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Transformation-Defective Mutants of Polyomavirus Middle T Antigen Associate with Phosphatidylinositol 3-Kinase (PI 3-Kinase) but Are Unable To Maintain Wild-Type Levels of PI 3-Kinase Products in Intact Cells

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Middle T antigen (MT) of polyomavirus causes transformation by associating with a number of cellular proteins. The association with and activation of two such proteins, phosphatidylinositol 3-kinase (PI 3-kinase) and pp60$^{c\text{-}src}$, appears to be necessary for transformation by MT. The tyrosine kinase activity of MT-associated pp60$^{c\text{-}src}$ is significantly increased when assayed in vitro, and levels of phosphotyrosine-containing proteins are elevated in vivo. Similarly, levels of the PI 3-kinase products phosphatidylinositol-3,4-bisphosphate [PI(3,4)P_2] and phosphatiylinositol-3,4,5-trisphosphate [PI(3,4,5)P_3] are constitutively elevated in MT-transformed cells. However, the formation of a complete MT/cellular protein complex and the activation of tyrosine kinase are not sufficient to cause transformation, since the transformation-defective mutants 248m and *dl*1015 associate with all wild-type MT-associated proteins, including PI 3-kinase and pp60$^{c\text{-}src}$, and neither mutant appears to be defective in MT-associated tyrosine kinase activity. Studies presented here compared (i) the amount of PI 3-kinase activity associated with the MT complex and (ii) levels of [^{3}H]inositol incorporation into PI 3-kinase products in cells expressing mutant or wild-type MT. The results show that *dl*1015 is defective in both assays, whereas 248m is defective only for incorporation of [^{3}H]inositol into PI(3,4,5)P_2 and PI(3,4)P_3. These findings identify a biochemical defect in the 248m mutant and corroborate previous results correlating transformation and elevated levels of PI 3-kinase products in vivo. In addition, they indicate that PI 3-kinase product levels are affected by factors other than simply the amount of PI 3-kinase activity associated with the MT complex.

Two of the earliest events in mitogenic signalling as well as two common attributes of cells transformed by activated tyrosine kinase oncogenes are an association of phosphatidylinositol 3-kinase (PI 3-kinase) with activated tyrosine kinases and an increase in the levels of PI 3-kinase products (4). This unique lipid kinase phosphorylates the D-3 position of the inositol ring on phosphatidylinositol, phosphatidylinositol-4-monophosphate, and phosphatidylinositol-4,5-bisphosphate [PI, PI(4)P, and PI(4,5)P_2, respectively], forming three products phosphatidylinositol-3-monophosphate, phosphatidylinositol-3,4-bisphosphate, and phosphatidylinositol-3,4,5-trisphosphate [PI(3)P, PI(3,4)P_2, and PI(3,4,5)P_3, respectively] (1, 6, 34–36).

In nontransformed cells, mitogenic stimulation through tyrosine kinase growth factor receptors (such as those for platelet-derived growth factor [PDGF], colony-stimulating factor 1, and insulin) is accompanied by several rapid occurrences: (i) the association of PI 3-kinase with the activated receptor, (ii) the phosphorylation of the p85 subunit of PI 3-kinase on tyrosine residues, and (iii) an increase in two PI 3-kinase products, PI(3,4)P_2 and PI(3,4,5)P_3 (1, 2, 13, 17, 27, 28, 33, 35). Similarly, oncogenic proteins with activated tyrosine kinase activity (such as pp60$^{v\text{-}src}$, pp160$^{gag\text{-}abl}$, pp130$^{gag\text{-}fps}$, and the complex of polyomavirus middle T antigen (MT) with Src family tyrosine kinases) have also been shown to associate with PI 3-kinase (8, 15, 14, 32, 36). Transformation by these oncogenes appears to stimulate PI 3-kinase activity, since studies of MT-, pp160$^{gag\text{-}abl}$-, and pp60$^{v\text{-}src}$-transformed cells show elevated levels of PI(3,4)P_2 and PI(3,4,5)P_3 (29, 29a, 31, 32). Cells expressing wild-type MT have increased amounts of these PI 3-kinase products not only during growth stimulation but also after serum starvation or growth to high density (29). These findings suggest that PI 3-kinase activation is involved in normal mitogenic signalling by growth factors as well as signalling of aberrant growth by oncogenic agents.

