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Phosphorylation of the PDGF receptor β subunit creates a tight binding site for phosphatidylinositol 3 kinase

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The β subunit of the platelet derived growth factor receptor (PDGFR) coprecipitates with a phosphatidylinositol 3 kinase activity (PI3K) following stimulation of cells by PDGF. Mutagenesis of a tyrosine (Y) phosphorylation site, Y751, in the PDGFR, greatly reduces PI3K, consistent with the possibility that phosphorylation of Y751 signals association of PI3K. To test this we have reconstituted the binding of the PDGFR β subunit and PI3K *in vitro*. Binding is rapid, saturable and requires phosphorylation of the PDGFR at Y751, but does not require PDGF-dependent phosphorylation of PI3K. To test which portions of the PDGFR are important for binding, we used an antibody to a small region of the receptor that includes Y751. This antibody blocked *in vitro* binding of PI3K to the receptor, while an antiserum to the C-terminus of the receptor had no effect on binding of PI3K. In addition, we found that PDGF stimulation of a cell results in the association of essentially all the PI3K activity with cellular PDGFRs. These data suggest that PI3K is a specific ligand for PDGF receptors that are phosphorylated at Y751.

Key words: PDGF receptor/phosphatidylinositol 3 kinase/phosphorylation/tyrosine kinase

Introduction

The cell surface receptors for platelet derived growth factor (PDGF), fibroblast growth factor, epidermal growth factor (EGF), insulin, insulin-like growth factor I, and macrophage colony stimulating factor (M-CSF) are transmembrane proteins possessing an intracellular tyrosine kinase domain (Ullrich and Schlessinger, 1990). In the case of the PDGF receptor (PDGFR), there are two distinct polypeptide subunits, α and β, both of which have tyrosine kinase activity. Considerable effort has been focused on elucidating the pathways by which these growth factor receptors relay a biological signal within a target cell. For all receptors tested, the intrinsic tyrosine kinase activity is required for signal transduction since kinase-negative mutant receptors are inactive (Yarden and Ullrich, 1988; Williams, 1989). Thus it seems likely that tyrosine phosphorylation of substrate proteins by the receptor kinase activity alerts the cell that a growth factor has bound. Specific substrates have been identified by their increase in phosphotyrosine content following stimulation with growth factor. They include the receptor itself, which becomes tyrosine phosphorylated at one or more tyrosine residues in response to growth factor binding (Yarden and Ullrich, 1988), the structural proteins calpactin and ezrein (Brugge, 1986; Gould *et al.*, 1989), phospholipase C γ (PLC γ) (Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989), Raf (a serine/threonine kinase) (Morrison *et al.*, 1988, 1989) and the GTPase activating protein (GAP) of Ras (Molloy *et al.*, 1989; Ellis *et al.*, 1990).

Recently, certain growth factor receptors have been reported to have a second ligand-stimulated activity, namely the binding of specific intracellular proteins. For the PDGFR these proteins include PLC γ (Kumjian *et al.*, 1989; Morrison *et al.*, 1990), Raf (Morrison *et al.*, 1989), GAP (Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990), a phosphatidylinositol 3 kinase (PI3K) activity (Kaplan *et al.*, 1987; Coughlin *et al.*, 1989; Kazlauskas and Cooper, 1989), cytoplasmic tyrosine kinases of the Src family (Courtneidge *et al.*, personal communication), and a series of polypeptides of known molecular sizes but unknown functions (Kazlauskas and Cooper, 1989). The identities of the bound proteins, as well as the requirement of kinase activity for binding, suggest that the bound proteins may be involved in signal transduction.

Currently, it seems most likely that there is not a single PDGFR complex, but a variety of complexes containing one or more different bound proteins, because specific receptor mutations can decrease the binding of some, but not other, bound proteins (Kaplan *et al.*, 1990; Kazlauskas and Cooper, 1989; Kazlauskas *et al.*, 1990). Also, not all activated receptor kinases bind the same set of proteins. PI3K activity binds to the receptors for M-CSF (Varticovski *et al.*, 1989) as well as PDGF. The activated insulin receptor apparently does not associate with a PI3K, but insulin stimulation of cells increases the amount of PI3K that can be immunoprecipitated with antiphosphotyrosine antibodies (Endemann *et al.*, 1990). PLC γ binds to EGFRs (Margolis *et al.*, 1989) as well as PDGFRs (Morrison *et al.*, 1990), but may not bind to M-CSFRs (Downing *et al.*, 1989).

PI3K activity has also been found complexed with a variety of intracellular, non-receptor types of tyrosine kinases, including c-Src associated with middle T antigen (mT) and transforming Src proteins (Whitman *et al.*, 1985; Courtneidge and Heber, 1987; Fukui and Hanafusa, 1989). It was found that all transforming Src mutants were complexed with an active PI3K, however, activating PI3K appears insufficient to drive cell proliferation, since some non-transforming Src mutants also associated with an active PI3K (Fukui and Hanafusa, 1989).

The specific protein that is responsible for PI3K activity has not been identified, but there is a correlation between activity and an 81-85 kd polypeptide (Courtneidge and Heber, 1987; Kaplan *et al.*, 1987). Unlike the major PI kinases in the cell, that phosphorylate the D4 and D5 positions on the inositol ring, the tyrosine kinase-associated PI kinase phosphorylates the D3 position of the insitol ring of PI, PI(4)P and PI(4,5)P_2 (Whitman *et al.*, 1988). The PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3 products cannot be

degraded by any of the known PLCs, and may play a special role in cell regulation (Lips *et al.*, 1989; Serunian *et al.*, 1989). Study of the regulation of PI3K activity as well as the roles of its products in growth regulation may provide insights into the mechanisms of signal transduction.

Previous studies have investigated the recruitment of signaling proteins by activated PDGFRs, using PDGFR β subunit mutants. Kinase-inactive mutants do not bind GAP, PLC γ, Raf, PI3K activity or polypeptides of 120, 84 and 72 kd (Kazlauskas and Cooper, 1989; Morrison *et al.*, 1989; Williams, 1989; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990; Morrison *et al.*, 1990). This raises the question of whether tyrosine phosphorylation of the PDGFR, the bound protein, or of a third protein is required for binding. Binding of GAP to the PDGFR is greatly reduced by mutation of either of the two known tyrosine (Y) phosphorylation sites in the PDGFR, Y751 and Y857. In contrast, binding of PI3K activity and of 120, 84 and 72 kd proteins is ablated by replacing the Y751 with either phenylalanine or glycine, but not by replacing Y857 with phenylalanine. Y751 lies in a region known as the kinase insert. A large deletion in the kinase insert (Everinsson *et al.*, 1990) reduces PDGFR kinase activity, but a smaller deletion (Escobedo and Williams, 1988) has little effect. The smaller deletion blocks binding of PI3K activity (Williams, 1989). These observations are consistent with the possibility that phosphorylation of Y751 triggers binding of PI3K to the receptor. However, even point mutations may cause unforeseen and largely untestable changes in protein conformation warranting caution in the interpretation of such experiments. Indeed, in the case of the mouse PDGFR β subunit, deletion of the kinase insert or mutation of the Y857 homolog prevents the induction by PDGF of an antigenic site near the C-terminus (Bishayee *et al.*, 1988; Keating *et al.*, 1988; Williams, 1989).

