Selective Inhibition of Growth Factor-stimulated Mitogenesis by a Cell-permeable Grb2-binding Peptide*

(Received for publication, March 11, 1997, and in revised form, June 24, 1997)

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The activation of the mitogen-activated protein kinase (MAPK) cascade by a variety of growth factors and other agents is central to a mitogenic response. In the case of polyepitope growth factor receptors such as the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), the steps leading to activation of MAPK require the function of the adaptor protein Grb2 (growth factor receptor binding protein 2), which can bind either directly or indirectly via its Src homology 2 domain to activated receptor tyrosine kinases. A cell-permeable mimetic of the EGF receptor Grb2 binding site has been investigated for its ability to inhibit biological responses stimulated by a variety of growth factors. Pretreatment of cells with this peptide results in the accumulation of the peptide in cells and its association with Grb2. This is associated with a complete inhibition of the mitogenic response stimulated by EGF and PDGF. In contrast, the peptide has no effect on the mitogenic response stimulated by fibroblast growth factor. The peptide could also inhibit the phosphorylation of MAPK stimulated with EGF and PDGF in the absence of an effect on the fibroblast growth factor response. These data demonstrate that cell-permeable mimetics of Src homology 2 binding sites can selectively inhibit growth factor-stimulated mitogenesis, and also directly demonstrate specificity in the coupling of activated receptor tyrosine kinases to the MAPK cascade.

Growth factors such as EGF, PDGF, and FGF stimulate cell proliferation by binding to and activating membrane-spanning receptors that have cytoplasmic tyrosine kinase domains (1, 2). Ligand binding induces receptor dimerization, and this is as-

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1 This work was supported by grants from the Medical Research Council of Great Britain, the Wellcome Trust, the Royal Society of Guy’s and St. Thomas’s Hospitals. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

4 This work was supported by grants from the Medical Research Council of Great Britain, the Wellcome Trust, the European Union BIOMED 2 program (Contract BMH 4 CT 950524), and the Special Trustees of Guy’s and St. Thomas’s Hospitals. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

5 The abbreviations used are: EGF, epidermal growth factor; A, anhydroamino acid; BrdUrd, 5-bromo-2-deoxyuridine/5-fluoro-2-deoxyuridine; CHAPS, 3-i-[3-cholamidopropyl]dimethylammonio]-1-propane sulfonate; DMEM, Dulbecco’s modified Eagle’s medium; Me₃SO, dimethyl sulfoxide; FCS, fetal calf serum; FGF, fibroblast growth factor; FITC, fluorescein isothiocyanate; Grb2, growth factor receptor-binding protein 2; GS, goat serum; GST, glutathione S-transferase; HRP, horse-radish peroxidase; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; PLCγ, phospholipase C gamma; Shc, Src homology and collagen protein; SH2, Src homology 2; Fmoc, N-(9-fluorenyl)methoxycarbonyl; FGGF, FGF receptor; EGFR, EGF receptor; bFGF, basic FGF; PBS, phosphate-buffered saline; AMEM, amphibian modified Eagle’s medium.

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7 This level of specificity was unexpected and might be related to lower levels of FGF receptors as compared with EGF receptors that have cytoplasmic tyrosine kinase domains (1, 2). Ligand binding induces receptor dimerization, and this is associated with a complete inhibition of the mitogenic response stimulated by EGF and PDGF. In contrast, the peptide has no effect on the mitogenic response stimulated by fibroblast growth factor. The peptide could also inhibit the phosphorylation of MAPK stimulated with EGF and PDGF in the absence of an effect on the fibroblast growth factor response. These data demonstrate that cell-permeable mimetics of Src homology 2 binding sites can selectively inhibit growth factor-stimulated mitogenesis, and also directly demonstrate specificity in the coupling of activated receptor tyrosine kinases to the MAPK cascade.

