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Epidermal Growth Factor Receptor Signaling Cascade as Target for Tyrphostin (RG 50864) in Epithelial Cells

PARADOXICAL EFFECTS ON MITOGEN-ACTIVATED PROTEIN KINASE KINASE AND MITOGEN-ACTIVATED PROTEIN KINASE ACTIVITIES

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ABSTRACT. Tyrphostins are synthetic compounds that have been described as *in vitro* inhibitors of epidermal growth factor receptor (EGF-R) tyrosine kinase activity. The inhibitory effect of tyrphostins in intact cells has been shown only after prolonged treatment. However, these compounds appear to be readily incorporated, which suggests that tyrphostin acts indirectly on EGF-R. We studied the effects of a tyrphostin derivative, RG 50864, without preincubation in intact epithelial cells. We selected two human cell lines differing in degree of expression of the $p185^{erbB2}$ protein, which is closely related to EGF-R. We showed that tyrphostin (RG 50864) had no effect on EGF-dependent EGF-R tyrosine phosphorylation in the parental cell line. On the contrary, it prolonged the EGF-dependent EGF-R and $p185^{erbB2(V-E)}$ tyrosine phosphorylation in $p185^{erbB2(V-E)}$-expressing cells. Because tyrphostin has been shown to be an inhibitor of $p185^{erbB2}$ and EGF-R *in vitro*, this finding indicates that the tyrphostin effect on $p185^{erbB2(V-E)}$ and EGF-R was the result of an indirect mechanism in transfected cells. Tyrphostin treatment alone led to the activation of mitogen-activated protein (MAP) kinase kinase or MAP kinase or extracellular signal-regulated kinase kinase (MEK), suggesting that one of the tyrphostin targets was upstream of MEK1. MAP kinase, however, was not activated after tyrphostin treatment. This finding indicates that tyrphostin had another target in intact cells because MEK1 activation by tyrphostin alone did not correlate with MAP kinase activation. In the two cell lines, tyrphostin modified the time course of EGF-dependent MEK and MAP kinase activation. We conclude that whereas tyrphostins were designed to inhibit EGF-R tyrosine kinase activity, under our conditions EGF-R is not a physiological target for tyrphostin, nor is one of its related protein tyrosine kinases, $p185^{erbB2(V-E)}$. On the contrary, our results show that tyrphostin targets are multiple, leading to complex effects on receptor signaling in these epithelial cells. *Copyright © 1997 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;3:287–298, 1997.

KEY WORDS. epithelial cells; oncogene; tyrphostin; signal transduction; MEK; MAP kinase

Many receptor tyrosine kinases are often overexpressed in tumor cells. EGF-R§ is overexpressed in many human tumors of epithelial and neuroepithelial origin [1]. In most cases, this amplification is accompanied by enhanced expression of the ligand, TGF α [2, 3]. In addition, the closely related *c-erbB2* gene is amplified in human breast and ovarian cancers. This overexpression is linked with a poor prognosis [4–6].

The *c-erbB2* overexpression leads to transformation of murine fibroblasts [7–9]. The rat homologue of *c-erbB2* (*neu*) was originally identified as a transforming gene in a chemically transformed rat neuroblastoma cell line [10]. The strong oncogenic potential of *neu* is conferred by a single point mutation Val 664 → Glu within the predicted transmembrane stretch of the protein [11]. In living cells, this mutated protein is highly phosphorylated on tyrosine and has rapid turnover [10]. The human homologue of the oncogenic *neu* gene, *erbB2(V-E)*, has not been associated with human neoplasia.

Many putative ligands for $p185^{erbB2}$ have been identified, and for some the corresponding genes have been cloned [12]. Nevertheless, none has yet been proven to be the real ligand of $p185^{erbB2}$. EGF cannot bind to $p185^{erbB2}$; how-

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§ *Abbreviations:* DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; FAK, focal adhesion kinase; FCS, fetal calf serum; GAP, GTPase-activating protein; JAK, Janus kinase; KI, protein kinase A inhibitor; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAP kinase or extracellular signal-regulated kinase kinase; NGF, nerve growth factor; PBS, phosphate-buffered saline; PLC, phospholipase C; PKC, protein kinase C; PPi, tetrasodium pyrophosphate; PP2A, protein phosphatase 2A; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; SH2, src homology 2; TCA, trichloroacetic acid; TGF, transforming growth factor.

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ever, activation occurs via the formation of heterodimers between the two receptors [13]. Activation of EGF-R or $p185^{erbB2}$ leads to the activation of several transducing cascades, in turn producing proliferation. One of these is the ras/MAP kinase cascade [14, 15]. MAP kinase is a serine/threonine kinase activated by MEK on phosphorylation of threonine and tyrosine residues [16].

The involvement of tyrosine kinases in the malignant process and in other proliferative disorders has stimulated the search for inhibitors of their catalytic function for use as pharmacologic probes and drug candidates. A large number of tyrosine kinase inhibitors have been obtained either as natural products or through chemical design [17, 18]. An important advance toward the use of tyrosine kinase activity inhibitors as drugs has been made by Levitzki and Gazit [18]. They synthesized the series of tyrphostins that inhibit EGF-R tyrosine kinase activity both *in vitro* and in intact cells. Some tyrphostins are highly selective *in vitro* for different tyrosine kinases [19], whereas others present a potential antitumor activity [20].

In cells expressing high levels of EGF-R, such as A431 or HER14 cells, inhibition of the kinase is observed only after approximately 16–24 hr of pretreatment [21, 22]. Under these conditions, inhibitory effects on transducing events located downstream of EGF-R such as phosphorylation of PLCγ, has been also reported [23, 24]. Similar pretreatment of A431 and B2A4 cells with the same tyrphostin is also required to observe an inhibition of EGF-dependent tyrosine phosphorylation of MAP kinase [25]. In PC12 cells, preincubation for only 10 min with another tyrphostin (AG 879) completely blocks the activation of MAP kinase by NGF, whereas in the same cells it has no effect on the stimulation of MAP kinase by EGF [19]. Tyrphostins can also inhibit nonreceptor tyrosine kinases, such as $p60^{src}$, but equally after several hours of preincubation of the cells with the drug [26]. Faaland *et al.* suggested that inhibition of tyrphostin could occur via an indirect mechanism because they showed that tyrphostin accumulation in the cells is maximal within 1 hr of treatment [27]. Tyrphostin metabolism occurs during preincubation, leading to more active compounds [28]. Treatment without preincubation with tyrphostin seems to have striking effects. Exposure to AG 879 alone causes a 50% enhancement of MAP kinase activity in PC12 cells [19]. Moreover, when NIH3T3 cells are briefly and simultaneously treated with EGF and tyrphostin (RG 50864), an enhancement of MAP kinase tyrosine phosphorylation and activity is observed, whereas no modification of MAP kinase tyrosine phosphorylation and activity is observed after the same treatment by tyrphostin alone [29].

In the present work, we have investigated the effect of tyrphostin without preincubation on the EGF-mediated transducing events in two human epithelial cell lines differing by the level of expression of the erbB2 protein. We transfected the *erbB2(V-E)* gene in an epithelial cell line derived from a human colon tumor, SW 613-S. In the transfected cell line, we showed that $p185^{erbB2(V-E)}$ was constitutively tyrosine phosphorylated and that the ras/MAP kinase signaling cascade was constitutively activated. We also demonstrated that neither EGF-R nor $p185^{erbB2(V-E)}$ were physiological targets of tyrphostin (RG 50864). Nevertheless, in the two cell lines, tyrphostin treatment led to the activation of MEK1 and modified the time course of the EGF-dependent MAP kinase activation, suggesting that tyrphostin had a target upstream of MEK1. Moreover, MEK1 activation by tyrphostin alone did not correlate with MAP kinase activation, suggesting that tyrphostin had at least two targets in intact cells.

