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v-Src Enhances Phosphorylation at Ser-282 of the Rous Sarcoma Virus Integrase

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The Rous sarcoma virus (RSV) integrase (IN) and the β polypeptide (β) of the reverse transcriptase are posttranslationally modified by phosphorylation on Ser at amino acid position 282 of IN. When IN was immunoprecipitated from RSV (Prague A strain) virions, approximately 30 to 40% of the IN molecules were phosphorylated. When IN was immunoprecipitated from a v-*src* deletion mutant (ΔMst-A) of RSV or from avian myeloblastosis virus (AMV), the percentage of IN molecules that were phosphorylated was significantly reduced. This reduction in phosphorylation of IN between virions was verified by [^{35}S]Met-[^{35}S]Cys or ^{32}P labeling of IN, followed by immunoprecipitation analysis using antisera directed to the amino or carboxyl terminus of IN. In ΔMst-A or AMV, a nonphosphorylated, slightly truncated (at the carboxyl terminus) polypeptide was the major species of IN. The enhanced phosphorylation of IN does not appear to be a general function of transformed cells, since enhanced phosphorylation was not detected in AMV derived from viremic chickens or from a v-*src* deletion mutant of RSV propagated in a chemically transformed quail cell line, QT6. From these data, we conclude that v-Src is necessary for efficient phosphorylation of IN and β.

Rous sarcoma virus (RSV) is a replication-competent, acute transforming retrovirus that transforms cells via its oncogene, v-*src* (Fig. 1). This oncogene codes for a 60-kDa protein that possesses tyrosine kinase activity and is associated with the inner face of the cytoplasmic membrane. The tyrosine kinase activity initiates a cascade of events that leads to DNA replication, cell division, and neoplasia. This cascade includes the activation of several serine/threonine kinases (for a review, see reference 2).

Two important steps of retrovirus replication are (i) reverse transcription of the RNA genome into a DNA copy and (ii) integration of the DNA copy into the host genome. These processes are performed by two *pol* gene products: reverse transcriptase (RT) (αβ in the avian retroviruses) and the integrase (IN). These proteins are produced by proteolytic processing of a *gag-pol* precursor, Pr180$^{gag\text{-}pol}$ (Fig. 1B) (3). Proteolytic processing occurs within the virion after budding and is the result of the virus-encoded protease.

The β subunit (β) of RT and IN are posttranslationally modified by phosphorylation (15). The major site of phosphorylation is the Ser at amino acid position 282 of IN and the corresponding Ser in β (7, 8). Like proteolytic processing, phosphorylation occurs within the virion after budding (3) but is the result of a host kinase. The virion-associated kinase responsible for phosphorylation of IN and β has not been identified, although several kinases have been purified from RSV and avian myeloblastosis virus (AMV) (14, 20). It is unclear whether phosphorylation directly affects the enzymatic properties of IN or RT (11, 18, 19).

When immunoprecipitated from RSV, IN exhibits polypeptide heterogeneity, depending on the state of phosphorylation and the actual site of processing at the carboxyl terminus. Three polypeptides are detected: (i) the full-length (286-amino-acid) phosphorylated species, (ii) the full-length nonphosphorylated species, and (iii) the truncated (approximately at residue 282) nonphosphorylated species (Fig. 2) (8). The exact carboxyl terminus of the truncated species has not been rigorously identified. We presume that β has similar heterogeneity at the carboxyl terminus. However, because of the large mass (94 kDa), the various species cannot be resolved electrophoretically. Mutations at the site of phosphorylation altered the proteolytic processing at the carboxyl terminus of IN. From these data, we proposed that phosphorylation directs proteolytic processing to the full-length site (Fig. 1). The nonphosphorylated form would be proteolytically cleaved at the truncated site. We now propose and provide evidence that v-Src indirectly promotes phosphorylation of the RSV IN. The major site of phosphorylation remains at Ser-282 in the presence or absence of v-Src. The enhanced phosphorylation appears to be dependent on v-Src and not a general function of transformed cells, since the effect was not observed in a chemically transformed avian cell line.