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able mimetics of the EGF receptor Grb2 binding site and FGF receptor PLCγ binding site for their ability to inhibit mitogenic responses stimulated by EGF, PDGF, and FGF. All three responses were inhibited by PD 098059, which inhibits MAPK kinase (13), and this is consistent with activation of the MAPK cascade being required for these responses. We find that the EGF receptor Grb2 binding site mimetic was able to bind to Grb2 in cells and inhibit the mitogenic response stimulated by EGF and PDGF, but had no effect on the response stimulated by FGF. This peptide could also inhibit the phosphorylation of MAPK stimulated by EGF and PDGF, but not FGF. In contrast, the distinct phosphopeptide corresponding to the FGF receptor PLCγ binding site had no effect on the mitogenic response stimulated by EGF, PDGF, or FGF. These data demonstrate for the first time that cell-permeable peptide mimetics of SH2 domain binding sites can selectively inhibit activation of the MAPK cascade and mitogenic responses stimulated by polypeptide growth factors.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—Peptides were synthesized on a 431A Applied Biosystem peptide synthesizer using p-hydroxymethylphenoxymethyl polystyrene resin and standard Fmoc chemistry. The EGF Grb2-binding peptide sequence was as follows, Biot-Ahx-Arg-Arg-Trp-Arg-Trp-Trp-Arg-Arg-Trp-Trp-Arg-Arg-Trp-Arg-Arg-Pro-Val-Pro-Glu-Tyr(P)-Ile-Asn-Gln-Ser, where the underlined sequence corresponds to the 9-amino acid sequence containing phosphotyrosine 1068 of the EGF receptor that has been established to bind to the SH2 domain of Grb2 (the full peptide is called the EGFR1068Y-P peptide throughout the study). A phosphorylated Fmoc tyrosine (Novabiochem) was used in this sequence. The rest of the peptide sequence corresponds to a functional analogue of a sequence derived from the third helix of the Antennapedia protein, which has been established to act as a vector that can carry other peptides across the cell membrane into the cytosol (for full details of internalization properties of Antennapedia-based peptides, see Refs. 11 and 14). This peptide also contained an aminohexanoic acid (Ahx) spacer (Novabiochem) and a biotin at the N terminus. Synthesis of the cell-permeable Antennapedia-FGFR PLCγ-binding peptide (called the FGFR766Y-P peptide throughout the study) was as
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**Fig. 2. Uptake of the EGFR<sup>1068Y</sup>-P peptide into cells.** In the left panel, representative examples are shown of A1 cells treated with the biotinylated peptide for 60 min (see “Experimental Procedures”). The cultures were washed and permeabilized and the peptides detected with FITC-conjugated streptavidin. The figure shows optical sections through the cells, which demonstrate that the biotinylated EGFR<sup>1068Y</sup>-P peptide can be localized to the interior of the cells. Similar results were obtained with other cell types, including mammalian fibroblasts and C2 muscle cells. In the right panel, results are shown for experiments were C2 myoblasts were treated for 60 min with either the EGFR<sup>1068Y</sup>-P peptide, or the FGFR<sup>766Y</sup>-P peptide and extensively washed (see “Experimental Procedures”). The cells were then lysed and the biotinylated peptides precipitated. The amount of Grb2 in the lysates and precipitates was then determined by Western blotting.

**Fig. 3. The MAPK kinase inhibitor PD 098059 blocks mitogenic responses induced by EGF, PDGF, and bFGF.** Following serum starvation cells were cultured in the presence or absence of EGF (10 ng/ml), PDGF (10 ng/ml), or bFGF (5 ng/ml) in control medium or medium further supplemented with PD 098059 (100 μM). The results show the percentage of cells in S-phase as determined by BrdUrd incorporation. The percentage of cells in S-phase was calculated by dividing the number of nuclei incorporating BrdUrd by the total number of nuclei (see “Experimental Procedures”). The data shown are from one representative experiment (mean ± S.E.). 1, control medium; Ⅲ, +PD098059.

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**Description of the text**

Described previously with the exception that this peptide also contained an N-terminal biotin (11). After synthesis and deprotection, the peptides were desalted on a Sephadex G-10 column and lyophilized. The peptides were analyzed for purity by analytical high pressure liquid chromatography, and in each case a single peak was observed. Peptides were made as stock solutions at 25 mg/ml in dimethyl sulfoxide (Me<sub>2</sub>SO) and stored at −20 °C before use.

**Biosensor Measurements**—All surface plasmon resonance experiments were carried out using a BLAcore X<sup>TM</sup> biosensor (Pharmacia Ltd., Biacore, Uppsala, Sweden), the principle of which has been described elsewhere (15). Parallel flow cells of CM 5 sensor chips were derivatized, 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 of ligand were immobilized, corresponding to a concentration of 2.1–2.6 ng/mm<sup>2</sup>. 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 sulfate (SDS) were carried out to remove any nonspecifically 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 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% SDS 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 control binding. The amount of Grb2 in the lysates and precipitates was then determined by Western blotting.

