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The Membrane-Binding Domain of the Rous Sarcoma Virus Gag Protein

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The Gag protein of Rous sarcoma virus (RSV) can direct particle assembly and budding at the plasma membrane independently of the other virus-encoded products. A previous deletion analysis has suggested that the first 86 amino acids of RSV Gag constitute a large membrane-binding domain that is absolutely required for these processes. To test this hypothesis, we inserted these residues in place of the N-terminal membrane-binding domain of pp60$^{v\text{-}src}$, a transforming protein whose biological activity requires plasma membrane localization. The ability of the Src chimera to induce cellular transformation suggests that the RSV sequence indeed contains an independent, functional domain.

Retroviral Gag proteins play dual roles in the virus life cycle. One role is in viral egress, when 2,000 to 3,000 molecules (33) direct the budding process to form a viral particle (36). Shortly thereafter, Gag is cleaved by the viral protease (PR) to yield the major structural components of the virion (matrix [MA], capsid [CA], and nucleocapsid [NC]), which are required for a second role, infectivity. Before initiating either event, Gag must travel from its site of synthesis in the cytosolic space and stably bind to the plasma membrane. Although the mechanism by which this occurs is largely unknown, it is believed that the MA sequence located at the N terminus of Gag provides the membrane-binding activity (Fig. 1), because it is intimately associated with the lipid bilayer of the mature virus (8, 21–24).

The features of Gag proteins that promote membrane binding are not certain. Most require the posttranslational modification of their N termini with myristic acid (29); however, this hydrophobic, 14-carbon fatty acid does not provide enough binding energy to anchor proteins to the lipid bilayer (16, 19). Moreover, not all Gag proteins are myristylated (29). Therefore, amino acids in Gag are certain to play a role in membrane binding, too. Attempts to identify these have typically employed MA mutants that exhibit a loss-of-function phenotype (for examples, see references 7, 9, 25, 38, 40, and 41). While the results obtained from such studies are highly suggestive, they do not rule out the involvement of residues outside MA for membrane binding. One way to address that possibility is to test the ability of a given putative binding domain to function in the context of a heterologous protein. The only Gag membrane-binding domain to be carefully mapped in this manner is that of human immunodeficiency virus (HIV) (42). That analysis revealed that the binding domain is located within the first 31 amino acids and is composed of two distinct elements: myristic acid and a cluster of basic residues. In vitro experiments suggest that the basic residues serve to enhance the membrane-binding energy by establishing electrostatic interactions with acidic phospholipids, which are located on the cytoplasmic face of the plasma membrane. The recently reported three-dimensional structure of HIV MA further supports this mechanism in showing that these residues project out from the protein surface in a manner that would allow them to physically contact a lipid bilayer (14, 15).

We have been investigating the Gag protein of Rous sarcoma virus (RSV). Its membrane-binding domain (previously named AD1 [18, 36]) appears to be very different from that of HIV. In particular, it lacks both myristate (6) and an obvious cluster of basic residues (34). Furthermore, recent deletion analyses have suggested that all of the first 86 amino acids are needed for membrane binding (see accompanying report by Nelle and Wills [17] and reference 38), which would make this domain much larger than that of HIV. Deletions in the putative binding domain do not appear to affect other Gag assembly functions because the mutants can be complemented in *trans* (i.e., rescued into particles) by other RSV Gag molecules and in *cis* by heterologous membrane-binding domains (38). While this implies that the membrane-binding domain is contained within residues 1 to 86, it was important to test this hypothesis.

Gag-Src chimeras. To determine whether AD1 is sufficient for membrane binding, we tested its ability to direct a heterologous protein, pp60$^{v\text{-}src}$, to the plasma membrane. The Src oncoprotein was selected for several reasons. First, its ability to cause cellular transformation is dependent upon membrane localization (4, 5, 10, 12), allowing membrane binding to be assayed in vivo by observing cellular transformation. Furthermore, the membrane-binding domain of Src is small (about 10 residues [11, 20, 38]) and well characterized. In fact, it is very similar to that of HIV Gag in that both depend on myristate and neighboring basic charges to establish stable membrane binding (3, 5, 31). Because myristate can be added to a glycine residue only when it immediately follows the initiator methionine (which is removed prior to myristylation), the natural membrane-binding capacity of pp60$^{v\text{-}src}$ is destroyed simply by fusing a foreign sequence to its N terminus.

