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Anisomycin-Activated Protein Kinases p45 and p55 but Not Mitogen-Activated Protein Kinases ERK-1 and -2 Are Implicated in the Induction of c-*fos* and c-*jun*

EVA CANO, CATHERINE A. HAZZALIN, AND LOUIS C. MAHADEVAN*

Nuclear Signalling Laboratory, Developmental Biology Research Centre, The Randall Institute, King's College London, London WC2B 5RL, United Kingdom

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Independent of its ability to block translation, anisomycin intrinsically initiates intracellular signals and immediate-early gene induction [L. C. Mahadevan and D. R. Edwards, Nature (London) 349:747–749, 1991]. Here, we characterize further its action as a potent, selective signalling agonist. In-gel kinase assays show that epidermal growth factor (EGF) transiently activates five kinases: the mitogen-activated protein (MAP) kinases ERK-1 and -2, and three others, p45, p55, and p80. Anisomycin, at inhibitory and subinhibitory concentrations, does not activate ERK-1 and -2 but elicits strong sustained activation of p45 and p55, which are unique in being serine kinases whose detection is enhanced with poly-Glu/Tyr or poly-Glu/Phe copolymerized in these gels. Translational arrest using emetine or puromycin does not activate p45 and p55 but does prolong EGF-stimulated ERK-1 and -2 activation. Rapamycin, which blocks anisomycin-stimulated p70/85^{S6k} activation without affecting nuclear responses, has no effect on p45 or p55 kinase. p45 and p55 are activable by okadaic acid or UV irradiation, and both kinases phosphorylate the c-Jun NH_2-terminal peptide 1–79, putatively placing them within c-Jun NH_2-terminal kinase/stress-activated protein kinase (JNK/SAPK) subfamily of MAP kinases. Thus, the EGF- and anisomycin-activated kinases p45 and p55 are strongly implicated in signalling to c-*fos* and c-*jun*, whereas the MAP kinases ERK-1 and -2 are not essential for this process.

When quiescent fibroblasts are stimulated with growth factors or phorbol esters, a complex series of intracellular signalling and immediate-early (IE) gene induction responses is rapidly initiated (reviewed in references 1, 2, 4, 9, 18, 24, 26, and 29). Concomitant with c-*fos*/c-*jun* induction, early mitogen-stimulated responses in chromatin include phosphorylation of histone H3, a high-mobility-group (HMG)-like protein (identified by protein microsequencing studies as HMG-14 [3a]), and a nucleolar fraction of S6 (3, 21, 31, 43). The hallmark of these early signalling and genetic responses is their transience; through concerted phosphatase action, key epidermal growth factor (EGF)-activated protein kinases are switched off within minutes and their substrates are dephosphorylated, producing a phosphorylation response that is rapidly terminated (29, 51). Similarly, transcription of c-*fos* and c-*jun* is switched off at about 30 min and their mRNAs are rapidly degraded, resulting in the transient presence of transcripts in the cell (reviewed in references 18, 24, 26, and 66).

By contrast, the combination of growth factors with translational inhibitors produces augmented, prolonged induction of IE genes, a process called superinduction (1, 18, 24, 66). At least three factors contribute to this (reviewed in reference 18). First, the normally labile IE gene transcripts become much more stable upon translational arrest (24, 66). Second, transcriptional shutoff is delayed in the presence of protein synthesis inhibitors, in some cases as a result of failure of autorepression (18, 40, 58). Third, some inhibitors, most potently anisomycin and to a lesser extent cycloheximide, can act as signalling agonists to induce chromatin-associated protein phosphorylation and IE gene induction similar to that induced by EGF or tetradecanoyl phorbol acetate (TPA) (3, 18, 31, 42, 43). The striking observation is that whereas growth factor-elicited responses are transient, those elicited by anisomycin are extremely sustained (18). Further, they remain demonstrable at subinhibitory anisomycin concentrations and are not a consequence of translational arrest (42). Finally, anisomycin stimulates an extremely restricted subset of the complex cytoplasmic signals elicited by EGF or TPA (reviewed in references 18 and 31) and will not, for instance, activate protein kinase C (43). However, it elicits chromatin-associated protein phosphorylation and gene induction responses virtually identical to those induced by EGF or TPA (3, 18, 31, 42, 43), suggesting its possible use to define an area of signalling from which the nuclear responses arise (31).

To establish how anisomycin initiates signals, we and others have shown that it produces prolonged activation of p70/85^{S6k} and S6 phosphorylation (6, 31, 34). However, ablation of p70/85^{S6k} activation by using rapamycin has no effect on anisomycin-stimulated nuclear signals or proto-oncogene induction (31). Thus, the point of anisomycin action and the point of bifurcation to histone H3/HMG-like protein phosphorylation and c-*fos*/c-*jun* induction both lie upstream of p70/85^{S6k} (31). Current indications are that a proline-directed protein kinase (PDPK) lies immediately upstream of and phosphorylates p70/85^{S6k} (19, 50). One group of PDPKs activated by EGF and TPA are the mitogen-activated protein (MAP) kinases (reviewed in reference 5, 7, 33, 37, 47, 54, and 55). The MAP kinase family comprises at least four distinct members (reviewed in references 47, 54, and 64). Three of these, p42^{mapk} (ERK-2), p44^{erk} (ERK-1), and p44^{mpk}, utilize microtubule-associated protein 2 and myelin basic protein (MBP) as in vitro substrates, whereas p54 MAP kinase, identified in cycloheximide-treated rat liver (35, 36), prefers microtubule-associated protein 2 to MBP. Sequencing and cloning studies have identified p54 MAP kinase as a member

* Corresponding author. Mailing address: Nuclear Signalling Laboratory, Developmental Biology Research Centre, The Randall Institute, King's College London, 26–29 Drury Ln., London WC2B 5RL, United Kingdom.

(SAPK-α) of the c-Jun NH_2-terminal kinase (JNK) or stress-activated protein kinase (SAPK) subfamily, comprising at least six closely related (88 to 90% identity) mRNA species with 40 to 45% sequence identity to the MAP kinases (17, 27, 35). Recent evidence implicates MAP kinases in conveying signals into the nucleus. First, ERK-1 and -2 activation is a common downstream response to a variety of initiating stimuli (47, 49, 52). Second, their translocation into the nucleus upon stimulation has been reported (10, 39). Third, an unknown PDPK, possibly a MAP kinase, has been directly implicated in c-*fos* induction via phosphorylation of $p62^{TCF}$ (22, 45, 67; reviewed in reference 65). Here, we have used in-gel renaturation and kinase assays (30), with various substrates copolymerized in these gels, to detect MAP kinase and other kinases activated by EGF and anisomycin. Further, because growth factors induce transient signalling and proto-oncogene induction whereas anisomycin produces prolonged responses, special emphasis has been placed on kinases that are activated transiently by EGF but sustainedly by anisomycin. The activation of these kinases would correlate well with that of c-*fos* and c-*jun* under inducing and superinducing conditions, and they would thus constitute part of restricted set of kinases strongly implicated in delivering growth factor- and anisomycin-stimulated signals into the nucleus.

MATERIALS AND METHODS

Cell culture and UV irradiation. Mouse C3H 10T1/2 fibroblasts were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum. Confluent cultures were rendered quiescent by incubation for 12 to 18 h in DMEM containing 0.5% (vol/vol) fetal calf serum. Cells were stimulated as indicated and lysed directly in buffers appropriate for the in-gel kinase assay or immunoprecipitation as described. For UV irradiation, 10-cm-diameter dishes of quiescent cells with 3 ml of DMEM were exposed to 400 J of UV radiation (254 nm) per m^2, using a Spectrolinker (XL-1000; Spectronics Corp.).