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**Cell Culture and Mitogenic Assays**—Newt A1 myoblast cells were grown in gelatin-coated plastic flasks in medium composed of 60% minimum essential medium with Earle’s salts (ICN), 27% distilled water (AMEM), 10% fetal calf serum (FCS) supplemented with insulin, glutamine, and antibiotics as described previously (16). Cells were maintained at 25 °C in a humidified atmosphere of 2% CO<sub>2</sub>. Mammalian C2 myoblast cells (17) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.) supplemented with 15% FCS and glutamine at 37 °C in 5% CO<sub>2</sub>. Mitogenetic assays were set up by seeding either 2000 A1 cells/well of 48-well plates (Nunc), previously coated with 0.75% gelatin, or 4000 C2 cells/well of 48-well plates. Cells were initially serum-starved by culture in serum-free medium for 3 days. Serum-free medium was then replaced with medium containing 0.5% FCS alone or further supplemented with basic FGF (bFGF, 5 ng/ml), EGF (10 ng/ml), or PDGF (10 ng/ml), (all from Collaborative), in the presence or absence of the EGFR<sup>1068Y</sup>-P peptide (10 μg/ml) or the MAPK kinase inhibitor PD 098059 (100 μM; Calbiochem). In some experiments cultures were treated with the EGFR<sup>1068Y</sup>-P peptide for 2 h before being washed three times and the medium replaced with growth factors in the absence of additional peptide (see “Results and Discussion”). After 24 h, cultures were labeled for another 24 h with 1 mg/ml 5-bromo-2-deoxyuridine/5-fluoro-2-deoxyuridine (BrdUrd) using the Amersham proliferation kit according to instructions. Cells were transferred to medium further supplemented with PD 098059 compound or the EGFR<sup>1068Y</sup>-P peptide and individual growth factors (see text for treatment schedules and concentrations). The cultures were then washed with ice-cold PBS before being lysed and collected in 200 μl of sample buffer (62.5 mM Tris-HCl at pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% (w/v) bromophenol blue) before being heated at 90 °C for 5 min. Equal protein amounts were then electrophoresed through polyacrylamide gel (10% T, 4% C) and transferred to nitrocellulose. The protein bands were then visualized using 125<sup>i</sup>-labeled protein A coupled to antibody specific for the EGFR<sup>1068Y</sup>-P peptide.
blue), again at the indicated time points. Samples were boiled for 5 min and sonicated for 30 s before being resolved on 12.5% SDS-polyacrylamide gels and Western blotted onto polyvinylidene difluoride membranes (Millipore). Membranes were blocked for 1 h at room temperature with gentle agitation in blocking buffer (5% nonfat dried milk, 0.1% Tween 20 in PBS), and then incubated for 24 h at 4 °C with a polyclonal rabbit anti-active MAPK antibody (1:20,000 from Promega) in PBS (containing 5% BSA, 0.05% Tween 20). Membranes were then washed three times with blocking buffer, incubated with HRP-conjugated donkey anti-rabbit secondary antibody (1:4000 in blocking buffer; Amersham) for 1 h at room temperature, and finally washed three times with blocking buffer before probing with enhanced chemiluminescence (ECL; Amersham).

Peptide Uptake into Cells— Cultures of A1 cells were established overnight on gelatin-coated wells of an eight-chamber tissue culture slide. The cultures were washed and media replaced with AMEM containing 10 μg/ml biotinylated EGF receptor 1068Y-P peptide. After 2 h the cultures were washed three times with AMEM and then fixed and permeabilized with ethanol/acetic acid (9:1 by volume) for 5 min at −20 °C. Non-specific protein binding sites were blocked for 30 min with 10% GS in PBS. The cultures were then incubated for 1 h with FITC-conjugated streptavidin (1:500; Amersham). The cultures were washed three times with PBS before being mounted for viewing by confocal microscopy.