To test directly whether phosphorylation of the PDGFR β subunit acts as the signal for PI3K binding, we have reconstituted the binding of PI3K activity to the PDGFR *in vitro*, and shown that phosphorylation of receptors is necessary for binding of PI3K. PDGF-induced phosphorylation of the PI3K is not required. In addition, phosphorylation of receptors which lack Y751 does not allow PI3K to bind. Furthermore, blocking the Y51 region of the PDGFR with an antipeptide antiserum prevented the binding of PI3K activity as well as the 120, 110, 84 and 72 kd proteins. These data suggest that phosphorylated Y751 and the surrounding region constitutes or regulates the binding site for PI3K. Thus the failure of kinase-inactive PDGF receptors to signal could be due to the lack of receptor autophosphorylation rather than (or as well as) the inability to phosphorylate other proteins.

Results

In vivo and in vitro binding of PI3K activity

We have shown that the β subunit of the human PDGFR, expressed in dog epithelial (TRMP) cells, has readily detectable associated PI3K activity when immunopurified from PDGF-stimulated cells, while very little PI3K was associated with the PDGFR from resting cells (Kazlauskas and Cooper, 1989; Figure 1, lanes 1 and 2). To determine whether changes in the PDGFR or in PI3K trigger the association of these two molecules in a PDGF-stimulated

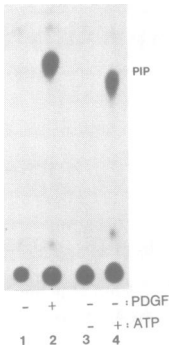

Fig. 1. Binding of PI3K *in vitro* and *in vivo*. Confluent quiescent cultures of dog epithelial cells (TRMP) expressing 10^5 human PDGFRs/cell were exposed (+) to 40 ng/ml BB-PDGF, or left unstimulated (−), and the PDGFR was immunoprecipitated as described in Materials and methods (6×10^5 cells in each case). Lanes 1 and 2: association of PI3K activity with the PDGFR *in vivo*. An autoradiogram of the thin layer chromatogram is shown. PIP marks the position of an unlabeled PI(4)P standard visualized by I_2 staining. Lanes 3 and 4: association of PI3K activity with the PDGFR *in vitro*. Receptor immunoprecipitates from unstimulated cells were incubated with ATP (+) or with buffer (−), washed and exposed to lysate from an equivalent number of TRMP cells which do not express PDGFRs. Associated PI3K was assayed as described in Materials and methods. The unequal chromatographic separation of the radiolabeled species in lanes 2 and 4 resulted from the solvent front running unevenly and was not reproducible in other experiments. The radioactivity in the PI3P spot is 702 c.p.m. (lane 2) and 466 c.p.m. (lane 4).

cell, we reconstituted the association of these two proteins *in vitro*. Human PDGFRs were immunoprecipitated with a monoclonal antibody to an extracellular epitope from unstimulated TRMP cells, incubated in the presence or absence of non-radioactive ATP, washed and exposed to lysates of TRMP cells not expressing PDGFRs. After 1 h, non-associated proteins were removed by washing, and receptor associated PI3K activity was determined. PI3K activity bound to the PDGFR *in vitro*, but only if the receptor had been incubated with ATP before exposure to the cell lysate (Figure 1, lanes 3 and 4). The amount of PI3K activity that bound *in vitro* was at least half as much as that bound to the same number of receptors *in vivo*. Given that PI3K activity from unstimulated cells bound to PDGFRs under conditions where the PDGFR kinase is inactive [in the presence of 5 mM EDTA (not shown)], it seems probable that PI3K molecules were not phosphorylated directly or indirectly by the PDGFR immunoprecipitates during the binding reaction. Thus changes in the receptor appear to be sufficient to promote the binding of an otherwise unmodified PI3K activity.

PI3K from lysates of resting human foreskin fibroblasts (AG1523) or Swiss 3T3 cells could also bind to PDGFR immunoprecipitates *in vitro*. Unless stated otherwise, all subsequent experiments were done with Swiss 3T3 cell lysates. This cell type was chosen because it is a good source of PI3K, it responds to PDGF, and because the human-specific anti-PDGFR monoclonal antibody used for the *in vitro* binding studies does not recognize mouse PDGFRs.

Requirement for ATP treatment of PDGFR

The dependence of PI3K binding on pre-incubation of the PDGFR immunoprecipitate with ATP most likely reflects

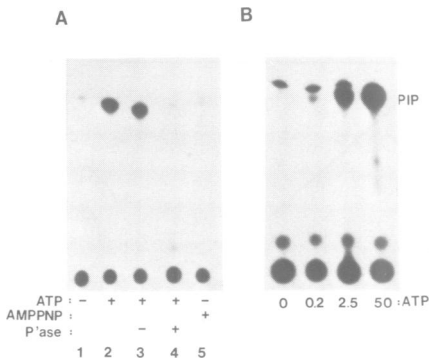

Fig. 2. *In vitro* binding of PI3K requires receptor phosphorylation. (**A**) PDGFR immunoprecipitates were prepared from unstimulated cells, then incubated with buffer (lane 1), 10 μM ATP (lanes 2−4) or 10 μM β-γ imido ATP (lane 5), for 10 min at 30°C. Following the phosphorylation step, immunoprecipitates were washed and some samples were exposed to phosphatase buffer (lane 3) or phosphatase buffer with phosphatase (P'ase) (lane 4), then used in an *in vitro* binding assay using Swiss 3T3 cell lysates as described in Figure 1. (**B**) Receptor immunoprecipitates from resting cells were incubated with the indicated concentration of ATP, washed and exposed to equivalent quantities of 3T3 cell lysates. Receptor-associated PI3K activity was assayed.