Reagents. All reagents were of analytical grade or higher from Sigma or ICN/Flow unless otherwise indicated. EGF (kindly provided by George Panayotou, Ludwig Institute for Cancer Research, London, United Kingdom) was used at a final concentration of 50 ng/ml. Stock cycloheximide, puromycin, and emetine solutions were prepared at 10 mg/ml in water, and anisomycin was prepared at 10 mg/ml in 40 mM HCl. Polyclonal rabbit antisera against ERK-1 and -2 (antisera 122 [anti-α ERK-2] and 124 [anti-ERK-1 and -2] [38]) were kindly provided by Chris Marshall, Chester Beatty Institute of Cancer Research, London, United Kingdom. Glutathione *S*-transferase (GST)–c-Jun*1-79* and control plasmids were generously provided by Michael Karin, Pharmacology Department, University of California, San Diego.

In-gel kinase assays. Quiescent C3H 10T1/2 cells (60-mm-diameter dishes) treated as indicated were lysed in 150 μl of Laemmli sample buffer (10% [wt/vol] glycerol, 5% [vol/vol] 2-mercaptoethanol, 2.3% [wt/vol] sodium dodecyl sulfate [SDS], 62.5 mM Tris-HCl [pH 6.8], 10 mM EDTA).

Kinase assays in SDS-polyacrylamide gels were carried out by using modifications of the procedures of Kameshita and Fujisawa (30) and Gotoh et al. (23). Polyacrylamide (14%) minigels (8 by 12 by 0.075 cm; Bio-Rad) were cast with or without 0.5 mg of MBP per ml or 0.2 to 0.3 mg of random copolymer of L-glutamic acid alone (poly-Glu) per ml or with tyrosine (4:1; poly-Glu/Tyr) or phenylalanine (4:1; poly-Glu/Phe) added to the SDS-polyacrylamide gel solution prior to polymerization. Bacterially expressed GST–c-Jun*1-79* fusion protein or GST (17, 27; plasmids kindly provided by Michael Karin) were purified by glutathione-agarose affinity chromatography as described previously (17, 27, 61), and 40 μg/ml was copolymerized for in-gel kinase assays. All subsequent steps were at room temperature unless otherwise indicated. After electrophoresis, SDS was removed by incubation in 20% isopropanol in 50 mM Tris-HCl (pH 8.0) (1 h, 250 ml), followed by 1 h in 1 mM dithiothreitol (DTT; Sigma)–50 mM Tris-HCl (pH 8.0) (250 ml). To denature proteins, gels were incubated (1 h) in 50 to 100 ml of 6 M guanidine-HCl (ultrapure; Sigma)–20 mM DTT–2 mM EDTA–50 mM Tris-HCl (pH 8.0). Proteins were renatured by incubation at 4°C, without agitation, in 250 ml of 1 mM DTT–2 mM EDTA–0.04% Tween 20 (ICN)–50 mM Tris-HCl (pH 8.0) for 12 to 18 h. For kinase assays, gels were equilibrated for 30 to 60 min in 10 ml of kinase buffer [1 mM DTT, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 20 mM $MgCl_2$, 40 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-NaOH (pH 8.0), 100 μM sodium vanadate], and the kinase reaction was carried out for 60 min in 10 ml of the same buffer with 30 μM ATP containing [γ-^{32}P]ATP (10 μCi/ml; NEN). The gels were then washed extensively in 5% (wt/vol) trichloroacetic acid plus 1% sodium PP_i (Sigma) until washes were free of radioactivity (usually four to five changes). Autoradiography of dried gels was performed with Kodak X-Omat films with two intensifying screens.

Immunoprecipitation and Western blotting (immunoblotting). For immunoprecipitation, cells were lysed in 0.5 ml of 20 mM Tris-HCl (pH 8.0)–40 mM sodium pyrophosphate–50 mM NaF–5 mM $MgCl_2$–100 μM sodium vanadate–10 mM EGTA–1% Triton X-100–0.5% sodium deoxycholate with protease inhibitors (41). Lysates (approximately 50 to 100 μg of protein per ml) were immunoprecipitated with 10 to 20 μl of rabbit antiserum 122 (38) attached to protein A-Sepharose, washed three times in lysis buffer without $MgCl_2$, and analyzed by in-gel kinase assays or Western blotting. For Western blot analysis, proteins were electrophoresed on 20-cm polyacrylamide gels (10% acrylamide, 0.165% bisacrylamide) and transferred overnight to polyvinylidene difluoride membranes (Immobilon P; Millipore); the transfer buffer consisted of 50 mM Tris, 380 mM glycine, 0.1% SDS, and 20% methanol. Membranes were blocked with 5% Marvel in 20 mM Tris (pH 8.0)–150 mM NaCl–0.05% Tween 20 (TBST) for 1 h and then probed with antiserum 124, which recognizes ERK-1 and -2 (38), diluted 1:10,000 in TBST for 1 h. The membranes were rinsed twice and washed three times for 5 min each time with TBST. After washing, blots were incubated with goat anti-rabbit antibody coupled to horseradish peroxidase (1:10,000 in TBST) for 1 h, washed as before, and visualized by using the ECL detection system (Amersham International).

Northern (RNA) blot studies and gene probes. C3H 10T1/2 cells were set up as described above and stimulated as indicated. Total cellular RNA was isolated by the method of Chomczynski and Sacchi (11). Aliquots containing 3 μg of RNA were resolved on formaldehyde-agarose gels (61) and transferred onto nylon membranes (Hybond-N^+; Amersham); hybridization was performed essentially as described by Church and Gilbert (13), using a ^{32}P-labelled *Bgl*II-*Sal*I fragment of v-*fos* (16) in pAT153. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe was a 1-kbp fragment of murine cDNA cloned into pBluescript KS^- (Stratagene). Mouse c-*jun* pAH119 (60) was generously provided by Rodrigo Bravo (Roche).

Phosphoamino acid analysis of p45 and p55. Bands from 10 lanes of in-gel (with poly-Glu/Tyr) kinase assays containing

