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ACTIVATION OF THE MITOGEN-ACTIVATED PROTEIN KINASE CASCADE BY TYRPHOSTIN (RG 50864)

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Abstract—Tyrphostins are synthetic compounds which have been described as *in vitro* inhibitors of epidermal growth factor (EGF)-receptor tyrosine kinase activity. In NIH3T3 cells, stimulation of EGF-receptor tyrosine kinase leads to an increase of intracellular protein phosphorylations, among them the phosphorylation of mitogen-activated protein (MAP) kinase and the S6 kinases $p90^{rsk}$ and p70S6K. Phosphorylation of these proteins, either on tyrosine or serine/threonine residues or on both residues increases their protein kinase activity. Unexpectedly, treatment of NIH3T3 cells with both tyrphostin (RG 50864) and EGF results in an increase in the level of tyrosine phosphorylation of the MAP kinase. During this treatment, we also observed an increase in MAP kinase and S6 kinase $p90^{rsk}$ activities. Tyrphostin treatment diminishes the level of c-fos mRNA but has no effect on c-myc mRNA expression nor on S6 kinase p70S6K activity. Mitogenic signalling induced by EGF in NIH3T3 cells was blocked by tyrphostin, suggesting that the target(s) for this event may be elements downstream from the MAP kinase or independent of this signal transduction.

Key words: MAP kinase; S6 kinases; tyrphostin; c-fos mRNA; protein tyrosine kinase; cell proliferation; EGF-receptor

Peptide growth factors elicit a burst of intracellular protein phosphorylations in cells, most of which occur on serine and threonine residues. Because tyrosine-specific growth factor receptor kinases initiate these phosphorylation events, they must regulate serine/threonine-specific protein kinases and phosphatases.

All receptor tyrosine kinases (RTKs)‡ studied so far phosphorylate themselves (autophosphorylation) in response to ligand binding. Several experiments in the past have highlighted an important function of autophosphorylation: individual phosphotyrosine residues of receptors appear to serve as highly selective binding sites specific to cytoplasmic signalling molecules. These signalling molecules mediate the pleiotropic responses of cells to growth factors [1].

Mitogen-activated protein (MAP) kinases, also called extracellular signal-regulated kinases (ERKs) [2] are serine/threonine protein kinases that are activated in response to a wide variety of stimuli. Activation of MAP kinases has been observed during growth factor stimulation, differentiation, secretion and insulin stimulation of glycogen synthesis [3]. MAP kinases therefore appear to be a central component of many different signal transduction pathways. The activation of MAP kinases has two consequences: the direct phosphorylation of substrates, such as transcription factors c-myc, c-jun and $p62^{TCF}$ [4–7], and the activation of other kinases, such as S6 kinase $p90^{rsk}$ [8, 9] and MAP KAP kinase 2 [10].

Two distinct families of growth factor-regulated S6 protein kinases have been identified as functioning in these signalling processes: the *rsk*-encoded 85–92 kDa S6 kinases referred to as $p90^{rsk}$ [11, 12] and the 70 kDa and 85 kDa S6 kinases [13, 14] referred to as p70S6K. Although both families of S6 kinases are activated rapidly after addition of growth factors to quiescent cells, several studies indicate that the activities of $p90^{rsk}$ and p70S6K [15–17] can be regulated by distinct pathways. The upstream participants in the signalling pathway leading to the activation of RSK are now being identified. However, little is known about the effectors participating in the regulation of 70 kDa and 85 kDa S6 kinases [19] and the physiological role of this signalling pathway.

The subsequent characterization and purification of a MAP kinase (MAP KK or MEK), that activates MAP kinase by concomittant phosphorylation on threonine and tyrosine and is itself regulated by serine/threonine phosphorylation [20–24], provided an additional member of the link between the RTK and the MAP kinase.

In vivo expression of oncogenically activated forms of the raf serine/threonine protein kinase results in the activation of MAP kinase ERK2 in Swiss 3T3 fibroblasts and in Cos-1 cells [25], suggesting that raf is upstream of the MAP kinase.

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‡ Abbreviations: BSA, bovine serum albumin; EGF, epidermal growth factor; SDS, sodium dodecyl sulfate; EGTA, ethyleneglycol-bis-β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MAP kinase, mitogen-activated protein kinase; ERK, extracellular regulated kinase; PMSF, phenyl methane sulfonyl fluoride; RTK, receptor protein tyrosine kinase.

In vitro raf is also able to activate the MAP KK [25]. Recent studies demonstrate that c-ras can mediate signal transduction from RTK to the MAP kinases, S6 kinases $p90^{rsk}$ and c-raf [26, 27]. Downstream of this event, the nuclear and cytoplasmic localisation of MAP kinases and S6 kinases $p90^{rsk}$ [28] and their temporal regulation [29, 30] are consistent with the possibility that these activated protein kinases can transmit growth-modulatory information in the nucleus and participate in the regulation of immediate early gene expression.

Protein tyrosine kinase inhibitors acting on ligand-activated growth factor receptors and on a number of oncogene products have been described (for review see Refs 31–34). Among these inhibitors, the most extensively studied are genistein, erbstatin and tyrphostins. Genistein, an ATP binding site competitor, inhibits EGF-RTK both *in vitro* and *in vivo* [35]. It also behaves as an S6 kinase [36] and DNA topoisomerase II [37] inhibitor. Erbstatin inhibits EGF-RTK activity both *in vitro* [38] and in intact cells [39] and also inhibits $pp60^{v\text{-}src}$ tyrosine kinase activity when added to Rous sarcoma virus infected cells [38]. Tyrphostins are synthetic compounds described for their inhibitory activity on the EGF-dependent proliferation of keratinocytes, A431/clone 15 and HER14 cells [40–42]. The antiproliferative actions of some tyrphostins are correlated with inhibition of EGF-R tyrosine kinase activity in intact cells and also *in vitro*. Some tyrphostins also block EGF-induced PLC γ phosphorylation, Ca^{2+} release in living cells [43] and phosphoinositide production [44]. Based on the information provided above, certain tyrphostins affect the EGF signal transduction systems [41, 43, 44]. The effect on intact cells requires a relatively long period of incubation and it was suggested by Faaland and coworkers that the inhibitory effect of tyrphostins on EGF-RTK activity could be an indirect mechanism [45].

In the present work, we have investigated the simultaneous treatment of NIH3T3 cells by EGF and one of the tyrphostins (RG 50864) on some early events observed in the EGF transduction signal and particularly on the different kinase activities, the activity of which is increased by the fixation of EGF to its receptor. We show that MAP kinase and S6 kinase $p90^{rsk}$ activities are enhanced in the cells treated by tyrphostin and EGF as compared to the EGF treated cells. Under the same conditions the S6 kinase p70S6K activity is not modified. Tyrphostin inhibits other events downstream from the MAP kinase leading to an antiproliferative effect in these cells.

MATERIALS AND METHODS

Materials and antibodies

Mouse EGF was purchased from Sigma (France). MAP kinase substrate peptide, S6 kinase substrate peptide, anti-mouse rsk kinase (rsk III) polyclonal and anti-P tyrosine monoclonal antibodies were from UBI (Lake Placid, U.S.A.) and mouse-anti MAP kinase (ERK 1 + 2) monoclonal antibody was from Zymed (San Francisco, U.S.A.). Ribosomal 40 S subunits were purified from rat liver as described [36].

Tyrphostin (RG 50864) was synthesized as described by Yaish *et al.* [40] and its physical characteristics (mass spectroscopy, NMR) were identical to a genuine sample.

