Evidence of a Role for Phosphatidylinositol 3-Kinase Activation in the Blocking of Apoptosis by Polyomavirus Middle T Antigen

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A polyomavirus mutant (315YF) blocked in binding phosphatidylinositol 3-kinase (PI 3-kinase) has previously been shown to be partially deficient in transformation and to induce fewer tumors and with a significant delay compared to wild-type virus. The role of polyomavirus middle T antigen-activated PI 3-kinase in apoptosis was investigated as a possible cause of this behavior. When grown in medium containing 1n-3-deoxy-3-fluoro-myoinositol to block formation of 3'-phosphorylated phosphatidylinositols, F111 rat fibroblasts transformed by wild-type polyomavirus (PyF), but not normal F111 cells, showed a marked loss of viability with evidence of apoptosis. Similarly, treatment with wortmannin, an inhibitor of PI 3-kinase, stimulated apoptosis in PyF cells but not in normal cells. Activation of Akt, a serine/threonine kinase whose activity has been correlated with regulation of apoptosis, was roughly twofold higher in F111 cells transformed by either wild-type virus or mutant 250YS blocked in binding Shc compared to cells transformed by mutant 315YF. In the same cells, levels of apoptosis were inversely correlated with Akt activity. Apoptosis induced by serum withdrawal in Rat-1 cells expressing a temperature-sensitive p53 was shown to be at least partially p53 independent. Expression of either wild-type or 250YS middle T antigen inhibited apoptosis in serum-starved Rat-1 cells at both permissive and restrictive temperatures for p53. Mutant 315YF middle T antigen was partially defective for inhibition of apoptosis in these cells. The results indicate that unlike other DNA tumor viruses which block apoptosis by inactivation of p53, polyomavirus achieves protection from apoptotic death through a middle T antigen–PI 3-kinase–Akt pathway that is at least partially p53 independent.

Programmed cell death occurs during normal development and under certain pathological conditions. In mammalian cells, apoptosis can be induced by a variety of stimuli, including DNA damage (45), virus infection (54, 57), oncogene activation (25), and serum withdrawal (34, 37). Apoptosis can also be blocked by a number of factors, including adenovirus E1B 55- or 19-kDa proteins (9, 16), baculovirus p35 and iap genes (10), Bcl-2 (36, 61), and survival factors (12, 21). DNA tumor viruses have evolved mechanisms that both trigger and inhibit apoptosis. These frequently involve binding and inactivation of tumor suppressor proteins. E7 in some papillomaviruses (22), E1A in adenovirus (31, 43, 64), and large T antigen in simian virus 40 (SV40) (17) bind Rb and/or p300 and lead to upregulation of p53, which is thought to trigger apoptosis in virus-infected cells. The same viruses also inhibit apoptosis by inactivating p53 by various mechanisms (44, 63, 67). In contrast, the mechanism by which polyomavirus interacts with apoptotic pathways in the cell is not known; no direct interaction with p53 by any of the proteins encoded by this virus has been demonstrated (19, 62).

The principal oncoprotein of polyomavirus is the middle T antigen. Neoplastic transformation by polyomavirus middle T antigen has as a central feature its association with and activation of members of the Src family of tyrosine kinases p60c-src (13) and p62c-src (42). The major known consequence of these interactions is phosphorylation of middle T antigen on specific tyrosine residues creating binding sites for other signaling proteins. Phosphorylation at tyrosines 250, 315, and 322 promotes binding to Shc (18), the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) (59), and phospholipase Cγ (58), respectively. Recognition of multiple signaling pathways emanating from middle T antigen has led to a keen interest in identifying their downstream biochemical effects, which collectively lead to the emergence of neoplastic transformation and presumably underlie the dramatic ability of the virus to induce many kinds of tumors in the mouse.

