T_H2-like cells. These results concern only the effector phase of the disease and do not bear on the role of T_H1-T_H2 cells in earlier events. However, they are in accord with the observation that artificial expression by islet cells of IL-10, an immunosuppressive lymphokine with documented effects on T_H1 cells, actually promotes insulitis and diabetes rather than inhibits them (*14*). Some of the major challenges remaining are to prove the role of T_H1 cells in spontaneous diabetes in rodents and humans, to understand the role of accessory cells or molecules in regulating the T_H1-T_H2 balance, and to find the least interventional means to divert T_H1 cells to a T_H2 phenotype in ongoing disease.

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24. Analysis by three-color flow cytometry was performed as described (*6*), after staining with either KT4-10 (anti-$V_\beta4$), 7D4 (anti-CD25), IM7 (anti-CD44), or MEL-14 (anti-CD62L), in conjunction with anti-CD4 and anti-CD8.
25. Cell suspensions were prepared from spleens of 3- to 5-week-old BDC2.5/Cα° mice. Red cells were lysed in 0.87% ammonium chloride. Remaining cells were washed and resuspended at 5×10^5 cells/ml in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 1× nonessential amino acids, 1 mM glutamine, penicillin and streptomycin, and 50 μM 2-mercaptoethanol, divided in half, and put into culture. T_H1-like cultures: concanavalin A (5 μg/ml), recombinant murine IL-2 (r-mulL-2, 100 U/ml), r-mulFN-γ (1000 U/ml), and mulL-4 mAb (11B11, 15 μg/ml). T_H2-like cultures: concanavalin A (5 μg/ml), r-mulL-4 (500 U/ml), and mulFN-γ mAb (AN-18.17.24 and R4-6A2, 15 μg/ml). Cells were used after culture for 4 days at 37°C and 10% CO_2.
26. For anti-$V_\beta4$ stimulation, enzyme-linked immunosorbent assay (ELISA)–grade 96-well plates were coated with 10-fold serial dilutions of affinity-purified mAb KT4-10, starting from 10 μg/ml, overnight at 4°C. After washing, 5×10^4 cultured cells were added to each well. The cultures were incubated for 48 hours at 37°C, 10% CO_2 including a 12-hour pulse with 1 μCi per well of [^{3}H]thymidine before harvesting.
27. IFN-γ ELISA was performed with paired mAb AN 18.17.24 and biotin-conjugated R4-6A2, and paired IL-4 mAbs (BVD4-1D11 and biotin-conjugated BVD6-24G2 were purchased from PharMingen). ELISAs were performed as described by the manufacturer's protocol. The concentration of lymphokines was determined by comparison with a standard curve of recombinant cytokine. IL-2 concentration was determined by bioassay with the IL-2 indicator line CTLL-2.

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Requirement for Phosphatidylinositol Transfer Protein in Epidermal Growth Factor Signaling

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Stimulation of phosphatidylinositol-4,5-bisphosphate (PIP_2) hydrolysis is a widespread mechanism for receptor-mediated signaling in eukaryotes. Cytosolic phosphatidylinositol transfer protein (PITP) is necessary for guanosine triphosphate (GTP)–dependent hydrolysis of PIP_2 by phospholipase C–β (PLC-β), but the role of PITP is unclear. Stimulation of phospholipase C–γ (PLC-γ) in A431 human epidermoid carcinoma cells treated with epidermal growth factor (EGF) required PITP. Stimulation of PI-4 kinase in cells treated with EGF also required PITP. Coprecipitation studies revealed an EGF-dependent association of PITP with the EGF receptor, with PI-4 kinase, and with PLC-γ.

The regulation of PLC activity by tyrosine kinases occurs by a signaling mechanism distinct from that of GTP-dependent pathways (*1*). Cytosolic PLC-γ associates with specific phosphotyrosine residues on activated receptor tyrosine kinases at the plasma membrane, including phosphotyrosine residues at the COOH-terminus of the human EGF receptor (*2*, *3*). Association in vitro is mediated by the Src homology 2 domains of PLC-γ and results in tyrosine phosphorylation of PLC-γ (*3*, *4*), but these events are insufficient to stimulate phosphoinositidase activity in intact cell membranes (*5*), and substrate presentation appears to be an important criterion (*6*). It is