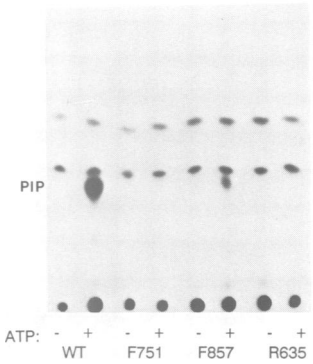

Fig. 3. *In vitro* binding of PI3K to PDGFR mutants. The PDGFR was immunoprecipitated from confluent quiescent cultures of TRMP cells expressing ~10^5 introduced mutant PDGFR β subunits/cell, incubated with 10 μM ATP (+) or buffer (−), then exposed to lysates of Swiss 3T3 cells. Associated PI3K activity was determined. Similar immunoprecipitates were phosphorylated with [γ-^{32}P]ATP and the relative amount of receptor autophosphorylation was quantitated. WT, wild-type; F751, F857, receptors in which phenylalanine replaces Y751 and Y857, respectively; R635, receptors in which arginine replaces lysine at position 635.

a need for phosphorylation. (Tyrosine phosphorylation in PDGFR immunoprecipitates does not require added PDGF). To test whether transfer of the γ phosphate of ATP is required, we used a non-hydrolyzable ATP analog. No binding of PI3K was detected (Figure 2A, lane 5). Furthermore, when ATP-treated PDGFRs were exposed to phosphatases prior to adding cell lysate, no PI3K activity bound (Figure 2A, lanes 3 and 4). Therefore, it appears that the signal for PI3K binding is phosphorylation of a protein in the immunoprecipitate.

The extent of receptor phosphorylation influenced the amount of PI3K bound. Incubation of the receptor immunoprecipitate in the presence of 10−50 μM ATP resulted in the phosphorylation of up to 30% of the receptors, whereas in the presence of 0.2 μM ATP only 1−2% of the receptors were phosphorylated (calculated from ATP specific activity and receptor number, data not shown). Using receptors phosphorylated to different extents we found that the more highly phosphorylated receptors bound more PI3K (Figure 2B).

Phosphorylating the PDGFR *in vitro* incorporates phosphate primarily at Y751 and very little at the major *in vivo* site (Y857) (Kazlauskas and Cooper, 1989). PDGFRs behave in this way when immunoprecipitated with two different antibodies (antiphosphotyrosine, or a monoclonal to the extracellular domain), when the immunoprecipitates are prepared from resting or activated cells, and whether or not PDGF is added to the phosphorylation reaction. Also, PDGFRs in intact or detergent-solubilized membranes, not bound to antibody, are phosphorylated primarily at Y751 (data not shown). Therefore we tested whether receptor phosphorylation at Y751 was required for PI3K binding using receptor mutants (Figure 3).

As expected, a kinase negative (R635) receptor failed to bind PI3K even if it was pre-incubated with ATP prior to binding. Receptors with phenylalanine in place of Y751 (F751) have kinase activity, and autophosphorylate to a low level at numerous undefined sites (unpublished results). However, F751 receptors failed to bind PI3K *in vitro* (Figure 3). PDGFRs with F857 bound PI3K, although at only 10% of the level of wild-type receptors (Figure 3). With immunoprecipitates prepared under these conditions, F857 PDGFRs have reduced *in vitro* kinase activity and the extent of autophosphorylation is only 13% of wild-type. Thus the reduction of PI3K binding to F857 PDGFRs is consistent with reduced phosphorylation of Y751.

In vitro binding of specific polypeptides

We have previously reported that in addition to PI3K activity and GAP (124 kd), proteins of 120, 84 and 72 kd coprecipitate with the human PDGFR from PDGF-treated TRMP cells (Kazlauskas and Cooper, 1989; Kazlauskas *et al.*, 1990). These associated proteins become radiolabeled when receptor immunoprecipitates are incubated with [γ-^{32}P]ATP (Figure 4, lanes 5 and 6). PDGF induces association of a similar complement of proteins with human PDGFRs expressed in NIH 3T3 (PA317) cells, but a 73 kd protein is detected instead of the 84 and 72 kd species (Figure 4, lanes 1 and 2). A 150 and several 64 kd proteins were also detected in these experiments. Attempts to detect associated proteins directly, by biosynthetic labeling, have been problematic. PDGFR immunoprecipitates of [^{35}S]-methionine labeled cells contain a number of labeled proteins which are detected irrespective of whether the cells were treated with PDGF before lysis. The proteins that are labeled specifically with [γ-^{32}P]ATP must be of very low abundance.

To test whether the proteins that associate in a living cell also do so *in vitro*, we incubated the receptor immunoprecipitate with [γ-^{32}P]ATP following the *in vitro* binding reaction. Proteins corresponding to those bound *in vivo*, with the exception of the 124 and 64 kd bands, were detected. Thus when TRMP cell lysates were used, 150, 120, 110, 84 and 72 kd species were detected (Figure 4, lanes 7 and 8), and with Swiss 3T3 cells lysates, 120, 110 and a strongly labeled 73 kd protein were detected (Figure 4, lanes 3 and 4). Using lysates of [^{35}S]methionine labeled Swiss 3T3 cells, 110 and 73 kd proteins were found to bind specific-

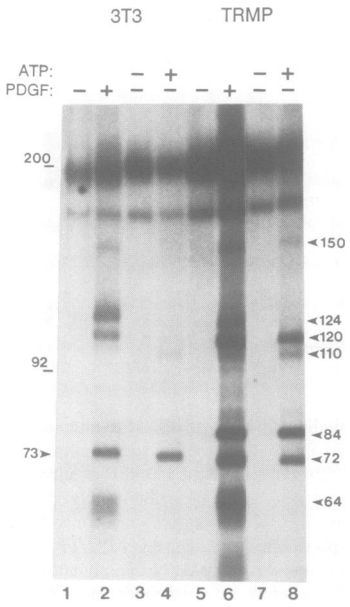

Fig. 4. Comparison of proteins that associate with the PDGFR *in vitro* and *in vivo*. The PDGFR was immunoprecipitated from resting (−) or PDGF-activated (40 ng/ml) (+) cells expressing the human PDGFR. Either NIH 3T3 (PA317) cells (lanes 1−4) or TRMP cells (lanes 5−8) were used. To form receptor complexes *in vitro*, receptor immunoprecipitates from resting cells were incubated with non-radioactive ATP (+) or buffer (−) and exposed to lysates of Swiss 3T3 (lanes 3 and 4) or TRMP cells (lanes 7 and 8). To radiolabel the receptors and associated proteins, samples were incubated with [γ-^{32}P]-ATP, resolved on a 7.5% acrylamide, 0.193% bisacrylamide SDS gel, the gel was exposed to 1 M KOH, and the radiolabeled proteins were visualized by autoradiography. The arrowheads indicate the proteins which consistently bind *in vivo*. Bars on the left indicate mol. wt markers.

ally to phosphorylated PDGFRs (see below, Figure 7B, lanes 5 and 6). The 110 kd polypeptide labeled more strongly with [^{35}S]methionine than with [γ^{32}P]ATP. Presumably it is a poorer substrate for the PDGFR kinase than the 73 kd protein.

PI3K as a ligand for the phosphorylated PDGFR

To examine the kinetics of binding, a PDGFR immunoprecipitate was incubated with excess Swiss 3T3 cell lysate for various times at 4°C, then the receptor immunoprecipitates were washed and the associated PI3K activity determined. We found that PI3K bound with pseudo first order kinetics, with a half time of ~15 min (Figure 5A). In a living cell, PI3K binding is maximal by 2−5 min after PDGF stimulation. The slower binding *in vitro* may be attributed to the lower temperature or to the dilution of the cell contents upon lysis.