Previous work has shown that the binding of PI 3-kinase to middle T antigen is essential for full transformation of rat fibroblasts in culture (8) and for rapid development of a broad spectrum of tumors in mice (30), for translocation of the GLUT1 transporter (68), and activation of p70 S6 kinase (14). While the mutant 315YF (blocked in PI 3-kinase activation) was able to induce some tumors, it did so at reduced frequencies and with an average latency three times longer than that of either the wild-type virus or a mutant, 250YS, blocked in binding Shc (4, 30). Recent studies have indicated a role of PI 3-kinase in blocking apoptosis in nonviral systems. Growth factor receptors acting through protein tyrosine kinases may prevent apoptosis by activating PI 3-kinase (14), and phosphatidylinositol 3-kinase (PI 3-kinase) (59), and phospholipase Cγ (58), respectively. Recognition of multiple signaling pathways emanating from middle T antigen has led to a keen interest in identifying their downstream biochemical effects, which collectively lead to the emergence of neoplastic transformation and presumably underlie the dramatic ability of the virus to induce many kinds of tumors in the mouse.

MATERIALS AND METHODS

Cells. Rat F111 cells, as well as PyF, PyF-315YF, and PyF-250YS cell lines derived from F111 cells, were described previously (14). Cells were routinely
cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% calf serum, 0.375% sodium bicarbonate, 100 U of penicillin, and 100 μg of streptomycin per ml in a 5% CO₂ atmosphere at 37°C. Rat-1 clones stably expressing vector alone (Neo), wild-type, 315YF, and 250YS middle T antigens were prepared by electroporation of Rat-1 cells with defective murine retroviral vectors containing the cloned middle T antigen genes (15, 68). Stably transfected cells were selected with G418 (G418) at 400 μg/ml, and clones were isolated by limiting dilution. To establish cell lines expressing temperature-sensitive p53 (p53×5T) p53sens was cotransfected with a 10-fold excess of pLTRCp53Val135 (47) by electroporation into Rat-1 clones stably expressing vector alone (Neo), wild-type, 315YF, and 250YS middle T antigens. Cells were selected with 2 μg of puromycin per ml and cloned by limiting dilution. Cell lines expressing similar level of middle T antigen or p53Val135 were maintained in DMEM supplemented with 10% calf serum, sodium bicarbonate, penicillin-streptomycin, 100 μg of G418 per ml, and 0.5 to 1 μg of puromycin per ml.

Cell growth assays. Cells plated at a density of 4 × 10⁵ cells per well in 96-well plates were grown for 4 days in myo-inositol-free DMEM (GIBCO), supplemented with 10% calf serum, 5 μM myo-inositol, and increasing levels of 1D-3-deoxy-3-fluoro-myoinositol (38). Viable cells were assessed with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (49). [3H]-1D-3-deoxy-3-fluoro-myoinositol was obtained from Moravek Biochemicals, Inc.

Ca²⁺ uptake assays. To measure Ca²⁺ uptake, cells grown in myo-inositol-free DMEM supplemented with 10% dialyzed calf serum, 5 μM myo-inositol and 2 mM analog, for 72 h were changed to 20 mM HEPES (pH 7.4), 150 mM NaCl, 0.1 mM CaCl₂, 1 mM MgCl₂, 50 μM K₂HPO₄, and 0.1% glucose (buffer A) containing 0.1 mM CaCl₂. The reaction was started by adding 2 μCi of ⁴⁰Ca²⁺ (NEN) per ml and 100 μM lysophosphatic acid. After 1 min, the cells were washed five times with buffer A containing 10 mM CaCl₂ and solubilized in 1% sodium dodecyl sulfate for scintillation counting and protein determination (bicinchoninic acid assay; Pierce).

Apoptosis assays. To measure the effect of the myo-inositol analog on apoptosis, F111 and PyF cells were plated on coverslips at a density of 5 × 10⁴ cells per well in 10-cm² plates and grown to 60 to 80% confluence. Cells were washed twice with serum-free medium and incubated at the appropriate temperature. Rat-1 clones expressing p53val135 were cultured at 38.5°C before temperature shift. 4 × 10⁴ F111 and 2 × 10⁴ PyF cells were cultured at 38.5°C before temperature shift. 4 × 10⁴ F111 and 2 × 10⁴ PyF cells were cultured in 8-cm² plates and grown to 60 to 80% confluence. Cells were washed five times with buffer A containing 10 mM CaCl₂ and solubilized in 1% sodium dodecyl sulfate for scintillation counting and protein determination (bicinchoninic acid assay; Pierce).

