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ANTI-PHOSPHOTYROSINE IMMUNOPRECIPITATION OF PHOSPHATIDYLINOSITOL 3' KINASE ACTIVITY IN DIFFERENT CELL TYPES AFTER EXPOSURE TO EPIDERMAL GROWTH FACTOR

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In previous studies from this laboratory, it was shown that mouse epidermal growth factor (mEGF) or insulin increased the labeling of phosphaditylinositol-3,4-bisphosphate (PI-3,4-P_2) in MA-10 cells prelabeled with different radioactive precursors (Pignataro, O.P., and Ascoli, M. (1990) *J. Biol. Chem.* 265, 1718-1723 and *Mol. Endocrinol.* (1990) 4, 758-765). In order to further characterize this phenomenon we sought to determine if we could use anti-phosphotyrosine antibodies to immunoprecipitate a phosphatidylinositol (PI) kinase activity from MA-10 cells treated with mEGF or insulin. Our data indicate that this is indeed the case, and that the PI kinase precipitated is a PI-3' kinase. A second cell type, A431 cells, in which we were unable to detect an increase in PI-3,4-P_2 labeling when stimulated with mEGF or insulin, was also studied. It was found that, as in MA-10 cells, A431 cells also contain an immunoprecipitable PI-3' kinase activity that is increased in response to mEGF or insulin.

Recent studies from a number of laboratories (including our own) have shown that mammalian cells contain phosphatidylinositols phosphorylated in the D3 position of the inositol ring, and that the turnover of these rare lipids increases upon transformation or exposure of cells to growth factors or other regulatory ligands (1-13). The molecular mechanisms responsible for the increased turnover of these lipids and their role (if any) in cell transformation or activation are just beginning to be explored. It seems clear, however, that one mechanism by which increased labeling of 3-phosphorylated phosphatidylinositols can be brought about is by activation of a PI-3' kinase. This activation, in turn, appears to involve tyrosine phosphorylation of the enzyme(s) (3,6,8,9,11).

In two recent publications (4,5) we reported that mEGF and insulin increased the labeling of PI-3,4-P_2 in MA-10 Leydig tumor cells that had been prelabeled with different radioactive precursors. We were, however, unable to demonstrate the same phenomenon in other cells types such as Balb/c/3T3 or A431 cells. These findings were in agreement with a previous paper showing that neither of these ligands (mEGF or insulin) increased the amount of PI-3' kinase activity recovered in anti-phosphotyrosine immunoprecipitates from 3T3 cells (3). On the other

Abbreviations and trivial names used are: mEGF, mouse epidermal growth factor; NP-40, Nonidet P-40; TLC, thin layer chromatography; PI, phosphatidylinositol; PI-3-P, phosphatidylinositol-3-phosphate; PI-4-P, phosphatidylinositol-4-phosphate; PI-3,4-P_2, phosphatidylinositol-3,4-bisphosphate; GPI-4-P, glycerophosphoinositol-4-phosphate; GPI-3-P, glycerophosphoinositol-3-phosphate; HPLC, high performance liquid chromatography; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

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hand, very recent publications have shown that insulin treatment of CHO cells transfected with the human insulin receptor increases the amount of PI-3' kinase activity recovered in anti-phosphotyrosine immunoprecipitates (1,6). Based on these considerations, we set out to determine if mEGF or insulin treatment leads to an increase in PI-3' kinase activity in anti-phosphotyrosine immunoprecipitates obtained from a cell type where these two ligands each induce a robust increase in the labeling of PI-3,4-P_2 (i.e., MA-10 cells) and a cell type where they do not (i.e., A-431 cells). In contrast to the results obtained with the metabolic labeling experiments (4,5), we find that both cell types respond to mEGF or insulin with an activation of PI-3' kinase. While this work was in progress, other investigators (14) reported that EGF treatment of NR6 cells transfected with the human EGF receptor leads to an increase in PI-3' kinase activity in immunoprecipitates prepared with anti-EGF receptor antibodies . The ability of mEGF treatment to increase PI-3' kinase activity in anti-phosphotyrosine immunoprecipitates, however, has not been previously shown.

