Genetic Analysis of Interactions between Gag Proteins of Rous Sarcoma Virus

XINGQIANG LI, BING YUAN, AND STEPHEN P. GOFF*

Howard Hughes Medical Institute and Department of Biochemistry and Molecular Biophysics, Columbia University College of Physicians and Surgeons, New York, New York 10032

Received 5 August 1996/Accepted 17 March 1997

The yeast two-hybrid system was used to characterize homomeric interactions between the Gag proteins of Rous sarcoma virus (RSV). The RSV Gag precursor was found to interact strongly with itself and not with various control proteins. The RSV Gag did not interact significantly with Gag proteins of a variety of other retroviruses, including murine leukemia viruses and primate lentiviruses. Deletion analysis suggested that two nonoverlapping regions are independently sufficient to mediate RSV Gag-Gag dimerization. One such region lies near the N terminus and contains p2, p10, and a large N-terminal part of the capsid (CA) domain; the other is localized in the C terminus and includes a small C-terminal portion of CA and the nucleocapsid protein. These interaction domains may play roles in viral assembly.

The major structural proteins of retroviral virion particles are encoded by the gag gene (37). Expression of the Gag precursor alone in cells results in the production of virus-like particles in cell culture medium, suggesting that Gag is sufficient, even in the absence of other viral proteins, to mediate the assembly and release of particles (9, 30, 38); for this reason, Gag has been termed a “particle-making machine” (11). The Gag proteins are synthesized as a polyprotein precursor that is posttranslationally cleaved by the viral protease into the separate proteins found in mature virions (2, 32, 33). The importance of various portions of the Gag precursor in viral assembly has been determined in several experimental systems (8, 28, 29, 35, 36). It is likely that Gag-Gag contacts are important both for the multimerization of the Gag precursor and, after proteolytic cleavage of the precursor into the mature products, for the infectivity of the virion particle.

One of the most intensively studied Gag proteins is the Gag of the Rous sarcoma virus (RSV), which has been subjected to mutational analysis to localize regions required for virion assembly and release (7, 35). At least three domains of the RSV Gag protein have been identified as required for the normal assembly and release of virion particles. First, the Gag precursor is targeted to the plasma membrane through interactions of a domain located in the MA protein, termed assembly domain 1, or the membrane-binding (M) domain (39). The second domain is proline rich, lies in p2, and is called the late (L) domain because it is required for late stages of the assembly process during budding and release of viral particles from the cellular membrane (36). The third domain is located in the nucleocapsid (NC) protein of Gag and contains the two Cys-His repeats which bind zinc (3, 7, 35). This region (assembly domain 3, or the I domain) is thought to mediate direct interactions between Gag proteins. Chimeric gag genes encoding portions of RSV and other Gag proteins have been used to demonstrate that these assembly domains can often be exchanged to form functional hybrids, and the function of these domains is in many cases remarkably position independent within the precursor protein in mediating assembly (3, 27).

The yeast two-hybrid system is a powerful tool in the analysis of protein-protein interactions (see references 6 and 12; for reviews, see references 13 and 24). Two proteins under investigation are expressed in yeast as fusion proteins, one with a DNA binding domain (BD) and the other with a transcriptional activation domain (AD); if the two proteins interact, a functional transactivator is formed that can activate expression of an appropriate indicator gene (often the lacZ gene encoding β-galactosidase). The system has been used to study the direct interactions between Gag proteins of a variety of murine and human retroviruses (1, 15, 22). The Gag proteins of various related retroviruses do not interact, while Gag proteins of very closely related viruses, which are known to be capable of formation of mixed virion particles, do interact. Mutational studies suggested that smaller fragments of the Gag precursor could also mediate multimerization. In the case of human immunodeficiency virus type 1 (HIV-1), a region near the C terminus, including the C terminus of the capsid (CA) and all of NC, was the only domain identified as capable of multimerization (15). Murine leukemia virus (MuLV) Gag contained a very similar domain at the C terminus and an additional region at the N terminus that could independently multimerize (1). Although the functions of RSV Gag in viral assembly have been studied extensively by mutagenesis, direct interactions between Gag proteins have not been investigated. In this report, we describe the use of the yeast two-hybrid system to monitor the multimerization of RSV Gag proteins and to map the regions responsible for these interactions.

