Localization of P protein binding sites on the Sendai virus nucleocapsid

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Previous studies have shown that the molecules of P protein associated with transcriptionally active Sendai virus nucleocapsids are arranged in discrete clusters. Our study investigates whether or not this localized distribution is due to the existence of only a few P protein binding sites on the nucleocapsid core. We used immunoelectron microscopy to examine whether additional P proteins could bind at locations between the groups of endogenous P proteins. To differentiate between endogenous and added proteins, we constructed a recombinant gene which instructs the in vitro synthesis of a chimeric protein containing the carboxyl-terminal nucleocapsid-binding region of P protein, fused to chloramphenicol acetyltransferase (CAT). Immunogold labelling, using an antibody to the CAT moiety, revealed at the electron microscope level, that the chimeric product bound to nucleocapsids at many sites located over the entire length of the nucleocapsid. This indicated that the localized distribution of P protein molecules is not due to a limited number of P protein binding sites on the nucleocapsid core.

Paramyxovirus P proteins are structural components of nucleocapsids which are required for viral RNA synthesis (Hamaguchi et al., 1983; Deshpande & Portner, 1985). Previous immunoelectron microscopic studies have shown that these P proteins are distributed uniformly on nucleocapsids obtained from Sendai virions. However, P proteins on nucleocapsids extracted from infected cells have a distinctly localized distribution: they are found attached to the nucleocapsid in groups, at four to 10 discrete locations at random intervals along the length of the nucleocapsid (Portner & Murti, 1986; Portner et al., 1988). Presumably the P protein distribution is related to the metabolic state of the nucleocapsid, as nucleocapsids in virions are functionally inert whereas those in infected cells are actively engaged in RNA synthesis. The localized distribution could be due to the existence on the transcriptionally active nucleocapsid of only a few discrete sites where P proteins may bind. Alternatively, it could be due to a function of the P protein itself, or to a function of some other factor present in the infected cell. In an attempt to discriminate among these possibilities, we have added chimeric P protein molecules to nucleocapsids extracted from infected cells and examined their topographical distribution by immunoelectron microscopy.

Exogenous P proteins can be added to nucleocapsids in vitro, even when the endogenous P proteins are not removed first (Ryan & Kingsbury, 1988). We could distinguish these added proteins from the endogenous molecules if we could make them immunologically distinct without altering their ability to bind. All the residues required for P protein binding to nucleocapsids are near the carboxyl terminus of P, and deletion of residues near the amino terminus has no effect on binding (Deshpande & Portner, 1985; Ryan & Kingsbury, 1988). Therefore, we removed a small part of the P gene cDNA from the in vitro transcription plasmid pGEM1-P31 (Ryan & Kingsbury, 1988). The removed sequences encoded the 77 amino-terminal residues of the P protein; these were replaced with most of the gene encoding chloramphenicol acetyltransferase (CAT), as depicted in Fig. 1(a). In vitro transcription and translation (Ryan & Kingsbury, 1988) produced the 3H-labelled protein shown in Fig. 1(b). The size of the protein was increased relative to normal P protein, because the recombinant gene had lost the nucleotides encoding 77 amino acids of P, and gained the nucleotides encoding 210 amino acids of the CAT protein. The chimeric product had epitopes specific to both P and CAT proteins, since it could be immunoprecipitated by rabbit antiserum to CAT protein (5Prime-3Prime, Incorporated), or by the monoclonal antibody M5 to the P protein (Deshpande & Portner, 1985), as shown in Fig. 1(c).

To confirm that the chimeric protein could attach to nucleocapsids, radiolabelled protein was mixed with lysates of mock-infected or virus-infected cells. Mixtures were fractionated on density step gradients, so that free protein remained at the top of the gradient and nucleocapsid-bound protein was found above the bottom density cushion (Ryan & Kingsbury, 1988). Gradient
Fig. 1. Chimeric P protein. (a) Construction of a recombinant P gene. Coding regions were cleaved at the indicated restriction enzyme sites. Scal cleavage generated a blunt end; AccI cleavage was followed by filling in with Klenow DNA polymerase which generated a blunt end. Ligation of these gene fragments fused the two coding regions in-frame.