Experimental Procedures

Cells

The origin and handling of MA-10 cells have been described (15). A431 cells were obtained from the American Type Culture Collection and were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Preparation of Cell Extracts and Assay of PI kinase Activity

Confluent 100 mm dishes of MA-10 or A431 cells were washed with Waymouth MB752/1 without added $NaHCO_3$, but containing 20 mM Hepes and 1mg/ml albumin, pH 7.4. Mouse EGF, insulin, or diluent (0.15 M NaCl, 20 mM Hepes, 1 mg/ml albumin, pH 7.4) were added to this medium, and cells were incubated at 37 C for 10 min. Extracts were then prepared and incubated with anti-phosphotyrosine antibodies (py20) prebound to Protein G-Sepharose exactly as described by Endemann *et.al.* (1). PI kinase activity was measured directly in immunoprecipitates as described by Endemann *et.al.* (1) except that the TLC procedure used to separate the lipid products was that previously described by us (4), and the concentration of [γ-^{32}P]ATP used was 2.0 mCi/ml.

Identification of the Products of the PI Kinase

Areas of interest were scraped from the TLC plate, eluted from the silica, deacylated and analyzed by HPLC as previously described (4). The GPI-4-P standard used was prepared from [^{3}H]inositol-prelabeled MA-10 cells as described previously (4). The GPI-3-P standard was prepared by deacylation (4) of a ^{32}P-labeled PI-3-P standard kindly provided by Dr. Lewis Cantley (Tufts University).

Hormones and Supplies

Mouse EGF was purified as described by Savage and Cohen (16) and was a generous gift of Dr. Graham Carpenter (Vanderbilt University). Crystalline porcine insulin was generously provided by Lilly Research Laboratories. PI, PI-4-P, NP-40, and Protein G-Sepharose were purchased from Sigma. [2-^{3}H]inositol (14.6-15.2 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were from Du Pont-New England Nuclear. A monoclonal antibody (py20), which recognizes phosphotyrosine residues (17) was purchased from ICN. All other reagents used were of the best grade available and were obtained from commonly used suppliers.

Results

Anti-phosphotyrosine Immunoprecipitation of PI-3' kinase activity from MA-10 Cells.

Previously, we have shown that mEGF or insulin treatment of MA-10 cells increases the labeling of PI-3,4-P_2 (4,5). In order to further investigate the effects of mEGF on PI metabolism

in MA-10 cells, we wished to determine if mEGF activates a PI-3' kinase in this cell type. Thus, MA-10 cells were treated with mEGF (5 ng/ml) or insulin (1 μg/ml) for 10 min. A detergent lysate was then prepared, and phosphotyrosine-containing proteins were immunoprecipitated with the monoclonal antibody, py20, which was prebound to Protein G-Sepharose. Assay of the immunoprecipitates for PI kinase activity showed that mEGF and insulin treatment of MA-10 cells clearly increased this activity compared to control cells as can be seen by the increase in the amount of radioactivity detected in the area of the PI-4-P standard (Fig. 1, left panel) . In this experiment the product of the reaction co-migrated with a PI-4-P standard. However, in other experiments (c.f. Fig. 3) the PI-4-P standard migrated further on the TLC plate. Therefore the co-migration seen in Fig. 1 should not be interpreted to mean that the product of this kinase reaction was PI-4-P.

At the concentrations tested, mEGF and insulin elicited a similar increase in PI-3,4-P_2 labeling in MA-10 cells during a 10 min incubation (4,5). However, it should be noted that in some experiments the increase in PI kinase activity induced by insulin (1 μg/ml) was somewhat greater than that produced by mEGF (5 ng/ml). Last, it should also be noted that when the TLC plates were exposed longer, other products which migrated more slowly than the major product of the reaction were also present. These products appeared to be increased in the mEGF- or insulin-treated cells as compared to control cells.