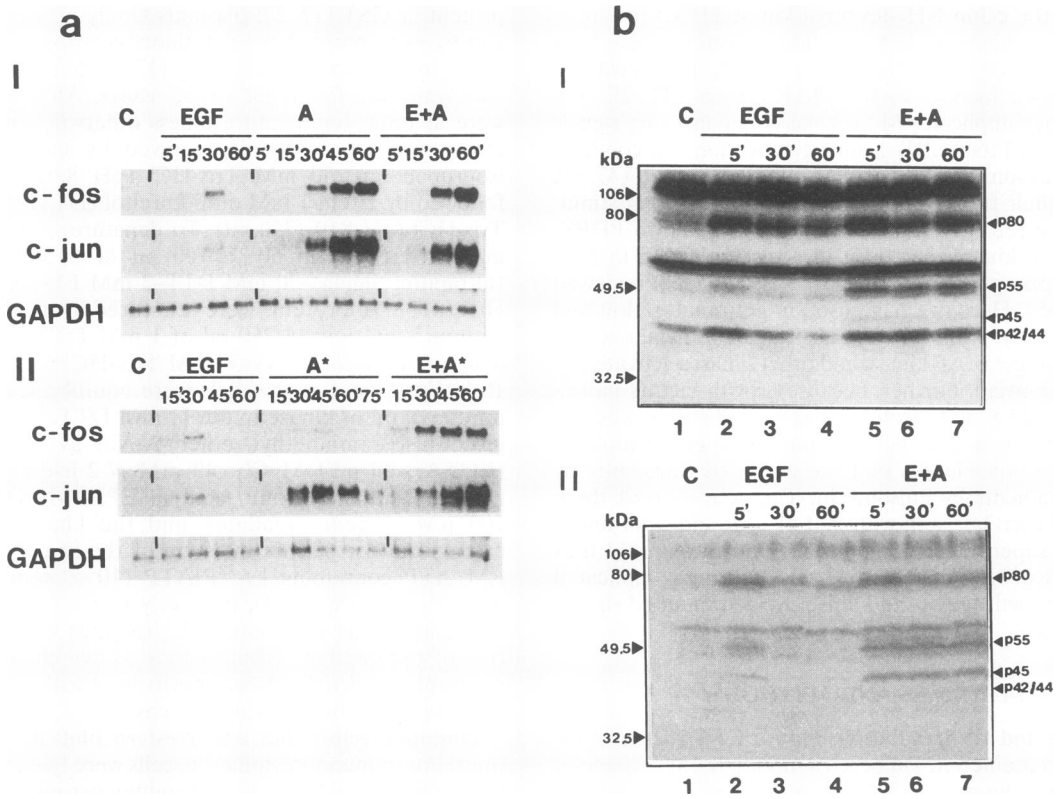

FIG. 1. Comparison of EGF- and EGF-anisomycin-stimulated genetic and signalling responses in C3H 10T1/2 cells. (a) Northern blot analysis of total RNA from quiescent confluent C3H 10T1/2 cells treated for 5 to 75 min as indicated. In panel I, anisomycin was used at an inhibitory concentration (10 μg/ml; A), while in panel II, a subinhibitory concentration of anisomycin (25 ng/ml; A*) was used. C, control unstimulated; A, anisomycin; E+A, EGF plus anisomycin. The EGF concentration was 50 ng/ml. Blots were hybridized to probes against v-*fos*, c-*jun*, and GAPDH as described in Materials and Methods. The GAPDH probe was used to confirm the equivalence of loading. (b) In-gel kinase assay of renatured proteins in a polyacrylamide gel containing MBP (panel I) or poly-Glu/Tyr (panel II) as the substrate. The cells were treated with EGF (50 ng/ml) alone or EGF (50 ng/ml) plus anisomycin (10 μg/ml) (E+A) for the times shown (5, 30, and 60 min). Lysates were prepared as described in Materials and Methods. The arrows indicate the five kinases activated by EGF: p42/44, p45, p55, and p80.

^{32}P-labelled p45 and p55 activated by stimulation with okadaic acid and anisomycin were located by autoradiography, excised, and subjected to *Staphylococcus aureus* V8 digestion (66 μg/ml) in 15 mM ammonium bicarbonate (pH 8.0) (overnight, 37°C). Supernatants containing labelled phosphopeptides were microcentrifuged and lyophilized to remove ammonium bicarbonate and gel fragments; this step was repeated thrice. Samples were hydrolyzed (110°C, 60 min) in 100 μl of constantly boiling HCl (Fisons) and repeatedly (three times) lyophilized to remove HCl. Two-dimensional thin-layer electrophoresis was performed exactly as described previously (15).

RESULTS

C3H 10T1/2 mouse fibroblasts were used for consistency with previous work, and EGF was used because it produces well-characterized, clear nuclear signalling responses (3, 18, 31) and activates MAP kinases (47, 54). To assay EGF- and anisomycin-activated kinases, in-gel renaturation and kinase assays have been used (23, 30, 57). This involves resolving proteins from control and stimulated cells on SDS-gels and renaturing them in the gel to recover kinase activity, which can then be detected by incubation with [γ-^{32}P]ATP (20, 23, 28, 30, 38). Further, if polypeptides such as MBP (30) or poly-Glu/Tyr (57) are copolymerized in these gels, the renatured kinases can, if appropriate, phosphorylate them, allowing detection of substrate-specific kinases. For brevity, we refer to this as an in-gel kinase assay.

Induction of c-*fos* or c-*jun* by EGF or anisomycin and their superinduction by EGF and anisomycin. To establish the characteristics of c-*fos*/c-*jun* induction and superinduction under various conditions, cells were treated with anisomycin at inhibitory (10 μg/ml; Fig. 1a, panel I) and subinhibitory (25 ng/ml; Fig. 1a, panel II) concentrations with or without EGF, and Northern blots were probed for c-*fos*/c-*jun* transcripts. EGF on its own produced rapid, transient induction of both genes, the response peaking at 30 min and terminating by 60 min. Anisomycin at the inhibitory concentration (Fig. 1a, panel I) elicited sustained induction of these mRNAs, high levels remaining at 60 min. The combination of EGF with anisomycin at the inhibitory concentration (Fig. 1a, panel I) superinduces c-*fos* and c-*jun*, high transcript levels remaining for up to 6 h (18) (not shown). With other translational inhibitors, c-*fos*/c-*jun* superinduction is strongly (10- to 50-fold) synergistic (18), whereas anisomycin on its own induces c-*fos* and c-*jun* to levels approaching superinduction by a combination of its potent signalling effect and the mRNA-stabilizing influence of translational arrest (18). When transcript stabilization is eliminated by using subinhibitory concentrations (42), anisomycin induces c-*fos* and c-*jun* more gradually, the response peaking at 45 min (Fig. 1a, panel II) and terminating by 75 min (18) (not shown).

