Abelson Murine Leukemia Virus P120: Identification and Characterization of Tyrosine Phosphorylation Sites

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Tryptic peptides containing two major *in vivo* P120^{*gag-abl*} tyrosine phosphorylation acceptor sites were identified, phosphorylated *in vitro*, and purified to homogeneity. The tyrosine site in peptide a is localized at a position six residues distal to its trypsin cleavage site, whereas the tyrosine acceptor site in peptide b is at residue seven. A third peptide, c, contains an amino-terminal phosphotyrosine residue; phosphorylation of this latter peptide only occurs to a significant extent *in vivo*.

Abelson murine leukemia virus (Abelson MuLV) is a replication-defective retrovirus which, in the presence of an appropriate type C helper virus, transforms embryo fibroblasts in cell culture (12) and induces a rapid B-cell lymphoid leukemia *in vivo* (1, 11). The major Abelson MuLV translational product has been identified as a 120,000-*M_r* polypeptide consisting of Moloney MuLV amino-terminal, *gag* gene-encoded structural proteins (p15 and p12) covalently linked to a transformation-specific, acquired sequence-encoded component (8, 10, 16, 21). P120^{*gag-abl*} is one of a growing list of retrovirus-encoded transforming proteins which possess an intrinsic or highly associated tyrosine-specific protein kinase activity (13, 17, 18). Moreover, overall cellular levels of phosphotyrosine are elevated 8- to 10-fold in Abelson MuLV-transformed cells as compared with uninfected control cells (2, 13). A further characteristic property of Abelson MuLV-transformed cells is the production of a transforming growth factor which transforms cells in culture and induces tyrosine phosphorylation of the epidermal growth factor receptor (2, 14a, 15). The involvement of P120^{*gag-abl*}, its associated protein kinase activity, and induced transforming growth factor in transformation has been established by use of transformation-defective mutants (9, 19).

Tyrosine-specific phosphopeptides have been identified within translational products of a number of transforming retroviruses. These include representative isolates containing each of the following acquired cellular oncogene sequences: *v-src*, *v-fes*, *v-fps*, and *v-yes* (for a review, see reference 5a). Generally, correspondence has been observed between phosphorylation of these acceptors in the presence of [γ -³²P]ATP in

vitro and that observed *in vivo*. In contrast, considerable variability has been observed in attempts to correlate *in vitro* and *in vivo* phosphorylation of Abelson MuLV tyrosine phosphate acceptor sites (7, 13, 20). In the present study, *in vitro* phosphorylation conditions optimized in studies of the *v-fes* gene product acceptor site (3; F. H. Reynolds, Jr., S. Oroszlan, J. Blomberg, and J. R. Stephenson, *Virology*, in press) were applied to identify and compare the P120^{*gag-abl*} tyrosine phosphate acceptor site(s) with those of other transforming retroviruses.

P120^{*gag-abl*} was purified from Abelson MuLV-transformed FRE 3A rat cells by immunoprecipitation, reacted with [γ -³²P]ATP, purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subjected to two-dimensional tryptic peptide analysis. Two major and several minor phosphopeptides were observed (Fig. 1). One of these, a, corresponds to the acceptor peptide previously described by our laboratory (9), whereas the second major peptide, b, migrates close to the base line in the chromatographic dimension and was not previously resolved (9). To obtain a more quantitative estimate of the extent of *in vitro* phosphorylation of individual Abelson MuLV acceptor sites, the total tryptic digest was analyzed by high-performance liquid chromatography (HPLC) with a 0-to-35% acetonitrile gradient. The radioactivity in 10% aliquots of each fraction was measured by liquid scintillation. Major peaks of ³²P label were observed at acetonitrile concentrations of 15 and 27% (Fig. 2A). Fractions corresponding to these peaks were pooled, and a sample of each was analyzed by two-dimensional peptide separation on thin-layer chromatography plates. The peak chromatographing at 27% acetonitrile contained a single well-resolved

FIG. 1. Identification of the P120^{gag-abl} acceptor sites phosphorylated in immunoprecipitates in vitro. P120^{gag-abl} was immunoprecipitated from Abelson MuLV-transformed FRE 3A cells by using a high-titer goat antiserum to detergent-disrupted Moloney MuLV, labeled with [γ -³²P]ATP on ice for 1 min, purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, trypsinized from gel slices, and analyzed by two-dimensional thin-layer electrophoresis and chromatography (A) as described previously (8, 17); HPLC fractions 322 to 325 (B) and 221 to 224 (C) containing [γ -³²P]ATP-labeled P120^{gag-abl} tryptic peptides (see Fig. 2A) were pooled and lyophilized, and samples were similarly analyzed.

phosphopeptide corresponding to peptide a (Fig. 1B), whereas the second peak (15% acetonitrile) corresponded to peptide b (Fig. 1C). The identification of these peptides was confirmed in appropriate mixing experiments, and, by phos-

phoaminoacid analysis, each was shown to contain tyrosine as its predominate phosphorylated residue (data not shown).

