Oncogenic Raf-1 Activates p70 S6 Kinase via a Mitogen-activated Protein Kinase-independent Pathway*

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Cell proliferation requires the co-ordinate triggering of several protein kinases of Ser/Thr specificity such as p70 S6 kinase (S6K), which phosphorylates the ribosomal S6 protein and thus increases translation of mRNAs with polypyrimidine tracts. The multiplicity of signaling pathways leading to p70 S6K activation is not fully elucidated. However, several reports have indicated that the activation of p70 S6K is independent of mitogen-activated protein kinase (MAPK) activation. Interestingly, we and others have shown that constitutive activation of the MAPK pathway promotes cell proliferation, suggesting that this cascade is able to activate p70 S6K, a key step to trigger cell cycle entry. In this report we demonstrate that transfection of constitutively active mitogen-activated protein kinase kinase 1, MAPK, and p70 S6K. Surprisingly, the activation of p70 S6K is not mediated by MAPK because blocking MAPK activation by expression of the phosphatase MKP-1 did not prevent p70 S6K activation by blocking MAPK activation by expression of the phosphatase MKP-1. Hence, it seems that several signaling pathways are able to activate p70 S6K.

An additional class of protein kinases rapidly activated by all mitogens are the p42/p44 MAPK. Previous work from our group has demonstrated that activation of p42/p44 MAPK is an absolute requirement for fibroblasts to progress from G0 to S-phase. This was shown by expressing either p44 MAPK an antisense or dominant-negative MAPK (p44 MAPK-TA) mutant (12), p42/p44 MAPK is activated by direct phosphorylation by MKK1 (13), which itself is phosphorylated and activated by Ras (reviewed in Refs. 14–16). Ras is recruited to the membrane by a protein-coupled receptors (17) or tyrosine kinase receptors (18). Activation of p42/p44 MAPK induces pleiotropic effects (reviewed in Ref. 16) ranging from the phosphorylation of membrane bound proteins such as c-phospholipase A2 (19) to the transcription factors such as Elk-1 (20) following nuclear translocation of both p42 and p44 isoforms (21).

Interestingly, we and others have shown that permanent activation of the p42/44 MAPK pathway upon expression of a constitutively active form of MKK1 (MKK1-SSDD) promotes cell cycle entry and oncogenicity (22–24). This finding suggests that this cascade should activate all the steps necessary to trigger cell proliferation, such as activation of p70 S6K. Indeed, the first experiment of this report will show that transfection of MKK1-SSDD is sufficient to activate p70 S6K. This result is in accordance with the observation that v-Ras transformed cells...
have increased phosphorylation of S6 due to p70 S6K activation (25), but it is in contradiction with several studies that led to the conclusion that the MAPK and p70 S6K signaling pathways are independent. It was shown that firstly MAPK does not phosphorylate in vitro p70 S6K (26), secondly some agonists that fully activate MAPK have little or no effect on the activation of p70 S6K and vice versa (26), thirdly the transfection of a dominant negative form of Ras prevents MAPK activation by epidermal growth factor or O-tetradeacanoylphorol 13-acetate without affecting p70 S6K activation (11), and fourthly, transfection of dominant negative mSOS1 is able to block insulin-mediated activation of Ras and of MAPK but has no effect on p70 S6K stimulation (27).

The activation of p70 S6K by the constitutively active form of MKK1 that we report could be due to the triggering of an autocrine loop. In order to exclude this possibility we have examined the activity of p70 S6K following the rapid activation of the Raf-MAPK cascade through an estradiol-regulated form of oncogenic Raf-1 (28, 29). We have demonstrated that Raf-1 is capable of activating p70 S6K by a MAPK-independent pathway.