RESULTS

1D-3-Deoxy-3-fluoro-myoinositol inhibits net growth and stimulates apoptotic death in polyomavirus transformed but not in nontransformed F111 rat fibroblasts. Analogs of myo-inositol with substitution at the D-3 position are potential antagonists for cells exhibiting a constitutively activated PI-kinase and are known to inhibit growth of v-sis-transformed NIH 3T3 cells (52). Several studies have shown that 1D-3-deoxy-3-fluoro-myoinositol acts as a substrate for mammalian phosphatidylinositol synthase and is incorporated into phosphatidylinositol (38, 51, 52). Signaling via phosphatidylinositol 4,5-bisphosphate and phospholipase C appears to be unaffected by myo-inositol analogs at the D-3 position (38). The data in Fig. 1 show that PyF cells exhibited marked growth inhibition compared to F111 cells when grown in the presence of 1D-3-deoxy-3-fluoro-myoinositol (up to 4 mM). Differences in viability were not due to differences in uptake, since studies showed that the analog was incorporated into phospholipid equally well by both cell types (data not shown).

The effect of the analog on phospholipase C-dependent pathways was studied by measuring Ca²⁺ uptake induced by lysophosphatic acid (40) and was found to be similar in cells grown in the absence or presence of 1D-3-deoxy-3-fluoro-myoinositol (Fig. 2). Taken together, the data show that while normal and transformed cells take up the 3-deoxy-3-fluoro-analog of myo-inositol equally, the growth inhibitory effect was more evident in transformed cells. The effect of the inositol analog on cell viability was apparently unrelated to signal transduction via phospholipase C. Previous work has shown that an increased level of inositol trisphosphate in cells expressing wild-type middle T antigen is dependent on p70S6k but independent of PI 3-kinase activation (33).

To determine whether the myo-inositol analog induced ap-
Optosis preferentially in PyF cells, F111 and PyF cells were subjected to DAPI staining and the TUNEL assay after growing on 2 mM analog (Fig. 3A). In the presence of 1D-3-deoxy-3-fluoro-myoinositol, the percentage of apoptosis of PyF cells rose nearly threefold over the level seen in the absence of analog, whereas F111 cells remained unchanged (Fig. 3B). These results show that blocking the PI 3-kinase pathway in cells transformed by wild-type polyomavirus drives cells toward apoptosis.

Wortmannin stimulates apoptosis in polyomavirus-transformed but not nontransformed F111 cells. Further evidence that PI 3-kinase helps to prevent apoptosis in polyomavirus-transformed cells was sought by using wortmannin, a potent inhibitor of PI 3-kinase both in vivo and in vitro (53, 60). F111 and PyF cells growing in 10% calf serum were treated with increasing concentrations of wortmannin for 2 h before fixation. TUNEL-positive PyF cells were observed with >10 nM wortmannin, whereas F111 cells failed to undergo apoptosis after treatment with wortmannin at concentrations up to 1,000 nM (Fig. 4A). Wortmannin was shown to inhibit in vitro PI 3-kinase of PyF cells with a similar sensitivity (Fig. 4B). The results support the involvement of PI 3-kinase in preventing apoptosis in cells transformed by polyomavirus but not in nonvirus-transformed F111 cells.