To test for Gag-Gag interactions, DNA segments encoding the whole RSV Gag precursor were amplified by PCR. Two plasmids, pSV.T10C.Pr− and pSV.H6MyroN (generous gifts of John Wills, Penn State), were used to prepare DNA templates for PCRs. pSV.T10C.Pr− contains a gag gene with 10 codons of c-src sequence at the N terminus, a deletion within Gag (Δ743 to 1387), and a mutation in the active site of the viral protease (39). pSV.H6MyroN contains a complete wild-type RSV gag gene. The XhoI-BsaHI fragment from pSV.H6MyroN was used to replace the corresponding fragment of pSV.T10C.Pr− to form plasmid pSV.H6, encoding the full-length Gag with the src sequence at the N terminus. DNA fragments encoding the whole RSV Gag with the src sequence were amplified by PCR with synthetic oligonucleotides as primers (Fig. 1A and B) and pSV.H6 as the template and subsequently were cloned into...
FIG. 1. Construction of mutants of the RSV Gag precursor protein. (A) Schematic diagram of the RSV Gag precursor protein. Individual domains released after cleavage of Gag are also illustrated. MA, matrix; SP, spacer peptide. The positions of the primers used in PCR amplification of various regions and of restriction sites used in construction of deletion mutants are indicated. (B) PCR primers used in construction of various deletion mutants. The name of each construct is designed to reflect the regions deleted. 5' or 3' indicates the position of the deletion. WT, wild type. (C) Positions of deletion and restriction sites used to generate deletions in various mutant constructs. Asterisk indicates that after religation of NcoI sites, the Gag reading frame is disrupted, resulting in a short stretch of sequence encoding random amino acids and stop codons.
yeast vectors to direct the synthesis of fusion proteins. The amplified RSV Gag sequences were fused to the C terminus of the DNA BD of transcription factors Gal4 (in plasmid pMA424 [25]) and LexA (in pSH2-1 [18]) or the activation domain of Gal4 (pGADNOT [23]). Yeast strains were cotransformed with various pairs of plasmids, encoding various Gal4AD-Gag fusion proteins, together with plasmids encoding various Gal4BD-Gag fusion proteins, and the resulting colonies were lifted to nitrocellulose and probed with a monoclonal antibody (Ab) that recognizes the Gal4 DNA BD, permeabilized, and stained with X-Gal as previously described (21). Yeast strains were cotransformed with plasmids encoding LexABD fusions of Gag proteins and Gal4AD-Gag fusions. The RSV-gag construct contains a mutation in the protease active site. Interactions between Gag proteins were scored as described for panel A.

The results are consistent with the idea that the RSV Gag protein can act as a dimer, with the two subunits being held together by an interaction between the BD and AD domains.
with genetic experiments showing that PR is not required for assembly (34) and with others that suggest that RSV Gag containing PR can interact with mutant Gag proteins lacking PR (26).

To map more precisely the regions important in mediating Gag-Gag interactions, deletion mutants were prepared for testing in the two-hybrid system. Many mutants were generated by PCR, with synthetic oligonucleotides as primers and the wild-type RSV gag DNA as the template (Fig. 1A and B). The primers contained restriction enzyme sites (at the 5' end, EcoRI for vector SH2-1 or NotI for vector GADN; at the 3' end, SacI) so that the amplified PCR products could be directly cloned into the yeast vectors (SH2-1 to make LexA DNA BD fusion proteins, or GADN to generate Gal4 transcriptional AD fusion proteins). In a few cases, natural restriction enzyme sites were used to remove portions of the gag gene (Fig. 1C). The various portions of Gag were expressed as LexABD fusions and Gal4AD fusions and were tested against the wild-type constructs or against each other (Table 1 and Fig. 3).

Removal of various portions of the RSV Gag protein at the N terminus showed that a small region near the C terminus was sufficient to mediate both interaction with the full-length Gag and also homodimerization. This region, which is retained in mutant 5'Δ1658-3'Δ2111, included the very C terminal of CA, a small spacer peptide, and all of NC (shown as region II [Fig. 3]). Similarly, removal of portions from the C terminus resulted in identification of a region near the N terminus that could at least interact with the wild-type Gag. This region, which is present in mutant 5'Δ845-3'Δ1636, included p2, p10, and a large N-terminal portion of CA (shown as region I in Fig. 3). These two nonoverlapping regions are independently sufficient to mediate Gag-Gag interactions when tested in at least one set of fusions between mutant and wild-type Gags. While they may well play very distinct roles in the virus life cycle, they provide redundant functions for interactions between the complete Gag precursors in yeast. The two domains are very similar in location to those defined for M-MuLV Gag (1), and the region II is similar to an interaction domain defined for HIV-1 Gag (15).

In some cases, the mutant Gag sequences in the DNA BD vectors induced β-galactosidase expression without a partner (termed self-activating [Table 1]) and so could not be scored for interaction. This behavior is presumably due to transcription activation activity inherent in the DNA binding fusion protein. When these constructs retained a positive interaction in the combinations that could still be tested, they were still considered to retain a functional interactional domain. The mutant constructs that scored negative with all partners were tested for expression of stable proteins by Western blot analysis with antibodies against Gal4BD (Santa Cruz) and against RSV proteins (generous gifts of John Wills; examples shown in Fig. 4). Mutants which failed to express fusion proteins were not included in our analysis, and thus all of the mutants listed in Fig. 3 encoded stable proteins of the expected sizes. The mutant 3'Δ1388 was not subjected to Western blot analysis because it was known to produce an interaction-competent protein in yeast, as judged by its interaction with a cellular Gag-interacting protein in the yeast two-hybrid system (20). Thus, the smaller domains failing to induce β-galactosidase activity are likely to be genuinely interaction negative.

