The p15 matrix protein of moloney murine leukemia virus is a phosphotyrosine protein

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Abstract Retroviruses have been etiologically implicated with leukemia in humans and animals. Understanding the virus life cycle, the proteins and enzymes involved in its replication, is essential for developing potent anti-viral drugs. Phosphorylation of retroviral proteins may alter their shape in such a way as to increase or inhibit their biological activities and thus influence the replication and pathogenic potential of the retroviruses. In our previous work, we demonstrated that non-cytotoxic doses of tyrphostins (protein tyrosine kinase blockers) inhibit moloney murine leukemia virus (Mo-MuLV) replication in acutely and chronically infected cells. In an attempt to understand their mode of action as anti-MoMuLV drugs, we examined the possibility that a viral protein is phosphorylated in tyrosine. Indeed, in our present work, we show that the p15 matrix protein (MA) of Mo-MuLV is a phosphotyrosine protein and is the only viral protein which is phosphorylated on tyrosine. Moreover, treatment of Mo-MuLV/NIH/ 3T3 chronically infected cells with tyrphostin AG-555 specifically inhibits the synthesis of p15 and other viral proteins but does not affect the synthesis of cellular proteins. Our results suggest that tyrosine phosphorylation of p15 MA protein may play a pivotal role in Mo-MuLV replication.

Key words: p15 Matrix protein; Tyrosine phosphorylation; Mo-MuLV

1. Introduction

The biological activity of proteins is often, but not always, influenced by phosphorylation on serine, threonine or tyrosine residue in the protein. Phosphorylation can alter the shape of the protein in such a way as to increase or inhibit its biological activity. Since certain retroviruses are the causative agents for severe diseases in humans and animals, regulation of their protein activity by phosphorylation may influence their replication and pathogenic potential. Phosphorylation of retrovirus proteins was demonstrated in several studies, i.e., in moloney murine leukemia virus proteins such as $pr65^{gag}$, p12 [1], p30 [2]; in rous sarcoma virus proteins such as the integrase [3]; and in human immunodeficiency virus proteins such as Nef [4,5], $pr55^{gag}$ and p17 [6,7], Vpu [8–10] and Rev [11]. The predominant phosphoamino acids in these proteins were phosphoserine and/or phosphothreonine. Tyrosine phosphorylation of retroviral protein was recently demonstrated in HIV matrix protein (MA) [12] and it was shown that a direct interaction between tyrosine-phosphorylated MA and the central domain of the integrase governed the HIV nuclear import [13].

We have recently demonstrated that tyrphostin, protein tyrosine kinase blockers, inhibited Mo-MuLV replication in acutely and chronically MoMuLV/NIH/3T3 infected cells [14]. We also showed that this compound inhibited the integration process of the proviral DNA into the host cell genome in acutely infected cells and viral protein synthesis in chronically infected cells (manuscript submitted). It was also recently demonstrated that certain tyrphostin derivatives inhibited the HIV-1 integrase activity in an in vitro assay [15]. Taking all these data into account, we suggest that tyrosine phosphorylation may play a crucial role in retrovirus replication. Therefore, we examined the possible existence of tyrosine phosphorylation in Mo-MuLV proteins and the effect of tyrphostin on this phosphorylation. We found that the Mo-MuLV $p15^{gag}$ is phosphorylated on tyrosine and treatment of the Mo-MuLV/ NIH-3T3 chronically infected cells with non-cytotoxic doses of tyrphostins resulted in the inhibition of viral protein synthesis and notably decreased the level of p15.

2. Materials and methods

2.1. Cells

NIH/3T3-Mo-MuLV chronically infected cells were grown in Dullbecco's modified Eagle medium supplemented with 10% fetal calf serum, 1% penicillin–streptomycin and 1% L-glutamine.

2.2. Virus preparation

The culture supernatant of NIH/3T3/Mo-MuLV chronically infected cells (10^6/ml) was collected and centrifuged (3000 rpm, 10 min). The virus was purified twice from the culture medium by precipitation using ultracentrifugation (30 000 rpm, 3 h, 4°C) through a cushion of 5% ficol in TE (10 mM Tris·HCl, pH 8, 1 mM EDTA). To remove traces of ficol, the virus pellet was resuspended in TE and precipitated by ultracentrifugation, resuspended again in TE and stored in aliquots at −70°C for further analysis.