The MT/pp60$^{c\text{-}src}$/PI 3-kinase transforming complex has been studied in detail to elucidate the mechanism of transformation by the MT protein. MT itself has no known enzymatic activity, but it associates with a number of cellular proteins, including PI 3-kinase and pp60$^{c\text{-}src}$ (11, 16, 19, 24, 26, 36). The association of pp60$^{c\text{-}src}$ and PI 3-kinase with MT appears to activate both enzymes. Activation of pp60$^{c\text{-}src}$ has been measured by comparing the tyrosine kinase activity of MT-associated versus unassociated pp60$^{c\text{-}src}$ (3). Activation of PI 3-kinase is inferred by the increase in PI 3-kinase products in MT-transformed cells (29). MT-associated tyrosine kinase activity is necessary for PI 3-kinase association, since the effective binding of PI 3-kinase to MT requires phosphorylation of tyrosine 315 (10). MT mutants which lack tyrosine 315 have less associated PI 3-kinase activity and are defective for transformation (9, 30, 36). However, these mutants do maintain the ability to associate with all other MT complex proteins, including pp60$^{c\text{-}src}$ (5, 16, 23, 24, 30). These results not only illustrate the mechanism by which PI 3-kinase associates with MT/pp60$^{c\text{-}src}$ but also strongly suggest that PI 3-kinase association is necessary for MT transforming ability.

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To test this hypothesis and the postulate that elevated levels of PI(3,4,5)P_3 and PI(3,4)P_2 in vivo correlate with transformation (29, 29a, 31, 32), we characterized two transformation-defective MT mutants, 248m and *dl*1015. These two mutants are paradoxical in that they form a complete MT complex containing all known MT-associated proteins, including the 85-kDa subunit of PI 3-kinase, and yet they are defective for transformation. Neither of these mutations causes any apparent defects in tyrosine kinase activity (12, 23). However, data presented here suggest that cells expressing either *dl*1015 or 248m are defective in the amount of [^{3}H]inositol incorporation into PI(3,4,5)P_3 and PI(3,4)P_2. These findings support the contention that the level of these two PI 3-kinase products in intact cells correlates with transformation. The amount of PI 3-kinase activity associated with the MT complex of each mutant was also determined, and the *dl*1015 MT complex was found to have less activity than did the wild-type MT complex (see also reference 24). In contrast, the level of PI 3-kinase activity in the 248m mutant MT complex was equal to or slightly greater than that associated with the wild-type complex. Taken together, these results indicate that although the association of high levels of PI 3-kinase activity with MT may be a prerequisite for transformation, it is neither sufficient to elevate levels of PI 3-kinase products in vivo nor sufficient to induce cellular transformation.

MATERIALS AND METHODS

Immunoprecipitation and determination of phospholipid kinase activity in MT complexes. MT complexes were immunoprecipitated with high-titer rabbit polyclonal anti-MT antiserum (25) from cell lysates as described previously (36). Antibody-antigen complexes were precipitated by the addition of Sepharose CL4B-protein A beads and incubation for 45 min at 4°C. Beads were washed once in lysis buffer, once in phosphate-buffered saline (PBS), twice in 0.5 M LiCl–0.1 M Tris (pH 7.4), and twice in 10 mM Tris–1 mM EDTA–100 mM NaCl. Excess liquid was removed from immunoprecipitates, and phospholipid kinase activity was assayed by adding phospholipid, ATP, and reaction buffer to final concentrations of 40 μg of PI (Avanti Polar Lipids) per ml, 40 μg of PI(4)P (Sigma) per ml, 40 μg of PI(4,5)P_2 (Sigma) per ml, 80 μg of phosphatidylserine (Avanti Polar Lipids) per ml, 10 μM ATP (10 μCi of [γ-^{32}P]ATP [New England Nuclear] per reaction), 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 10 mM $MgCl_2$, and 1 mM EDTA (pH 7.5). Assay mixtures were incubated for 5 min at 25°C and extracted with equal volumes of 1:1 1 N HCl–methanol and $CHCl_3$. The $CHCl_3$ phase was washed once with 1:1 1 N HCl–methanol then dried under N_2. The phospholipids were resuspended in 2:1:0.01 $CHCl_3$-methanol-water and spotted onto 1% potassium oxalate-treated silica gel 60 thin-layer chromatography plates (EM Science). Plates were developed for 4 to 5 h in 65:2:33 (vol/vol) *n*-propanol–glacial acetic acid–water. Labeled products were visualized by autoradiography, and unlabeled phospholipids were visualized by iodine vapor development. ^{32}P-labeled standards were synthesized by phosphorylating PI, PI(4)P, and PI(4,5)P_2 at the D-3 position with either purified PI 3-kinase (kind gift of Chris Carpenter and Rosana Kapeller) or immunoprecipitated wild-type MT complex. The amount of ^{32}P incorporated into each lipid was quantitated with a Molecular Dynamics Phosphorimager. High-pressure liquid chromatography (HPLC) analysis of the labeled species was performed by excising the area of the thin-layer sheet containing the lipid, deacylating the lipid in situ, and separating the glycerophosphoinositide products on a Partisphere SAX ion-exchange column. These procedures are described in detail elsewhere (1, 29).