MATERIALS AND METHODS

Materials and Antibodies

Mouse EGF was purchased from Sigma (St. Louis, MO, USA). MAP kinase substrate peptide, antiphosphotyrosine monoclonal antibody, anti-Shc polyclonal antibody and anti-MEK 1 polyclonal antibody were obtained from UBI (Lake Placid, NY, USA). Anti-MAP kinase (ERK 1 + 2) was obtained from Zymed (San Francisco, CA, USA). Anti-$p185^{erbB2}$ monoclonal antibody (Ab3) and anti-EGF-R monoclonal antibody (Ab1) were obtained from Oncogene Science (Cambridge, MA, USA). Tyrphostin (RG 50864) was synthesized as previously described [29, 30].

Kinase inactive MAP kinase was purified from BL21(DE3) LysS strain according to Gardner *et al.* [31].

Cell Culture

The SW613-S cell line, previously referred to as being derived from a human breast carcinoma, has in fact the same cellular origin as the SW 480 cell line and is thus derived from a colon carcinoma [32]. In this cell line, subclones of heterogenous ability to promote tumors in nude mice have been isolated [33, 34]. The clone B3, used as parental cells for the transfection, is a nontumorigenic clone of SW 613-S cells.

Cells were cultured in DMEM supplemented with 10% FCS under an atmosphere of 5% CO_2 at 37°C. Exponentially growing cells were starved in DMEM containing 0.1% FCS for 16 hr. Serum-starved cells were stimulated or not with 100 ng/mL EGF in the presence or absence of 100 μM of tyrphostin (RG 50864) for different lengths of time. Cells were rinsed with ice-cold PBS.

Construction and Transfection of SW 613-S Cells, Clone B3

Full-length erbB2(V-E) cDNA was isolated from pSV2erbB2(V-E) vector. The cDNA was blunt ended with Kleenow polymerase (Amersham, Little Chalfont, Buckinghamshire, England) and inserted into the blunt-ended BamH1 site of the retrovirus expression vector pZip-

NeoSV(X). This process generated the pZipNeoSV(X)-erbB2(V-E) vector.

Transfection of SW 613-S cells, clone B3, was performed by the calcium phosphate technique [35].

Western Blotting and Immunoprecipitation

Cellular extracts, prepared as previously described [26], were directly fractionated by SDS-PAGE or immunoprecipitated. For immunoprecipitation, cellular extracts were incubated at 4°C for 4 hr with the appropriate immunoprecipitating antibody. Immunocomplexes were recovered by the addition of 10% (v/v) protein A–Sepharose beads (Sigma), separated by SDS-PAGE and transferred to nitrocellulose. Immunoblotting was performed as previously described [26].

Kinase Assays

MAP KINASE ACTIVITY. Cytosolic extracts (12.5 μL), prepared as previously described [29], were mixed with 4.2 μL of MAP kinase substrate peptide, corresponding to residues 93–98 of MBP (final concentration: 1 mM). The phosphorylation reaction 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 (DTT), 3.7 mM EGTA Na_2 and 0.3 mM ATP (0.5 μCi of [γ-^{33}P] ATP, specific activity 3000 Ci/mmol).

Reactions were carried out for 10 min at 30°C and terminated by adding 17 μL of a solution of 24% trichloroacetic acid and 10 μL of 10 mg/mL of bovine serum albumin. After a 5-min centrifugation at 4°C at 10,000*g*, an aliquot of 25 μL was spotted onto P-81 phosphocellulose filter paper squares (Whatman), which were washed several times with 75 mM phosphoric acid.

Phosphate incorporation levels, measured in the absence of substrate, were subtracted from values obtained in the presence of substrate to correct for nonspecific phosphorylation.

MEK ACTIVITY. Cellular extracts were prepared by lysing the cells in a solution of 50 mM Tris-HCl (pH 7.5), 50 mM NaF, 5 mM EDTA, 1% Triton X-100, 40 mM β-glycerophosphate, 1 mM DTT, 1 mM PMSF, 1 μg/mL of aprotinin and 1 μg/mL of leupeptin. Lysates were cleared of insoluble material by centrifugation at 15,000*g* for 15 min.

The 0.25 μg of kinase inactive MAP kinase was phosphorylated for 10 min at 30°C by 6 μg of cellular extracts in a buffer containing 25 mM β-glycerophosphate (pH 7.3), 10 mM $MgCl_2$, 1.25 mM EGTA Na_2, 1 mM Na_3VO_4, 6 μM KI peptide, 33 μM ATP and 5 μCi [γ-^{32}P] ATP. Anti-MEK1 antibody was used to immunoprecipitate MEK1. The immunoprecipitated proteins were washed twice in lysis buffer and twice in buffer (50 mM HEPES, pH 7.4, 10 mM magnesium acetate). Immune-complex kinase assays were performed in a solution of 50 mM HEPES (pH 7.4), 10 mM magnesium acetate, 1 mM DTT, 5 μM ATP, 20 μCi of [γ-^{32}P] ATP and 2 μg of kinase inactive MAP kinase per sample for 30 min at 30°C.

In Gel Kinase Assay

Polyacrylamide (14%) minigels were cast with 0.5 mg/mL of MBP. 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 hr, 250 mL), followed by 1 hr in 250 mL of buffer (1 mM DTT, 50 mM Tris-HCl, pH 8.0). To denature proteins, gels were incubated 1 hr in 50 mL of 6 M guanidine-HCl, 20 mM DTT, 2 mM EDTA and 50 mM Tris-HCl (pH 8.0). Proteins were renatured by incubation at 4°C, without agitation, in 250 mL of buffer (1 mM DTT, 2 mM EDTA, 0.04% Tween 20, 50 mM Tris-HCl, pH 8.0) for 18 hr. For kinase assays, gels were equilibrated for 30–60 min in 10 mL of kinase buffer (1 mM DTT, 0.1 mM EGTA Na_2, 20 mM $MgCl_2$, 40 mM HEPES, pH 8.0, 100 μM Na_3VO_4), 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). The gels were then washed extensively in 5% (wt/vol) TCA plus 1% sodium PPi until washes were free of radioactivity. Autoradiography of dried gels was then performed.

In vitro *Dephosphorylation of MAP Kinase*

Cellular extracts were prepared by lysing the cells in a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 8 mM 2-mercapthoethanol, 10 μg/mL leupeptin and 10 μg/mL aprotinin. Samples were then subjected to a 15-min centrifugation at 15,000*g*. The 0.25 μg of kinase-inactive MAP kinase [31] was phosphorylated for 10 min at 30°C by 6 μg of extracts from cells stimulated by EGF for 20 min in a buffer containing 25 mM β-glycerophosphate (pH 7.3), 10 mM $MgCl_2$, 1.25 mM EGTA Na_2, 1 mM Na_3VO_4, 6 μM KI peptide, 33 μM ATP and 5 μCi [γ-^{32}P] ATP. ATP was removed by passing the solution containing the phosphorylated MAP kinase through a G-25 Sephadex column equilibrated in the lysis buffer. The MAP kinase was then incubated for 40 min at room temperature in the presence of 60 μg of cellular extracts in lysis buffer, according to the method of Peraldi *et al.* [36]. The reaction was stopped by adding sample buffer. Proteins were then submitted to SDS-PAGE under reducing conditions. After electrophoresis, the gel was dried and autoradiographed.