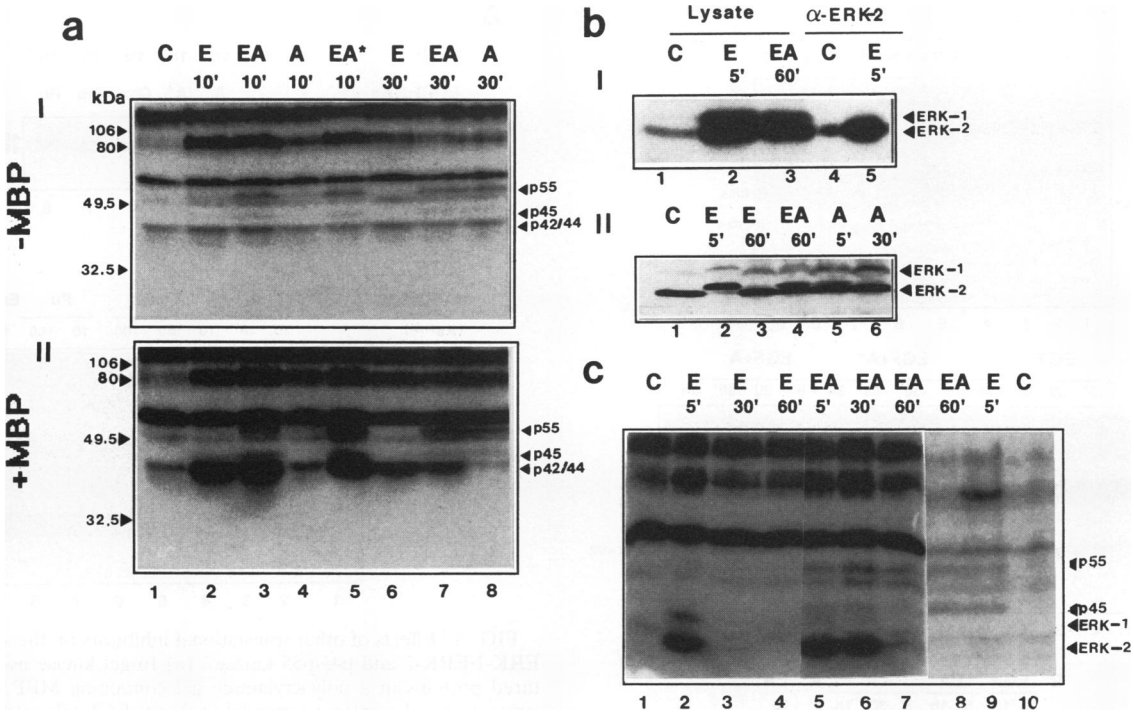

FIG. 2. Substrate specificities and identities of kinases activated under superinducing conditions. (a) In-gel kinase assay of renatured proteins in polyacrylamide gels in the presence or absence of MBP as the substrate. Lanes represent control unstimulated cells (C) and cells stimulated with EGF (50 ng/ml; E), anisomycin (10 μg/ml; A), and combinations of EGF and anisomycin at inhibitory (EA; 10 μg of anisomycin per ml) or subinhibitory (EA*; 50 ng of anisomycin per ml) concentrations for the times (in minutes) indicated. (b) Panel I, in-gel kinase assay of lysed cells (lanes 1 to 3) and immunoprecipitates (lanes 4 and 5), using antiserum 122 against ERK-2. C, control unstimulated; E, EGF (50 ng/ml, 5 min); EA, EGF plus anisomycin (10 μg/ml), 60 min. Duplicates of control and EGF-stimulated cells were immunoprecipitated with antiserum 122 as described, and immunoprecipitates were analyzed by in-gel kinase assays (lanes 4 and 5). Panel II, Western blot analysis of control unstimulated cells (C) and cells treated with EGF (E; 50 ng/ml, 5 and 60 min), anisomycin (A; 10 μg/ml, 5 and 30 min), and EGF plus anisomycin (10 μg/ml) (EA; 60 min). Antiserum 124, which detects both ERK-1 and ERK-2, was used. The positions of ERK-1 and ERK-2 are indicated. (c) In-gel kinase assay of renatured proteins in polyacrylamide gels containing as the substrate MBP (lanes 1 to 7) or Glu/Tyr (lanes 8 to 10). Lanes represent control unstimulated cells (C) and cells stimulated with EGF (E; 50 ng/ml); anisomycin (A; 10 μg/ml), and a combination of EGF and anisomycin at an inhibitory concentration (10 μg/ml) (EA) for the times (in minutes) indicated. The position of p55, p45, ERK-1, and ERK-2 are indicated.

At subinhibitory concentrations, superinduction is not sustained as at inhibitory concentrations (spike superinduction [18]). At both concentrations, anisomycin induces c-*jun* much more strongly than c-*fos*, a transcriptional effect previously observed by nuclear run-on assays (18).

Anisomycin produces prolonged activation of kinases activated transiently by EGF. Activation of kinases in response to EGF or EGF plus anisomycin was analyzed by in-gel kinase assays with copolymerized MBP (Fig. 1b, panel I) or poly-Glu/Tyr (Fig. 1b, panel II). With poly-Glu/Tyr, the clarity of signals from three activable kinases, labelled p45, p55, and p80, is enhanced by a combination of strengthening of signals, particularly that of p45, and suppression of background signals from nonactivable kinases close to p55 and p80. Apart from poly-Glu/Tyr acting as a substrate, the presence of highly charged amino acid polymers is known to selectively stimulate (e.g., polylysine [35, 46]) or inhibit (poly-Glu/Tyr [57]) specific kinases (see Fig. 6 for phosphoamino acid analysis). EGF activated five kinases. On MBP-containing gels, EGF activates a doublet at 42/44 kDa (Fig. 1b; labelled p42/44), similar in size and substrate utilization to ERK-1 and ERK-2 (5, 47, 54). The 42- and 44-kDa kinases virtually comigrate on the small gels used here but resolve on longer gels to represent two distinct kinases (see Fig. 2b, panel I, and 2c, lanes 5 to 7). These kinases are not detectable on poly-Glu/Tyr-containing gels or on gels devoid of a substrate (Fig. 1b, panel II). Three other EGF-activated kinases of 45, 55, and 80 kDa are also seen, with enhanced clarity on poly-Glu/Tyr-containing gels (Fig. 1b; labelled p45, p55, and p80). EGF produces rapid, transient activation of all five kinases, peaking 5 min after stimulation and terminating within 30 min. In the presence of anisomycin, activation of four of these, p42/44, p45, and p55 is prolonged, remaining detectable after 1 h. Further study of p80 is deferred because the sustaining effect is not consistently observed (see below). Thus, the activation of four kinases, p42/44, p45, and p55, correlates well with c-*fos*/c-*jun* activation under inducing and superinducing conditions (Fig. 1a).

Substrate specificity and anisomycin sensitivity of kinases activated under inducing and superinducing conditions. To determine which of the five kinases seen in Fig. 1b phosphorylate MBP, in-gel kinase assays were performed without (Fig. 2a, panel I) and with (Fig. 2a, panel II) copolymerized MBP. Only p42/44 kinase was highly dependent on the presence of MBP to produce a phosphorylation signal. The signal from the other kinases, p45, p55, and p80, is only slightly augmented with MBP in the gel, suggesting that these are autophosphorylating kinases, whereas p42/44 represents MBP kinases. Anisomycin on its own does not activate p42/44 kinase (Fig. 2a, lanes 4 and 8). Although it does seem to activate p80, this effect is not always observed (see Fig. 3a) and is often compromised

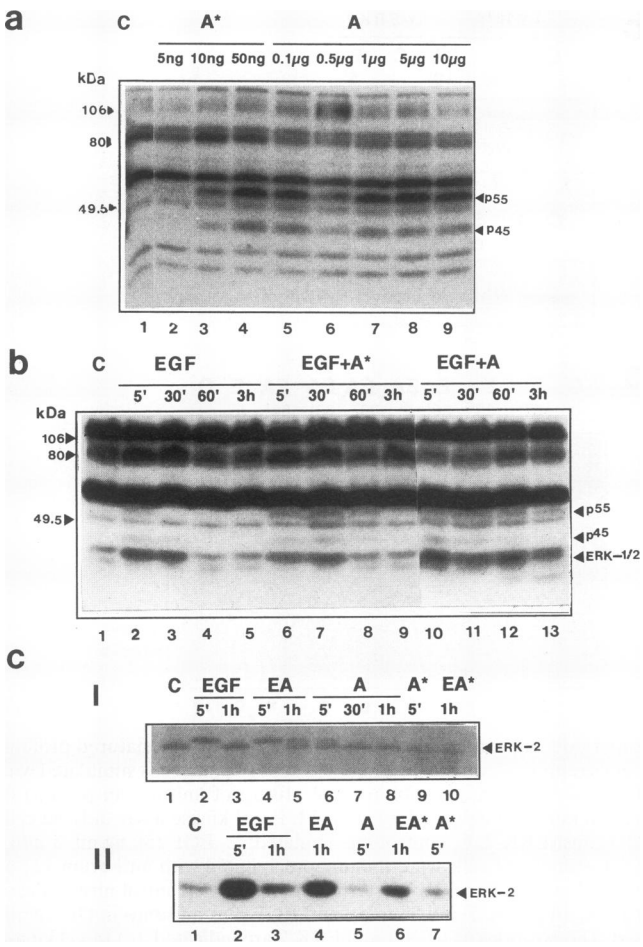

FIG. 3. Distinguishing between the translational arrest-related and signalling effects of anisomycin on kinases. (a) Untreated (C) C3H 10T1/2 cells or cells treated with subinhibitory (A*) or inhibitory (A) concentrations of anisomycin (10 μg/ml to 1 ng/ml) for 1 h as indicated were subject to in-gel kinase assays with copolymerized poly-Glu/Tyr. The positions of p55 and p45 are indicated. (b) In-gel kinase assay of renatured proteins in polyacrylamide gels containing MBP as the substrate. Lanes: 1, control unstimulated; 2 to 5 EGF (50 ng/ml); 6 to 9, EGF plus anisomycin (25 ng/ml); 10 to 13, EGF plus anisomycin (10 μg/ml). Cells were stimulated for 5 min to 3 h as specified. The positions of p55, p45, ERK-1, and ERK-2 are indicated. (c) Panel I, immunoprecipitation of ERK-2 using antiserum 122, followed by Western blotting using antiserum 124. Lanes: 1, control unstimulated; 2 and 3, EGF (50 ng/ml) for 5 min (lane 2) and 1 h (lane 3); 4 and 5, EGF plus anisomycin (10 μg/ml) for 5 min (lane 4) and 1 h (lane 5); 6 to 8, anisomycin (10 μg/ml) for 5 min (lane 6), 30 min (lane 7), and 1 h (lane 8); 9, anisomycin (25 ng/ml) for 5 min; 10, EGF plus anisomycin (25 ng/ml) for 1 h. The position of ERK-2 is indicated; the doublet representing phosphorylated and nonphosphorylated forms was identified by immunostaining. Panel II, in-gel kinase assay of antiserum 122 ERK-2 immunoprecipitates, using gels containing MBP as the substrate. Lanes: 1, unstimulated control; 2 and 3, EGF (50 ng/ml) for 5 min (lane 2) and 1 h (lane 3); 4, EGF plus anisomycin (10 μg/ml) for 1 h; 5, anisomycin (10 μg/ml) for 5 min; 6, EGF plus anisomycin (25 ng/ml) for 1 h; 7, anisomycin (25 ng/ml) for 5 min. The single band indicated (ERK-2) represents the active fraction of ERK-2.