A chimeric *gag-src* gene was constructed by fusing the first 94 codons for RSV MA (encoding the putative Gag membrane-binding domain plus an 8-amino-acid spacer peptide) to the entire coding sequence of v-*src* (Fig. 1). This was accomplished by amplifying the *gag* sequence by PCR with two synthetic primers. One primer, 5′-CTCAGAAGTCGACGAGCTC TACT, corresponds to a sequence ending 116 nucleotides upstream of *gag* and introduces a *Sal*I restriction endonuclease site (underlined). The reverse PCR primer, 5′-AACCAAAC

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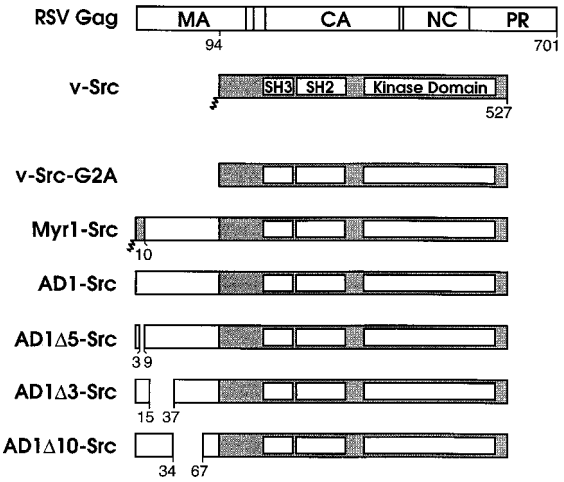

FIG. 1. Schematic diagrams of chimeras. The wild-type Gag and Src proteins are depicted at the top by white and gray bars, respectively. Vertical lines in Gag represent cleavage sites recognized by the viral protease (PR), whose action brings about the release of the indicated mature proteins. The white boxes within the Src protein represent conserved domains. Squiggles at the N terminus of the Src and Myr1-Src proteins represent myristate. Numbers below each molecule refer to amino acid residues. The proteins were expressed by using vectors described in the text.

CATGGCTTGCTCAGAT, corresponds to a sequence starting 269 nucleotides downstream of the first nucleotide of *gag* and introduces an *Nco*I site. This *Nco*I site was positioned to allowed the sequence from *gag* to be linked in frame to the *Nco*I site at the first codon of v-*src*. The PCR product produced with these two primers was digested with *Sal*I and *Nco*I and directly ligated into the Src expression vector, pMX2122 (2), between its *Xho*I and *Nco*I sites to generate pMX-ad1-src. For a control, a second chimera (pMX-myr1-src [Fig. 1]) was generated in an analogous manner by using the *myr1* allele of RSV *gag* (37), in which the first 10 codons have been replaced with those of v-*src*.

These two expression plasmids, in parallel with the same vectors carrying the wild-type v-*src* gene or the v-*src*-G2A allele (which encodes an unmyristylated form of Src and cannot transform cells [10]), were transfected into NIH 3T3 cells by a standard calcium phosphate coprecipitation method (28). Dense foci were scored 14 to 21 days later. Initially, two independent plasmid clones of *ad1-src* (i.e., encoding the putative membrane-binding domain of Gag linked to Src) were tested in this assay. One was able to induce transformation of mouse fibroblasts in a manner that was as clear and reproducible as was that for the v-*src* and *myr1-src* controls (Fig. 2, left *ad1-src* panel). The other clone did not induce foci (Fig. 2, right *ad1-src* panel), but it was later discovered to encode two amino acid substitutions in the Gag sequence (S22P [an S-to-P mutation at position 22] and E87K) unintentionally introduced during amplification or cloning. To further confirm these results, a third clone of *ad1-src* was tested in a separate experiment and was also able to induce transformation (data not shown). These observations were not cell type specific in that similar results were obtained with Rat-2 fibroblasts (data not shown).