Cell Proliferation

Cells were seeded at 2×10^4 cells per well of a 96-well plate in a chemically defined medium composed of a mix of DMEM medium and F12 Ham medium (50/50, v/v) supplemented with 5 μg/mL of transferrin.

Cells were stimulated with 100 ng/mL of EGF or 10% FCS in the presence or absence of 100 μM of tyrphostin

(RG 50864) for 24 hr. Six hours after stimulation, the cells were labeled with [^{3}H]-thymidine (1 μCi per well) for 18 hr. Cells were harvested using a cell harvester (Skatron Instruments, Norway), and the incorporated radioactivity was determined by scintillation counting [29].

RESULTS

Effect of Tyrphostin (RG 50864) on Serum-Starved and EGF-Stimulated Cells

We examined the effect of tyrphostin on the level of tyrosine phosphorylation of serum-starved and EGF-stimulated cells after a short-time scale.

The level of phosphotyrosine was first evaluated in extracts from either the parental or p185$^{erbB2(V-E)}$ expressing cells in Western blots probed with antiphosphotyrosine antibodies. Virtually no tyrosine phosphorylation was observed in serum-starved parental cells, whereas in the serum-starved p185$^{erbB2(V-E)}$ expressing cells we observed a constitutive phosphorylation of several proteins, i.e., p185$^{erbB2(V-E)}$ itself, a group of proteins of 130 kDa, Shc proteins and MAP kinase (Fig. 1).

Stimulation of these two cell lines by EGF led to the same enhancement of the EGF-R, of the Shc proteins and of MAP kinase tyrosine phosphorylation (Fig. 1). Tyrosine phosphorylation of MAP kinase and Shc proteins was correlated with a shift of their electrophoretic mobility (Fig. 2C,D).

The effect of 100 μM tyrphostin on either the serum-starved parental or p185$^{erbB2(V-E)}$ expressing cells after 5 min was to enhance slightly the tyrosine phosphorylation of

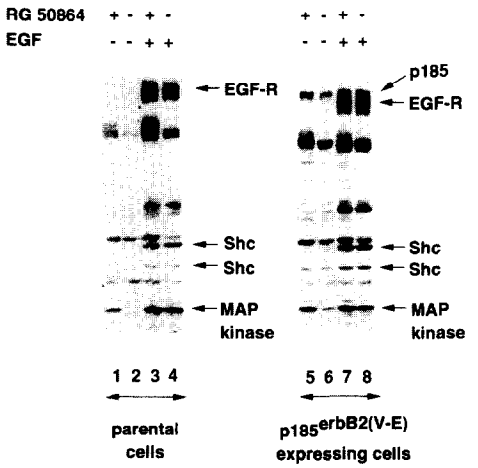

FIG. 1. Effect of tyrphostin on the level of tyrosine phosphorylation in the proteins of parental and p185$^{erbB2(V-E)}$-expressing cells. Serum-starved parental and p185$^{erbB2(V-E)}$-expressing cells were stimulated or not with EGF (100 ng/mL) in the presence or absence of 100 μM of tyrphostin for 5 min. Cell lysates were prepared as described in Materials and Methods. Proteins were directly separated on an 8% SDS-PAGE, transferred onto a nitrocellulose membrane and blotted with a monoclonal antibody to phosphotyrosine. Reprobing a blot with appropriate antibody allowed us to locate EGF-R, p185$^{erbB2(V-E)}$, Shc proteins and MAP kinase.

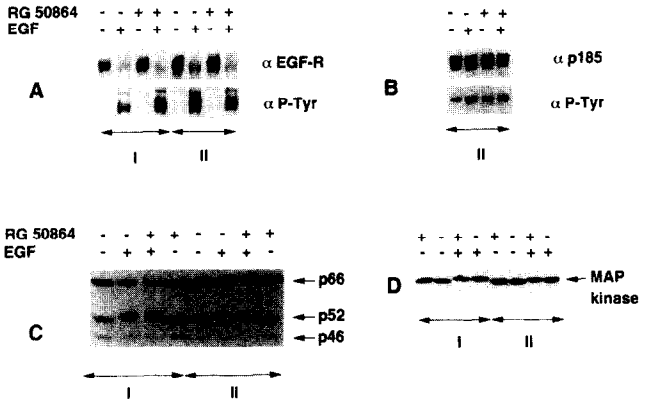

FIG. 2. Tyrosine phosphorylation of EGF-R, p185$^{erbB2(V-E)}$ and Shc proteins and MAP kinase after a 5-min tyrphostin treatment. Cells were stimulated or not with EGF in the presence or absence of 100 μM of tyrphostin for 5 min. (A) One milligram of cellular proteins from either parental (I) or p185$^{erbB2(V-E)}$-expressing (II) cells was immunoprecipitated with a monoclonal antibody to EGF-R. Proteins were separated on an 8% SDS-PAGE, transferred onto a nitrocellulose membrane and blotted with a monoclonal antibody to phosphotyrosine and then with a polyclonal antibody to EGF-R (RK2). (B) One milligram of cellular proteins from p185$^{erbB2(V-E)}$-expressing cells was immunoprecipitated with a monoclonal antibody to p185^{erbB2}. Proteins were separated on an 8% SDS-PAGE, transferred onto a nitrocellulose membrane and blotted with a monoclonal antibody to p185^{erbB2} and then with a monoclonal antibody to phosphotyrosine (4G10). (C) Cell lysates from either parental (I) or p185$^{erbB2(V-E)}$-expressing (II) cells were directly separated on an 8% SDS-PAGE, transferred onto a nitrocellulose membrane and blotted with a polyclonal antibody to Shc proteins. (D) Cell lysates from either parental (I) or p185$^{erbB2(V-E)}$-expressing (II) cells were directly separated on an 8% SDS-PAGE, transferred onto a nitrocellulose membrane and blotted with a monoclonal antibody to MAP kinase.

MAP kinase and of the group of proteins of 130 kDa (Figs. 1, 2D). Immunoprecipitation experiments revealed that these 130-kDa proteins were neither PLCγ, JAK, FAK, nor GAP (data not shown). No modification of the tyrosine phosphorylation of EGF-R or of p185$^{erbB2(V-E)}$ was observed in the two cell lines (Figs. 1, 2A,B). Moreover, no modification of the constitutive tyrosine phosphorylation of Shc proteins was observed in the p185$^{erbB2(V-E)}$-expressing cells (Figs. 1, 2C).

Simultaneous treatment by EGF and tyrphostin for 5 min potentiated the effect of EGF on MAP kinase tyrosine phosphorylation in the two cell lines (Fig. 1). In addition, mobility of MAP kinase was more reduced in EGF- and tyrphostin-treated cells than in EGF-stimulated cells (Fig. 2D). The resulting tyrosine phosphorylation of MAP kinase was also higher than in the cells treated with tyrphostin alone. Under the same conditions (5 min), no modification of the tyrosine phosphorylation of EGF-R, p185$^{erbB2(V-E)}$ or Shc proteins was observed (Figs. 1, 2A–C).