Quantitation of MT-associated PI 3-kinase activity and MT protein. Cells were labeled for 5 to 6 h at 37°C in 10% CO_2 in Dulbecco's modified Eagle's medium lacking methionine and supplemented with 10% donor calf serum and 170 mCi of ^{35}S-Translabel (methionine, cysteine) (New England Nuclear) per ml. The cells were washed with PBS and lysed in buffer containing 1% Nonidet P-40, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM Tris, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, and 4 mM leupeptin (pH 7.5). PI 3-kinase activity in these ^{35}S-labeled cell lysates was assayed after immunoprecipitation as described above. Quantitation of MT protein was done by preparing identical immunoprecipitations from ^{35}S-labeled cell lysates with the exception that these were washed four times with RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 137 mM NaCl, 20 mM Tris [pH 8], 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol) and analyzed by 10% SDS-polyacrylamide gel electrophoresis (PAGE). Amounts of ^{35}S-MT in SDS-PAGE and ^{32}P-PI 3-kinase products in thin-layer chromatography plates were quantitated by using a Molecular Dynamics Phosphorimager.

Cell culture and [^{3}H]myoinositol labeling. Cells were grown at 37°C in 10% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% donor calf serum (GIBCO). Mutant 248m was expressed in BALB/3T3 (clone A31) in the vector pLJ (12). Mutants *dl*1015 and NG59 were expressed in NIH 3T3 cells, using the vector pZIPneo (23). Phosphoinositides were labeled by incubating cells for 2 days in 10 μCi of [^{3}H]myoinositol (New England Nuclear) per ml in inositol-free Dulbecco's modified Eagle's medium (GIBCO) containing 10% dialyzed donor calf serum. After 2 days of growth to confluence, the medium was changed to fresh [^{3}H]myoinositol (10 μCi/ml) in Dulbecco's modified Eagle's medium with 0.5% dialyzed donor calf serum. Cells were allowed to become quiescent for 1 to 2 days and were then harvested. Cells were washed three times in ice-cold PBS prior to lysis with 1:1 1 N HCl–methanol. Cells were scraped from the plate, and lipids were extracted with $CHCl_3$. The $CHCl_3$ layer was reextracted with 1:0.9 methanol–0.1 M EDTA and dried under N_2. Phospholipids were deacylated in 3.7:4.1 (vol/vol) 25% methylamine–methanol–*n*-butanol for 50 min at 50°C, dried in vacuo, and extracted with distilled water and 20:4:1 *n*-butanol–light petroleum ether–ethyl formate. Glycerophospholipids in the aqueous layer were then separated by HPLC as previously described (29). Standards for glycero PI(3)P, glycero PI(3,4)P_2, and glycero PI(3,4,5)P_3 were prepared as described above.

RESULTS

PI 3-kinase activity associated with mutant or wild-type MT in anti-MT immunoprecipitations. Wild-type, 248m, and *dl*1015 MT-associated PI 3-kinase activities were compared in terms of substrate preference and relative amounts of activity. PI 3-kinase activity was measured in anti-MT immunoprecipitations from cells expressing wild-type MT, *dl*1015, or 248m, using equimolar amounts of PI, phosphatidylinositol phosphate (PIP), and phosphatidylinositol bisphosphate (PIP_2) as substrates (Fig. 1). The three major labeled species formed were confirmed by HPLC analysis to

