MOLECULAR AND CELLULAR BIOLOGY, Sept. 1995, p. 4930–4938 Vol. 15, No. 9
0270-7306/95/$04.00+0

Protein Synthesis Inhibitors Reveal Differential Regulation of Mitogen-Activated Protein Kinase and Stress-Activated Protein Kinase Pathways That Converge on Elk-1

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Received 10 February 1995/Returned for modification 14 March 1995/Accepted 2 June 1995

Inhibitors of protein synthesis, such as anisomycin and cycloheximide, lead to superinduction of immediate-early genes. We demonstrate that these two drugs activate intracellular signaling pathways involving both the mitogen-activated protein kinase (MAPK) and stress-activated protein kinase (SAPK) cascades. The activation of either pathway correlates with phosphorylation of the c-*fos* regulatory transcription factor Elk-1. In HeLa cells, anisomycin stabilizes c-*fos* mRNA when protein synthesis is inhibited to only 50%. Under these conditions, anisomycin, in contrast to cycloheximide, rapidly induces kinase activation and efficient Elk-1 phosphorylation. However, full inhibition of translation by either drug leads to prolonged activation of SAPK activity, while MAPK induction is transient. This correlates with prolonged Elk-1 phosphorylation and c-*fos* transcription. Elk-1 induction and c-*fos* activation are also observed in KB cells, in which anisomycin strongly induces SAPKs but not MAPKs. Purified p54 SAPKα efficiently phosphorylates the Elk-1 C-terminal domain in vitro and comigrates with anisomycin-activated kinases in in-gel kinase assays. Thus, Elk-1 provides a potential convergence point for the MAPK and SAPK signaling pathways. The activation of signal cascades and control of transcription factor function therefore represent prominent processes in immediate-early gene superinduction.

Treatment of quiescent cells with various growth factors, phorbol esters, or UV light results in the transient transcriptional activation of a class of immediate-early genes whose induction is independent of new protein synthesis (2). Many of these genes, as exemplified by the c-*fos* proto-oncogene, share a common promoter element, the serum response element (SRE), which is sufficient to confer induction by immediate-early signal transduction (41, 42). The c-*fos* SRE appears to be constitutively occupied in vivo, and the pattern observed is reproduced in vitro by a transcription factor protein complex including a dimer of the serum response factor and one molecule of an accessory ternary complex factor (TCF) (20, 41, 42). Proteins with TCF activity have been identified as members of the Ets protein family and appear to be important in activating SRE-dependent transcription upon activation of the mitogen-activated protein kinase (MAPK) signal transduction pathway (20, 41, 42). $p62^{TCF}$, the major TCF activity in HeLa cells, is biochemically indistinguishable from Elk-1 (17, 31), and in transient transfection assays, Elk-1 has been identified as a growth factor-activated transcription factor (18, 22, 28). The C-terminal activation domain of Elk-1 is hyperphosphorylated upon signaling (42), which can be assayed in electrophoretic mobility shift assays (EMSA). This change in the mobility of Elk-1 complexes has been shown to correlate temporally with induction of the MAPK cascade (15, 16, 32) as well as the transcriptional activity of c-*fos* (15, 32, 45).

In a phenomenon called superinduction, treatment of cells with protein synthesis inhibitors results in the prolonged accumulation of c-*fos* mRNA stemming from persistent transcriptional activity and stabilization of the otherwise short-lived c-*fos* transcripts (8, 9, 11, 12, 26, 27, 34, 38, 43). Superinduction also occurs after treatment of cells with the phosphatase inhibitor okadaic acid (35, 36), an effect due to deregulation of signal transduction pathways by inhibition of some regulatory phosphatase, possibly phosphatase 2A (15, 45). In contrast, the cause of superinduction by protein synthesis inhibitors is not clear. The existence of a labile repressor molecule, which would maintain genes like c-*fos* in an inactive state, has been hypothesized. In this model, protein synthesis inhibitors deplete the cell of the short-lived repressor, causing superinduction (11, 30, 38). However, no such repressor molecules have been identified.

Moreover, protein synthesis inhibitors like anisomycin and cycloheximide can activate intracellular kinases similar to those activated by signal transduction cascades. In the case of anisomycin, this occurs independently of the ability to inhibit protein synthesis (5, 27). This could potentially explain superinduction via direct promoter activation without the existence of a labile repressor. c-*fos* induction by cycloheximide depends on the SRE (38), suggesting a mode of c-*fos* transcriptional activation by signaling kinases similar to that described for growth factors (42). These observations led us to address whether the protein synthesis inhibitors anisomycin and cycloheximide exert effects directly on transcription factors bound to the c-*fos* promoter or on upstream signaling pathways. We find that TCF/Elk-1 is hyperphosphorylated upon superinduction by anisomycin and cycloheximide. This effect correlates with the transcriptional activation of c-*fos*. Using in-gel kinase assays, we identify activation of specific Elk-1 kinases, representing members of both the MAPK and stress-activated protein kinase (SAPK) families, after treatment of cells with pro-

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tein synthesis inhibitors, tetradecanoyl phorbol acetate (TPA), epidermal growth factor (EGF), or UV light (UVC).

MATERIALS AND METHODS

Additions. Anisomycin, cycloheximide, and actinomycin D were purchased from Sigma. Stock solutions of anisomycin (10 mg/ml) and of actinomycin D (5 mg/ml) were prepared in 100% ethanol. Stock solutions of cycloheximide (30 mg/ml) and recombinant human EGF (0.5 mg/ml) were prepared in water. EGF was purchased from ProGen (Heidelberg, Germany), and methionine-free medium was purchased from CC Pro (Karlsruhe, Germany).

Cell culture and treatment of cells. HeLa tk^- cells (3) or KB cells were grown to near confluence at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 U/ml). Cells were starved for 16 h in DMEM supplemented with 0.5% fetal calf serum. EGF (50 ng/ml), TPA (60 ng/ml), and the protein synthesis inhibitors anisomycin and cycloheximide (for concentrations, see figure legends) were added to the culture medium from concentrated stock solutions. UV irradiation (30 J/m^2) of HeLa cells has been described previously (32). For determination of the stability of the c-*fos* RNA, cells were first treated with either EGF or protein synthesis inhibitors for 30 min. Actinomycin D (10 μg/ml) was added subsequently, and cells were harvested after further incubation for 10, 30, 60, 90, 150, or 210 min. In a control experiment, cells were incubated for 30 min in the presence of both EGF and actinomycin D.

