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Use of Site-Specific Antipeptide Antibodies to Perturb the Serine Kinase Catalytic Activity of p37^{mos}

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The *mos* oncogene of Moloney murine sarcoma virus encodes a protein of approximately 37,000 daltons (designated p37^{mos}). We have detected a serine protein kinase activity which is closely associated with p37^{mos} in immune complexes obtained with antibodies [anti-mos(37-55) serum] that were generated with a peptide containing amino acids 37 through 55 of the v-*mos* protein (S. A. Maxwell and R. B. Arlinghaus, Virology 143:321–333, 1985). Immune complexes that were derived with antibodies generated against peptides representing the C-terminal 8 or 12 amino acids of v-*mos* (anti-C2 and anti-C3 serum, respectively) exhibited very little kinase activity capable of phosphorylating p37^{mos}. Treatment of anti-*mos*(37-55) complexes containing active v-*mos* kinase with anti-C3 or anti-C2 serum resulted in a dramatic reduction of the in vitro phosphorylation of p37^{mos}. Antiserum blocked with the appropriate C-terminal peptide had no inhibitory effect on the phosphorylation of p37^{mos} in anti-*mos*(37-55) complexes which indicated that the inhibition of v-*mos* kinase activity was a specific effect of these antibodies. The specific inhibition of the in vitro phosphorylation of p37^{mos} by antibodies directed against the C terminus of the v-*mos* protein provides strong evidence that the v-*mos* gene encodes a serine protein kinase. In addition, the extreme C terminus of p37^{mos} may be critical for an active v-*mos* kinase.

A number of retrovirus oncogene-coded proteins have been shown to be closely associated with protein kinases that phosphorylate tyrosine residues (1, 4–6, 9, 15, 25). The *src* and *abl* protein kinase activities are two of the most widely studied examples (1, 3, 4, 15, 19, 25). Several types of evidence indicate that their tyrosine protein kinase activities are intrinsic properties of their respective gene products (10, 23). The v-*mos* gene has been placed within this group of oncogenes because it contains sequences that are homologous to the catalytic site of the tyrosine kinase group (22). In addition, the v-*mos* gene contains sequences that are homologous to the ATP binding site present in bovine cyclic AMP-dependent protein kinase (11). Our laboratory has provided strong evidence that the *gag-mos* proteins encoded by *ts*110 Moloney murine sarcoma virus and its revertant are closely associated with a protein kinase activity that phosphorylates at predominantly serine residues with some activity at threonine (12, 13). We have shown that p37^{mos} encoded by Moloney murine sarcoma virus also has an associated serine protein kinase (16).

The methodology used by many investigators requires the use of an immune complex to isolate and assay the protein kinase activity. Because of the presence of contaminating proteins in such immunoprecipitates, it is difficult to prove that the kinase is an intrinsic property of the protein in question. In this report we have used antibodies to a synthetic peptide from the N-terminal domain of p37^{mos} to isolate the active v-*mos* kinase capable of phosphorylation at serine residues (16). Such immune complexes were specifically inhibited by addition of antibodies to the extreme C terminus of the p37^{mos}. This result provides strong evidence that the v-*mos* gene encodes a serine protein kinase.