(b) In vitro translation products. The recombinant gene and the normal P gene were transcribed by SP6 RNA polymerase, and the transcripts were translated in a reticulocyte lysate containing [3H]leucine. Products were fractionated in 10% polyacrylamide-SDS gel and fluorographed. (Lane 1, normal P protein; lane 2, chimeric protein.) The positions of Mr marker proteins are shown on the left. (c) Immunoprecipitation of the chimeric protein. Normal P protein and chimeric protein were mixed together and immunoprecipitated with antibody to P protein (lane 1) or to CAT protein (lane 2). The chimeric product possessed epitopes specific to each protein moiety.

Fig. 2. Binding of chimeric protein to nucleocapsids. 3H-labelled chimeric protein was mixed with viral nucleocapsids (lanes T2 and B2) or with a mock-infected cell lysate (lanes T1 and B1). After density gradient separation of free and nucleocapsid-bound protein, samples were fractionated in a 10% polyacrylamide-SDS gel and fluorographed. Free protein was found at the top (T) of the density gradient (lane T1), and nucleocapsid-bound protein was found on the bottom (B) density cushion (lane B2).

samples containing free or bound chimeric protein were analysed by SDS–PAGE, and fluorographed (Fig. 2). When mixed with a mock-infected cell lysate, the radiolabelled protein remained soluble and was recovered from the top of the density gradient (lane T1). When mixed with a lysate of virus-infected cells, the chimeric protein was able to attach to nucleocapsids, and was found in the bottom gradient fraction (lane B2). CAT protein alone did not bind to nucleocapsids (data not shown).

To detect the location of endogenous P protein molecules and exogenous chimeric molecules on nucleocapsids, samples were examined by the immunogold
Fig. 3. Immunogold localization of bound P protein on nucleocapsids. (a) Endogenous P protein. Nucleocapsids from infected cells were adsorbed to EM grids and treated with monoclonal antibody to P protein, followed by gold-conjugated goat anti-mouse antibodies. (b) Chimeric P protein. Nucleocapsids from infected cells were mixed with an in vitro translation mixture containing chimeric P protein, and were then adsorbed to EM grids. Samples were treated with rabbit antiserum to CAT protein, followed by gold-conjugated goat anti-rabbit antibodies. (c) Negative control. Nucleocapsids without chimeric protein added were adsorbed to EM grids and treated with rabbit antiserum to CAT protein, followed by gold-conjugated goat anti-rabbit antibodies. (d) Full-size P protein. Nucleocapsids from infected cells were mixed with normal P protein translated in vitro, adsorbed to EM grids, and treated with monoclonal antibody to P protein followed by gold-conjugated goat anti-mouse antibodies. This procedure marked the locations of both the endogenous P proteins and the added molecules. Bar marker represents 0.2 μm.

Labelling technique. Nucleocapsids with or without chimeric protein added were first incubated with rabbit antiserum to CAT or with monoclonal antibody to P, respectively. Samples were then incubated with gold-conjugated anti-rabbit or anti-mouse antibodies. A variety of controls were maintained to ensure that all the reagents used were specific. These included the elimination of the primary antibody from the procedure, or substituting it with an irrelevant antibody. Fig. 3(a) shows the distribution of the endogenous P proteins localized by the immunogold labelling method. As described previously (Portner & Murti, 1986), the endogenous P proteins had a localized distribution on the nucleocapsid. In contrast, Fig. 3(b) shows that the added chimeric molecules were able to bind at many other sites, as revealed by the more uniform distribution of gold particles. Fig. 3(c) shows a control experiment in which the nucleocapsids were not incubated with the chimeric protein prior to immunogold labelling with anti-CAT antibody: non-specific labelling of nucleocapsids was not detected.