Preparation of cell extracts. Preparation of nuclear salt extracts and detection of TCF modifications were performed as described previously (45). Whole cell extracts were prepared as described by Baccarini et al. (4), with slight modifications. Before harvesting, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) supplemented with 50 mM NaF and 2 mM Na_3VO_4. Residual PBS was removed by aspiration from plates which had been tilted on ice. Subsequently cells were scraped off in 400 μl of lysis buffer (10 mM Tris-HCl [pH 7.05], 30 mM $NaPP_i$, 50 mM NaCl, 5 μM $ZnCl_2$, 1% Triton X-100) containing protease inhibitor cocktail (0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg of leupeptin per ml, 2 μg of aprotinin per ml, 0.5 μg of pepstatin per ml, 1 U of α_2-macroglobulin per ml, 0.5 mM benzamidine) and phosphatase inhibitor cocktail (2 mM Na_3VO_4, 50 mM NaF, 20 mM β-glycerophosphate, 10 mM *p*-nitrophenyl phosphate, 400 nM okadaic acid). The addition of these phosphatase inhibitors is essential to maintain the phosphorylation status of all proteins under investigation. The extracts were vortexed for 1 min and subsequently clarified by centrifugation at 10,000 × *g* for 15 min at 4°C. Extracts were frozen in liquid nitrogen and stored at −80°C.

RNA preparation and Northern (RNA) blot hybridization. RNA was prepared from postnuclear supernatants (Fig. 3 and 4) or whole cell extracts (Fig. 8) and transferred to hybridization membranes as described previously (45). For Northern hybridization, a modification of the chemiluminescence RNA detection assay described previously (7) was used. All reagents except the digoxigenin (DIG) labeling mix (Boehringer Mannheim) were part of the Promega in vitro transcription kit. The reaction mixture contained 1 μg of linearized plasmid encoding the respective gene probe, and the reaction was performed as instructed by the manufacturer. The gene probes used for c-*fos* and *GAPDH* have been described previously (13, 37). For Northern hybridization, the blot was prehybridized for 2 h at 69°C in 10 ml of DIG hybridization buffer (50% [vol/vol] deionized formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 2% [wt/vol] blocking reagent [Boehringer Mannheim], 0.1% *N*-lauryl sarcosine, 0.02% sodium dodecyl sulfate [SDS]) and then hybridized for 15 h at 69°C in 10 ml of the same buffer containing 300 ng of DIG-labeled probe per ml. Hybridization against c-*fos* and *GAPDH* probes was performed simultaneously. After removal of the hybridization solution, the blot was washed twice for 10 min with 2× SSC at room temperature and twice for 15 min in 0.1× SSC–0.1% SDS at 69°C.

Detection of DIG-labeled nucleic acids. The blot was washed for 2 min in wash buffer (100 mM maleic acid [pH 7.5], 150 mM NaCl, 0.6% [vol/vol] Tween 20), and nonspecific binding was subsequently blocked by incubation for 30 min at room temperature in buffer 2, consisting of 100 mM maleic acid (pH 7.5), 150 mM NaCl, and 1% (wt/vol) blocking reagent (Boehringer Mannheim). pH values were adjusted by addition of NaOH. Alkaline phosphatase-conjugated anti-DIG Fab (Boehringer Mannheim) was diluted 1:10,000 in buffer 2, and the blot was incubated for 30 min and then subjected to two washes of 15 min each with wash buffer. The blot was then equilibrated to alkaline pH in buffer 3 (100 mM Tris [pH 9.5], 100 mM NaCl, 50 mM $MgCl_2$) for 3 min. The chemiluminescence reagent AMPPD (Boehringer Mannheim) was diluted 1:100 in buffer 3 and applied to the blot for 5 min. Subsequently the membrane was incubated for 30 min at 37°C, and the film was exposed for 2 h at room temperature.

[^{35}S]methionine labeling of HeLa cells. Cells were grown in a 24-well plate, starved for 16 h in DMEM supplemented with 0.5% fetal calf serum, and washed twice with PBS and once with methionine-free medium (CC Pro). After incubation in this medium at 37°C for 10 min, the medium was replaced by 1 ml of medium containing [^{35}S]methionine (15 μCi/ml) and either anisomycin or cycloheximide at concentrations as indicated in the legend to Fig. 2. Cells were incubated for 10 min at 37°C, washed twice with DMEM containing 10 μg of cycloheximide per ml, and then lysed in 200 μl of whole cell lysis buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 0.1% deoxycholate, protease inhibitor cocktail). Debris were pelleted for 10 min at 10,000 × *g*, and 100 μl of the supernatant was trichloroacetic acid precipitated and collected onto glass fiber filters (Whatman). The filters were washed with 10 ml of 5% trichloroacetic acid and then with 3 ml of 100% ethanol. Radioactivity on the dried filter was determined by Cerenkov counting.

In-gel kinase assays. In-gel kinase assays were performed as described by Hipskind et al. (15), with modifications as indicated below. ^{14}C-labeled molecular weight markers (Amersham) or 15 μg of total cell lysate as described above was loaded onto a Laemmli gel (180 by 180 by 1 mm) and electrophoresed at 150 V for 10 to 15 cm into the separating gel. The separating gel contained either no copolymerized substrate or one of the following: 40 μg of c-Jun per ml (40), 40 μg of a glutathione *S*-transferase (GST)–Elk fusion protein containing Elk-1 amino acids 307 to 428 (GST-$Elk_{307–428}$), or 20 μg of GST per ml (45). After electrophoresis, the separating gel was detached, washed twice for 10 min in 250 ml of 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4; AppliChem)–10 mM dithiothreitol (DTT; AppliChem)–20% isopropanol, and then equilibrated for 1 h in 250 ml of 50 mM HEPES (pH 7.4)–10 mM DTT. Proteins were denatured with two 30-min changes of 6 M guanidine HCl (AppliChem) in 50 mM HEPES (pH 7.4)–10 mM DTT. This was followed by renaturation in two changes of 500 ml of 50 mM HEPES (pH 7.4)–10 mM DTT–0.04% Tween 20 at 4°C (for a total of 16 to 20 h). The gel was equilibrated for 30 min in 250 ml of 25 mM HEPES (pH 7.4)–10 mM DTT–10 mM $MgCl_2$–90 μM Na_3VO_4 at room temperature. Twelve milliliters of the same buffer containing 250 μCi of [γ-^{32}P]ATP (4,500 Ci/mmol; ICN) was layered onto the gel, which was then incubated at 25°C for 60 min, with manual agitation every 5 min to prevent the gel surface from dehydrating. The gel was immersed in 100 ml of 5% TCA–10 mM $NaPP_i$ and then incubated with gentle shaking over several days in multiple changes of 500 ml for at least 6 h in the same buffer until a 1-ml aliquot of the wash exhibited background radioactivity after Cerenkov counting. Dried gels were exposed on a Fujix BAS1000 Bio-Imaging Analyzer (Fuji). Na_3VO_4 was prepared fresh by adjusting the pH of a 200 mM solution to between 9.5 and 10, boiling the solution until it was colorless, and then readjusting the pH. Bacterially expressed substrate proteins were purified by Ni^{2+}-nitrilotriacetic acid affinity chromatography via six-histidine tags under denaturing conditions of 6 M guanidine hydrochloride, and concentrations were estimated by comparison with a bovine serum albumin standard.