Association of Grb2 and Peptide in Intact Cells—C2 myoblast cells were grown to 70% confluence on collagen-coated plates, serum-starved in DMEM supplemented with l-glutamine (2 mM), insulin (10 μg/ml), transferrin (5 μg/ml), and sodium selenite (10 nM) for 4 h and then treated with or without peptide (50 μg/ml) for 1 h at 37 °C. The cell monolayers were washed extensively with ice cold PBS (three times), 2 M NaCl at pH 7.4 (three times), and 2 M NaCl/20 mM sodium acetate at pH 4 (two times) to remove extracellular peptide before being lysed in 20 mM Tris-HCl (pH 7.5) containing 1 M NaCl, 1% CHAPS, and complete protease inhibitors from Boehringer Mannheim (according to manufacturer's instructions). Lysates were clarified by centrifugation and the biotinylated peptides precipitated from the supernatant with streptavidin-agarose following gentle mixing at 4 °C for 2 h. Precipitates were washed extensively with ice cold PBS (three times), 2 M NaCl at pH 7.4 (three times), and 2 M NaCl/20 mM sodium acetate at pH 4 (two times) to remove extracellular peptide before being lysed in 20 mM Tris-HCl (pH 7.5) containing 1 M NaCl, 1% CHAPS, and complete protease inhibitors from Boehringer Mannheim (according to manufacturer's instructions). Lysates were clarified by centrifugation and the biotinylated peptides precipitated from the supernatant with streptavidin-agarose following gentle mixing at 4 °C for 2 h. Precipitates were washed extensively with ice cold PBS (three times), 2 M NaCl at pH 7.4 (three times), and 2 M NaCl/20 mM sodium acetate at pH 4 (two times) to remove extracellular peptide before being lysed in 20 mM Tris-HCl (pH 7.5) containing 1 M NaCl, 1% CHAPS, and complete protease inhibitors from Boehringer Mannheim (according to manufacturer's instructions). Lysates were clarified by centrifugation and the biotinylated peptides precipitated from the supernatant with streptavidin-agarose following gentle mixing at 4 °C for 2 h. Precipitates were washed extensively with ice cold PBS (three times), 2 M NaCl at pH 7.4 (three times), and 2 M NaCl/20 mM sodium acetate at pH 4 (two times) to remove extracellular peptide before being lysed in 20 mM Tris-HCl (pH 7.5) containing 1 M NaCl, 1% CHAPS, and complete protease inhibitors from Boehringer Mannheim (according to manufacturer's instructions). Lysates were clarified by centrifugation and the biotinylated peptides precipitated from the supernatant with streptavidin-agarose following gentle mixing at 4 °C for 2 h. Precipitates were washed extensively with ice cold PBS (three times), 2 M NaCl at pH 7.4 (three times), and 2 M NaCl/20 mM sodium acetate at pH 4 (two times) to remove extracellular peptide before being lysed in 20 mM Tris-HCl (pH 7.5) containing 1 M NaCl, 1% CHAPS, and complete protease inhibitors from Boehringer Mannheim (according to manufacturer's instructions). Lysates were clarified by centrifugation and the biotinylated peptides precipitated from the supernatant with streptavidin-agarose following gentle mixing at 4 °C for 2 h. Precipitates were washed extensively with ice cold PBS (three times), 2 M NaCl at pH 7.4 (three times), and 2 M NaCl/20 mM sodium acetate at pH 4 (two times) to remove extracellular peptide before being lysed in 20 mM Tris-HCl (pH 7.5) containing 1 M NaCl, 1% CHAPS, and complete protease inhibitors from Boehringer Mannheim (according to manufacturer's instructions).

RESULTS AND DISCUSSION

We have synthesized a biotinylated peptide consisting of a functional analogue of the 16-amino acid sequence derived from the third helix of the Antennapedia protein that acts as an internalization vector for other peptide sequences (see Ref. 14 for a detailed description of the internalization properties of Antennapedia-related peptides and Ref. 11 for an example) in tandem with the well characterized Grb2 docking site on the activated EGFR receptor (EGFR1068Y-P peptide; see “Experimental Procedures” for details). Fig. 1 shows characterization of the binding properties of this peptide using BiAcore technology. The peptide readily bound a GST fusion protein containing the full Grb2 molecule and also one containing only the single SH2 domain. The peptide did not bind a control GST fusion protein or a GST fusion protein containing the N-terminal p85 SH2 domain, which are very poorly bound by peptides containing only the N-terminal SH2 domain of PLCγ (Fig. 1A). In contrast, a peptide mimetic of the FGF receptor PLCγ binding site (the FGFR766Y-P peptide) specifically bound PLCγ, and showed only background binding of Grb2 and p85 (Fig. 1B). In addition, a range of control phosphopeptides that do not share the consensus sequence for PLCγ or Grb2 binding bound neither molecule (data not shown). Thus the peptides show the predicted specificity in terms of SH2 domain interactions (see Ref. 6 for details) despite the presence of the internalization sequence.