Once bound, the complex appears to be very stable. This was shown by following the fate of bound 110 and 73 kd proteins, detected by [^{35}S]methionine and [γ-^{32}P]ATP labeling. When the receptor with bound proteins was washed, then incubated for 15 min in NANPD buffer or solutions containing ionic detergents (RIPA buffer), 2 M NaCl, 1 M urea or 0.2% SDS, no dissociation was detected (data not shown). More strongly dissociating conditions disrupted the antibody−PDGFR complex, so the effects on the PDGFR-associated protein complex could not be determined. These incubations were performed with 6×10^{10} receptors in 250 μl, from which we estimate that

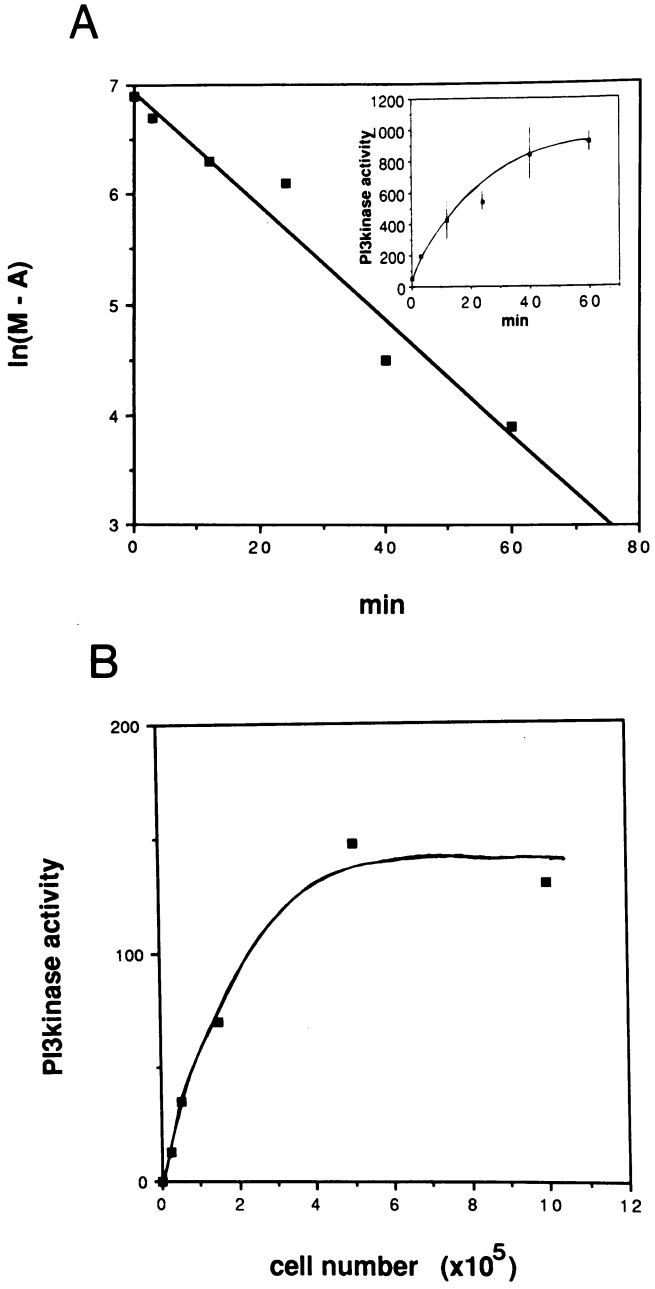

Fig. 5. The *in vitro* binding of PI3K to the PDGFR is rapid and saturable. (**A**) PDGFR immunoprecipitates were prepared from resting cells (4×10^5 cells), incubated with 10 μM ATP and then exposed to excess Swiss 3T3 cell lysate (1×10^5 cells) for the indicated length of time, after which the immunoprecipitate was washed and the associated PI3K assayed. The data were fitted to the equation for a first order reaction, $\ln(M-A) = kT$, where M is the maximal binding (1000 c.p.m.) and A is the binding at time T. The inset plots the PI3K activity [c.p.m. in PI(3)P] bound at time *T*. Similar results were obtained in two other independent experiments. (**B**) 1.0 ml of NANPD buffer containing lysate from the indicated number of Swiss 3T3 cells was exposed to a fixed quantity of PDGFR (6×10^{10} receptors) which had been pre-incubated with ATP. Associated PI3K is expressed as the radioactivity (c.p.m.) incorporated into PI(3)P. Similar results were obtained in two other independent experiments.

the K_d of the complex is $<10^{-10}$ M. Interestingly, incubation of the complex under *in vitro* phosphorylation conditions did not lead to loss of the associated [^{35}S]-methionine labeled 110 or 73 kd proteins or of PI3K activity,

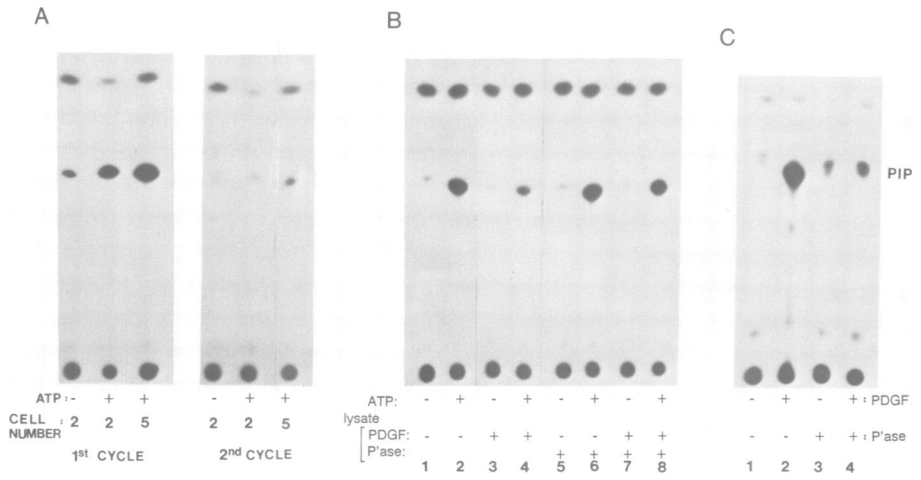

Fig. 6. The majority of cellular PI3K activity binds to PDGFRs following exposure to PDGF. (**A**) PDGFR immunoprecipitates were isolated from resting cells, incubated with either 10 μM ATP (+) or buffer alone (−), then exposed to lysate from the indicated number of Swiss 3T3 cells ($\times 10^5$) (1st CYCLE). Receptor immunoprecipitates were removed by centrifugation, the same cell lysate was then exposed to a fresh set of PDGFR immunoprecipitates (2nd CYCLE), and then the receptor-associated PI3K activity was determined. (**B**) PDGFRs prepared from unstimulated cells were exposed to buffer (−) or 10 μM ATP (+), then incubated with a saturating amount of Swiss 3T3 cell lysate prepared from resting (−) or PDGF-activated cells (+). In some cases (lanes 5−8), the cell lysate was phosphatase treated (P'ase) prior to incubation with PDGFR immunoprecipitates. (**C**) Resting (−) or PDGF-activated cells (+) were lysed, exposed to phosphatases (lanes 3 and 4) or left untreated (lanes 1 and 2), the PDGFR was immunoprecipitated and the associated PI3K activity determined.

suggesting that phosphorylation does not signal the dissolution of the complex *in vitro*.