Virtually no modification of EGF-R tyrosine phosphorylation was observed after up to a 60-min treatment by EGF and tyrphostin in parental cells (Fig. 3A). On the contrary,

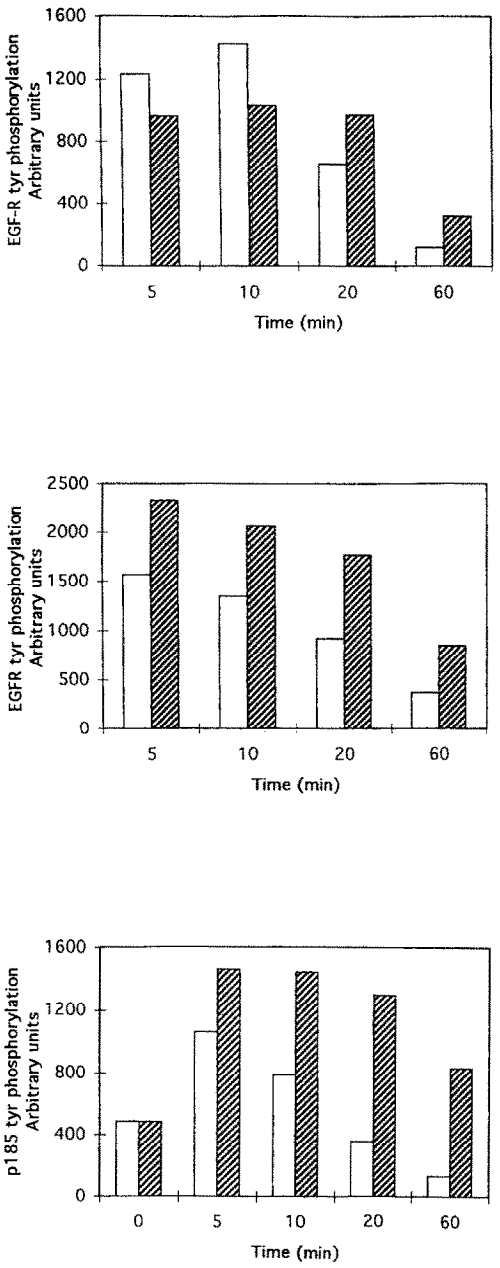

FIG. 3. Time-course of EGF-dependent EGF-R and p185$^{erbB2(V-E)}$ tyrosine phosphorylation in the presence of tyrphostin. Serum-starved parental and p185$^{erbB2(V-E)}$-expressing cells were stimulated with EGF (100 ng/mL) in the presence (open bars) or absence (striped bars) of 100 μM of tyrphostin. Cell lysates were prepared as described in Materials and Methods. Proteins were directly separated on an 8% SDS-PAGE, transferred onto a nitrocellulose membrane and blotted with a monoclonal antibody to phosphotyrosine. The level of EGF-R tyrosine phosphorylation of either parental (A) or p185$^{erbB2(V-E)}$-expressing (B) cells and the level of p185$^{erbB2(V-E)}$ tyrosine phosphorylation of transfected cells (C) were determined by density scanning. The results presented are representative of at least two separate experiments.

in p185$^{erbB2(V-E)}$-expressing cells, tyrphostin greatly prolongated p185$^{erbB2(V-E)}$ tyrosine phosphorylation and, to a lesser extent, EGF-dependent EGF-R tyrosine phosphorylation (Fig. 3B,C). Thus, tyrphostin treatment either had no effect or enhanced EGF-R and p185$^{erbB2(V-E)}$ phosphorylation. In any case, under our experimental conditions, tyrphostin did not inhibit their catalytic activity.

Equally in these two cell lines, tyrphostin's long-term effect did not seem to be related to inhibition of EGF-R. We examined the effect of 100 μM tyrphostin on the EGF- or serum-dependent stimulation of [^{3}H]-thymidine incorporation. Cells were seeded in a chemically defined medium and 24 hr later, EGF or FCS was added in the presence or absence of 100 μM of tyrphostin.

The incorporation of [^{3}H]-thymidine in untreated cells was approximately fourfold higher in the parental than in the p185$^{erbB2(V-E)}$-expressing cells (Fig. 4A). Stimulation by EGF led to a twofold increase in [^{3}H]-thymidine incorporation in the two cell lines. Simultaneous treatment of EGF and tyrphostin led to a complete inhibition of DNA synthesis in the two cell lines with regard to stimulation by EGF. In fact, in the two cell lines, the incorporation of [^{3}H]-thymidine was the same in the tyrphostin-treated cells as in the EGF- and tyrphostin-treated cells.

Only a slight inhibition (30%) of DNA synthesis was observed, when the cells were stimulated by FCS (Fig. 4B), indicating that FCS stimulation could suppress the inhibitory effect of tyrphostin alone, whereas EGF could not. Because EGF-R was not activated in the cells treated with tyrphostin alone, the inhibition of DNA synthesis in the cells treated with EGF and tyrphostin seems to be unrelated to the inhibition of EGF-R catalytic activity itself.

Tyrphostin Activates EGF-Dependent MAP Kinase Activity

We observed an enhancement of the tyrosine phosphorylation of MAP kinase after a brief EGF and/or tyrphostin

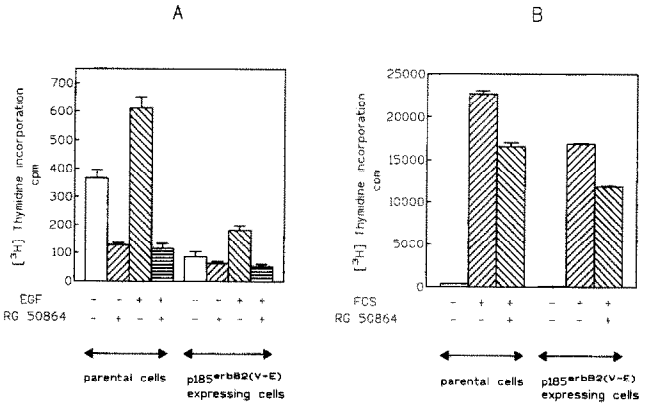

FIG. 4. Effect of tyrphostin on the onset of DNA synthesis in parental and p185$^{erbB2(V-E)}$-expressing cells. Parental and p185$^{erbB2(V-E)}$-expressing cells were stimulated for 24 hr prior to harvest with EGF (100 ng/mL) (A) or 10% FCS (B) in the presence or absence of 100 μM of tyrphostin. Cells were labeled with [^{3}H]-thymidine for 18 hr and then harvested to determine the incorporation of isotope into the DNA of the growing cells. Each point represents the average of three samples ± the standard deviation.

treatment and sought to determine if it was correlated with a modification in its catalytic activity.

MAP kinase activity from cellular extracts was measured by *in vitro* kinase assays on the MAP kinase substrate peptide. Treatment of the cells with 100 μM tyrphostin for different periods of time did not modify the MAP kinase activity of either parental or $p185^{erbB2(V-E)}$-expressing cells (Fig. 5A) after up to 1 hr of incubation. We observed that MAP kinase activity was twofold higher in the $p185^{erbB2(V-E)}$-expressing cells than in the parental cells either by an in gel kinase assay (Fig. 5B, lanes 1 and 5) or by immunoprecipitating MAP kinase and using MBP as a substrate (data not shown). Whereas renatured MAP kinase from EGF-treated parental and $p185^{erbB2(V-E)}$-expressing cells was able to phosphorylate MBP, renatured MAP kinase from tyrphostin-treated cells was unable to do so (Fig. 5B). Thus, tyrphostin treatment had no effect either on MAP kinase activity in parental cells or on the constitutive MAP kinase activation observed in $p185^{erbB2(V-E)}$-expressing cells, even after 1 hr of treatment.