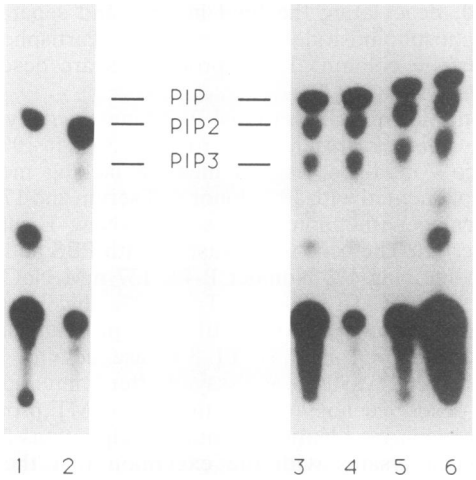

FIG. 1. Phosphoinositide kinase activity in MT immunoprecipitates. PI 3-kinase activities were measured in MT complexes from cells expressing wild-type MT (lane 5), 248m (lane 4), dl1015 (lane 3), NG59 (lane 2), or cells with vector alone (lane 1). Standards prepared as described in Materials and Methods are shown in lane 6. Anti-MT antiserum immunoprecipitations as described in Materials and Methods were incubated with 0.4 mg each of PI, PIP, PIP_2 per ml and 0.8 mg of phosphatidylserine per ml in 10 μM [γ-^{32}P]ATP–50 mM HEPES–10 mM $MgCl_2$–1 mM EGTA–0.1 mM EDTA (pH 7.5) for 5 min at 25°C. Phospholipids were extracted with $CHCl_3$-methanol-HCl and analyzed by thin-layer chromatography.

be D-3-phosphorylated PI 3-kinase products PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3 (data not shown). Immunoprecipitations from cells containing vector alone or a mutant MT which does not associate with PI 3-kinase (NG59) showed only a small amount of background PI(4,5)P_2 and none of the PI 3-kinase products (Fig. 1 and data not shown).

By comparing the percentage of PI(3)P, PI(3,4)P_2, and PI(3,4,5)P_3 produced in each reaction, the substrate preference of the PI 3-kinase associated with mutant versus wild-type MT was assessed. As shown in Table 1, the proportions of label in each product were very similar for wild type, 248m, and *dl*1015. Under these assay conditions, phosphorylation of PI to PI(3)P is the preferred reaction for both the wild-type and mutant MT-associated PI 3-kinases. (It should be noted that the same results were obtained when lysates were prepared from cells which were serum starved as in the [^{3}H]myoinositol labeling experiments discussed below.)

TABLE 1. PI activity in wild-type and mutant MT[a]

Cell line	PI 3-kinase activity[b]	% Phosphorylation of[c]:		
		PI(3)P	PI(3,4)P_2	PI(3,4,5)P_2
Wild type	100	70 ± 5	10 ± 1	21 ± 4
248m	180 ± 80	65 ± 10	17 ± 6	19 ± 4
*dl*1015	50 ± 20	61 ± 9	16 ± 4	22 ± 7
NG59	0			
Vector alone	0			

[a] Data for all cell lines are means and standard errors of the means from three experiments.

[b] Percentage of PI 3-kinase activity/MT, where wild-type MT-associated PI 3-kinase activity/MT is 100%. PI 3-kinase activity/MT is the sum of counts per minute in PI(3)P plus PI(3,4)P_2 plus PI(3,4,5)P_3 divided by the counts per minute in ^{35}S-labeled MT (see Materials and Methods).

[c] Percentage of total counts per minute in PI(3)P plus PI(3,4)P_2 plus PI(3,4,5)P_3. The amount of radioactivity in each phosphoinositide was determined as described in Materials and Methods. Incorporation into PI(3)P plus PI(3,4)P_2 plus PI(3,4,5)P_3 was typically between 30,000 and 90,000 cpm.

To compare the amounts of PI 3-kinase activity associated with wild-type and mutant MT complexes, the total activity was normalized with respect to the amount of MT in each immunoprecipitation. As shown in Table 1, the amount of PI 3-kinase activity associated with 248m MT immunoprecipitations was equal to or greater than the amount of PI 3-kinase activity associated with the wild-type MT immunoprecipitations. In contrast, the *dl*1015-associated activity was approximately half that found associating with wild-type or 248m MT. Taken together, these results show that the relative proportion of PI, PI(4)P, and PI(4,5)P_2 phosphorylating, PI 3-kinase activities is unaffected by the 248m and *dl*1015 mutations. However, the total amount of MT-associated PI 3-kinase activity is decreased in *dl*1015 MT complexes and similar to that in wild-type or increased in the 248m MT complexes.