MATERIALS AND METHODS

Plasmids, viruses, and cell culture. pΔMSTA and pJD100 were kindly provided by J. T. Parsons. pΔMSTA was derived from pJD100 (13, 21). Virus (ΔMst-A) derived from pΔMSTA lacked v-*src* sequences from positions 7150 to 8635 (16). Stocks of wild-type RSV Prague A (PrA) virus and Mst virus were obtained by DNA transfections of chicken embryo fibroblast (CEF) cells (C/E, leukosis free, Chf$^-$ Gs$^-$) (SPAFAS, Inc.). Chemically transformed quail cells (QT6) were also used to grow virus. [^{35}S]Met-[^{35}S]Cys (Tans[^{35}S]-label, ICN Biochemicals) and $^{32}P_i$-labeled viruses were propagated and purified as described previously (7, 8).

Immunoprecipitation analysis. Immunoprecipitation of radiolabeled PrA, AMV, and mutant viruses was previously described (7). Rabbit polyclonal antisera directed against peptides generated to the amino-terminal 10 residues or the last 11 residues at the carboxyl terminus of IN were used (8). The carboxyl-terminal antiserum (C28) was used for these studies. The immunoprecipitated ^{32}P- or ^{35}S-labeled *pol* products were analyzed on analytical or preparative sodium dodecyl sulfate (SDS)–10% polyacrylamide gels or on 8 to 22% polyacrylamide-SDS gels (8). The labeled proteins were

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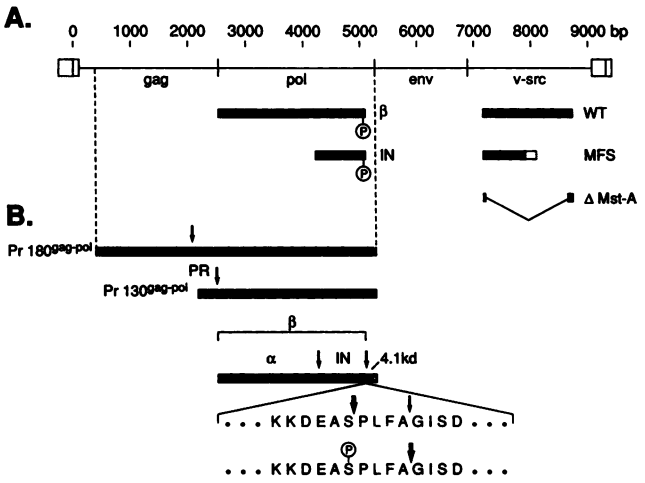

FIG. 1. (A) Schematic of the RSV genome, the mature β and IN protein, and the wild-type and mutant v-Src proteins. The circled P indicates the major site of phosphorylation on β and IN. (B) Proteolytic processing pathway of the *pol* gene products. Arrows indicate sites of proteolytic processing. The larger arrows indicate preferential processing sites at the carboxyl terminus of IN (8). WT, wild type.

autoradiographed from dried gels by using Kodak X-Omat film with or without an intensifying screen. Tryptic peptide analysis of ^{32}P-labeled IN was previously described (7).

RESULTS

Is the phosphorylation state of IN dependent on the oncogene carried by virus? We initially examined the various polypeptide forms of IN from three different avian retroviruses propagated in CEF cells. These viruses included (i) RSV, PrA strain, which carries v-*src*; (ii) AMV, which carries the oncogene v-*myb*; and (iii) ΔMst-A, a deletion mutant in the v-Src coding region of RSV PrA (Fig. 1). The viruses were propagated in CEF cells, labeled with [^{35}S]Met-[^{35}S]Cys, immunoprecipitated with antiserum directed against the amino-terminal 10 amino acids of IN, and fractionated by high-resolution SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2). IN from PrA was resolved into three distinct bands: the full-length phosphorylated species (slowest migration), the full-length nonphosphorylated species (middle band), and the truncated nonphosphorylated species (fastest migration). Data confirming the identities of these species are presented below. The phosphorylated

