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# Gag Influences Transformation by Abelson Murine Leukemia Virus and Suppresses Nuclear Localization of the v-Abl Protein▽

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**Like the v-Onc proteins encoded by many transforming retroviruses, the v-Abl protein is expressed as a Gag-Onc fusion. Although the Gag-derived myristoylation signal targets the v-Abl protein to the plasma membrane, the protein contains the entire MA and p12 sequences and a small number of CA-derived residues. To understand the role of Gag sequences in transformation, mutants lacking portions of these sequences were examined for the effects of these deletions on v-Abl function and localization. Deletion of the N-terminal third of p12 or all of p12 enhanced the transformation of both pre-B cells and NIH 3T3 cells. In contrast, deletions in MA or a deletion removing all of Gag except the first 34 amino acids important for myristoylation highly compromised the ability to transform either cell type. Although all of the mutant proteins retained kinase activity, those defective in transformation were reduced in their ability to activate Erk, suggesting a role for Gag sequences in v-Abl signaling. Immunofluorescence analysis revealed that a v-Abl protein retaining only the first 34 amino acids of Gag localized to the nucleus. These data indicate that Gag sequences are important for normal v-Abl signaling and that they suppress nuclear localization of the molecule.**

Abelson murine leukemia virus (Ab-MLV) is a replication-defective retrovirus that arose spontaneously from a recombination between Moloney MLV (Mo-MLV) and the c-*abl* proto-oncogene. As a result, sequences from the retroviral *gag* gene and the cellular c-*abl* gene were fused, leading to the production of a single gene product. The v-Abl protein encoded by this gene is a nonreceptor tyrosine kinase, and its expression induces pre-B-cell lymphoma in mice and transforms NIH 3T3 and pre-B cells in vitro (26, 29). The *abl*-derived sequences contain the catalytic domain, the SH2 domain that is important for binding to tyrosine-phosphorylated proteins, and the carboxyl terminus that functions to strongly enhance the transformation of pre-B cells (16, 17). Intact tyrosine kinase activity and the SH2 domain are required for transformation by Ab-MLV (12, 14, 26).

Despite the importance of *abl*-encoded sequences in Ab-MLV transformation, the Gag domain, which contains the entire MA and p12 regions, a small portion of CA, and a myristoylation signal, has long been appreciated as playing an important role in this process. An analysis of mutants with insertions in *gag* showed that the integrity of the region is important for Ab-MLV-mediated transformation of NIH 3T3 cells (22). A v-Abl protein that lacks the myristoylation signal fails to transform NIH 3T3 cells but still stimulates the growth of a factor-dependent hematopoietic cell line (3), a phenotype associated with loss of plasma membrane localization. Other experiments have also highlighted the importance of the myristoylation signal and the residues at the extreme amino terminus of the protein. For example, mutants that encode v-Abl proteins containing only the first 34 amino acids of Gag, including the myristoylation signal, or those that retain these sequences but lack the rest of MA fail to transform pre-B cells, but not NIH 3T3 cells (18, 19). However, for reasons that were never clarified, these mutants were unstable in the presence of the full carboxyl terminus of the protein and cells transformed by these viruses expressed v-Abl proteins that lacked some portion of this region. Since the carboxyl terminus is required for high levels of lymphoid transformation (16, 17), the absence of these residues may have influenced the behavior of the mutants.

To understand the contribution of Gag residues to Ab-MLV transformation more fully, a panel of deletion and alanine substitution mutants of Ab-MLV were constructed and tested for transformation in the presence of the carboxyl terminus of the protein. These analyses revealed that mutations affecting the Gag residues in v-Abl could enhance or decrease the transformation of both NIH 3T3 cells and pre-B cells. Further studies of the transformation-defective mutants revealed that Gag sequences affect both v-Abl-mediated growth signals and the subcellular localization of the molecule. These analyses reinforce the role of Gag in transformation and suggest that Gag sequences play an important role in the trafficking of the oncoprotein, perhaps mimicking its role in replicating retroviruses (31, 41).

## MATERIALS AND METHODS

**Cells and viruses.** 293T, NIH 3T3, and Ab-MLV-transformed pre-B cells were grown as described previously (36). Viral stocks were prepared by transfection of 293T cells with the pMIG vector (12) encoding various Ab-MLV *gag* mutants and the pSV-$\psi^-$-E-MLV retroviral packaging plasmid (15) as described previously (35). To determine the infectious titer of the viral stocks, $1 \times 10^5$ NIH 3T3 cells were plated in 60-mm dishes and infected 24 h later with dilutions of virus containing 8 μg/ml Polybrene. The cells were collected 24 h postinfection and analyzed for the frequency of green fluorescent protein (GFP)-positive cells by flow cytometry. To characterize the mutants in a pre-B-cell setting, the temperature-sensitive P70/H590 Ab-MLV strain-transformed pre-B-cell line 7C411 (6) was infected with viral stocks in the presence of 8 μg/ml Polybrene by centrifug-

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ing the mixture at 1,000 × *g* for 1.5 h at room temperature. Derivatives of 7C411 cells expressing wild-type or mutant Ab-MLV were obtained by sorting for GFP-positive cells using a MoFlo instrument the day after the cells were infected. The cells were maintained at 34°C, the permissive temperature for the 7C411 cells; the nonpermissive temperature used to test the activity of Ab-MLV mutants was 39.5°C. Growth analyses were performed by seeding the 7C411 derivatives at $5 \times 10^5$ cells/dish in 60-mm dishes and incubating them at 39.5°C. The proliferation of the cells was monitored by counting cells using a hemocytometer; viability was determined by trypan blue exclusion.

