Reduced Requirement of Mitogen-activated Protein Kinase (MAPK) Activity for Entry into the S Phase of the Cell Cycle in Swiss 3T3 Fibroblasts Stimulated by Bombesin and Insulin*

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Bombesin induced a marked and persistent activation of the mitogen-activated protein kinase kinase-1 (MEK-1), p42^mapk and p90^rsk in Swiss 3T3 cells by a pathway that was independent of p74^raf-1 but dependent on the activity of protein kinase C. Pretreatment of the cells with a specific inhibitor of MEK-1, PD 098059, markedly reduced the early and abolished the sustained phase of bombesin-induced p42^mapk activation. In addition, PD 098059 prevented bombesin-induced DNA synthesis and progression of the cells through the cell cycle, indicating that the mitogenic effect of bombesin is dependent on the activation of p42^mapk. However, in the presence of insulin, which neither stimulated p42^mapk activation nor DNA synthesis on its own in Swiss 3T3 cells, bombesin potently stimulated DNA synthesis even at concentrations of PD 098059 (15 μM) that completely abolished the mitogenic effect of bombesin alone. Furthermore, Swiss 3T3 cells stably transfected with interfering mutants of MEK-1 showed a marked decrease in the mitogenic effect of bombesin. In contrast, the combination of bombesin and insulin strongly stimulated DNA synthesis in these cells to levels comparable with that obtained in the wild type cells. Thus, our data demonstrate that insulin dramatically reduced the requirement for the mitogen-activated protein kinase pathway for reinitiation of DNA synthesis in bombesin-treated Swiss 3T3 cells and consequently indicate that the contribution of the mitogen-activated protein kinase cascade to mitogenesis depends on the combination of extracellular signals that are used to stimulate these cells.

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‡ The abbreviations used are: PKC, protein kinase C; BrdUrd, 5-bromodeoxyuridine; DMEM, Dulbecco’s modified Eagle’s medium; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; G protein, guanylate nucleotide-binding protein; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK kinase; PAGE, polyacylamide gel electrophoresis; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; GST, glutathione S-transferase.

Neuropeptides stimulate DNA synthesis and proliferation in cultured cells and are implicated as growth factors in embryogenesis, tissue regeneration, and tumorigenesis (1, 2). In particular, bombesin is a potent mitogen for quiescent Swiss 3T3 cells (3), a useful model to elucidate signal transduction pathways leading to cell proliferation (4). Bombesin binds to a seven-transmembrane receptor (5-7) and induces rapid polyphosphoinositide hydrolysis, Ca^2+ mobilization, PKC activation and tyrosine phosphorylation of focal adhesion-associated proteins, including p125^FAK (8-10). Further downstream, bombesin induces the expression of immediate early genes and subsequently stimulates DNA synthesis via PKC-dependent and -independent pathways (11).

The mitogen-activated protein (MAP) kinases are a family of highly conserved serine/threonine kinases that are activated by a range of extracellular signals (12, 13). The two best characterized isoforms p42^mapk and p44^mapk are directly activated by phosphorylation on specific tyrosine and threonine residues by the dual specificity MAPK kinase (or MEK) of which at least two isoforms have been identified in mammalian cells (14-16). Several pathways leading to MEK activation have been described. Tyrosine kinase receptors induce MAPK activation via SOS-mediated accumulation of p21^ras-GTP, which then activates a kinase cascade comprising p42^mapk, MEK, and MAPK (12, 13). In contrast, the mechanisms by which G protein-coupled receptors induce MAPK activation remain less well defined though p21^ras-GTP, and PKC-dependent pathways have been implicated (17-21). MAPK has various substrates, including transcription factors (22) and other protein kinases such as p90^rsk (23). Recent reports indicate that sustained activation of p42/p44^mapk is both necessary and sufficient to induce proliferation or differentiation of various cell lines (24-27). However, the contribution of the activation of the MAPK cascade to bombesin-stimulated DNA synthesis has as yet not been defined.

Here we report that bombesin stimulates MEK-1, p42^mapk, and p90^rsk activity in Swiss 3T3 cells through a PKC-dependent pathway. Using the selective inhibitor of MEK-1 activation, PD 098059, and Swiss 3T3 cells stably transfected with interfering mutants of MEK-1, we show that MAPK activation is essential for bombesin-stimulated DNA synthesis. However, using both experimental approaches we also show that insulin strikingly reduces the requirement of MAPK activity for reinitiation of DNA synthesis in bombesin-stimulated cells. Thus, our results demonstrate, for the first time, that the level of MAPK activity required for the transition of quiescent cells to S phase of the cell cycle depends on the combination of growth factors used to stimulate these cells.
Reduced Requirement of MAPK Activity

Membrane-Precipitable 

Microcystin LR.