NIH 3T3 cells acutely infected with Moloney murine sarcoma virus were prepared and processed as described previously (16). The treated 3T3 cells exhibited morphology of transformed cells 2 to 3 days after infection. A separate flask of infected 3T3 cells was used for 4-h [^{35}S]methionine labeling as described previously (16). The radioactive medium was then removed, and the [^{35}S]methionine samples were prepared identically to those for the kinase assay (described below). For immunoprecipitation, medium was drained thoroughly from the infected 3T3 cells, and 0.5 ml of lysis buffer (1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 10 mM sodium phosphate [pH 7.2], 100 kIU of trasylol per ml) was added for each 40 to 60% confluent 25-cm^2 flask of cells. The cells were lysed, and the lysate was clarified by a high-speed spin (60,000 × *g* for 30 min) as described previously (16). The clarified extract was then incubated on ice for 20 to 30 min with 1 ml of Pansorbin (10% Formalin-fixed *Staphylococcus aureus*; Calbiochem-Behring, La Jolla, Calif.) that had been previously complexed with 0.5 normal rabbit serum immunoglobulin G for each 0.5-ml cell extract. After centrifugation (10,000 × *g* for 10 min) to remove the Pansorbin-immunoglobulin G, 20 μl of heat-inactivated antiserum was added per 0.5 ml of cell extract and the immune reaction was allowed to proceed for 1 to 2 h on ice. Blocking of the antipeptide antibody was done with an excess of synthetic peptide: 2 μg of cyclic v-*mos*(37-55) peptide (composed of amino acids 37 through 55 of the predicted v-*mos* sequence [22]) per 20 μl of anti-*mos*(37-55) sera [directed against cyclic v-*mos*(37-55) peptide (7)]; 3 μg of C3 peptide per 20 μl of anti-C3 sera (directed against the C-terminal 12 amino acids of v-*mos* [18]). The immune complexes were then precipitated with an equal volume (to that of antiserum) of Pansorbin on ice for 15 to 20 min. The Pansorbin-precipitated immune complexes were washed twice with wash buffer (0.1% Nonidet P-40, 150 mM NaCl, and 10 μM sodium phosphate [pH 7.2]). For the anti-C3 serum inhibition of the v-*mos* kinase, anti-*mos*(37-55) complexes containing active v-*mos* kinase were resuspended in 100 μl of wash buffer. Heat-inactivated (56°C for 30 min) anti-C3 serum (20 μl) was added to the suspension of anti-*mos*(37-55) complexes, and the mixture was incubated on ice for 1 h. The anti-C3-treated anti-*mos*(37-55) complexes were then

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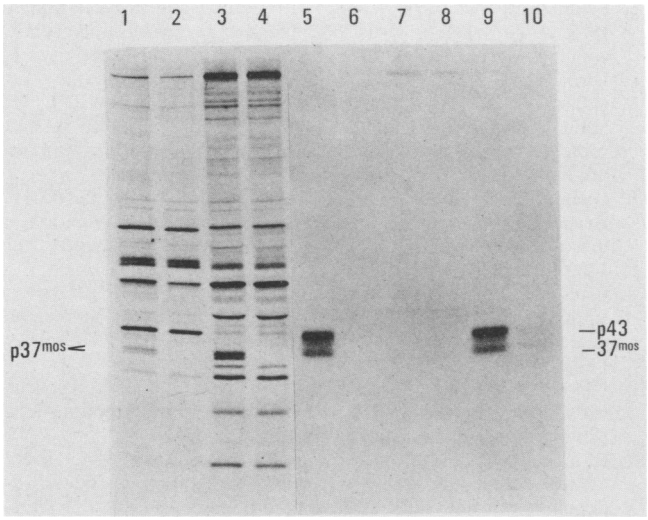

FIG. 1. Inhibition of the p37^{mos}-associated kinase with antibody to a synthetic peptide from the C terminus of the v-*mos* protein. The gel was treated with En^{3}Hance (New England Nuclear Corp; Boston, Mass.) for 45 min and subjected to autoradiography on Kodak XAR-5 film by using an enhancing screen. [^{35}S]methionine immune complexes (lanes 1 through 4) and ^{32}P-labeled immune complex kinase samples (lanes 5 through 10) were exposed for 18 and 2 h, respectively. Lanes: 1 and 5, anti-*mos*(37-55) immune complex; 2 and 6, cyclic v-*mos*(37-55) peptide-blocked anti-*mos*(37-55) immune complex; 3 and 7, anti-C3 complex; 4 and 8, C3 peptide-blocked anti-C3; 9, anti-*mos*(37-55)–p37^{mos} complexes treated with anti-C3 serum that had been blocked with C3 peptide before assay; 10, anti-*mos*(37-55)–p37^{mos} complexes treated with anti-C3 before assay.

washed once with wash buffer. Washed Pansorbin pellets were thoroughly drained and disrupted in 45 μl of wash buffer containing 1 mM sodium pyrophosphate. A 5-μl portion of a solution containing 2 mM Quercetin in dimethyl formamide (12.8 M) was then added, and the disrupted pellets were incubated on ice for 10 min. Quercetin inhibits background protein kinases but not the v-*mos* kinase (16).