To establish whether the two in vitro ³²P-labeled P120^{gag-abl} peptides were phosphorylated in vivo, Abelson MuLV-transformed FRE 3A cells were metabolically labeled by overnight incubation in ³²P_i-containing medium. P120^{gag-abl} was immunoprecipitated, purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, trypsinized, and analyzed by HPLC. Multiple peaks of radioactivity were observed (Fig. 2B). By phosphoaminoacid analysis, three of these were found to contain phosphotyrosine as their predominant phosphorylated component, whereas the remaining peaks of radioactivity consisted of either phosphoserine or phosphothreonine. Two of the phosphotyrosine-containing peaks were designated a and b based on their HPLC elution positions relative to the two in vitro-phosphorylated peptides. The third in vivo-phosphorylated peptide, c, did not show obvious correspondence to a major in vitro-phosphorylated species. In a mixing experiment, samples of in vitro-phosphorylated peptides a and b were added in equal proportion to the total in vivo ³²P_i-labeled P120 tryptic digest and rechromatographed by HPLC. The results (Fig. 2C) indicate a proportional increase in the labeling of peptides a and b and thus further establish a correlation between their in vivo and in vitro phosphorylation.

The availability of HPLC-purified preparations of the two major P120^{gag-abl} peptides phosphorylated in vitro made possible a determination of the positions of their phosphotyrosine residues relative to trypsin cleavage sites. Accordingly, samples of both purified peptides were analyzed by sequential Edman degradation. The phosphotyrosine residue in acceptor peptide a phosphorylated in vitro was localized at a position at six amino acid residues distal to its trypsin cleavage site (Fig. 3A), whereas the peptide b phosphotyrosine was localized in residue seven (Fig. 3B). Upon a similar analysis of peptides containing in vivo-phosphorylated tyrosine residues, the phosphotyrosine residue in peptide b was localized at position seven, (Fig. 3C), whereas in peptide c the phosphotyrosine was identified in the first cycle after the trypsin cleavage site (Fig. 3D). Although peptide a was subjected to a similar analysis, the extent of ³²P incorporation was insufficient to accurately identify the position of the phosphotyrosine site.

The results described above define two Abelson MuLV phosphotyrosine acceptor sites, a and b, which are subject to phosphorylation both in vitro in the presence of [γ -³²P]ATP and in vivo. An additional in vivo phosphotyrosine-containing peptide, c, was identified which is not

FIG. 2. HPLC fractionation of in vitro and in vivo ^{32}P -labeled P120^{gag-abl} tryptic peptides (A). In vitro-labeled P120^{gag-abl} tryptic peptides were prepared as described in the legend to Fig. 1. (B) Alternatively, Abelson MuLV-transformed FRE 3A cells were labeled in vivo by overnight incubation in medium containing ^{32}P (0.1 mCi/ml); P120^{gag-abl} was purified by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and tryptic peptides were prepared. (C) ^{32}P -labeled tryptic peptides were applied to a μ -Bondapak C₁₈ column and eluted with a linear gradient of acetonitrile (0 to 35%) in water containing 0.05% trifluoroacetic acid, 0.1-ml fractions were collected at a flow rate of 1 ml/min, and the radioactivity in 10% of each fraction was measured by liquid scintillation (5; Reynolds et al., in press). Total ^{32}P -labeled P120^{gag-abl} peptides were mixed with an equivalent concentration of purified peptides a and b labeled in vitro and were rechromatographed over a μ bondapak C₁₈ column, and the radioactivity in the entire fraction was measured. The results of a phosphoamino-acid analysis of ^{32}P -labeled peaks a (fractions 322 to 325) and b (fractions 221 to 224) are shown as inserts in panel B. Samples of purified tryptic peptides were lyophilized, hydrolyzed in hydrochloric acid, and compared with authentic phosphothreonine (lower left), phosphotyrosine (top), and phosphoserine (lower right) by thin-layer electrophoresis and chromatography (9).

