Tyrosine Phosphorylation of Measles Virus Nucleocapsid Protein in Persistently Infected Neuroblastoma Cells

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Subacute sclerosing panencephalitis is a slowly progressing fatal human disease of the central nervous system which is a delayed sequel of measles virus (MV) infection. A typical pathological feature of this disease is the presence of viral ribonucleocapsid structures in the form of inclusion bodies and the absence of infectious virus or budding viral particles. The mechanisms governing the establishment and maintenance of a persistent MV infection in brain cells are still largely unknown. To understand the mechanisms underlying MV persistence in neuronal cells, a tissue culture model was studied. Clone NS20Y/MS of the murine neuroblastoma C1300 persistently infected with the wild-type Edmonston strain of MV secretes relatively high levels of alpha/beta interferon (IFN). As shown previously, treatment of the persistently infected cultures with anti-IFN serum converted the persistent state into a productive infection indicated by the appearance of multinucleated giant cells. In this study, we have investigated whether alpha/beta IFN produced by NS20Y/MS cells activates cellular protein tyrosine kinases which will induce tyrosine phosphorylating activity specific to virus-infected cells. We present data to show augmented protein tyrosine kinase activity in the persistently infected cells. We demonstrate that the MV N protein is phosphorylated on tyrosine in addition to serine and threonine in the persistent state but not in NS20Y cells acutely infected with MV.

Subacute sclerosing panencephalitis (SSPE) is a slowly progressing fatal human disease of the central nervous system which is a delayed sequel of measles virus (MV) infection. It occurs years after MV infection consequent to unnoticeable latent persistence of the virus and mostly affects young adults between 5 and 14 years old (5, 25, 49). Although the exact pathogenesis of this disease is largely unknown, MV has been isolated and its presence was consistently demonstrated in brain biopsy specimens from these patients (5, 32, 46, 48). A typical pathological feature of this disease is the presence of viral ribonucleocapsid structures in the form of inclusion bodies in different cell types in both the gray matter and the white matter in the absence of infectious virus or budding viral particles (11, 31). Electron microscopy of infected cells during acute MV infection reveals budding of the assembled viral particles from the cell surface. In brain specimens from patients with SSPE, nucleocapsid particles can be seen inside the cells but they are not seen to assemble and bud from the cell surface, thus indicating that there may be mechanisms which promote latency or persistence by interfering with the productive life cycle of the virus (30, 34, 35).

Measles virus is a member of the family Paramyxoviridae and has a nonsegmented, negative-sense RNA genome encapsidated by the virus-encoded nucleocapsid (N) protein to form a helical ribonucleoprotein complex termed the nucleocapsid. The nucleocapsid is required as a template for viral RNA synthesis and for both the phosphoprotein (P) and the large or polymerase protein (L) (23).

Despite the intense molecular analysis performed on autopsy brain material which revealed that MV persistence is characterized by alterations of viral gene expression (1, 6, 8, 10), the mechanisms governing the establishment and maintenance of a persistent MV infection in brain cells are still largely unknown. Studies of persistent MV infection in neuronal tissue cultures suggested that a variety of factors specific to neuronal cell-MV interactions play a role in the establishment and maintenance of the persistent infection of the central nervous system (4, 9, 37, 41, 43).

To understand the mechanisms underlying MV persistence in neuronal cells, a tissue culture model was developed (33). Clone NS20Y of the murine neuroblastoma C1300 was infected with the wild-type Edmonston strain of MV and after a transition to a carrier culture became a stable persistently infected cell line termed NS20Y/MS. It was found in these studies that the infected cells secreted relatively high levels of alpha/beta interferon (IFN). When the persistently infected cultures were exposed to anti-IFN serum, the persistent state was converted into a productive infection indicated by the appearance of multinucleated giant cells (33). It therefore appears that alpha/beta IFN is a contributory factor to the maintenance of the persistent state.