In vitro phosphorylation of proteins. Five micrograms of recombinant GST fusion proteins or GST in a total volume of 5 μl was incubated with 5 μl (0.3 U) of p55 SAPKα purified to homogeneity from livers of rabbits injected with interleukin-1 as described previously (24). The phosphorylation reaction was started by adding 5 μl of kinase buffer, yielding final concentrations of 50 mM Tris (pH 7.4), 10 mM $MgCl_2$, and 20 μM ATP, including 1.5 μCi of [γ-^{32}P]ATP. After 20 min of incubation at room temperature, sample buffer was added and proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% polyacrylamide gel. Phosphorylated substrates were visualized by autoradiography, and phosphorylation was quantitated on a Fujix BAS1000 Bio-Imaging Analyzer (Fuji). One unit is defined as incorporation of 1 pmol of ^{32}P per min into GST-$Jun_{1–135}$ as described previously (24).

RESULTS

Anisomycin and cycloheximide induce Elk-1 modification in a concentration-dependent manner. Anisomycin has been shown to efficiently induce intracellular signaling, apparently independently of its ability to inhibit translation (5, 27). To ascertain the effects of anisomycin on the phosphorylation status of the Ets transcription factor TCF/Elk-1, we made nuclear extracts from HeLa cells treated with anisomycin or cycloheximide and measured in EMSA the migration behavior of Elk-1–DNA complexes. This assay provides a highly sensitive indication of the phosphorylation status of Elk-1, which in turn correlates with the transcriptional activity of the c-*fos* gene. The E74 DNA binding sequence used can be bound by TCF proteins directly and independently of an accessory protein. Binding is greatly stimulated by TCF phosphorylation and drastically impaired by TCF dephosphorylation (45). Anisomycin at a low concentration (25 ng/ml) induced a rapid and transient modification of Elk-1 (Fig. 1, lanes 6 to 8) that lagged only slightly behind the EGF-stimulated response (lanes 2 to 5). In contrast, at high concentrations of anisomycin (10 μg/ml), the rapid induction of Elk-1 modification was long-lived and was not reversed within 90 min (lanes 9 to 11). Cycloheximide however, did not significantly increase Elk-1 phosphorylation at a low concentration (51 ng/ml) (lanes 12 to 14) but

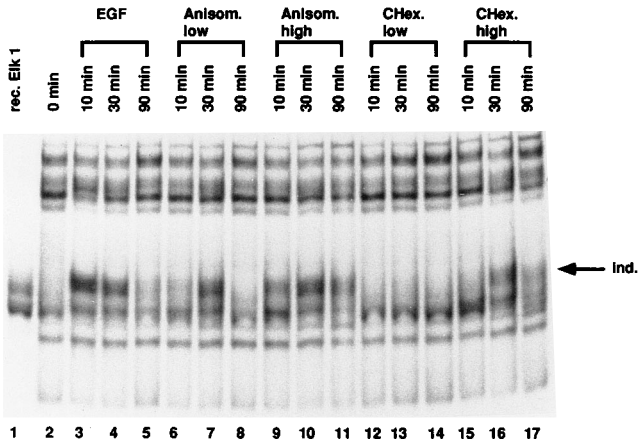

FIG. 1. Nuclear extracts from HeLa cells treated with protein synthesis inhibitors display altered mobility of Elk-1-DNA complexes. Shown are results of EMSA of direct binding complexes formed by nuclear extracts prepared from starved cells (lane 2) and cells stimulated with EGF, low (25 ng/ml) or high (10 μg/ml) doses of anisomycin (Anisom.), or low (51 ng/ml) or high (10 μg/ml) doses of cycloheximide (CHex.) for the indicated times. The ^{32}P-labeled E74 DNA sequence (18) was used to detect binding. The control reaction (lane 1) contained recombinant human Elk-1 (19). The induced (ind.), hyperphosphorylated Elk-1 complex is marked with an arrow.

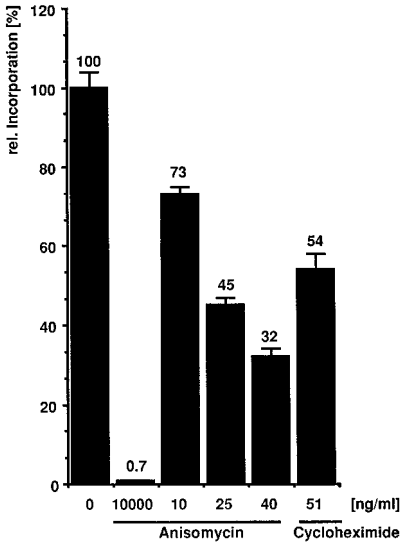

FIG. 2. Protein synthesis levels in HeLa cells change in response to protein synthesis inhibitors. To determine the inhibitory effects of various concentrations of protein synthesis inhibitors, starved HeLa cells were treated for 10 min without (0) or with 10,000, 10, 25, and 40 ng of anisomycin per ml or 51 ng of cycloheximide per ml in the presence of [^{35}S]methionine. Incorporation of [^{35}S]methionine was determined in quadruplicate, and the average results were normalized to control values. Error bars show standard deviations.

caused a slower and prolonged Elk-1 response at a high concentration (10 μg/ml) (lanes 15 to 17). Results obtained with extracts prepared from A431 cells were indistinguishable (not shown). The stimulation of A431 cells with EGF in the presence of low doses of either inhibitor generated the expected rapid and transient response, whereas these effects on Elk-1 were persistent when EGF was supplemented with high doses of either anisomycin or cycloheximide (not shown). These induced complexes from both HeLa and A431 cells contained the ternary complex factor Elk-1, or an immunologically related molecule, since they were blocked by anti-Elk-1 antisera (data not shown) (45).

These results suggested that low doses of anisomycin, but not cycloheximide, can activate one or more kinases which target the nuclear transcription factor Elk-1. As demonstrated below, the low doses used here represent conditions that permit protein synthesis to occur at only half its normal rate. Fully inhibitory doses of either cycloheximide or anisomycin also lead to this induction, but without the subsequent reversal of the Elk-1 modification, thereby generating a long-lived response. The transition from hyper- to hypophosphorylated Elk-1 correlates with the amount of protein synthesis, and not signaling activity, and therefore probably depends on de novo protein synthesis. This putative newly synthesized activity, which may or may not be a phosphatase, may act directly on Elk-1 or, in conjunction with a constitutive Elk-1 phosphatase, may down-regulate an upstream component.

Quantitative effects of anisomycin and cycloheximide on protein synthesis. To assess the correlation between protein synthesis inhibition and the Elk-1 modification status seen in Fig. 1, the influence of anisomycin and cycloheximide on protein synthesis was measured in HeLa cells by pulse-labeling with [^{35}S]methionine. Complete inhibition of protein synthesis occurred at 10 μg of anisomycin per ml (Fig. 2). Anisomycin at 25 ng/ml or cycloheximide at 51 ng/ml lowered protein synthesis to half-maximal levels. Anisomycin at 10 or 40 ng/ml reduced protein synthesis to 70 or 30%, respectively. Thus, the transient signaling seen in Fig. 1, induced by anisomycin at 25 ng/ml, occurred under conditions of half-maximal levels of protein synthesis. Full inhibition of protein synthesis, obtained at a high concentration (10 μg/ml) of either drug, correlated not only with gene induction but also with inhibition of Elk-1 dephosphorylation. These effects were comparable in HeLa and A431 cells. Note that in the mouse 10T1/2 cell line, 70 ng of anisomycin per ml was subinhibitory to protein synthesis (27), suggesting that the dose response to protein synthesis inhibitors varies between cell lines or species.