The reaction was terminated by resuspending the pellet in 25 μl of kinase assay mixture containing kinase buffer, 100 μM ATP, 100 μCi/ml [γ-32P]ATP, 100 nM microcystin LR, and either 1 mg/ml MBP-peptide (APRTPGGRR) or 56 peptide (RRRLSSLRA) for the assay of p42 MAPK and p90 S6 kinase, respectively. Incubations were performed for 10 min (linear assay conditions) at 30 °C and terminated by spotting 20 μl of the supernatant onto P81 chromatography paper (Whatman). Filters were washed four times for 5 min in 0.5% orthophosphoric acid, immersed in acetone, and dried before Cerenkov counting. The average radioactivity of two blank samples containing no immune complex was subtracted from the result of each sample. The specific activity of [γ-32P]ATP used was 900-1200 cpmmol−1.

p42MAPK and p90S6 Kinase Assays—Quiescent Swiss 3T3 cells were treated as indicated and lysed in lysis buffer as above with the addition of 100 μM microcystin LR, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Immunoprecipitations were performed incubating the lysates with a polyclonal anti-p42 MAPK antibody or a 1:1 mixture of monoclonal anti-MEK-1 and anti-MEK-2 antibody or either monoclonal anti-MEK-1 or anti-MEK-2 antibody for 2 h with 40 μl of protein A-agarose (1:1 slurry) added for the second hour. Immune complexes were collected by centrifugation and washed three times in lysis buffer without phenylmethanesulfonyl fluoride and once with buffer A (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.5 mM NaF, and 1% mercaptoethanol). Pellets were then resuspended in 30 μl of MEK/MAPK buffer (30 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1% 2-mercaptoethanol, 6.5 μg/ml GST-MEK, 100 μg/ml GST-MAPK, 0.03% Brij-35, 10 mM Mg-ATP and 20 mM Na3VO4) and incubated at 30 °C for 30 min. The MEK kinase buffer did not contain GST-MEK. The reaction was then terminated by diluting the supernatant in 40 μl of buffer A containing 1 mg/ml of bovine serum albumin and, after mixing, 10 μl of the supernatant was removed to a fresh tube. MAPK activation was then measured using the MBP-peptide phosphorylation assay, essentially as described above. The specific activity of [γ-32P]ATP used was 900-1200 cpmmol−1.

Phosphorylation Incorporation Assay and Detection of BrdUrd Incorporation into Cellular DNA—Confluent and quiescent cultures of Swiss 3T3 cells were washed twice with DMEM and incubated with DMEM/Waymouth's medium (1:1 (v/v)) containing [3H]thymidine (0.25 μCi/ml, 1 μM) and various additions as described in the figure legends. After 40 h of incubation at 37 °C, cultures were washed twice with PBS and incubated in 5% trichloroacetic acid at 4 °C for 30 min to remove acid-insoluble radioactivity, washed with methanol, air-dried, and solubilized in 1 ml of 2% Na2CO3, 0.11 M NaOH, 1% SDS. The acid-insoluble radioactivity was determined by scintillation counting in 6 ml of Ultima Gold (Packard).

For detection of BrdUrd incorporated into cellular DNA, confluent and quiescent cultures of Swiss 3T3 cells were washed twice with DMEM and incubated with DMEM/Waymouth's medium (1:1 (v/v)) containing 10 μM BrdUrd and various additions as described in the figure legends. After 40 h of incubation at 37 °C, cultures were washed twice with PBS, fixed in 70% ethanol for 20 min, and incubated with anti-BrdUrd monoclonal antibody followed by labeling the monoclonal antibody with anti-mouse Ig-fluorescein isothiocyanate. Cells were examined using a Zeiss Axioshot immunofluorescence microscope, and data are expressed as the percentage of BrdUrd-labeled nuclei.

FACS Analysis—The number of cells in G0/G1, S, or G2/M phase was determined by FACS analysis. After washing three times with PBS containing 4 mM EDTA, cells were detached by treatment with trypsin (0.025%), suspended in DMEM containing 10% FBS, centrifuged at 15,000 g for 10 min at 4 °C. Immunoprecipitation was performed using a polyclonal anti-p42 MAPK antibody or a monoclonal anti-p90 S6 antibody, incubating the samples on a rotating wheel for 2 h. Protein A-agarose beads (40 μl; 1:1 slurry) were added for the supernatant hour. Immune complexes were collected by centrifugation and washed twice in lysis buffer and twice in kinase buffer (15 mM Tris-HCl, pH 7.4, 15 mM MgCl2). The kinase reaction was performed by resuspending the pellet in 25 μl of kinase assay mixture containing kinase buffer, 100 μM ATP, 100 μCi/ml [γ-32P]ATP, 100 nM microcystin LR, and either 1 mg/ml MBP-peptide (APRTPGGRR) or 56 peptide (RRRLSSLRA) for the assay of p42 MAPK and p90 S6 kinase, respectively. Incubations were performed for 10 min (linear assay conditions) at 30 °C and terminated by spotting 20 μl of the supernatant onto P81 chromatography paper (Whatman). Filters were washed four times for 5 min in 0.5% orthophosphoric acid, immersed in acetone, and dried before Cerenkov counting. The average radioactivity of two blank samples containing no immune complex was subtracted from the result of each sample. The specific activity of [γ-32P]ATP used was 900-1200 cpmmol−1.