To determine whether the binding of PI3K to the PDGFR was saturable, PDGFRs from 6×10^5 TRMP cells were incubated in a final volume of 1.0 ml containing increasing amounts of cell lysate prepared from TRMP cells lacking PDGFRs. As the concentration of cell lysate was increased, PI3K binding increased until saturation was reached (Figure 5B). Under these conditions, lysate from 18×10^5 cells was required for saturation, suggesting an excess of receptors relative to PI3K molecules. Assuming that each phosphorylated PDGFR binds one molecule of PI3K, that the antibody precipitation is quantitative, and that 30% of PDGFRs are phosphorylated *in vitro* at Y751 (data not shown), there may be one-ninth as many PI3K molecules as PDGFRs in TRMP cells. A similar titration with lysate from Swiss 3T3 cells revealed a 12-fold higher content of P13K activity normalized to cell number. Swiss cells have $\sim 10^5$ endogenous mouse PDGFRs per cell, so in these cells there may be approximately equal numbers of PDGFRs and PI3K molecules.

To ascertain whether the *in vitro* binding reaction proceeds to completion, we tested whether a lysate could be depleted of all its PI3K activity by incubating with excess PDGFRs. At subsaturating levels of Swiss 3T3 cell lysate no additional PI3K activity would bind to fresh PDGFR after the initial incubation with PDGFRs (Figure 6A). This suggested that the *in vitro* binding reaction can be used to measure the amount of free PI3K activity in a cell lysate. Direct measurement of PI3K activity in cell lysates is not possible because the PI4 kinase activity obscures PI3K activity.

Given that PI3K binding *in vitro* is quantitative, and that the quantities of PDGFRs and PI3K molecules per cell are similar, we suspected that PDGF stimulation might lead essentially all the cellular PI3K to become bound to PDGFRs *in vivo*. In this case, lysate from PDGF-activated cells would be a poor source of PI3K activity to bind to PDGFRs *in vitro*. To test this possibility we compared lysates of control or PDGF-treated Swiss 3T3 cells as sources of PI3K activity for binding to ATP-treated PDGFRs. Only 15% as much PI3K activity was bound from lysates of PDGF-stimulated cells relative to control cells (Figure 6B, compare lanes 2 and 4), while lysates of EGF-stimulated cells are as good a source of PI3K as unstimulated cells (data not shown). In parallel with the PI3K activity, the amounts of 110 and 73 kd ^{35}S-labeled proteins that bound specifically to the PDGFR *in vitro* were also dramatically reduced from lysates of PDGF-activated cells (data not shown).

Since phosphorylation of Y751 appears to be the signal for association of PI3K with the PDGFR, would dephosphorylating the receptors liberate PI3K from lysates of PDGF-activated cells? We found that phosphatase effectively (>90%) removed phosphate from Y751, and when lysates of PDGF-stimulated cells were phosphatase treated prior to immunoprecipitation, the endogenous PDGFRs no longer had an associated PI3K activity (Figure 6C, compare lanes 2 and 4). Furthermore, phosphatase-treated lysates of PDGF activated cells released PI3K activity that could subsequently bind the PDGFR *in vitro* (Figure 6B, lanes 4 and 8). These data suggest that the majority of a cell's PI3K activity binds to the PDGFR upon PDGF stimulation *in vivo*, and confirms that PI3K phosphorylation is not required for binding.

Antibody to Y751 region blocks binding of PI3K and other proteins

To address which region of the PDGFR may be involved in the binding of PI3K we used two polyclonal antisera to mask different regions of the PDGFR, and then assayed the binding of PI3K *in vitro*. The 18.5 kd C-terminal tail of the PDGFR was expressed in bacteria as a glutathione-S-transferase fusion protein, and was used to immunize rabbits. The resulting antiserum precipitates PDGFR kinase activity, and receptors from PDGF-activated cells have an associated PI3K activity (data not shown). Binding this antiserum to the prephosphorylated PDGFR did not affect the *in vitro* binding of either PI3K activity (Figure 7A, lane 3) or of the

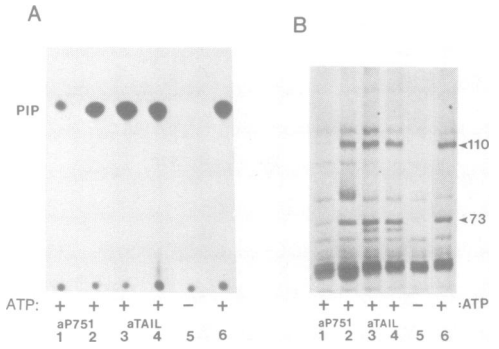

Fig. 7. An antibody to the Y751 region blocks *in vitro* binding of PI3K activity. PDGFR immunoprecipitates prepared from resting cells were incubated with excess non-immune rabbit serum to block all available antibody binding sites of the *S.aureus*, washed, incubated with ATP (+) or buffer (−), then exposed to an antipeptide antiserum to the 13 amino acid peptide containing Y751 (lanes 1 and 2) or an antiserum recognizing the C-terminal tail of the PDGFR (lanes 3 and 4). In some cases the antibody was blocked with immunizing antigen (lanes 2 and 4). The samples in lanes 5 and 6 were not treated with any antiserum. These receptor immunoprecipitates were then exposed to lysates from [^{35}S]methionine labeled Swiss 3T3 cells, and washed. Either the associated PI3K activity was determined (**A**), or (**B**) the proteins were resolved on a 7.5% acrylamide, 0.193% bisacrylamide SDS gel and the associated proteins visualized by fluorography. The arrowheads to the right of panel B point to a 110 and 73 kd protein.

120, 110, 84 or 72 kd proteins detected by labeling with [^{35}S]methionine (Figure 7B, lane 3) or [γ^{32}P]ATP (data not shown). Antiserum raised against the 13 residue tryptic peptide which includes Y751 (Kazlauskas and Cooper, 1989) also immunoprecipitated a kinase-active PDGFR, but receptors from PDGF-treated cells did not have any of the associated proteins or a detectable associated P13K activity (data not shown). Importantly, this antiserum was able to immunoprecipitate receptors whether or not they had phosphate at Y751 (data not shown). When prephosphorylated receptors were masked with the peptide antibody to the Y751 region, then the binding of P13K activity (Figure 7A, lane 1) and of the 120, 110, 84 and 72 kd proteins detected by labeling with [^{35}S]methionine (Figure 7B, lane 1) or [γ^{32}P]ATP (data not shown) was consistently reduced by at least 80% (Figure 7A, lanes 1 and 6). Preimmune serum or immune serum blocked with the Y751 peptide did not interfere with the ability of receptor to bind P13K activity (Figure 7A, lane 2 and data not shown). This result confirms the importance of the region including Y751 for binding of P13K.