Levels of c-*fos* mRNA upon induction by EGF, anisomycin, or cycloheximide. A tight temporal correlation between Elk-1 modification status and immediate-early gene activity has previously been seen upon cell stimulation by EGF, serum, and TPA (15, 45). Therefore, we analyzed the effects of high and low doses of anisomycin and cycloheximide on the kinetics of c-*fos* induction. Such Northern blot analysis demonstrated that in contrast to the transient presence of c-*fos* mRNA upon EGF treatment (Fig. 3, lanes 2 to 4), a significant fraction of c-*fos* mRNA induced by low doses of anisomycin was still present after 90 min (lanes 5 to 7). This prolonged presence of c-*fos* mRNA (lane 7) was seen at times when Elk-1 modification had

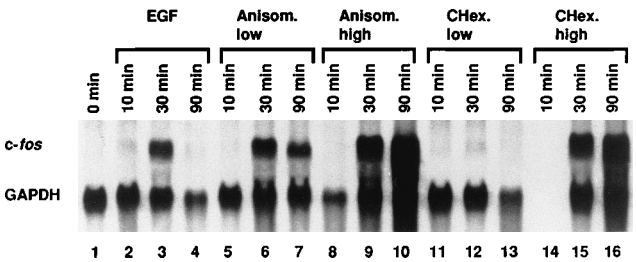

FIG. 3. Induction of c-*fos* transcription by EGF, low or high doses of anisomycin (Anisom.), or low or high doses of cycloheximide (CHex.). Starved HeLa cells were treated as indicated in the legend to Fig. 1 for 10, 30, or 90 min. Total RNA was prepared from postnuclear supernatants and assayed for c-*fos* and *GAPDH* mRNAs by Northern analysis. The RNA in lane 14 was lost during preparation.

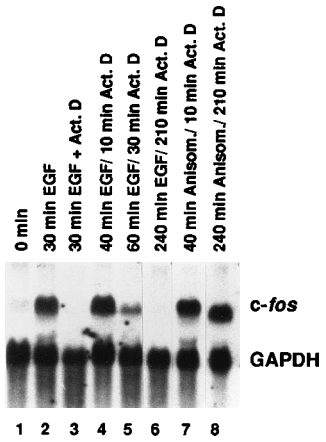

FIG. 4. Low doses of anisomycin increase c-*fos* mRNA stability. Starved HeLa cells were treated with EGF or anisomycin (Anisom.) in the presence of actinomycin D (Act. D), and total RNA was prepared and analyzed for c-*fos* and *GAPDH* transcripts by Northern analysis. Cells were preincubated with EGF or anisomycin (25 ng/ml) for 30 min. Actinomycin D was added after this period of time, and then the cells were further incubated for the time periods indicated. Cells were harvested 10 min (lanes 4 and 7), 30 min (lane 5), or 210 min (lanes 6 and 8) after addition of actinomycin D. As a control, EGF and actinomycin D were added simultaneously (lane 3).

been completely reversed (Fig. 1, lane 8) and, as shown below, reflects an increase in mRNA stability under these conditions of half-normal protein synthesis. High doses of anisomycin (Fig. 3, lanes 8 to 10) or cycloheximide (lanes 14 to 16) led to strong superinduction of c-*fos* mRNA levels, whereas low doses of cycloheximide caused barely detectable gene induction. In contrast to anisomycin treatment, c-*fos* mRNA induced by low doses of cycloheximide was no longer detectable after 90 min (lane 13).

c-*fos* mRNA is stabilized after induction by a low dose of anisomycin. High doses of protein synthesis inhibitors not only prevent translation but also lead to stabilization of short-lived mRNAs (8, 9, 11, 43). We observed the persistence of c-*fos* mRNA in HeLa cells after treatment with high doses of anisomycin and also with low doses that inhibited protein synthesis by only 50% (Fig. 3). c-*fos* mRNA was still detectable after 90 min (Fig. 3, lane 7), a time at which Elk-1 modification was no longer measurable (Fig. 1, lane 8). This effect could potentially be due to increased mRNA stability or to activation of the promoter after Elk-1 hyperphosphorylation had been reversed. To investigate mRNA stability at low anisomycin concentrations, we treated cells for 30 min with either EGF or low doses of anisomycin and then blocked transcription with actinomycin D (Fig. 4, lanes 3 to 8). Induction of c-*fos* mRNA by EGF (lane 2) was fully inhibited by actinomycin D (lane 3), confirming that the drug inhibited transcription under our experimental conditions. c-*fos* mRNA levels decayed rapidly after 30 min of EGF treatment in the presence of actinomycin D (lanes 4 to 6). In contrast, the c-*fos* mRNA levels present at 30 min upon induction by low concentrations of anisomycin were unaffected by actinomycin D (lanes 7 and 8). This finding demonstrates that c-*fos* mRNA is stabilized by anisomycin at 25 ng/ml, a dose which permits 50% of normal protein synthesis to occur (Fig. 2). Thus, the presence of c-*fos* mRNA at 90 min after induction by low-dose anisomycin treatment (Fig. 3, lane 7) does not reflect continued c-*fos* promoter activity but rather reflects the stabilization of previously transcribed mRNA. The underlying mechanism of mRNA stabilization remains to be clarified.