p42MAPK and p90S6 Kinase Assays—Quiescent Swiss 3T3 cells were treated with various combinations of bombesin for 5 min (left) or with 10 nM bombesin for various times (right) as indicated. Cells were lysed and mobility shift assays (upper panels) or p42 MAPK immune complex kinase assays (lower panels) were performed as described under "Experimental Procedures." The results shown in each case are representative of three independent experiments. The positions of nonphosphorylated p42 MAPK and the slower migrating phosphorylated form of p42 MAPK in the mobility shift assay are indicated. Results of the kinase assays are the means of duplicates and are expressed as percentage of the maximum bombesin-stimulated p42 MAPK activation (5000–6000 cpm/1.5 × 106 cells at 5 min); left panel, inset: Swiss 3T3 cells were treated with either 10 nM bombesin (Bom) or 10 ng/ml PDGF (P) for 5 min, lysed, and p42 MAPK immune complex kinase assays were performed as described under "Experimental Procedures." Data shown are representative of three independent experiments each performed in duplicate and are expressed as counts/min × 104/1.5 × 106 cells. B, quiescent Swiss 3T3 cells were stimulated with 10 nM bombesin (Bom) or 10 ng/ml PDGF for 3 min. Cells were lysed, and immune complex kinase assays were performed as described under "Experimental Procedures" using anti-MEK-1/MEK-2 monoclonal antibodies. Results are expressed as percentage of the maximum PDGF-stimulated activation of MEK-1/MEK-2 (19,000–25,000 cpm/1.5 × 106 cells at 3 min), and the data shown are the mean ± S.E. of three independent experiments each performed in duplicate. C, quiescent Swiss 3T3 cells were treated with 10 nM bombesin (Bom) or 10 ng/ml PDGF for 3 min, cells were lysed, and two-step immune complex kinase assays were performed using an anti-p42 MAPK monoclonal antibody as described under "Experimental Procedures." Results are expressed as percentage of the maximum PDGF-stimulated activation of p42 MAPK (5000–5000 cpm/1.5 × 106 cells at 3 min) and are the mean ± S.E. of five independent experiments each performed in duplicate. D, quiescent Swiss 3T3 cells were treated with 10 nM bombesin for various times (left) or with various concentrations of bombesin for 5 min (right) and p90 S6 immune complex kinase assays were performed as described under "Experimental Procedures." Results are the means of duplicates, and are expressed as percentage of the maximum bombesin-stimulated activation (10,000–15,000 cpm/1.5 × 106 cells at 5 min), and are representative of three independent experiments. The specific activity of [γ-32P]ATP used in all experiments was 900-1200 cpmmol−1.
Activation of p42
mediated MAPK activation remains unclear (31). Here we
activation (32–35), but the role of this pathway in bombesin-
lation has been identified as a major pathway leading to MAPK
A, lower left panel, inset
mote p21
mobilization from intracellular stores (6, 8), but does not pro-
rase assays (data not shown).

in response to bombesin was mainly due to activation of MEK-1
/MEK-2 activity in Swiss 3T3 cells, reaching 36% of the activ-
C ). However, bombesin stimulated MEK-
kinase activity (Fig. 1

malyzed in mobility shift assays using a specific polyclonal anti-
body against p90
COOH-terminal peptide (EETARFQPGYRS) and the polyclonal anti-
body against p90
were from Amersham Corp., Amersham, UK. The MEK-1

gent of Signal Transduction, Parke Davis Research Division, Ann
Arbor, MI. The polyclonal anti-p42
mapk antibody was obtained from Santa Cruz Biotechnology Ltd., Santa
Cruz, CA. The monoclonal anti-MEK-1 and anti-MEK-2 antibodies
were obtained from Affiniti Research Products Ltd., Nottingham, UK.

Materials—The PKC inhibitor GF 109203X and microcystin LR were
obtained from Calbiochem-Novabiochem Ltd., Nottingham, UK, PDGF
(BB homodimer), 125I-labeled protein A (15 mCi/mg), and [γ-32P]ATP
(370 MBq/ml) were from Amersham Corp., Amersham, UK. The MEK-1
inhibitor PD 098059 was the generous gift of Alan R. Saltiel, Depart-
mant of Signal Transduction, Parke Davis Research Division, Ann
Arbor, MI. The polyclonal anti-p42
mapk antibody raised against a
COOH-terminal peptide (EETARFQPGYRS) and the polyclonal anti-
body against p90
were from Dr. J. Van Lint,
Katholieke Universiteit Leuven, Belgium. The polyclonal anti-p74
antibody was obtained from Santa Cruz Biotechnology Ltd., Santa
Cruz, CA. The monoclonal anti-MEK-1 and anti-MEK-2 antibodies
were obtained from Affiniti Research Products Ltd., Nottingham, UK.

GST-MEK and GST-MAPK fusion proteins were the generous gift from
Professor C. Marshall, Institute of Cancer Research, London, UK. All
other reagents were of the purest grade available.