Cells expressing 248m or *dl*1015 have lower levels of ^{3}H-PI(3,4,5)P_3 and ^{3}H-PI(3,4)P_2 than do cells expressing wild-type MT. The levels of PI 3-kinase products in intact cells expressing wild-type MT, mutant MT, or vector alone were measured by labeling the cells with [^{3}H]myoinositol and analyzing the labeled phosphoinositides by HPLC. Since all cells contain elevated levels of PI 3-kinase products during log-phase growth (29), the cells were made quiescent by serum starvation prior to harvest. The quiescence and viability of each set of cells was tested by adding PDGF prior to harvest to identically treated plates of cells and confirming a mitogenic response by an increase in incorporation into PI(3,4)P_2 and PI(3,4,5)P_3 (data not shown). Lipids from labeled cells were extracted, deacylated, and analyzed by HPLC. Figure 2 shows representative HPLC tracings of ^{3}H-labeled species from wild-type MT cells and 248m cells.

Since 248m and *dl*1015 were expressed in BALB/3T3 and NIH 3T3 cells, respectively, the mutants were compared with wild-type MT and vector-alone control cells of the appropriate background. Compared with wild-type MT-transformed cells, cells expressing mutants 248m and *dl*1015 had significantly lower incorporation of [^{3}H]inositol into PI(3,4,5)P_3 and PI(3,4)P_2 (Fig. 3). 248m-expressing BALB/3T3 cells had near control levels of ^{3}H-PI(3,4,5)P_3 which were fivefold lower than levels found in wild-type MT-expressing cells (Fig. 3A). Comparison of *dl*1015-expressing cells with wild-type MT and vector-alone-transformed NIH 3T3 cells showed similar results, although the basal level of ^{3}H-PI(3,4,5)P_3, as seen in the vector alone, was slightly higher in the NIH 3T3 cell background. The *dl*1015-expressing cells showed levels of ^{3}H-PI(3,4,5)P_3 which were twofold lower than those in wild-type MT-expressing cells, and the levels were indistinguishable from the amounts in cells with NG59 or vector alone. Incorporation of [^{3}H]inositol into PI(3,4)P_2 in cells expressing either 248m, *dl*1015, or NG59 was also decreased in comparison with the amount of labeled PI(3,4)P_2 in wild-type MT-expressing cells (Fig. 3B). These mutant MT cell lines showed levels of ^{3}H-PI(3,4)P_2 similar to those in cells with vector alone.

The levels of [^{3}H]inositol incorporation into phosphoinositides of the classical PI turnover pathway, PI, PI(4)P, and PI(4,5)P_2, show no correlation to transformation in these MT-expressing cell lines (Fig. 4). Also, as previously shown (29), steady-state incorporation of [^{3}H]inositol into PI(3)P does not correlate to the transforming ability of MT (data not shown). Thus, both nontransforming mutants, 248m and *dl*1015, are defective in the ability to elevate intracellular