Most biological activities of IFN are mediated by a variety of IFN-induced proteins generally known as the IFN system. Two of the best-characterized proteins are (2-5) oligoadenylate synthetase (2-5A synthetase) and a double-stranded RNA-activated protein kinase (p68 kinase) (2, 47, 50), which were shown not only to suppress virus infection but also to regulate cell growth (20, 24, 36). The IFN-induced genes are activated by a number of transcription factors, the most important being IFN-stimulated gene factor 3 (ISGF3). This factor consists of four proteins: a 48-kDa component termed ISGF3γ and three additional components of 84, 91, and 113 kDa which form a complex termed ISGF3α (22, 28). It has been recently demonstrated that following treatment with IFN-α an IFN receptor-associated protein tyrosine kinase(s) (PTK) is activated,
resulting in phosphorylation of the ISGF3α components. This phosphorylation leads to the association of both α and γ components and to the translocation of the active ISGF3 protein to the nucleus (12, 14, 39, 51).

In this study, we have investigated whether alpha/beta IFN produced by NS20Y/MS cells activates cellular PTKs which will induce tyrosine phosphorylating activity specific to virus-infected cells. We present data to show augmented PTK activity in the persistently infected cells. We demonstrate that the MV N protein is phosphorylated on tyrosine in the persistent state but not in acutely infected NS20Y cells.

**MATERIALS AND METHODS**

**Cells and viruses.** The C1300 neuroblastoma clone NS20Y of the A/J mouse strain was used. The establishment of persistently infected NS20Y cells termed NS20Y/MS cells is described in detail elsewhere (33). Cells were routinely grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, glutamine, and antibiotics. The Edmonston strain of MV was doubly plaque purified and purified on Vero cells, and stock virus was produced by growth in Vero cells with an initial multiplicity of infection (MOI) of 0.01 PFU per cell.

**Infection of NS20Y cells.** Cells were infected with 5 × 10⁶ PFU of the Edmonston strain of MV (MOI, 0.01) and were harvested 72 h later.

**Antibodies.** Polyclonal anti-MV hyperimmune serum was raised in rabbits by immunization with antigen in complete Freund’s adjuvant followed by repeated intramuscular injections with solubilized virus alone (31). Monoclonal MAbs (MoAbs) which recognize the N protein of MV were described earlier by Birrer et al. (7). MAbs against phosphotyrosine (poly[Ala,Gly]) (anti-p-Tyr) were kindly provided by B. M. Selton, Salk Institute, La Jolla, Calif. (18).

**Treatment of cells with anti-MV antibodies.** For antibody treatment, the medium of semiconfluent cultures was replaced with fresh medium containing polyclonal anti-MV serum sufficient to neutralize 10⁶ PFU of MV. Every third day, the cells were split and fresh medium containing antibodies was added. Antibody-treated or untreated cells were harvested at different times with respect to antibody treatment. For reactivation, the cells were cultured for an additional 14 days in medium without antibodies.

**Preparation of total-cell extracts and Western immunoblot analysis.** For protein analysis, NS20Y/MS or NS20Y cells were washed twice with phosphate-buffered saline (PBS), resuspended, and lysed in ice-cold RIPA buffer containing 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 0.5% Nonident P-40, 0.1% sodium dodecyl sulfate (SDS), and 0.25% sodium deoxycholate. Extracts were clarified by centrifugation for 30 min at 4°C. The supernatants were transferred to nitrocellulose filters. After 1 h of blocking in 10% dry milk in PBS containing 10 mM HEPES (pH 7.5), the resolved proteins were electroblotted to nitrocellulose sheets (Merck, Darmstadt, Federal Republic of Germany) and immersed in water. The blotted proteins were incubated with peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin in 1% milk or 4% BSA, respectively, for 1 h. Filters were washed three times with PBS and developed with 4-chloronaphthol (0.3 mg/ml) and H₂O₂.