EGF stimulation of the parental and the $p185^{erbB2(V-E)}$-expressing cells led to a peak of MAP kinase activation occurring after 20 min of EGF stimulation (Fig. 6A,B). In the parental cell line, MAP kinase activity returned to the basal level after 2 hr of stimulation. A second less intense wave of activation then occurred 5 hr after EGF stimulation (Fig. 6A). In the $p185^{erbB2(V-E)}$-expressing cells, there was no return even after 8 hr of stimulation (Fig. 6B). When the two cell lines were simultaneously treated by EGF and tyrphostin (100 μM), we observed a modification in MAP kinase time-course activation, without any modification of the maximal level of MAP kinase activity. Maximal MAP kinase activity occurred within 5 min of treatment rather than within 20 min. Whereas MAP kinase activity decreased rapidly in the EGF-stimulated cells, it remained maximal for at least 1 hr of treatment, then slowly decreased in the simultaneously EGF- and tyrphostin-treated cells. The level of MAP kinase activity remained higher in the EGF- and tyrphostin-cells than in the EGF-stimulated cells for at least 3 hr. After 4 hr of treatment and up to 8 hr, MAP kinase activity in the EGF-stimulated cells and in the EGF- and tyrphostin-treated cells was not significantly different. These results suggest that tyrphostin led to a modification in the time course of the EGF-dependent MAP

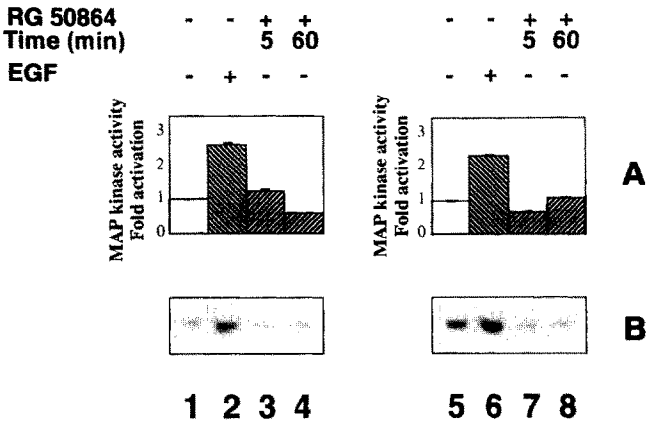

FIG. 5. MAP kinase activity after tyrphostin treatment. Serum-starved parental cells (lanes 1–4) or $p185^{erbB2(V-E)}$-expressing cells (lanes 5–8) were stimulated with 100 ng/mL of EGF for 20 min or treated with 100 μM of tyrphostin for the indicated times. (A) Determination of MAP kinase activity using peptide MAP as a substrate. Cellular extracts were prepared and MAP kinase activity determined as described in Materials and Methods. MAP kinase activity is represented with regard to the level of MAP kinase activity of the unstimulated cells of each cell line. (B) Determination of MAP kinase activity by an in gel kinase assay, using immobilized MBP as a substrate. Cell lysates were prepared as described in Materials and Methods. Proteins were directly separated on a 14% SDS-PAGE containing 0.5 mg/mL of MBP. The in gel kinase assay was performed as described in Materials and Methods.

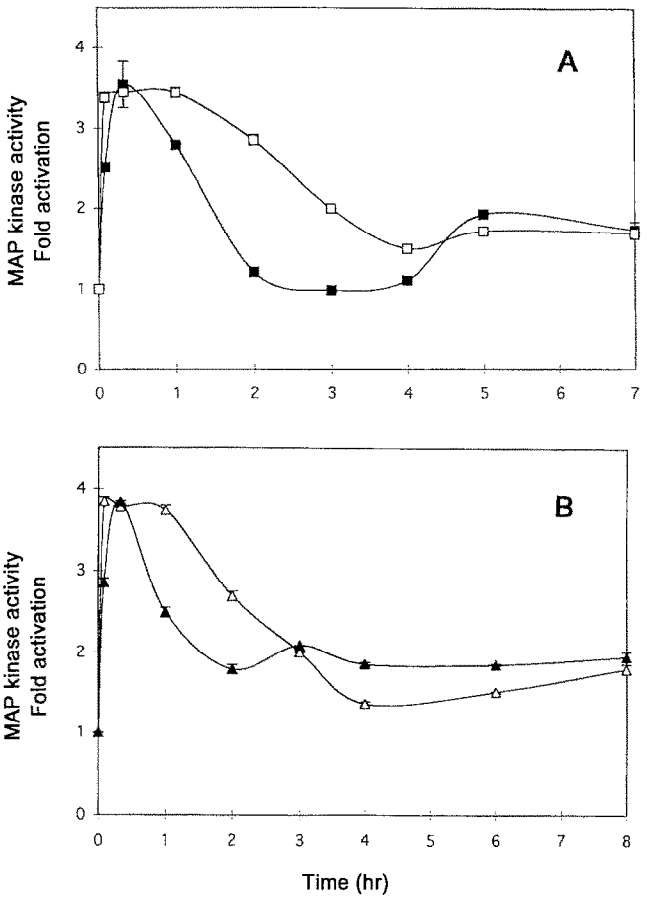

FIG. 6. Modification of the EGF-dependent time-course of MAP kinase activity in tyrphostin-treated cells. Serum-starved parental cells (A) were stimulated with EGF (100 ng/mL) (filled square) and with EGF (100 ng/mL) and 100 μM of tyrphostin (open square) for the indicated times. Cellular extracts were prepared and MAP kinase activity determined as described in Materials and Methods. Serum-starved $p185^{erbB2(V-E)}$-expressing cells (B) were stimulated with EGF (100 ng/mL) (filled triangle) and with EGF (100 ng/mL) and 100 μM of tyrphostin (open triangle) for the indicated times. Cellular extracts were prepared and MAP kinase activity determined as described in Materials and Methods. The results presented are representative of at least three separate experiments (error bars represent standard deviation from the mean).

kinase activation rather than an enhancement of its catalytic activity.

Tyrphostin Treatment has no Effect on Phosphatases Acting on MAP Kinase

We observed a modification in the phosphotyrosine content of MAP kinase in intact cells after tyrphostin treatment. One possibility was that the administration of tyrphostin led to the inhibition of a protein phosphatase acting on MAP kinase, and a second possibility was that tyrphostin stimulated a protein kinase that phosphorylated MAP kinase.

Kinase inactive MAP kinase was phosphorylated *in vitro* by cellular extracts of EGF-stimulated parental cells and used as a substrate to examine whether cellular extracts were able to dephosphorylate MAP kinase. Phosphorylated MAP kinase was then incubated for 40 min in the presence of extracts from EGF- and/or tyrphostin-treated parental cells. The level of MAP kinase phosphorylation was lowered after incubation with extracts from serum-starved cells (Fig. 7, lanes 1 and 2). Incubation of phosphorylated MAP kinase with extracts from vanadate (an inhibitor of tyrosine phosphatase)-treated cells promoted only a slight dephosphorylation of MAP kinase (Fig. 7, lane 3). These results suggested that cell extracts from unstimulated cells contained a phosphatase activity able to dephosphorylate an *in vitro* phosphorylated MAP kinase. This phosphatase activity was strongly inhibited in vanadate-treated cells, as shown by Peraldi *et al.* [36]. Incubation of phosphorylated MAP kinase with cellular extracts from EGF-stimulated cells promoted similar MAP kinase dephosphorylation as with extracts from serum-starved cells. In fact, incubation of phosphorylated MAP kinase with cellular extracts from cells stimulated with EGF, treated with tyrphostin alone or treated with EGF and tyrphostin at any point on the time course led to the same remaining level of MAP kinase phosphorylation. Similar results were obtained with cellular extracts from $p185^{erbB2(V-E)}$-expressing cells (data not shown). These results exclude the possibility that, under our conditions, tyrphostin treatment could inhibit a protein phosphatase acting on phosphorylated MAP kinase in either cell line.