**EXPERIMENTAL PROCEDURES**

**Materials—**Wortmannin, estradiol, horseradish peroxidase-conjugated anti-rabbit IgG, and myelin basic protein were obtained from Sigma. Thrombin was kindly provided by D. J. W. Fenton II (New York State Department of Health, Albany NY), and [γ-32P]ATP was purchased from ICN; ECL and Hybond C extra supported membranes were from Radiochemical Centre (Amersham Corp.). Protein A-Sepharose CL4B was obtained from Pharmacia Biotech Inc.; 12CA5 monoclonal antibody that recognizes the HA epitope was from BABCO (Emeryville, CA). PS04 monoclonal antibody against the VSVG epitope (30) was provided by Dr. B. Gould, Institut Pasteur, Paris; 9E10 monoclonal antibody that recognizes the Myc epitope was provided by Dr. G. Evan, ICRF London. All other chemicals were of the highest purity available.

The polyclonal antibody M5 that immunoprecipitates specifically p70 S6K and the p70 S6K substrate (Ribosomal S6 protein) was kindly provided by Dr. G. Thomas (Friedrich Miescher Institute, Basel, Switzerland). Dr. F. R. McKenzie kindly provided the rabbit antibody that recognizes specifically p42 and p44 MAPK on Western blot (E18, directed against the peptide coding for the 14 amino acids of the C-terminal end of hamster p44-MAPK) and the rabbit antibody that immunoprecipitates MAPK (Kelly, directed against bacterially produced GST-hamster p44 MAPK). The rabbit antibody that immunoprecipitates specifically MKK1 (MKK1(16)) is identical to that previously reported (12).

**Cell Lines and Culture—**Chinese hamster Lung fibroblasts CCL39 were maintained in Dulbecco's modified Eagle's medium (H21 reference number 52100, Life Technologies, Inc.) containing 25 mM NaHCO3. The derived CCL39-r Raf-1:ER done was maintained in H21 medium without phenol red, supplemented with glutamine and glucose to reach the concentrations of normal H21 (H21 without phenol red, reference number 11880). Both culture medium were supplemented with 7.5% fetal calf serum (Life Technologies, Inc.), penicillin (50 units/ml), and streptomycin (50 μg/ml). Cells were maintained at 37°C in the presence of 5% CO2.

The CCL39-r Raf-1:ER clonal cell line was obtained by transfection of CCL39 cells with the plasmid pLNCr Raf-1:ER (28) and selection of clones resistant to Geneticin (G418). The clone that displayed the highest stimulation of MAPK activity upon estradiol addition was selected and re-cloned.

**Expression Vectors and Transfection Procedures—**The plasmid p44 MAPK-VSVG is constructed from the hamster cDNA of p44 MAPK tagged with the VSVG epitope at its C terminus (21). The plasmids p44 MAPK and p44 MAPK-T192A are derived from the hamster cDNA of p44 MAPK as described previously (12). The plasmid p70 S6K-Myc kindly provided by Dr. G. Thomas (Friedrich Miescher Institute, Basel, Switzerland) is constructed from the human cDNA of p70 S6K tagged with the Myc-9E10 epitope at its C terminus (31). The human cDNA of the phosphatase MKP-1 kindly provided by Dr. S. Keyse (32) was subcloned in the expression vector pcDNAneo (Invitrogen, San Diego, CA) by J.-M. Bronsello.

The cells were stably transfected using the calcium phosphate method and selected in Geneticin (500 μg/ml). Cells were seeded at 7 × 10^4 in a 35-mm dish for transient transfection. 24 h later, cells were incubated with the calcium-phosphate DNA precipitate, the precipitate was removed, and the cells were allowed to recover for 12 h in the presence of 7.5% FCS. Finally the cells were serum deprived for 4 h prior to subsequent stimulation.

**Western Blotting and SDS-PAGE Analysis of Proteins—**Confluent cells were lysed in lysis buffer (0.1% Triton, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 40 mM paranitrophosphophosphate, and 200 μg/ml orthovanadate). 25 μg of detergent-extracted proteins were separated by SDS-PAGE on 10% (w/v) polyacrylamide gel (29:1) and electrophoretically transferred to Hybond-C membranes and immuno-probed as described previously (33).