**Determination of 2-5A synthetase and p68 kinase activities.** The assays of these enzymatic activities were described previously by Gopas et al. (13). For quantitation of 2-5A synthetase activity, the radioactive spots containing 2-5A oligomers were cut out of polyethyleneimine cellulose thin-layer chromatography sheets (Merck, Darmstadt, Federal Republic of Germany) and immersed in toluene-based scintillation fluid and the radioactivity was determined.

**Immunoprecipitation.** Cells were washed twice in PBS and lysed in 1 ml of ice-cold RIPA buffer. Lysates were centrifuged at 10,000 × g for 20 min at 4°C. Ten microliters of rabbit anti-MV antibodies or 10 μl of anti-MV N protein MAbs was incubated with 1 ml of supernatant at 1 h at 4°C, and then 100 μl of 10% protein A-Sepharose beads was added. These immune complexes were incubated for 1 h before being washed three times with washing buffer containing 10 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; pH 7.5)-150 mM NaCl-0.5% Nonident P-40-5 mM EDTA and were then resuspended in SDS sample buffer before being boiled for 4 min. Proteins were separated by electrophoresis and subjected to Western blot (immunoblot) analysis.

**In vivo labeling.** Confluent plates of NS20Y/MS cells were washed in Tris-buffered saline and incubated in phosphate-free Dulbecco’s modified Eagle medium containing 10% (vol/vol) dialyzed fetal bovine serum and 1 μCi of ³²P (carrier free; ICN) per ml. After a 14-h incubation at 37°C, MV proteins were immunoprecipitated as described above.

**RESULTS**

**2-5A synthetase and p68 kinase activities are dependent on the presence of MV N protein.** To establish a linkage between the activation of the IFN system in NS20Y/MS cells and the synthesis of MV proteins, we determined the activities of 2-5A synthetase and double-stranded RNA-activated p68 kinase of these cells in parallel with the appearance of viral proteins. Downregulation of the expression of MV mRNAs and proteins was achieved by treating NS20Y/MS cells with polyclonal anti-MV antibodies for 23 days (42). Then, antibodies were removed and de novo synthesis of MV N protein was monitored by Western blotting with MAb anti-N protein (Fig. 1A). Consequently, the levels of both 2-5A synthetase and p68 kinase activities in the same cell extracts were assayed (Fig. 1B and C, respectively). It is clearly demonstrated that while very low basal amounts of 2-5A oligomers and phosphorylated p68 were observed in NS20Y cell extracts (125 cpm; Fig. 1B and C, lanes 1), high levels of oligomers and of phosphorylated p68 kinase were detected in NS20Y/MS cell extracts (42,100 cpm; Fig. 1B and C, lanes 2). In acutely infected cells, the levels of 2-5A synthetase were considerably reduced (750 cpm). As expected, MV N protein was detected only in NS20Y/MS and not in NS20Y cell extracts (Fig. 1A; compare lanes 1 and 2). The addition of anti-MV antibodies to NS20Y/MS cultures for 23 days resulted in a profound decrease in the levels of the N protein expression and 2-5A synthetase and p68 kinase activities (2,200 cpm; Fig. 1, lanes 3). When the cells were cultured in fresh growth medium without antibodies, the synthesis of the N protein was gradually reactivated. The N protein was first detected 10 days after the removal of the antibodies (Fig. 1A, lane 5), and at this time phosphorylated p68 could be observed (Fig. 1C, lane 5). By day 14, N protein synthesis and both enzymatic activities reached levels comparable to those detected in untreated NS20Y/MS cells (Fig. 1, lanes 6). These results indicate that the activity of IFN-induced enzymes is correlated with the presence of MV N protein. In contrast to findings with the persistently infected cells, no activation of 2-5A synthetase and p68 kinase could be detected in acutely infected NS20Y cells (data not shown).

