Controlling Epidermal Growth Factor (EGF)-stimulated Ras Activation in Intact Cells by a Cell-permeable Peptide Mimicking Phosphorylated EGF Receptor*†

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Epidermal growth factor (EGF)-stimulated Ras activation involves specific interactions between the EGF receptor (EGFR), the adaptor proteins Grb2 and Shc, and the nucleotide exchange factor Sos-1. Study and control of these protein-protein interactions in vivo can be greatly promoted by introducing intracellular reagents that mimic EGFR functions. Here, we showed that a synthetic phosphopeptide encompassing the autophosphorylation site 1068 of EGFR formed a complex with endogenous Grb2 after this peptide was delivered into intact cells by a cell-permeable peptide import technique. Consequently, this intracellular peptide inhibited EGF-induced EGFR/Grb2 associations but not EGFR/Shc or Shc/Grb2 associations. Peptide-mediated disruption of the EGF/Grb2/Sos-1 cascade led to reduced Ras activation and mitogen-activated protein kinase activation. These results indicate that the binding of Grb2 to the phosphorylated Tyr-1068 of EGFR is crucial to the EGF-induced Ras/mitogen-activated protein kinase signaling pathway. The application of cell-permeable peptides to this study demonstrates a useful biochemical tool to probe and control various intracellular processes involved in signal transduction and gene transcription.

Mitogenic signaling stimulated by epidermal growth factor (EGF) requires the intrinsic tyrosine kinase activity of its transmembrane receptor (for review see Refs. 1 and 2). A number of intracellular proteins including the EGFR receptor itself are phosphorylated on tyrosine residues in EGF-stimulated cells. Several autophosphorylation sites have been identified in the carboxyl-terminal region of the EGFR. These sites and their flanking regions constitute specific motifs that can be recognized by the Src homology 2 (SH2) domains of many intracellular signaling proteins (for review see Refs. 3 and 4).

Ras activation originating from the EGF receptor comprises a cascade of protein-protein interactions involving a Grb2/Sos-1 signaling protein complex (5–12). The Grb2 protein, which functions as an adaptor, binds to tyrosine-phosphorylated EGFR either directly by its SH2 domain or indirectly via the Shc protein (13). Recent studies using in vitro peptide competition assays and EGFR mutants have suggested that the photophorysine (Tyr(P)) 1068 of activated EGFR is a direct binding site for the Grb2 SH2 domain (11, 14–17). To determine the functional consequence of disrupting the EGFR/Grb2 protein-protein association in vivo, we delivered a peptide mimicking this EGFR autophosphorylation site into intact cells by using a nondestructive cell-permeable peptide import method (see Ref. 18). We showed in this report that this outside-in peptide formed an intracellular peptide-protein complex with endogenous Grb2, resulting in a substantial inhibition of EGF-stimulated EGFR/Grb2 association and Ras/MAP kinase activation. Our results demonstrate that intracellular tyrosine kinase signaling pathways can be studied and regulated in living cells by cell-permeable peptides carrying specific functional motifs, such as photophosphorysine-containing sequences.

MATERIALS AND METHODS

Peptides, Antibodies, and Cell Lines—Peptides were synthesized by a stepwise solid-phase peptide synthesis method (19) using the tert-butyloxycarbonyl (Boc) protection. For photophosphorysine-containing peptides, Boc-Tyr(PO3Me2) was used. Synthetic peptides were purified by C18 reverse-phase high pressure liquid chromatography as described (18, 20). The molecular weights of the purified peptides were verified by mass spectrometry analysis. A polyclonal anti-SP1068 peptide antibody was raised against the SP1068 peptide-keyhole limpet hemocyanin conjugate in rabbits and recognized in enzyme-linked immunosorbent assay not only SP1068 but also P1068 and SY1068 peptides in enzyme-linked immunosorbent assay. SAA cells are the NIH 3T3 cells overexpressing human EGF receptor (21). They were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum.

Indirect Immunofluorescence Assay—Subconfluent SAA cells grown on the chamber slides (Nunc) were serum-starved for 18 h and then treated with or without 0.5 ml of peptide solution (100 μg/ml) in DMEM containing 20 mM HEPES and 0.1% bovine serum albumin at 37°C for 30 min. The cells were washed and fixed, and intracellular peptide was detected by an indirect immunofluorescence assay using anti-SP1068 peptide antibody and rhodamine-labeled anti-rabbit antibody as described (18, 20). Coverslips with stained cells were mounted in Poly/Mount (Polyscience) and analyzed in a fluorescence microscope using a ×100 immersion lens.

