slightly less effective than those from VSV-IND. In contrast, only minimal changes in fluorescence of the labeled G protein from VSV-NJ were observed upon addition of nucleocapsid-M protein complexes from either serotype. These results indicate that the interaction of the VSV-IND G protein with M protein is not serotype specific. The lack of response of the VSV-NJ G protein is presumably due to the fact that it remains monomeric in octyl glucoside even in the presence of M protein.

Even though the VSV-NJ G protein remained monomeric, it was still capable of binding to M protein as shown by its ability to compete for the binding of labeled VSV-IND G protein. Fluorescein-labeled G protein from VSV-IND was incubated at a G protein subunit concentration of 4 nM for 10

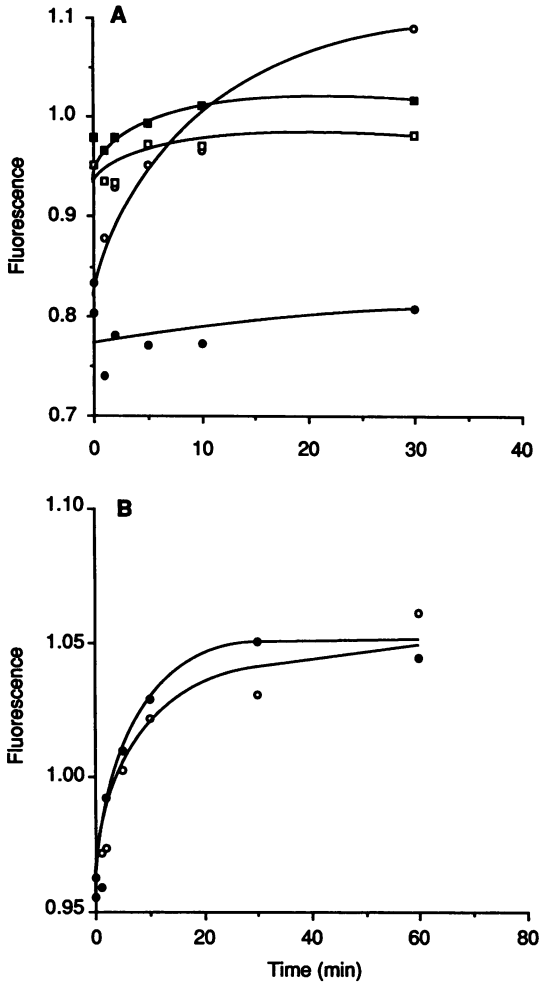

FIG. 8. Association of VSV-IND G protein with M protein in the presence of VSV-NJ G protein (A) or influenza virus HA (B). (A) Fluorescein-labeled G protein from VSV-IND was diluted to a subunit concentration of 4 nM either in the presence (●, ■) or absence (○, □) of unlabeled G protein from VSV-NJ (80 nM). Nucleocapsid-M protein complexes isolated from VSV-NJ (50 nM M protein) (○, ●) or serum albumin (3 μg/ml) (□, ■) were added, and the fluorescence intensity was determined as a function of time. Intensities were normalized to the initial fluorescence immediately after dilution of labeled G protein. (B) Fluorescein-labeled G protein from VSV-IND was diluted to a subunit concentration of 5 nM either in the presence (●) or absence (○) of unlabeled influenza virus HA (300 nM). Purified M protein from VSV-IND (20 nM) was added, and the fluorescence intensity was determined as a function of time after addition of M protein.

min in the presence of unlabeled G protein from VSV-NJ (80 nM) and then mixed with nucleocapsid-M protein complexes isolated from VSV-NJ (Fig. 8A). The unlabeled VSV-NJ G protein prevented the fluorescence increase of the labeled VSV-IND G protein in response to addition of nucleocapsid-M protein complexes from VSV-NJ. This result indicates that the VSV-NJ G protein can bind to M protein even though it is monomeric. Similar inhibitory activity was observed by using nucleocapsid-M protein complexes from VSV-IND (not shown). The interaction of serum albumin with labeled G protein from VSV-IND was not affected by excess unlabeled G protein from VSV-NJ (Fig. 8A).