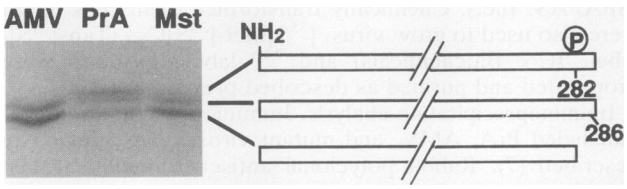

FIG. 2. Proportionality of IN species in PrA, ΔMst-A, and AMV. ^{35}S-labeled RSV PrA, ΔMst-A, and AMV were immunoprecipitated by antisera directed against the NH_2-terminal 10 amino acids of IN. The samples were subjected to electrophoresis on 8 to 22% polyacrylamide–SDS, and the dried gel was subjected to autoradiography. The schematic on the right depicts the three species of IN.

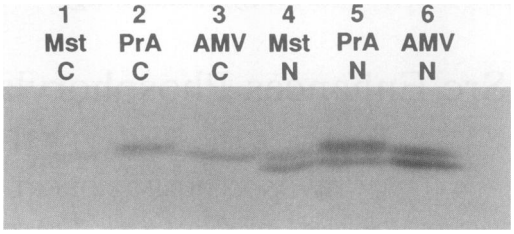

FIG. 3. Immunoprecipitation of ^{35}S-labeled AMV, RSV, and ΔMst-A. A total of 10^6 cpm of purified ^{35}S-labeled virions was immunoprecipitated with anti-IN sera and subsequently fractionated by SDS-PAGE (8 to 22% polyacrylamide). Antisera directed against the COOH-terminal 11 amino acids (C) and against the NH_2-terminal 10 amino acids (N) of IN were used.

species is usually the major form of PrA IN (Fig. 2 and 3) (8). In contrast, ΔMst-A virus, which lacks v-*src*, has a smaller percentage of phosphorylated IN molecules. Instead, the truncated nonphosphorylated species was the most prominent. AMV IN was similar to that of the ΔMst-A virus, with the majority of AMV IN being truncated. The phosphorylated species was barely detectable. Further, as defined by comigration experiments, purified AMV IN isolated from virions derived from viremic chickens was similar to ^{35}S-labeled AMV IN propagated in cultured CEF cells (data not shown).

The pΔMSTA clone was originally derived from pJD100, which was used for wild-type PrA in this study (13). To confirm that no subtle alterations had occurred in the IN gene or elsewhere in pΔMSTA, resulting in the lack of phosphorylation of IN, the v-*src* gene of our pJD100 clone was also mutated. The plasmid was digested at the unique *Mlu*I site in the v-*src* gene of pJD100, and the site was filled in by Klenow DNA polymerase, creating a frameshift mutation (pMFS) in v-Src near the middle of the protein (Fig. 1). Transfection of pMFS into CEF cells resulted in virus production as monitored by RT activity. ^{35}S-labeled MFS virus was prepared and analyzed by immunoprecipitation. MFS IN (data not shown) had the same profile as ΔMst-A IN (Fig. 2). Further passage of MFS virus-infected cells resulted in apparent transformation of some cells, suggesting that the mutated v-*src* reverted to wild-type PrA (13, 21). Further analysis of these MFS virus-infected cells was terminated because of the potential of a mixed-virus population.