Discussion

Our previous studies, examining the binding of proteins to mutant human PDGFR β subunits *in vivo*, demonstrated that PDGFR kinase activity and phosphorylation sites are needed for binding. Maximal GAP binding was observed only to receptors with tyrosine at both positions 751 and 857, while Y751 but not Y857 was required for the binding of PI3K activity and 120, 84 and 72 kd proteins (Kazlauskas and Cooper, 1989; Kazlauskas *et al.*, 1990). Kinase negative receptors failed to bind any proteins following PDGF addition, suggesting that the phosphorylation of the receptor or a cell protein was the signal for complex formation. The *in vitro* assay has allowed us to test this theory directly.

Binding of PI3K activity to PDGFRs *in vitro* appears to be high affinity, specific and saturable; hallmarks of a ligand−receptor interaction. In the *in vitro* system, phosphorylation of Y751 appears to signal the association of PI3K, and masking of the Y751 region with an antipeptide antibody blocks PI3K binding. Furthermore, PDGF-induced phosphorylation of the PI3K is not required for binding, since PI3K from unstimulated cells or from phosphatase-treated lysates binds to PDGFRs under conditions that do not allow *in vitro* phosphorylation. Also, *N*-ethylmaleimide treatment of phosphorylated PDGFRs inhibits their kinase activity but does not block subsequent binding of PI3K activity (data not shown). Since these modified receptors are unable to phosphorylate bound proteins, P13K phosphorylation may not be required for activity. Binding of activity correlates with the association of 4 specific polypeptides. However, we do not know which of these proteins may be responsible for P13K activity. It is formally possible that an inactive P13K is constitutively bound to the PDGFR, and Y751 phosporylation triggers the binding of a subunit required for activity.

Because PI3K can bind to PDGFRs *in vitro*, it is possible that the binding observed upon PDGF stimulation of intact cells actually occurs upon cell lysis. However, the stability and specificity of complex formation indicate that it probably occurs in the cell. Also, the observation of similar complexes with different receptor tyrosine kinases is an argument for biological relevance.

We have observed seven polypeptides which associate specifically with human PDGFRs in PDGF-treated TRMP cells, and are detected when immunoprecipitates are incubated with [γ-^{32}P]ATP (Figure 4). When prephosphorylated receptors are allowed to bind proteins *in vitro*, all of the proteins which bind in a living cell, with the exception of 124 kd GAP and ~64 kd proteins, were subsequently detected by the *in vitro* kinase assay (Figure 4). The requirements for *in vitro* binding of GAP and the 64 kd proteins may include phosphorylation of other sites in the receptor. Previous mutagenesis studies had suggested that binding of GAP might require phosphorylation of both Y751 and Y857 in the receptor (Kazlauskas *et al.*, 1990), and phosphorylation of Y857 occurs inefficiently in our *in vitro* system (Kazlauskas and Cooper, 1989). Using murine 3T3 instead of canine TRMP cells gives slightly different results. The canine 84 and 72 kd polypeptides are replaced by a 73 kd murine polypeptide, sometimes resolved as a doublet. We do not know whether these are actually different proteins or murine and canine homologs. Expression of the human PDGFR in TRMP cells at 10^5 receptors/cell enables these cells to synthesize DNA in response to PDGF stimulation, and F751 mutant receptors display a diminished biological response (unpublished results), suggesting that complex formation is biologically significant.

The use of antibodies recognizing different regions of the PDGFR have revealed that a region within the kinase insert that includes phosphorylated Y751 participates in the binding of all the proteins that can bind to the PDGFR *in vitro* including the PI3K activity. Antibody specific for residues 746−758 of the PDGFR, but not to the C-terminal or extracellular domains, blocked binding of PI3K. Interestingly, antibodies to the mouse PDGFR β subunit, recognizing the region homologous to residues 770−792 of the human

PDGFR, can immunoprecipitate a mouse PDGFR−PI3K complex from PDGF-treated cells (Coughlin *et al.*, 1989). This suggests that only the part of the kinase insert close to Y751 may be important. Whether phosphate at Y751 is itself enough of a binding site, or this modification of Y751 induces a conformational change resulting in the unveiling of a PI3K binding site elsewhere, remains to be determined. PDGF binding does induce a conformational change within the region immediately carboxy terminal to the end of the kinase domain (Bishayee *et al.*, 1988; Keating *et al.*, 1988), and a kinase-insert deletion or an autophosphorylation site mutant fail to execute this conformational change, suggesting a requirement for receptor autophosphorylation (Williams, 1989). Recently, J.Escobedo, D.Kaplan and L.T.Williams have found that a phosphorylated 20 amino acid peptide including Y751 can compete for binding of PI3K to the PDGFR (personal communication). Since this peptide can compete for binding, the sequence including phosphorylated Y751 may interact directly with PI3K.

Talmage *et al.* (1989) reported that the phosphorylation of Y315 of mT is required for the binding of a PI3K to the c-*src*−mT complex. The sequence surrounding Y315 of mT shares limited homology to the sequence surrounding Y751 of the PDGFR. Very recently, Cohen *et al.* (1990) have found that ^{32}P-labeled SDS-denatured mT can bind to an SDS-denatured 85 kd polypeptide on Western blots. This polypeptide is probably a component of PI3K and may correspond to our 73 kd protein. Binding of mT in this assay requires that it be phosphorylated, and a F315 mutant does not bind. The ability of SDS-denatured proteins to interact suggests a strong interaction between small epitopes.

The question arises of how protein tyrosine kinases that lack regions of homology with Y751 of the PDGFR are able to bind PI3K activity. In the case of the M-CSFR, binding of PI3K activity requires M-CSF stimulation, which causes phosphorylation of two tyrosines in the kinase insert region as well as the Y857 homolog (Tapley *et al.*, 1990; Van der Geer and Hunter, 1990). However, there is no apparent sequence similarity between the phosphorylated residues in the M-CSFR and Y751 of the PDGFR. Perhaps there exists a family of PI3Ks, and each member binds to a limited number of the various activated protein tyrosine kinases. Alternatively, there may be a single PI3K which requires a phosphorylated Y751 homology region for binding, provided by an undiscovered cell protein that binds to the activated tyrosine kinase. Finally, a single PI3K may have alternate binding sites for recognition of different tyrosine kinases.