**Transformation assays.** The NIH 3T3 cell transformation assay (27) was used to determine the titer of transforming virus in the different viral stocks. Cells were infected with serial dilutions of virus as described above and fed every 5 days with fresh medium. The foci of morphologically distinct, transformed cells were counted 13 to 15 days postinfection. BALB/cJ bone marrow cells were used for pre-B-cell transformation assays as described previously (24). For liquid transformation assays (2), bone marrow cells were infected with viral stocks and plated directly into 35-mm dishes. The cultures were fed every 5 days and were scored as transformed when the density of transformed lymphoid cells reached $2 \times 10^6$ cells/ml.

**Viral plasmids.** Mutations were introduced into Ab-MLV coding sequences by using the QuikChange mutagenesis (Stratagene) method. The Ab-MLV coding region was cloned into the TA cloning vector (Stratagene) for use as a template, and all mutagenized fragments were sequenced before they were reinserted into the pMIG vector (12). The oligonucleotide sequences used to generate the deletion and alanine substitution mutants are available on request.

**Protein analysis.** The expression of the proteins encoded by the mutants was confirmed by Western blot analysis of lysates prepared from 293T cells transfected with the viral plasmids. For experiments examining Erk phosphorylation, 293T cells transfected with the different viral plasmids were incubated in serum-free medium 24 h after transfection; lysates were prepared after an additional 24 h and analyzed by gel electrophoresis and Western blotting as described previously (2). The antibodies used include anti-Abl (24-21) (28), anti-phospho-Erk (Cell Signaling Technology), anti-Erk (Cell Signaling Technology), antiphosphotyrosine (Upstate), and alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit antibodies (Promega).

**Immunofluorescence staining.** The localization of v-Abl proteins was assayed by immunofluorescence staining of 7C411 derivatives that expressed wild-type or mutant Ab-MLV. Cells were centrifuged onto glass slides by using a Cytospin instrument (Shandon). The slides were fixed in 3% formaldehyde in phosphate-buffered saline (PBS) for 10 min, permeabilized in 0.1% Triton X-100 in PBS for 10 min, and blocked in 10% fetal calf serum in PBS for 30 min at room temperature. Samples were stained in a humidified chamber with anti-Abl antibody (28) for 1 h at room temperature. After the slides were washed three times with PBS for 10 min each, the cells were stained with Alexa 594-conjugated goat anti-mouse antibody (Molecular Probes) for 30 min at room temperature. The slides were washed three times with PBS for 10 min each, stained with 300 nM 4′,6-diamidino-2-phenylindole (DAPI) in PBS for 5 min, and washed three times with PBS for 5 min each. The slides were mounted using Bacto FA mounting fluid (pH 7.2) (Difco), and the cells were visualized by using a fluorescence E400 Nikon microscope. The fluorescence intensities of the digitized images were analyzed by using ImageJ 1.36b (http://rsb.info.nih.gov/ij).

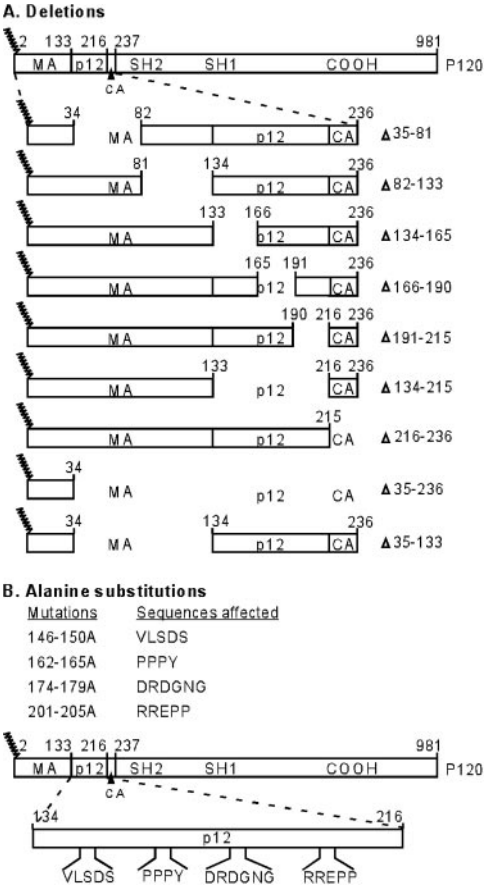


FIG. 1. v-Abl proteins encoded by the wild-type virus and *gag* mutants. The different domains of the wild-type P120 v-Abl protein are depicted. (A) The Gag domains of the mutant v-Abl proteins are expanded to show the amino acids that are deleted as a result of each mutation. (B) The p12 region of Gag is expanded to show the positions of residues in p12 that were replaced with alanines. The numbers above the diagrams denote amino acid numbers, and the jagged lines indicate the myristoylation sites.