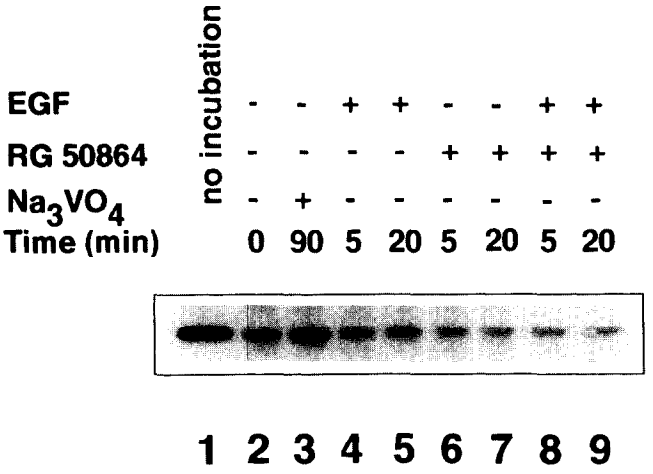

FIG. 7. Dephosphorylation of kinase inactive MAP kinase by various cell extracts. Kinase inactive MAP kinase was phosphorylated by extracts from parental cells stimulated by EGF for 20 min. MAP kinase was incubated for 40 min with various extracts from parental cells as described in Materials and Methods. A representative autoradiogram of four separate experiments with similar results is shown.

Tyrphostin Treatment Leads to the Activation of MEK1

The enhancement of tyrosine phosphorylation of MAP kinase after tyrphostin treatment could not be explained by the inhibition of a protein phosphatase acting on MAP kinase. Alternatively, we wished to know whether tyrphostin treatment could lead to the activation of a protein kinase acting on MAP kinase. The ability of cellular extracts from EGF- and/or tyrphostin-treated parental and $p185^{erbB2(V-E)}$-expressing cells to phosphorylate a kinase inactive MAP kinase was examined *in vitro*. As shown in Fig. 8A, MAP kinase was phosphorylated twice as much by cellular extracts from EGF-stimulated cells as by cellular extracts from serum-starved cells. This phosphorylation was

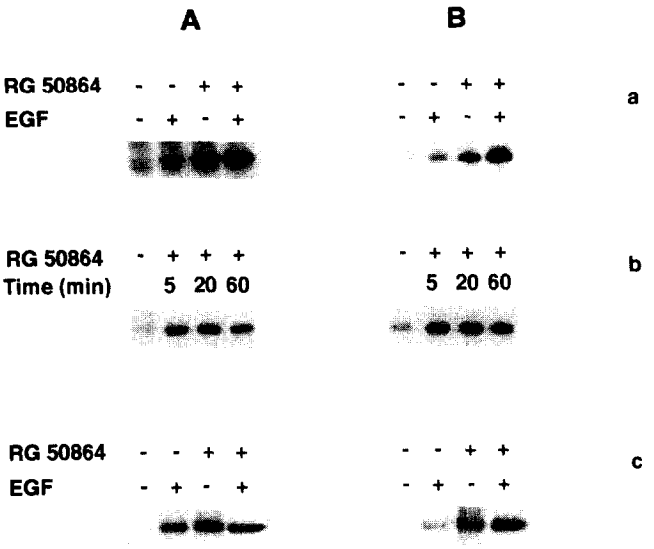

FIG. 8. Activation of MEK1 by EGF and/or tyrphostin treatment. Serum-starved parental (A) and $p185^{erbB2(V-E)}$-expressing (B) cells were stimulated or not with EGF (100 ng/mL) in the presence or absence of 100 μM of tyrphostin for 5 min or for the indicated times. Cell lysates were prepared as described in Materials and Methods. (a) Kinase inactive MAP kinase was phosphorylated *in vitro* by extracts from EGF and/or 100 μM tyrphostin-treated cells for 10 min as described in Materials and Methods. (b) Kinase inactive MAP kinase was phosphorylated *in vitro* by extracts from 100 μM tyrphostin-treated cells for 10 min as described in Materials and Methods. (c) Cellular extracts from EGF- and/or tyrphostin-treated cells were subjected to immunoprecipitation using an anti-MEK1 antibody. Immunecomplex kinase assays were performed as described in Materials and Methods.

maximal within 5 min of stimulation and then decreased to the basal level after 60 min of stimulation (Fig. 9). Extracts from cells treated with tyrphostin alone exhibited an enhanced ability to phosphorylate MAP kinase, compared with extracts from serum-starved cells (Fig. 8A, lanes 3 and 7). This effect was time independent, at least for up to a 2-hr incubation with tyrphostin (Figs. 8B, 9). Extracts from cells simultaneously treated with EGF and tyrphostin exhibited enhanced phosphorylation of MAP kinase in comparison with cellular extracts from either EGF- or tyrphostin-treated cells (Fig. 8A, lanes 4 and 8). MAP kinase phosphorylation was maximal after 5 min of stimulation and then slowly decreased. After 2 hr of stimulation, MAP kinase still remained phosphorylated to a threefold greater extent than did MAP kinase from serum-starved cells (Fig. 9). Thus, tyrphostin treatment led to the activation of a protein kinase able to phosphorylate MAP kinase. Because MEK1 phosphorylates and activates MAP kinase *in vivo*, MEK1 was immunoprecipitated from cellular extracts from EGF- and/or tyrphostin-treated cells and an *in vitro* kinase assay was performed. MAP kinase was phosphorylated 10 times as much by cellular extracts from EGF-stimulated cells and from tyrphostin-treated cells as by cellular extracts from serum-starved cells (Fig. 8C). MAP kinase was phosphorylated 15 times more by cellular extracts from simultaneously EGF- and tyrphostin-treated cells than by cellular extracts from serum-starved cells. Thus, tyrphostin activated MEK1 and potentiated the activation of MEK1 by EGF. The phosphorylation patterns of kinase inactive MAP kinase were slightly different depending on whether kinase inactive MAP kinase was phosphorylated by cellular extracts or by immunoprecipitated MEK1. More particularly, cellular extracts from tyrphostin-treated cells phosphorylated kinase inactive MAP kinase more than those from EGF-stimulated cells. Immunoprecipitated MEK1 from tyrphostin-treated cells phosphorylated kinase inactive MAP kinase, as did MEK1 from immunoprecipitated EGF-stimulated cells. This finding suggested that tyrphostin could act not only on MEK1 but also on another kinase able to phosphorylate MAP kinase.