RESULTS

Bombesin Induces Activation of MAPK, MEK-1, and
p90
To examine the effects of bombesin on p42
mapk activity, lysates of Swiss 3T3 cells treated with various concentrations of
bombesin for 5 min were analyzed by Western blotting using a specific polyclonal antibody against p42
mapk. Activation of
p42
mapk was determined by the appearance of slower migrating
forms, which results from the phosphorylation of specific threo-
line and tyrosine residues within subdomain VIII (28). Bomb-
esin induced p42
mapk activation in a concentration-dependent
manner as judged by the mobility shift assay (Fig. 1A, top left
panel). Half-maximum and maximum effects in immune com-
plex kinase assays were achieved at 0.15 and 1 nm bombesin
(Fig. 1A, lower left panel). The maximum effect of bombesin on
p42
mapk activation was comparable with that induced by 10
nm PDGF in Swiss 3T3 cells (Fig. 1A, lower left panel, inset).
Activation of p42
mapk peaked after 5 min of bombesin stimula-
tion and remained elevated above baseline levels after 3 h of
stimulation (Fig. 1A, right panel). Similar results were obtained
in mobility shift assays using a specific polyclonal antibody against p44
mapk (data not shown).

Tyrosine kinase receptors induce p42
mapk activation by a
signaling cascade involving p21
ras , p74
rsk , and MEK (12, 13).
In contrast, bombesin failed to significantly induce p74
rsk kinase activity (Fig. 1C). However, bombesin stimulated MEK-
1/MEK-2 activity in Swiss 3T3 cells, reaching 36% of the activ-
ity induced by 10 ng/ml PDGF (Fig. 1B). The activation of MEK
in response to bombesin was mainly due to activation of MEK-1
as bombesin failed to induce MEK-2 activation in MEK-2 ki-
nase assays (data not shown).

Bombesin also markedly stimulated the activity of p90
sk , a
major downstream target of MAPK (23, 29). As shown in Fig.
1D (left panel), p90
sk activation in response to bombesin was
rapid reaching its maximum after 5 min of incubation and
remained elevated to 30% of its maximum value after 1 h of
stimulation. Bombesin-induced p90
sk activity in a concentra-
tion-dependent manner; maximum stimulation in immune
complex kinase assays was achieved at 1–3 nm bombesin (Fig.
1D, right panel).

Role of PKC and MEK-1 in Bombesin-induced p42
mapk Activation—Bombesin potently stimulates PKC activation and Ca2+
 mobilization from intracellular stores (6, 8), but does not pro-
mote p21
ras activation (30, 31) in Swiss 3T3 cells. PKC stimu-
lation has been identified as a major pathway leading to MAPK
activation (32–35), but the role of this pathway in bombesin-
mediated MAPK activation remains unclear (31). Here we
show that pretreatment of Swiss 3T3 cells with the selective

FIG. 2. Role of PKC and MEK-1 in bombesin-induced p42
mapk activation. A, left: quiescent Swiss 3T3 cells were incubated with various concentrations of GF 109203X (GF) for 1 h or received an equivalent amount of solvent (–). Upper panel, the cells were subsequently stimulated with 10 nm bombesin for 5 min and lysed; the lysates were subjected to SDS-PAGE followed by Western blotting with anti-p42
mapk antibody. Shown is a representative of three independent experiments. The positions of nonphosphorylated p42
mapk and the slower migrating phosphorylated form of p42
mapk in the mobility shift assay are indicated. Lower panel, cells were stimulated with bombesin as above, lysed in lysis buffer, and lysates were immunoprecipitated with an anti-p42
mapk polyclonal antibody, and immune complex kinase
assays were performed as described under "Experimental Procedures." Results are representative of three independent experiments, are the means of duplicates, and are expressed as percentage of the maximum bombesin-stimulated p42
mapk activity (5000–6000 cpm/1.5 106 cells at 5 min). Middle and right, quiescent Swiss 3T3 cells were incubated with 3.5 μM GF 109203X for 1 h, stimulated with 10 nm bombesin for 5 min (middle, filled bars) or 3 min (right, filled bars) and p90
sk (middle) or MEK-1 (right) immune complex kinase assays were performed as described under "Experimental Procedures." Results are the means of three independent experiments ± S.E. (for p90
sk) or two independent experiments (for MEK-1), each performed in duplicate and are expressed as percentage of the maximum bombesin-stimulated activity (10,000–15,000 cpm/1.5 106 cells at 5 min) for p90
sk and 19,000–25,000 cpm/1.5 106 cells for MEK-1). B, upper panel: quies-
cent Swiss 3T3 cells were washed and incubated for 2 h at 37°C with
various concentrations of PD 098059 (PD) as indicated or received an
equivalent amount of solvent (–). The cells were subsequently stimu-
lated with 10 nm bombesin for 5 min and lysed. The lysates were then
subjected to SDS-PAGE followed by Western blotting with anti-p42
mapk antibody. Shown is a representative of three independent experiments. The positions of nonphosphorylated p42
mapk and the slower migrating phosphorylated form of p42
mapk are indicated. Lower panel, quiescent cells were treated with 15 μM PD 098059 for 2 h (triangles) or received an equivalent amount of solvent (circles). Cells were subsequently stimu-
lated with 10 nm bombesin for various times as indicated. Lower panel,
insert: quiescent Swiss 3T3 cells were incubated for 2 h with various
concentrations of PD 098059 (PD) as indicated and subsequently stimu-
lated with 10 nm bombesin (Bom) for 5 or 60 min. In all cases cells were
lysed and p42
mapk immune complex kinase assays were performed as
described under "Experimental Procedures." The results are the means
of duplicates, are expressed as percentage of the maximum bombesin-
stimulated p42
mapk activation (5000–6000 cpm/1.5 106 cells at 5
min), and are representative of three independent experiments.
PKC inhibitor GF 109203X (9, 36, 37) prevented both the mobility shift of p42\textsuperscript{mapk} (Fig. 2A, upper left panel) and the increase in p42\textsuperscript{mapk} activity in immune complex kinase assays in response to bombesin. The effect of GF 109203X was concentration-dependent; a complete inhibition of bombesin-induced p42\textsuperscript{mapk} activation was observed at 5 \( \mu \text{M} \) GF 109203X (Fig. 2A, left). Similar results were obtained when PKC was down-regulated by chronic treatment with PDB (results not shown), in agreement with previous data (38).