## RESULTS

**Intact Gag sequences are not required for v-Abl protein expression and kinase activity.** To explore the way in which Gag-derived sequences influence v-Abl-mediated transformation, a panel of mutants missing portions of the protein or containing substitutions within Gag were constructed (Fig. 1). All of the mutants retained the first 34 amino acids of Gag that include the myristoylation sequence shown to be important for transformation (3). To examine the effects of the mutations on v-Abl expression and kinase activity in the absence of selection for transformation, the mutants were expressed in 293T cells and the lysates were prepared. Western blotting analysis using an anti-Abl antibody that reacts with an epitope in the carboxyl terminus of the protein revealed the expression of v-Abl proteins of the sizes expected based on the mutations that were present (Fig. 2A and B). Western blots probed with antiphosphotyrosine antibodies revealed that all of the v-Abl proteins and some cellular proteins were phosphorylated in all cases, except in lysates expressing the kinase-inactive D484N mutant (20). These data indicate that the alterations in Gag do not abolish kinase activity or affect protein expression in a marked way.

**Some mutations affecting p12 can enhance transformation of cells.** To determine if deletions removing portions of Gag affect the ability of Ab-MLV to transform NIH 3T3 and pre-B cells, virus stocks were prepared and titered by flow cytometry using the GFP expressed by the virus. To monitor transformation, viruses were tested in the NIH 3T3 focus assay (27) and in the soft-agar pre-B-cell transformation assay (24). Two mutants, the Δ166–190 mutant, lacking sequences encoding the central portion of p12, and the Δ216–236 mutant, lacking sequences encoding CA, had results similar to those of wild-type virus in both transformation assays (Tables 1 and 2). In contrast, the Δ134–165 mutant, lacking sequences encoding the N-terminal third of p12, and the Δ134–215 mutant, missing sequences encoding the entire p12 region, both transformed

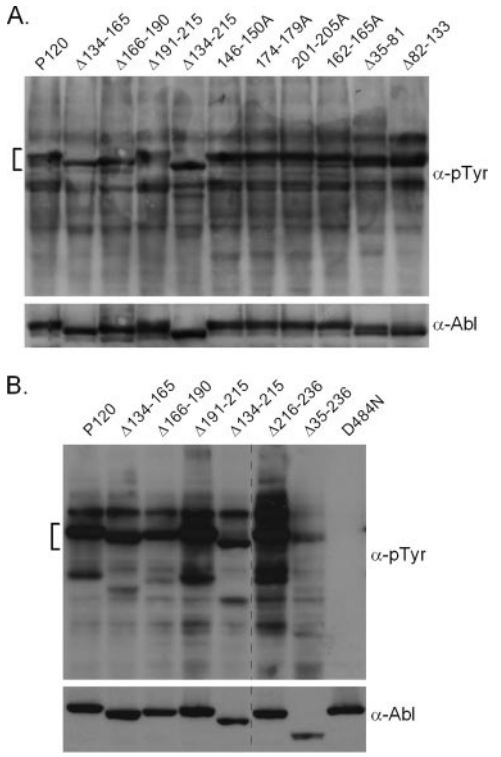


FIG. 2. Ab-MLV mutants express v-Abl proteins that retain kinase activity. 293T cells were transiently transfected with plasmids expressing the different mutants, and 48 h later, the lysates were prepared and analyzed by Western blotting with antibodies directed against phosphotyrosine. The blots were stripped and reprobed with an anti-Abl antibody that recognizes a determinant in the carboxyl terminal region of the v-Abl protein (28). The results shown in panels A and B were obtained in independent experiments; the dotted line in panel B indicates that lanes containing samples irrelevant to the experiment were deleted using Adobe Photoshop. The brackets indicate the positions of the v-Abl proteins expressed by the different viruses. α, anti.

NIH 3T3 cells more readily than wild-type virus (Table 1). The increase observed with these mutants, ranging from 6- to more than 20-fold, was statistically significant ($P < 0.05$; paired $t$ test). An increase was also observed when these two mutants were compared to wild-type virus in the pre-B-cell transformation assay (Table 2), although the magnitude of the increase was not as great. Although deletion of the C-terminal third of p12 decreased NIH 3T3 cell transformation 2.5- to 3.5-fold, this mutant did not consistently differ from wild-type virus in the pre-B-cell assay. Alanine substitutions of residues in p12 that play important roles in the Mo-MLV life cycle, such as the PPPY motif or the arginine-rich regions (39, 40), did not affect transformation in NIH 3T3 cells (Table 3), indicating that functions associated with these residues do not play a major role in mediating the biological properties of Ab-MLV.

TABLE 1. Mutations affecting Gag alter transformation of NIH 3T3 cells[a]

| Virus | Transformation titer (FFU/ml)[b] | | | |
|---|---|---|---|---|
| | Expt 1 | Expt 2 | Expt 3 | Expt 4 |
| P120 | $5.3 \times 10^3$ | $8.8 \times 10^3$ | $1.2 \times 10^3$ | $1.1 \times 10^4$ |
| Δ35–81 | $1.9 \times 10^1$ | $1.6 \times 10^1$ | NT | NT |
| Δ82–133 | $2.0 \times 10^1$ | $1.7 \times 10^1$ | NT | NT |
| Δ134–165 | $4.2 \times 10^4$ | $1.4 \times 10^5$ | $2.7 \times 10^4$ | NT |
| Δ166–190 | $5.8 \times 10^3$ | $1.1 \times 10^4$ | NT | NT |
| Δ191–215 | $1.6 \times 10^3$ | $3.5 \times 10^3$ | NT | NT |
| Δ134–215 | $3.8 \times 10^4$ | $5.3 \times 10^4$ | NT | NT |
| Δ216–236 | NT | NT | $1.6 \times 10^4$ | $3.4 \times 10^4$ |
| Δ35–236 | NT | NT | <0.5 | <0.5 |

[a] The rate of transformation was assayed by focus-forming assay by infecting NIH 3T3 cells with serial dilutions of virus stocks of similar titers.