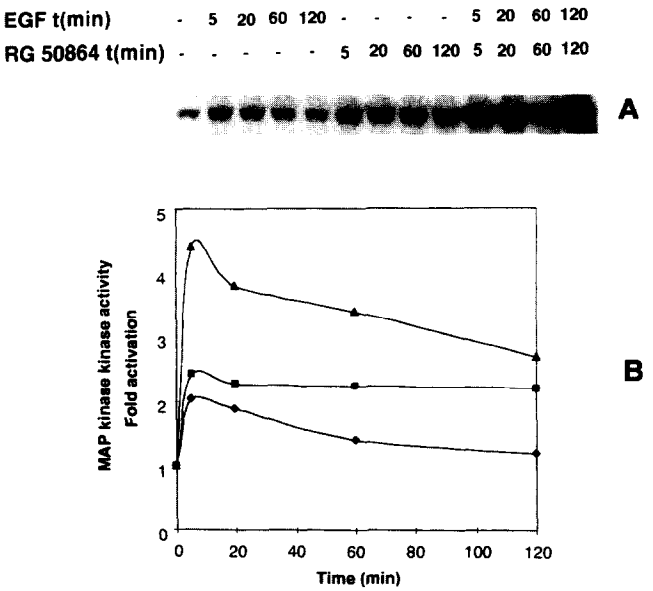

FIG. 9. Time course of MEK activation in tyrphostin-treated cells. Serum-starved parental cells were stimulated with EGF (100 ng/mL) in the presence (square) or absence (triangle) of 100 μM of tyrphostin or treated with tyrophostin alone (circle). Cell lysates were prepared as described in Materials and Methods. (A) Results presented are representative of at least two separate experiments. (B) Densitometric scanning of the experiment presented in A. Kinase inactive MAP kinase was phosphorylated *in vitro* by these cellular extracts for 10 min as described in Materials and Methods.

Thus, tyrosine phosphorylation of MAP kinase after incubation of cells with tyrphostin could be explained by activation of MEK1 and not by inhibition of tyrphostin phosphatase acting on MAP kinase.

MAP kinase is activated by MEK by dual phosphorylation on tyrosine and threonine. In our cells, phosphoaminoacid analysis revealed that immunoprecipitated MEK1 from EGF-stimulated cells phosphorylated MAP kinase both on threonine and on tyrosine residues (data not shown). Immunoprecipitated MEK1 from tyrphostin- and simultaneously EGF- and tyrphostin-treated cells were able to phosphorylate MAP kinase *in vitro* both on tyrosine and threonine residues. We also observed a nonspecific phosphorylation on serine residues. The observed ratio of phosphorylated tyrosine:phosphorylated threonine was 90:10 in the EGF-stimulated and in tyrphostin-treated cells and 70:30 in the simultaneously EGF- and tyrphostin-treated cells. Thus, MEK1 was effectively activated after incubation with tyrphostin as measured by activity determination performed *in vitro*.

In vitro *Inhibition of MAP Kinase Kinase Activity by Tyrphostin*

MEK1 was activated by tyrphostin; however, this activation did not correlate with an enhancement of MAP kinase catalytic activity. Tyrphostin could inhibit MAP kinase catalytic activity. However, we have previously shown that tyrphostin did not inhibit MAP kinase catalytic activity *in vitro* [29]. However, tyrphostin could disrupt the interaction between activated MEK1 and MAP kinase. To test this hypothesis, we measured the ability of cellular extracts from either parental or $p185^{erbB2(V\text{-}E)}$-expressing cells to phosphorylate kinase inactive MAP kinase *in vitro* in the presence of increasing concentrations of tyrphostin. Cellular extracts from EGF- and/or tyrphostin-treated cells were able to phosphorylate kinase inactive MAP kinase (Fig. 10). Cellular extracts from EGF- and/or tyrphostin-treated cells exhibited reduced activity in the presence of increasing concentrations of tyrphostin. This inhibitory effect was dose dependent, with an IC_{50} of approximately 50 μM. These results suggest that tyrphostin could have at least two distinct targets in intact cells: one upstream of MEK1, leading to its activation, and the second possibly MEK1 itself, preventing MAP kinase activation by MEK1.

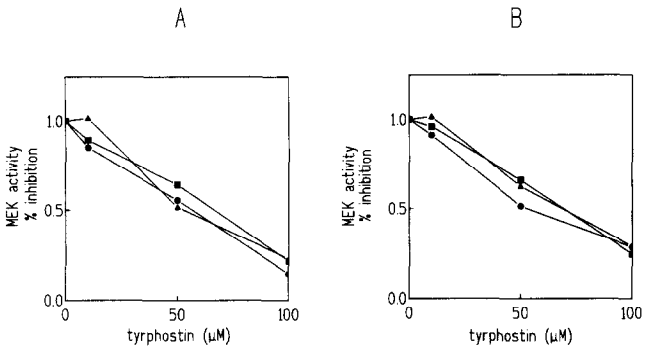

FIG. 10. Tyrphostin inhibition of MAP kinase kinase activity *in vitro*. Serum-starved parental (A) and p185$^{erbB2(V-E)}$-expressing (B) cells were stimulated with EGF (100 ng/mL) in the presence (triangle) or absence (circle) of 100 μM of tyrphostin or treated with tyrphostin alone (square) for 5 min. Cell lysates were prepared as described in Materials and Methods. Kinase inactive MAP kinase was phosphorylated *in vitro* by cellular extracts in the presence of increasing concentrations of tyrphostin. Results presented are representative of at least two separate experiments.

DISCUSSION

Tyrphostins are a family of protein tyrosine kinase inhibitors that block receptor autophosphorylation *in vitro* [21]. Some of these compounds can inhibit receptor and nonreceptor tyrosine kinases, but they are not that selective [26]. Some tyrphostins selectively block postreceptor effects of growth factors, but this requires several hours of pretreatment [21, 23, 24]. Faaland *et al.* suggested that the tyrphostin effect was not directly related to the inhibition of the tyrosine kinase activity of EGF-R because it enters the cells rapidly [27]. Tyrphostin (RG 50864) has already been described as an inhibitor of both EGF-R and p185^{erbB2} tyrosine kinase activity [30, 37]. The purpose of this study was to analyze the consequences of the treatment by one of the tyrphostins (RG 50864) on some events observed in EGF and p185^{erbB2} signaling.

Tyrphostin (RG 50864) has been described as an inhibitor of EGF-dependent proliferation [21, 22, 25]. Nevertheless, previous studies have never considered the inhibitory effect of tyrphostin alone on cell proliferation. Under our experimental conditions, tyrphostin treatment also led to a strong inhibition of the EGF-induced DNA synthesis. However, the level of [^{3}H]-thymidine incorporation was the same in the tyrphostin- as in the EGF- and tyrphostin-treated cells and was lower than in untreated cells: stimulation by EGF did not suppress the inhibitory effect of tyrphostin within cells, whereas stimulation by FCS did. Because EGF-R is not activated after a 24-hr treatment (data not shown), inhibition of DNA synthesis in cells treated with EGF and tyrphostin does not seem to be related to the inhibition of EGF-R catalytic activity itself.

The inhibitory effect observed after a 24-hr treatment with tyrphostin could be the result of multiple events. Therefore, we wanted to know whether EGF-R and p185$^{erbB2(V-E)}$ are targets of tyrphostin after short-term incubations with this drug. After simultaneous EGF and tyrphostin treatment, tyrphostin did not modify the EGF-dependent EGF-R tyrosine phosphorylation after up to a 60-min treatment in parental cells. On the contrary, tyrphostin prolonged the EGF-dependent tyrosine phosphorylation of p185$^{erbB2(V-E)}$ and to a lesser extent that of EGF-R in p185$^{erbB2(V-E)}$-expressing cells. Thus, whereas tyrphostin (RG 50864) was designed to inhibit EGF-R tyrosine kinase activity, we observed either no effect or an activating effect after coincubation with EGF and tyrphostin. Moreover, incubation with tyrphostin alone slightly enhanced p185$^{erbB2(V-E)}$ tyrosine phosphorylation, but it had no effect on EGF-R tyrosine phosphorylation (data not shown). It seems unlikely that tyrphostin could directly activate p185$^{erbB2(V-E)}$ because RG 50864 has been described as an inhibitor of p185^{erbB2} tyrosine kinase activity *in vitro* [37]. Tyrphostin could inhibit a protein tyrosine phosphatase that dephosphorylates p185^{erbB2}. Incubation of SKBR3 cells that overexpress p185^{erbB2} with vanadate leads to an enhancement of p185erbB2 tyrosine phosphorylation [38]. A similar result has been obtained in our p185$^{erbB2(V-E)}$-expressing cells (data not shown). This phosphatase would be p185^{erbB2} specific because tyrphostin has no effect on the EGF-dependent EGF-R tyrosine phosphorylation in parental cells. However, tyrphostin could inhibit a protein kinase that specifically downregulates p185^{erbB2} by phosphorylation. This mechanism of regulation could be similar to that of PKC, which has been described as downregulating EGF-R by phosphorylation on residue Thr 654.