Pretreatment of Swiss 3T3 cells with GF 109203X or down-regulation of PKC by prolonged pretreatment with PDB also prevented MEK-1 and p90\textsuperscript{rsk} activity in response to bombesin (Fig. 2A and results not shown). In contrast, inhibition of Ca\textsuperscript{2+} influx or mobilization from intracellular stores did not affect bombesin-mediated activation of p42\textsuperscript{mapk} or p90\textsuperscript{rsk} (results not shown). Thus, PKC activation is a major signaling pathway leading to MAPK activation in bombesin-treated cells, possibly through a MEK-1-dependent pathway.

To further examine whether MEK-1 is the main upstream regulator of bombesin-induced MAPK activity, quiescent Swiss 3T3 cells were treated with PD 098059, a recently identified compound that selectively inhibits MEK-1 activation (39, 40). PD 098059 prevented the mobility shift of p42\textsuperscript{mapk} in response to bombesin (Fig. 2B, upper panel) and markedly decreased the early (5 min) and the late phase (60 min) of bombesin-stimulated p42\textsuperscript{mapk} activity in immune complex kinase assays in a concentration-dependent manner (Fig. 2B, lower panel, inset). A maximum effect was achieved at 15 \( \mu \text{M} \) PD 098059; at this concentration PD 098059 reduced the early phase of bombesin-induced p42\textsuperscript{mapk} activation by 60% and caused an almost complete inhibition of the sustained activation of p42\textsuperscript{mapk} (Fig. 2B, lower panel and inset). These findings indicate that PD 098059 is a potent inhibitor particularly of the sustained phase of bombesin-mediated p42\textsuperscript{mapk} activation in Swiss 3T3 cells.

Role of Activation of p42\textsuperscript{mapk} in Bombesin-stimulated DNA Synthesis—It has been reported that the sustained phase of p42\textsuperscript{mapk} activation is necessary for the induction of DNA synthesis by growth factors in fibroblasts (24, 27). Therefore, we examined the contribution of the MAPK pathway to bombesin-stimulated reinitiation of DNA synthesis in Swiss 3T3 cells using PD 098059. Quiescent cultures of these cells were stimulated with 10 \( \text{nm} \) bombesin in the presence of increasing concentrations of PD 098059. Cumulative \([\text{H}]\) thymidine incorporation was measured after 40 h of incubation. As shown in Fig. 3A (closed circles), PD 098059 dramatically decreased bombesin-induced DNA synthesis in a concentration-dependent manner. Half-maximum inhibition was achieved at 1 \( \mu \text{M} \) PD 098059, and treatment of the cells with 15 \( \mu \text{M} \) PD 098059 completely blocked DNA synthesis induced by bombesin (Fig. 3A, closed circles). We verified that \([\text{H}]\) thymidine incorporation in response to bombesin was virtually abolished by 15 \( \mu \text{M} \) PD 098059 at all times examined up to 48 h of incubation (results not shown). Thus, the activity of MEK-1-dependent MAPKs is essential for bombesin-induced mitogenesis.