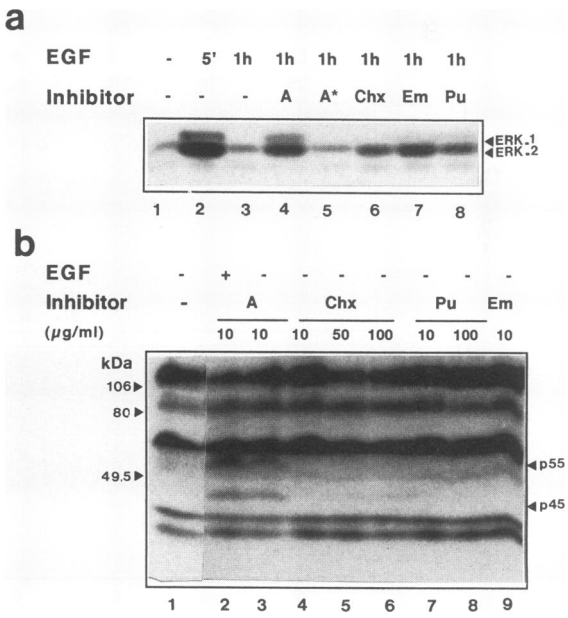

FIG. 4. Effects of other translational inhibitors on the activation of ERK-1/ERK-2 and p45/p55 kinases. (a) In-gel kinase assay of renatured proteins in a polyacrylamide gel containing MBP as the substrate. Lanes: 1, control unstimulated; 2 to 8, EGF (50 ng/ml) for 5 min (lane 2) or 1 h (lane 3) alone or for 1 h in the presence of the following translational inhibitors at the indicated concentrations: anisomycin (10 μg/ml) (A), anisomycin (25 ng/ml) (A*), cycloheximide (Chx) (10 μg/ml), emetine (Em) (10 μg/ml), and puromycin (Pu) (100 μg/ml). Apart from A*, all treatments produce complete translational arrest (18). (b) In-gel kinase assays of renatured proteins in a polyacrylamide gel containing poly-Glu/Tyr as the substrate, all samples being obtained in a single experiment. Cells were treated with the different agents for 1 h. Lane 1, control unstimulated; 2, as a positive control, treatment with a combination of EGF (50 ng/ml) plus anisomycin (10 μg/ml); 3, anisomycin (10 μg/ml) alone; 4 to 6, cycloheximide (Chx) at 10 (lane 4), 50 (lane 5), and 100 (lane 6) μg/ml; 7 and 8, puromycin (Pu) at 10 (lane 7) and 100 (lane 8) μg/ml; 9, emetine (Em) at 10 μg/ml.

by high background levels in control lysates; until this variability is eliminated, valid analysis of p80 is not possible. Anisomycin on its own clearly activates the p45 and p55 kinases (Fig. 2a, lane 8; see also Fig. 3a). Whereas EGF activation of p45 and p55 kinase is extremely rapid and transient (Fig. 2a, lane 2; Fig. 2c, lane 9; see also Fig. 1b), anisomycin activates them more gradually but sustainedly, activity becoming detectable at 30 min (Fig. 2a, lane 8) and remaining for at least 3 h (not shown). Thus, of the five kinases, anisomycin activates two, p45 and p55, with stronger and more prolonged activation than that elicited by EGF, correlating well with their relative effects on IE genes (Fig. 1a).

Identification of p42/44 kinases as ERK-1 and ERK-2. The activation, size, and MBP-phosphorylating activity of p42/44 kinases are reminiscent of MAP kinases ERK-1 and -2 (references 47 and 54 and references therein). This possibility was investigated by subjecting ERK-2 immunoprecipitated from these cells to in-gel kinase assays. An antibody specific for ERK-2 (38) immunoprecipitated the lower part of p42/44 kinases, identifying p42 unequivocally as ERK-2 (Fig. 2b, panel I, lanes 4 and 5). Because antibodies for immunoprecipitating ERK-1 were unavailable, a Western blotting antibody recognizing both ERK-1 and -2 (38) was used, exploiting the mobility shift of these kinases to indicate their phosphorylation and activation (Fig. 2b, panel II). Here, it was possible to use high-resolution gels in which the separation between ERK-1 and -2 as well as the mobility shift due to phosphorylation are clear. Both kinases were quantitatively activated within 5 min

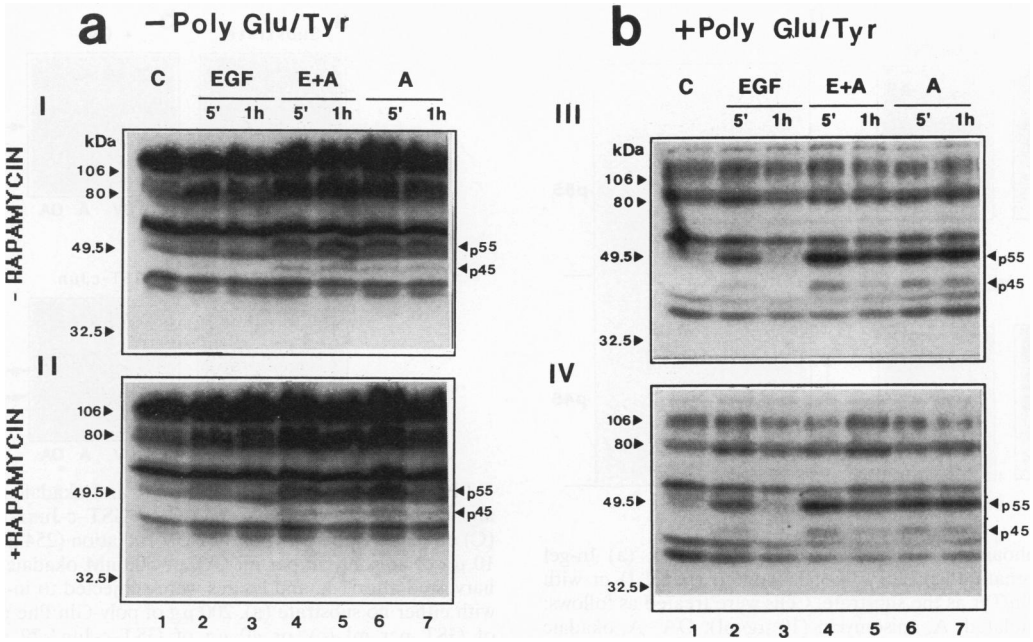

FIG. 5. Effect of rapamycin on the autophosphorylation of p45 and p55. (a) In-gel kinase assay of renatured proteins in a polyacrylamide gel without substrate. (b) In-gel kinase assay of renatured proteins in a polyacrylamide gel containing Glu/Tyr as the substrate. Untreated cells (panels I and III) or cells pretreated for 30 min with 20 ng of rapamycin per ml (panels II and IV) were stimulated with EGF (50 ng/ml) for 5 min (lane 2) or 1 h (lane 3), with EGF plus anisomycin (10 μg/ml) (E+A) for 5 min (lane 4) and 1 h (lane 5), and with anisomycin alone (10 μg/ml) (A) for 5 min (lane 6) or 1 h (lane 7). The positions of p45 and p55 are indicated. The detection of activated p45 and p55 by EGF alone at 5 min is evident in gels without poly-Glu/Tyr (a) but is enhanced in gels with poly-Glu/Tyr (b).