The uptake of the biotinylated EGFR1068Y-P peptide into cells was monitored by confocal microscopy. Cells were treated with the biotinylated peptide at 10 μg/ml for 60 min before being washed, fixed, and permeabilized (see “Experimental Procedures”). Optical sections through the cell confirmed the presence of the peptide within the cytoplasm (Fig. 2, left panel). Similar results were obtained with the other phosphopeptides (e.g. see Ref. 11). To determine if the EGFR1068Y-P peptide could complex with Grb2 inside cells, cultures of C2 myoblasts were treated with the peptide at 50 μg/ml for 60 min before being extensively washed to remove noninternalized peptide. The cells were then lysed and the biotinylated peptide precipitated with streptavidin-agarose. Grb2 co-precipitated with the EGFR1068Y-P peptide and the peptide essentially depleted all of the Grb2 from the cell lysate (Fig. 2, right panel). Other phosphopeptides, including the FGFR766Y-P peptide, failed to precipitate Grb2 or deplete it from the cell lysates (Fig. 2, right panel, and data not shown).

EGF, PDGF, and FGF all stimulate proliferation of newt A1 myoblasts as indexed by BrdUrd incorporation. It was established that a maximal response was elicited by 10, 10, and 5 ng/ml EGF, PDGF, and FGF, respectively, and that these responses could be fully inhibited by the MAPK kinase inhibitor PD 098059 (Fig. 3). At the lower concentrations of growth factors (1 ng/ml), the peak activation of MAPK was seen at 30 min, and this was inhibited by treatment with PD 098059 (Fig. 4, top). However, at the higher concentrations of growth factors (5–10 ng/ml activation of the MAPK cascade was apparent even after 4 h of treatment with the growth factors (shown for EGF and FGF in Fig. 4, bottom), and EGF in Fig. 6 (top)). Addition of PD 098059 2 h after the addition of the growth factors brought the MAPK activity down to control levels (Fig. 4, bottom) and also fully inhibited the mitogenic response (data not shown), suggesting that this sustained activation of the MAPK cascade is required for the full mitogenic response.

The effect of the EGFR1068Y-P peptide on the mitogenic
EGFR1068Y-P peptide. The results show data pooled from three independent experiments (mean ± S.E.). In B the experiment was repeated; however, the EGFR1068Y-P peptide (10 μg/ml) was only added to cultures for 2 h prior to the addition of growth factors; the results are from a single representative experiment. [ ], control medium; III, Tyr(P)1068 peptide.

activity of EGF, PDGF, and FGF on A1 cells was also determined. Results pooled from three independent experiments showed that the continued presence of 10 μg/ml peptide over the 2-day assay period completely inhibited the mitogenic response stimulated by EGF and PDGF, but had no effect on the response to FGF (Fig. 5A). The peptide also had no obvious effect on the viability or morphology of control cells (data not shown), suggesting that its effects on mitogenesis are the result of a specific rather than toxic action, and this is in accord with the failure of the peptide to affect the FGF response. The uptake studies show that the peptide will accumulate in cells following a 60-min treatment period (Fig. 2) and in this respect we find that a 2-h pretreatment of cells with the peptide is sufficient to fully inhibit the mitogenic responses stimulated by EGF and PDGF (Fig. 5B). Pretreatment of C2 myoblasts with 50 μg/ml peptide was also associated with a full inhibition of the mitogenic response stimulated by EGF and PDGF in the absence of any effect on the response stimulated by FGF (data not shown). This result also suggests that the peptide has a relatively long effective half-life in cells, and this might reflect the slow dissociation of the peptide from Grb2, which would be expected to protect it from dephosphorylation and degradation; it also confirms that the peptide is effective in both mammalian and newt cells.

The Grb2-binding peptide was initially tested for its ability to inhibit the activation of MAPK stimulated by EGF. At the maximally active concentration of EGF (10 ng/ml) 10 μg/ml peptide substantially inhibited the activation of the MAPK cascade, and this was seen at all time points tested (Fig. 6, top). The competitive nature of the inhibition was demonstrated by showing that 100 μg/ml peptide could fully inhibit the activation of MAPK stimulated by 1 ng/ml EGF (measured at 30 min; see Fig. 6, bottom). Under the same conditions the peptide also fully inhibited the activation of MAPK by PDGF, but had no effect on the response stimulated by FGF (Fig. 6, bottom).