[b] The number of transformed foci formed per ml of virus added was determined. Values with a less-than symbol represent the limit of detection. FFU, focus-forming unit; NT, not tested.

TABLE 2. Mutations affecting Gag alter pre-B-cell transformation[a]

| Virus | Transformants per million cells[b] | |
|---|---|---|
| | Expt 1 | Expt 2 |
| P120 | 10 ± 0.1 | 46 ± 0.4 |
| Δ35–81 | <0.5 ± 0.5 | <0.5 ± 0.5 |
| Δ82–133 | <0.5 ± 0.5 | <0.5 ± 0.5 |
| Δ134–165 | 25 ± 0.3 | 70 ± 0.5 |
| Δ166–190 | 22 ± 0.2 | 39 ± 0.3 |
| Δ191–215 | 5 ± 0.1 | 36 ± 0.3 |
| Δ134–215 | 43 ± 0.3 | 59 ± 0.5 |

[a] The rate of transformation was tested using a soft-agar assay (24) by infecting bone marrow cells with undiluted virus stocks of similar titers.

[b] Infected bone marrow cells were plated in agar, and the number of macroscopic colonies of transformants was determined 10 days postinfection. The mean number of transformed colonies per $10^6$ bone marrow cells ± the standard error of the mean is shown. Under these conditions, no macroscopic colonies form in the absence of virus infection. Values with a less-than symbol represent the limit of detection.

**Mutations affecting MA or the majority of Gag sequences decrease transformation.** In contrast to mutations affecting the p12 portion of the v-Abl protein, loss of MA sequences reduced NIH 3T3 cell transformation by more than 200-fold (Table 1). Despite this marked reduction in transformation, both the Δ35–81 mutant and the Δ82–133 mutant gave rise to a small number of transformants; recovery of the viral sequences from these cells and PCR analysis revealed that the original deletions were still present. These rare transformants could have arisen because of compensating mutations elsewhere in the virus or through the effects of complementary mutations present in a minority of NIH 3T3 cells. However, no changes that altered protein size, such as those observed by Prywes and coworkers (18, 19) that affected large portions of the carboxyl terminus, were detected (data not shown). No transformants were recovered from NIH 3T3 cells infected with the Δ35–236 mutant, a mutant that encodes a v-Abl pro-

TABLE 3. Alanine substitutions in p12 do not affect transformation of NIH 3T3 cells by Ab-MLV[a]

| Virus | Transformation titer (FFU/ml) | |
|---|---|---|
| | Expt 1 | Expt 2 |
| P120 | $6.8 \times 10^3$ | $1.7 \times 10^4$ |
| 146–150A | $5.5 \times 10^3$ | $6.3 \times 10^4$ |
| 162–165A | $5.7 \times 10^3$ | $3.2 \times 10^4$ |
| 174–179A | $3.5 \times 10^3$ | $6.0 \times 10^3$ |
| 201–205A | $1.9 \times 10^4$ | $4.9 \times 10^4$ |

[a] NIH 3T3 cells were infected with serial dilutions of virus stocks of similar titers, and the number of foci formed per ml of virus added was determined by focus-forming assay. FFU, focus-forming unit.

TABLE 4. Ab-MLV *gag* mutants with deletions in MA are defective for pre-B-cell transformation[a]

| Virus | Liquid transformation assay[b] | | | |
|---|---|---|---|---|
| | Expt 1 | | Expt 2 | |
| | Frequency | Day | Frequency | Day |
| P120 | 3/3 | 8 | 5/5 | 9 |
| Δ35–81 | 0/4[c] | * | NT | NT |
| Δ82–133 | 0/4[c] | * | NT | NT |
| Δ134–165 | 4/4 | 7 | 5/5 | 8 |
| Δ166–190 | 4/4 | 8 | NT | NT |
| Δ191–215 | 4/4 | 8 | NT | NT |
| Δ134–215 | 4/4 | 8 | NT | NT |
| Δ216–236 | NT | NT | 4/4[c] | 9 |
| Δ35–236 | NT | NT | 0/4[c] | * |

[a] The frequency of transformation was assayed by infecting nucleated bone marrow cells with undiluted virus stocks of similar titers.

[b] Bone marrow cells were infected and plated in liquid medium. Cell cultures were scored as transformed when the density of transformed lymphoblastoid cells reached $2 \times 10^6$ cells/ml. The frequency of transformed cultures out of the total number of cultures plated is shown. Also listed is the day when all of the cultures were transformed. An asterisk indicates cases in which there was no evidence of transformation during the entire observation period of approximately 25 days. Unless indicated (see footnote *c*), the result shown is representative of the results of at least three independent experiments. NT, not tested.