There are two known PI kinase activities which can be distinguished by their physical properties and by the products generated when given PI as a substrate (18). A Type II or PI-4' kinase is stimulated by NP-40 and produces PI-4-P from PI. In contrast, a Type I or PI-3' kinase is inhibited by NP-40 and converts PI to PI-3-P. To help determine which kinase was immunoprecipitated we did two additional experiments. We first performed the PI kinase assay in the presence of NP-40. As shown in the right panel of Fig.1, this detergent clearly inhibited the reaction, suggesting the presence of a PI-3' kinase. Secondly, we scraped the areas of the TLC plate that contained the radioactive product that migrates near the PI-4-P standard. After elution from the silica and deacylation, the resulting glycerophosphoinositols were separated by HPLC. The results presented in Figure 2 clearly show that the product of the PI kinase reaction comigrates with GPI-3-P.

In summary, the results presented show an increased amount of PI-3' kinase activity recovered in MA-10 cells treated with mEGF or insulin and immunoprecipitated with anti-phosphotyrosine antibodies. This correlates with our previous finding that mEGF or insulin increase the labeling of PI-3,4-P_2 in this cell type (4,5).

Anti-phosphotyrosine Immunoprecipitation of PI-3' kinase activity from A431 Cells.

In contrast to the results obtained with MA-10 cells, we were unable to show increased labeling of PI-3,4-P_2 in A431 cells treated with mEGF or insulin (4,5). However, Ruderman *et. al.* have reported that insulin increased the labeling of PI-3,4-P_2 in CHO cells expressing a large number of human insulin receptors (6). In that study and one other (1) it was also shown that insulin stimulation increases the amount of PI-3' kinase activity precipitated with anti-phosphotyrosine antibodies. Thus, we decided to determine if we could immunoprecipitate PI-3' kinase activity from A431 cells treated with mEGF or insulin. As shown in Fig. 3 it is clear that mEGF or insulin