The lack of serotype specificity in the interaction of the VSV G and M proteins raises the question of whether the VSV M protein can interact with other viral glycoproteins whose structures are similar to that of the G protein, such as the influenza virus HA. The HA protein is also a trimer of identical subunits, but in contrast to the G protein, the HA subunit interaction is sufficiently strong that subunit dissociation and reassociation do not occur in vitro (2). Thus, it is not practical to measure the effect of the VSV M protein on the subunit interaction of HA, nor is it certain that if the VSV M protein were to bind to HA the effect would be to enhance the subunit interaction. Therefore, the ability of HA to compete with G protein for M protein binding was tested. Fluorescein-labeled G protein was incubated at a concentration of 5 nM for 10 min in the presence or absence of excess purified HA (300 nM). Purified VSV M protein was added to each sample, and the time course of the fluorescence increase was determined (Fig. 8B). A minimal concentration of M protein (20 nM) was used in a range where the fluorescence change is dependent on the M protein concentration (Fig. 5A; note the expanded scale in Fig. 8) so that a decrease in the effective M protein concentration due to binding to HA would be readily observed. Despite the large molar excess of HA above the concentration of both the M protein and the G protein, there was no inhibitory effect of HA on the M protein-induced reassociation of G protein subunits. Thus, if the VSV M protein binds to HA, it neither occupies the same site as the G protein nor does it sequester the M protein from effective interaction with the G protein.

DISCUSSION

The conclusion that the VSV M protein enhances the association of G protein subunits critically depends on the validity of the fluorescence assays as a measure of the G protein subunit interaction. Essentially, there were two different assays used in the present study, resonance energy transfer between fluorescein- and rhodamine-labeled subunits and fluorescence quenching of fluorescein-labeled G protein upon subunit dissociation. In order for resonance energy transfer to occur, fluorescein and rhodamine probes must be within about 5 nm, which means that the labeled G protein subunits have to be in contact. This assay is, therefore, a direct measurement of subunit interaction. Its validity was shown in our previous study of G protein subunit exchange by comparison with more traditional measurements of subunit interaction such as sedimentation velocity and chemical cross-linking (13).

The second assay, quenching of fluorescein fluorescence upon subunit dissociation, was not predicted but rather was an empirical observation of the properties of fluorescein-labeled G protein. The mechanistic basis for this quenching has not been fully established. Presumably, it is an indirect effect of subunit dissociation that alters the environment surrounding the fluorescein label so as to make it more susceptible to naturally occurring (presumably nonspecific) quenching mechanisms. Similar changes have been observed with other fluorescein-labeled proteins, as well as a variety of other protein-linked fluorophores, when the proteins undergo changes in conformation (see references 1 and 16 for recent examples). The validity of this phenomenon as an assay for G protein subunit interaction is supported by the following observations. The fluorescence change is correlated with the extent of dilution of the labeled G protein in the range of the trimer dissociation constant (Fig. 2). It is reversible upon elevation of the G protein concentration to

promote subunit association (Fig. 3). Quenching is not reversed by the VSV-NJ G protein, which is incapable of subunit interaction (Fig. 3); it is not reversed by association with rhodamine-labeled subunits, in which quenching by resonance energy transfer compensates for the reversal of the nonspecific quenching mechanisms (Fig. 3).

Even though an interaction between viral matrix proteins and envelope glycoproteins has been postulated for many years as a basic mechanism of virus assembly that is common to a wide variety of enveloped viruses, there has been surprisingly little direct evidence for such an interaction. A few reports of in vitro binding of viral glycoproteins to internal virion components have been published (e.g., references 8 and 22). However, attempts to study the interaction of isolated components have traditionally been plagued by difficulty in maintaining viral matrix proteins in a soluble form and by doubts as to whether the native structure is maintained after isolation. Finally, in retrospect, since the interaction between the VSV M and G proteins appears to be readily reversible, it is not easily studied by assays that require lengthy times for separation (such as sedimentation) and that are qualitative rather than quantitative (such as chemical cross-linking). The present study was made possible by the development of procedures for isolation of VSV nucleocapsid-M protein complexes in which the native structure was clearly retained (17), procedures for maintaining the purified M protein in a soluble form (14), and the fluorescence assays for G protein subunit interaction, which have a quantitative basis and measure the interactions in real time. In addition, recent progress in defining the specificity of glycoprotein incorporation into VSV virions in vivo (21) made possible the proper specificity controls for this in vitro study. The following evidence (discussed below) supports the contention that the interaction between the VSV G and M proteins in vitro is relevant to the mechanism of virus assembly and does not represent nonspecific interaction resulting from disruption of the native virus structure: (i) M protein binding had the same specificity as virus assembly in vivo, (ii) the interaction between the G and M proteins was reversible, and (iii) it required M protein in a native configuration and did not require nucleocapsids.