Cell culture and preparation of extracts

NIH3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Cells were usually seeded at a density of 2×10^6 cells/15 cm diameter dish and left to grow for 3 days. The medium was then replaced with DMEM containing 0.1% fetal calf serum. After 48 hr, quiescent cells were either treated or not with EGF (12 ng/mL) in the presence of different concentrations of tyrphostin at different incubation times. After varying lengths of time at 37°, cells were rinsed twice with 5 mL of ice-cold phosphate-buffered saline and once with ice-cold buffer A (80 mM β-glycerophosphate, 15 mM $MgCl_2$, 20 mM EGTA Na_2). Cells were scraped into 0.3 mL/dish of ice-cold buffer A containing 1 mM Na_3VO_4, 10 μg/mL leupeptin, 10 μg/mL aprotinin and 1 mM PMSF homogenized with a tissue homogenizer, and then centrifuged at 100,000 *g* for 30 min at 4° and stored at −80°.

Purification of the MAP kinase

Cells were prepared as previously described except that cell lysis was accomplished in 50 mM β-glycerophosphate (pH 7.2), 100 μM sodium vanadate, 2 mM $MgCl_2$, 1 mM Na_3EDTA, 0.5% Triton X-100, 10 μg/mL aprotinin and 1 mM dithiothreitol. The cell lysates were centrifuged for 5 min in a microcentrifuge to remove insoluble cell components. The supernatants were normalised for proteins and 0.5 mL was loaded onto a 0.2 mL Q Sepharose column equilibrated in 50 mM β-glycerophosphate (pH 7.2), 100 μM sodium vanadate, 1 mM EGTA and dithiothreitol (buffer A). After rinsing the column once with 2 mL of buffer A and a second time with 2 mL of buffer A containing 0.2 M NaCl, the MAP kinase was eluted with 0.5 mL buffer A containing 0.4 M NaCl. Fractions (0.5 mL each) were collected and 12.5 μL of the fractions assayed using the MAP kinase assay.

Kinase assays

Assay for MAP kinase. Cytosolic extracts (12.5 μL) were mixed with 4.2 μL substrate (MAP kinase substrate peptide, final concentration = 1 mM) and the phosphorylation was initiated by adding 8.3 μL of assay buffer containing 75 mM β-glycerophosphate, 6 μM KI peptide, 30 mM $MgCl_2$, 0.15 mM Na_3VO_4, 1.5 mM dithiothreitol, 3.7 mM $EGTANa_2$ and 0.3 mM ATP (0.5 μCi of [γ-^{32}P]ATP, specific activity ~3000 Ci/mmol). Reactions were carried out for 10 min at 30° and terminated by spotting aliquots (20 μL) onto P-81 phosphocellulose filter paper squares (Whatman) which were washed several times with 175 mM phosphoric acid. Phosphate incorporation levels, measured in the absence of substrate, were substracted from values obtained in the presence of substrate to correct for non-specific phosphorylation.

Assay for S6 kinase $p90^{rsk}$. Cell-free lysate (50 μg of protein) prepared as described above, was

incubated with 5 μg anti-rsk III on ice for 1 hr and then absorbed onto protein A Sepharose (Sigma). The immunocomplexes were washed twice with buffer B (1% Nonidet P-40, 0.5% deoxycholate, 0.1 M NaCl, 10 mM Tris–HCl, pH 7.2, 1 mM EDTA, 1 mM Na_3VO_4, 2 mM dithiothreitol, 40 μg/mL PMSF), twice with buffer C (buffer B containing 1 M NaCl), and once with ST (150 mM NaCl, 50 mM Tris–HCl pH 7.2). Following the final wash, the immunocomplexes were suspended in 20 μL of 1.5 × $p90^{rsk}$ reaction buffer minus ATP and substrate. The reaction was started by the addition of 10 μL of ATP and substrate. The final reaction concentrations were 20 mM Hepes–KOH pH 7.2, 10 mM $MgCl_2$, 50 μM ATP (20 μCi of [γ-^{32}P]ATP), 3 mM 2-mercaptoethanol, 0.1 mg of bovine serum albumin per mL and 0.75 mM of S6 kinase peptide. The reaction was allowed to proceed for 15 min at 30° (linear assay conditions) and terminated by centrifugation at 15,000 *g* for 15 min at 4°. Aliquots (20 μL) were spotted on P-81 phosphocellulose papers and proceeded as described above.

Assay for S6 kinase p70S6K. S6 kinase p70S6K was measured in the cellular extracts by using the 40 S ribosomal subunit as previously described [36]. Under this condition less than 5% of the S6 kinase p70S6K activity corresponds to the $p90^{rsk}$.

Immunoblot and immunoprecipitation analysis

Cell-free lysates were prepared in buffer containing 1% Nonidet P-40, 50 mM Tris–HCl, pH 7.6, 2 mM EDTA, 100 mM NaCl, 1 mM Na_3VO_4, 1 mM PMSF, 20 μg/mL aprotinin and 20 μg/mL leupeptin (buffer C), clarified by centrifugation at 15,000 *g* for 15 min at 4°. Protein concentrations were determined using bicinchoninic acid reagent (Pierce).

Polypeptides were resolved by SDS–10% polyacrylamide gel electrophoresis (4 hr at 30 mA) and then transferred electrophoretically to nitrocellulose membranes (BAS-85 Schleicher et Schuell) in the transfer buffer (192 mM glycine, 25 mM Tris base) using a Bio-Rad semi-dry blotter (4 mA/cm^2, 2 hr at 4°).

Following blocking in TBS containing 0.05% Tween 20 and 5% of fat-free dried milk, the membrane was incubated overnight at 4° in blocking buffer containing antiserum to MAP kinase (1:4000 dilution, Zymed) or antiserum to phosphotyrosine (1:2000, UBI). Membranes were washed three times in blocking buffer (10 min) and further incubated in the same buffer containing a second antibody conjugated with horseradish peroxidase. The specific interaction was visualised with the enhanced chemiluminescence procedure (Amersham).

For immunoprecipitation, 450 μg of cellular extracts in 600 μL of buffer C were incubated with RK2 1/1000 [46] or with mAb # sc 154 (Santa Cruz) 1/1000 at 4° for 2 hr. Immunocomplexes were recovered by the addition of 10% (v/v) protein A Sepharose (Sigma). The beads were washed three times in buffer C and proteins were analysed as previously described.

EGF-receptor tyrosine kinase assay

A431 cells (ATCC #CRL 1555) were grown in DMEM supplemented with 5% fetal calf serum. The cells, collected by centrifugation at 1200 rpm at 4°, were homogenized (20 strokes) in 20 mM Hepes–KOH pH 7.2, 2.5 mM $MgCl_2$, 1 mM PMSF, 10 μg/mL pepstatin, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM benzamidin and 2 μg/mL Soybean Trypsin Inhibitor. The homogenate was centrifuged at 2500 *g* for 5 min at 4°. The supernatant was centrifuged at 23,000 *g* for 30 min. The pellet was used as membrane fraction.

For the *in vitro* kinase assay, the reaction mixture contained 25 μg of membrane fraction in 20 mM Hepes–KOH pH 7.4, 0.1 mM $MnCl_2$, 75 μg/mL BSA, 0.1 mM Na_3VO_4 with or without prior incubation for 10 min with EGF (1.2 μg/mL). Phosphorylation in the presence or absence of tyrphostin was initiated by adding 15 μM ATP (5 μCi of [γ-^{32}P]ATP). Reaction was carried out for 10 min at 0° and terminated by the addition of 3 × sample buffer (0.375 M Tris–HCl pH 8.8, 6% SDS, 30% glycerol and 3% 2-mercaptoethanol). Phosphorylated polypeptides were then resolved by SDS gel electrophoresis followed by autoradiography. The level of ^{32}P incorporated into EGF receptor was determined by densitometric scanning of the autoradiogram.