2.3. Antibodies

Anti-$p15^{gag}$, anti-gp70, anti-total Mo-MuLV proteins antibodies were obtained from Donald G. Blair, NCI/ NIH Frederick Cancer Research and Development Center, Frederick, MD, USA. Anti-phosphotyrosine antibodies were purchased from Transduction Laboratories. Phosphotyrosine, phosphoserine and phosphothreonine were purchase from Sigma. Tyrphostin AG-555 was received from A. Levitzki and A. Gazit, the Hebrew University, Jerusalem, Israel. Stock solutions of the drugs, 10 mM in 100% DMSO were stored at −20°C.

2.4. Immunoprecipitation and Western blot analysis

Viral particles were lysed with 0.3% NP-40 at 37°C for 20 min and 50 μg of viral proteins was incubated overnight at 4°C with anti-p15 antibodies (1 : 500 dilution), or anti-total antibodies (1 : 500 dilution). Protein A-sepharose (0.1 gr/ml) was added for 1 h at 4°C and the immunocomplexes were precipitated by centrifugation with microcentrifuge (13 000 rpm, 2 min). The pellet was washed 3 times with RIPA buffer (50 mM Tris·HCl, 0.5 M Na_2HPO_4, 0.5% NP-40, 0.25% sodium deoxycholate, 0.5% SDS). Samples were analyzed on 10% SDS-polyacrylamide gel electrophoresis (PAGE) and the proteins were transferred to a nitrocellulose membrane as described [16]. The membrane was reacted with anti-phosphotyrosine antibodies or after stripping, it was reprobed with anti-p15 antibodies (1 : 5000 dilution). The

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blot was developed by the Western blotting detection reagents; enhanced chemiluminescence (ECL) (Amersham).

2.5. Two-dimensional gel analysis

The two-dimensional gel analysis (2D-gel) was performed as described by O'Farell [17]. Viral proteins (50 μg) were immunoprecipitated by anti-total antibodies and subjected to 2D-gel analysis. The proteins were visualized by silver staining or were transferred to nitrocellulose membrane for Western blot analysis.

2.6. Metabolic labeling of viral proteins with [^{32}P]orthophosphate

NIH/3T3/Mo-MuLV chronically infected cells (10^6 cells/flask) were incubated with a medium depleted from phosphate for 24 h followed by incubation with fresh medium containing [^{32}P]orthophosphate (2.5 mCi) for 18 h. The culture medium was collected after 24 h and the virus particles were precipitated by ultracentrifugation (30000 rpm, 3 h, 4°C). The virus particles were suspended in TE (10 mM Tris·HCl pH 8, 1 mM EDTA) and lysed by 0.03% NP-40. Viral proteins were immunoprecipitated with anti-total antibodies and subjected to SDS-PAGE and autoradiography.

3. Results

3.1. The Mo-MuLV p15^{gag} protein is recognized by anti-phosphotyrosine antibodies

Purified Mo-MuLV particles were lysed and immunoprecipitation with anti-p15 or anti-Mo-MuLV total proteins or anti-phosphotyrosine antibodies was performed. The immunocomplexes were subjected to Western blot analysis using anti-phosphotyrosine antibodies (Fig. 1, panel A). The same nitrocellulose membrane underwent the stripping procedure to remove the anti-phosphotyrosine antibodies and was reprobed with anti-p15 antibodies (Fig. 1, panel B). We found that the phosphotyrosine antibodies immunoprecipitated a protein of 15 kDa (Fig. 1, lane 1A) which was also recognized by anti-p15 antibodies (Fig. 1, lane 1B). When the viral proteins were immunoprecipitated with anti-p15 or anti-total antibodies and the Western blot was performed with anti-phosphotyrosin antibodies, a 15 kDa protein was again recognized by the anti-phosphotyrosine antibodies (Fig. 1, lanes 2A and 3A). This 15 kDa protein is also recognized by the anti-p15 antibodies (Fig. 1, lanes 2B and 3B). The protein bands of 43 kDa and 28 kDa observed in Fig. 1A are the heavy and light chains of the immunoglobulins that are phosphorylated on tyrosine.