To further explore the ability of the chimeras to transform cells, several clonal cell lines were isolated after cotransfection of Rat-2 cells with each expression plasmid and a selectable-marker plasmid (pMX1112-*neo*) and selection in G418. Control lines expressing wild-type v-*src*, v-*src*-G2A, or no *src* allele (i.e., selectable plasmid only) were generated at the same time. The morphologies of representative clones are shown in Fig. 3. Again, cells transfected only with the selectable plasmid exhibited no transformation, as was the case for cells cotransfected with v-*src*-G2A (data not shown). Cells transfected with *ad1-src* had an obvious transformed morphology in comparison with that of control cells expressing only the selectable marker; however, this morphology was readily distinguishable from that induced by wild-type v-*src*. Cells transformed by v-*src* had a rounded morphology, did not adhere tightly to the culture

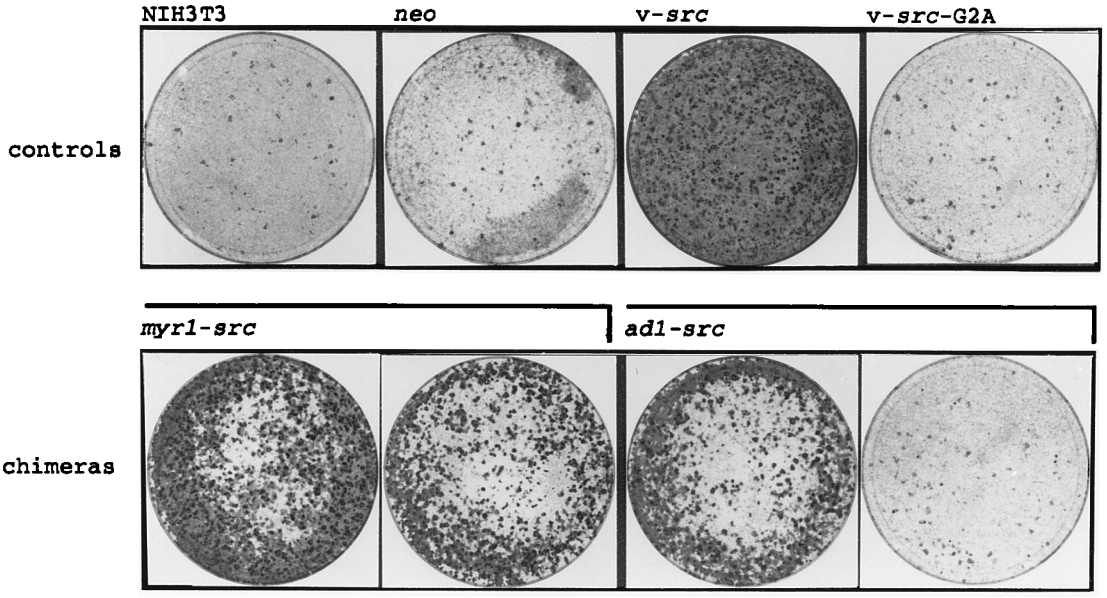

FIG. 2. Focus assay. NIH 3T3 cells were transfected with expression plasmids carrying the indicated genes. The transfected cells were maintained in culture for 2 weeks, fixed, and stained with crystal violet. Representative plates are shown for each gene tested. In the case of the chimeras, two independently isolated clones of the indicated DNAs were tested. Note: the second clone of *ad1-src* encoded two amino acid substitutions in the Gag portion of the molecule and appeared inactive; however, subsequent transfections with a third clone produced foci similar to those of the first clone.