**Kinase Assays—**MKK1 activity was assayed as described previously (22). Briefly, the substrate is provided by immunoprecipitated p44 MAPK-HA from a non-stimulated over-expressor cell line. The substrate is incubated for 30 min with immunoprecipitated endogenous MKK1 (with the specific antibody MKK1(16) (21)) at 30°C in presence of 30 μM ATP (3 μCi of [γ-32P]ATP). Proteins were separated on SDS-PAGE (10% acrylamide gel). Measure of MAPK (33) and p70 S6K (34) activity was assayed as described previously:

Cells were lysed in 0.5 ml of lysis buffer (120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 15 mM NaPPi, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.5 mM dithiothreitol, and 0.2 mM orthovanadate). Cells remaining attached on the plate were scraped, and the extract was sonicated and centrifuged at 12,000 x g for 10 min. 350 μl of the supernatant wa h of incubation with the specific antibody and protein A-Sepharose, the immune complex was washed five times in lysis buffer and one time in kinase buffer without substrate. Immunoprecipitated MAPK was incubated 20 min at 30°C in the following buffer: 20 mM Hapes, pH 7.4, 10 mM MgCl2, 1 mM MnCl2, 1 mM dithiothreitol, 20 mM paranitrophosphophate, 0.25 mg/ml myelin basic protein, and 30 μM ATP (3 μCi of [γ-32P]ATP). Immunoprecipitated p70 S6K was incubated for 30 min at 37°C in the following buffer: 50 mM MOPS, pH 7.2, 5 mM MgCl2, 1 mM dithiothreitol, 10 mM paranitrophosphophate, 30 μM ATP (3 μCi of [γ-32P]ATP), and 2 mg/ml 40 S ribosomal subunit (35).

**RESULTS**

Expression of Constitutively Active MKK1 (MEK) Stimulates p70 S6K Activity—We have shown previously that mutation of MKK1 on its two phosphorylation sites Ser18 and Ser22 to two aspartic residues (MKK1-SSDD) is sufficient to permanently activate p42/44 MAPK (22). Expression of MKK1-SSDD into the established cell line CCL39 (Chinese hamster lung fibroblasts) increased the probability of cell cycle entry in the absence of exogenously added growth factors (22). It was therefore of interest to analyze the effects of MKK1-SSDD on the activity of p70 S6K as activated of this protein kinase is required for cell proliferation. As shown in Fig. 1, growth-arrested CCL39 cells co-transfected with MKK1-WT and epitope-tagged reporter kinases, display a low level of both p44 MAPK (see Fig. 3A) and p70 S6K (see Fig. 3B) activities (lane 1), whereas the addition of serum for 1 h markedly increased their activities (lane 2). Interestingly, serum-deprived cells (lane 3) co-transfected with MKK1-SSDD display an elevated level of p44 MAPK and p70 S6K activities equivalent to the levels reached by stimulation with 10% FCS for 1 h (lane 4). Hence, the expression of the constitutively active MKK1-SSDD is sufficient to promote activation of both p42/44 MAPK and p70 S6K.

The stimulation of p70 S6K by MKK1-SSDD could be either a primary effect mediated by its only known substrate p42/44 MAPKs or a secondary effect due to the triggering of an autocrine loop subsequent to the persistent activation of the MAPK cascade.

To resolve this question, we sought a means to conditionally and rapidly stimulate MAPK in vivo by acting downstream of membrane receptors. An interesting approach is provided by fusion of the hormone binding domain of the estradiol receptor to oncogenic forms of Raf kinases, which gives rise to fusion proteins whose activities are exquisitely controlled by the con-
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Concentration of estradiol in the growth medium (28, 29). As shown previously in a variety of cell types, activation of ΔRaf-1:ER leads to the activation of MKK1 and MAPK minutes after the addition of estradiol to cells (28).