The specificity of assembly of viral glycoproteins into virions in vivo has remained a question for many years. The envelope glycoproteins of many viruses can be incorporated into the VSV envelope to produce phenotypically mixed particles upon coinfection. This apparent lack of specificity is paradoxical considering the fact that host proteins are largely excluded from the VSV envelope (reviewed in reference 24). Using viral glycoproteins expressed from recombinant vectors, Whitt et al. (21) showed that G proteins from either VSV-IND or VSV-NJ could complement the temperature-sensitive VSV-IND G protein mutant *ts*045. The influenza virus HA, which can complement *ts*045 in a coinfection, is not able to complement the mutant when it is expressed in the absence of other influenza virus proteins. The reasons for this apparent discrepancy are not clear. However, the results presented here show that the in vitro interactions of the VSV M protein have a specificity similar to that observed by Whitt et al. (21) for virus assembly in vivo; namely, the interaction with the G protein was not serotype specific, but no functional interaction with the influenza virus HA could be detected. These results suggest that the VSV M protein binds specifically to the G protein and that phenotypic mixing between VSV and influenza virus is due either to a low-affinity interaction between HA and the VSV M protein or else to interaction of HA with some other viral component.

The results of this study show that binding of M protein stabilizes the G protein subunit interaction. The key data indicating that M protein binding induces association of G protein subunits are from resonance energy transfer experiments (Fig. 6), in which the subunits must be associated in order for energy transfer to occur. This conclusion was supported by the lack of effect of M protein on the VSV-NJ G protein (Fig. 7), which appeared to be unable to form trimers under these conditions. It is not clear why the VSV-IND and VSV-NJ G proteins differ in the stability of their subunit interactions. One possibility is that the subunit affinity of the VSV-NJ G protein may be too weak to be detected under the conditions used here. It is likely that binding of M protein to dissociated G protein subunits induces the formation of G protein trimers, since that appears to be the native form in virions. However, the resonance energy transfer experiments did not provide information on the stoichiometry of the interaction, so that the formation of G protein dimers or oligomers of an order higher than trimers upon M protein binding remains a formal possibility.

The observation that M protein binding enhanced the G protein subunit interactions is not, in itself, an argument for the specificity of M protein binding. The interaction of G protein subunits can be altered by binding different detergents (13) and lipids (unpublished data) as well as proteins such as serum albumin (Table 2), which presumably acts by binding to detergent or lipid molecules associated with the G protein. However, in contrast to agents that act indirectly on the G protein, the binding of M protein could not be competed with by excess detergent (Table 2). The ability of the VSV-NJ G protein to compete with the VSV-IND G protein for binding to M protein (Fig. 8A) provides further evidence that the M protein does not act indirectly by binding to detergents or lipids.

Expression of M protein in vivo is not essential for formation of G protein trimers, since expression of G protein in the absence of other VSV proteins can lead to trimer formation (5). However, the reversible dissociation and reassociation of G protein subunits observed in vitro have also been shown to occur in vivo (23). A mechanism for stabilization of the G protein subunit interaction would have obvious advantages for the stability of the virion. G protein monomers appear to be inherently less stable than trimers and undergo presumably irreversible changes that prevent reassociation (Fig. 4). This probably accounts for the fact that G protein isolated under a variety of conditions is often found to be monomeric (3, 4).

As in the case of the G protein subunit interaction, the interaction between the G and M proteins was found to be reversible. This was shown by the ability of fluorescein-labeled G protein subunits bound to M protein to undergo exchange with rhodamine-labeled G protein (Fig. 6B), which requires dissociation and reassociation of labeled subunits (13). Other less direct evidence for the reversibility of the interaction was obtained when we tried to analyze the binding of G protein and the nucleocapsid-M protein complex by sedimentation in sucrose gradients. The G protein was distributed throughout the gradient rather than cosedimenting with the nucleocapsid-M protein complexes, suggesting that it had dissociated during sedimentation (unpublished experiments). The reversibility of the interaction between the G and M proteins has interesting biological implications. While it might seem that the process of virus