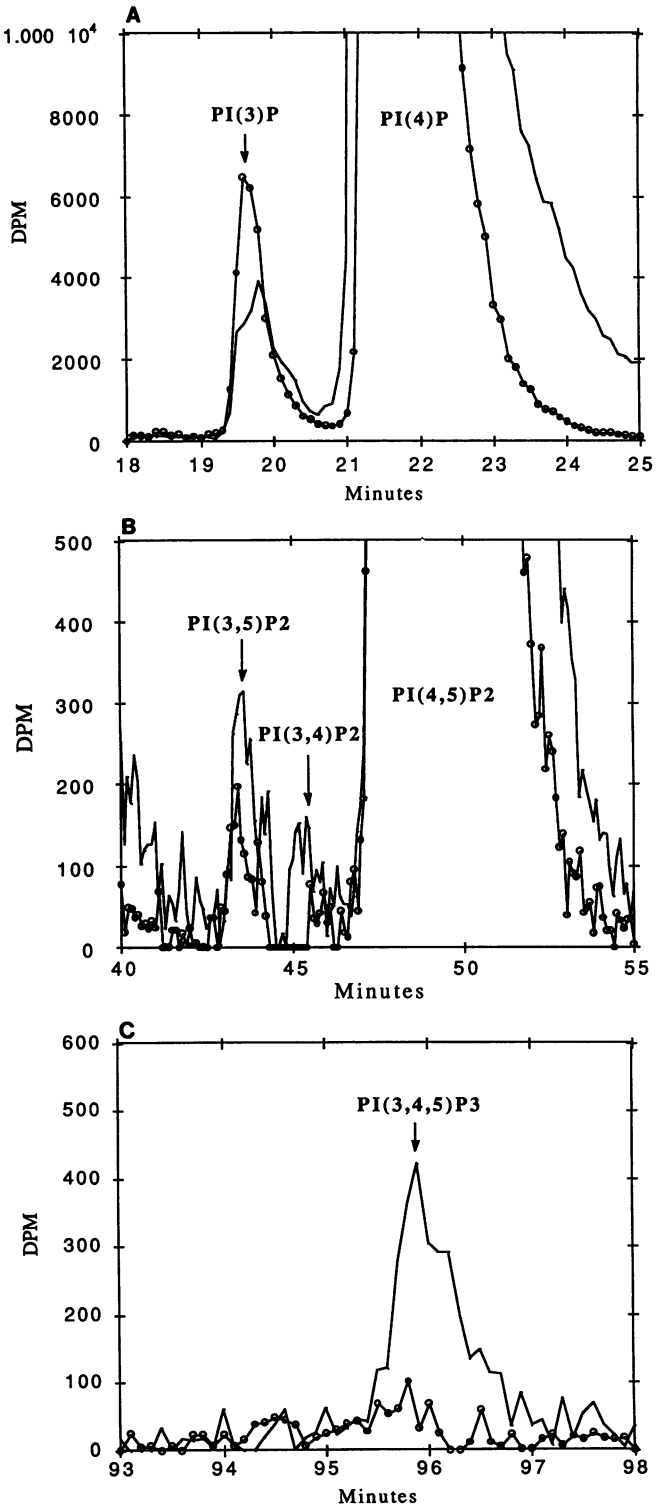

FIG. 2. Identification of [^{3}H]myoinositol-labeled phosphoinositides in intact cells. Cells were labeled with [^{3}H]myoinositol as described in Materials and Methods. Labeled phospholipids were rapidly extracted from intact cells with $CHCl_3$, methanol, and HCl. The extracted phospholipids were deacylated and analyzed by HPLC in parallel with ^{32}P-labeled PI(3)P, PI(3,4)P_2, and PI(3,4,5)P_3 standards. PI, PI(3)P, phosphatidylinositol-3,5-bisphosphate [PI(3,5)P_2], PI(3,4)P_2, PI(4,5)P_2, and PI(3,4,5)P_3 are indicated by arrows. (A) Comparison of steady-state-labeled phosphoinositides from wild-type (solid line)- and 248m (circles)-expressing cells showing the region near PIP. (B) Comparison of steady-state-labeled phosphoinositides from wild-type (solid line)- and 248m (circles)-expressing cells showing the region near PIP_2. (C) Comparison of steady-state-labeled phosphoinositides from wild-type (solid line)- and 248m (circles)-expressing cells showing the region near PI(3,4,5)P_3. PI(3,4,5)P_3 reproducibly eluted at 96 min between Ins(1,4,5)P_3 at 75 min and Ins(1,3,4,5)P_4 at 107 min (the latter compounds used as standards), using the HPLC conditions described in the Materials and Methods.