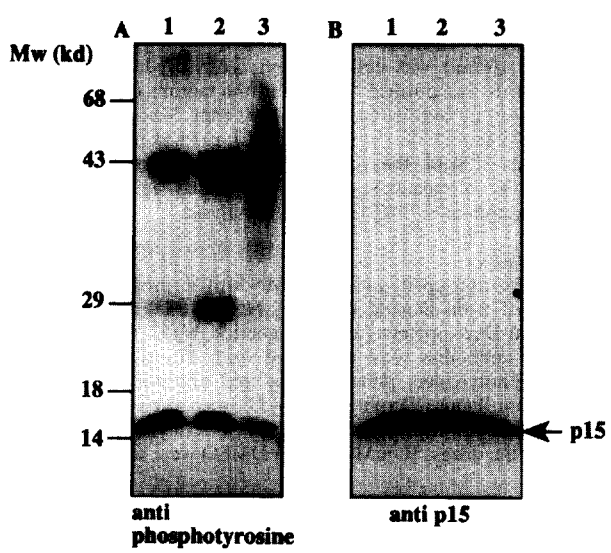

Fig. 1. The p15^{gag} protein from Mo-MuLV is recognize by anti-phosphotyrosine antibodies. Purified virus particles were lysed with 0.03% NP-40 at 37°C for 20 min and 50 μg of viral proteins were subjected to immunoprecipitation by anti-total Mo-MuLV antibodies (lanes 1A and 1B), or anti-p15 antibodies (lanes 2A and 2B), or anti-phosphotyrosine antibodies (lanes 3A and 3B). Western blot analysis was performed using anti-phosphotyrosine antibodies (panel A). The nitrocellulose membrane was subjected to stripping procedure followed by reprobing with anti-p15 antibodies (panel B). The immuno-complexes were detected by ECL-Western blotting detection reagents (Amersham).

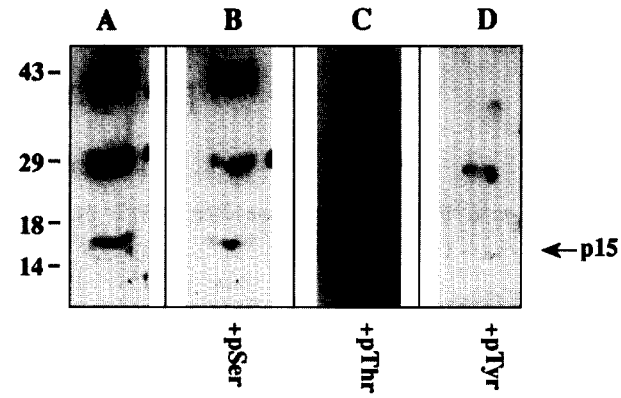

Fig. 2. Phosphotyrosine compete out the binding of anti-phosphotyrosine antibodies to p15 protein. Viral proteins were immunoprecipitated with anti-p15 antibodies and the immunocomplexes were subjected to Western blot analysis using anti-phosphotyrosine antibodies (lane A) and in the presence of 1 mM phosphoserine (lane B) or 1 mM phosphothreonine (lane C) or 1 mM phosphotyrosine (lane D).

To rule out the possibility that a 15 kDa phosphotyrosin protein from a cellular origin is present in the culture medium, a mock preparation of virus particles from NIH/3T3 uninfected cells was performed and underwent the same imunoprecipitation and Western blot procedures as the virus preparation. No protein bands were detected in the mock preparation from uninfected cells (data not shown).

To examine the binding specificity of the anti-phosphotyrosine antibodies to the p15, we performed competition experiments using phosphotyrosine, phosphothreonine and phosphoserine. The anti-phosphotyrosine antibodies were incubated with 1 mM of each of the phosphoamino acids followed by Western blot assay. The results depicted in Fig. 2 demonstrated that only phosphotyrosine can compete out the binding of the anti-phosphotyrosine antibodies to the p15 protein (compare Fig. 2, lanes D to A, B, and C) indicating that this protein is phosphorylated on tyrosine.