Preparation of RNA and northern-blot analysis

Cells were seeded at 10^6 cells per 15 cm diameter dish. Three days later, cell growth was arrested by 48 hr serum deprivation. Cells were then exposed to increasing drug concentration just prior to EGF addition, or treated with a unique dose of tyrphostin for different times of EGF (12 ng/mL) treatment. The preparation of RNA was carried out as previously described [36]. The different probes were obtained from Dr F. Dautry (Institut Gustave Roussy, Villejuif) and proceeded as described [47].

Cell proliferation

NIH3T3 fibroblasts were seeded at 1.6 × 10^4 cells per well of a 96-well plate and grown for 2 days in DMEM supplemented with 10% fetal calf serum. Cells were cultured for another 2 days in DMEM with 0.1% fetal calf serum. They were then stimulated with 12 ng/mL of EGF or 10% fetal calf serum in the presence of different concentrations of tyrphostin for different lengths of time. After treatment, cells were pulse labelled with [^{3}H]thymidine (0.1 μCi per well) for 1 hr, rinsed twice with PBS, and then trypsinized. Cells were harvested using a cell harvester (Skatron Instruments, Norway) and the incorporated radioactivity was determined by scintillation counting.

Alternatively, cells plated at 4 × 10^3 cells per well in a 24-well plate in DMEM with 10% fetal calf serum were allowed to attach for 24 hr and then incubated with increasing concentrations of tyrphostin. Cells grown in the same medium were then counted at 24 hr intervals.

RESULTS

Effect of tyrphostin (RG 50864) on the level of EGF-stimulated tyrosine phosphorylation

We have examined the effect of a brief treatment by tyrphostin on the level of tyrosine phosphorylation

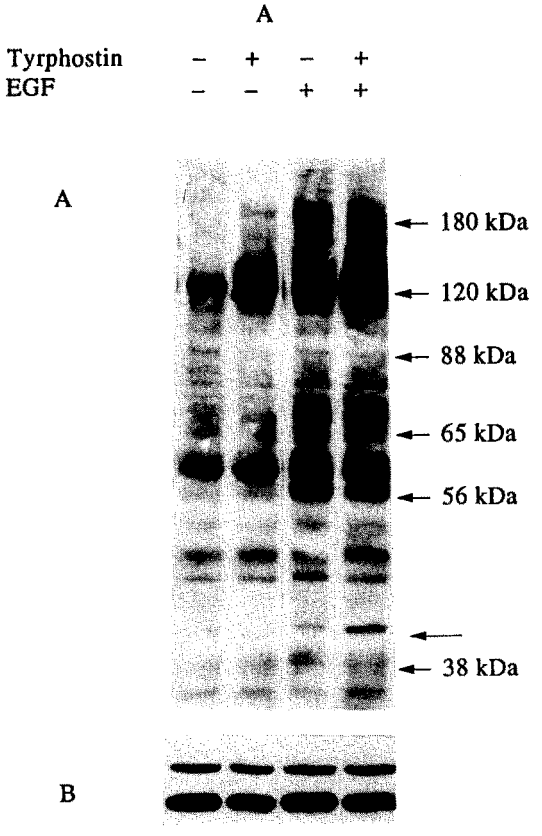

Fig. 1. Effect of tyrphostin on the level of tyrosine phosphorylation in the proteins of NIH3T3 cells. Quiescent NIH3T3 cells were treated either with or without EGF in the presence or absence of tyrphostin (100 μM) for 5 min. Cell lysates were prepared in buffer containing 1% NP40 as described in Materials and Methods. Polypeptides were resolved by SDS–10% polyacrylamide gel electrophoresis. After blotting, the membrane was incubated either with antibodies to phosphotyrosine (1:2000) (A) or with antibody directed to MAP kinase (1:4000) (B). The specific interaction was visualized with the enhanced chemiluminescence procedure (Amersham).

induced by EGF in NIH3T3 cells by western blot analysis using anti-phosphotyrosine antibodies. As shown in Fig. 1A, treatment of cells with 12 ng/mL of EGF for 5 min led to an increase in phosphorylation of some proteins, especially a 42 kDa protein (4.5-fold) and proteins of 54 kDa and 70 kDa, which appear as new bands in the EGF-treated cells. Immunoblotting with an anti-MAP kinase antibody identified the 42 kDa protein as a MAP kinase (Fig. 1B). An increase of tyrosine phosphorylation in a protein of 120 kDa corresponding to the ras GTPase-activating protein was also observed.

Treatment of quiescent cells with 100 μM of tyrphostin did not modify the phosphorylation level of the proteins which are phosphorylated in the absence of EGF stimulation (Fig. 1), except for a slight increase in 120 kDa and 60 kDa proteins.

When growth arrested NIH3T3 cells were stimulated by EGF in the presence of 100 μM of tyrphostin we observed a modification of the tyrosine phosphorylation of the 42 kDa MAP kinase (2.5-fold stimulation as compared to the level in the EGF treated cells) (Fig. 1). No modification in the level of phosphotyrosine in other protein bands was observed when the cells were treated simultaneously by EGF and tyrphostin. Longer exposure time (6 or 24 hr) with tyrphostin along the EGF did not result in any modification in the tyrosine phosphorylation level in the different proteins except for a group of proteins of 120 kDa, where we observed a slight decrease in the tyrosine phosphorylation level in a 6 hr treatment (data not shown).

In order to further ensure that the 42 kDa corresponds to the MAP kinase, we immunoprecipitated the MAP kinase from cellular extracts with an antibody directed to the C-terminal part of ERK2. As shown in Fig. 2A, when immunoprecipitated MAP kinase was blotted with an anti-P-tyr, we observe the same increase in the MAP kinase tyrosine phosphorylation. In contrast, immunoprecipitation of the EGF-receptor by RK2 antibody and further blotting with anti-P-tyr antibody showed no modification in the phosphotyrosine in

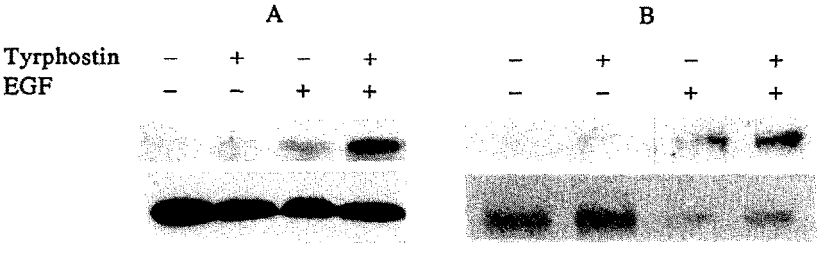

Fig. 2. Tyrosine phosphorylation of MAP kinase and EGF receptor in NIH3T3 cells. NIH3T3 cells stimulated or not stimulated by EGF in the presence or absence of 100 μM tyrphostin for 5 min were lysed and proteins immunoprecipitated with anti-MAP kinase mAb# sc154 (A) or with anti-EGF receptor RK2 (B). Immunoprecipitates were separated by gel electrophoresis, transferred to nitrocellulose and immunoblotted either with anti P-tyr (A, B upper part) or with mAb #sc154 (A, bottom part) or with RK2 (B, bottom part).