of EGF treatment, returning to baseline within 1 h. With EGF and anisomycin, a proportion of ERK-1 and -2 remained active at 1 h, agreeing with results from in-gel kinase assays. With anisomycin on its own, a slight mobility shift of ERK-2 but not ERK-1 is seen at 5 min, although no ERK-2 activation is detected by in-gel kinase assays (see Fig. 3c, panel II, lane 5). This slight mobility shift at early time points, but no ERK-2 activation, was observed with other translational inhibitors (unpublished data). Thus, either anisomycin-stimulated ERK-2 activation is so slight as to be undetectable by in-gel kinase assays or the mobility shift is not associated with its activation (discussed below; see Fig. 3c).

Distinction between anisomycin-activated kinase p45 and ERK-1. Because p45 kinase migrates close to ERK-1 on these gels, we performed in-gel kinase assays using longer gels to prove that these are clearly two distinct kinases (Fig. 2c). The position of ERK-1 is clear on the gel with copolymerized MBP (lanes 2 and 5 to 7), whereas the position of p45 is evident on the gel run in parallel containing poly-Glu/Tyr (lanes 8 to 10). On these longer gels (Fig. 2c), the separation between the two kinases is obvious. Their clearly distinct migration on these gels, taken together with the fact that ERK-1 is seen only on gels containing MBP and is not detectable with poly-Glu/Tyr as a substrate, whereas the reverse is true for p45, rules out the possibility the p45 may be ERK-1. In efforts to purify p45 for further analysis, we have also shown that its chromatographic properties are distinct from those of the ERKs (not shown).

Distinguishing between translational arrest-related and signalling effects of anisomycin on protein kinases. The anisomycin-stimulated effects described above could arise from translational arrest or from the intrinsic capacity of anisomycin to act as a signalling agonist. We recently showed that it is possible to dissociate the two; at concentrations below 70 to 80 ng/ml, anisomycin no longer blocks translation but continues to produce signalling responses (42). First, we examined whether anisomycin-stimulated activation of p45 and p55 occurs at subinhibitory concentrations. This analysis showed that anisomycin continued to activate these kinases at 10 and 50 ng/ml (Fig. 3a, lanes 3 and 4), concentrations at which no translational inhibition is seen (42). Thus, anisomycin-stimulated activation of p45 and p55 is not the consequence of translational arrest and arises from its signalling agonist effect.

Next, the ability of anisomycin to prolong EGF-stimulated activation of ERK-1 and -2 was analyzed at subinhibitory (25 ng/ml; Fig. 3b, lanes 6 to 9) and inhibitory (10 μg/ml; Fig. 3b, lanes 10 to 13) concentrations (18, 42). At the inhibitory concentration, anisomycin prolonged EGF-stimulated activation of ERK-1 and -2; this was not apparent at the subinhibitory concentration, suggesting its origin in translational arrest. By contrast, anisomycin prolongs p45 and p55 activation at both inhibitory and subinhibitory concentrations. The identity of ERK-2 in these experiments was confirmed by immunoblotting analysis (Fig. 3c, panel I), proving that anisomycin prolongs EGF-stimulated activation of ERK-2 only with translational arrest (panel I, lanes 4, 5, and 10). In addition, at the inhibitory (panel I, lanes 6 to 8) but not at the subinhibitory (panel I, lane 9) concentration, a slight ERK-2 mobility shift was observed with anisomycin alone at 5 min, although no ERK-2 activation was detected by kinase assays (Fig. 2a). To test if this was due to limited sensitivity of in-gel kinase assays, ERK-2 was quantitatively immunoprecipitated and then subjected to these assays (Fig. 3c, panel II). As before, no ERK-2 activation was seen in response to anisomycin alone, suggesting that the slight ERK-2 mobility shift observed with translational arrest is not associated with activating phosphorylation. This issue is now under investigation.

Effects of other translational inhibitors on activation of ERK-1 and -2 and on p45 and p55 kinases. The findings

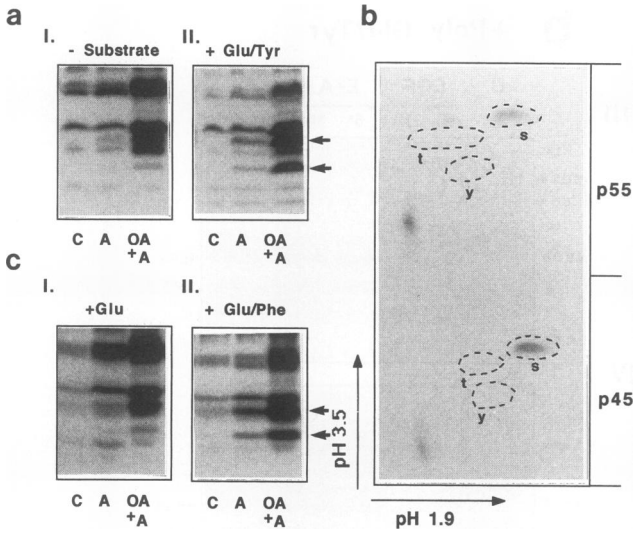

FIG. 6. Phosphoamino acid analysis of p45 and p55. (a) In-gel kinase assay of renatured proteins in gels without (panel I) or with (panel II) poly-Glu/Tyr as the substrate. Cells were treated as follows: C, control unstimulated; A, anisomycin (10 μg/ml); OA+A, okadaic acid (500 nM) plus anisomycin (10 μg/ml) to obtain maximal activation. p55 and p45 are indicated by arrowheads. (b) Phosphoamino acid analysis of p55 and p45 phosphoproteins stimulated with okadaic acid (500 nM) plus anisomycin (10 μg/ml) for 1 h. p45 and p55 bands were excised from 10 lanes of in-gel kinase assays as in panel a, part II (OA+A), labelled peptides were proteolytically released and hydrolyzed, and phosphoamino acids were resolved by two-dimensional thin-layer electrophoresis. Phosphoamino acid standards stained with ninhydrin were used to locate the positions of phosphoserine (s), phosphothreonine (t), and phosphotyrosine (y). (c) In-gel kinase assay of renatured proteins in gels with poly-Glu (panel I) or poly-Glu/Phe (panel II) as the substrate. Cells were treated exactly as in panel a. C, control; A, anisomycin (10 μg/ml); OA+A, okadaic acid (500 nM) plus anisomycin (10 μg/ml). p55 and p45 are indicated by arrowheads.