We also examined the effect of PD 098059 on \([\text{H}]\) thymidine incorporation in Swiss 3T3 cells stimulated by bombesin in the presence of insulin, which is not a sole mitogen for these cells, but potentiates the mitogenic activity of bombesin (3). A salient feature shown in Fig. 3A (open circles) is that \([\text{H}]\) thymidine incorporation induced by the combination of bombesin and insulin was only slightly inhibited by PD 098059 at a concentration (15 \( \mu \text{M} \)) that completely abolished bombesin-induced DNA synthesis. The kinetics of \([\text{H}]\) thymidine incorporation stimulated by bombesin and insulin was comparable with that induced by these factors in the presence of 15 \( \mu \text{M} \) PD 098059, reaching a maximum after 36-40 h of incubation (results not shown). Reduced Requirement of MAPK Activity

Fig. 3. Role of activation of p42\textsuperscript{mapk} in bombesin-stimulated DNA synthesis. A, confluent and quiescent cultures of Swiss 3T3 cells were washed and incubated at 37 °C in 2 ml of DMEM/Waymouth’s medium containing 1 \( \mu \text{Ci/ml} \) [\text{H}] thymidine and either 10 \( \text{nm} \) bombesin (closed circles) or 10 \( \text{nm} \) bombesin together with 0.5 \( \mu \text{g/ml} \) insulin (open circles) in the presence of increasing concentrations of PD 098059 as indicated. After 40 h, DNA synthesis was assessed by measuring the [\text{H}] thymidine incorporated into acid-precipitable material. The solvent dimethyl sulfoxide (up to 0.1%) did not affect the measurements of thymidine incorporation. Results are expressed as percentage of the maximum incorporation of [\text{H}] thymidine induced by 10 \( \text{nm} \) bombesin (45,000–50,000 cpm/3 \( \times \) 10\textsuperscript{5} cells, closed circles) or 10 \( \text{nm} \) bombesin with 0.5 \( \mu \text{g/ml} \) insulin (110,000–130,000 cpm/3 \( \times \) 10\textsuperscript{5} cells, open circles), respectively, and are the means ± S.E. of four independent experiments each performed in duplicate. Where no error bars are shown they lie within the boundaries of the symbol. B, quiescent Swiss 3T3 cells were preincubated either with 15 \( \mu \text{M} \) PD 098059 (PD, +) or with an equivalent amount of solvent for 2 h (–). The cultures were either lysed immediately (open bars) or stimulated with either 0.5 \( \mu \text{g/ml} \) insulin (Ins, shaded bars), 10 \( \text{nm} \) bombesin (Bom, filled bars), or 10 \( \text{nm} \) bombesin with 0.5 \( \mu \text{g/ml} \) insulin (Bom + Ins, hatched bars) for 5 or 60 min as indicated. Cells were lysed, and p42\textsuperscript{mapk} (immunecomplex kinase assays were performed as described under “Experimental Procedures.” Results are the means of three independent experiments ± S.E., each performed in duplicate and are expressed as percentage of the maximum bombesin-stimulated p42\textsuperscript{mapk} activation (5000–6000 cpm/1.5 \( \times \) 10\textsuperscript{5} cells at 5 min). C, quiescent cultures of Swiss 3T3 cells were washed and incubated at 37 °C in 2 ml of DMEM/Waymouth’s medium containing 1 \( \mu \text{Ci/ml} \) [\text{H}] thymidine and either 10 \( \text{nm} \) bombesin (closed circles) or 10 \( \text{nm} \) bombesin together with 0.5 \( \mu \text{g/ml} \) insulin (open circles) in the presence of various concentrations of PD 098059 as indicated (lines). After 40 h, DNA synthesis was assessed as described above. Parallel cultures of Swiss 3T3 cells were incubated with various concentrations of PD 098059 as indicated for 2 h and subsequently stimulated with 10 \( \text{nm} \) bombesin for 5 min (left panel) or 60 min (right panel) in the absence (closed circles) or in the presence (open circles) of 0.5 \( \mu \text{g/ml} \) insulin. Cells were lysed and p42\textsuperscript{mapk} (immunecomplex kinase assays were performed as described under “Experimental Procedures.” Data are expressed as percentage of the maximum [\text{H}] thymidine incorporation induced by 10 \( \text{nm} \) bombesin (45,000–50,000 cpm/3 \( \times \) 10\textsuperscript{5} cells) as a function of the percentage of the maximum bombesin-stimulated p42\textsuperscript{mapk} activation at 5 min (5000–6000 cpm/1.5 \( \times \) 10\textsuperscript{5} cells). The figures are the means of duplicate determinations and are representative of three independent experiments.
Reduced Requirement of MAPK Activity

A. quiescent cultures of Swiss 3T3 cells in 33-mm dishes were washed with DMEM and incubated at 37°C with DMEM/Waymouth’s medium containing 10 μM BrdUrd and either 10 nM bombesin (Bom) or 10 nM bombesin with 0.5 μg/ml insulin (Ins) in the presence or absence of 15 μM PD 098059 (PD) for 40 h. Detection of BrdUrd incorporated into cellular DNA was performed as described under “Experimental Procedures.” The images are representative of three independent experiments. B. cells were treated as described in A. The data shown are expressed as the percentage of BrdUrd-positive nuclei in at least three independent microscopic fields per condition and are the means ± S.E. of three independent experiments. Where no error bars are shown they lie within the boundaries of the columns. C. quiescent Swiss 3T3 cells in 100-mm dishes were washed and incubated at 37°C for 10 ml of DMEM/Waymouth’s medium containing 10 nM bombesin (Bom), 0.5 μg/ml insulin (Ins), or both in the absence or presence (+) of 15 μM PD 098059 (PD) for 40 h. Cells were then analyzed by FACS as described under “Experimental Procedures.” The number of cells is represented on the y axis, and the relative DNA content in each cell (fluorescence intensity) is shown on the x axis. The brackets (from left to right) indicate the position of the cells in the cell cycle: G0/G1, S, and G2/M, respectively. Similar results were obtained in three independent experiments. The fraction of cells in S + G2 + M (mean ± S.E.) in each experimental condition was: 5.7 ± 0.7 control (-); 4.2 ± 0.3, insulin; 36.7 ± 1.1, bombesin; 13.4 ± 0.7, bombesin + PD 098059; 69.5 ± 1.1, bombesin + insulin; 70.5 ± 0.6, bombesin + insulin + PD 098059.

