BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **251,** 148–152 (1998)
ARTICLE NO. RC989444

Stimulation of Mitogenesis by a Cell-Permeable PI 3-Kinase Binding Peptide

Daniele Derossi, Emma J. Williams, Paula J. Green, Dara J. Dunican, and Patrick Doherty[1]
Department of Experimental Pathology, King's College London GKT Medical School, Guy's Hospital Campus, London, SE1 9RT, United Kingdom

Received August 31, 1998

The binding of small phosphopeptides to the SH2 domains of the p85 regulatory subunit of PI 3-kinase can activate the enzyme *in vitro.* In the present study a cell-permeable peptide that binds specifically to the SH2 domains of p85 has been evaluated for its ability to stimulate a mitogenic response in the C2 muscle cell line. This peptide, in contrast to four other SH2-binding peptides, was as effective as serum, EGF, and FGF at stimulating entry into S-phase. The response to the p85 binding peptide, but not FGF, was inhibited by wortmannin and rapamycin, indicating that the peptide activates the PI 3-kinase/S6 kinase signalling pathway. The peptide response was not inhibited by the MEK inhibitor (PD098059) and did not stimulate Erk phosphorylation. Thus, there would appear to be no direct cross-talk between the pathway activated by the p85 binding peptide and the p42/p44 MAPK cascade.

Key Words: **PI 3-kinase; cell-permeable peptides; cell proliferation; FGF.**

By phosphorylating the D3 position of phosphatidylinositol, phosphatidylinositol-3-OH kinase (PI 3-kinase) stimulates the production of lipid derived second messengers that regulate many aspects of cell function including cell survival and proliferation (1). Several species of PI 3-kinase have been identified, and these include a family of heterodimeric enzymes consisting of a 110-kDa catalytic subunit and an 85-kDa regulatory subunit (2). This family has been implicated in the transduction of both the anti -apoptotic and cell proliferation responses that growth factors can deliver to cells (1–4). In many instances the enzyme can be directly activated by binding to defined motifs, containing phosphorylated tyrosine, that are found in activated growth factor receptors or adaptor molecules that bind to activated growth factor receptors. In this context the two SH2 domains of the p85 subunit of PI 3-kinase can bind specifically to phosphorylated YXXM motifs present in the cytoplasmic domain of, for example, the activated platelet derived growth factor (PDGF) receptor, the colony stimulating factor receptor and the insulin receptor and insulin receptor substrate 1 (5–7). Small phosphopeptide mimetics of these binding sites have been shown to effectively activate PI 3-kinase to a level comparable to that attained by stimulation with a growth factor providing that they bind with high affinity to the p85 SH2 domains (8–10). However, there are other ways in which PI 3-kinase can be activated including the direct binding of Ras to the p110 catalytic subunit of some PI 3-kinases (11), and the activation of γ forms of the enzyme (which do not interact with p85) by G proteins (12). Activation by the above effectors might have very distinct effects on cell function by virtue of the kinetics of enzyme activation, as well as compartmentalisation of the activated enzyme in cells. Additional complicating factors are likely to include the possibility that certain feedback pathways might impinge on one method of activation but not another. For example, PtdIns (3, 4, 5)P3 can bind to the SH2 domain of p85 and might thereby directly compete for the binding of PI 3-kinase to tyrosine phosphorylated proteins (1), however, this mechanism would not directly affect the activation of PI 3-kinases by Ras or G-proteins.

There are a number of ways to study the direct consequences of PI 3-kinase activity on cell function that range from the expression of constitutively active forms of the catalytic subunit of the enzyme (13), to the introduction of small phosphopeptides into cells that can activate the enzyme by binding to the SH2 do-

[1] To whom correspondence should be addressed. Fax: 0171 403 8883. E-mail: p.doherty@umds.ac.uk.

Abbreviations used: Ahx, amino hexanoic acid; BrdU, 5-Bromo-2-deoxyuridine/5-fluro-2-deoxyuridine; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; EGF(R), epidermal growth factor (receptor); FCS, foetal calf serum; FGF(R), fibroblast growth factor (receptor); Grb2, growth factor receptor binding protein 2; GST, glutathione-S-transferase; MAPK, mitogen activated protein kinase; PDGF(R), platelet derived growth factor (receptor); PLCγ, phospholipase C gamma; RU, resonance units.