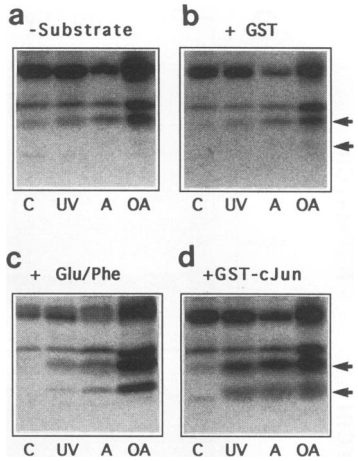

FIG. 7. UV irradiation, anisomycin, and okadaic acid activate p45 and p55 kinases which phosphorylate GST–c-Jun*1-79*. Control cells (C) or cells treated with 400 J of UV radiation (254 nm) per m^2 (UV), 10 μg of anisomycin per ml (A), or 500 nM okadaic acid (OA) were harvested after 1 h, and lysates were subjected to in-gel kinase assays with either no substrate (a), 200 μg of poly-Glu/Phe per ml (b), 40 μg of GST per ml (c), or 40 μg of GST–c-Jun*1-79* per ml (d). The positions of p45 and p55 kinases are indicated.

presented above suggest that prolonged activation of ERK-1 and -2 arises from translational arrest, whereas anisomycin activates p45 and p55 by acting as a signalling agonist. This implies that any translation inhibitor administered together with EGF would prolong ERK-1 and -2 activation, whereas no other inhibitor, apart from cycloheximide, which has a weaker signalling effect, would activate p45 and p55. The effects of cycloheximide, emetine, and puromycin on EGF-stimulated activation of ERK-1 and -2 were studied (Fig. 4a). We have previously shown that with EGF, all four inhibitors superinduce c-*fos* and c-*jun* principally through transcript stabilization arising from translational arrest (18). In in-gel kinase assays (Fig. 4a), all four inhibitors were capable, to different extents, of maintaining EGF-stimulated activation of ERK-1 and -2 (Fig. 4a, lanes 4 and 6 to 8), confirming that this is a consequence of translational arrest. Anisomycin at subinhibitory concentrations is negative in this respect (Fig. 4a, lane 5). In agreement with the literature (6), none of these translational inhibitors activated ERK-1 or -2 on its own (not shown); the presence of growth factor to activate the kinase is essential. Thus, translational arrest retards the deactivation of ERK-1 and -2 but cannot itself activate this kinase. This agrees with recent findings that ERK-1 and -2 deactivation is mediated by an inducible phosphatase, CL100/3CH134 (32, 51, 63). However, current work in our laboratory shows that translational (Fig. 3b) or transcriptional (not shown) arrest delays but does not completely abrogate deactivation of ERK-1 and -2, implying that an existing phosphatase is partly responsible for deactivation (8a).

The effects of these compounds on p45 and p55 kinases were assessed by in-gel kinase assays using poly-Glu/Tyr-containing gels (Fig. 4b). Neither puromycin (lanes 7 and 8) nor emetine (lane 9) produced p45 and p55 activation. Cycloheximide, which we previously showed to have a weaker signalling agonist effect (18, 43), did cause very weak p45 and p55 activation (lanes 4 to 6). Of these inhibitors, only anisomycin used on its own produced strong activation of p45 and p55 (lane 3). Taken together, these results confirm earlier indications that rapid deactivation of ERK-1 and -2 requires ongoing translation, whereas the activation of the p45 and p55 kinases arises from the signalling agonist effect of anisomycin.

Rapamycin sensitivity of anisomycin-activated kinases. In C3H 10T1/2 cells, anisomycin stimulates S6 phosphorylation via the $p70/85^{S6k}$ pathway (reference 31 and references therein). However, inhibition of anisomycin-stimulated $p70/85^{S6k}$ by rapamycin (12) has no effect on histone H3/HMG-like protein phosphorylation and c-*fos*/c-*jun* induction or superinduction (31). To locate the position of anisomycin-activated p45 and p55 kinases in this context, we tested the sensitivities of these two kinases to inhibition with rapamycin (12, 31). Untreated cells (Fig. 5a, panel I; Fig. 5b, panel III) or cells pretreated for 30 min with rapamycin (Fig. 5a, panel II; Fig. 5b, panel IV) were stimulated as indicated, and lysates were analyzed by in-gel kinases assays in the absence (Fig. 5a) or presence (Fig. 5b) of poly-Glu/Tyr. As described above, the presence of poly-Glu/Tyr (Fig. 5b) increases the clarity of p45 and p55 signals. Pretreatment with rapamycin does not block EGF (lanes 2 and 3)-, anisomycin (lanes 6 and 7)-, or EGF-anisomycin (lanes 3 and 4)-stimulated activation of p55 or p45 kinases. This result locates these two kinases upstream of $p70/85^{S6k}$, or on the bifurcation to nuclear signalling and proto-oncogene induction which is not affected by rapamycin treatment. Other experiments performed with MBP in in-gel kinase assays showed that ERK-1 and -2 activation is not inhibited by rapamycin (12) (not shown).

p45 and p55 are serine kinases showing enhanced activity in the presence of poly-Glu/Tyr or poly-Glu/Phe. A major problem in determining amino acid specificities of p45 and p55 kinases was the small quantity of ^{32}P-labelled material extractable after in-gel kinase assays, mainly because the denaturing and renaturing manipulations greatly decreased efficiency of proteolytic or electrophoretic release of labelled protein from these gels. To strengthen the p45 and p55 signals, we discovered that costimulation of these cells with anisomycin and the phosphatase inhibitor okadaic acid (14) greatly enhanced p45 and p55 signals (Fig. 6a). Okadaic acid alone also elicited activation of p45 and p55 kinases (see Fig. 7), implicating the okadaic acid-sensitive phosphatase 1 or 2A (14) in negative regulatory control at or upstream of these kinases. Costimulation with anisomycin and okadaic acid enabled sufficient label to be incorporated and proteolytically released from p45 and p55 for phosphoamino acid analysis (Fig. 6b). Under these conditions, only labelled phosphoserine (Fig. 6b) is obtained from both p45 and p55 bands, showing that they are serine kinases. Thus, the tyrosine in poly-Glu/Tyr is not acting as substrate for these kinases, but the presence of this polymer enhances p45 and p55 serine kinase activity. Unequivocal confirmation of this was obtained by using poly-Glu/Phe in place of poly-Glu/Tyr in these gels (Fig. 6c, panel II), which also enhanced p45 and p55 kinase signals, although it cannot act as substrate. Note that poly-Glu (Fig. 6c, panel I) slightly augmented p55 activity but had no effect on p45, indicating that the tyrosine or phenylalanine, in an as yet unknown way, provides preferable conditions for these kinases. Although other kinases, such as casein kinase II (reference 46 and references therein) and p54 MAP kinase/SAPK-α (35, 36), are known to be activated by charged amino acid polymers (polylysine), this represents the first demonstration of enhancement of kinase activity by poly-Glu/Phe.

Anisomycin, okadaic acid, or UV irradiation activates p45 and p55 kinases, which phosphorylate the c-Jun NH_2-terminal peptide 1–79. p45 and p55 kinases are similar in size and renaturability to the recently described UV-activated kinases JNK-1 and JNK-2, which strongly phosphorylate the amino terminus of c-Jun on serines 63 and 73. To assess their similarity, C3H 10T1/2 cells were treated with anisomycin, UV irradiation, and okadaic acid (Fig. 7) and subjected to in-gel kinase assays without substrate (Fig. 7a) or with either poly-Glu/Phe (Fig. 7c), GST protein (Fig. 7b), or GST–c-Jun*1-79* (Fig. 7d) copolymerized in these gels. This analysis showed first that UV irradiation and okadaic acid activate p45 and p55 kinases which are similar to those activated by anisomycin in that their detection is enhanced in the presence of poly-Glu/Phe, an effect clearer for p45 than for p55 (compare Fig. 7a and c). Second, anisomycin-activated p45 and p55 are identical to the UV- and okadaic acid-activated kinases in being able to strongly phosphorylate GST–c-Jun*1-79* (Fig. 7d). GST alone (Fig. 7b) does not enhance the signals. This indicates that the EGF- and anisomycin-activated p45 and p55 kinases in C3H 10T1/2 cells are very similar to the UV-activated kinases of the JNK/SAPK subfamily, although their exact identification must await sequencing, as there are at present six mRNA species in this subfamily (17, 35). A comprehensive comparison of UV, okadaic acid, and anisomycin effects on cytoplasmic and chromatin-associated signalling and on IE gene induction is to be presented elsewhere.