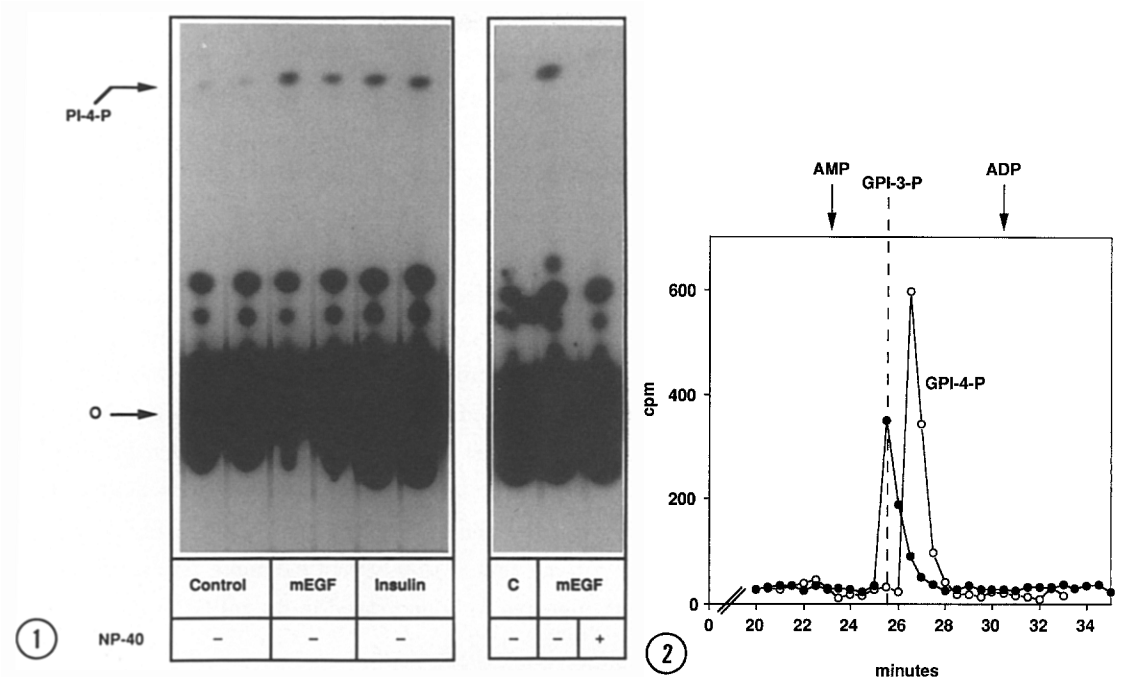

Figure 1. Anti-phosphotyrosine Immunoprecipitation of PI Kinase Activity from MA-10 Cells Treated with mEGF or Insulin and Inhibition by Nonidet P-40.

Left panel. MA-10 cells were incubated with diluent, 5 ng/ml mEGF, or 1 μg/ml insulin for 10 min as indicated. After cell lysis and immunoprecipitation with anti-phosphotyrosine antibodies, the immunoprecipitates were assayed for PI kinase activity as described in Experimental Procedures.

Right panel. MA-10 cells were incubated with or without mEGF and immunoprecipitated as described above. The PI kinase reaction was performed in the presence or absence of 0.6% NP-40 as indicated.

Both autoradiograms shown were from overnight exposures (12-15h) with two intensifying screens. The arrow indicates the position of the PI-4-P standard and "O" represents the origin. The results of a representative experiment are shown. The upper third of the TLC plate is not shown because there were no radioactive products that migrated beyond the PI-4-P standard.

Figure 2. HPLC Analysis of Deacylated PI Products Formed by Anti-phosphotyrosine Immunoprecipitates obtained from mEGF or Insulin Treated MA-10 Cells.

MA-10 cells were incubated with mEGF or insulin as described in the legend to Fig. 1. After immunoprecipitation and assay of PI kinase activity, the appropriate areas of the thin-layer plate were scraped, eluted, deacylated, and separated by HPLC as described in Experimental Procedures. The open symbols show the elution profile of [^{3}H]GPI-4-P. The closed symbols show the elution profile of the major product (c.f. arrow in Fig. 1) of the PI-kinase reaction. The product obtained from immunoprecipates prepared from mEGF- or insulin-treated cells were pooled and chromatographed. Although only a selected area of the chromatogram is shown, we estimate that 70-80% of the radioactivity applied to the column eluted in the area shown. The position of two internal standards (i.e., AMP and ADP) and of GPI-3-P is also shown.

increased the incorporation of radioactivity into several lipid products, the most prominent of which migrates just below PI-4-P. At the concentrations used in this experiment, insulin induced a larger increase in PI kinase activity. We estimate (by scintillation counting) that the radioactivity incorporated into the most prominent spot increased 5.4- compared to 3.4-fold in the insulin and mEGF-treated cells, respectively. When a higher concentration of mEGF (200 ng/ml) was used, however, the amount of PI kinase activity immunoprecipitated was approximately equal to that precipitated from cells treated with 1 μg/ml insulin (data not shown).