The specificity of action of the EGFR1068Y-P peptide was confirmed by showing that the FGER1068Y-P peptide, which inhibits FGF-stimulated inositol phosphate accumulation and axonal growth responses (11), had no effect on the FGF, EGF, or PDGF mitogenic response (Table I). In addition, we have tested three other cell-permeable phosphopeptides that do not bind to the SH2 domain of Grb2 as measured by BIAcore, and found that they also have no effect on the mitogenic response stimulated by EGF and PDGF (data not shown). Thus the inhibitory activity of the EGFR1068Y-P is very specific and can most probably be accounted for by its ability to bind to the SH2 domain of Grb2 and inhibit activation of the MAPK cascade. This is best explained by the known ability of peptide mimetics of the Grb2 binding site on the EGF receptor to compete out binding of Grb2 to the activated receptor in vitro (8), as well as in live cells (10). Although we have demonstrated that our peptide will bind to Grb2 in intact cells, and that it does not interact with PLCγ, p85 (this study), or Shc (data not shown), we cannot exclude the possibility that the peptide interacts with other targets. However, it is clear from the results that its effects are highly specific and that they can be best explained by the peptide’s ability to bind to the SH2 domain of Grb2.

The fact that the EGFR1068Y-P peptide can inhibit both EGF and PDGF responses is perhaps not surprising, as the SH2 domain of Grb2 is required for both the direct and indirect interactions of this adaptor with the EGF receptor (see Introduction) and an indirect association via Syp with the activated PDGF receptor (19, 20). The possibility that the failure of the peptide to inhibit the FGF response reflects the use of a com-
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A1 cells were serum-starved before culture in control media or media supplemented with EGF (10 ng/ml), PDGF (10 ng/ml), or FGF (5 ng/ml) in the presence or absence of 10 μg/ml FGFR766Y-P peptide. The results show the percentage of cells in S-phase (mean ± S.E.) pooled from three independent experiments.

| Control media | EGF | PDGF | FGF |
|---------------|-----|------|-----|
| 10.2 ± 1.8    | 29.1 ± 2 | 30 ± 1 | 41.7 ± 6.7 |
| FGFR766Y-P peptide | 9.9 ± 1.4 | 29.9 ± 2 | 31.8 ± 1 | 37.7 ± 4.5 |

Completely different mitogenic pathway is unlikely for a number of reasons. For example, FGF activated the MAPK cascade, and both this and the FGF-stimulated mitogenic response were inhibited by the PD 098059 MAPK kinase inhibitor. The alternative possibility, that the selectivity of the Grb2-binding peptide reflects differences in the coupling of the EGF/PDGF receptors and the FGF receptor to the MAPK cascade, is a more probable and interesting explanation. An emerging view is that a novel ~90-kDa protein might couple the FGF receptor to the Ras-MAPK pathway via Grb2 (21–24), and in this context a new lipid anchored FGF receptor substrate, called FRS2, has recently been cloned; following tyrosine phosphorylation, FRS2 can bind Grb2 and link the FGF receptor to MAPK activation (25). Shc can also act as an adaptor molecule that can link Grb2 to the activated FGF receptor (21, 25). Thus, the inability of the EGFR1068Y-P peptide to inhibit the FGF response might be accounted for by its inability to inhibit Grb2 binding to FRS2 and/or Shc. In fact, it has been reported previously that the EGFR1068Y-P peptide is relatively ineffective at inhibiting the association of Grb2 with Shc (10).

A number of control peptides that do not bind to the Grb2 SH2 domain had no effect on the mitogenic response stimulated by EGF and PDGF. The most conspicuous of these is a cell-permeable peptide mimetic of the PLCγ binding site on the FGF receptor that inhibits phospholipid hydrolysis and axonal growth responses stimulated by FGF (11). It is interesting that this peptide had no effect on the mitogenic response stimulated by FGF, and this is in accord with a large body of work that has reported that whereas mutation of Tyr766 can prevent the FGF receptor from activating PLCγ, it has no effect on the mitogenic response stimulated by this factor (4).

In summary, we have shown that cell-permeable peptides that have unique binding profiles for SH2 domain-containing molecules can be used to inhibit the mitogenic activity of polypeptide growth factors. This confirms our prediction (11) that the ability of cell-permeable phosphopeptides to interact with high affinity and relative specificity to the SH2 domains of adaptors and effectors can be readily exploited to design novel tools for the dissection of signaling pathways in living cells.

Acknowledgments—The functional analogue of the internalization sequence derived from the third helix of the Antennapedia protein was developed by Drs. Alain Prochiantz and Gerard Chassaing and their colleagues, and we thank them for sharing this sequence with us.

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