[c] A total of 8 to 10 dishes were evaluated for each virus in two independent experiments.

tein in which the first 34 amino acids of MA are fused directly to v-*abl*-derived sequences.

Because no transformants were recovered from bone marrow cells infected with these mutants in the agar assay (Table 2 and data not shown), they were also examined in a liquid transformation assay that allows infected cells to be monitored for several weeks and thereby can reveal the transforming potential of weakly oncogenic viruses (2, 35). Bone marrow cells were infected with undiluted virus stocks of similar titers and plated in 35-mm dishes. The cultures were monitored for growth and scored as transformed when the density of transformed lymphoblastoid cells reached $2 \times 10^6$ cells/ml (2, 35). As expected, bone marrow cells infected with P120 or the Δ134–165 mutant became transformed within 7 to 9 days (Table 4). However, cultures infected with the Δ35–81, Δ82–133, and Δ35–236 mutants showed no evidence of transformation during the entire observation period. Taken together, these data reveal that the loss of MA sequences or all of the Gag sequences except for the first 34 amino acids affects in a marked way the ability of the virus to induce transformation.

**Cells expressing v-Abl with deletions in MA have decreased levels of Erk phosphorylation.** The v-Abl protein activates multiple signaling pathways that ultimately result in transformation of the cell (reviewed in reference 29). One central downstream circuit that is activated in v-Abl-expressing cells is the mitogen-activated protein kinase pathway. Erk is a key component of this pathway and is activated by phosphorylation in many mitogenic and oncogenic responses (reviewed in reference 13). To determine if the *gag* mutants retain the ability to activate this pathway, 293T cells were transfected with viral plasmids and serum starved and the lysates were analyzed by Western blotting using anti-Erk and anti-phospho-Erk antibodies (Fig. 3). Mutants lacking p12 or CA sequences were similar to the wild-type P120 protein in their ability to stimulate the activation of Erk. In contrast, mutants with deletions of MA sequences and the Δ35–236 mutant were compromised in their ability to stimulate Erk phosphorylation. Densitometric quantitation revealed that Erk1 and Erk2 phosphorylation in cells expressing the Δ35–81 mutant was decreased about 20-fold and 10-fold, respectively, when the samples were normalized for v-Abl and total Erk levels. In cells expressing the Δ82–133 mutant, Erk1 and Erk2 phosphorylation were both reduced by more than 30-fold. A similar decrease was observed in cells expressing the Δ35–236 mutant. The inability to stimulate Erk phosphorylation indicates that these mutants have defects in their ability to signal to at least one downstream pathway that is critical for transformation (reviewed in references 29 and 42).

**The MA and the Δ35–236 mutants do not support the growth and survival of pre-B cells.** To determine if the mutants that fail to transform pre-B cells are capable of affecting the growth or survival of pre-B cells, the ability of these proteins to substitute for the v-Abl protein encoded by the temperature-sensitive P70/H590 mutant Ab-MLV strain was examined (5, 8). 7C411 cells, which grow normally at the permissive temperature but not at 39.5°C, the nonpermissive temperature for the P70/H590 mutant, were infected with wild-type virus and the different mutants, and the infected cells were recovered by sorting the populations on the basis of GFP expression. As expected, all of the 7C411 derivatives grew well at the permissive temperature (data not shown). When the cells were shifted to the nonpermissive temperature, cells expressing the wild-type P120 protein and those expressing the hypertransforming Δ134–165 mutant grew rapidly and maintained high viability (Fig. 4A and B). However, cells expressing the Δ35–81, Δ82–133, and Δ35–236 mutants did not grow at the nonpermissive temperature and their viability decreased. Thus, these mutants fail to transmit signals required for either the growth or viability of pre-B cells.

Because some mutations affecting Gag have been reported to affect the stability of the v-Abl protein in lymphoid cells (19), the expression of the mutants that failed to support the growth of 7C411 cells was examined by immunofluorescence staining. Cells were stained with an anti-Abl antibody (28) that reacts with an epitope in the carboxyl terminus of the protein, a portion of the protein that is not present in the P70/H590 v-Abl protein expressed by 7C411 cells but is found in the P120 wild-type strain and all the mutants. To compare the intensities of staining, the images were analyzed with the image process-

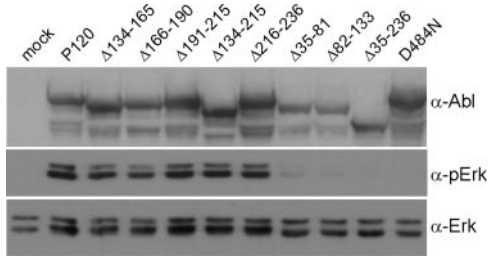


FIG. 3. Ab-MLV *gag* mutants that have deletions in MA sequences are defective for Erk activation. 293T cells were transiently transfected with plasmids expressing wild-type virus, the different *gag* mutants, or the kinase-defective P120/D484N mutant. The cells were incubated in serum-free medium, and the lysates were prepared and analyzed by Western blotting using the indicated antibodies. α, anti.