In the p185$^{erbB2(V-E)}$-expressing cells, Shc proteins were constitutively tyrosine phosphorylated and MAP kinase was constitutively activated. Treatment of these latter cells with tyrphostin for up to 60 min had no effect on the constitutive p185$^{erbB2(V-E)}$ and Shc protein tyrosine phosphorylation. Nevertheless, in the two cell lines, tyrphostin treatment led to a slight tyrosine phosphorylation of MAP kinase that does not correlate with MAP kinase activation. Thus, tyrphostin has no effect on constitutive MAP kinase activity in p185$^{erbB2(V-E)}$-expressing cells. The time course of MAP kinase catalytic activity after EGF stimulation is modified in the presence of tyrphostin: maximal MAP kinase activity occurred after only 5 min of stimulation and tyrphostin (RG 50864) prolonged MAP kinase activity without modifying its maximal level of activation. This result contrasts with that we previously obtained with NIH3T3 cells, where the same treatment led to a real enhancement of MAP kinase phosphorylation and activity without modification of the time course of its catalytic activity [29]. This difference indicates that tyrphostin has some cell type-specific effects. In parental cells, the time course of MAP kinase activity after EGF stimulation is modified in the presence of tyrphostin, whereas that of EGF-R tyrosine kinase activity is not. Tyrphostin effect on MAP kinase activity must be mediated by effectors located downstream of the two receptors.

We have been unable to acquire any evidence that tyr-

phostin regulates a protein phosphatase able to dephosphorylate MAP kinase. Conversely, we have shown that treatment by tyrphostin alone leads to the effective activation of MEK1. MEK1 is not directly activated by tyrphostin because MEK1 is not activated by tyrphostin *in vitro*. These results indicate that tyrphostin acts either on a step downstream of EGF-R and $p185^{erbB2(V-E)}$ or on a step independent of EGF-R and $p185^{erbB2(V-E)}$, which would connect upstream of MEK1. Activation of MEK1 occurring after tyrphostin treatment is still persistent after a 2-hr treatment. Moreover, tyrphostin treatment enhances EGF-dependent MEK1 activation and modifies its time course: activation of MEK is still persistent after a 2-hr treatment, which indicates that tyrphostin target is not downregulated.

Within cells, more than one signaling pathway has been shown to lead to MEK activation, and tyrphostin target(s) could be located on one of them. For example, PKC activates Raf-1 kinase and therefore MEK1 and MAP kinase [39]. Tyrphostin could lead to the activation of PKC or to a related enzyme *in vivo* and thus to the enhancement of the MAP kinase cascade. However, tyrphostin (RG 50864) has been described as an effective inhibitor of the GTPase activity of transducin, this effect being related to the unsubstituted hydroxyl groups at the 3-, 4-position of the phenyl ring [40]. Tyrphostin could inhibit the GTPase activity of p21ras and therefore upregulate the MAP kinase cascade. Alternatively, several of the components of the MAP kinase pathway are subject to regulation by protein phosphatases. Inhibition of PP2A by SV40 small t antigen, which can substitute for the B subunit of PP2A and therefore inhibit its catalytic activity, results in the activation of MEK1/2 and MAP kinase without any effect on Raf1 activity [41]. MEK1/2 seems the most likely target for PP2A. A similar effect has been observed in adipocytes treated with okadaic acid, an inhibitor of serine/threonine phosphatase and especially for PP2A [42]: it stimulates MAP kinase activity, probably by inhibiting a serine/threonine phosphatase acting upstream of MAP kinase. Tyrphostin does not seem to inhibit a phosphatase acting on MAP kinase; nevertheless, it could inhibit a serine/threonine phosphatase able to dephosphorylate a kinase lying upstream of MAP kinase.

MEK1 activation after tyrphostin treatment does not correlate with MAP kinase activation, reflecting a more complicated mechanism of action of tyrphostin, and implies that tyrphostin must have at least one other target in intact cells. Our results suggest that tyrphostin could disrupt the interaction between activated MEK1 and MAP kinase and thus prevent activation of MAP kinase by MEK1. We have shown that MAP kinase kinase catalytic activity *in vitro* is inhibited by increasing tyrphostin concentrations. When no tyrphostin is added to the reactional medium, MEK1 from tyrphostin-treated cells is able to phosphorylate and to activate MAP kinase *in vitro*. These results imply that tyrphostin's effect on the interaction between activated MEK1 and MAP kinase is reversible. Tyrphostin's effects on MAP kinase activity would also be the result of two contradictory effects: activation of MEK and inhibition of the interaction between activated MEK and MAP kinase. The more MEK is activated, the more MAP kinase is activated. On the contrary, the more tyrphostin concentration in cell cytoplasm is elevated, the less MAP kinase is activated. In cells treated with tyrphostin alone, these contradictory effects could cancel each other out: MAP kinase activity remains unaffected. In the EGF- and tyrphostin-treated cells, MEK is strongly activated and remains activated for at least 2 hr. On the contrary, in the EGF-stimulated cells, MEK activity returned near its basal level after 60 min of stimulation. Thus, MEK could be activated enough to prevail over tyrphostin inhibitory effect. The resulting MAP kinase activation would be higher in EGF- and tyrphostin-treated cells than in EGF-stimulated cells. This hypothesis could explain why tyrphostin (RG 50864) prolongs EGF-dependent MAP kinase activation.

After tyrphostin treatment, MAP kinase is not activated but is nevertheless slightly phosphorylated on tyrosine residues. MAP kinase is activated by a dual phosphorylation both on threonine and on tyrosine residues. Tyrosine phosphorylation of MAP kinase has been postulated by Zhang *et al.* [43] as being a prerequisite for the modification of MAP kinase conformation and, consequently, MAP kinase threonine phosphorylation. The structure of inactive MAP kinase has been solved [43] but not that of MEK and active MAP kinase. Under these conditions, it is rather difficult to understand the precise mechanism of action of tyrphostin on the MAP kinase–MEK complex. Understanding this mechanism would be of great interest to solve the conformation of these enzymes because it would reveal whether tyrphostin interacts with the active sites. Tyrphostin could prevent the modification of MAP kinase conformation after tyrosine phosphorylation and thus MAP kinase threonine phosphorylation. Another protein kinase inhibitor may have a similar mechanism of action: it would inhibit MEK activation without interacting with the active site of Raf or MEK but would interact instead at another site, thereby blocking access to activating enzymes [44].

In conclusion, whereas tyrphostins were designed to inhibit EGF-R tyrosine kinase activity, we have shown that tyrphostin (RG 50864) has several targets in the transducing pathway stimulated by EGF in epithelial cells that are neither EGF-R nor its related protein, $p185^{erbB2(V-E)}$. These results indicate that one must be cautious when using tyrphostin and other inhibitors of protein tyrosine kinases because their precise mechanism of action is still not yet well understood.

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