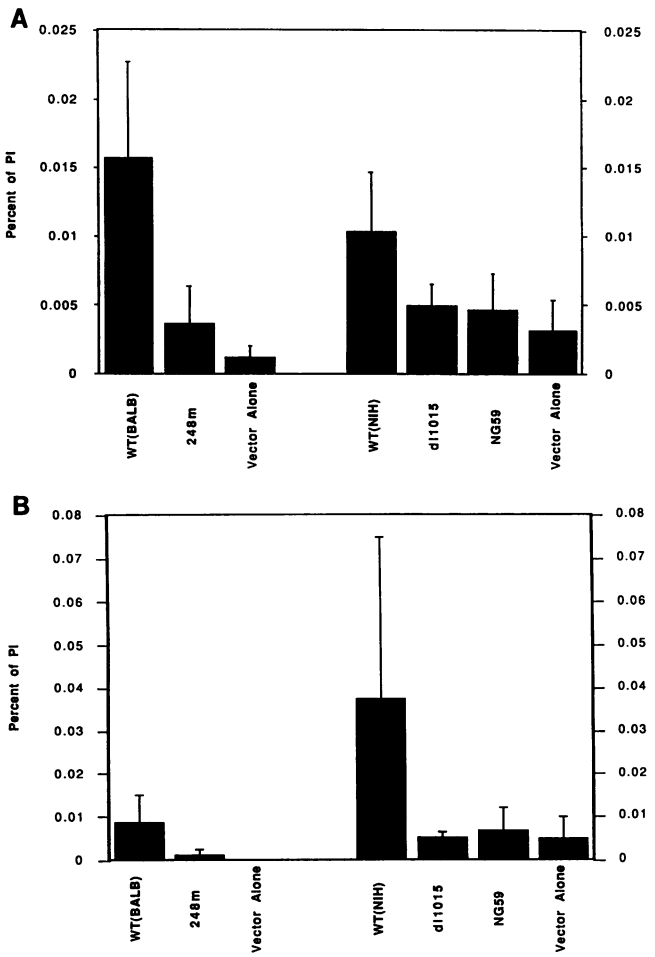

FIG. 3. Steady-state levels of ^{3}H-PI(3,4)P_2 (A) and ^{3}H-PI(3,4,5)P_3 (B) in intact cells. Cells were labeled as described in Materials and Methods, and phospholipids were extracted and analyzed by HPLC as for Fig. 2. Values for means and standard errors of the means were derived from three experiments except for the wild-type (NIH 3T3) PI(3,4)P_2 data, which were from two experiments. Values are normalized to those for PI, which constitutes approximately 85% of counts in all phosphoinositides. The average incorporation into PI was $1.71 \times 10^7 \pm 0.02 \times 10^7$ dpm.

PI(3,4,5)P_3 and PI(3,4)P_2 levels compared with wild-type MT.

DISCUSSION

The results presented here identify a biochemical defect in the MT 248m mutant and quantitatively characterize a similar defect in mutant *dl*1015. In addition, the decreased levels of ^{3}H-PI(3,4)P_2 and PI(3,4,5)P_3 in cells transfected

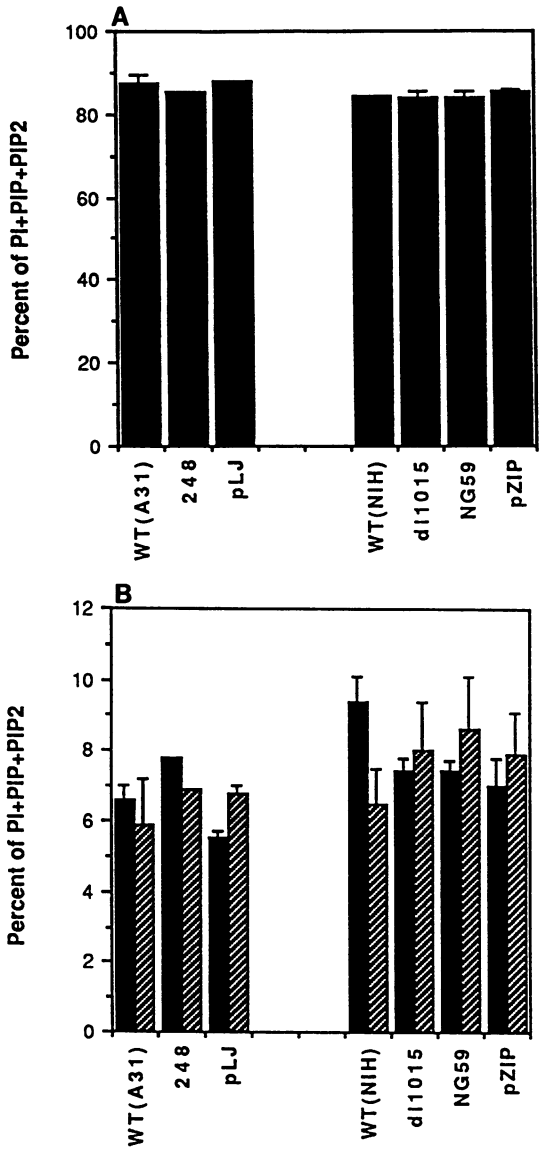

FIG. 4. Steady-state levels of ^{3}H-PI, ^{3}H-PI(4)P, and ^{3}H-PI(4,5)P_2 in intact cells. Cells were labeled as described in Materials and Methods, and phospholipids were extracted and analyzed by HPLC as for Fig. 3. Values for means and standard errors of the means were derived the same experiments as were those in Fig. 3. The average incorporation into PI plus PIP plus PIP_2 was $2.1 \times 10^7 \pm 0.8 \times 10^7$ dpm. (A) PI; (B) PI(4)P (hatched bars) and PI(4,5)P_2 (solid bars).

with 248m or *dl*1015 are consistent with the hypothesis that elevated levels of these PI 3-kinase products correlate with transformation. Previous studies of cells transformed by MT or other tyrosine kinase oncogenes as well as cells stimulated with tyrosine kinase-activating growth factors yielded results similar to those reported here (1, 29, 31, 32). In each case, cells that are transformed or growth factor stimulated exhibit significantly higher levels of [^{3}H]inositol-labeled PI(3,4)P_2 and PI(3,4,5)P_3 than do their untransformed and quiescent counterparts.