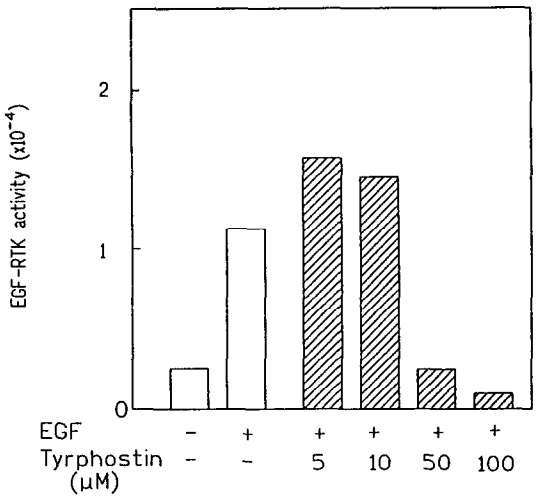

Fig. 3. Effect of tyrphostin on the EGF receptor tyrosine kinase activity. *In vitro*, autophosphorylation of EGF-receptor tyrosine kinase was determined as described in Materials and Methods in the presence of different concentrations of tyrphostin. After separation of phosphorylated proteins by SDS–polyacrylamide gel electrophoresis, autoradiography was performed. The quantification of ^{32}P into EGF receptor was done by densitometric scanning of the autoradiogram.

the EGF-receptor occurred during the tyrphostin treatment.

As we observed an unexpected effect of tyrphostin on the tyrosine phosphorylation level in the MAP kinase and did not observe any modification in EGF-RTK phosphorylation *in vivo*, we therefore verified that tyrphostin was able to inhibit the EGF-RTK in an *in vitro* assay as was previously described [40]. As shown in Fig. 3, tyrphostin inhibited the EGF-RTK; the remaining activity of the kinase activity in the presence of 50 and 100 μM of tyrphostin was 16 and 6%, respectively.

It has previously been shown that phosphorylation of the MAP kinase, which occurs on both tyrosine and threonine residues in response to growth factors, led to an increase in MAP kinase activity. Since we observed (in tyrphostin and EGF simultaneous treated cells) an increase in the MAP kinase phosphorylation level, we examined whether MAP kinase activity was also increased under the same conditions. We also examined the effect of this treatment on the S6 kinase activity, $p90^{rsk}$, which is activated by MAP kinase.

Tyrphostin activates MAP kinase activity and S6 kinase activity $p90^{rsk}$ but has no effect on the S6 kinase p70S6K

MAP kinase activation was measured by *in vitro* kinase assays on the MAP kinase substrate peptide corresponding to amino acids 93 to 98 of the myelin basic protein. In NIH3T3 cells treatment with EGF led to a 5.3-fold increase in MAP kinase activity as compared to the non-stimulated cells. Treatment of the cells with EGF for 5 min in the presence of 100 μM of tyrphostin resulted in a further increase of MAP kinase activity. MAP kinase was partially purified by chromatography through Q Sepharose. The fractionation of MAP kinase through this support revealed a peak of activity eluted at 0.4 M NaCl and which corresponded to the 42 kDa MAP kinase polypeptide (data not shown). Table 1 shows that MAP kinase is indeed activated 7.4-fold in EGF-treated cells and 27-fold in EGF and tyrphostin treated cells as compared to the non-stimulated cells or tyrphostin-treated cells. These results are in agreement with the above observation showing that tyrphostin increased the level of tyrosine phosphorylation in the MAP kinase protein. Maximal activity of MAP kinases occurred within 5 min of stimulation by EGF and then rapidly decreased [17]. No modification in this time course activation of MAP kinase was observed in the cells treated with EGF and tyrphostin as compared to the EGF-treated cells (data not shown).

Table 1. Tyrphostin enhances the MAP kinase activity and the S6 kinase $p90^{rsk}$ activity stimulated by EGF and not the S6 kinase p70S6K activity

	Quiescent cells	EGF-treated cells	EGF- and tyrphostin-treated cells
MAP kinase cpm incorporated in MAPK peptide /μg of protein	49 ± 9	370 ± 12	1375 ± 25
$p90^{rsk}$ cpm incorporated into S6 peptide /μg of protein	352 ± 8	709 ± 11	1197 ± 61
p70 S6K cpm incorporated into ribosomal protein S6 /μg of protein	56 ± 2	154 ± 6	179 ± 10

Quiescent NIH3T3 cells were treated for 5 min (MAP kinase and S6 kinase $p90^{rsk}$) or for 60 min (S6 kinase p70 S6K) with EGF in the presence or absence of 100 μM tyrphostin.

Lysates were prepared and assayed for the different enzyme activities as described in Materials and Methods.

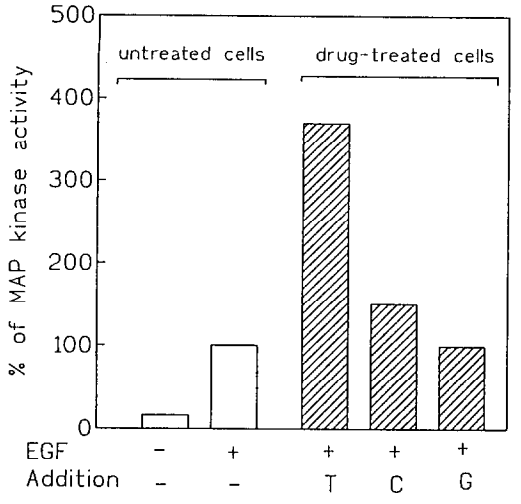

Fig. 4. Modification of MAP kinase activity by tyrphostin, genistein and chelerythrine. Quiescent NIH3T3 cells were treated for 5 min with EGF in the presence of 100 μM tyrphostin (T), 15 μM genistein (G) or 25 μM chelerythrine (C). MAP kinase activity was measured as described in Materials and Methods.

EGF-stimulated MAP kinase was used to examine the *in vitro* effect of tyrphostin on this enzymatic activity. In contrast to the *in vivo* effect of the tyrphostin, only a slight decrease of kinase activity was observed at 100 μM. This result suggested that the increase in MAP kinase activity must be a result of an effect of the tyrphostin on one or several steps of the transduction signal leading to the increase in MAP kinase activity and not to a direct effect of the tyrphostin on MAP kinase activity.

Other protein kinase inhibitors, genistein [35] and chelerythrine [48], were ineffective in MAP kinase activity stimulated by EGF in NIH3T3 cells (Fig. 4). These results reinforce the idea that the mechanism of action of tyrphostin is unique.

It has been described that, *in vitro*, MAP kinase phosphorylates and substantially restores the enzymatic activity of *Xenopus* $p90^{rsk}$ S6 kinase which has been deactivated with protein phosphatase 2A or 1 [8]. Activation of $p90^{rsk}$ S6 kinase by MAP kinase has also been demonstrated by Price and co-workers [49].

To determine if the increase in the tyrosine phosphorylation of MAP kinase leading to an enhanced enzyme activity in EGF- and tyrphostin-treated cells (Fig. 1 and Table 1) promotes an increase in S6 kinase $p90^{rsk}$ activity, we have measured S6 kinase $p90^{rsk}$ activity by immune complex kinase assay. As shown in Table 1, treatment of cells with EGF results in a two-fold increase of S6 kinase $p90^{rsk}$ activity. Treatment with EGF in the presence of 100 μM tyrphostin resulted in an increase in $p90^{rsk}$ S6 kinase activity by a factor of 3.2 as compared to the basal value. As was observed with the MAP kinase, no modification in S6 kinase $p90^{rsk}$ activity was observed when the cells were treated only with tyrphostin (data not shown). These results show that the increase in MAP kinase activity is associated with an increase in $p90^{rsk}$ S6 kinase activity.