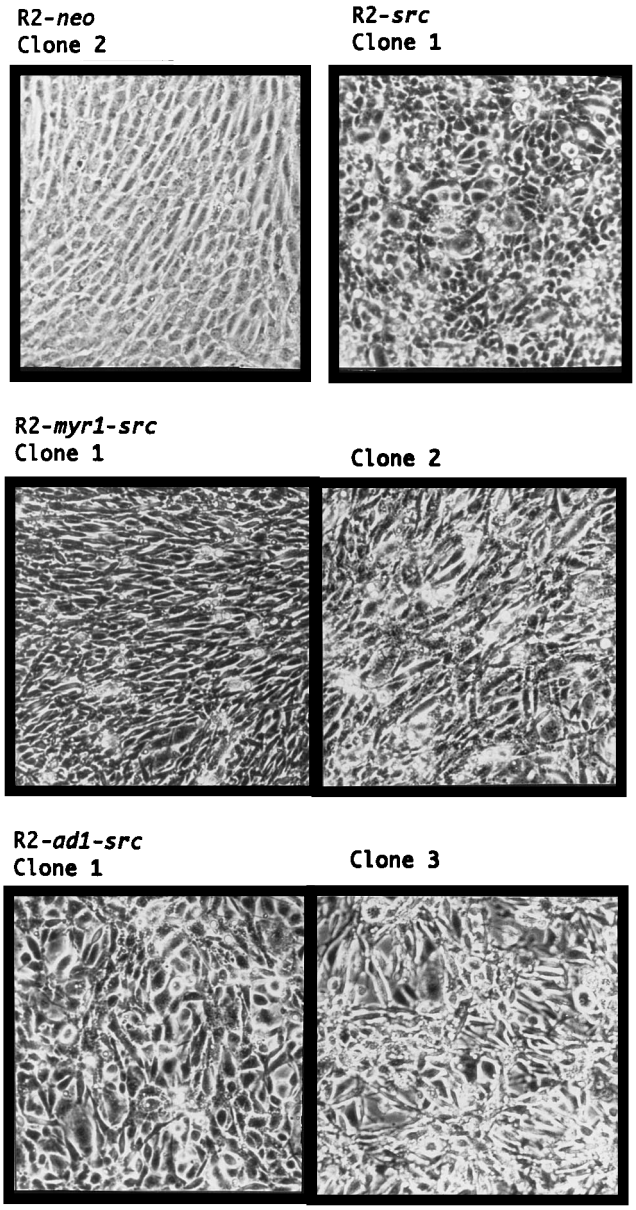

FIG. 3. Cell morphology. Rat-2 cells were transfected with the indicated DNAs and selected with neomycin. The resulting clonal cell lines were plated, allowed to grow to confluence, and photographed by using a phase-contrast microscope. The control, R2-*neo*, received only the neomycin resistance plasmid, while the other clones were derived from cotransfections of this selectable plasmid and the indicated expression plasmids.

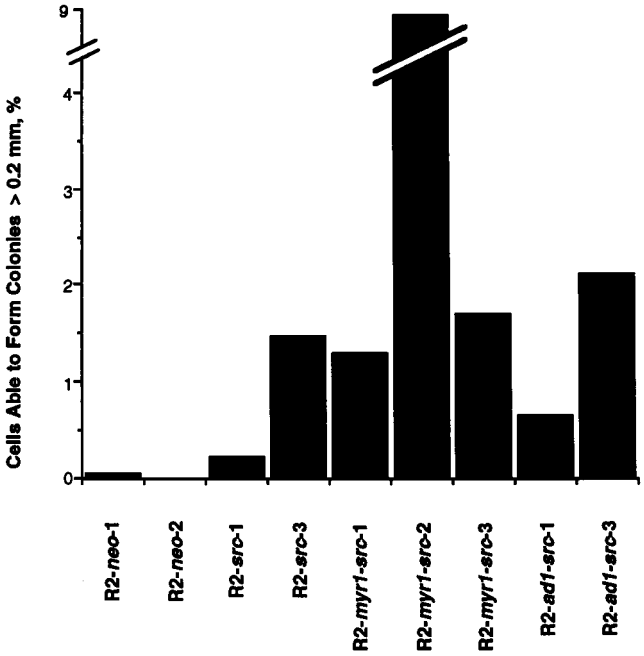

FIG. 4. Anchorage-independent growth assay. Clonal cell lines were plated in 0.35% agarose as described elsewhere (32). The plates were incubated for 22 days, and colonies >0.2 mm in diameter were counted. Results from a typical experiment are shown. Cell line designations are as in Fig. 3.

dishes, were highly refractile, and when grown to high density packed tightly together. In contrast, cells transformed by *ad1-src* were not as evenly round and appeared to have a larger cross-sectional area. Moreover, the cells were more firmly attached to the plates and did not pack together as tightly as did v-*src*-transformed cells.

The cell lines also were tested for their ability to grow in an anchorage-independent manner, which is a strong predicator of tumorigenic potential (30). We found that each line derived from *ad1-src* transfections was able to form colonies in agarose with efficiencies rivaling those obtained with wild-type v-*src* (Fig. 4). Cell lines derived either with v-*src*-G2A (data not shown) or without any *src* allele did not efficiently form colonies under identical conditions, as expected. Similar results were obtained with all of the constructs when cell lines were derived by using Rat-1 rather than Rat-2 cells (data not shown).