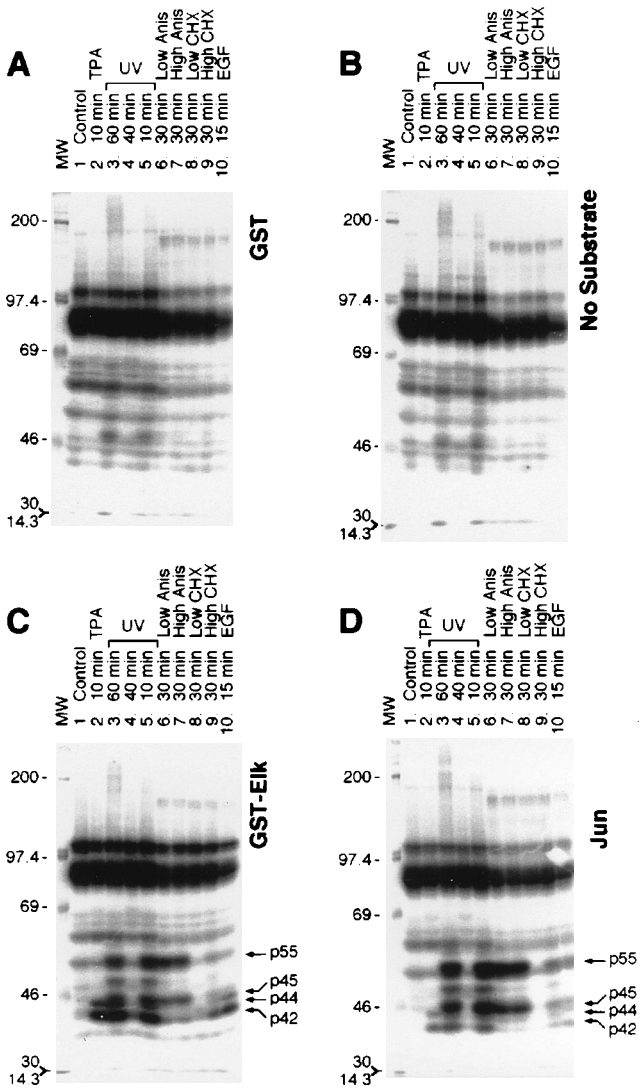

FIG. 5. Protein synthesis inhibitors induce Elk-1 and c-Jun kinases. HeLa whole cell extracts were analyzed by an in-gel kinase assay using 7.5% acrylamide separating gels after the indicated treatments (Low Anis, High Anis, Low CHx, and High CHx refer to low- and high-dose anisomycin and cycloheximide treatments as specified in the legend to Fig. 1). Acrylamide separating gels contained the copolymerized substrate GST (A), no substrate (B), GST-Elk$_{307–428}$ (C), or c-Jun (D). Positions of molecular size standards (lane MW) are shown in kilodaltons to the left of each panel. Arrows depict the migration of inducible kinases of approximately 55, 45, 44, and 42 kDa. These kinases show phosphorylation activity in the gels containing GST-Elk or Jun but exhibit negligible phosphorylation of gels either without substrate or containing GST. All panels represent computer-generated images from the program MacBAS version 2.0 (Fuji) which were manipulated identically, using the program Adobe Photoshop 1.25. Induced kinase activity has been lost from the 40-min UV sample (lanes 4). Note that an approximately 48-kDa band is observed in all gels after UV treatment. It is most probably the product of autophosphorylation of an unidentified kinase and is distinct from the Elk-1/Jun kinases.

Elk-1 kinase activities induced by anisomycin and cycloheximide. Since Elk-1 phosphorylation is induced upon treatment of cells with high and low doses of protein synthesis inhibitors, we performed in-gel kinase assays using as the substrate a GST-Elk fusion protein containing the C-terminal activation domain of Elk-1 (GST-Elk$_{307–428}$). For control purposes, all extracts were also loaded on gels containing either the GST fusion component alone (Fig. 5A) or no substrate at all (Fig. 5B) to monitor autophosphorylation activities. Extracts of cells

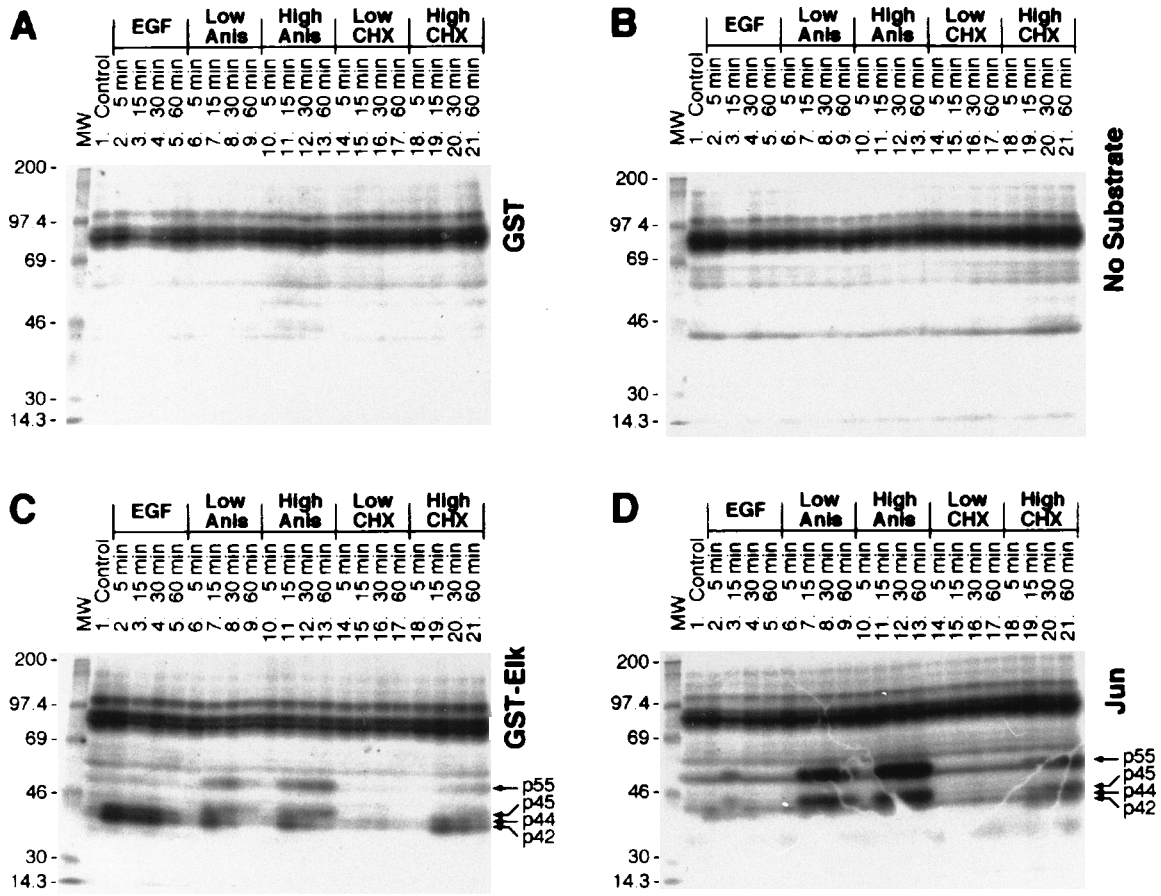

FIG. 6. Activation kinetics of MAPKs and SAPKs by protein synthesis inhibitors. HeLa whole cell extracts prepared after the indicated treatments were analyzed as described in the legend to Fig. 5 by in-gel kinase assay using 8% acrylamide separating gels. Notation is as specified in the legend to Fig. 5.

treated with either low or high concentrations of anisomycin displayed very efficient stimulation of two Elk-1 kinases, migrating at positions of 45 and 55 kDa (Fig. 5C, lanes 6 and 7). Comigrating kinases were also activated very efficiently by stimulation of cells with UVC (Fig. 5C, lanes 3 to 5). This treatment is known to stimulate the SAPKs JNK1 and JNK2, which also exhibit apparent molecular masses in SDS-PAGE of approximately 45 and 54 kDa, respectively (6, 14, 25). These data suggest that the anisomycin- and UVC-induced SAPK p45 and p55 are also Elk-1 kinases. Since the identity of the larger anisomycin-induced kinase remains unconfirmed, we continue to refer to it as p55 as originally described (5). However, it probably corresponds to p54 SAPKα (see below).