^{32}P labeled to an appreciable extent under the in vitro phosphorylation conditions described in the present study. Of the two in vitro-labeled phosphotyrosine peptides, one, peptide a, corresponds to the phosphotyrosine acceptor which we described previously (9). In contrast, peptide

b was not initially resolved by two-dimensional thin-layer chromatography analysis due to the presence of a number of minor peptides migrating in the same region of the plate (9). In a previous study, Patschinsky et al. (7) analyzed a total tryptic digest of in vitro-phosphorylated

FIG. 3. Sequential Edman degradation of purified $P120^{gag-abl}$ phosphotyrosine acceptor peptides. Automated Edman degradations were performed with a Beckman sequencer model 890C (4). Apomyoglobin (0.8 mg) and Polybrene (1.2 mg; Sigma Chemical Co., St. Louis, Mo.) were included in the spinning cup with in vitro ^{32}P -labeled peptides from (A) Fig. 2A, peak a and (B) Fig. 2A, peak b. In vivo-labeled peptides, including those from (C) Fig. 2B, peak b and (D) Fig. 2B, peak c, were similarly analyzed. Fractions corresponding to each Edman degradation cycle were lyophilized, and radioactivity was measured by liquid scintillation counting.

$P120^{gag-abl}$ and found the major tyrosine phosphorylation site at residue six. This finding is consistent with the present results in that a mixture of phosphopeptides a and b would result in a sequential Edman degradation profile which might be difficult to distinguish from that of a single phosphorylation site at position six. On the basis of the position of its phosphotyrosine site at residue seven, peptide b appears to correspond to one of two previously reported in vivo phosphotyrosine-containing peptides, whereas peptide c resembles the second previously de-

scribed in vivo tyrosine phosphorylation acceptor site in that the phosphotyrosine residue is directly adjacent to a trypsin cleavage site (7). In contrast, peptide a represents a relatively minor in vivo phosphotyrosine acceptor which was not resolved in previous studies.

Although the Abelson MuLV P120 in vitro phosphorylation patterns observed in the present study differ from those previously reported by Sefton et al. (13) and Witte et al. (20), the reaction conditions used in these studies differ significantly. In particular, as previously reported in studies of the Gardner and Snyder-Theilen feline sarcoma virus phosphotyrosine acceptors (Reynolds et al., in press), temperature and reaction duration significantly influence observed tyrosine phosphorylation patterns. When in vitro phosphorylation reactions are carried out for 1 min on ice and in the presence of 5 mM Mg^{2+} , patterns of tyrosine phosphorylation reproducibility reflect those observed in vivo. In contrast, upon increase of either reaction temperature or time, in vitro labeling of secondary phosphotyrosine sites is observed (Reynolds et al., in press).

It is of interest that major phosphotyrosine acceptor sites identified within each of several virus-encoded transforming proteins examined to date have been localized at a position seven residues distal to a trypsin cleavage site (6, 7, 14; Reynolds et al., in press). Moreover, *fes*-, *fps*-, and *src*-encoded acceptors each contain glutamic acid residues at position four (6, 7, 14), and considerable similarity has been observed between tryptic peptides containing *src*- and *yes*-encoded phosphotyrosine acceptor sites (6). As shown in the present study, one of the Abelson MuLV tyrosine acceptors subject to phosphorylation in vitro is also located at position seven, whereas the second is at position six. Whether the similarities in phosphotyrosine acceptor sites of these otherwise unrelated transforming proteins reflect structural requirements for their acceptor activity or, alternatively, are due to divergent evolution from a common associated sequence remains to be determined.

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LITERATURE CITED

1. Abelson, H. T., and L. S. Rabstein. 1970. Lymphosarcoma virus-induced thymic independent disease in mice. *Cancer Res.* 30:2213-2222.
2. Blomberg, J., F. H. Reynolds, Jr., W. J. M. Van de Ven, and J. R. Stephenson. 1980. Abelson murine leukaemia virus transformation involves loss of epidermal growth factor-binding sites. *Nature (London)* 286:504-507.
3. Blomberg, J., W. J. M. Van de Ven, F. H. Reynolds, Jr., R. P. Nalewaik, and J. R. Stephenson. 1981. Snyder-