Activation of Akt requires binding of PI 3-kinase to middle T antigen in F111 cells transformed by polyomavirus. Phosphatidylinositol 3,4-bisphosphate, a product of PI 3-kinase, directly regulates Akt (27), the serine/threonine kinase also designated PKB or Rac (2, 11, 39). Akt has been implicated in inhibition of apoptosis by serum and certain growth factors (5, 20, 41). Receptors blocked in PI 3-kinase binding fail to activate Akt (28), and dominant negative Akt expression induces apoptosis (20). To determine whether transformation by polyomavirus leads to activation of Akt, in vitro kinase assays of Akt immunoprecipitates were performed with extracts from serum-starved cells. Akt activity, shown in Fig. 5, was roughly twofold higher in cells transformed by wild-type and 250YS polyomavirus than in parental F111 cells or cells transformed by mutant 315YF. The level of constitutive activation in PyF approached maximal levels achieved by adding back 15% serum. The levels of phosphatidylinositol 3,4-bisphosphate are known to be elevated in 250YS and wild-type middle T antigen-expressing F111 cells compared to 315YF middle T antigen-expressing cells (14). When F111 cells expressing wild-type or mutant middle T antigens were serum starved and examined for apoptosis, cells expressing 315YF showed a twofold elevation in the percentage of apoptotic cells over the percentage seen in either wild-type or 250YS middle T antigen-expressing cells. These data suggest that activation of Akt depends on binding of PI 3-kinase to middle T antigen and correlates with inhibition of apoptosis.

Wild-type middle T antigen blocks apoptosis induced by serum withdrawal in Rat-1 cells by a p53-independent process. Rat-1 cells are dependent upon serum for survival and undergo apoptosis when treated with wortmannin (65). To extend the

**FIG. 3.** Effect of 3-deoxy-3-fluoro-myoinositol on apoptosis. (A) DAPI and TUNEL staining of PyF cells showing DNA condensation and fragmentation. (B) Percentage of apoptotic cells in cultures grown for 2 days on medium containing 5 μM myoinositol (open bars) or 5 μM myoinositol and 2 mM 1D-3-deoxy-3-fluoro-myoinositol (hatched bars) and quantitated by counting >1,000 cells in duplicate experiments.

**FIG. 4.** Effect of wortmannin on apoptosis of F111 (○) and PyF (■) cells (A) and in vitro PI 3-kinase activity in anti-T-antigen immunoprecipitates of PyF (■) cell extracts (B). Cells growing on DMEM containing 10% calf serum were treated with wortmannin for 2 h before fixation for DAPI and the TUNEL assay. The percentage of apoptotic cells was quantitated by counting >1,000 cells in duplicate experiments.
finding of a role for PI 3-kinase in the prevention of apoptosis by middle T antigen. Rat-1 cells were transformed with retroviral vectors encoding wild-type or mutant middle T antigens and selected with G418. In an attempt to determine if the apoptotic response of these cells is p53 dependent, clones stably expressing the middle T antigens were also transfected with a temperature-sensitive p53 gene, p53val135 (47), and selected for puromycin resistance. p53val135 behaves like a dominant inhibitory mutant at the restrictive temperature (38.5°C) and exhibits wild-type function at 32°C. At 38.5°C, p53val135 cooperates with E1A or ras to transform primary cells which also express wild-type endogenous p53 (16, 35, 47). p53val135 is impaired in transcription activation (23), repression (1), and nuclear translocation (6, 32, 46) at the nonpermissive temperature.

To determine the effect of p53 on apoptosis, all clones were grown with 10% calf serum at 38.5°C and either fixed for apoptosis assays at 0 h or grown for 2 days more either at 38.5°C in serum-free medium or at 32°C in medium with or without calf serum and then fixed (Fig. 6). On shifting to 32°C in the presence of serum, there was only a slight increase in the percentage of apoptotic cells in all clones. However, when normal cells or cells expressing 315YF mutant middle T antigen were shifted down and the serum was removed, there was roughly a 10-fold increase in the percent of apoptotic cells after 2 days. Cells expressing either wild-type middle T antigen or mutant 250YS middle T antigen showed a roughly threefold increase in apoptotic cells under the same conditions, indicating an effect of middle T antigen-activated PI 3-kinase in partially blocking the apoptotic response brought on by serum withdrawal. Protection against apoptotic death upon serum withdrawal by wild-type and 250YS middle T antigens was not significantly temperature sensitive in any of the clones. The protection afforded by middle T antigen–PI 3-kinase interaction thus appeared to be p53 independent, at least to a large extent.