DISCUSSION

Growth factors elicit extremely complex early responses involving sequential activation of tyrosine, dual-specificity, and serine/threonine kinases and diverse other signals, rendering it difficult to distinguish events crucial to nuclear signalling from those associated with simultaneous responses such as membrane and cytoskeletal effects, receptor regulation, and recycling (5, 9, 23, 29, 44, 53, 54). In this and our recent work (3, 18, 31), we show that anisomycin may be useful in defining events involved in delivery of signals into the nucleus, as it activates a very restricted subset of cytoplasmic kinases but virtually all of the chromatin-associated protein phosphorylation and IE gene induction responses. Here, we have shown that EGF transiently activates the kinases p45 and p55, likely to be of the JNK/SAPK subfamily, whereas anisomycin activates them strongly and sustainedly. Further, anisomycin does not activate the MAP kinases ERK-1 and -2 but is clearly capable of inducing c-*fos* and c-*jun*, implicating p45 and p55, but not the ERKs, in this process.

Anisomycin-activated protein kinases p45 and p55 and the JNK/SAPK kinases. Several properties of the p45 and p55 kinases, such as their activation by anisomycin (35) and UV irradiation (17, 27), their apparent sizes (17, 35) and renaturability (17, 27), and their ability to phosphorylate the NH_2-terminus peptide 1–79 from c-Jun (17, 27, 35), place them within the JNK/SAPK subfamily, but there are differences that we discuss here. First, although p45 and p55 are activable by UV irradiation, a major point shown here is that they also respond to EGF in virtually the same way as the MAP kinases ERK-1 and -2. These kinases are therefore not restricted to stress responses but are activable by growth factor stimulating. An important consideration here is their very rapid and transient activation by EGF, peaking at 5 min and terminating within 10 to 15 min (Fig. 2a, lane 2); under conditions used by Kyriakis et al. (20-min stimulation [35]), we would not detect p45 and p55 activation in C3H 10T1/2 cells. Second, we find that the activation of p45 and p55 by anisomycin is not strictly related to the stress of translational arrest, as emetine or puromycin is ineffective, whereas anisomycin at sub-translational inhibitory concentrations is effective. Kyriakis et al. (36) find that emetine and puromycin activate very weakly compared with anisomycin, although all three agents ablate translation equally well (18, 42), supporting our observation that anisomycin has additional activating effects on these kinases (18). Third, using anti-p54 MAP kinase/SAPK-α antibodies (the kind gift of John Kyriakis, Harvard University), we immunoprecipitated an anisomycin-activated MAP kinase from C3H 10T1/2 cells that phosphorylated c-Jun in vitro (not shown), but this kinase, as previously reported by these authors (references 35 and 36 and references therein), was very poorly renaturable. We could not detect the immunoprecipitated p54 MAP kinase/SAPK-α by in-gel kinase assays, whereas anisomycin-activated p55 was readily detectable as the positive control on the same gel (not shown). This may find explanation in that distinct members of the JNK/SAPK subfamily may have different susceptibilities to renaturation, and that although the anisomycin-activated p55 reported here is of this subfamily, it may not be identical to p54 MAP kinase/SAPK-α (35). It is already clear that there are differences between p45 and p55 kinases because the presence of poly-Glu/Tyr or poly-Glu/Phe enhances p45 activity more markedly than p55 (Fig. 6 and 7). There being at least six closely related mRNAs within this subfamily (27, 35), resolution of these issues must await sequence information from p45 and p55.

The signalling pathway within which anisomycin acts. In addition to the ERK-1 and -2 activation cascade, there is emerging evidence of a poorly understood pathway involving an unknown PDPK that phosphorylates p70/85^{S6k} concomitant with its activation (19, 50). The MAP kinase kinase (MEK)

that phosphorylates and activates MAP kinase on the motif T-E-Y does not activate JNK/SAPKs, where the corresponding motif is T-P-Y (17, 35), indicating that anisomycin activates a distinct upstream kinase. As anisomycin also elicits strong prolonged phosphorylation and activation of $p70/85^{S6k}$ (3, 31, 34), the anisomycin-stimulated signal must originate upstream of both these and the JNK/SAPK kinases. The macrolide rapamycin, which specifically blocks anisomycin-stimulated $p70/85^{S6k}$ activation and S6 phosphorylation (12, 31), has no effect on histone H3/HMG-like protein phosphorylation or c-*fos*/c-*jun* induction (31), placing the point of bifurcation to these latter nuclear events upstream of $p70/85^{S6k}$. Likewise, the demonstration here that anisomycin activates two kinases, p45 and p55, which are unaffected by rapamycin places these kinases either upstream of $p70/85^{S6k}$ or along the bifurcation to nuclear signalling that is impervious to rapamycin. Phosphorylation of c-Jun on serines 63 and 73 by JNK/SAPK kinases potentiates its transactivating capacity and activates the c-*jun* proto-oncogene through upstream TRE sites (references 17 and 27 and references therein) which are constitutively occupied by AP-1 complexes (59). It is noted that apart from c-Jun, the JNK/SAPK kinases may phosphorylate and activate other downstream kinases, as seen with the MAP kinases ERK-1 and -2.

Role of MAP kinases in nuclear signalling. In addition to identifying two kinases that are tightly correlated with IE gene induction and superinduction, this work shows that contrary to suggestions in recent work (reviewed in references 47, 48, and 65), the activation of the MAP kinases ERK-1 and -2 is clearly not essential for the delivery of signals to IE genes. Recent evidence does suggest a role for ERK-1 and -2 in the nucleus but does not prove their direct involvement in IE gene induction. First, members of this family such as ERK-1/2 and its activable substrate the serine/threonine kinase $pp90^{rsk}$ have been shown to translocate to the nucleus upon stimulation (10, 39). However, the translocation lags behind the peak of c-*fos*/c-*jun* activation. Second, several transcription factors including c-Jun are substrates for MAP kinases (47, 55, 56). p54 and p42/44 MAP kinases both phosphorylate c-Jun within the A1 transactivation domain, which is phosphorylated in response to mitogens (55, 56). However, the kinase that executes this function in vivo may in fact be the JNK/SAPKs described above which may be physically complexed to c-Jun (17, 27). The main evidence implicating MAP kinases in IE gene activation comes from studies of the transcription factor $p62^{TCF}$ (22, 45, 67; reviewed in reference 65). Mitogens stimulate rapid phosphorylation of $p62^{TCF}$ on sites adjacent to proline that can be phosphorylated by MAP kinases in vitro, modulating ternary complex characteristics on the serum response element. But the identity of the PDPK responsible for $p62^{TCF}$ phosphorylation in vivo remains unknown (see reference 65). We show here that anisomycin does not activate ERK-1 or -2 but will clearly induce c-*fos*/c-*jun* expression, implying that phosphorylation of $p62^{TCF}$ by ERK-1 or -2 cannot be necessary for anisomycin-stimulated c-*fos* induction. We are currently investigating the ability of anisomycin-activated p45 and p55 to phosphorylate $p62^{TCF}$, as this would connect anisomycin stimulation with c-*fos* induction without involving the MAP kinases. Note that the uncoupling of c-*fos* induction from p42/44 MAP kinase is also supported by converse experiments using kinase-negative EGF receptor mutants, in which clear activation of p42/44 MAP kinase is not associated with c-*fos* gene activation (8, 62); similar observations having been reported for c-*jun* (25). Thus, a distinct PDPK activated by EGF and anisomycin, for which the JNK/SAPKs p45 and p55 are now candidates, although other kinases may emerge, might be responsible for $p62^{TCF}$ phosphorylation in vivo.

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