It is possible that the molar ratio of P protein to nucleocapsid may influence the specificity of binding, so that a localized distribution could form only near a certain concentration of P. To address this point we estimated the molar ratios of chimeric protein and nucleocapsid present in our in vitro binding experiments. Since Fig. 2 shows that nearly all radiolabelled protein becomes bound, this estimate can be made by counting the gold-labelled P proteins in the immunogold electron micrographs. Fig. 3(a) shows 130 gold grains marking the locations of native P protein molecules, whereas Fig. 3(b) shows 170 grains marking the locations of chimeric molecules. We assume that after fixation of the nucleocapsids to the electron microscope (EM) grid, only half of the P proteins are accessible to antibody labelling.
Therefore we estimate that about 340 molecules of the chimeric protein bind to a nucleocapsid, compared to about 260 molecules of native P protein detected by the same technique. If 95% of the \(^{3} \text{H}\)-labelled P protein bound (suggested by the band intensities in Fig. 2), then the ratio of all chimeric protein (bound and unbound) to each nucleocapsid would be about 360 : 1. The molar ratio determined by electron microscopy of chimeric molecules to nucleocapsids is similar to the biochemically determined value of 300 P molecules per nucleocapsid (Lamb et al., 1976). This indicates that the observed uniform distribution is not caused simply by a chimeric P protein : nucleocapsid ratio which is either far too high or too low.

Portner & Murti (1986) observed that P protein molecules were clustered on infected cell nucleocapsids, and the location of the P proteins differed on individual nucleocapsids. These observations suggested that P functions in a concerted fashion, and that the proteins can move along the length of the nucleocapsid. It was then unclear whether this P protein distribution was due to the existence of a few movable binding sites on the nucleocapsid core, or to mobile P proteins which could bind to the core at any location. It appears from our current results that the localized distribution is not due to the existence of only a few discrete P protein binding sites on the transcriptionally active nucleocapsid core, since the added chimeric proteins were able to attach at many sites between the groups of endogenous P proteins.

If P proteins are not restricted to bind only at discrete sites on the nucleocapsid, what then might cause their localized distribution? Our data show that this distribution does not rely solely on the amino acids near the carboxyl terminus of the P protein which are responsible for binding to nucleocapsids. Since the chimeric protein was able to bind but did so at many sites, the nucleocapsid-binding region of the P protein is not by itself sufficient to form a localized distribution. It is possible that some other part of the P protein may be involved, instead of or in addition to the nucleocapsid-binding regions near the carboxyl terminus. If this is the case, the deletion of amino-terminal P protein residues and their replacement with CAT residues may have abolished this activity. To test this possibility, we added full-size P protein synthesized in vitro to nucleocapsids. These added molecules, as well as the native P proteins already on the nucleocapsids, were visualized by immunogold staining with an antibody to the P protein (Fig. 3d). The full-size protein bound in a dispersed fashion, filling in the regions between clusters of endogenous P protein as the chimeric protein had done. This showed that the distribution of chimeric protein was not due to the absence of amino-terminal residues, as the full-size product displayed the same distribution. The presence of the amino-terminal end of P protein is not sufficient to confer the localized distribution to proteins added in vitro.

Alternative explanations of the localized distribution are that it may result from P protein interactions during the infectious cycle with other proteins present in the infected cell such as the L protein, or it may be due to changes in P which occur at some time during infection. Whether the distribution is due to the P protein itself or to some other factor, our results show that the availability of P protein binding sites on the nucleocapsid is not the determining element.

Mary Ann Buchanan and Curtis Herring provided expert technical assistance. This work was supported by Research Grant AI 05343 from the National Institute of Allergy and Infectious Diseases, by Cancer Center Support Grant CA 21765 from the National Cancer Institute, by American Cancer Society Grant CD 253, and by American Lebanese Syrian Associated Charities of St Jude Children's Research Hospital.

References


(Received 2 August 1989; Accepted 3 January 1990)