0006-291X/98 $25.00

mains of p85 (10). These complementary techniques both have advantages and limitations. For example, the constitutively active catalytic domain of the enzyme might better reflect on pathological rather than physiological processes as there is no scope for regulation via the p85 subunit, and such versions of the enzyme can be oncogenic (14). The peptide approach has the advantage that it will activate physiological levels of the endogenous enzyme via the p85 regulatory subunit in a manner that shares some features with activation by receptor tyrosine kinases, however, one obvious limitation with this method is that phosphopeptides will not freely enter into cells.

A 16-amino-acid peptide derived from the third helix of the Antennapedia protein will freely enter into the cell cytosol from the extracellular compartment, and moreover this sequence can act as an internalisation vector for other peptide sequences (see Ref. 15 for a detailed description of the internalisation properties of Antennapedia-related peptides). We have demonstrated that this vector can be used to internalise small tyrosine phosphorylated peptides into cells and that these peptides retain the ability to specifically bind to the SH2 domains of molecules such as Grb2 and PLCγ within the cell (16, 17). To date, we have used these peptides as specific antagonists of biological responses such as axonal growth and cell proliferation (16–18). In the present study we have investigated the use of a cell permeable p85 binding peptide as an agonist for the stimulation of cell growth.

EXPERIMENTAL PROCEDURES

Peptide Synthesis

Peptides were synthesised on a 431A Applied Biosystem peptide synthesiser using p-hydroxymethylphenoxymethyl polystyrene resin and standard Fmoc chemistry as previously described (16, 17). All peptides were made as linear sequences of the following configuration: Biot–Ahx–Antennapedia sequence–phosphopeptide.

The amino hexanoic acid (Ahx) functions as a spacer and the biotin (Novabiochem) at the N-terminus allows for immunoprecipitation and capture to Biosensor chips (17). In general the Antennapedia internalisation sequence was RQIKIWFQNRRMKWKK, with the exception of the EGFR^{1068}Y-P peptide where a functional analogue of this sequence was used (see Ref. 17 for details). The following phosphopeptides, derived from natural sequences present in the PDGF, EGF and FGF receptors, were synthesised in tandem with the Antennapedia sequence.

Peptide name	Sequence of peptide	SH2 target
PDGFR 740Y-P	SDGGY(P)MDMS	p85
EGFR 1068Y-P	PVPEY(P)INQS	Grb2
FGFR 463Y-P	GVSEY(P)ELPE	Not known
FGFR 730Y-P	TNELY(P)MMMR	Not known
FGFR 766Y-P	SNQEY(P)LDLS	PLCγ

The established SH2 targets for the phosphopeptides is given (again for details see Refs. 6, 16, 17). Phosphorylated Fmoc tyrosines (Novabiochem) were used in the sequences.

After synthesis and deprotection the peptides were de-salted on a Sephadex G-10 column and lyophilised. The peptides were analysed for purity by analytical high-pressure liquid chromatography, and in each case a single peak was observed. Peptides were generally made as stock solutions at 25 mg/ml in dimethyl sulphoxide (DMSO) and stored at −20°C before use.

Biosensor Measurements

All surface plasmon resonance experiments were carried out as previously described (17) using a BIAcore X Biosensor (Pharmacia Ltd., BIAcore, Uppsala, Sweden), the principle of which has been described elsewhere (19). Parallel flow cells of CM 5 sensor chips were derivatised, using the amine coupling method, with streptavidin (200 μg/ml) in 10 mM Sodium Acetate, pH 4.0, according to the manufacturer's protocol. Approximately 2100–2600 resonance units (RU) of ligand were immobilized, corresponding to a concentration of 2.1–2.6 ng/mm^2. Biotinylated peptides were coupled via streptavidin to one flow cell only (the other remaining as a control) by injection at 1 μg/ml in running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% P20) at a flow rate of 5 μl/min and at 25°C. Five short (4 μl) pulses of 0.1% sodium dodecyl sulphate were carried out to remove any non-specifically bound peptide and also to test regeneration conditions. Glutathione-*S*-transferase (GST) fusion proteins were subsequently injected simultaneously over both flow cells at 100 nM in running buffer (see above) at a flow rate of 5 μl/min. After a period of free buffer flow any analyte remaining bound to the surface was removed with a pulse of 0.1% sodium dodecyl sulphate bringing the signal back to baseline. Specific binding of the GST fusion proteins to each peptide was determined automatically by the system by subtraction of test from control flow cell responses, measured in arbitrary units called resonance units (RU). The GST fusion proteins used in the present study included the SH2 domain of Grb2, the N- terminal SH2 domain of PLCγ, and both the N- and C-terminal SH2 domains of the p85 subunit of PI 3-kinase (17).