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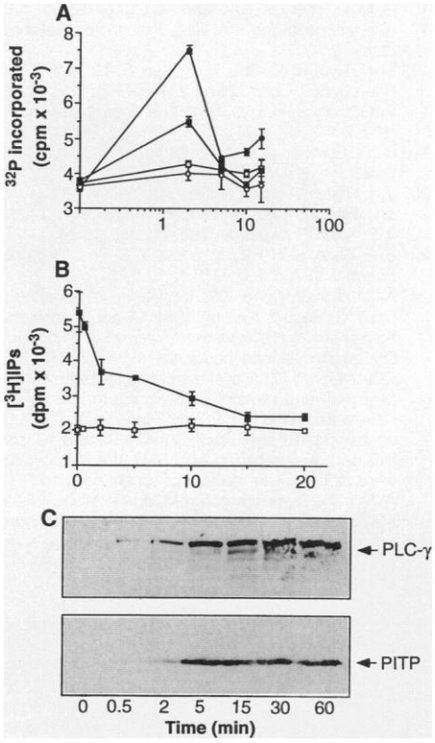

Fig. 1. Loss of EGF receptor signal transduction in A431 cells after permeabilization. (**A**) After permeabilization for 0.5 (solid circles), 5 (solid squares), 10 (open squares), and 20 (open circles) min (*24*), the time course of EGF stimulation of PI phosphorylation was measured with endogenous substrate (*25*). (**B**) After treatment of cells with SLO for the times indicated, the time courses of EGF-dependent (solid squares) and unstimulated (open squares) inositol phosphate production were measured with the use of endogenous substrate (*26*). (**C**) Time course of the effusion of PLC-γ and PITP into the extracellular medium after permeabilization was analyzed by immunoblotting (*27*).

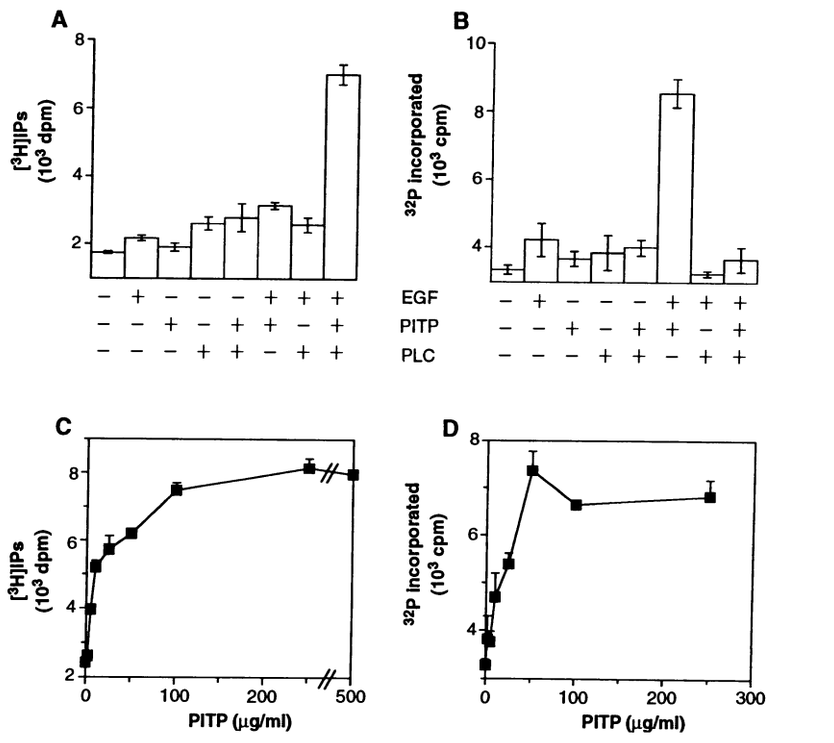

Fig. 2. Regeneration of signal transduction in permeabilized A431 cells. Restoration of the stimulation of PLC activity (**A**) and PI phosphorylation (**B**) by EGF was measured in cytosol-depleted A431 cells. A431 cells were permeabilized with SLO (0.6 IU/ml) for 20 min and incubated with or without 100 nM EGF, PITP (50 μg/ml), and PLC-γ1 (2.3 μg/ml) as indicated (*28*). Samples were incubated for 15 min (A) or 2 min (B) and analyzed for inositol phosphates or polyphosphoinositides essentially as described in Fig. 1. After permeabilization of cells and their treatment with EGF, reconstituted PLC activity (**C**) and PI phosphorylation (**D**) were measured in the presence of different concentrations of exogenous PITP. Exogenous PLC-γ1 (2.3 μg/ml) was included in PLC assays only.

not yet clear which enzymatic steps besides PIP_2 hydrolysis, if any, are regulated by tyrosine kinases. The PI-4 kinase and PI-4-phosphate-5 kinase (PIP-5 kinase) activities, which convert PI to PIP_2 (the substrate for PLC-γ), are also stimulated in cells treated with EGF (*7–10*). Because PITP increases the rate of GTP-dependent PIP_2 hydrolysis by PLC-β (*11*), and PITP cannot transport PI phosphates (*12*), it is possible that intracellular PI transport by PITP from its site of biosynthesis in the endoplasmic reticulum to the plasma membrane is also regulated. Thus PI metabolism may be compartmentalized in the cell, and phosphoinositides in the plasma membrane may not be freely available substrates for PI kinases or for PLC in intact cells (*5, 13, 14*). Such complexity can be studied in intact membranes, but would not be so evident in solubilized preparations with exogenous substrates.