assembly should be driven by essentially irreversible interactions in order to enhance the stability of the virion, it is also necessary for the virion to undergo disassembly during virus penetration upon initiation of a new infection. Rigaut et al. (18) have recently shown that following penetration of VSV by fusion of the virus envelope with endosome membranes, the M protein dissociates from the other virion components and is distributed throughout the cell cytoplasm, indicating that the binding of M protein to other virion components must be reversible in vivo. They proposed that this dissociation might be driven by the laws of mass action due to the dilution of M protein into a cellular environment devoid of preexisting M protein. Our in vitro results would be entirely consistent with such a model.

By using purified components, it could be shown that the G protein interacts directly with the M protein and does not require the participation of the nucleocapsid (Fig. 5). The purity of the M protein preparation is quite high (e.g., see reference 14), so that its activity was unlikely to be due to contaminating components. However, it is difficult to completely remove the M protein in the nucleocapsid preparation, and the activity of nucleocapsids (Fig. 5C) is easily accounted for by the residual M protein. Nucleocapsid-M protein complexes were slightly more effective at interacting with G protein than was the purified M protein on a molar M protein basis. However, this difference could be accounted for by the fact that the nucleocapsid-M protein complex provides multivalent binding and by the fact that the purified M protein is partially aggregated under the conditions at which the two preparations could be compared. Thus, it appears likely that the M protein is the internal virion component that is solely responsible for interacting with the G protein in vitro, although it is possible that binding of M protein to nucleocapsids may enhance this interaction.

Finally, the availability of an in vitro system for studying the interaction of the G and M proteins allows mechanistic aspects of this interaction to be addressed. The dissociation of G protein subunits and their reassociation induced by M protein binding behave like simple reversible reactions in dynamic equilibrium. However, this system is complicated by the fact that it is actually made up of a series of separate equilibria as follows:

$$G_3 \overset{1}{\rightleftharpoons} G_2 + G \overset{2}{\rightleftharpoons} 3G \overset{3}{\rightleftharpoons} 3(GM_n) \overset{4}{\rightleftharpoons} (GM_n)_2 + GM_n \overset{5}{\rightleftharpoons} (GM_n)_3 + 3n(M)$$

where G_3, G_2, and G are G protein trimers, dimers, and monomers, respectively, and n is the ratio of M protein to G protein subunits in the complex (GM_n). The fluorescence techniques cannot show the stoichiometry of the different interactions, only that the interactions exist. Thus, the G protein dissociation experiments (e.g., Fig. 2, 3, and 4) measure a combination of reactions 1 and 2, and the experiments with M protein measure reactions 3, 4, and 5 as well as possible higher-order structures such as dimers of the G protein-M protein complex, etc. The data show that M protein binds to dissociated G protein subunits in order to induce subunit association (i.e., that reactions 3, 4, and 5 occur), rather than binding only to G protein trimers and inducing subunit association by mass action. The G protein subunit exchange rate is governed by the rate of dissociation of G protein trimers (13) and is unchanged by M protein binding (Fig. 6B). Therefore, M protein must bind to dissociated G protein subunits and promote the formation of trimers by increasing the rate of subunit association.

Despite the complexity of these interactions, the experiments presented here allow the equilibrium constants for these reactions to be estimated. For example, from Fig. 2 and 3, it can be estimated that the concentration of G protein at which 50% dissociation occurs is in the range of 12 nM total G protein subunits. This corresponds to a trimer concentration of approximately 2 nM. Thus, the trimer dissociation constant is likely to be approximately 10^{-9} to 10^{-8} M. Likewise, from Fig. 5, it can be estimated that 50% reactivity with M protein occurs at a concentration of around 20 nM total M protein subunits, so that the dissociation constant for the G protein-M protein complex must also be in this order of magnitude. We have estimated that the intracellular concentration of M protein during the peak times of virus assembly is in the range of 10^{-6} to 10^{-5} M (unpublished data), so that the affinities measured in vitro would be sufficient to drive the association of G and M proteins in vivo.

In conclusion, all of the properties of the in vitro reaction of M and G proteins described so far are consistent with the function of M protein in vivo. These properties include specificity, reversibility, and affinity. These results strongly suggest that the interactions described here are, in fact, the ones that govern virus assembly.

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