Characterization of the Stable Cell Line CCL39-ΔRaf-1:ER—A stable clone of CCL39 cells expressing ΔRaf-1:ER was isolated and is hereafter referred to as CCL39-ΔRaf-1:ER. The addition of estradiol to the parental cell line CCL39 did not affect the activity of MAPK nor p70 S6K, even at concentrations as high as 10 μM (data not shown). In contrast the addition of 1 μM of estradiol to CCL39-ΔRaf-1:ER cell leads to the rapid activation of MKK1 activity, as can be seen by the increase in phosphorylation of its substrate p44 MAPK (Fig. 2A, lanes 3–8). The stimulation of MKK1 activity is detectable as early as 2 min following ΔRaf-1:ER activation (lane 4), reaches its maximum 15 min following agonist addition (lane 6) at a level comparable with that of serum stimulation (compare lane 2 and lane 6). The level of MKK1 activation following activation of ΔRaf-1:ER is maintained for at least 1 h (lane 8).

In Fig. 2B, activation of ΔRaf-1:ER induces the mobility shift-up of both p42 and p44 MAPK in a time-dependent manner. This retardation of MAPK mobility in SDS-PAGE has been shown to result from phosphorylation of the two activation sites of MAPK and strictly correlates with MAPK activation (36). The activation of MAPK is detected 5 min following activation of ΔRaf-1:ER (Fig. 2B, lane 4) and reaches its maximum 15 min later (lane 5). A number of experiments indicated that MAPK activation was maximal after 15–30 min. In most experiments, such as this one, the level of MAPK activation upon ΔRaf-1:ER activation is comparable with that observed following serum stimulation (Fig. 2B compare lanes 2 and 7) and persists for several hours (data not shown). Concentrations of estradiol as low as 10 nM elicited an upward mobility shift of p42 and p44 MAPK in SDS-PAGE (data not shown). The maximal shift of MAPK is observed with concentrations of 100 nM or higher (data not shown). This effect is specific because the addition of estradiol to the parental CCL39 cell line does not affect MAPK activity (data not shown).

Time Course of p70 S6K and MAPK Activation Following ΔRaf-1:ER Activation by Estradiol—The enzymatic activities of p44 MAPK (Fig. 3A) and p70 S6K (Fig. 3B) were measured in a single cell lysate obtained from CCL39-ΔRaf-1:ER cells exposed to either 10% FCS or 1 μM estradiol for increasing periods of time. Induction of p44 MAPK activity following serum stimulation is rapid, reaching its maximum within 5 min (Fig. 3A, lane 2). Maximal activation persists for 60 min (Fig. 3A, lane 5) and decreases markedly 3 h post-stimulation (lane 6). The induction of p70 S6K activity by serum shows a similar profile; maximum activation is obtained 5 min following stimulation (Fig. 3B, lane 2) and remains activated for at least 3 h (Fig. 3B, lane 6).

One must note that in this particular experiment, cells with high passage number were used, and the level of MAPK activation reached by ΔRaf-1:ER activation is lower than that obtained with serum (Fig. 3B, compare lane 9 with lane 3). Nonetheless ΔRaf-1:ER stimulates MAPK within 15 min, and it is worth emphasizing that ΔRaf-1:ER activation is sufficient to activate p70 S6K. This effect is detectable 30 min post-stimulation (Fig. 3B, lane 10). In this experiment the level of p70 S6K stimulation obtained by activation of ΔRaf-1:ER is lower than that obtained by serum (Fig. 3B, compare lanes 5 and 11, for example). In fact, the extent of p70 S6K activation by ΔRaf-1:ER compared with that obtained upon stimulation with FCS varied somewhat within experiments, but the appearance of p70 S6K activation by ΔRaf-1:ER activation was reproducibly detected within 30 min, and the extent of MAPK and p70 S6K activation seemed to be coordinated (data not shown).