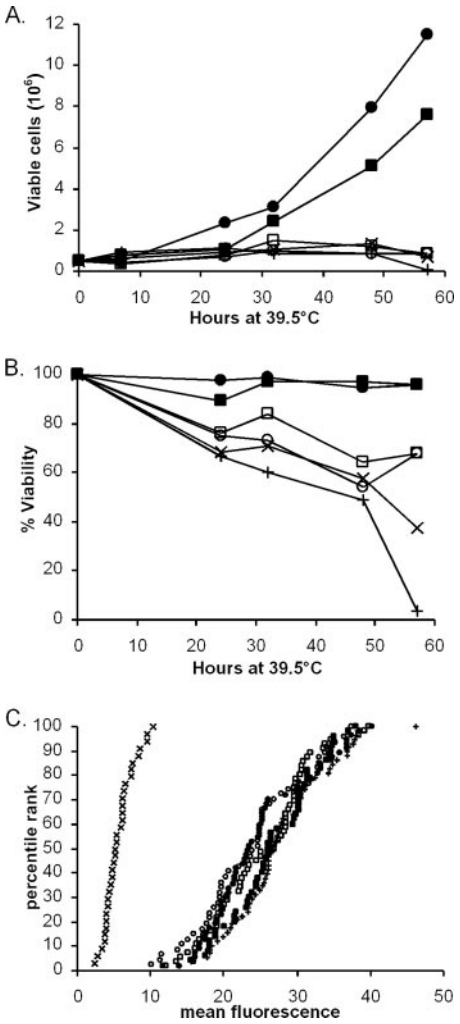


FIG. 4. Ab-MLV *gag* mutants that are defective for transformation do not support the growth and survival of 7C411 cells at the nonpermissive temperature. Temperature-sensitive 7C411 cells (6) that had been superinfected with wild-type virus or one of several of the *gag* mutants were assayed for growth (A) and viability (B) at the nonpermissive temperature (39.5°C). (A) The growth of the cells was determined by counting the number of live cells by trypan blue exclusion using a hemocytometer after shifting to the nonpermissive temperature. (B) Viable cells were identified by trypan blue staining, and the percent viability was calculated. (C) Expression of the v-Abl proteins encoded by the mutants that fail to support growth of 7C411 cells was confirmed by analysis with ImageJ 1.36b software, an image analysis program that calculates the mean fluorescence per cell. The values were plotted by percentile rank; each point represents an individual cell. Symbols represent the following: ×, uninfected; ●, wild type; ■, Δ134–165 mutant; ○, Δ35–81 mutant; □, Δ82–133 mutant; +, Δ35–236 mutant. The data are representative of the results of at least three independent experiments.

ing software ImageJ 1.36b (http://rsb.info.nih.gov/ij), and the mean fluorescence per cell was plotted on a graph based on the percentile rank (Fig. 4C). These analyses reveal that the levels of expression of the Δ35–81, Δ82–133, and Δ35–236 mutants are comparable to the level of expression of the wild-type protein. Thus, protein instability does not explain the failure of these mutants to stimulate the growth of pre-B cells.

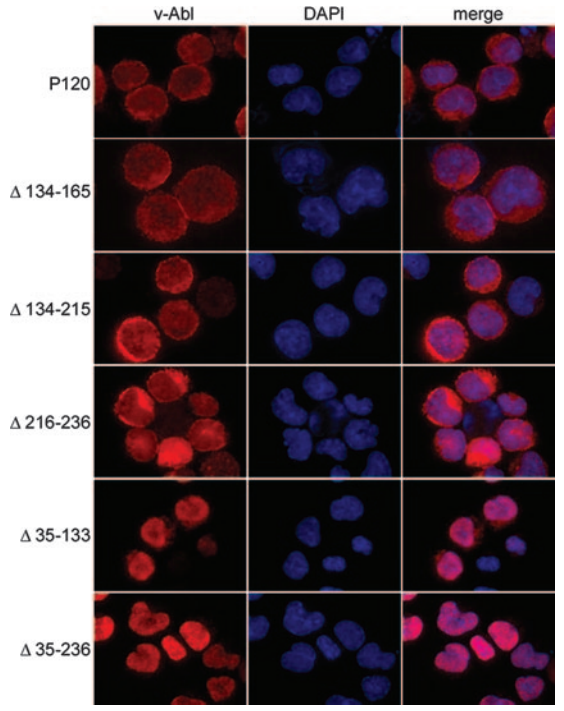


FIG. 5. Gag sequences influence subcellular localization of the v-Abl protein. The localization of the v-Abl proteins was monitored by immunofluorescence staining of 7C411 cells with anti-Abl antibodies and DAPI. The anti-Abl antibody used in these studies reacts with a determinant in the carboxyl terminus of the protein and does not stain the P70/H590 protein. The images shown are representative of the results of a minimum of three independent experiments in which at least 250 cells were observed.