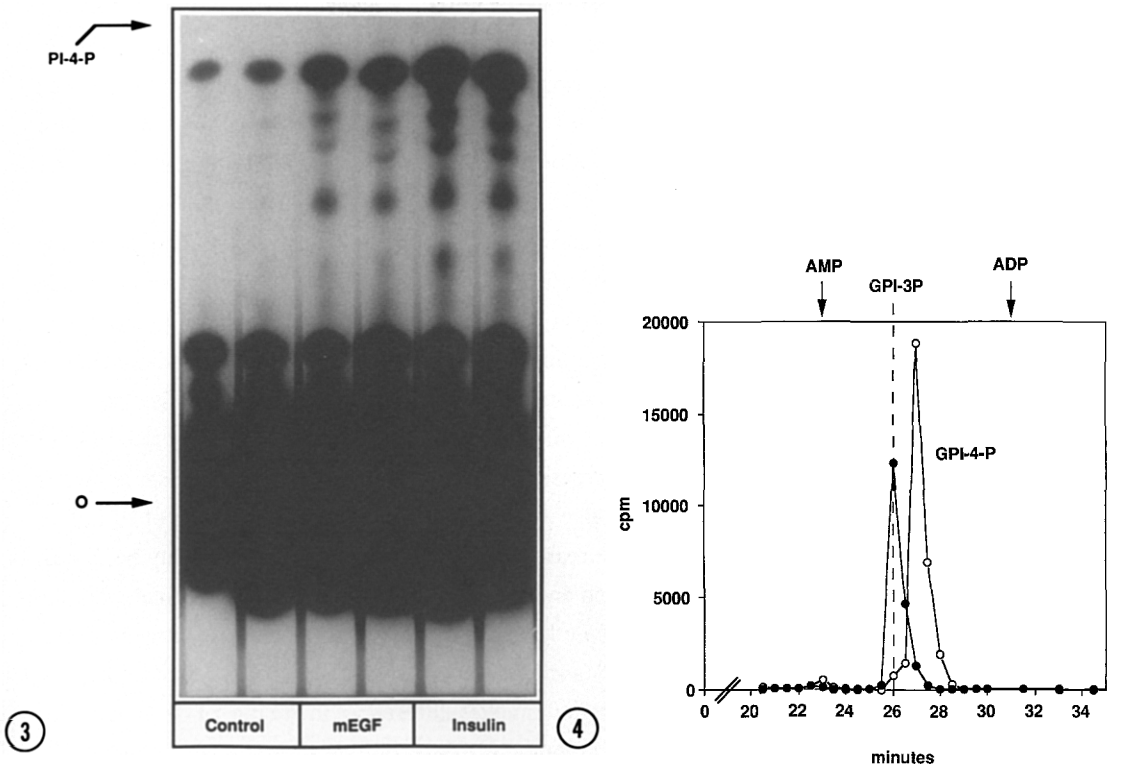

Figure 3. Anti-phosphotyrosine Immunoprecipitation of PI Kinase Activity from A431 cells treated with mEGF or insulin.

A431 cells were incubated with diluent, 5 ng/ml mEGF, or 1μg/ml insulin for 10 min. See the legend to Fig. 1 for experimental details.

Figure 4. HPLC Analysis of Deacylated PI Products Formed by Anti-phosphotyrosine Immunoprecipitates from EGF or Insulin Treated A431 Cells.
A PI kinase assay was performed in immunoprecipitates prepared from A431 cells that had been incubated with mEGF or insulin. The appropriate areas of the thin-layer plate were processed and analyzed as described in the legend to Fig. 2.

In order to further characterize the product of the PI kinase activity in A431 cells, HPLC analysis of the deacylated compound was performed as described above. The glycerophosphoinositol generated from the product of the PI kinase assay of immunoprecipitates prepared from A431 cells also co-eluted with the GPI-3-P standard (Fig. 4). We therefore conclude that in spite of our inability to detect an increase in the labeling of PI-3,4-P_2 in A431 cells incubated with mEGF or insulin (4,5) these ligands do indeed activate a PI-3' kinase in A431 cells.