BrdU Labelling

Mammalian C2 myoblast cells (20) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% FCS and glutamine at 37°C in 8% CO_2. Mitogenic assays were set up by seeding 4000 C2 cells per well of 48-well plates (Nunc). Cells were initially serum starved by culture in low serum (0.5%) media for one day. The media was then replaced with media containing 0.5% FCS alone or further supplemented with either basic FGF (bFGF, 5 ng/ml), EGF (10 ng/ml) (all from Collaborative Res.), or with various peptides as described. In some experiments (as indicated) peptides were added in serum free media that contained 10 μM Insulin. The effects of PD098059 (100 μM), wortmannin (10 μM) and rapamycin (10 or 100 nM) (all from Calbiochem) were determined on cell growth by either adding these reagents at the same time as the growth factors and peptides (rapamycin and PD098059), or in the case of wortmannin, 2 h before the addition of growth factors and peptides. After 24 h, cultures were labelled for a further 24 h with 5-Bromo-2-deoxyuridine/5-fluoro-2-deoxyuridine (BrdU) using the Amersham proliferation kit according to instructions. Cells were fixed for 5 min with ice-cold methanol and stained for BrdU as previously described (21). Briefly, following fixation cells were treated for 10 min with 1M HCl followed by 5 min with 100 mM Tris (pH 7.8), then blocked for 10 min with phosphate buffered saline (PBS) containing 10% goat serum, the primary and secondary antibodies were mouse anti-BrdU and anti-mouse fluorescein isothiocyanate respectively (diluted in PBS/10% goat serum). Cell nuclei were counterstained with 1 mg/ml Hoechst dye and visualised with UV epifluorescent optics. The percentage of cells in S-phase was calculated by dividing the number of nuclei stained with the BrdU antibody by the total number of nuclei stained with the Hoechst dye. One hundred fifty to 300 cells were routinely scored in three replicate wells for each condition.

Affinity Precipitation Using Phosphopeptides

(a) From cell extracts. biotinylated peptides were pre-coupled to agarose-streptavidin beads as follow: the beads were washed once in NP40 lysis buffer (150 mM NaCl, 20 mM Tris–Cl, 1% NP40, 1 mM Pyrophosphate, 1 mM NaF, 1 mM Vanadate, Protease inhibitors "complete" cocktail from Boehringer-Mannheim). A 2.5-fold molar excess of peptide was added in 1 ml of NP40 lysis buffer and incubated by rotation at 4°C for 30 min and subsequently the beads were washed 4 times in lysis buffer. Aliquots of cell lysate (300–500 μg of protein) were added to 20 μl of packed beads and rotated for 1h at 4°C. The beads were then washed 3 times in NP40 lysis buffer supplemented with 0.5% deoxycholate and 0.1% SDS and boiled in SDS sample buffer. Samples were resolved by SDS–PAGE on a 12% gel. Samples were transferred to nitrocellulose and immunoblotted with either a p85 (a gift from D. Cantrell, ICRF, London; 1:500 dilution) or a Grb2 (Transduction Laboratories; 1:1000 dilution) monoclonal antibody.

(b) From intact cells. NIH 3T3 cells (2×10^6) were incubated in suspension at 37°C in Ca^{2+} and Mn^{2+} free HBSS (Gibco BRL), 10% FCS containing either 50 μg/ml of 740Y-P peptide or an equal volume of PBS as a control. After 2h cells were centrifuged, washed and trypsinised to degrade non-internalised peptide. Cells were then resuspended in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 10% Glycerol, 2% NP40, 0.25% deoxycholate, 1 mM EDTA, 1 mM Vanadate, Protease inhibitors "complete" cocktail from Boehringer-Mannheim) for 1 h at 4°C. Lysates were clarified by centrifugation at 1.4×10^4g for 5 min and the supernatants were incubated for 1h with streptavidin-agarose beads. Beads were subsequently washed three times in lysis buffer and finally boiled in SDS sample buffer and resolved by SDS–PAGE on a 12% gel, transferred to nitrocellulose and immunoblotted with the p85 monoclonal antibody.