The carboxyl-terminal sequence of full-length AMV IN purified from virions isolated from viremic chickens is $SPLFA_{-COOH}$ (5), which is the same as that predicted for PrA IN (12). The exact carboxyl terminus of truncated AMV IN is unknown and was apparently undetected in the original carboxylpeptidase Y analysis (5). Therefore, the lack of the major phosphorylated species for AMV IN is not due to the absence of Ser-282. To confirm the identities of the various forms of IN, AMV, PrA, and ΔMst-A viruses were propagated in CEF cells, labeled with [^{35}S]Met-[^{35}S]Cys, and immunoprecipitated by antiserum directed against the carboxyl terminus of full-length IN (8). Figure 3 demonstrates that full-length IN (middle band of PrA) of ΔMst-A, PrA, and AMV was immunoprecipitated by this carboxyl-terminal antiserum. The full-length phosphorylated species of PrA IN was also immunoprecipitated by this antiserum. The fastest-migrating band with each virus IN preparation (Fig. 3, lanes 4 to 6) was not precipitated by the carboxyl-terminal antiserum (Fig. 3, lanes 1 to 3), suggesting that this species of IN was truncated in all three viruses.

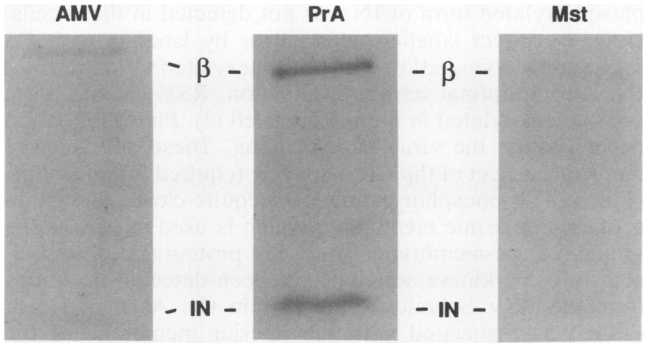

FIG. 4. Preparative immunoprecipitation of ^{32}P-labeled AMV, RSV, and ΔMst-A. A total of 1.6×10^7 cpm of ^{32}P-labeled virions was immunoprecipitated with antiserum directed against the NH_2-terminal 10 amino acids of IN and fractionated by SDS-PAGE (10% polyacrylamide).

Is Ser-282 of ΔMst-A and AMV phosphorylated at a low level? A small percentage of ΔMst-A and AMV IN appears to be the full-length phosphorylated form (Fig. 2 and 3). To confirm the identity of the phosphorylated species, RSV, ΔMst-A, and AMV virions were labeled with ^{32}P or ^{35}S. For each virus, a single ^{32}P-labeled species of IN comigrated with the slowest-migrating ^{35}S-labeled IN polypeptide (data not shown).

We wanted to directly compare the quantities of ^{32}P-labeled β and IN for equivalent amounts of PrA, ΔMst-A, and AMV. First, each purified virus preparation was standardized by determining trichloroacetic acid (TCA)-precipitable counts per minute of ^{32}P (the majority of the ^{32}P in virions is incorporated into viral RNA). The same quantity of TCA-precipitable counts per minute of each virus was fractionated by SDS-PAGE. The ^{32}P-labeled *gag* matrix protein (MA) from each virus was excised and quantitated by scintillation counting. The quantity of ^{32}P-labeled MA was directly related to the quantity of TCA-precipitable counts per minute of each virus preparation. This is in agreement with previous reports (4, 10) showing that the degree of phosphorylation of MA was not dependent on the presence of v-Src. We did not positively identify ^{32}P-labeled MA by immunoprecipitation but only by size analysis on SDS-PAGE.

Equivalent quantities of ^{32}P-labeled AMV, PrA, and ΔMst-A were subjected to preparative immunoprecipitation with NH_2-terminal-specific antiserum (7, 8). The immunoprecipitated samples were subjected to preparative SDS-PAGE, and the dried gels were autoradiographed (Fig. 4). Both β and IN of AMV and ΔMst-A were significantly less labeled than PrA β or IN. Cerenkov counting of the extracted gel bands for β or IN demonstrated that these AMV and ΔMst-A proteins ranged from 17 to 31% of the respective PrA proteins. When subjected to tryptic digestion, ^{32}P-labeled PrA IN and β exhibit two major ^{32}P-labeled peptides and several minor ^{32}P-labeled peptides. Both major peptides arise from phosphorylation at Ser-282, presumably because of partial tryptic digestion (8). Tryptic peptide analysis of AMV and Mst IN revealed that both ^{32}P-labeled tryptic peptides found to be associated with PrA IN (7, 8) were present in these viruses, although at a reduced intensity (data not shown). No conclusions for AMV or ΔMst-A regarding the presence or absence of the minor ^{32}P-labeled tryptic peptides found in PrA IN could be made, because of insufficient labeled materials. Combined, these data suggest