Comparison between ΔRaf-1:ER Activation and FCS Stimulation to Activate p70 S6K—We compared the activation of MAPK and p70 S6K by ΔRaf-1:ER with that obtained by the stimulation with increasing amounts of FCS (Fig. 4). In several experiments such as this one (with cells of lower passage num-
The kinase assays were performed as described in the legend to Fig. 3. The two kinase assays were performed from the same cell extract as activity was assayed on myelin basic protein. MAPK was immunoprecipitated with the antibody Kelly, and its kinase activity of the co-expressed reporter kinase p70 S6K-Myc (2 μg). Serum stimulation of p70 S6K-Myc activity is not affected by expression of the dominant-negative kinase p44 MAPK-T192A (Fig. 5, compare lane 2 with lane 5). This result is consistent with reports indicating that p70 S6K activation is independent from that of MAPK (11, 26). Similarly the level of p70 S6K stimulation by ΔRaf-1:ER activation is not impeded by expression of p44 MAPK-T192A (Fig. 5, compare lanes 3 and 6). These results indicate that serum and ΔRaf-1:ER activation increase p70 S6K activity independently of MAPK activation.

MKP-1 Over-expression Blocks MAPK Stimulation by ΔRaf-1:ER without Affecting p70 S6K Activation—As an independent and perhaps more potent approach to block the MAPK cascade, we co-transfected the MAPK-specific phosphatase, MKP-1, in conjunction with the reporter kinases p44 MAPK-VSVG and p70 S6K-Myc. MKP-1 has been shown in vivo and in vitro to dephosphorylate and inactivate MAP kinases (37).

In cells co-transfected with the control vector (Fig. 6A, lanes 1–3) estradiol or 10% FCS induced a marked activation of p44 MAPK-VSVG over control (compare lane 1 with lanes 2 or 3). However, the co-transfection of the phosphatase MKP-1 (Fig. 6A, lanes 4–6) reduced MAPK activity stimulated by ΔRaf-1:ER activation or 10% FCS nearly to control levels (compare lanes 4 with lane 5 or 6). Interestingly, expression of MKP-1 reduced the basal level of p70 S6K activity in comparison with that obtained with the control transfection (Fig. 6B, compare lane 1 with lane 4). More importantly, expression of MKP-1 had no effect on the stimulation of p70 S6K by either ΔRaf-1:ER activation (Fig. 6B, lane 5) or by serum (lane 6).

Clearly activation of p70 S6K by ΔRaf-1:ER still occurs despite the abolition of MAPK activation. Thus the activation of p70 S6K by Raf-1 is mediated by a MAPK-independent pathway.

Raf-mediated Activation of p70 S6K Is Insensitive to Low Concentrations of Wortmannin—Does p70 S6K activation involve PI3K activation, one of the pathways that activate p70 S6K (38–41)? CCL39-ΔRaf-1:ER cells were pre-treated 30 min with wortmannin, a specific PI3K inhibitor (reviewed in Ref. 42), and subsequently stimulated for 1 h with estradiol or α-thrombin (1 unit/ml), a potent mitogen in CCL39 cells known to activate p70 S6K (43). At concentrations ranging from 30 nM to 1 μM, wortmannin had no effect on the stimulation of MAPK by α-thrombin or ΔRaf-1:ER activation (Fig. 7A, lanes 3–5 and lanes 8–10, respectively). In contrast, α-thrombin-mediated stimulation of p70 S6K was abolished when cells were pre-treated with as low as 30 nM of wortmannin (Fig. 7B, lane 3), whereas the activation of p70 S6K by ΔRaf-1:ER was unaffected by pre-treatment with 30 or 100 nM wortmannin (Fig.
FIG. 6. MKP-1 expression blocks MAPK activation without affecting p70 S6K stimulation by serum or Raf-1. CCL39-ΔRaf-1:ER cells were transiently transfected as described under "Experimental Procedures" with the plasmid coding for the reporter proteins: p70 S6K-Myc (0.6 μg) and the plasmid p44 MAPK-VSVG (0.6 μg). In addition the cells were transfected with 30 μg of the control vector pcDNAneo (lanes 1–3) or with the pcDNAneo-MKP-1 (lanes 4–6). The kinase assays were performed as described in Fig. 3. A, MAPK assay. B, p70 S6K assay. This experiment is representative of three qualitatively similar ones. MBP, myelin basic protein; NS, nonstimulated; Estr, estradiol.