RESULTS AND DISCUSSION

The SH2 domains of the p85 subunit of PI 3-kinase can specifically recognise and bind to molecules that contain a tyrosine phosphorylated motifs with a general consensus sequence of YXXM, and small peptides containing such sequences can activate the enzyme *in vitro* and in permeabilised cells providing that they bind to the p85 SH2 domain with high affinity (see introduction). Phosphorylation of Y-740 in the PDGF receptor creates a high affinity binding site for PI 3-kinase, and peptide mimetics of this site bind with high affinity to the SH2 domains of p85 and activate the enzyme (7, 8, 22). We have synthesised a biotinylated peptide consisting of the 16-amino acid sequence derived from the third helix of the Antennapedia protein in tandem with a 9 amino acid phosphopeptide mimetic of the high affinity p85 binding site on the PDGF receptor (called the 740 Y-P peptide, see methods for details). The peptide was immobilised to a Biosensor chip and tested for its ability to bind p85 using BIAcore technology. The peptide readily bound GST fusion proteins containing both the N- and C- terminal SH2 domains of p85 (Fig. 1a). The peptide failed to bind GST alone, or a GST fusion protein containing the N- terminal SH2 domain of PLCγ and showed relatively weak binding of a GST fusion proteins containing the SH2 domain of Grb2 (Fig. 1a). In a previous series of experiments we have shown that the fusion

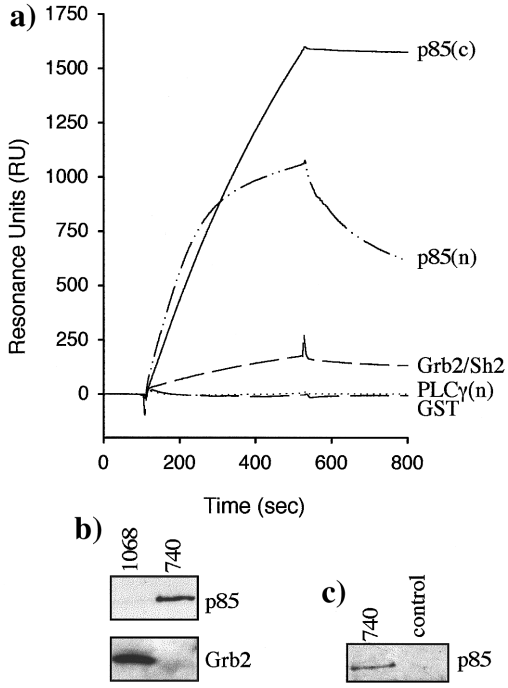

FIG. 1. Binding of the PDGFR 740Y-P peptide as assessed on a BIAcore Biosensor and by affinity precipitation. (a) GST fusion proteins of either Grb2-SH2 domain, p-85 N and C-terminal SH2 domains, PLCγ N-terminal SH2 domain, or GST alone were passed over two flow cells, one coated with streptavidin alone (the control) and one coated with streptavidin and the biotinylated PDGFR 740Y-P peptide as the test flow cell. The specific binding (test minus control) is shown. (b) Cell lysates were incubated with 740Y-P or 1068Y-P peptide pre-coupled to streptavidin-agarose beads. Proteins affinity captured on the beads were subsequently analysed by western blot using either a p85 monoclonal antibody (upper panel) or a Grb2 monoclonal antibody (lower panel). (c) Intact cells were incubated in suspension with or without the 740Y-P biotinylated peptide, trypsinised, washed and lysed. Lysates were then incubated with streptavidin–agarose beads. Proteins captured on the beads were analysed by Western blot using a p85 monoclonal antibody.

protein containing the N- terminal p85 SH2 domain will not bind to Biosensor chips coated with Antennapedia peptides made in tandem with 9 amino acid phosphopeptides that bind to the SH2 domain of PLCγ and Grb2 (17). Thus the 740 Y-P peptide shows the predicted specificity in terms of SH2 domain interactions despite the presence of the internalisation sequence. The 740 Y-P peptide is also able to specifically interact with p85 in cell lysates as shown by affinity precipitation (Fig. 1b). Moreover, we have been able to affinity precipitate p85 from intact cells incubated with the biotinylated 740 Y-P peptide using streptavidin-agarose beads, thus showing that the peptide is not only internalised in living cells but can also interact with p85 *in vivo*. The specificity of this interaction is confirmed by the fact that a phosphopeptide corresponding to the EGFR Grb2 (1068 Y-P) binding site cannot affinity precipitate p85 from cell extracts (Fig. 1b). We have also used immunocytochemistry to fur-