shown). Insulin also partially reversed the inhibition of DNA synthesis by 15 μM PD 098059 in Swiss 3T3 cells stimulated with bombesin at 1 nM instead of 10 nM (results not shown).

Recently, Alessi et al. (40) demonstrated that the inhibitory effect of PD 098059 on the activation of p42 MAPK depends on the strength of activation of p74 raf-1 and MEK by growth factors. We therefore examined whether insulin could synergize with bombesin in MAPK activation, thereby reversing the inhibition of p42 MAPK activity and DNA synthesis by PD 098059. As shown in Fig. 3B, insulin neither induced p42 MAPK activation in Swiss 3T3 cells nor potentiated p42 MAPK activation induced by 10 nM bombesin. Insulin slightly reversed the inhibitory effect of PD 098059 on the early phase of p42 MAPK activation induced by bombesin. However, PD 098059 inhibited the late phase of bombesin-stimulated p42 MAPK activation virtually to the same degree in the absence or presence of insulin (Fig. 3B).

The conclusions drawn from Fig. 3, using [3H]thymidine incorporation, were further substantiated by experiments in which DNA synthesis in Swiss 3T3 cells was determined using either an immunofluorescence assay to detect BrdUrd incorporated into cellular nuclei or FACS analysis. As shown in Fig. 4A, treatment with 15 μM PD 098059 markedly reduced the proportion of BrdUrd-labeled nuclei in response to bombesin (from 64 to 15%). In contrast, DNA synthesis induced by bombesin and insulin (98% stained nuclei) was virtually undiminished by treatment with 15 μM PD 098059 (Fig. 4, A and B).

DNA Synthesis in Swiss 3T3 Cells Stably Overexpressing...
Reduced Requirement of MAPK Activity

Fig. 5. Interfering mutants of MEK-1 impair DNA synthesis in response to bombesin, but not in response to bombesin and insulin. Top of each panel: confluent and quiescent cultures of untransfected Swiss 3T3 cells (3T3) or those overexpressing wild type (WT) or mutant MEK-1 with alanine for serine substitutions (Ala217 and Ala221) were washed and treated with 4 nM bombesin (Bom) for 5 min at 37 °C. Cells were subsequently lysed in SDS sample buffer and analyzed by Western blotting with anti-p42\textsuperscript{mapk} polyclonal antibody. The positions of nonphosphorylated p42\textsuperscript{mapk} and the slower migrating phosphorylated form of p42\textsuperscript{mapk} are indicated. Lower part of each panel: confluent and quiescent cultures of untransfected Swiss 3T3 cells (3T3) or those overexpressing wild type (WT) or mutant MEK-1 (Ala217 and Ala221) were washed and incubated at 37 °C in 2 ml of DMEM/Waymouth’s medium containing 1 μCi/ml \[^{3}H\]thymidine and either 2 or 4 nM bombesin (Bom, filled bars) or 2 or 4 nM bombesin together with 0.5 μg/ml insulin (Ins, +, hatched bars). Control cells received an equivalent amount of solvent (−). After 40 h, DNA synthesis was assessed by measuring the \[^{3}H\]thymidine incorporated into acid-precipitable material. Results are expressed as percentage of the maximum incorporation of \[^{3}H\]thymidine induced by 10% FBS (110,000–130,000 cpm/3 × 10\(^5\) cells) and are representative of three independent experiments each performed in duplicate.

Interfering Mutants of MEK-1 Is Blocked in Response to Bombesin, but Not in Response to Bombesin and Insulin—Expression of interfering MEK-1 mutants with alanine substitutions at serine 217 or serine 221 have been shown to block MAPK activation in vivo (27). If, as indicated by the preceding results, bombesin induces DNA synthesis through a MEK-1-dependent MAPK pathway in Swiss 3T3 cells, expression of interfering mutants of MEK-1 should prevent bombesin-induced \[^{3}H\]thymidine incorporation. To test this prediction, we employed Swiss 3T3 cells overexpressing wild type MEK-1 and Ala\(^{217}\) and Ala\(^{221}\) mutants to comparable levels (41). To verify that the level of expression of these mutants interfered with p42\textsuperscript{mapk} activation in Swiss 3T3 cells, we performed mobility shift assays using lysates of these three cell subtypes treated with bombesin or solvent. As shown in Fig. 5, p42\textsuperscript{mapk} activation in cells overexpressing wild type MEK-1 was similar to that achieved with bombesin in untransfected cells. However, both the Ala\(^{217}\) and Ala\(^{221}\) mutants partially inhibited bombesin-induced p42\textsuperscript{mapk} activation in the mobility shift assays.