To investigate whether PITP (*15*) is involved in signaling through PLC-γ, we studied the EGF-dependent generation of inositol polyphosphates. Streptolysin O (SLO) was used to permeabilize the plasma membrane of A431 human epidermoid carcinoma cells, thereby allowing cytosolic components to diffuse out of the cell. Treatment of cells with SLO caused a progressive decrease in the magnitude of the EGF-induced phosphorylation of PI, whereas the time required for maximal stimulation (approximately 2 min) did not appear to change (Fig. 1A). Depletion of cytosol by permeabilization with SLO was time-dependent; in cells stimulated with EGF for 2 min after increasing times of permeabilization, a decline in the amount of lipid hydrolysis was seen (Fig. 1B) that, although certainly not identical, occurred at a similar rate to that of the loss of PLC-γ and PITP from the cells (Fig. 1C). The rate at which PLC-γ and PITP appeared in the culture medium was consistent with their release by free diffusion (*16*).

The addition of a rat brain cytosol preparation to the culture medium of permeabilized cells restored EGF-dependent production of inositol phosphates (IPs) (*17*). We therefore investigated the effects of replacing PITP and PLC-γ individually and in combination. We measured PLC activity after treating cytosol-depleted cells with various combinations of EGF, PITP, and PLC-γ in the presence of adenosine triphosphate (ATP) (Fig. 2A). Treatment of permeabilized cells with EGF and PLC-γ gave little increase in the production of IPs. Because PLC-γ associates with the EGF receptor and is tyrosine-phosphorylated in response to EGF under these conditions (*17*), regulation of PLC-γ alone appears to be insufficient for signaling by EGF. Addition of PITP to this system restored EGF-mediated activation of PLC-γ. Therefore, signaling through inositol lipid hydrolysis by both PLC-γ and PLC-β (*16*) pathways requires PITP as an essential component.

PITP carries PI, but the preferred substrate for PLC is phosphorylated PI (PIP and PIP_2). This fact and the requirement for PITP in the reconstitution of EGF-dependent PI phosphorylation and hydrolysis indicate that the presence of PI kinases may also be necessary. The type II PI-4 kinase regulated by EGF in A431 cells is membrane-associated and would therefore not be lost from permeabilized cells (*7, 8*). Reagents for the characteriza-

tion of PIP-5 kinase activities are not available. The EGF-dependent isozyme in A431 cells has not been identified, but EGF-dependent PIP-5 kinase activity has been shown to be present in a plasma membrane fraction prepared from A431 cells (*8*). We measured the dependence of endogenous PI-4 kinase activity on exogenously added EGF, PITP, and PLC-γ (Fig. 2B). Although each individual component was ineffective, the addition of EGF and PITP synergistically increased the amount of phosphorylated PI (PIP and PIP_2). When purified PLC-γ was also added, this stimulation was barely detected, presumably because PIP and PIP_2 generated by PI-4 kinase and PIP-5 kinase activity were hydrolyzed by the phospholipase (*18*). PITP alone was unable to increase the amount of phosphorylated PI. Therefore, PI-4 kinase activity appears to be regulated by EGF (*7–10*), and this regulation requires the presence of PITP.

A widely accepted role for PITP is to freely exchange its bound PI and phosphatidylcholine (PCh) with PI or PCh in different subcellular membranes (*15, 19*). However the dose response of PLC-γ activity to PITP is similar to that of PI phosphorylation (Fig. 2, C and D), which is consistent with another model in which PITP, PI-4 kinase, and PLC-γ are complementary parts of an integral EGF receptor signaling complex (*20*). This latter model also explains, at least in part, the reported compartmentation of PI metabolism into agonist-sensitive and -insensitive pools (*5, 14*). Although reagents are not yet available to fully test this model, we investigated whether PITP could be coprecipitated with relevant signaling molecules. In unstimulated cells, PITP was only weakly detected in immunoprecipitates prepared with antibodies to the EGF receptor, PLC-γ or PI-4 kinase (Fig. 3), as compared with the amount detected in immunoprecipitates from cells treated with EGF for 5 min. Longer treatment of cells with EGF (up to 30 min) caused no further increase in association of these proteins (*17*). Similar experiments showed that both PITP and the EGF receptor could be coprecipitated with antibodies to PLC-γ after EGF stimulation of intact A431 cells (*17*). EGF treatment of A431 cells also causes a rapid recruitment of PI-4 kinase to activated EGF receptors (*10*).