Within minutes of addition of EGF to quiescent NIH3T3 cells, there was also an enhancement of S6 kinase p70S6K activity [16]. The effect of tyrphostin was investigated on this EGF-stimulated S6 kinase. As shown in Table 1, S6 kinase activity increased in the presence of EGF as compared to untreated cells and no modification in this enzyme activity was observed in the tyrphostin- and EGF-treated cells, but tyrphostin did not promote an additional effect.

Effect of tyrphostin on the expression of c-fos and c-myc mRNA after EGF stimulation

Treatment of quiescent NIH3T3 cells by EGF increases the expression of early genes and this expression requires the tyrosine kinase activity of EGF-R. The protooncogene c-fos is one of these early genes [50]. Critical to this response is the serum-response element which forms *in vivo* a ternary complex with the transcription factors $p67^{SRF}$ and $p62^{TCF}$ [51]. One of these factors ($p62^{TCF}$) is phosphorylated by the MAP kinase *in vitro* and this phosphorylation results in an enhanced ternary complex formation [52]. If phosphorylation of $p62^{TCF}$ by MAP kinase and the consequent enhancement of ternary complex formation are part of the signal cascade leading to a rapid induction of c-fos expression, enhanced c-fos expression might be detectable in tyrphostin- and EGF-treated NIH3T3 cells as compared to the EGF-treated cells.

To address this question, we examined the effect of 100 μM tyrphostin treatment on the EGF induced

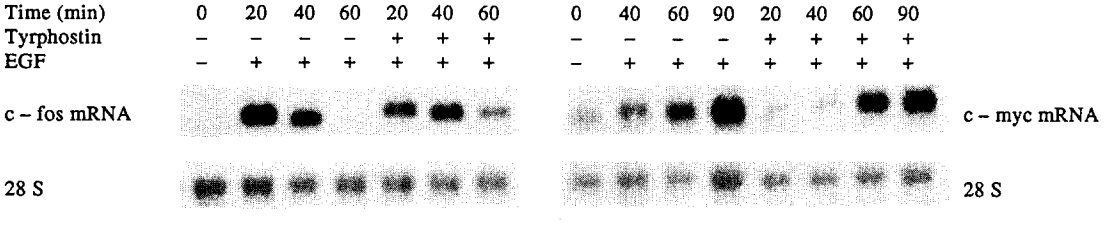

Fig. 5. Effect of tyrphostin on the c-fos and c-myc mRNA stimulated by EGF. Total RNA was isolated from quiescent cells stimulated with EGF for different lapses of time in the presence or absence of tyrphostin (100 μM). After agarose gel electrophoresis and transfer to Nylon membrane, the blot was sequentially probed with c-fos RNA probe, c-myc RNA probe and 28S oligonucleotide probe.

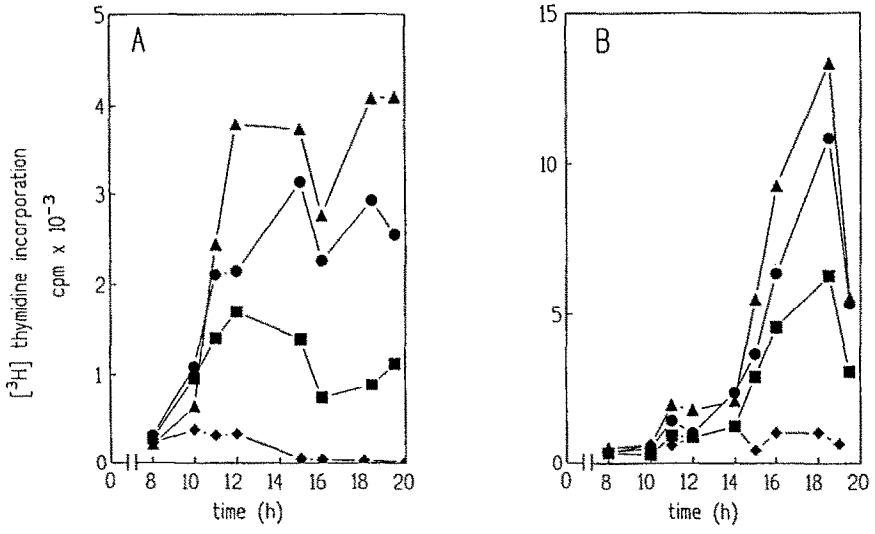

Fig. 6. Effect of tyrphostin on the onset of DNA synthesis in NIH3T3 cells. Cells were plated in the microtitration plates and allowed to grow for 2 days. At approximately 50% confluence, medium was changed to low serum (0.1%) for 2 additional days and then stimulated with EGF (12 ng/mL) (part A) (▲) or with 10% FCS (part B) (▲) with the addition of different concentrations of tyrphostin [(●) 10 μM, (■) 30 μM, (◆) 100 μM]. Cells were pulsed for 1 hr with [^{3}H]thymidine and harvested at different intervals of time to determine the incorporation of isotope into the DNA of the growing cells.

c-fos expression. Treatment for increasing times showed that the maximal enhancement by EGF of c-fos mRNA was observed after a 20 min stimulation (Fig. 5). A treatment of quiescent cells with EGF and tyrphostin for 20 min reduced the level of c-fos mRNA. To quantify the inhibitory effect, we compared the level of the c-fos mRNA to the level of the 28S RNA probe used to normalize the gels by densitometric tracing (data not shown). Under these conditions, the level of c-fos mRNA was reduced by 25% and, after a 40 min treatment, the same c-fos mRNA level as in the control cells was observed (Fig. 5).

Experiments performed to examine the effect of tyrphostin on the level of c-myc mRNA showed that tyrphostin along with EGF did not modify the level of c-myc mRNA (Fig. 5).

Effect of tyrphostin on late events observed in the EGF-stimulated cells

A late event leading to cell multiplication after treatment of the cells with EGF is the stimulation of DNA synthesis. We then examined whether this event is modified by tyrphostin treatment in NIH3T3.

Changes in DNA synthesis were monitored following serum or EGF addition in the presence of increasing concentrations of tyrphostin to quiescent cells with 1 hr pulses of [^{3}H]thymidine. The inhibition of EGF or serum-stimulated DNA synthesis increased with the concentration of tyrphostin (Fig. 6). No modification in the delay in the entry into S phase was observed in the drug-treated cells as compared to the non-treated cells. A complete inhibition of DNA synthesis was observed at 100 μM.

In addition, the effect of tyrphostin on cell growth was determined by cell counts. A 72 hr treatment with tyrphostin (RG 50864) at 100 μM reduced the rate of serum-stimulated cell proliferation by 40% (Fig. 7).

DISCUSSION

Tyrphostins are a family of protein tyrosine kinase blockers that selectively inhibit receptor autophosphorylation [41]. Some of these compounds can inhibit receptor and non-receptor tyrosine kinases [53]. In several cell types expressing a high level of EGF-R, the antiproliferative action of tyrphostin has been correlated to EGF-R kinase activity inhibition *in situ* [40–42]. In cells expressing

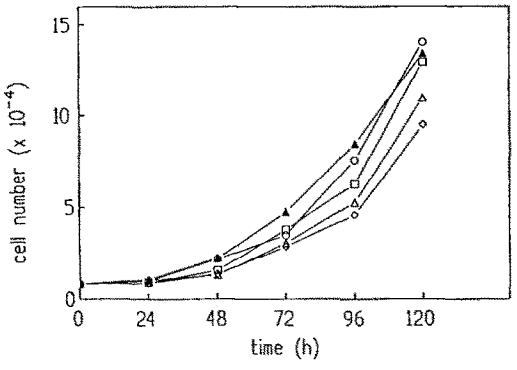

Fig. 7. Effect of tyrphostin on cell growth. Cells were grown in the absence of (▲) or the presence of different concentrations of tyrphostin (○) 10 μM, (□) 20 μM, (△) 50 μM and (◇) 100 μM for different times. All values represent mean values of three cell counts for each time.

human EGF-receptor, tyrphostins block some post-receptor effects of growth factors, such as EGF-induced PLC-γ phosphorylation, the association between PLC-γ and EGF-R, EGF-induced Ca^{2+} release and EGF-induced DNA synthesis [43]. This effect was only observed after a 18 hr pretreatment of the cells with the drug. It has been suggested by Faaland *et al.* that the tyrphostin effect could be an indirect effect, not directly related to the inhibition of the tyrosine kinase activity of the EGF-receptor [45].