Anisomycin also induced kinases of 42 and 44 kDa. These kinases comigrated with those activated by treatment with EGF (Fig. 5C; compare lanes 6, 7, and 10) and the phorbol ester TPA (lane 2). The p42 and p44 kinases are recognized in Western blots (immunoblots) by pan-ERK antisera (15) and likely represent the MAPK ERK2 and a close relative (20, 42). In contrast to p42 and p44, the p45 and p55 kinases were not activated by TPA. The nonoverlapping suite of kinases activated by TPA, EGF, and anisomycin confirms that MAPKs and SAPKs are differentially activated (6, 14, 29, 44). Under high concentrations of cycloheximide, all four kinases are activated (lane 9).

The identity of p45 and p55 as c-Jun kinases was tested by in-gel kinase assays using c-Jun as the substrate (Fig. 5D). All agents that induced p45 and p55 activities in Elk-1 substrate gels induced apparently identical kinase activities in the c-Jun substrate gels (compare Fig. 5C and D). Comparison of the relative efficiencies of c-Jun and Elk-1 phosphorylation indicated that the p45 and p55 kinases were more efficient at phosphorylating c-Jun than Elk-1, while the p42 and p44 kinases efficiently phosphorylated Elk-1 but not c-Jun. From this point on, we designate p42 and p44 as MAPKs and p45 and p55 as SAPKs. Elk-1, unlike c-Jun and myelin basic protein, provides a potent substrate for both MAPK and SAPK phosphorylation.

Kinetics of Elk-1 kinase activities. We next used in-gel kinase assays to investigate the kinetic profile of Elk-1 kinase activity after stimulation by various treatments. No induced kinases were observed when either GST alone (Fig. 6A) or no substrate (Fig. 6B) was used. Figure 6C demonstrates that EGF stimulated p42 and p44 Elk-1 kinase activities within 5 min. This activity decreased between 30 and 60 min postinduction (lanes 2 to 5). The activation profiles of MAPKs displayed similar transient kinetics when induced by either low concentrations of anisomycin (lanes 6 to 9), high concentrations of anisomycin (lanes 10 to 13), or high concentrations of cycloheximide (lanes 18 to 21). In the latter cases, activation lagged somewhat behind that seen upon EGF treatment, whereas down-regulation appeared comparably efficient under all treatments. Low concentrations of cycloheximide induced minimal activation of p42 and p44 (lanes 15, 16) associated with marginal activation of c-*fos* (Fig. 3, lane 12). The p45 and p55 SAPKs were also rapidly induced within 15 min by both low

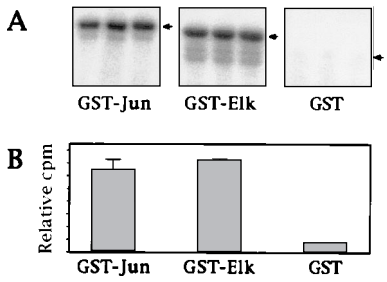

FIG. 7. Purified SAPK phosphorylates GST-Elk in vitro. (A) Bacterially expressed GST-Jun$_{1-135}$, GST-Elk$_{307-428}$, or GST was phosphorylated by purified SAPK and subjected to SDS-PAGE, and ^{32}P incorporation was visualized on a Fujix BAS1000 Bio-Imaging Analyzer (Fuji). Computer-generated images (see the legend to Fig. 5) show the results of three independent experiments for each substrate. Dark arrows mark the positions of major Coomassie blue-stained bands, and light arrows mark the positions of minor breakdown products of GST-Elk$_{307-428}$. (B) Quantitation of ^{32}P incorporation from bands indicated by dark arrows in panel A performed by the program MacBAS version 2.0 (Fuji). Error bars show standard deviations.

and high concentrations of anisomycin (Fig. 6C, lanes 6 to 13). However, a significant difference was seen with regard to the kinetics of SAPK down-regulation. Whereas low anisomycin levels did not hinder the down-regulation of these kinases within 60 min, the p45 and p55 kinases remained maximally active at 60 min when concentrations fully inhibitory to protein synthesis were used (lane 13). High concentrations of cycloheximide led to a rapid induction within 15 min and subsequent decay of MAPK activities, while a more gradual increase in p45 and p55 kinase activities was observed (lanes 18 to 21). The same results were seen with c-Jun as the substrate (Fig. 6D). As noted above, c-Jun did not serve as an efficient MAPK substrate.

The fully inhibitory, high-cycloheximide and high-anisomycin treatments demonstrated the kinetically distinct activation profiles of MAPKs and SAPKs. Our kinetic measurements further suggest that the mechanisms of down-regulation of MAPKs and SAPKs differ in an important respect, namely, that SAPK down-regulation appears to be strictly dependent on de novo protein synthesis while MAPK inactivation is not.

Purified SAPKα phosphorylates GST-Elk in vitro. To verify that SAPKα could phosphorylate Elk-1, we performed an in vitro phosphorylation of GST-Elk$_{307-428}$ (Fig. 7). The highly purified SAPKα fraction used contained two kinases, both of which were identified as SAPKα by amino acid sequencing of tryptic peptides (24). GST-Elk$_{307-428}$ was as efficient a substrate as GST-Jun$_{1-135}$, whereas GST alone was not. This kinase fraction was also used in an in-gel kinase assay, in which the major band comigrated with approximately 55-kDa bands activated by anisomycin in KB and HeLa cells and by UV light in HeLa cells (Fig. 8C).

Stimulation of KB cells with anisomycin preferentially activates the SAPK pathway and induces c-*fos*. To address the role of Elk-1 phosphorylation by SAPK in transcriptional activation, we sought conditions in which SAPK could be activated without significant MAPK stimulation. Differential activation of these kinases has been observed in KB cells (23). We found that high doses of anisomycin led to accumulation of both hyperphosphorylated Elk-1 molecules (Fig. 8A) and c-*fos* mRNA (Fig. 8B). As in mouse 10T1/2 cells (5), anisomycin appreciably stimulated only p45 and p55 SAPKs and not p42