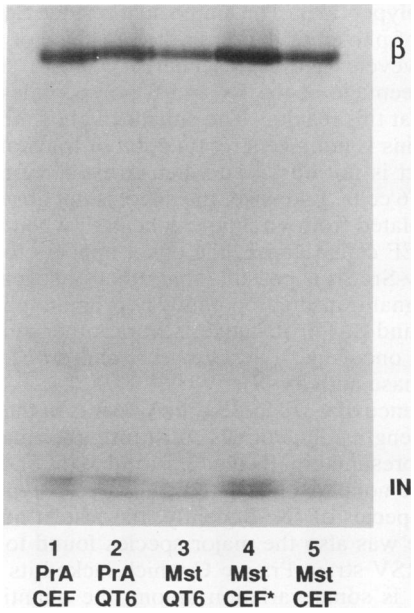

FIG. 5. Propagation of PrA and ΔMst-A in quail QT6 cells. ^{35}S-labeled RSV PrA and ΔMst-A virions grown in QT6 or CEF cells were immunoprecipitated by NH_2-terminal IN antiserum and fractionated by SDS-PAGE (8 to 22% polyacrylamide). In lane 4, the ΔMst-A virus stock was previously passaged through QT6 cells prior to infection of CEF cells (*).

that Ser-282 is the major site of phosphorylation in PrA, AMV, and ΔMst-A when the virus is propagated in CEF cells. Further, the percentage of IN polypeptides that are phosphorylated is enhanced when the virus carries v-*src*.

Is the phosphorylation of PrA and ΔMst-A IN independent of the cell line used to propagate virus? The phosphorylation difference between PrA and ΔMst-A IN may be a nonspecific function of transformed CEF cells and not a v-Src-specific function. To test this possibility, PrA and ΔMst-A viruses were propagated in CEF cells or the chemically transformed quail cell line QT6. The phosphorylation of PrA IN (the slowest-migrating species) appears equivalent in virus grown in either CEF or QT6 cells (Fig. 5, lanes 1 and 2), suggesting that if a nonspecific serine kinase is incorporated into PrA virus, it is cell independent. The structure of ΔMst-A IN propagated in CEF cells is not significantly different from that of virus derived from QT6 cells (Fig. 5, lanes 3 and 5), demonstrating that the transformed state existing with QT6 cells does not appear to influence phosphorylation of IN at Ser-282. As a control, a stock of ΔMst-A virus was isolated from QT6 infected cells. This ΔMst-A virus stock was then used to infect CEF cells. Again, there was no apparent increase in the quantity of phosphorylated ΔMst-A IN, whether or not the virus was previously passaged through QT6 cells prior to infection of CEF cells (Fig. 5, compare lanes 4 and 5).

DISCUSSION

We have previously shown that RSV IN and β of RT are phosphorylated on Ser at amino acid position 282 of IN (7, 8). Mutational analysis at the site of phosphorylation suggests that phosphorylation directs proteolytic processing at the carboxyl termini of IN and β (Fig. 1) (8). We now present evidence that v-Src promotes phosphorylation of the RSV

IN and β polypeptides. The amino acid residue Ser-282 is the major site of phosphorylation in the presence or absence of v-Src. However, in the presence of v-Src, a significantly greater percentage of the IN and β polypeptides are phosphorylated at this residue. The enhanced phosphorylation of these proteins is not a general function of transformed cells, as the effect is not observed when ΔMst-A virus is propagated in QT6 cells. Likewise, the effect is not observed when AMV is isolated from viremic chickens or when it is propagated in CEF cells. Hence, the effect appears to be dependent upon v-Src. It is possible that other oncogenes derived from the signal transduction pathway promote phosphorylation of IN and β. For instance, several other avian retroviruses carry oncogenes (*yes*, *fps*, *ros*, and *erbB*) possessing tyrosine kinase activity (9).