Immunoprecipitation and Immunoblot Analysis—SAA cells were grown to 95% confluency in DMEM containing 10% fetal calf serum, serum-starved for 18 h, and then treated with or without peptides (100 μg/ml or as specified) in DMEM containing 20 mM HEPES and 0.1% bovine serum albumin for 30 min. Subsequently, cells were stimulated with or without human EGF (50 ng/ml, Intergen) for 10 min and then extensively washed with cold phosphate-buffered saline, 2 mM NaCl, pH 7.4, and 2% NaCl, 20 mM sodium acetate, pH 4, to remove extracellularly associated peptides. In each case, 0.5 ml of cell lysates (0.3 mg/ml) was incubated with 0.5–1 μg of antibodies as indicated or with 20 μl of anti-SP1068 peptide serum. The immune complexes were precipitated with protein A-Sepharose (Sigma). Immunoprecipitates were separated on SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies as indicated followed by horseradish peroxidase-linked anti-rabbit or anti-mouse antibodies according to ECL protocol (18).

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‡ The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; DMEM, Dulbecco’s modified Eagle’s medium; MAP, mitogen-activated protein; SH2, Src homology 2; PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid.

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Inhibition of EGF-induced Ras and MAP Kinase Activation

RESULTS

Cellular Import of a Synthetic Phosphotyrosine-containing Peptide Carrying the Autophosphorylation Site 1068 of EGFR—To study and influence intracellular protein-protein interaction between EGFR and Grb2 protein, we designed a peptide (referred as to SP1068, Fig. 1) comprised a cell membrane-translocating sequence (18, 20) at its amino terminus and the phosphotyrosine 1068-containing region of EGFR at its carboxyl terminus. Two control peptides (Fig. 1), a non-cell-permeable version (P1068) and an unphosphorylated version (SY1068) of the SP1068 peptide, were also prepared. We first examined whether cell-permeable SP1068 peptide can be taken up efficiently by living NIH 3T3 cells overexpressing human EGFR receptors (SAA cells (21)). Intracellular peptide was observed in a punctate staining pattern in cells treated with SP1068 peptide, as detected by an indirect immunofluorescence assay using anti-peptide antibodies (Fig. 2). Immunodetection of the cell-permeable peptide was specific because untreated cells or cells incubated with non-cell-permeable P1068 peptide did not showed intracellular fluorescence signals (Fig. 2). The same assay showed that SY1068 peptide was also taken up by SAA cells (not shown). Cell-permeable peptides within the concentration range used were not cytotoxic as determined by staining with fluorescein diacetate/ethidium bromide (18, 20).

Disruption of EGF-induced Ras Activation Cascade by Cell-permeable Phosphopeptide SP1068—To determine whether intracellular import of SP1068 phosphopeptide has any effects on EGF-induced EGFR/Grb2 protein-protein association in vivo, serum-starved SAA cells were incubated with SP1068 peptide for 30 min prior to EGF stimulation, and the Grb2 protein associated with the activated EGFR was determined in a number of communoprecipitation assays. Upon the EGF stimulation (see Fig. 3A), the Grb2 protein was coprecipitated with the phosphorylated EGFR in anti-EGFR immunoprecipitates. However, the amount of associated Grb2 protein was significantly reduced in cells pretreated with SP1068 peptide (Fig. 3A), indicating that intracellularly imported SP1068 peptide can inhibit the EGFR/Grb2 association by mimicking a specific EGFR autophosphorylation site. A similar inhibition pattern by this phosphopeptide was also seen in anti-Grb2 immunoprecipitates (Fig. 3B). Neither non-cell-permeable P1068 peptide nor unphosphorylated SY1068 peptide treatment showed any
significant inhibition of the inducible EGFR/Grb2 association. Inhibition by SP1068 peptide was concentration-dependent, plateauing at about 50 $\mu$g/ml (18 $\mu$M, extracellular concentration) (Fig. 3B). The guanine nucleotide-releasing factor Sos-1 is involved in EGF-stimulated Ras activation cascades by forming a constitutive complex with Grb2 (5–8, 11, 12, 22). It is thus expected that SP1068 peptide treatment would have a similar inhibitory effect on the association of Sos-1 with EGFR in EGF-stimulated cells. To address this possibility, we analyzed the amount of tyrosine-phosphorylated EGFR coprecipitated with Sos-1 in anti-Sos-1 immunoprecipitates. As shown in Fig. 3C, the association of activated EGFR with Sos-1 in EGF-stimulated cells was significantly attenuated by SP1068 peptide but not by SY1068 or P1068 peptide.