**Detection of proteins phosphorylated on tyrosine in NS20Y/MS cells.** It was recently shown that alpha/beta IFN binding to its cellular receptor triggers a specific PTK (39); therefore, we investigated whether the activation of the IFN system in NS20Y/MS cells leads to tyrosine phosphorylation. Total-cell extracts were prepared from persistently infected NS20Y/MS cells or uninfected NS20Y cells, and tyrosine phosphorylation was analyzed by Western blotting with an anti-p-Tyr MAb (Fig. 2). In the initial experiments, only faint positive bands of phosphorylated proteins recognized by the antibodies could be detected in cell extracts of NS20Y/MS cells (Fig. 2, lane 3). These bands were enhanced upon overnight incubation of the cells with 50 or 100 μM Na₃VO₄ (Fig. 2, lanes 4 and
5, respectively), a known inhibitor of tyrosine phosphatases. Hence, in the following experiments the cells were routinely incubated overnight with Na$_3$VO$_4$. The results shown in Fig. 2 demonstrate that the level of phosphorylation of proteins on tyrosine residues was substantially higher for cell extracts of NS20Y/MS cells (Fig. 2, lanes 4 and 5) than for NS20Y cells (Fig. 2, lane 2). The molecular masses of the phosphorylated proteins in NS20Y/MS cells were 106, 90, 80, and 60 kDa (Fig. 2, lanes 4 and 5). In some experiments, low levels of tyrosine phosphorylation of p106 or p80 could also be detected in NS20Y cells.

MV N protein is phosphorylated on tyrosine in NS20Y/MS cells but not in acutely infected NS20Y cells. Next, we tested whether tyrosine phosphorylation in NS20Y/MS cells is associated with the viral proteins. Extracts from NS20Y/MS cells (Fig. 3, lane 1) or NS20Y cells (Fig. 3, lane 2) were immunoprecipitated with polyclonal anti-MV antibodies or with anti-N protein MAb (Fig. 3, lane 3). The resulting precipitates were then subjected to Western blot analysis with specific anti-p-Tyr antibodies. The results obtained with the MAbs clearly indicate that the MV N protein is phosphorylated on tyrosine. When polyclonal anti-MV antibodies were used, an additional 49-kDa protein, probably a degradation product of the N protein, was observed (Fig. 3, lane 1). In the uninfected cells, no tyrosine-phosphorylated proteins could be detected in anti-MV precipitates (Fig. 3, lane 2). Next, we investigated whether tyrosine phosphorylation can be demonstrated in N protein immunoprecipitated after acute infection of NS20Y cells. Cells were infected with 5 × 10$^4$ PFU of MV (MOI, 0.01) and were harvested 72 h later when the newly synthesized MV N protein was detected by anti-N protein MAb (Fig. 3, lane 7). When these immunoprecipitates were subjected to immunoblotting with anti-p-Tyr MAb (Fig. 3, lane 5), no phosphorylation of p106 or p80 could also be detected in NS20Y cells.

FIG. 1. Correlation of 2-5A synthetase and p68 kinase activities in NS20Y/MS cells with MV N protein synthesis. NS20Y/MS cells were cultured for 23 days with rabbit polyclonal anti-MV serum and for various times without antibodies. Cell extracts were subjected to Western blot analysis using MAb anti-MV N protein. The activity of 2-5A synthetase or p68 kinase was determined as described in Materials and Methods. (A) Expression of N protein (NP, 60-kDa marker). (B) Activity of 2-5A synthetase. (C) Activity of p68 (68-kDa) kinase. Lanes: 1, uninfected control NS20Y cells (125 cpm); 2, NS20Y/MS cells (42,100 cpm); 3, NS20Y/MS cells after antibody treatment for 23 days (2,200 cpm); 4 to 6, NS20Y/MS cells after treatment with the antibody for 23 days and then without an antibody for 7 days (lanes 4), 10 days (lanes 5), or 16 days (lanes 6). In acutely infected NS20Y/MS cells, levels of 2-5A synthetase activity were 750 cpm.