The presence of v-*src* in RSV PrA results in the phosphorylated full-length polypeptides constituting the major species of IN and, presumably, β (Fig. 2, 3, and 5) (5, 8). In AMV or ΔMst-A, the nonphosphorylated truncated polypeptides are the major species of IN. Recently, the fastest-migrating IN polypeptide was also the major species found to be associated with RSV strain Prague C which lacked its v-*src* gene (17). There is some variation among the quantities of the three species of PrA IN with different virus preparations. However, in all cases the ratio of phosphorylated PrA IN to phosphorylated ΔMst-A or AMV IN is high. It is still unclear whether these differences in phosphorylation and in polypeptide length of IN and β directly affect reverse transcription or integration functions. In vitro, phosphorylation of purified RT increased RT activity two- to fivefold (11). The majority (>95%) of AMV IN purified from virions derived from viremic chickens is either proteolytically processed or is nonphosphorylated (see Results). Therefore, no conclusions regarding the possible role that phosphorylation or truncation of IN has on IN catalytic functions (endonuclease or integration) can be made. The emphasis of oncogene research has traditionally been directed at the effect of the oncogene on the cell. This report suggests that an oncogene may also directly modulate viral functions.

Several serine kinases are activated by v-Src. These include $pp74^{c\text{-}raf}$ and a 42-kDa serine/threonine kinase (2). Several kinases of various molecular weights and substrate preferences have been isolated from avian retroviruses (1, 6, 14, 20). There is currently not enough evidence to predict whether any of these kinases is responsible for phosphorylation of IN and β. However, the phosphorylation of purified AMV IN or β may provide a useful assay for identifying the responsible kinase. Development of this assay and identification of the kinase may lead to a greater understanding of signal transduction via the activity of v-Src.

While we have emphasized the role of a serine kinase, phosphatase(s) may play an important role in the phosphorylation status of IN and β. ΔMst-A and AMV may nonspecifically incorporate more protein phosphatase than PrA, thereby resulting in less phosphorylated IN because of dephosphorylation by these phosphatases. It was not possible to determine whether virion-associated phosphatase played a role in determining the quantity of phosphorylated IN in each virus. However, all of the immunoprecipitation experiments were performed in the presence of 50 mM sodium PP_i and 1 mM EDTA, which are general inhibitors of protein phosphatases.

The RSV PrA IN was expressed independently in mouse C127 cells by using a bovine papillomavirus vector. IN was localized primarily to nuclei and in some cells appeared to concentrate at discrete loci within the nuclei (12a). The phosphorylated form of IN was not detected in these cells, either by direct labeling with ^{32}P or by labeling with ^{35}S followed by comigration with phosphorylated virion-derived IN. During normal retroviral infection, RSV IN and β are not phosphorylated in the infected cell (3). Phosphorylation occurs within the virus after budding. These data suggest that some aspect of the virion may be required for phosphorylation. This phosphorylation may require close association of the cytoplasmic membrane, which is used in part as the viral envelope membrane. The v-Src protein and its associated tyrosine kinase activity have been detected in virions from the RSV Schmidt-Ruppin strain (1). As in the cell, v-Src was associated with the interior membrane of the virus. Further, serine/threonine kinase activity was detected in the RSV virions but not in nontransforming Rous-associated virions. Therefore, we propose that v-Src enhances phosphorylation of IN and β by packaging of a serine kinase into virions or by activation of a serine kinase or both.

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