Identification of an Intracellular Peptide-Protein Complex between SP1068 Peptide and Endogenous Grb2 Protein—It appears that cell-permeable SP1068 peptide is active because it contains a specific binding site for Grb2 protein that allows it to compete with endogenous EGFR for Grb2 protein binding. To determine whether an intracellular complex of SP1068/Grb2 can be isolated from SP1068 peptide-treated living cells, serum-starved SAA cells were incubated with SP1068 peptide, and the cell lysate was precipitated with anti-SP1068 peptide antibody. Immunoblotting analysis with anti-SP1068 peptide antibody showed that intracellular SP1068 peptide was isolated from the immunoprecipitates (Fig. 4). When these anti-SP1068 peptide immunoprecipitates were blotted with anti-Grb2 antibody, coprecipitated Grb2 protein was detected (Fig. 4), indicating that an intracellular complex of SP1068/Grb2 was indeed formed in the SP1068 peptide-treated SAA cells.

The same intracellular peptide-protein complex was also observed in the SP1068 peptide-treated parental NIH 3T3 cells (not shown). The isolated SP1068 peptides represented roughly 1% of the extracellularly added peptide as estimated by cell-free immunoprecipitation of the SP1068 peptide. To further verify that the identified SP1068-Grb2 complex is not formed in vitro, we examined non-cell-permeable P1068 peptide-treated cells in the same assay and found no sign of a P1068-Grb2 complex (Fig. 4). Successful isolation of the SP1068-Grb2 complex from SP1068 peptide-treated cells demonstrates a feasible approach.
for studying various protein-protein interactions in living cells.

Specificity of the Inhibitory Activity of SP1068 Peptide—To determine whether the inhibition by SP1068 peptide is specific for the direct EGFR/Grb2 protein-protein association, we first examined the effect of SP1068 peptide on EGF-induced EGFR/Grb2 association. Shc is an intracellular protein substrate for several protein tyrosine kinases, including the EGF receptor (23–25). It contains an SH2 domain and a phosphotyrosine binding/phosphotyrosine interaction domain, which have been suggested to bind to EGFR autophosphorylation sites 1173 and 1148, respectively. Shc is also known to bind to Grb2 to mediate the indirect association of EGF receptor with Grb2-Sos signaling complex. Immunoprecipitation with anti-Shc antibodies demonstrated that SP1068 peptide treatment did not disrupt EGF-induced EGFR/Shc association (Fig. 5A). Correspondingly, cell-permeable phosphopeptides carrying autophosphorylation sites 1148 or 1173 (Fig. 1) were without significant effects on inducible EGFR/Grb2 association (Fig. 5B). These results thus indicate that the activated EGF receptor uses specific autophosphorylation sites to interact with distinct signaling substrates in vivo. We also examined the effect of SP1068 peptide on Grb2/Shc association. As shown in Fig. 5C, some Shc proteins formed a constitutive complex with Grb2 in SAA cells. SP1068 peptide treatment did not alter the Grb2/Shc association.

Inhibition of EGF-induced Ras Guanine Nucleotide Exchange by Cell-permeable SP1068 Peptide—EGF stimulates Ras guanine nucleotide exchange activity through signaling pathways that involve EGFR and Grb2/Sos-1 proteins (5–12). To determine whether the SP1068 peptide-mediated dissociation of EGFR from the Grb2-Sos complex has any effects on Ras activation, serum-starved SAA cells were pretreated with SP1068 peptide, washed, permeabilized with streptolysin O, and then stimulated with EGF in the presence of [α-32P]GTP. As shown in Fig. 6A, the α-32P-labeled nucleotide bound to Ras was significantly increased by EGF stimulation. This EGF-induced increase was substantially, although not completely, inhibited by pretreatment of cells with SP1068 peptide in a dose-dependent manner (Fig. 6B). In contrast, no significant inhibition was seen in non-cell-permeable P1068 peptide-treated or unphosphorylated SY1068 peptide-treated cells (Fig. 6A). These results suggest that the SP1068 peptide-mediated disruption of the EGFR/Grb2/Sos-1 protein-protein association leads to reduced Ras activation in EGF-stimulated cells.