3.2. Separation between the p15 proteins by two-dimensional gel electrophoresis

The 15 kDa protein band that was recognized by the anti-p15 and anti-phosphotyrosine antibodies could have more than one polypeptide protein in this size. To examine this possibility, immunoprecipitation of viral proteins with anti-total antibodies was performed and the immunocomplexes were subjected to 2D-gel electrophoresis. The protein bands were visualized by silver staining (Fig. 3A) or transfered to nitrocellulose membrane for further analysis. The membrane was first reacted with anti-phosphotyrosine antibodies (Fig. 3B) and after stripping procedure the membrane was reprobed with anti-p15 antibodies (Fig. 3C). Two basic proteins in the

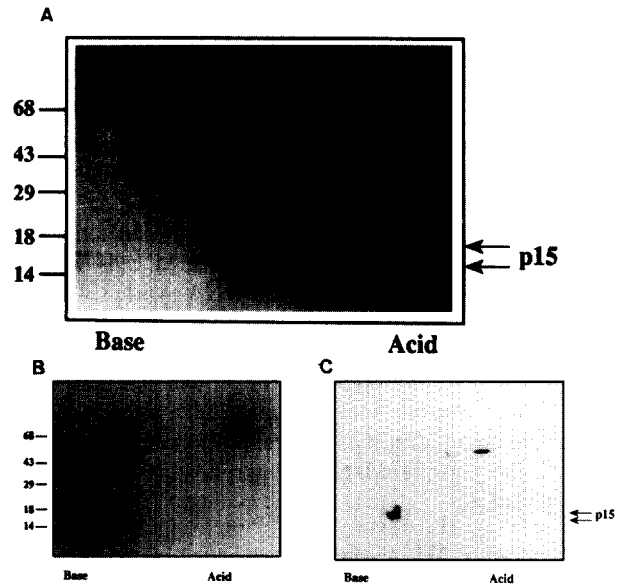

Fig. 3. Two forms of tyrosine phosphorylated p15 were observed by two-dimensional gel analysis. Viral proteins were immunoprecipitated with anti-total Mo-MuLV protein antibodies and subjected to 2D-gel analysis. Panel A demonstrates the viral proteins visualized by silver staining. Western blot analysis was performed using anti-phosphotyrosine antibodies (panel B). The nitrocellulose membrane was subjected to a stripping procedure and reprobed with anti-p15 antibodies (panel C).

size of 15 kDa and ~15.5 kDa were recognized by the anti-phosphotyrosine (Fig. 3A) and anti-p15 antibodies (Fig. 3B). In most mammalian retroviruses the MA domain of the Gag precursor is cotranslationally modified at the N-terminus by attachment of myristic acid [18]. Therefore, it is possible that the two basic proteins are two forms of the Mo-MuLV MA (p15) protein in which one is modified by myristic acid and both forms are probably phosphorylated on tyrosine.

3.3. Metabolic labeling of the p15 protein

Metabolic labeling by ^{32}P of Mo-MuLV proteins was performed previously [1,2]. It was shown that the phosphorylation event of viral proteins, except the p12, was difficult to detect because p12 the major virion phosphoprotein incorporated almost all the ^{32}P label added to infected cells [1]. It was calculated that the degree of phosphorylation at the p30 domain of the $pr65^{gag}$ was only 0.22–0.54%, relative to phosphorylation at the p12 domain [2]. Indeed we found that the major phosphoprotein is the viral p12, but in addition a faint radioactive band of 15 kDa protein was observed (Fig. 4, lane A). The identification of this radioactive band as p15 was carried out by immunoblotting with anti-p15 and anti-total antibodies (Fig. 4, lanes C and D, respectively) and we also proved that it is the only protein band recognized by the anti-phosphotyrosine antibodies (Fig. 4, lane B).