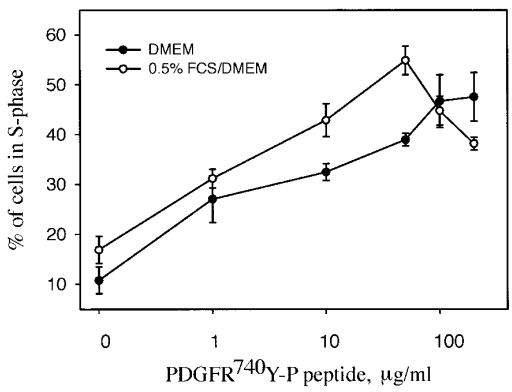

FIG. 2. The PDGFR 740Y-P peptide stimulates a mitogenic response. Following serum starvation, C2 cells were cultured in the presence of the PDGFR 740Y-P peptide at the given concentrations for 48 h. The mitogenic response was assessed by measurement of the percentage of cells in S-phase. The peptide was added in either serum free media, or media supplemented with 0.5% FCS as indicated. The results show the mean ± SEM measured in three replicate cultures in a single representative experiment.

ther demonstrate that the Antennapedia sequence can carry the 740 Y-P peptide into cells (not shown) as has been previously established for a range of other phosphopeptides (16, 17).

Constitutively active forms of PI 3-kinase are oncogenic (14). We therefore tested the cell-permeable p85 binding peptide for its ability to stimulate cell proliferation. C2 cells were grown for 2 days in the presence of the peptide (0–200 μg/ml) in either serum free media or media containing 0.5% serum with the results from individual experiments shown in Fig. 2, and pooled results from a number of experiments shown in Table 1. The peptide stimulated mitogenesis, as indexed by BrdU incorporation, at the lowest concentration tested (1 μg/ml). The peptide stimulated mitogenesis in both the presence and absence of serum (0.5%), and in the former instance a maximal response observed at 50 μg/ml.

We have tested 4 similar cell permeable phosphopeptides for their ability to stimulate mitogenesis: these included a Grb2 binding peptide from the EGF receptor (17), a PLCγ binding peptide from the FGF receptor (16, 17), and two peptides that correspond to additional tyrosine phosphorylation sites on the activated FGFR (23). None of these peptides stimulated a mitogenic response (Table 1). Thus the ability of the 740Y-P peptide to stimulate mitogenesis is highly specific and not a general feature of a cell permeable SH2 domain binding peptides. Table 1 also shows the effects of 10% serum and maximally active concentrations of FGF and EGF on cell proliferation: it is apparent that the 740 Y-P peptide is as good as 10% serum, and better than EGF or FGF, at stimulating entry into S-phase.

Binding of tyrosine phosphorylated peptides to the SH2 domains of p85 activates PI 3-kinase both *in vitro* and in permeabilised cells (8–10), and this is the most probable mechanism underlying the mitogenic activity of the 740 Y-P peptide. Although it does not have absolute specificity as a PI 3-kinase inhibitor, wortmannin remains a powerful tool for determining if a cellular response requires PI 3-kinase activity (1, 24). In the present study we found that wortmannin could fully inhibit the mitogenic response stimulated by the 740 Y-P peptide (Fig. 3). The mitogenic response stimulated by FGF in C2 muscle cells depends upon the activation of the MAPK cascade with no evidence for the involvement of the PI 3-kinase cascade (e.g., see 17, 25); in accord we found that wortmannin had no effect on the mitogenic response stimulated by FGF (Fig. 3). These data demonstrate that wortmannin is acting in a highly specific manner, and this makes it more likely that it is acting via inhibition of PI 3-kinase. A second PI 3-kinase inhibitor, LY294002, also substantially inhibited entry into S-phase stimulated by the 740Y-P peptide (~60% inhibition at 1 μM) further indicating an involvement of this enzyme in the response (data not shown). PI 3-kinase activates the p70 S6 kinase possibly via Akt/PKB (3, 26, 27) and the p70 S6 kinase inhibitor, rapamycin, has been used to demonstrate that this pathway is not involved in the anti-apoptotic actions of PI 3-kinase (3, 4). In the present study we found that rapamycin could fully inhibit the mitogenic activity of the 740 Y-P peptide (Fig. 3). In contrast we found that rapamycin had no effect on the response stimulated by FGF (Fig. 3). Given the established binding properties of the peptide, inhibition of the peptide response by wortmannin and rapamycin, and the fact that p70 S6 kinase is an established downstream effector of PI 3-kinase, the results suggest that the pep-