As shown in Fig. 5 (upper panels), the levels of \[^{3}H\]thymidine incorporation induced by bombesin at either 2 or 4 nM in cells overexpressing the wild-type MEK-1 were comparable with those in untransfected cells and further enhanced by the presence of insulin. In contrast, the ability of bombesin to stimulate DNA synthesis was completely abolished in cells overexpressing the interfering mutants (Fig. 5, lower panels). The striking finding, however, was that in the presence of insulin, which did not induce significant \[^{3}H\]thymidine incorporation on its own, bombesin-stimulated \[^{3}H\]thymidine incorporation in these cells was comparable with that in untransfected cells or those overexpressing wild-type MEK-1 (Fig. 5, lower panels).

DISCUSSION

While a large number of studies have been dedicated to the dissection of the upstream pathways leading to MAPK activation by a wide variety of extracellular stimuli, the precise role of this kinase cascade in the transition of quiescent cells to the S phase of the cell cycle induced by defined mitogens added singly or in combination has been much less explored.

In the present study we examined the contribution of the MAPK pathway to the stimulation of DNA synthesis induced by bombesin in Swiss 3T3 cells using two different experimental approaches. First, we found that the specific MEK-1 inhibitor, PD 098059, dramatically inhibited bombesin-stimulated DNA synthesis and progression through the cell cycle as judged by three different assays, including FACs analysis. In fact, a modest inhibition of p42\textsuperscript{mapk} activation severely reduced bombesin-mediated DNA synthesis. Second, interfering mutants of MEK-1 stably transfected into Swiss 3T3 cells also provided convincing evidence that MEK-1 activation is essential for DNA synthesis induced by bombesin. Given that bombesin predominantly stimulates MEK-1 activity in Swiss 3T3 cells (Fig. 1) and p42\textsuperscript{mapk} and p44\textsuperscript{mapk} are the only known substrates for MEK-1 (27), the inhibitory effects on cellular DNA synthesis described above occur most likely at the level of activation of MAPK. Thus, our results show that a high level of p42\textsuperscript{mapk} activity is crucial for bombesin-stimulated mitogenesis.

Phorbol ester-sensitive PKC isoforms play a critical role in bombesin-mediated DNA synthesis (3). Since activation of MEK-1, p42\textsuperscript{mapk}, and p90\textsuperscript{rsk} is downstream of PKC (Fig. 2) and MEK-1 activation is essential for DNA synthesis (Figs. 3–5), it is conceivable that a major function of PKC in bombesin-stimulated mitogenesis is to activate the MAPK cascade. Interestingly, bombesin, in the presence of insulin, is known to stimulate DNA synthesis through a PKC-independent pathway (11), although insulin neither induces p42\textsuperscript{mapk} activation nor potentiates the stimulation of this pathway by bombesin (Fig. 3). These considerations raise important questions regarding the requirement of p42\textsuperscript{mapk} activity for the stimulation of DNA synthesis induced by the combination of bombesin and insulin.

Our results demonstrate that DNA synthesis induced by bombesin and insulin was only slightly inhibited by PD 098059 added at concentrations that completely abolished DNA synthesis in response to bombesin alone. Furthermore, bombesin failed to induce DNA synthesis in Swiss 3T3 cells stably transfected with interfering mutants of MEK-1, whereas addition of insulin together with bombesin produced a marked stimulation of DNA synthesis in these cells. Thus, using two independent experimental approaches, our results indicate that the requirement of the MAPK cascade for DNA synthesis is strikingly reduced in Swiss 3T3 cells stimulated with bombesin and insulin.
The results presented here have several important implications. Recently, Cowley et al. (27) emphasized the importance of the kinetics of MAPK activation and the cellular context in defining the role of the MAPK pathway in the production of biological responses. Our results provide a novel insight into the role of the MAPK cascade in cellular mitogenesis, demonstrating, for the first time, that the level of MAPK activity required for the transition of quiescent cells to the S phase of the cell cycle depends on the combination of growth factors used to stimulate the cells. Since each cell in a multicellular organism is exposed physiologically to multiple growth regulatory and differentiation signals, we suggest that the level of MAPK activity required for cellular mitogenesis is likely to depend on the repertoire of extracellular signals that interact with the cell at any given time. This concept is relevant for the development of antiproliferative drugs directed against the MAPK pathway. It has been proposed that MEK-1 could be a useful target to select drugs or other agents capable of inhibiting cell proliferation (27). In view of the results presented in this study, we predict that the potency of MEK-1 inhibitors as blockers of cell proliferation will depend dramatically on the combination of mitogenic signals that interact with the target cell.

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Reduced Requirement of Mitogen-activated Protein Kinase (MAPK) Activity for Entry into the S Phase of the Cell Cycle in Swiss 3T3 Fibroblasts Stimulated by Bombesin and Insulin

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