3.4. Effect of tyrphostin treatment on p15 synthesis and phosphorylation

We previously demonstrated that tyrphostins, protein tyrosine kinases blockers, inhibited Mo-MuLV replication [14]. We examined the effect of these compounds on the synthesis and phosphorylation of the viral p15. NIH/3T3/Mo-MuLV chronically infected cells were treated with non cytotoxic doses of tyrphostin AG-555 [14] for 20 h. The viral particles were precipitated from the culture media and Western blot analysis using anti-phosphotyrosine antibodies or anti-p15 antibodies was performed. The results depicted in Fig. 5 demonstrate that tyrphostin treatment inhibited the synthesis of the p15 viral protein (Fig. 5B, compare lanes 3 and 4 to lanes 1 and 2) and significantly inhibited the tyrosine phosphorylation of this protein (Fig. 5A, compare lanes 3 and 4 to lanes 1 and 2). The inhibitory effect of tyrphostin treatment is not unique to the viral p15 since we could observe this inhibitory effect on other viral proteins such as gp70 (Fig. 5C, compare lanes 2 and 3 to lane 1). This inhibitory effect of tyrphostin treatment appears to be specific to viral proteins since no effect on cellular protein such as β-actin was observed (Fig. 5D).

4. Discussion

Matrix protein of retroviruses carries two motifs, a myristoylation signal and a nuclear localization signal. These two signals exert conflicting influences on the subcellular localization of MA. It was shown by Gallay et al. [13] that phosphorylation of MA protein of HIV-1 on the C-terminal tyrosine enable the transportation of this protein to the nucleus. In our present work, we showed that the $p15^{gag}$, which is the Mo-MuLV MA protein, is a phosphotyrosine protein. We also demonstrated that the MA protein is the only viral protein which is phosphorylated on tyrosine since: (1) Immunoprecipitation of Mo-MuLV proteins by anti-total Mo-MuLV antibodies and Western blot analysis using anti-phosphotyrosine antibodies or anti-p15 antibodies revealed that the $p15^{gag}$ protein was the only protein recognized by the anti-phosphotyrosine antibodies. (2) Metabolic labeling of the viral proteins by ^{32}P in combination with Western blot analysis using anti-phosphotyrosine antibodies showed that among the phosphorylated viral proteins, only the $p15^{gag}$ protein was both phosphorylated with ^{32}P and recognized by the anti-phosphotyrosine antibodies. Our data are compatible with other stud-

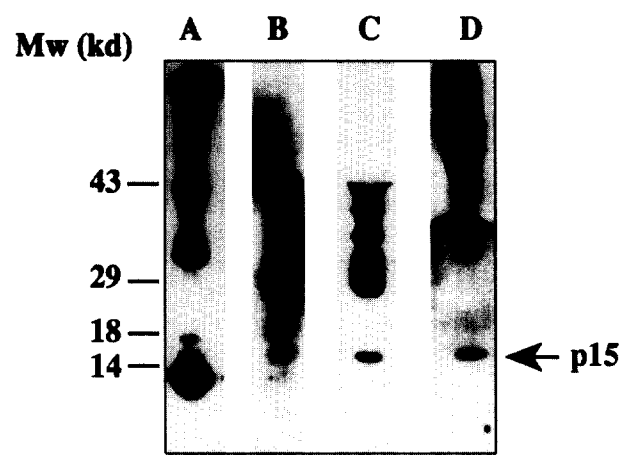

Fig. 4. Metabolic labeling of viral proteins with [^{32}P]orthophosphate. Viral proteins were labeled with ^{32}P as described in Section 2. Viral lysates were performed and the proteins were immunoprecipitated with anti-total Mo-MuLV proteins antibodies and the immunocomplexes were separated on 15% SDS-polyacrylamide gel followed by transfer to nitrocellulose membrane and autoradiography (A) or probed with anti-phosphotyrosine antibodies (B) or with anti-p15 antibodies (c), or with anti-total Mo-MuLV antibodies (D).