The kinase assay was performed as described previously (16) by using 20 μCi of [γ-^{32}P]ATP, 10 μM ATP, 7.5 mM $MnCl_2$, and 1 mM sodium pyrophosphate in 100 μl (final volume). The reaction proceeded for 10 min at 22°C and was terminated by adding 3 ml of RIPA buffer (1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 1% deoxycholate, 1 mM EDTA, 150 mM NaCl, and 20 mM sodium phosphate [pH 7.2]) containing 2 mM ATP and 1 mM sodium pyrophosphate, and the immune complexes were washed twice with RIPA buffer. Washed pellets were drained and disrupted in 40 to 50 μl of sample buffer (2% sodium dodecyl sulfate, 10% 2-mercaptoethanol, 10% glycerol, and bromophenol blue in 20 mM Tris [pH 6.8]). Suspended pellets were then heated in a boiling-water bath for 3 to 5 min and centrifuged at 4,000 × *g* for 5 min to remove the Pansorbin. The supernatants were then applied to an 8% sodium dodecyl sulfate-polyacrylamide gel, and electrophoresis was performed for 3.5 h at 30 mA (constant current) per gel.

The experiment shown in Fig. 1 involves a comparison of two antisera made against different v-*mos* peptides for their ability to immunoprecipitate p37^{mos} and catalyze its autophosphorylation. Both anti-*mos*(37-55) sera (7) and anti-C3 sera (18) recognized p37^{mos} (Fig. 1, lanes 1 and 3, respectively). The recognition of p37^{mos} by each of the antipeptide sera is specific, since blocking the sera with peptide before immunoprecipitation failed to yield any p37^{mos} (lanes 2 and 4). In agreement with the work of Papkoff et al. (18), p37^{mos} is detected as a doublet, the upper band being the phosphorylated form of p37^{mos}.

Under assay conditions used to selectively enhance the p37^{mos}-associated kinase (16), anti-*mos*(37-55) complexes produced both phosphorylated p37^{mos} and phosphorylated 43-kilodalton protein (p43) (lane 5). Both proteins were found to contain only phosphoserine (16). Blocking the anti-*mos*(37-55) serum with cyclic v-*mos*(37-55) peptide before harvesting the immune complexes prevented the formation of phosphorylated products (lane 6). Within the limits of V8 protease partial digestion (2), both metabolically and in vitro-phosphorylated p37^{mos} generated identical phosphopeptides (16). p43 is believed to be a super-phosphorylated form of p37^{mos} on the basis of its delayed formation during the kinase assay and its pattern of V8 protease-generated peptides (16). In contrast to the results obtained with anti-*mos*(37-55)–p37^{mos} complexes, anti-C3–p37^{mos} complexes allowed only an extremely weak phosphorylation of the v-*mos* proteins to occur. In fact, after a 2-h exposure (lanes 5 through 10) no p37^{mos} or p43 was detected (lane 7). Faint p37^{mos} and p43 bands were only seen after 48 h of exposure. These results are consistent with previous studies which detected no kinase activity associated with p37^{mos} in the anti-C3 immune complex kinase assay (17). The lack of in vitro phosphorylation of p37^{mos} in anti-C3 complexes could be due either to recognition of v-*mos* proteins not activated for kinase activity or to the fact that C-terminal v-*mos* antibodies inhibit phosphotransferase activity contained within the v-*mos* protein.