In this work, we have analysed the effect of one of the tyrphostins (RG50864) on the MAP kinase activated by EGF, when both the drug and the growth factor were added simultaneously and for a short period of time. We examined the effect of this treatment on MAP kinase activity, S6 kinase $p90^{rsk}$ and p70S6K activities and on the stimulation of c-fos and c-myc transcription observed in the NIH3T3 cells in response to EGF.

First, we have shown that treatment of the cells by tyrphostin and EGF enhances MAP kinase tyrosine phosphorylation as compared to the EGF-treated cells without affecting the EGF-RTK. MAP kinase is activated by a dual phosphorylation on threonine and on tyrosine. The enhancement of tyrosine phosphorylation observed in the MAP kinase in the cells treated by tyrphostin and EGF also leads to an increase in MAP kinase activity. This enhancement is 7.4-fold the level observed in the EGF-stimulated cells and 27-fold the level of unstimulated cells. This effect requires that the signal transduction be mediated by the activation of the EGF-receptor, since it is not observed in the cells treated only by tyrphostin. Treatment of the cells with other PTK inhibitors such as genistein or chelerythrine in the presence of EGF did not result in a significant modification in MAP kinase activity. These results reinforce the idea that this stimulating effect on the MAP kinase activity and phosphorylation is specific to tyrphostin. *In vitro* no modification in MAP kinase activity is promoted by tyrphostin. These results strongly suggest that tyrphostin acts at one particular step between the EGF receptor and the MAP kinase. MAP kinase is activated by phosphorylation on tyrosine and threonine and this activation can be reversed by dephosphorylation either by tyrosine (PTPase) or serine/threonine (PP2A) phosphatases [54, 55]. *In vivo*, it has been shown that okadaic acid, an inhibitor of PP2A, stimulates MAP kinase activity [56]. An inhibitory effect of tyrphostin on these phosphatases could result in an enhancement of MAP kinase activity. It will be of interest to examine whether there are protein phosphatases, whose activity could be modified by the tyrphostin treatment. A recent report shows that in PC12 cells, exposure to tyrphostin AG 879 alone causes a 50% increase in MAP kinase activity [57], while direct addition of AG 879 to the lysate has no effect on MAP kinase activity [57]. In addition, preincubation with AG 879 for 10 min completely blocks the stimulation of the MAP kinase caused by NGF, whereas it has no effect on the stimulation of this enzyme caused by EGF [57]. Discrimination among tyrosine kinases and eventually tyrosine phosphatases could be observed with tyrphostins and may help to dissect the precise signalling that leads to the mitogenic effects of growth factors in fibroblasts.

Second, in addition to the increase in activation of MAP kinase, we also observed an increase in S6 kinase $p90^{rsk}$ activity by cell treatment with tyrphostin. With a 100 μM tyrphostin treatment, we observed a 2.5-fold stimulation of MAP kinase and a 1.6-fold stimulation of S6 kinase $p90^{rsk}$. *In vitro*, studies have shown that S6 kinase $p90^{rsk}$ could be directly regulated by serine/threonine phosphorylation/dephosphorylation. Activation of S6 kinase $p90^{rsk}$ by MAP kinase *in vitro* is associated with threonine phosphorylation of the S6 kinase [8]. *In vivo* S6 kinase contains both phosphoserine and phosphothreonine [58]. The phosphopeptide map of S6 kinase $p90^{rsk}$ radiolabelled *in vivo* is more complex than that of the same S6 kinase radiolabelled *in vitro* with MAP kinase. Additional S6 kinase(s) stimulated by growth factors may account for the *in vivo* phosphorylation pattern of S6 kinase $p90^{rsk}$. The fact that other S6 kinase(s) could participate in the activation of S6 kinase $p90^{rsk}$ can explain why we cannot observe the same level of activation between MAP kinase and S6 kinase in the tyrphostin treated cells.

Third, tyrphostin has no effect on the activation of S6 kinase p70S6K activity mediated by EGF. Previous studies have shown that both S6 kinases $p90^{rsk}$ and p70S6K are differentially regulated [6, 29, 59] and that serum stimulation of HeLa cells provides evidence that S6 kinase $p90^{rsk}$ and p70S6K lie on distinct signalling pathways [29] whereas S6 kinase $p90^{rsk}$ and the MAP kinase lie on the same signalling pathway [8, 17]. Corroborating these observations are studies showing the differential activation of MAP kinase activity and S6 kinase p70S6K activity in insulin-treated 3T3 cells and the inability of MAP kinase to activate S6 kinase p70S6K *in vitro* [16]. Protein synthesis inhibitors such as cycloheximide and puromycin stimulate S6 kinase p70S6K activity but not S6 kinase $p90^{rsk}$ or MAP kinase activities [17]. The macrolide rapamycin blocks the phosphorylation and activation of S6 kinase p70S6K but not the S6 kinase $p90^{rsk}$ in a variety of animal cells [60]. In this report, we provide additional evidence that S6 kinase p70S6K lies on a distinct growth factor activated signalling pathway with MAP kinase and S6 kinase $p90^{rsk}$.

Fourth, we have shown that c-fos mRNA expression is reduced in EGF- and tyrphostin-treated cells as compared to EGF-stimulated cells (25% reduction). The tyrphostin treatment has no effect on the increase in c-myc mRNA observed in EGF-treated cells. The inhibition of c-fos transcription cannot be explained by the stimulation of the MAP kinase promoted by tyrphostin. It has been shown that stimulation of c-fos transcription occurs by fixation of several factors at the serum response element (SRE), the major ones being $p67^{SRF}$ and $p62^{TCF}$ [61]. *In vitro*, it has been shown that $p62^{TCF}$ is phosphorylated by the MAP kinase and that phosphorylation results in enhanced ternary complex implicating the SRE [51]. In addition p42 MAP kinase has been shown to phosphorylate serine 63 of bacterial c-jun *in vitro* [62–64]. *In vivo*, it is

difficult to identify which kind of protein kinase is the c-jun NH_2 terminal protein kinase. Although a variety of pharmacological agents, including PMA, cycloheximide, AlF_4 and okadaic acid stimulated c-jun phosphorylation, cycloheximide and okadaic acid had no effect on p42 MAP kinase phosphorylation, suggesting that MAP kinase activation was not necessary for c-jun phosphorylation *in vivo* [64]. Moreover, it has been shown very recently that transcription factor p91 contains an SH2 domain and is activated rapidly by tyrosine phosphorylation stimulated by EGF receptor. p91 is a necessary component of an EGF-induced DNA-binding factor that recognizes the SIE (c-sis inducible element) in the c-fos gene promotor. EGF also uses a direct signalling pathway to control nuclear transcriptional events [65].