FIG. 7. Wortmannin pretreatment inhibits p70 S6 kinase stimulation. CCL39 cells (lanes 1–5) and CCL39-ΔRaf-1:ER cells (lanes 6–10) were serum-deprived for 24 h and pretreated for 30 min with increasing nanomolar concentrations of wortmannin (lanes 3–5 and 8–10) or vehicle (0.1% Me2SO, lanes 1, 2, 6 and 7). Cells were left unstimulated (NS) or stimulated for 1 h with α-thrombin (1 unit/ml, lanes 2–5) or estradiol (1 μM, lanes 7–10) for 1 h. The kinase assays were performed as described in Fig. 3. A, MAPK assay. B, p70 S6K assay. MBP, myelin basic protein.

DISCUSSION

The triggering of cell proliferation requires the activation of p70 S6K (5, 7); it is therefore important to understand the mechanisms by which this kinase is activated. An array of evidence suggests that a multiplicity of signaling pathways activate p70 S6K. For example, Chung et al. (39) have demonstrated that PDGF receptor activation triggers at least two signaling pathways leading to p70 S6K activation; the single most important path is mediated by phosphotyrosines at positions 740 and 751, which are required for PI3K activation, and a minor contribution is attributed to phosphotyrosine 1021, which mediates phospholipase C-γ activation. Recently Weng et al. (8) have shown that PI3K indirectly stimulates p70 S6K on tyrosine 252. Indeed, activation of PI3K may be the preeminent pathway that activates p70 S6K because blocking PI3K activation by wortmannin abrogates the stimulation of p70 S6K by numerous agonists (interleukin-2 (10, 44), insulin (38), and PDGF (39)). However, p70 S6K can be stimulated by O-tetradecanoylphorbol 13-acetate without PI3K activation (10). Furthermore, Ming et al. (11) have shown that the mutation of tyrosine 740 of the PDGF receptorabolished the activation of P13K in transfected 293 cells but did not alter the stimulation of p70 S6K by PDGF. At present it is impossible to know at what level the wortmannin-dependent and -independent signaling pathways converge to activate p70 S6K. Because p70 S6K is multiphosphorylated (reviewed in Ref. 2), it is likely that several independent kinase cascades mediate the direct activation of p70 S6K. However, whatever the putative pathways mediating p70 S6K activation may be, so far they have been demonstrated to be independent of the MAPK signaling cascade (11, 26, 27).

Surprisingly, our first results indicate that permanent activation of the MAPK pathway via the sole transfection of MKK1-SSDD is sufficient to stimulate p70 S6K. Because the only substrates of MKK1 known so far are the p42/p44 MAPK isoforms, we had predicted that MAPK should mediate the activation of p70 S6K elicited by MKK1-SSDD. Alternatively, this effect could result from an autocline loop triggered by the prolonged effect of transfected MKK1-SSDD.

To circumvent the problem of long-term stimulation of the MAPK pathway before assessing its action on p70 S6K, we have used the ΔRaf-1:ER inducible system (28). The addition of estradiol in the culture medium of the cell line CCL39-ΔRaf-1:ER activates within minutes the Raf-1 downstream MAPK cascade and is sufficient to stimulate p70 S6K within 30 min and maximally after 1 h. These effects are specific because the addition of estradiol to the parental cell line CCL39 did not modify the basal nor the growth factor-stimulated state of both MAPK and p70 S6K.

The delay in activation of p70 S6K versus MAPK has been observed with other stimuli such as α-thrombin (43). This delay (30 min versus 5 min) may be shorter, but our measurement of p70 S6K activity is insufficiently sensitive to detect an increase earlier than 30 min, because even potent mitogens do not produce a robust response. The rapidity of p70 S6K activation by Raf:ER (30 min) tends to eliminate the possibility that it is due to the triggering of an autocline loop. In order to completely eliminate this possibility, we tested whether inhibitors of protein synthesis affected p70 S6K activation by estradiol. Unfortunately, it was shown that inhibitors of protein synthesis activate p70 S6K (46). Indeed in our system, the protein synthesis inhibitors cycloheximide and anisomycin by themselves stimulated markedly p70 S6K and rendered an additional stimulation difficult to detect. Nonetheless, estradiol further stimulated mildly p70 S6K when added after cycloheximide pretreatment (data not shown), which indicates that ΔRaf-1:ER stimulates p70 S6K independently of protein synthesis. The mechanism by which inhibitors of protein synthesis activate p70 S6K is unknown. It was therefore crucial to analyze whether ΔRaf-1:ER-mediated activation of p70 S6K could be attributed to a slight inhibition of protein synthesis. In fact estradiol stimulated protein synthesis in the cell line CCL39-ΔRaf-1:ER at a rate equivalent to that of serum for at least 4 h following stimulation (data not shown). This increase of protein synthesis is likely not to be due solely to the activation of p70
S6K, because it was shown that blocking p70 S6K affected only mildly global protein synthesis (4).