Inhibition of EGF-induced MAP Kinase Activation by Cell-permeable SP1068 Peptide—To examine whether partial inhibition of Ras activation by SP1068 peptide would affect EGF-induced activation of MAP kinases (26, 27), we employed a quantitative MAP kinase assay using [γ-32P]ATP and myelin basic proteins as substrate. As shown in Fig. 7A, MAP kinases were fully activated in SAA cells treated with EGF (10 ng/ml) as assessed by their ability to phosphorylate myelin basic protein. Pretreatment of cells with SP1068 peptide substantially inhibited the inducible MAP kinase activation (59% inhibition, see Fig. 7A). However, the peptide was not inhibitory when a higher dose (50 ng/ml) of EGF was applied. Similar results were obtained from SP1068 peptide-treated parental NIH 3T3 cells (Fig. 7A).

Peptide-mediated inhibition was verified in a MAP kinase gel mobility shift assay. As shown in Fig. 7B, two MAP kinase proteins (p42R2 and p44R2) were activated in EGF-treated SAA cells as assessed by their slower migrating electrophoretic mobilities due to EGF-induced phosphorylation. Consistent with the results of the kinase assay shown in Fig. 7A, pretreatment of cells with SP1068 peptide did not inhibit the inducible phosphorylations of the MAP kinases when cells were stimu-
lated with 50 ng/ml EGF (Fig. 7B). However, in cells stimulated with a lower dose (10 ng/ml) of EGF, the peptide partially inhibited the phosphorylations of the MAP kinases as determined by their slightly faster migrating electrophoretic mobilities (Fig. 7B).

It has been suggested that Sos-1 can be phosphorylated by the activated MAP kinases (5–8, 11, 28, 29), which may lead to the dissociation of Sos-1 from membrane-bound receptor complex involved in Ras activation. As also characterized by electrophoretic mobility shift (Fig. 7B), the phosphorylation of Sos-1 can be stimulated with EGF (10 or 50 ng/ml). However, this Sos phosphorylation was not affected by SP1068 peptide treatment even in the cells stimulated with 10 ng/ml EGF (Fig. 7B), suggesting that a low level of MAP kinase activation is sufficient to stimulate Sos-1 phosphorylation.

**DISCUSSION**

The results reported here indicate that the phosphorylated tyrosine 1068 of EGFR is a major site for initiating EGF-induced Ras activation and MAP kinase activation in vivo. The intracellular interaction of this autophosphorylation site and its flanking region with the downstream signaling protein Grb2 is demonstrated by isolating from intact cells a high affinity complex between endogenous Grb2 and an outside-in cell-permeable phosphopeptide, which mimics the EGFR motif. The competition of this intracellular peptide with endogenous EGFR for Grb2 results in a significant, but not complete, disruption of EGFR/Grb2 association and reduced Ras activation and MAP kinase activation in EGF-stimulated cells. However, when a higher dose of EGF is used to stimulate cells, the intracellular peptide concentration is insufficient to block MAP kinase activation, suggesting that maximal activation of MAP kinase requires only a few activated EGF receptors. It is also possible that in the presence of high concentrations of EGF, alternative signaling pathway(s) involving the autophosphorylation sites of EGFR receptor other than Tyr-1068 play important roles in EGF-induced MAP kinase activation. These alternative pathways could be mediated by Shc or other signaling proteins.

Our findings using cell-permeable phosphopeptides demonstrate a feasible biochemical approach for studying and influencing the EGFR tyrosine kinase-initiated signal transduction pathway in intact cells. The precise key-lock in vivo interactions between phosphorylated EGF receptor and its signaling
substrates are dissected by our study using cell-permeable peptides carrying different autophosphorylation sites. By introducing functionally distinct domains into living cells, it is expected that this peptide import approach can be employed to study and control various intracellular processes involved in signal transduction and gene transcription. The resulting information may constitute a basis for the design of therapeutic molecular drugs for tumors related to oncogenes.

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