Assuming that there is a single form of PI3K in a cell, then our data indicate that the vast majority binds to the PDGFR following activation by PDGF. Binding may play some role in activating PI3K. The ability of PDGF to stimulate the synthesis of PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3 in cells expressing mutant PDGFRs which do not bind PI3K may shed light on this question.

Studies with the EGFR have shown that the PLC γ which associates with the EGFR lacks phosphotyrosine (Margolis *et al.*, 1990). Tyrosine phosphorylation of PLC γ may signal its dissociation from the EGFR. In contrast, our studies suggest that phosphorylation of the 73 kd protein, which is a putative component of the PI3K activity does not detectably affect its affinity for the PDGFR. In a living cell, the activated, tyrosine phosphorylated PDGFRs are subject to dephosphorylation by endogenous phosphatases which may dissociate the receptor−PI3K complex. In addition, within 15−30 min the receptor is degraded. The fate of receptor associated PI3K remains unknown−it could either be liberated by phosphatase action, be carried into the cell on the surface of endosomes, or may be proteolyzed as a complex with the receptor. Changes in the localization of PI3K could be important for modulating its activity, or for the production of PIPs with phosphate at the D3 position in a specific subcellular compartment.

Materials and methods

Cells

Swiss 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum (FCS). Human PDGFR β subunits were expressed at $\sim 10^5$/cell in mass populations of PA317 cells (an NIH 3T3 cell line for packaging amphotropic retrovirus; Miller *et al.*, 1986) or dog kidney epithelial cells (TRMP; Kazlauskas *et al.*, 1990). Cells were maintained in DME, supplemented with 10% FCS and 0.25 mg/ml G418. 12−24 h prior to either immunoprecipitation or the preparation of cell lysates, confluent cell cultures were switched to medium containing 0.1% calf serum (CS).

Antisera

PR7212 is a monoclonal antibody specific for the human PDGFR β subunit (Hart *et al.*, 1987). A synthetic peptide containing Y751 (DESVDYVPMLDMK) was coupled to bovine serum albumin (BSA) with glutaraldehyde (Cooper and King, 1986), and the resulting antigen was used to immunize rabbits. A second rabbit was immunized with a bacterially expressed fusion of *Schistosoma japonicum* glutathione-S-transferase (Smith and Johnson, 1988) to residues 939−1108 of the human PDGFR. The immune sera of both of these rabbits, but neither the pre-immune nor immune blocked with the appropriate antigen, were able to immunoprecipitate kinase active PDGFR from living cells (unpublished data). Antiserum to the Y751 peptide was also able to recognize the PDGFR when Y751 was phosphorylated, as evidenced by the ability to immunoprecipitate receptors phosphorylated *in vitro* (unpublished data).

In vitro binding assay

Dishes (5 cm) of cells were either exposed for 5 min at 37°C to 40 ng/ml pure yeast recombinant BB type PDGF, or left unstimulated. The cultures were then washed twice wth ice-cold H/S and lysed in 1.0 ml of EB (Kazlauskas and Cooper, 1988). The insoluble debris was cleared by centrifugation at 15 000 *g* for 30 min at 4°C, and 1.6 μg of PR7212 was added to the cleared supernatant. After a 1.5 h incubation on ice, 3 μg of rabbit antiserum to mouse IgG was added. 15 min later 30 μl of a 10% suspension of formalin-fixed *Staphylococcus aureus* was added and the incubation was continued for 30 min at 0°C. Cell lysates were spun through a 600 μl cushion of EB containing 10% sucrose, then washed twice with 1.0 ml of EB, and twice with 1.0 ml PAN (0.1 M NaCl, 10 mM PIPES pH 7.0, 20 μg/ml aprotinin). The immunoprecipitate was resuspended in 50 μl PAN and frozen at −70°C.

To phosphorylate the PDGFR, 17 μl of immunoprecipitate (one-third of a 5 cm dish or 6×10^5 TRMP cells) was mixed with 8 μl of 60 mM PIPES, 30 mM $MnCl_2$, 60 μg/ml aprotinin and ATP at 0, 30 or 150 μM. After incubating at 30°C for 10 min, the immunoprecipitates were washed twice with PAN. The PDGFR was radiolabeled under the same conditions by incubating with 5 μCi [γ-^{32}P]ATP, 3000 Ci/mmol.

Lysates for binding of PI3K *in vitro*, were prepared from confluent, serum-starved cultures of either Swiss 3T3 cells (5×10^5 cells per 5 cm dish) or TRMP cells lacking PDGFRs (2×10^6 cells per 5 cm dish). To label cell proteins with [^{35}S]methionine, confluent quiescent cultures were incubated overnight in modified Eagle's medium lacking methionine, supplemented with 0.1% CS and 50 μCi/ml Tran-^{35}S-label (ICN Biomedicals Inc.). Cultures were either left unstimulated, or exposed to 40 ng/ml PDGF or 50 ng/ml EGF for 5 min at 37°C, then were washed twice with H/S, and lysed in 1.0 ml NANPD (150 mM NaCl, 10 mM PIPES pH 7.0, 0.1% NP-40, 20 μg/ml aprotinin, 3 mM DTT, 2 mM Na_3VO_4, 1 mM PMSF). The insoluble debris was removed by centrifugation at 15 000 *g* for 30 min at 4°C, and the cleared supernatant was used in the *in vitro* binding assay.

To bind PI3K to the PDGFR, ATP-treated receptor immunoprecipitates [from 6×10^5 TRMP cells (6×10^{10} receptors)] were resuspended in

25 μl PAN, then exposed to 100 μl of lysate from ~5 × 10^4 Swiss 3T3 or 6 × 10^5 TRMP cells. After 1 h at 4°C, during which time the tubes were mixed end-over-end, the immunoprecipitate was washed twice with RIPA (Kazlauskas *et al.*, 1988), twice with PAN containing 0.5% NP-40, twice with PAN and resuspended in 25 μl PAN. 5 μl of the sample was assayed for associated PI3K activity as previously described (Kazlauskas and Cooper, 1989). Note that the assay was performed in substrate excess, since <1% of the substrate was routinely converted to product.

Phosphatase treatment

Receptor immunoprecipitates were phosphorylated with 10 μM ATP, washed, then resuspended in 10 μl phosphatase buffer (PAN containing 1 mM $MgCl_2$, 20 μM leupeptin, 20 μg/ml aprotinin) or phosphatase buffer supplemented with 0.08 units of bacterial alkaline phosphatase (BAP) (Sigma) and 0.1 units of potato acid phosphatase (Sigma) and incubated for 15 min at 30°C. The receptor immunoprecipitates were washed once with PAN, once with PAN containing 2 mM Na_3VO_4, 1 mM *p*-nitrophenylphosphate (PNPP) and then used in the *in vitro* binding assay without any other manipulations.