**Deletion of Gag results in nuclear localization of the v-Abl protein.** The Gag portion of v-Abl influences the localization of the protein to the plasma membrane as a result of myristoylation of the glycine residue at position 2 in the MA-derived portion, and proper localization has been correlated to transformation (3). To determine if the v-Abl proteins encoded by the transformation-deficient mutants displayed altered subcellular localization, 7C411 cells expressing one of several of the mutants or the wild-type P120 protein were analyzed by using immunofluorescence staining with an anti-Abl antibody (28) that reacts with an epitope in the carboxyl terminus of the protein. The P120 protein displayed the expected localization (3, 23, 30); staining decorated the areas under the plasma membrane, and punctate staining was also observed in the cytoplasm (Fig. 5). In contrast, analysis of cells expressing the Δ35–236 v-Abl, a protein that lacks all but the first 34 amino acids of Gag, revealed that v-Abl and DAPI staining were completely overlapping, indicating that the protein was localized to the nucleus. These results suggest that the myristoylation signal and the basic residues at the N terminus, features retained by the Δ35–236 v-Abl protein, are not sufficient for proper localization of the protein and reveal an additional role of Gag in delivering and maintaining the molecule in the appropriate cellular compartment.

To explore more fully the way in which Gag sequences contribute to localization, we constructed the Δ35–133 mutant, which encodes a v-Abl protein missing all of MA except the

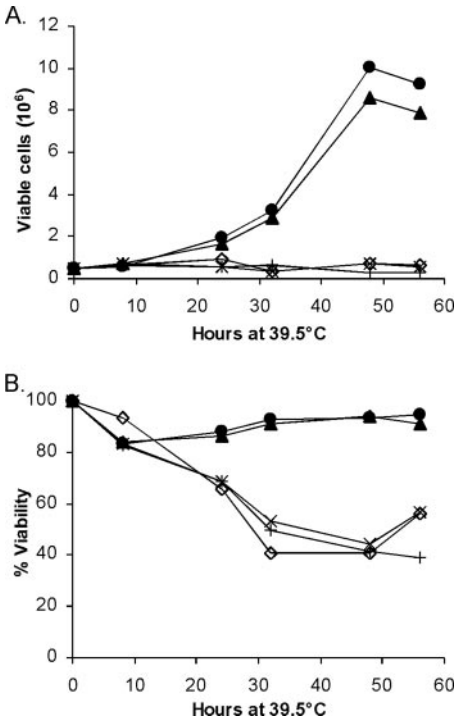


FIG. 6. The Δ35–133 mutant, lacking most of MA, does not support growth and survival in 7C411 cells. Temperature-sensitive 7C411 cells (6) that had been superinfected with wild-type virus or one of several of the *gag* mutants were assayed for growth (A) and viability (B) at the nonpermissive temperature (39.5°C). (A) The growth of the cells was determined by counting the number of live cells by trypan blue exclusion using a hemocytometer after shifting to the nonpermissive temperature. (B) Viable cells were identified by trypan blue staining, and the percent viability was calculated. Symbols represent the following: ×, uninfected; ●, wild type; ▲, Δ134–215 mutant; ◇, Δ35–133 mutant; +, Δ35–236 mutant. The data shown are representative of the results of three independent experiments.

first 34 amino acids (Fig. 1A). The v-Abl protein encoded by this mutant, along with the v-Abl proteins encoded by the Δ134–215 mutant, which lacks all of p12, and by the Δ216–236 mutant, which lacks all of CA, was examined by immunofluorescence staining. The localization of the Δ134–215 and Δ216–236 v-Abl proteins was identical to that observed for cells expressing the wild-type P120 protein (Fig. 5). In contrast, a significant portion of the Δ35–133 protein was found in the nucleus, but unlike the Δ35–236 mutant, a diffuse signal was also consistently observed in the cytoplasm (Fig. 5). These data suggest that sequences within MA play an important role in suppressing nuclear localization of the protein. However, the presence of staining throughout the cell, a pattern reminiscent of GFP expression (data not shown), could indicate that this protein lacks signals that target it to a specific compartment.

Because the localization of the Δ35–133 protein was abnormal, the mutant was tested for its ability to rescue the temperature sensitivity of 7C411 cells. Consistent with the results of earlier experiments, cells expressing wild-type virus or those expressing the Δ134–215 protein grew well and maintained high viability at the nonpermissive temperature (Fig. 6). In contrast, cells expressing the Δ35–133 protein did not grow and showed low viability at the nonpermissive temperature, a pattern similar to that observed for other mutants with mutations affecting MA sequences. These results reinforce the importance of plasma membrane localization in transformation and indicate that sequences within MA play a prominent, but not exclusive, role in mediating the appropriate localization of the v-Abl protein.

## DISCUSSION

Our study has revealed that the Gag portion of v-Abl influences viral transformation by suppressing nuclear localization of the oncoprotein. While wild-type v-Abl protein is targeted to the inner surface of the plasma membrane (3), deletion of all but the first 34 amino acids of the Gag region results in a kinase-active molecule that is found in the nucleus. The MA portion of Gag appears most important in mediating this phenomenon because a significant portion of the Δ35–133 protein is also found in the nucleus. Consistent with this idea, mutants lacking p12 or CA sequences encode proteins that are indistinguishable in localization from the wild-type P120 protein. The ability of Gag residues to influence v-Abl localization is critical for transformation because mutants lacking these sequences fail to transform either NIH 3T3 or pre-B cells and cannot sustain the growth of the temperature-sensitive pre-B-cell transformant 7C411.