To provide further support for the abilities of these domains to interact, an in vitro binding assay was established to assay the interactions between Gag proteins. The full-length gag gene was cloned into a pBS/SK vector (Stratagene) and transcribed in vitro with T7 polymerase, and the resulting RNA was translated with a rabbit reticulocyte lysate system (Promega) in the presence of [35S]methionine. Sequences corresponding to three mutants (3'Δ2211, 5'Δ845-3'Δ1636, and 5'Δ1658-3'Δ2111) were amplified by PCR and cloned into pGEX vectors to direct the synthesis of glutathione S-transferase (GST) fusion proteins in bacteria (14). Bacterial lysates were prepared, the GST fusion proteins were recovered on glutathione-agarose beads (Pharmacia Biotech), and the bound proteins were incubated with the full-length 35S-labelled RSV Gag protein (19). After extensive washing, the labelled proteins bound to agarose beads were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography (Fig. 5). The GST-Gag fusion corresponding to mutant 3'Δ2111 (Gag protein without protease) interacted strongly with the wild-type Gag. The GST fusion of Gag mutant 5'Δ1658-3'Δ2111 (region II) also interacted with the wild-type Gag in this system, while 5'Δ845-3'Δ1636 (region I) mediated a weak interaction. The control GST protein alone did not bind Gag. These results confirm the results of the yeast two-hybrid study that two nonoverlapping domains of RSV Gag can independently mediate multimerization and also suggest that region II mediates a strong interaction, while region I mediates a weaker interaction.

In summary, we have analyzed the interactions between RSV Gag proteins using the yeast two-hybrid system and using in vitro binding. These readouts define regions essential for the direct protein-protein interactions and are not complicated by the other requirements that the Gag protein may have for virus replication. The assays obviously do not allow any determination of when in the normal life cycle these interactions might be important. However, based on the fact that the domains often cross cleavage sites that separate the mature Gag products, it may be more likely that these interactions are used at the time of assembly, when the Gag precursor is still uncleaved. It is noteworthy that the wild-type RSV Gag has previously been expressed in yeast and was myristoylated and processed, which is indicative of some multimerization, although virion particles did not form (4).

### Table 1. Interactions of deletion mutants of RSV Gag

<table>
<thead>
<tr>
<th>Mutant</th>
<th>AD mutant vs BD WT</th>
<th>BD mutant vs AD WT</th>
<th>AD mutant vs BD mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>3'Δ2111</td>
<td>+ + + +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>Δ743-1387</td>
<td>+ +</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Δ743-1387-3'Δ2111</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>5'Δ1387-3'Δ2111</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Δ155-1658-3'Δ2111</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>5'Δ1658-3'Δ2111</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>5'Δ1816-3'Δ2111</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>3'ΔG1817</td>
<td>+</td>
<td>SA</td>
<td>SA</td>
</tr>
<tr>
<td>3'Δ1636</td>
<td>+ +</td>
<td>SA</td>
<td>SA</td>
</tr>
<tr>
<td>3'Δ1556</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3'Δ1388</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>3'Δ1555</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>3'Δ1097</td>
<td>NE</td>
<td>SA</td>
<td>SA</td>
</tr>
<tr>
<td>3'Δ911</td>
<td>NE</td>
<td>SA</td>
<td>SA</td>
</tr>
<tr>
<td>3'Δ743</td>
<td>NE</td>
<td>SA</td>
<td>SA</td>
</tr>
<tr>
<td>5'Δ595-3'Δ1636</td>
<td>+ +</td>
<td>SA</td>
<td>SA</td>
</tr>
<tr>
<td>5'Δ845-3'Δ1636</td>
<td>+</td>
<td>SA</td>
<td>SA</td>
</tr>
</tbody>
</table>

* AD, activation domain; BD, binding domain.
The data demonstrate that RSV Gag interacts strongly with itself and not with the Gag proteins of several other retroviruses, including HIV-1 and M-MuLV. These results suggest that the Gag of each virus has evolved a very specific set of contact surfaces that are generally not conserved well enough to mate with other viruses. Surprisingly, RSV Gag proteins interacted weakly with M-PMV Gag and SIV Gag proteins under some circumstances, although these sequences are only very distantly related. This finding suggests that some interaction surfaces are conserved among the viruses and may help account for the observations that several chimeric Gag proteins could function normally in assembly (3, 10, 27, 34).