The stimulation of p70 S6K by Raf-1 is rapid and independent of protein synthesis. Nonetheless, the effect of estradiol remained constantly lower than serum, about one third of the extent of protein synthesis. Nonetheless the effect of estradiol mildly global protein synthesis (4).

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Surprisingly, expression of an excess of dominant negative MAPK (p44MAPK-T192A) did not affect the stimulation of the co-expressed reporter p70 S6K-Myc by ΔRaf-1:ER activation (Fig. 5), whereas we have demonstrated previously that expression of p44MAPK-T192A at this level inhibited endogenous MAPK up to 70% (12). This result was confirmed by the expression of the dual specificity phosphatase MKP-1, which nearly abolished MAPK activation by ΔRaf-1:ER without affecting p70 S6K stimulation (Fig. 6). These results demonstrate unambiguously that p70 S6K stimulation by ΔRaf-1:ER is independent of MAPK activity, which confirms reports from Thomas' group indicating that p70 S6K activation by growth factors is independent of MAPK (11, 26). Raf-1 could activate p70 S6K via MKK1, which is very unlikely because MKK1 is a very specific kinase that activates p42/p44 MAPK without any effect on p38 MAPK and Jun N-terminal kinase, two kinases structurally and functionally similar to p42/p44 MAPK. Our results indicate for the first time that the MAPK and P70 S6K signaling pathways can branch out at the level of Raf-1.

Interestingly, long term expression of MKP-1 reduces the basal level of MAPK and of p70 S6K (Fig. 6), whereas long term expression of the constitutively active form of MKK1 stimulates MAPK and p70 S6K (Fig. 1). One common explanation for these results could be that the modulation of long term MAPK activity modifies the release of a factor able to activate p70 S6K. Thus the opposite effects of MKK1-SSD and MKP-1 on MAPK activity are mirrored on p70 S6K activation. This hypothesis could also explain the opposite effects of these two proteins on cell proliferation; MKP-1 expression blocks cell growth (37, 47), and MKK1 expression promotes cell proliferation (32, 34). In conclusion, we have demonstrated that activation of the chimeric ΔRaf-1:ER by estradiol is sufficient to activate p70 S6K independently of MAPK activation and insensitivity to PI3K inhibition by low concentrations of wortmannin. However, in our situation we have forced the stimulation of Raf-1 to high levels because MAPK is fully activated, but we have observed solely activation of p70 S6K equivalent to one-third of 10% serum (or to 0.3% FCS). This indicates that Raf-1 is certainly not the major pathway leading to activation of p70 S6K, and indeed in some cells it may be lacking because it was shown that dominant negative Raf-1 did not affect the stimulation of p70 S6K by epidermal growth factor and O-tetradecanoylphorbol 13-acetate in human 293 cells (11).

Identification of the links between the activation of Raf-1 to that of p70 S6K may be provided by the discovery of substrates of Raf-1 other than MKK1. For example, recent work has shown that the phosphatase CDC25 (49) is a substrate of c-Raf, and previously it was shown that Raf-1 activates the NF-κB transcription factor by dissociating the cytoplasmic NF-κB/IB complex (50). Alternatively, the most promising approach to reveal the signaling pathway between Raf-1 and p70 S6K will reside in the identification of the members of the kinase cascades that activate directly p70 S6K.

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