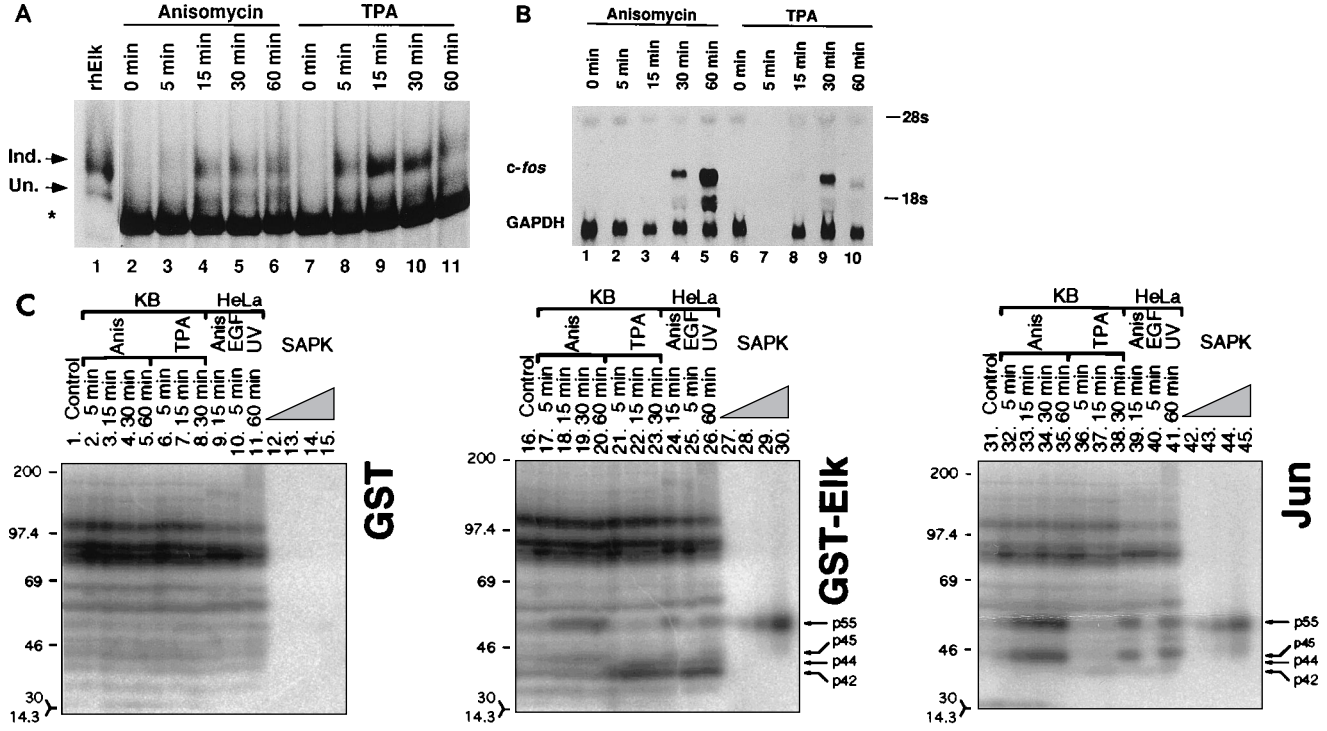

FIG. 8. TCF modification and c-*fos* induction correlate with activation of SAPKs but not MAPKs by anisomycin in KB cells. Whole cell extracts from KB cells treated with either anisomycin (Anis; 10 μg/ml) or TPA for the indicated times were analyzed by EMSA as in Fig. 1 (A), by Northern blotting as in Fig. 3 (B), and by an in-gel kinase assay as in Fig. 5 (C). Uninduced (Un.) and induced (Ind.) TCF complexes are indicated in panel A; positions of c-*fos* and *GAPDH* mRNAs and 28S and 18S rRNAs are indicated in panel B. RNA from lane 7 was lost during preparation. The asterisk in panel A denotes a nonspecific EMSA complex. In panel C, SAPK refers to serial fivefold dilutions of 3 μl of the purified SAPK from Fig. 7, and HeLa cell extracts are the same samples as used for Fig. 5 and 6. rhElk, recombinant human Elk. Sizes in panel C are indicated in kilodaltons.

and p44 MAPKs in KB cells (Fig. 8C), in contrast to the situation in HeLa cells (Fig. 5 and 6). TPA induction showed that KB cells do possess functional p42 and p44 Elk-1 kinases (Fig. 8C, lanes 21 to 23). These results correlate Elk-1 phosphorylation by SAPK with transcriptional activation of the c-*fos* gene upon anisomycin treatment of KB cells. However, we cannot exclude the possibility that Elk-1 activation is mediated by another kinase which cannot renature and is therefore not detected by our assay conditions.

DISCUSSION

In this study, we document that intracellular signaling is induced by the protein synthesis inhibitory drugs anisomycin and cycloheximide. We monitored the consequences of drug treatment at the levels of gene (c-*fos*) induction, mRNA stability, de novo protein synthesis, transcription factor (Elk-1) phosphorylation, and stimulation of protein kinases modifying Elk-1. The use of anisomycin and cycloheximide at concentrations partially or fully inhibiting protein synthesis revealed differential effects on both the activation and down-regulation of MAPKs and SAPKs. This finding further led to the identification of the C-terminal activation domain of Elk-1 as an efficient substrate for phosphorylation by SAPKs, demonstrating that the MAPK and SAPK pathways potentially converge at the level of the nuclear transcription factor Elk-1.

Anisomycin induces in HeLa cells signaling pathways activating both MAPKs and SAPKs. In-gel kinase assays provide a sensitive assay for the identification of intracellular kinases activated upon stimulation of signal cascades. Concordant with the results of Cano et al. (5), this assay permitted us to confirm that low concentrations of anisomycin, which reduce incorporation of [^{35}S]methionine into new proteins by not more than 50%, initiate intracellular signaling and kinase activation. Using Elk-$1_{307–428}$ and c-Jun as in-gel substrates, we identify the kinases p42 and p44 in HeLa cells, and p45 and p55 in both HeLa and KB cells, as anisomycin-stimulated kinases. The p42 and p44 kinases are probably members of the MAPK family, i.e., ERK2 and a close relative, as judged by their apparent molecular masses, by their induction with EGF and TPA, and by their substrate selectivity (highly active on Elk-1 and poorly active on c-Jun). The p45 and p55 kinases are likely members of the SAPK family, as shown by strong induction by anisomycin and UVC, very poor induction by EGF and TPA, high activity on c-Jun, and comigration with purified active SAPKα. Since SAPKα phosphorylates GST-$Elk_{307–428}$ (Fig. 8) and SAPKα is activated by anisomycin (25, 33), it is likely that the 55-kDa anisomycin-induced kinase is p54 SAPKα as proposed by Cano et al. (5). The primary sensor/receptor for anisomycin-induced activation of p42/p44 and p45/p55 kinases remains unknown at present.

Our findings in HeLa cells contrast with those of Cano et al. (5), who demonstrated that in mouse 10T1/2 cells, anisomycin was unable to stimulate MAPKs and therefore concluded that MAPKs were not essential for induction of the c-*fos* and c-*jun* genes. The profile of anisomycin-activated kinases that we observe in KB cells was similar if not identical to that reported by Cano et al. for 10/T1/2 cells and suggests that SAPK activation leads to c-*fos* induction. However, TPA induction shows that activation of MAPKs in the absence of SAPKs is sufficient for TCF phosphorylation and c-*fos* induction. Thus, cell-type-specific differences exist in the relative regulation of the MAPK and SAPK pathways.

Aided by the use of Elk-1 as an in-gel kinase substrate, we were also able to observe activation of both MAPKs and SAPKs upon treatment with cycloheximide, although this effect was significant only with cycloheximide concentrations that were fully inhibitory to protein synthesis. We cannot determine whether this is the result of protein synthesis inhibition per se or whether cycloheximide can directly activate signal pathways at this concentration. At low concentrations of anisomycin or cycloheximide, leading to 50% protein synthesis inhibition, significant kinase activation is seen only with anisomycin, not with cycloheximide. This finding indicates either that anisomycin-induced signaling is separable from protein synthesis inhibition or that at 50% inhibition levels the two drugs differentially affect the translation of one or more labile repressors of signaling.