Discussion

Recent studies from several laboratories have shown that activation of mammalian cells with platelet derived growth factor, colony stimulating factor, or insulin results in an increase in the amount of PI-3' kinase activity that can be recovered in immunoprecipitates prepared with anti-phosphotyrosine antibodies, or with antibodies to the appropriate ligand receptor (1,3,6,8,11,19,20). These findings have led to the belief that a primary event resulting from the

binding of these growth factors to their cognate receptors is a physical association of the PI-3' kinase with the receptor, which is then followed by increased phosphorylation (in tyrosyl residues) and activation of the PI-3' kinase(s) (21,22). It is important to note, however, that it has not been rigorously established that the PI-3' kinase(s) is a substrate for these growth factor receptor/kinases, or that tyrosine phosphorylation of the PI-3' kinase(s) is directly responsible for the activation of the enzyme.

To our knowledge, the results presented herein represent the first demonstration that incubation of intact cells with EGF leads to an increase in the amount of PI-3' kinase activity recoverable in anti-phosphotyrosine immunoprecipitates. Also, our studies show that the increased recovery of PI-3' kinase activity in anti-phosphotyrosine immunoprecipitates can be demonstrated not only in MA-10 cells, but also in A431 cells. This is an important finding because MA-10 cells respond to mEGF with increased labeling of PI-3,4-P_2, while A431 cells which are exceedingly rich in EGF receptors, do not respond to mEGF with increased labeling of PI-3,4-P_2 (4,5). The same results were obtained with insulin, a hormone that also provokes an increase in the labeling of PI-3,4-P_2 in MA-10, but not in A431 cells (4,5). The reason for this apparent discrepancy may be trivial. Thus, because of the radioactive isotopes used and their specific activities, it is very likely that the method used to measure PI-3' kinase activity in the immunoprecipitates is more sensitive than that used in the metabolic labeling experiments (see Experimental Procedures and refs 4 and 5) for experimental details). An alternative explanation invokes differences in the rate of degradation of the 3-phosphorylated phosphatidylinositols among these two cell types. It is possible that A431 cells are particularly rich in the enzyme(s) that degrades these lipids and that the high rate of degradation precludes measurements of the incorporation of [^{3}H]inositol into the 3-phosphorylated phosphatidylinositols of A431 cells. It should be emphasized, however, that in spite of the apparent discrepancy between the results obtained by the immunoprecipitation and the metabolic labeling experiments, the results presented here, together with those of Bjorge and coworkers (14), clearly show that the ability of EGF to affect the metabolism of 3-phosphorylated phosphatidylinositols is not restricted to MA-10 cells. In fact, it appears that the molecular mechanisms by which EGF activates this pathway (i.e., by stimulating PI-3' kinase activity) are very similar to those described for other growth factors (reviewed in refs 21 and 22). This is an important conclusion that is not necessarily expected from the results obtained with other systems because some of the ligands that affect the metabolism of 3-phosphorylated phosphatidylinositols do not appear to activate tyrosine kinases (7,10,12,13). Furthermore, a given protein need not be a substrate for all growth factor receptors (23,24). The observation that EGF stimulates PI-3' kinase activity in A431 cells is also important because this cell type is widely used to study the actions of EGF. Thus, the findings reported herein should be useful to many investigators in this field.

Earlier reports from other laboratories indicated that the EGF receptor associates with and/or stimulates a phosphatidylinositol kinase activity in A431 cells (25,26). It is unclear whether the enzyme(s) described in these reports is a PI-4' or a PI-3' kinase because the product of the reaction was not unambiguously identified. As assumed by the authors, it is likely that it is a PI-4' kinase because the enzyme assays were done in the presence of Triton X-100, a condition that is known to inhibit PI-3' kinase and stimulate PI-4' kinase activity (18). These results should now be

considered together with our results showing that incubation of PI and [g-^{32}P]ATP with anti-phosphotyrosine immunoprecipitates prepared from A431 cells pretreated with EGF or insulin leads not only to the formation of PI-3-P, but other products as well (Fig. 3). Since these products are more polar than PI-3-P, it is possible that EGF and insulin activate more than one phospholipid kinase, and that these products arise by further phosphorylation of PI-3-P by these other kinases.

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