FIG. 2. Tyrosine phosphorylation of proteins in NS20Y/MS cells. Total-cell extracts were prepared from confluent cultures of NS20Y/MS or NS20Y. Extracts were separated by SDS-PAGE, and immunoblot analysis was performed first with anti-p-Tyr antibodies and then with anti-mouse immunoglobulin peroxidase-conjugated antibodies. When appropriate, Na$_3$VO$_4$ was included for the last 16 h of incubation. Lanes: 1, uninfected control NS20Y cells; 2, NS20Y cells with 100 μM Na$_3$VO$_4$; 3, NS20Y/MS cells; 4, NS20Y/MS cells with 50 μM Na$_3$VO$_4$; 5, NS20Y/MS cells with 100 μM Na$_3$VO$_4$. The sizes of the proteins detected are indicated on the left.

FIG. 3. Tyrosine phosphorylation of MV N protein in persistent infection but not in acute infection. For acute infection, NS20Y cells were infected with 5 × 10$^4$ PFU of the wild-type Edmonston strain of MV (MOI, 0.01) for 72 h. Na$_3$VO$_4$ (100 μM) was added to NS20Y/MS cells and acutely infected NS20Y cells 16 h before harvesting. Cell extracts from persistently and acutely infected cells were separated by SDS-PAGE after immunoprecipitation as described in Materials and Methods. NS20Y/MS cells (lane 1) or NS20Y cells (lane 2) were immunoprecipitated with polyclonal rabbit anti-MV serum prior to immunodetection with anti-p-Tyr antibodies; NS20Y/MS cells (lane 3) were immunoprecipitated with MAb anti-MV N protein prior to immunodetection with anti-p-Tyr antibodies; NS20Y cells (lane 4) or acutely MV-infected NS20Y cells (lane 5) were immunoprecipitated with MAb anti-MV N protein antibodies prior to immunodetection with anti-p-Tyr antibodies; and NS20Y cells (lane 6) or acutely MV-infected NS20Y cells (lane 7) were immunoprecipitated with MAb anti-MV N protein prior to immunodetection with MAb anti-MV N protein. The N protein is indicated by the 60-kDa marker.
lution on tyrosine residues could be detected. The fuzzy bands seen in Fig. 3 are the immunoglobulin light and heavy chains.

Phosphorylation of MV N protein in vivo occurs on serine, threonine, and tyrosine. To study the phosphorylation state of the N protein in vivo, cell lysates from 32P-labeled NS20Y/MS cells were immunoprecipitated with anti-N protein MAb. Two phosphoproteins, p190 and p60, were immunoprecipitated (Fig. 4A). To examine which amino acid residues were phosphorylated in the 60-kDa protein, the 32P-labeled 60-kDa protein was subjected to PAA. This analysis revealed mainly phosphothreonine and phosphoserine, with a small amount of phosphotyrosine (Fig. 4B). The phosphorylation of N protein on tyrosine was confirmed by competition with cold phosphotyrosine, phosphoserine, and phosphothreonine in an immunoblotting assay (data not shown). The tryptic phosphopeptide mapping of the 60-kDa phosphoprotein revealed that this protein contains five different highly phosphorylated peptides (Fig. 4C).