MAPKs and SAPKs function as Elk-1 kinases. In this study, SAPKs are identified for the first time as targeting the transcription factor Elk-1. Since Elk-1 has been demonstrated to represent a potent substrate for MAPKs (10, 15, 18, 28; for reviews, see references 20 and 42), Elk-1 can now be considered a point of convergence for MAPK and SAPK signaling pathways. This characteristic distinguishes Elk-1 from c-Jun, which displays a high substrate preference for SAPKs over MAPKs (14, 25). While we can correlate phosphorylation of Elk-1 by SAPK with transcriptional activation, this remains to be verified in vivo. It is possible that members of the two classes of kinases modify overlapping sets of Ser and Thr residues within the Elk-1 molecule and thus exert differential effects on the activity of Elk-1.

Under all conditions tested here, the temporal correlation between TCF/Elk-1 phosphorylation status and c-*fos* gene activity that we detected previously (15, 45) is maintained. This finding strengthens the notion that Elk-1 phosphorylation functionally contributes to gene induction, while its reversal, Elk-1 dephosphorylation, contributes to gene inactivation. Elk-1 binds to the c-*fos* SRE upon serum response factor recruitment and thereby very likely contributes in vivo to c-*fos* activation upon signal activation. With our demonstration that Elk-1 represents a good substrate for the MAPK and SAPK signaling cascades, this transcription factor can be expected to play a major role in coupling the c-*fos* promoter to different signal transduction pathways. This could thereby explain how a plethora of extracellular stimuli can regulate this immediate-early gene. Conceivably other members of the TCF subfamily of Ets proteins, like SAP-1 and SAP-2/Erp/Net (42), may similarly act as integrators of different signals. In addition, different signaling pathways may selectively target the members of the TCF family (16).

MAPKs and SAPKs are differentially regulated by anisomycin and cycloheximide. Both MAPKs and SAPKs were rapidly induced by low and high concentrations of anisomycin in HeLa cells. High doses of cycloheximide also rapidly and preferentially stimulated the MAPKs. In contrast to anisomycin, high doses of cycloheximide led to a slow, steadily increasing activation of the SAPKs. Our data additionally reveal that MAPKs and SAPKs are apparently also down-regulated differently. Full inhibition of protein synthesis blocks down-regulation of p45 and p55 SAPKs, whereas SAPK inhibition occurs within 60 min under normal circumstances, or at conditions of 50% protein synthesis levels. In contrast, efficient down-regulation of MAPKs was seen within 60 min irrespective of the status of cellular protein synthesis. This finding indicates that the components involved in MAPK down-regulation are probably preexisting and do not require de novo synthesis, while an important component responsible for SAPK inactivation seems to be newly synthesized. MAPK phosphatase 1 (MKP-1) has been shown to be encoded by an immediate-early gene that is stimulated by extracellular stimuli with kinetics similar to those for c-*fos* induction (21, 39). From our data, it seems unlikely that

MKP-1 synthesis is required for MAPK down-regulation. Indeed, p42 MAPK inactivation seems to depend on protein phosphatase 2A and another unidentified tyrosine phosphatase (1). Given our observation that the SAPKs require protein synthesis for down-regulation, this raises the interesting possibility that in vivo, MKP-1 acts more efficiently on SAPKs than on MAPKs.

Role of protein synthesis inhibition in immediate-early gene superinduction. Superinduction of immediate-early genes by protein synthesis inhibitors is manifested in at least three ways: (i) mRNA stabilization, (ii) activation of intracellular signaling cascades, and (iii) interference with transcriptional down-regulation (5, 8, 9, 11, 27, 30, 38, 43). The relationship of these phenomena to protein synthesis inhibition remains unclear. We measured an increase in stability of c-*fos* mRNA upon gene activation by low anisomycin concentrations that only partially inhibited protein synthesis. These conditions also led to very efficient activation of both MAPKs and SAPKs, as well as the c-*fos* gene. In contrast, when cycloheximide was used at concentrations that resulted in a 50% inhibition of protein synthesis, we observed only marginal kinase activation and c-*fos* stimulation. Thus, the effects on mRNA stability of cycloheximide at low doses could not be reliably assessed. Cycloheximide induced significant MAPK and SAPK activities only at concentrations fully inhibitory to protein synthesis, with MAPK activation preceding that of SAPKs. Under these conditions, c-*fos* mRNA stability is also increased (11). Generally, anisomycin was a more potent inducer of c-*fos* transcription (Fig. 3) and of kinases (Fig. 6) than was cycloheximide at concentrations producing similar levels of protein synthesis inhibition.

The final aspect of gene superinduction concerns protein synthesis inhibitors interfering with postinduction repression of immediate-early genes (down-regulation). From our analyses (references 15 and 45 and this study), we conclude that immediate-early gene down-regulation involves at least two mechanisms: first, inactivation of signal-stimulated kinases like the MAPKs and SAPKs, and second, deactivation via dephosphorylation of transcription factors like Elk-1. This may occur via a regulated Elk-1 phosphatase activity or a constitutive Elk-1 phosphatase activity so that regulation is at the level of Elk-1 kinases. In the latter case, Elk-1 phosphorylation would be seen only when net phosphorylation exceeded phosphatase activity, as caused by the transient signal-stimulated activation of Elk-1 kinase activities such as MAPKs and SAPKs.

Interestingly, the rapid down-regulation of MAPKs occurs in the absence of protein synthesis, while down-regulation of the SAPKs does not occur when protein synthesis is fully inhibited. This differential sensitivity to inhibition of protein synthesis of the two classes of kinases suggests that the SAPKs play a more important role in mediating immediate-early gene superinduction by protein synthesis inhibitors. Furthermore, as discussed above, inducible kinase-specific phosphatases like MKP-1 may be more important for the down-regulation of SAPKs than for the down-regulation of MAPKs.

A labile repressor has been proposed to explain the disturbance of c-*fos* SRE regulation by protein synthesis inhibitors (34). If such a labile repressor exists, it must be a repressor of signal transduction activity. Furthermore, its mRNA would have to belong to a subpopulation that is preferentially inhibited by low doses of anisomycin but not by cycloheximide. Our results indicate that protein synthesis inhibitors disrupt the balance in the phosphorylation status of Elk-1 by activating signaling molecules such as MAPKs and SAPKs.

ACKNOWLEDGMENTS

R.Z. and M.A.C. contributed equally to this work.

We are grateful to Frank Schnieders and Tanja Vogel for advice and assistance concerning the nonradioactive detection method of RNA, to Dirk Bohmann for the gift of the c-Jun expression clone, to Ralf Janknecht for helpful comments on the manuscript, and to Manuela Baccarini for advice and assistance concerning in-gel kinase assays.

This work was supported by the DFG (grants 120/7-2 and SFB 265/A8) and the Dr. Mildred Scheel-Stiftung (grant W 37/92/No 1).

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