Our results demonstrate that PITP associates with the EGF receptor in an agonist-dependent manner. The requirement for PITP for both activation of PLC-β mediated by heterotrimeric GTP-binding proteins and activation of PLC-γ mediated by tyrosine kinases indicates that PITP may function at a common point in these

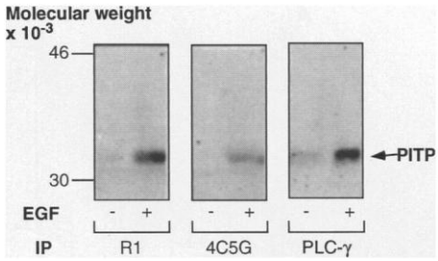

Fig. 3. EGF-dependent formation of intermolecular complexes with PITP. The association of PITP with EGF-dependent signaling enzymes was studied in serum-starved, intact A431 cells. PITP was detected by immunoblotting after immunoprecipitation (*29*) of cellular proteins with antibodies to the EGF receptor (EGF-R1) (*30*), to PLC-γ, or to PI-4 kinase (4C5G) (*31*).

two signaling pathways. Because PITP restored PI phosphorylation, PITP may present PI directly to PI-4 kinase (*20*). Thus, interaction of PITP with PI-4 kinase may be a general phenomenon and may also occur in the process of calcium-activated noradrenalin secretion in PC12 rat pheochromocytoma cells, for which PITP is also required (*21*).

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24. A431 cells grown to 50% confluence in Dulbecco's modified Eagle's medium (DMEM) containing fetal bovine serum (10%) were transferred to DMEM [or DMEM minus inositol, containing [^{3}H]inositol (3 μCi/ml) for PLC assays] supplemented with insulin (5 μg/ml) and transferrin (5 μg/ml) for 24 hours. Cells were rinsed with 20 mM Pipes buffer (pH 6.8) containing 137 mM NaCl, 3 mM KCl, and 10 mM LiCl and permeabilized with SLO [0.6 IU/ml; units were defined with the use of an antibody to SLO (*22*)] in the presence of 2 mM MgATP at pCa 7 for the indicated times at 37°C, during which there was no cell loss. Permeabilized cells were washed with Pipes buffer at 4°C and treated with 100 nM EGF at 37°C in the presence of 2 mM MgATP, 10 mM LiCl, and 2 mM $MgCl_2$ at pCa 6.
25. For PI phosphorylation assays, cells were washed with Pipes buffer and incubated for 5 min at 37°C in PI-4 kinase assay buffer [10 mM $MgCl_2$, 25 mM tris-HCl (pH 7.5), and 70 μM ATP) containing [γ-^{32}P]ATP (10 μCi/ml). Reactions were quenched with an equal volume of assay buffer containing Triton X-100 (3%). Methanol (300 μl) and 1N HCl (200 μl) were added, and the mixture was transferred to tubes containing chloroform (300 μl). The lower phase was washed with 1N HCl (400 μl). There was no increase in the amount of PI 3- or 3,4-bisphosphate in A431 cells after treatment with EGF (*8*).
26. For PLC assays, cells were treated with EGF for 15 min. Reactions were quenched with ice-cold 0.9% NaCl (1 ml), and the supernatants were analyzed for IPs as described (*16*).
27. For immunoblot analyses, A431 cells were permeabilized, the supernatants were recovered, and proteins in them were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). PLC-γ and PITP were identified by immunoblotting with antibodies to PLC-γ and PITP (*11*).
28. PITP was cloned from rat brain first-strand complementary DNA by polymerase chain reaction and transferred to pTZ19R (Pharmacia) for inducible expression of the native protein in BL21(DE3) cells (Novagen) (*11*). After cell lysis, recombinant PITP was purified from the soluble fraction by sequential chromatography on Hi-Trap heparin (Pharmacia) and gel filtration media (*16*). PLC-γ was prepared from bovine brain (*23*). Insufficient amounts of PLC-γ could be prepared to determine the concentration at which saturation occured.
29. Intact A431 cells deprived of serum were treated with or without 100 nM EGF for 5 min and lysed, and the indicated proteins were immunoprecipitated. The washed immunoprecipitates were resolved by SDS-PAGE, electroblotted onto a polyvinylidene difluoride membrane, and incubated with antibody to PITP.
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