Results obtained by Ulug et al. (31) with *dl*1015 were slightly different from those reported here. The [^{3}H]inositol labeling of PI(3,4,5)P_3 in *dl*1015-expressing cells was intermediate between the levels of the vector control and wild type in those studies rather than indistinguishable from the vector-alone control as shown here. The discrepancy is most likely due to differences in cell quiescence, since in the previous case, cells were labeled for 2 days in [^{3}H]inositol-containing medium immediately after plating and were not serum starved. Thus, the cells may not have been completely quiescent. This possibility is also supported by the 10-fold-higher levels of PI(3,4,5)P_3 in the vector-alone control cells and the generally lower levels of PI(4,5)P_2 and PI(4)P measured by Ulug et al. (31). Both of these differences suggest increased PI 3-kinase activity and turnover of PI(4,5)P_2 and PI(4)P associated with mitogenesis.

Previous studies also indicate that the amount of [^{3}H] inositol incorporation into other phosphoinositides, including the PI 3-kinase product PI(3)P, does not correlate to either the transformed state or mitogenesis (1, 29, 31, 32). Although PI(3)P is a direct product of PI 3-kinase in vitro, the less abundant PI(4)P and PI(4,5)P_2 appear to be targeted by PI 3-kinase during PDGF stimulation of intact cells or in transformed cells in vivo (1, 2, 13, 17, 27, 28, 33, 35). The mechanism of this substrate specificity is not clear, but evidence for a regulator of substrate preference has been noted during the purification of PI 3-kinase (6). The utilization of phosphoinositide subpopulations during signal transduction has also been observed during phospholipase C-mediated PI turnover (18, 21, 22).

The findings reported here taken together with previous data show that in several cases, the association of PI 3-kinase with MT correlates well to an elevated level of PI 3-kinase products in cells expressing that MT variant. For example, MT mutants which display negligible (NG59) or deficient (*dl*1015) amounts of associated PI 3-kinase activity result in low levels of ^{3}H-PI(3,4,5)P_3 and PI(3,4)P_2 in vivo, similar to findings for mock-transfected control cells. Mutant 248m represents a caveat in this trend. The immunoprecipitation assay and [^{3}H]inositol incorporation data for mutant 248m clearly demonstrate that the presence of wild-type amounts of MT-associated PI 3-kinase activity do not necessarily portend wild-type levels of incorporation into the two PI 3-kinase products in vivo.

Several explanations can be given for the increased PI 3-kinase activity in 248m MT complexes but decreased PI 3-kinase products in 248m-expressing cells. The mutation may promote the activity of a PI(3,4,5)P_3 and PI(3,4)P_2 degradative activity. It is also possible that the mutation affects the metabolism of these lipids indirectly through an unidentified mechanism. Finally, the effect of the 248m mutation may be to prohibit the interaction of MT-associated PI 3-kinase with substrates in vivo. Currently, neither the degradative pathways of these lipids nor the effects of MT or other oncogenic proteins on them have been clearly defined. However, access to lipid substrates does appear to be regulated by the subcellular location of oncogene-associated PI 3-kinase. Myristylation-minus mutants of v-*abl*, *bcr-abl*, and v-*src* which are cytoplasmic rather than membrane bound still associate with PI 3-kinase but no longer cause elevated levels of PI 3-kinase products in vivo (15a, 32). Some evidence does suggest that the 248m mutation may also affect subcellular localization, since the mutation occurs at the proline residue within the sequence NPXY. The cytoplasmic NPXY motif was recently found to be necessary for internalization of the low-density lipoprotein receptor through coated pits (7), and similar motifs were found to affect internalization of a number of transmembrane proteins

(20). Experiments to determine whether MT associates with distinct membrane microenvironments and whether the NPXY domain affects such localization are currently in progress.

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