DISCUSSION

In the present study, we show augmented levels of tyrosine phosphorylation in a persistently MV-infected neuroblastoma cell line (NS20Y/MS). In addition, we demonstrate that the N protein of MV is phosphorylated on serine, threonine, and tyrosine. These cells, which carry a persistent infection of the wild-type Edmonston strain of MV, secrete alpha/beta IFN and an activated IFN system represented by the high activity levels of 2-5A synthetase and p68 kinase that were detected. In our previous studies, IFN has been implicated as a contributory factor in the establishment and maintenance of the persistent state, since treatment of NS20Y/MS cells with anti-IFN antibodies converted the persistent state to an acute infection (33). The activation of the two enzymes 2-5A synthetase and p68 kinase in NS20Y/MS but not in NS20Y cells indicates that the IFN system in these cells is induced and active. Indeed, we have shown an IFN-dependent enhanced expression of major histocompatibility complex class I glycoproteins on NS20Y/MS cells, suggesting that the IFN produced by these cells acts in an autocrine manner by forming an autocrine loop which regulates gene transcription (3). The ability of IFN-α to induce an autocrine regulatory loop has been demonstrated in a study by Jourdan et al., who showed that treatment of myeloma cell lines with IFN-α induced interleukin-6 mRNA accumulation and an autocrine production of interleukin-6, which supported the proliferation of these cell lines independent of exogenous interleukin-6 (16). To establish the linkage between the presence of viral proteins and induction plus activation of the IFN system, NS20Y/MS cells were treated with anti-MV antibodies. This treatment was used because it has been shown to down-regulate both transcription and expression of MV structural genes (42). In the experiments reported here, a significant reduction in the levels of the intracellular MV N protein, accompanied by the ablation of both 2-5A synthetase and p68 kinase activities, was obtained. Upon removal of the antibodies from the growth medium, both synthesis of MV N and IFN-dependent enzymatic activities were reactivated. The time courses of the reactivation were the same for viral proteins and for the IFN-induced enzymes. From these experiments, it appears that there is a strong correlation between the transcription and translation of the MV genome and activation of the IFN system.

Recently, it has been demonstrated that, unlike processes for other ligands that use a second messenger for signal transduction, binding of IFN to the appropriate membrane receptors directly activates specific IFN-induced cellular PTK(s) (12, 21, 51). A tyrosine kinase termed TYK 2 and the related nonreceptor JAK 1 have been implicated in phosphorylation of ISGF3α transcription factor in cells treated with alpha/beta IFN (29, 39). The data reported in the present study suggest that ISGF3α is not the sole target protein substrate to be phosphorylated by these alpha/beta IFN-activated tyrosine kinases. The viral protein MV N may be a bystander protein substrate for the IFN-activated tyrosine kinases. Since no phosphorylated tyrosine residues could be detected on MV N protein synthesized during the acute infection of NS20Y, it appears that this phosphorylation occurs in the persistent state of MV infection. By in vivo labeling with 32P, we were able to detect two major phosphorylated proteins in the persistently infected cells. One was a 180- to 200-kDa protein of cellular or viral origin, and the other was the 60-kDa N protein (46). PAA of the 60-kDa band demonstrated that MV N protein, as expected for viruses from the Morbillivirus group, is highly phosphorylated on serine and threonine (3). In comparison, the extent of phosphorylation on tyrosine was relatively low in spite of the unequivocal results of the immunoblotting. These seemingly conflicting results can be attributed to the finding that immunoblotting with anti-p-Tyr antibodies is far more sensitive than the PAA technique for detection of tyrosine phosphorylation (27). The tryptic map of the N protein revealed the existence of five peptides. In further studies, we intend to investigate which of these peptides contain(s) the phosphorylated tyrosine and to identify the site(s) of tyrosine phosphorylation on the N polypeptide. On the basis of the studies of persistent MV infection in vivo and in vitro, a number of underlying (by no means exclusive) mechanisms leading to the establishment of persistent infection have been described. These mechanisms include the generation of viral mutants (1, 5, 9, 10, 33, 52), host cell restrictions (37, 40, 41, 43, 45), and immunological restrictions (4, 28, 36, 42). The absence of infectious virus or budding virus particles in tissue from patients with SSPE has been attributed for many years to the absence of, or a defect in, the M protein, which plays a key role in the formation of virions (23). Although multiple defects of the MV M gene have been described, it is nevertheless clear....
that SSPE cannot be explained simply by the elimination of the MV M protein. Indeed, there is compelling evidence to show that factors intrinsic to the neuronal host cell downregulate MV gene expression (37, 41, 42). This downregulation may be crucial for the establishment of persistent infection in human brain tissue. In addition, treatment with anti-MV antibodies has been shown to affect the early stages of viral transcription, which leads to an almost complete repression of viral gene transcription by IFN-α (15). It is therefore tempting to speculate that the phosphorylation of IFN-α induces autocrine production of IL-6 in myeloma cell lines. J. Immunol. 147:4402–4407.