Expression of the chimeric proteins was confirmed by subjecting cell extracts to immunoblot analysis with a monoclonal antibody specific to v-Src (Mab127 [13]). As predicted, lysates from control v-*src* and v-*src*-G2A cell lines contained a 60-kDa protein, while cell lines expressing chimeric genes expressed a protein of approximately 69 kDa, consistent with the size expected for the fusion protein (Fig. 5B; data for v-Src-G2A not shown). Additional analyses of these extracts by immune complex kinase assays demonstrated that AD1-Src had approximately the same level of kinase activity as did wild-type Src (Fig. 5A), consistent with the previous observation that N-terminal alterations in $pp60^{v\text{-}src}$ do not affect kinase activity (4). The differences in the transformation abilities between v-Src and AD1-Src suggest that the chimera might have a slight change in substrate accessibility, possibly due to a subtle change in subcellular localization in vivo. Whether such differences can be detected remains to be seen.

Despite several attempts, we were unable to biochemically verify the presence (Myr1-Src) or absence (AD1-Src) of myristate on the Src chimeras by labeling with [^{3}H]myristic acid. Unfortunately, the cells rapidly catabolized the [^{3}H]myristic acid (within an hour) and subsequently incorporated the label into amino acids, leading to a nonspecific labeling of all proteins (data not shown). Labeling for shorter time periods was not helpful since the level of expression of the controls (v-Src and Myr1-Src) dropped below our limit of detection. Nevertheless, it was important to establish that the transformation observed was not a result of DNA contamination. It seemed unlikely that contaminating v-*src* DNA was responsible, since all the *ad1-src* cell lines produced proteins of the larger size predicted for the chimera (Fig. 5B). To address the possibility

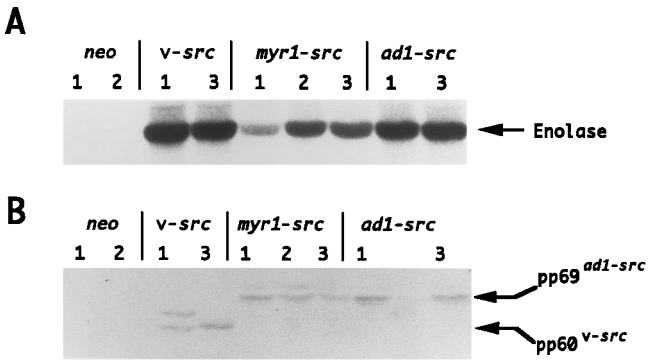

FIG. 5. Biochemical analysis of chimeric proteins. Different clonal Rat-2 cell lines (numbers above lanes) expressing the indicated *src* alleles were obtained as described in the text. (A) Proteins were immunoprecipitated from cell lysates by using a Src-specific monoclonal antibody, and kinase assays were performed by using acid-denatured enolase as a substrate, as previously described (39). (B) Whole-cell detergent lysates were separated on a 10% acrylamide gel, transferred to nitrocellulose, and probed with a monoclonal antibody specific for Src and then with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G. The blot was developed with 5-bromo-4-chloro-3-indolylphosphate toluidinium (BCIP) and nitroblue tetrazolium. The positions of the Src ($pp60^{v\text{-}src}$) and chimeric Src ($pp69^{ad1\text{-}src}$) proteins are indicated. Cell line designations are as in Fig. 3. (Occasionally, each cell line produced a more slowly migrating species that specifically cross-reacted with the primary antibody. The origin of these additional bands is uncertain.)

of contamination by the *myr1-src* allele, PCR was used to amplify the *gag* sequences from both the *ad1-src* and the *myr1-src* cell lines. DNAs of the expected size were obtained (providing further evidence of the presence of chimeric genes), and these were analyzed with restriction endonuclease digestions. As expected, only DNA amplified from the *myr1-src* cell lines was cut with *Mlu*I, a site for which is absent in the *ad1-src* allele (data not shown).