In conclusion, our results suggest that in NIH3T3 cells the tyrphostin (RG 50864) does not inhibit the EGF-RTK, since some of the early events are either enhanced (MAP kinase and S6 kinase $p90^{rsk}$) or inhibited (c-fos mRNA expression) or not modified (S6 kinase p70S6K and c-myc mRNA expression). A late event (DNA synthesis) is inhibited. These different effects on the EGF-stimulated events show that this tyrphostin can have several targets in the cells. The effect on the MAP kinase could be compatible with an inhibitory effect on phosphatase(s), whereas the inhibitory effect on the c-fos mRNA transcription would involve target(s) located downstream or independent of the MAP kinase. This latter effect could lead to the inhibition of DNA synthesis and to the anti-proliferative effect of the tyrphostin.

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REFERENCES

1. Pelech SL, Sanghera JS and Daya-Makin M, Protein kinase cascades in meiotic and mitotic cell cycle control. *Biochem Cell Biol* **68**: 1297–1330, 1990.
2. Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotos N, Cobb MH and Yancopoulos GD, ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* **65**: 663–675, 1991.
3. Crews C, Alessandri A and Erikson RL, Erks: their fifteen minutes has arrived. *Cell Growth Diff* **3**: 135–142, 1992.
4. Alvarez E, Northwood IC, Gonzalez FH, Latour DH, Seth A, Abate C, Curran T and Davies RJ, Pro-Leu-Ser/Thr-Pro is a consensus primary sequence for substrate protein phosphorylation. Characterization of the phosphorylation of c-*myc* and c-*jun* proteins by an epidermal growth factor receptor threonine 669 protein kinase. *J Biol Chem* **266**: 15277–15285, 1991.
5. Pulverer BJ, Kyriakis JM, Avruch J, Nikolakaki E and Woodgett JR, Phosphorylation of c-*jun* mediated by MAP kinases. *Nature* **353**: 670–674, 1991.
6. Seth A, Alvarez E, Gupta S and Davis RJ, A phosphorylation site located in the NH_2-terminal domain of c-myc increases transactivation of gene expression. *J Biol Chem* **266**: 23521–23524, 1991.
7. Gille H, Sharrocks AD and Shaw PE, Phosphorylation of transcription factor $p62^{TCF}$ by MAP kinase stimulates ternary complex formation at c-*fos* promoter. *Nature* **358**: 414–417, 1992.
8. Sturgill TW, Ray LB, Erikson E and Maller JL, Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature* **334**: 715–718, 1988.
9. Chung J, Pelech SL and Blenis J, Mitogen-activated Swiss mouse 3T3 RSK kinases I and II are related to $pp44^{mpk}$ from sea star oocytes and participate in the regulation of $pp90^{rsk}$ activity. *Proc Natl Acad Sci USA* **88**: 4981–4985, 1991.
10. Stokoe D, Campbell P, Nakielny S, Hidaka H, Leevers SJ, Marshall CJ and Cohen P, MAPKAP kinase-2; a novel protein kinase activated by mitogen-activated protein kinase. *EMBO J* **11**: 3985–3994, 1992.
11. Jones SW, Erikson E, Blenis J, Maller JL and Erikson RL, A *Xenopus* ribosomal protein S6 kinase has two apparent kinase domains that are each similar to distinct protein kinases. *Proc Natl Acad Sci USA* **85**: 3377–3381, 1988.
12. Alcorta DA, Crews CM, Sweet LJ, Bankston L, Jones SW and Erickson RL, Sequence and expression of chicken and mouse *rsk*: homologs of the *Xenopus laevis* ribosomal S6 kinase. *Mol Cell Biol* **9**: 3850–3859, 1989.
13. Banarjee P, Ahmad MF, Grove JR, Kozlosky C, Price DJ and Avruch J, Molecular structure of a major insulin mitogen activated 70-kDa S6 protein kinase. *Proc Natl Acad Sci USA* **87**: 8550–8554, 1990.
14. Kozna SC, Ferrari S, Bassand P, Siegmann M, Totty N and Thomas G, Cloning of the mitogen-activated S6 kinase from rat liver reveals an enzyme of the second messenger family. *Proc Natl Acad Sci USA* **87**: 7365–7369, 1990.
15. Chen R-H and Blenis J, Identification of *Xenopus* S6 protein kinase homologs ($pp90^{rsk}$) in somatic cells: phosphorylation and activation during initiation of cell proliferation. *Mol Cell Biol* **10**: 3204–3215, 1990.
16. Ballou LM, Luther H and Thomas G, MAP2 kinase and 70K S6 kinase lie on distinct signalling pathways. *Nature* **349**: 348–350, 1991.
17. Blenis J, Chung J, Erikson E, Alcorta DA and Erikson RL, Distinct mechanisms for the activation of the RSK kinase/MAP2 kinase/$pp90^{rsk}$ and pp70-S6 kinase signaling systems are indicated by inhibition of protein synthesis. *Cell Growth Diff* **2**: 279–285, 1991.
18. Jurivich DA, Chung J and Blenis J, Heat shock induces two distinct S6 protein kinase activities in quiescent mammalian fibroblasts. *J Cell Physiol* **148**: 252–259, 1991.
19. Grove JR, Banerjee P, Balasubramanyam A, Coffer PJ, Price DJ, Avruch J and Woodgett JR, Cloning and expression of two human p70 S6 kinase polypeptides differing only at their amino termini. *Mol Cell Biol* **11**: 5541–5550, 1991.
20. Gomez N and Cohen P, Dissection of the protein kinase cascade by which nerve growth factor activates MAP kinases. *Nature* **353**: 170–173, 1991.
21. Kosako H, Gotoh Y, Matsuda S, Ishikawa M and Nishida E, *Xenopus* MAP activator is a serine/threonine/tyrosine kinase activated by threonine phosphorylation. *EMBO J* **11**: 2903–2908, 1992.
22. Matsuda S, Kosako H, Takenaka K, Moriyama K, Sakai H, Akiyama T, Gotoh Y and Nishida E, *Xenopus* MAP kinase activator: identification and function as a key intermediate in the phosphorylation cascade. *EMBO J* **11**: 973–982, 1992.
23. Nakielny S, Cohen P, Wu J and Stugrill T, MAP kinase activator from insulin-stimulated skeletal muscle is a protein threonine/tyrosine kinase. *EMBO J* **11**: 2123–2129, 1992.