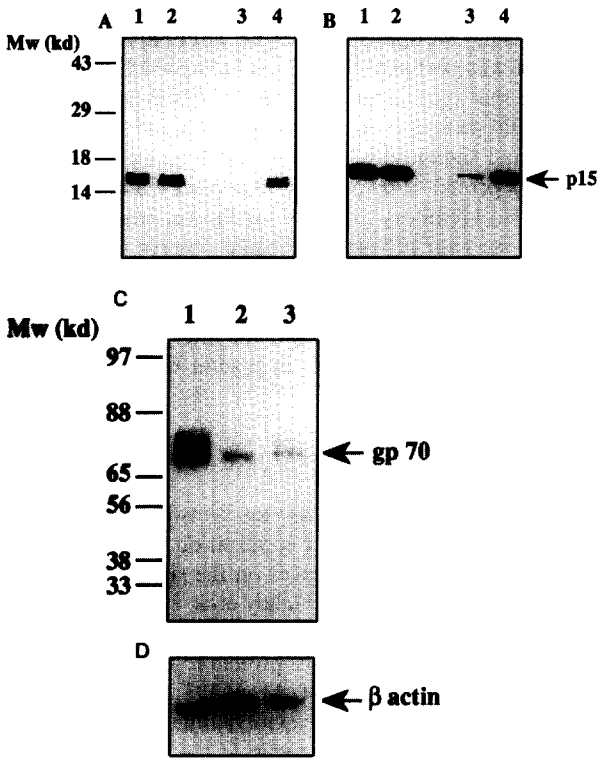

Fig. 5. Treatment of Mo-MuLV/NIH/3T3 chronically infected cells with tyrphostin AG-555 inhibit viral protein synthesis. The chronically infected cells were treated with 100 μM (lanes 3A, 3B and 2C) or with 50 μM (lanes 4A, 4B and 3C) of tyrphostin AG-555 or with 4% DMSO (lanes 2A and 2B) for 20 h. Lanes 1A,1B: untreated cells. Protein extract from the cells was prepared and 50 μg of proteins were subjected to 15% SDS-PAGE and Western blot analysis was performed using anti-phosphotyrosine antibodies (panel A) or anti-p15 antibodies (panel B) or anti-gp 70 antibodies (panel C) or anti-β-actin antibodies (panel D).

ies that demonstrated that the predominant phosphoamino acid in viral phosphoproteins is serine and/or threonine but not tyrosine [1–11] and tyrosine phosphorylation was shown only on the HIV-1 MA protein [12,13]. The role of the p15 MA protein in Mo-MuLV life cycle is not clear but studies performed with other retroviruses suggest that it has an important role in virus replication and infectivity. For instance, it was shown that the MA protein from HIV is involved in the virus nuclear import in nondividing cells [13], and in RSV it is involved in membrane binding during particle assembly and has yet undefined function required for viral infectivity [19]. In addition, it was demonstrated that the phosphorylation of tyrosine on the C-terminal residue of HIV MA protein enhanced the incorporation of MA into the virus core and is required for its association with the uncoated viral nucleoprotein complex [13]. Therefore, inhibition of the tyrosine phosphorylation of MA protein may affect virus replication in the infected cells. Indeed, we found that tyrphostin derivatives, which are protein tyrosine kinase blockers, inhibited Mo-MuLV replication in chronically infected Mo-MuLV/NIH 3T3 cells. Moreover, we showed that tyrphostin AG-555 specifically inhibited the synthesis of viral proteins including the p15 MA protein. A similar phenomenon was also demonstrated by other investigators who showed that certain tyrphostin derivatives specifically reduced the level of pp60F527 protein (encoded by the *c-src/F527* gene) but did not affect the synthesis of other proteins [20]. Since treatment of Mo-MuLV/NIH 3T3 chronically infected cells with tyrphostin AG-555 did not affect viral RNA synthesis (Priel et al., manuscript submitted), we may presume that a specific protein tyrosine kinase and/or the tyrosine phosphorylation of MA protein are involved in viral protein synthesis. However, it is also possible that certain cellular phosphotyrosine proteins are involved in the virus replication and inhibition of their tyrosine phosphorylation by tyrphostins may influence virus replication.

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