TABLE 1

Comparison of Growth Factor, Serum, and Peptide Effects on Mitogenesis

Treatment	% cells in S-phase
None:control cultures	14.2 ± 1.8 (7)
PDGFR 740Y-P peptide	48.3 ± 4.8 (7)
EGFR 1068Y-P peptide	14.6 ± 2.8 (3)
FGFR 766Y-P peptide	17.7 ± 2.0 (1)
FGFR 730Y-P peptide	13.7 ± 1.9 (4)
FGFR 463Y-P peptide	13.2 ± 1.7 (4)
10% FCS	42.5 ± 12.7 (3)
bFGF (5 ng/ml)	35.7 ± 1.5 (3)
EGF (10 ng/ml)	32.8 ± 1.6 (3)

Note. Following serum starvation, C2 cells were cultured for 48 h in media containing the above peptides (at 50 μg/ml), FCS (at 10%), bFGF (at 5 ng/ml), or EGF (10 ng/ml). The percentage of cells in S-phase was determined as described. The results show the mean values ± SEM for the given number of independent experiments. The FGFR 766Y-P peptide was tested in a single experiment, and the given value represents the mean ± SEM for three replicate cultures.

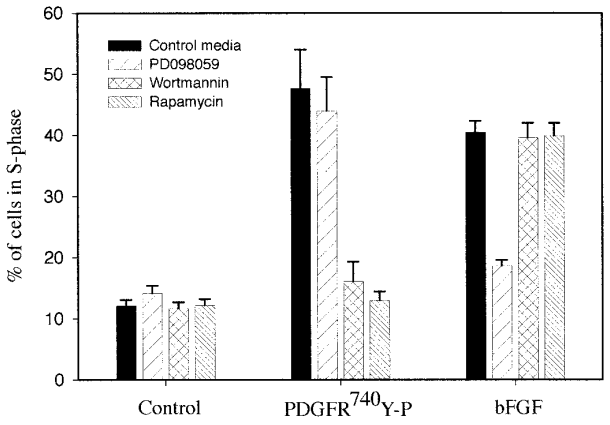

FIG. 3. Effects of PD098059, wortmannin and rapamycin on the mitogenic response stimulated by the PDGFR 740Y-P peptide and FGF. Following serum starvation, C2 cells were cultured in control media, media containing the PDGFR 740Y-P peptide (50 μg/ml) or media containing bFGF (5 ng/ml) as indicated. The experiments were conducted in the absence of additional reagents (control) or in the presence of PD098059 (100 μM, 4 experiments), wortmannin (10 μM, 6 experiments) or rapamycin (2 experiments at 10 nM and 2 at 100 nM, same result obtained and the data pooled), again as indicated. The results show the percentage of cells in S-phase as determined by BrdU incorporation, and each value is the mean ± SEM calculated from measurements made in 4–10 independent experiments.

tide is stimulating mitogenesis by activating a PI 3-kinase cascade.

The MAPK cascade is the major PI 3-kinase independent pathway for the stimulation of mitogenic responses in cells. Although there is some evidence for the γ isoforms of PI 3-kinase can activate the MAPK cascade, it would appear that the p85 activatable forms of PI 3-kinase will not activate this cascade (12). In the present study we found that inhibition of the MAPK cascade with PD098059 (28) which fully inhibits the mitogenic response stimulated by FGF (Fig. 3) and other growth factors (e.g., see Ref. 17), had no effect on the response stimulated by the 740 Y-P peptide. Immunoblotting experiments with an anti- active MAPK antibody (17) confirmed that the peptide did not activate the MAPK cascade in C2 cells (data not shown).

In conclusion, we have described for the first time stimulation of a mitogenic response in cells by a cell permeable p85 binding peptide. Pharmacological evidence is consistent with the notion that this peptide stimulates the response by activating a PI 3-kinase/p70 S6 kinase cascade, in the absence of any effect on the MAPK cascade. This demonstrates that cell-permeable peptides that can bind with high affinity to the SH2 domain of p85 can be exploited as novel tools for the study of PI 3-kinase function in cells, and also developed as agonists to stimulate PI 3-kinase dependent responses in living cells.

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