**DISCUSSION**

Apoptosis in F111 rat fibroblasts transformed by polyomavirus is shown to be enhanced when PI 3-kinase activity is inhibited by either growth of cells on 1d-3-deoxy-3-fluoro-myo-inositol or treatment with wortmannin, indicating involvement of PI 3-kinase in blocking apoptosis. In contrast, normal F111 cells are much less dependent on PI 3-kinase for survival, as indicated by their resistance to death induced by the myo-inositol analog and by wortmannin. Transformation of F111 by polyomavirus mutant 315YF blocked in activation of PI 3-kinase significantly enhances the susceptibility of these cells to apoptosis induced by serum withdrawal compared to that in cells transformed by the wild-type or 250YS polyomavirus that encodes middle T antigens that activate PI 3-kinase. Cells transformed by mutant 315YF also have a lower constitutive activity of Akt, a serine/threonine kinase regulated by phosphatidylinositol 3,4-bisphosphate (27) and known to be associated with regulation of apoptosis (20, 41). This suggests that signal transduction via middle T antigen through PI 3-kinase and Akt is important for survival and protection from apoptosis.

In contrast to F111 rat fibroblasts, nontransformed Rat-1 fibroblasts show a marked serum dependence for survival and are susceptible to wortmannin-induced apoptosis (65). Apoptosis induced by serum withdrawal in Rat-1 cells expressing p53val135 appears to be principally p53 independent, since the level of apoptosis at 32°C is not significantly higher than that at 38.5°C. In Rat-1 cells expressing wild-type or 250YS middle T antigen, there is significant protection against apoptosis upon serum withdrawal and the degree of protection is largely temperature independent. Rat-1 cells expressing 315YF mutant middle T antigen show less protection against apoptotic death, indicating a role of PI 3-kinase in blocking apoptosis under these conditions. While Rat-1 cells stably transfected with pLTRCgp53val135 possibly retain a low level of functional p53 at the nonpermissive temperature, which might arise either from unquenched endogenous wild-type p53 or p53val135 that retains partial wild-type activity, the absolute amount of functional p53 should be markedly lower at 38.5°C than at 32°C. Consistent with our findings, p53 independence of apoptosis induced by serum withdrawal has also been observed in other cell types (3, 24, 37, 55).

The ability of a virus to delay host cell death is thought to be essential for virus growth (54). Whether polyomavirus can
block apoptosis has been unclear. Other DNA tumor viruses, such as adenovirus and SV40, inhibit programmed cell death by inactivation of p53. Since polyomavirus has no known direct interaction with p53 (19), a separate antiapoptotic mechanism is indicated. The results presented here indicate that in polyomavirus-infected cells, middle T antigen, acting through PI 3-kinase and Akt, blocks apoptosis. Interestingly, viruses that handle p53 directly by inactivation or degradation (i.e., adenovirus, SV40, and papillomavirus) lack direct mechanisms for activating PI 3-kinase. Recent evidence suggests that PI 3-kinase and Akt block apoptosis by inhibiting the CED3/ICE-like activity (41), and CED3/ICE-like proteases are thought to lie on apoptotic pathways downstream of both p53 (43) and the FAS pathway (50).

Mutant 315YF polyomavirus, whose middle T antigen fails to bind PI 3-kinase, is associated with delayed appearance of tumors in neonatally infected mice (8, 14, 29). In contrast, the 250YS mutant, whose middle T antigen binds PI 3-kinase but fails to bind p53, induces tumors broadly and with little or no delay compared to wild-type virus (4). The delay in tumor induction by the mutant 315YF may be due to failure to protect against apoptosis in the lytic phase of viral growth or in tumor development.

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