24. Nakielny S, Campbell DG and Cohen P, MAP kinase from rabbit skeletal muscle; a novel dual specificity enzyme showing homology to yeast protein kinases involved in pheromone-dependent signal transduction. *FEBS Lett* **308**: 183–189, 1992.
25. Howe LR, Leevers SJ, Gomez N, Nakielny S, Cohen P and Marshall CJ, Activation of the MAP kinase pathway by the protein kinase raf. *Cell* **71**: 335–342, 1992.
26. Wood KW, Sarnecki C, Roberts TM and Blenis J, Ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. *Cell* **68**: 1041–1050, 1992.
27. Thomas SM, DeMarco M, D'Arcangelo G, Halegoua S and Brugge JS, Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. *Cell* **68**: 1031–1040, 1992.
28. Chen R-H, Sarnecki C and Blenis J, Nuclear localization and regulation of the *erk-* and *rsk*-encoded protein kinases. *Mol Cell Biol* **12**: 915–927, 1992.
29. Chen R-H, Chung J and Blenis J, Regulation of $pp90^{rsk}$ phosphorylation and S6 phosphotransferase activity in Swiss 3T3 cells by growth factor-, phorbol ester-, and cAMP-mediated signal transduction. *Mol Cell Biol* **11**: 1861–1867, 1991.
30. Chung J, Chen R-H and Blenis J, Coordinate regulation of $pp90^{rsk}$ and a distinct protein-serine/threonine kinase activity that phosphorylates recombinant $pp90^{rsk}$ *in vitro*. *Mol Cell Biol* **11**: 1868–1874, 1991.
31. Powis G, Signalling targets for anticancer drug development. *Trends Pharmacol Sci* **12**: 188–194, 1991.
32. Levitzki A and Gilon C, Tyrphostins as molecular tools and antiproliferative drugs. *Trends Pharmacol Sci* **12**: 171–174, 1991.
33. Burke Jr TR, Protein-tyrosine kinase inhibitors. *Drugs of the Future* **17**: 119–131, 1992.
34. Levitzki A, Tyrphostins: tyrosine kinase blockers as novel antiproliferative agents and dissectors of signal transduction. *FASEB J* **6**: 3275–3282, 1992.
35. Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M and Fukami Y, Genistein: a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem* **262**: 5592–5595, 1987.
36. Linassier C, Pierre M, Le Pecq J-B and Pierre J, Mechanisms of action in NIH-3T3 cells of genistein, an inhibitor of EGF-receptor tyrosine kinase activity. *Biochem Pharmacol* **39**: 187–193, 1990.
37. Markovits J, Linassier C, Fossé P, Couprie J, Pierre J, Jacquemin-Sablon A, Saucier J-M, Le Pecq J-B and Larsen AK, Inhibitory effects of the tyrosine kinase inhibitor genistein on mammalian topoisomerase II. *Cancer Res* **49**: 5111–5117, 1989.
38. Imoto M, Umezawa K, Sawa T, Takeuchi T and Umezawa H, *In situ* inhibition of tyrosine protein kinase by erbstatin. *Biochem Int* **15**: 989–995, 1987.
39. Pierre J, Mousset S, Pierre M, Umezawa K and Jacquemin-Sablon A, Effect of erbstatin on EGF mediated events in NIH3T3 cells. *J Cell Pharmacol* **1**: 8–11, 1990.
40. Yaish P, Gazit A, Gilon C and Levitzki A, Blocking of EGF-dependent cell proliferation by EGF receptor kinase inhibitors, *Science* **242**: 933–935, 1988.
41. Lyall RM, Zilberstein A, Gazit A, Gilon C, Levitzki A and Schlessinger J, Tyrphostins inhibit epidermal growth factor (EGF)-receptor tyrosine kinase activity in living cells and EGF stimulated cell proliferation. *J Biol Chem* **264**: 14503–14509, 1989.
42. Dvir A, Milner Y, Chomsky O, Gilon C, Gazit A and Levitzki A, The inhibition of EGF-dependent proliferation of keratinocytes by tyrphostin tyrosine kinase blockers. *J Cell Biol* **113**: 857–865, 1992.
43. Margolis B, Rhee SG, Felder S, Mervic M, Lyall R, Levitzki A, Ullrich A, Zilberstein A and Schlessinger J, EGF induces tyrosine phosphorylation of phospholipase C-II: A potential mechanism for EGF receptor signaling. *Cell* **57**: 1101–1107, 1989.
44. Posner I, Gazit A, Gilon C and Levitzki A, Tyrphostin inhibit the epidermal growth factor receptor breakdown of phosphoinositides. *FEBS Lett* **257**: 287–291, 1989.
45. Faaland CA, Mermelstein FH, Hayash J and Laskin JD, Rapid uptake of tyrphostin into A431 human epidermoid cells is followed by delayed inhibition of epidermal growth factor (EGF)-stimulated EGF receptor tyrosine kinase activity. *Mol Cell Biol* **11**: 2697–2703, 1991.
46. Kris RM, Lax I, Gullick W, Waterfield MD, Ullrich A, Fridkin M and Schlessinger J, Antibodies against a synthetic peptide as a probe for the kinase activity of the avian EGF receptor and v-erbB protein. *Cell* **40**: 619–625, 1985.
47. Barbu V and Dautry F, Mevalonate deprivation alters the induction of fos and myc by growth factors. *Oncogene* **5**: 1077–1080, 1990.
48. Herbert JM, Augereau JM, Gleye J and Maffrand JP, Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun* **172**: 993–999, 1990.
49. Price DJ, Gunsalus JR and Auruch J, Insulin activates a 70-kDa S6 kinase through serine/threonine-specific phosphorylation of the enzyme polypeptide. *Proc Natl Acad Sci USA* **87**: 7944–7948, 1990.
50. Herschman HR, Primary response genes induced by growth factors and tumor promoters. *Annu Rev Biochem* **60**: 281–319, 1991.
51. Herrera RE, Shaw PE and Nordheim A, Occupation of the c-*fos* serum response element *in vivo* by a multi-protein complex is unaltered by growth factor induction. *Nature* **340**: 68–70, 1989.
52. Gille H, Sharrocks AD, Shaw PE, Phosphorylation of transcription factor $p62^{TCF}$ by MAP kinase stimulates ternary complex formation at c-fos promoter. *Nature* **358**: 414–417, 1992.
53. Agbotounou WK, Levitzki A, Jacquemin-Sablon A and Pierre J, Effect of tyrphostins on the activated c-*src* protein in NIH3T3 cells. *Mol Pharmacol* in press.
54. Anderson NG, Maller JL, Tonks NK and Sturgill TW, Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature* **343**: 651–653, 1990.
55. Boulton TG, Gregory JS and Cobb MH, Purification and properties of extracellular signal-regulated kinase 1 an insulin-stimulated microtubule-associated protein 2 kinase. *Biochemistry* **30**: 278–286, 1991.
56. Haystead TAJ, Weiel JE, Litchfield DW, Tsukitani Y, Fischer EH and Krebs EG, Okadaic acid mimics the action of insulin in stimulating protein kinase activity in isolated adipocytes. *J Biol Chem* **265**: 16571–16580, 1990.
57. Ohmichi M, Pang L, Ribon V, Gazit A, Levitzki A and Saltiel AR, The tyrosine kinase inhibitor tyrphostin blocks the cellular actions of nerve growth factor. *Biochemistry* **32**: 4650–4658, 1993.
58. Erickson E and Maller JL, *In vivo* phosphorylation and activation of ribosomal protein S6 kinases during *Xenopus* oocyte maturation. *J Biol Chem* **264**: 13711–13717, 1989.
59. Sweet LJ, Alcorta DA and Erikson RL, Two distinct enzymes contribute to biphasic S6 phosphorylation in serum-stimulated chicken embryo fibroblasts. *Mol Cell Biol* **10**: 2787–2792, 1990.
60. Chung J, Kuo CJ, Crabtree GR and Blenis J, Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. *Cell* **69**: 1227–1236, 1992.
61. Hipskind RA, Rao VN, Mueller CGF, Reddy ESP and

Nordheim A, Ets-related protein Elk-1 is homologous to the c-*fos* regulatory factor p62^{TCF}. *Nature* **354**: 531–533, 1991.
62. Pulverer BJ, Kvriakis JM, Avruch J, Nikolakaki E and Woodgett JR, Phosphorylation of *c-jun* mediated by MAP kinases. *Nature* **353**: 670–674, 1991.
63. Baker SJ, Kerppola TK, Luk D, Vandenberg MT, Marshak DR, Curran T and Abate C, Jun is phosphorylated by several protein kinases at the same sites that are modified in serum-stimulated fibroblasts. *Mol Cell Biol* **12**: 4694–4705, 1992.
64. Franklin CC, Unlap T, Adler V and Kraft AS, Multiple signal transduction pathways mediate *c-jun* protein phosphorylation. *Cell Growth Differ* **4**: 377–385, 1993.
65. Fu X-Y and Zhang J-J, Transcriptional factor p91 interacts with the epidermal growth factor receptor and mediates activation of the *c-fos* gene promotor. *Cell* **74**: 1135–1145, 1993.