To phosphatase treat cell lysates, cells were lysed as usual except NANPD lacking Na_3VO_4 was used, $MgCl_2$ was added to 1 mM final, 0.3 units/ml of BAP and 3 units/ml of potato acid phosphatase were added and the lysate was incubated at 30°C for 30 min. Control lysates were incubated without added phosphatases. The phosphatases were inhibited by adding Na_3VO_4 and PNPP to 2 mM and 2 mM, respectively. Phosphatase-treated and control lysates were incubated wtih PDGFR immunoprecipitates in the *in vitro* binding assay as usual except that the concentrations of Na_3VO_4 and PNPP were boosted to 4 mM and 2 mM, respectively, half way through the *in vitro* binding reaction.

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References

Bishayee,S., Majumdar,S., Scher,C.D. and Khan,S. (1988) *Mol. Cell. Biol.*, **8**, 3696–3702.

Brugge,J.S. (1986) *Cell*, **46**, 149–150.

Cohen,B., Liu,Y., Druker,B. Roberts,T.M. and Schaffhausen,B.S. (1990) *Mol. Cell. Biol.*, **10**, 2909–2915.

Cooper,J.A. and King,C.S. (1986) *Mol. Cell. Biol.*, **6**, 4467–4477.

Coughlin,S.R., Escobedo,J.A. and Williams,L.T. (1989) *Science*, **243**, 1191–1194.

Courtneidge,S.A. and Heber,A. (1987) *Cell*, **50**, 1031–1037.

Downing,J.R., Margolis,B.L., Zilberstein,A., Ashmum,R.A., Ullrich,A., Sherr,C.J. and Schlessinger,J. (1989) *EMBO J.*, **8**, 3345–3350.

Ellis,C., Moran,M., McCormick,F. and Pawson,T. (1990) *Nature*, **343**, 377–381.

Endemann,G., Yonezowa,K. and Roth,R.A. (1990) *J. Biol. Chem.*, **265**, 396–400.

Escobedo,J.A. and Williams,L.T. (1988) *Nature*, **335**, 85–87.

Fukui,Y. and Hanafusa,H. (1989) *Mol. Cell. Biol.*, **9**, 1651–1658.

Gould,K.L., Bretscher,A., Esch,F.S. and Hunter,T. (1989) *EMBO J.*, **8**, 4133–4112.

Hart,C.E., Seifert,R.A., Ross,R. and Bowen-Pope,D.F. (1987) *J. Biol. Chem.*, **262**, 10780–10785.

Kaplan,D.R., Whitman,M., Schaffhausen,B., Pallas,D.C., White,M., Cantley,L. and Roberts,T.M. (1987) *Cell*, **50**, 1021–1029.

Kaplan,D.R., Morrison,D.K., Wong,G., McCormick,F. and Williams,L.T. (1990) *Cell*, **61**, 125–133.

Kazlauskas,A. and Cooper,J.A. (1988) *J. Cell Biol.*, **106**, 1395–1402.

Kazlauskas,A. and Cooper,J.A. (1989) *Cell*, **58**, 1121–1133.

Kazlauskas,A., Bowen-Pope,D., Seifert,R., Hart,C.E. and Cooper,J.A. (1988) *EMBO J.*, **7**, 3727–3735.

Kazlauskas,A., Ellis,C., Pawson,T. and Cooper,J.A. (1990) *Science*, **247**, 1578–1581.

Keating,M.T., Escobedo,J.A. and Williams,L.T. (1988) *J. Biol. Chem.*, **263**, 12805–12808.

Kumjian,D.A. Wahl,M.I., Rhee,S.G. and Daniel,T.O. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 8232–8236.

Lips,D.L., Majerus,P.W., Gorga,F.R., Young,A.T. and Benjamin,T.L. (1989) *J. Biol. Chem.*, **264**, 8759–8763.

Margolis,B., Rhee,S.G., Felder,S., Mervic,M., Lyall,R. Levitzki,A., Ullrich,A., Zilberstein,A. and Schlessinger,J. (1989) *Cell*, **57**, 1101–1107.

Margolis,B., Bellot,F., Honegger,A.M., Ullrich,A., Schlessinger,J. and Zilberstein,A. (1990) *Mol. Cell. Biol.*, **10**, 435–441.

Meisenhelder,J,. Suh,P.-G., Rhee,S.G. and Hunter,T. (1989) *Cell*, *57*, 1109–1122.

Miller,D.A., Trauber,D.R. and Buttimore,C. (1986) *Somat. Cell Mol. Genet.*, **12**, 175–183.

Molloy,C.J., Bottaro,D.P., Fleming,T.P., Marshall,M.S., Gibbs,J.B. and Aaronson,S.A. (1989) *Nature*, **342**, 711–714.

Morrison,D.K., Kaplan,D.R., Rapp,U. and Roberts,T.M. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 8855–8859.

Morrison,D.K., Kaplan,D.R., Escobedo,J.A., Rapp,U.R., Roberts,T.M. and Williams,L.T. (1989) *Cell*, **58**, 649–657.

Morrison,D.K., Kaplan,D.R., Rhee,S.G. and Williams,L.T. (1990) *Mol. Cell. Biol.*, **10**, 2359–2366.

Serunian,L.A. Haber,M.T., Fukui,T., Kim,J.W., Rhee,S.G., Lowenstein,J.M. and Cantley,L. (1989) *J. Biol. Chem.*, **264**, 17809–17815.

Severinsson,L., Ek,B., Mellstrm,K., Claesson-Welsh,L. and Heldin, C.-H. (1990) *Mol. Cell. Biol.*, **10**, 801–809.

Smith,D.B. and Johnson,K.S. (1988) *Gene*, **67**, 31–40.

Talmage,D.A., Freund,R., Young,A.T., Dahl,J., Dawe,C.J. and Benjamin,T.L. (1989) *Cell*, **59**, 55–65.

Tapley,P., Kazlauskas,A., Cooper,J.A. and Rohrschneider,L.R. (1990) *Mol. Cell. Biol.*, **10**, 2528–2538.

Ullrich,A. and Schlessinger,J. (1990) *Cell*, **61**, 203–212.

Van Der Geer,P. and Hunter,T. (1990) *Mol. Cell. Biol.*, **10**, 2991–3002.

Varticovski,L., Druker,B., Morrison,D., Cantley,L. and Roberts,T. (1989) *Nature*, **342**, 699–702.

Wahl,M.I., Olashaw,N.E., Nishibe,S., Rhee,S.G., Pledger,W.J. and Carpenter,G. (1989) *Mol. Cell. Biol.*, **9**, 2934–2943.

Whitman,M., Kaplan,D.R., Schaffhausen,B., Cantley,L. and Roberts,T.M. (1985) *Nature*, **315**, 239–242.

Whitman,M., Downes,C.P., Keeler,M., Keller,T. and Cantley,L. (1988) *Nature*, **332**, 644–646.

Williams,L.T. (1989) *Science*, **243**, 1564–1570.

Yardin,Y. and Ullrich,A. (1988) *Annu. Rev. Biochem.*, **57**, 443–478.

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