Deletion mutants. Because the RSV membrane-binding domain was originally mapped with deletion mutants in a loss-of-budding assay (1, 17, 38), it was possible that the actual domain is smaller than 86 residues. In an attempt to further delineate the domain, three of the previously described deletions were introduced into the MA portion of the AD1-Src chimera (Fig. 1). These were inserted into the pMX2122 expression vector by using the cloning strategy described above and assayed for their ability to induce transformation in a focus assay. None of the deletion mutants were able to induce foci on a monolayer of normal cells (data not shown). To assess the level of expression, cell lines resistant to G418 were isolated after cotransfection of each expression plasmid with pMX1112-*neo*; however, none of these cell lines expressed detectable amounts of protein (data not shown). To circumvent problems of low cotransfection efficiencies and to ensure that G418-resistant cell lines would carry the chimeric genes, all of the *src* alleles were cloned into pLXSN, which carries its own *neo* resistance gene (35). Unfortunately, cell lines isolated after transfection with these new constructs still did not contain detectable levels of Src-related protein, except in the case of the wild type, suggesting that the deletions may have rendered them unstable (data not shown). In an attempt to investigate protein stability directly, pulse-chase experiments were performed; however, the level of expression of the deletion mutants was not high enough to allow detection after a brief labeling period.

As an alternative approach, we examined the effects of the MA deletions on protein stability in the context of the Gag protein expressed in COS-1 cells using previously described methods (1). After a 10-min pulse with L-[^{35}S]methionine, cells expressing the deletion mutants or wild-type Gag were either lysed immediately or chased in the presence of excess methionine for 1, 2, or 4 h and then lysed. The Gag proteins were immunoprecipitated with rabbit antiserum against RSV, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and visualized by fluorography, as described previously (1, 37, 38). Relative protein levels were determined by laser-scanning densitometry. Each of the deletions caused a dramatic decrease in protein stability in the context of Gag (i.e., their half-lives were 30 min or less), in a manner reminiscent of that seen for MA deletions in the Gag proteins of Akv murine leukemia virus (9) and Mason-Pfizer monkey virus (25). It seems reasonable to suggest that our deletions have a similar effect in the context of the *ad1-src*-encoded proteins. Nonetheless, the instability of the mutant chimeras does not change the major finding of our studies, that the first 86 amino acids of RSV Gag function as a membrane-binding domain when fused to a heterologous protein. Whether this domain can be more finely mapped remains to be seen.

When considered in combination with previously described deletion analyses (see accompanying report by Nelle and Wills [17] and reference 38), the data presented here strongly support the hypothesis that the membrane-binding domain of the RSV Gag protein is contained entirely within the first half of its MA sequence. The distinct morphology of cells transformed with the *ad1-src* allele (versus v-*src*) argues that the sequence from RSV Gag did not in some way recreate the membrane-binding properties of Src. This in turn raises the possibility that the RSV Gag protein may be targeted to sites on the plasma membrane that are distinct from that of Src. Normally, $pp60^{v\text{-}src}$ is localized in focal adhesions (27), and mutations that disrupt this localization (but are still transforming) give rise to cells with a fusiform morphology rather than the typical round morphology (26). Future studies with AD1-Src may provide insights into the relationship between subcellular localization of $pp60^{v\text{-}src}$ and the regulation of cellular morphology in the transformed state.

The mechanism by which the membrane-binding domain of the RSV Gag protein works seems certain to be different from those previously described for other proteins targeted to the cytoplasmic face of the plasma membrane. In particular, the RSV domain seems significantly larger than the membrane-binding domain of $pp60^{v\text{-}src}$ (7 amino acids [11, 20, 38]) and of HIV Gag (31 amino acids [42]). Additionally, it does not require myristic acid, does not have an identifiable cluster of basic amino acids, and does not have a large hydrophobic moment. Although we have been unable to map the domain to a region smaller than 86 amino acids, we suspect that not all of the residues in the domain are needed for interactions with the membrane. Rather, it seems likely that folding of the protein brings together discrete, noncontiguous determinants, which form a surface which interacts with the membrane. While there is not an obvious cluster of basic residues, there are 10 such residues scattered throughout the first half of the MA sequence. It is possible that these residues could be positioned close together on the surface of the folded protein. Efforts to elucidate the three-dimensional structure of the RSV membrane-binding domain (now referred to as the M domain [18]) are currently in progress.

M.F.V. performed all of the transfections, cell cloning, and kinase assays. T.D.N. constructed and reconstructed all of the plasmid clones, labeled cell cultures, and performed the pulse-chase experiments.

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