To test the latter possibility, anti-C3 serum was incubated with active Pansorbin-precipitated anti-*mos*(37-55) complexes containing p37^{mos}(37-55). The treated complexes were washed by pelleting to remove extraneous serum components and then assayed for kinase activity. The in vitro phosphorylation of p37^{mos} was dramatically reduced on treatment of active anti-*mos*(37-55) complexes with anti-C3 serum (lane 10). If the anti-C3 serum was blocked with C3 peptide before addition to active anti-*mos*(37-55) complexes, no inhibition of in vitro phosphorylation of p37^{mos} occurred (lane 9). We have obtained similar inhibitory results with antibodies (kindly supplied by Tony Hunter of the Salk Institute, La Jolla, Calif.) obtained from a peptide (C2) composed of the eight C-terminal amino acids (18) of p37^{mos} (results not shown). Again, blocking the antibody with the C2 peptide prevented the inhibitory activity. Of interest is the observation that anti-C2 immunoprecipitates containing p37^{mos} show significantly more kinase activity than do anti-C3 immunoprecipitates, in that with anti-C2 complexes p37^{mos} and p43 phosphorylated forms are seen as weak bands in 6- to 8-h exposures, whereas with anti-C3 complexes at least 2-day exposures are needed to detect weak bands. However, anti-*mos*(37-55) is much better for kinase activity than is anti-C2 sera.

The experiments described here provide convincing evidence that p37^{mos} is itself a protein kinase and that antipeptide antibodies directed toward its C-terminal domain can interfere with the autophosphorylation activity of the p37^{mos} kinase. Thus, these results suggest that the C terminus of the v-*mos* protein plays an important role in the activity of the v-*mos* kinase and possibly in cell transformation.

Antipeptide antibodies have been shown to inhibit the tyrosine kinases of several viral oncogenes. Antibodies against an internal peptide (42 residues from the C terminus)

of the *fes* portion of the *gag-fes*-associated kinase were found to be inhibitory (20). Similarly, antibody against a peptide containing the sequence of amino acids 498 through 512 in v-*src* has been shown to inhibit kinase activity of $pp60^{src}$ (8), whereas antibody reactive for the extreme C-terminal six amino acids of $pp60^{src}$ had no effect on tyrosine kinase activity. In the *abl* system, antibodies reactive with a peptide representating an 11-amino-acid sequence immediately amino terminal to one of the in vivo sites of tyrosine phosphorylation of v-*abl* P160 and a second antibody to a larger sequence encompassing the other site of in vivo tyrosine phosphorylation (both contained within the N-terminal half of the v-*src* homologous region of v-*abl*) were found to be inhibitory for autophosphorylation of the v-*abl* protein present in cells transformed by Abelson murine leukemia virus (14). In other studies, C-terminal sequences have been found to be important for the transforming function of the v-*ras* transforming protein. The v-*ras* oncogene product requires its C-terminal sequence for lipid incorporation and association with the cytoplasmic membrane (24).

The mechanism of the inhibition of v-*mos* kinase by C-terminal-directed antibodies is unclear, but conformational changes induced by reactivity of the antibodies to the C terminus of the protein may be a likely possibility. The reactivity of antipeptide antibodies toward the expected native protein appears to be a function of the atomic mobility of sites in proteins (21). The C terminus of $p37^{mos}$ may be a highly mobile site that moves through a range of conformations. Antibodies to the C-terminal domain may fix $p37^{mos}$ in a conformational state that perturbs the catalytic site of the transphosphorylase domain. Our structural studies indicate that the C-terminal-directed antibodies are not blocking the site of phosphorylation of $p37^{mos}$ during autophosphorylation, since only the N-terminal half of $p37^{mos}$ is phosphorylated (unpublished results). We note that the proposed ATP binding site in $p37^{mos}$ is near its N terminus. However, since one $p37^{mos}$ molecule is thought to be phosphorylated by another $p37^{mos}$ molecule in an autophosphorylation reaction, it is possible that the C-terminal-directed antibodies interfere with binding of one $p37^{mos}$ molecule to the catalytic site of another. It will now be of interest to generate v-*mos* deletion mutants to see whether C-terminal sequences of v-*mos* are necessary for kinase activity and possibly for cell transformation.

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