The results of this mutational study define two domains which can independently mediate RSV Gag-Gag interactions. One domain lies in the C-terminal region of Gag and includes the C-terminal one-third of CA and NC; this region contains the AD3 or I domain, which had been previously shown to be essential for particle formation in vivo (35). The interaction of this region as scored in yeast thus may correspond to the major role of the I domain in assembly. This portion of Gag is also able to assemble into particles in vitro; a recombinant form of the uncleaved CA-NC protein can form a cylindrical structure in the presence of RNA (5). Taken together, these results further support the idea that this region plays an important role in viral assembly through Gag-Gag interactions.

The role of the N terminus-proximal region I in viral assembly is less clear. The smallest region that retained interaction capability was still very large, including large sections of CA; it is possible that CA is the more important section for dimerization. However, the region also includes the L domain, which is required for a late step in the assembly and release of the virion (27, 36). Mutants affected in this region are arrested, or at least delayed, in a very late stage of the budding process. There are several indications that this domain interacts with specific host proteins in virus replication. The p2 sequence contains a proline-rich motif, PPPPY (40), that is recognized by SH3 domains and the related WW motif (16). Whether the interaction between Gag proteins in this region could occur...
was synthesized in vitro by using the coupled transcription-translation rabbit panel A.

formed with pGADNOT/RSV Gag mutants. Cell lysates were similarly prepared
subjected to SDS-PAGE and Western blotting analysis by using antisera against
pSH2-1/RSV Gag. Cell lysates were prepared from these transformants and were
transformed individually with pSH2-1/RSV Gag mutants as well as with
relate well with observations of virus replication and with in
phology (35). Further genetic analysis of this region may shed
mutants in the L region can show alterations in virion mor-
simultaneously with a Gag-host protein interaction is unclear.

VOL. 71, 1997 NOTES 5629

3
50 mM Tris (pH 8.0), 1 mM dithiothreitol, 120 mM NaCl, and 1

by binding to glutathione-Sepharose 4B beads (14). Labelled RSV Gag proteins
Gag mutants were produced as GST fusion proteins in bacteria and were isolated
proteins. Proteins bound to GST fusion proteins were eluted and analyzed by
sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography.

simultaneously with a Gag-host protein interaction is unclear. One possible scenario is that a host protein could modulate or regulate Gag-Gag multimerization in a competitive way through interactions with the L domain. It is also known that mutants in the L region can show alterations in virion morphology (35). Further genetic analysis of this region may shed light on these important virus functions.

The results obtained with the yeast two-hybrid system correlate well with observations of virus replication and with in vitro biochemical analysis of Gag behavior. These findings suggest that the system may score for meaningful interactions

and at least partially reflect the contacts that occur in the intact virus. The system should prove useful in screening for mutants affecting these contacts, in isolating revertants of such mutants, and in identifying inhibitors that prevent the interactions.

We thank John Wills for providing plasmids pSV.H6Myr0N and pSV.T10C.Pr−, for anti-RSV serum, and for helpful discussions; and Jianghao Chen for help with in vitro translation of RSV Gag.

X. L. is an Associate and S.P.G. is an Investigator of the Howard Hughes Medical Institute.

REFERENCES

26. Oertle, S., N. Bowles, and P.-F. Spahr. 1992. Complementation studies with FIG. 4. Analysis of protein expression in yeast. (A) Yeast strain CTY5d-10 was transformed individually with pSH2-1/RSV Gag mutants as well as with pSH2-1/RSV Gag. Cell lysates were prepared from these transformants and were subjected to SDS-PAGE and Western blotting analysis by using antisera against pSH2-1/RSV Gag. Cell lysates were prepared from these transformants and were subjected to SDS-PAGE and Western blotting analysis by using antisera against pSH2-1/RSV Gag. Cell lysates were similarly prepared and analyzed with antibodies against Gal4AD (Santa Cruz) as described for panel A.

FIG. 5. In vitro binding between RSV Gag and mutants. RSV Gag protein was synthesized in vitro by using the coupled transcription-translation rabbit reticulocyte lysate kit (Promega), forming [35S]methionine-labelled protein. RSV Gag mutants were produced as GST fusion proteins in bacteria and were isolated by binding to glutathione-Sepharose 4B beads (14). Labelled RSV Gag proteins were incubated with Sepharose 4B beads coupled with RSV Gag mutants at 4°C for 2 to 4 h in the presence of the binding buffer containing 0.5% Nonident P-40, 50 mM Tris (pH 8.0), 1 mM dithiothreitol, 120 mM NaCl, and 1 μM PR inhibitors (19). The binding buffer was used to wash the beads six times to remove unbound proteins. Proteins bound to GST fusion proteins were eluted and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography.