- Theilen feline sarcoma virus P85 contains a single phosphotyrosine acceptor site recognized by its associated protein kinase. *J. Virol.* **38**:886-894.
4. Copeland, T. D., D. P. Grandgenett, and S. Oroszlan. 1980. Amino acid sequence analysis of reverse transcriptase subunits from avian myeloblastosis virus. *J. Virol.* **36**:115-119.
 5. Henderson, L. E., R. Sowder, and S. Oroszlan. 1981. Protein and peptide purification by reversed-phase high pressure chromatography using volatile solvents, p. 251-260. *In* D. T. Liu, A. N. Schechter, R. Heinrikson, and P. G. Condiff (ed.), *Chemical synthesis and sequencing of peptides and proteins*. Elsevier/North Holland Publishing Co., Amsterdam.
 - 5a. Klein, G. (ed.). 1982. *Advances in viral oncology*, vol. 1: *Oncogene studies*, p. 1-262. Raven Press, New York.
 6. Neil, J. E., J. Ghysdael, P. K. Vogt, and J. E. Smart. 1981. Homologous tyrosine phosphorylation sites in transformation specific gene products of distinct avian sarcoma viruses. *Nature (London)* **291**:675-677.
 7. Patschinsky, T., T. Hunter, F. S. Esch, J. A. Cooper, and B. M. Sefton. 1982. Analysis of the sequence of amino acids surrounding sites of tyrosine phosphorylation. *Proc. Natl. Acad. Sci. U.S.A.* **79**:973-977.
 8. Reynolds, F. H., Jr., T. S. Sacks, D. N. Deobagkar, and J. R. Stephenson. 1978. Cells nonproductively transformed by Abelson murine leukemia virus express a high molecular weight polyprotein containing structural and nonstructural components. *Proc. Natl. Acad. Sci. U.S.A.* **75**:3974-3978.
 9. Reynolds, F. H., Jr., W. J. M. Van de Ven, and J. R. Stephenson. 1980. Abelson murine leukemia virus transformation-defective mutants with impaired P120-associated protein kinase activity. *J. Virol.* **36**:374-386.
 10. Reynolds, R. K., W. J. M. Van de Ven, and J. R. Stephenson. 1978. Translation of type C viral RNAs in *Xenopus laevis* oocytes: Evidence that the 120,000-molecular-weight polyprotein expressed in Abelson leukemia virus-transformed cells is virus coded. *J. Virol.* **28**:665-670.
 11. Rosenberg, N., D. Baltimore, and C. D. Scher. 1975. *In vitro* transformation of lymphoid cells by Abelson murine leukemia virus. *Proc. Natl. Acad. Sci. U.S.A.* **72**:1932-1936.
 12. Scher, C. D., and R. Seigler. 1975. Direct transformation of 3T3 cells by Abelson murine leukemia virus. *Nature (London)* **253**:729-731.
 13. Sefton, B. M., T. Hunter, and W. C. Raschke. 1981. Evidence that the Abelson virus protein functions *in vivo* as a protein kinase that phosphorylates tyrosine. *Proc. Natl. Acad. Sci. U.S.A.* **78**:1552-1556.
 14. Smart, J. E., H. Opperman, A. P. Czernilofsky, A. F. Purchio, R. L. Erikson, and J. M. Bishop. 1981. Characterization of sites for tyrosine phosphorylation in the transforming protein of Rous sarcoma virus (pp60^{v-src}) and its normal cellular homologue (pp60^{c-src}). *Proc. Natl. Acad. Sci. U.S.A.* **78**:6013-6017.
 - 14a. Stephenson, J. R., and G. J. Todaro. 1982. Viral-encoded transforming proteins and transforming growth factors, p. 59-82. *In* G. Klein (ed.), *Advances in viral oncology*, vol. 1: *Oncogene studies*. Raven Press, New York.
 15. Twardzik, D. R., G. J. Todaro, H. Marquardt, F. H. Reynolds, Jr., and J. R. Stephenson. 1982. Abelson MuLV induced transformation involves production of a polypeptide growth factor. *Science* **216**:894-897.
 16. Van de Ven, W. J. M., F. H. Reynolds, Jr., R. P. Nalewaik, and J. R. Stephenson. 1979. The nonstructural component of the Abelson murine leukemia virus polyprotein P120 is encoded by newly acquired genetic sequences. *J. Virol.* **32**:1041-1045.
 17. Van de Ven, W. J. M., F. H. Reynolds, Jr., and J. R. Stephenson. 1980. The nonstructural components of polyproteins encoded by replication-defective mammalian transforming retroviruses are phosphorylated and have associated protein kinase activity. *Virology* **101**:185-197.
 18. Witte, O. N., A. Dasgupta, and D. Baltimore. 1980. Abelson murine leukaemia virus protein is phosphorylated *in vitro* to form phosphotyrosine. *Nature (London)* **283**:826-831.
 19. Witte, O. N., S. Goff, N. Rosenberg, and D. Baltimore. 1980. A transformation defective mutant of Abelson murine leukemia virus lacks protein kinase activity. *Proc. Natl. Acad. Sci. U.S.A.* **77**:4993-4997.
 20. Witte, O. N., A. Ponticelli, A. Gifford, D. Baltimore, N. Rosenberg, and J. Elder. 1981. Phosphorylation of the Abelson murine leukemia virus transforming protein. *J. Virol.* **39**:870-878.
 21. Witte, O. N., N. Rosenberg, M. Paskind, A. Shields, and D. Baltimore. 1978. Identification of an Abelson murine leukemia virus-encoded protein present in transformed fibroblasts and lymphoid cells. *Proc. Natl. Acad. Sci. U.S.A.* **75**:2488-2492.