Both the Δ35–133 and Δ35–236 mutants retain the first 34 amino acids that include sequences shown by others to be sufficient for myristoylation (1, 10, 31), a modification that is important for targeting to the plasma membrane, and the basic amino acids that are important for membrane association of the Pr65Gag precursor (30). Consistent with these functions, v-Abl proteins in which an aspartic acid (3) or an alanine (our unpublished results) has been substituted for the glycine that is targeted for myristoylation display a diffuse cytoplasmic localization pattern and are excluded from the nucleus. Thus, while myristoylation appears important for plasma membrane localization, this modification is not sufficient to properly direct the protein to this compartment when other Gag sequences are missing.

The Δ134–165 mutant that lacks sequences encoding the N-terminal third of p12 displayed enhanced transformation of both NIH 3T3 and pre-B cells. The mechanism by which this is accomplished requires further study. Because the v-Abl protein encoded by this mutant is similar to the wild-type protein in the Erk assay, the alteration might affect signaling through one of the other pathways critical for v-Abl-mediated transformation. The sequences deleted in this mutant include the PPPY motif that comprises the L domain of Mo-MLV (39). The L domain interacts with the cellular endosomal sorting machinery to facilitate the release of viruses during the budding process (reviewed in reference 4). However, because alanine substitutions affecting these residues do not affect transformation, a role for the L domain interactions in suppressing transformation seems unlikely. Since its original isolation, Ab-MLV has undergone multiple rounds of replication during passage in mice and rats, experiments that had the possibility of selecting for enhanced oncogenic potential. Nonetheless, natural loss of p12 sequences has not been reported. The effect of p12 deletion is not as pronounced in pre-B-cell transformation assays, and this fact may contribute to the stability of p12

sequences within the virus. Alternatively, perhaps the retention of the sequences encoding p12 affects virus replication in some fashion.

The Δ35–81, Δ82–133, and Δ35–236 mutants are highly compromised in their ability to transform both NIH 3T3 cells and pre-B cells and fail to stimulate Erk phosphorylation, a downstream signal required for transformation (reviewed in reference 29). Previous reports suggested that the sequences deleted in these mutants are required for the transformation of pre-B cells, but not NIH 3T3 cells (18, 19). Although the specific amino acids missing in the different panels of mutants are not absolutely identical, this difference may not explain the discrepant outcome. The earlier mutants were prepared on the P160 background, and for reasons that were not investigated, cells transformed by these mutants always expressed v-Abl proteins that had undergone additional mutations that deleted sequences in the carboxyl terminus. Because the earlier experiments involved transfection and subsequent virus replication, these mutations presumably arose during this process and were selected for their ability to transform cells. Our experiments involved helper virus-free stocks and monitored transformation in the absence of replication, greatly reducing the potential to undergo additional mutation. When viewed in this context, it seems probable that the ability of Gag sequences to suppress nuclear localization of the protein occurs only in the presence of the full carboxyl terminus. This phenomenon likely influenced the selection for additional mutation in the Prywes experiments (18, 19).

The v-Abl protein arose through a recombination event that joined *gag* to c-*abl*. This recombination event generated a protein with constitutive kinase activity, but also changed the localization of the molecule. The importance of these changes is emphasized by the observation that the overexpression of a c-Abl protein containing a myristoylation signal or the first 34 amino acids of Gag and lacking the c-Abl regulatory SH3 region can transform NIH 3T3 cells (7), albeit at a low level. These results highlight the role that the loss of c-Abl regulatory sequences and acquisition of the amino terminal portion of MA played in activating the transforming potential. Our data illustrating that Gag residues suppress nuclear localization reveal an additional feature acquired during recombination and broaden the way in which the function of the fusion protein is modulated.

The c-Abl protein can shuttle between the nucleus and the cytoplasm (32), a feature that was lost as a result of the recombination event that generated the v-Abl protein. In normal myeloid cells, the majority of endogenous c-Abl appears to be cytoplasmic (38), where this protein and the related BCR/ABL oncoprotein important in chronic myeloid leukemia (reviewed in reference 33) bind F-actin through sequences at the extreme carboxyl terminus (34). This region also has been reported to contain a nuclear export signal (NES) (32), as well as three nuclear localization signals (NLS) (37). However, recent nuclear magnetic resonance studies, examining the portion of the carboxyl terminus of c-Abl that interacts with F-actin and contains the NES, have suggested that the NES is buried and nonfunctional when the molecule is in its normal conformation (9). Thus, other mechanisms are likely to influence the movement of c-Abl protein within cells.

An understanding of how Gag impacts mechanisms controlling the localization of c-Abl requires additional experimentation. For example, the three NLS are missing in the P120 protein that is used in our studies. Even though Ab-MLV P120 is considered a wild-type strain, the virus has an in-frame deletion that removes 263 amino acids, including all three NLS, from the kinase-proximal portion of the carboxyl terminus (11, 21). The extreme carboxyl terminus of P120 is identical to that of murine c-Abl and shares the F-actin and NES sequences with human c-Abl and BCR/ABL. However, several Ab-MLV mutants that lack these sequences retain the ability to transform NIH 3T3 cells at levels similar to that of wild-type virus and can still transform pre-B cells, albeit at reduced levels (16, 25, 35). Perhaps a portion of this phenotype reflects the abilities of sequences in the Gag portion of the molecule to affect the function or structure of sequences at the extreme carboxyl terminus. Combining mutations affecting carboxyl-terminal sequences with those affecting Gag could help in understanding the mechanisms by which sequences in both parts of the protein influence transformation.

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