Regulation of ribosomal protein S6 kinase in human mammary tumor cells: Effect of estrogen, growth factors and phorbol ester

by I. Novak-Hofer, W. Küng, D. Fabbro, A. Matter and U. Eppenberger

Laboratory of Biochemistry-Endocrinology, Dept of Research and Dept of Gynecology, University Clinic Medical School, CH-4031 Basel, and Pharmaceutical Research Laboratories of Ciba-Geigy Ltd., CH-4002 Basel (Switzerland)

Summary. Growth of human mammary tumor cells ZR-75-1 is stimulated by estradiol (E_2), epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I). In these cells ribosomal protein S6 kinase is activated by EGF, IGF-I, insulin and phorbol 12-myristate 13-acetate (TPA) but not by E_2. The human mammary tumor cell line MDA-MB 231, which is E_2-receptor negative, has receptors for EGF, IGF-I and insulin but is unresponsive to these factors in terms of growth and S6 kinase activation. The role of protein kinase C (PKC) in the activation of S6 kinase by growth factors and TPA was investigated in ZR-75-1 cells. Down regulation of PKC activity by treatment with TPA for 48-h blocks the stimulation of S6 kinase by TPA but leaves the activation by EGF, IGF-I and insulin unaffected. In intact ZR-75-1 cells staurosporine blocks activation of S6 kinase by EGF and TPA, however with different IC_{50}.
The results show that S6 kinase is not activated by estradiol, that its activation by EGF, IGE-I and insulin does not depend on the presence of PKC activity and that its activation by TPA is mediated by a different (PKC-dependent) pathway.
Key words. Human mammary tumor cells; ribosomal protein S6 kinase; growth factors; phorbol ester; staurosporine.

For many years it has been known that steroid hormones such as estrogen stimulate the growth of human breast cancer. However, recently there is an increasing awareness that growth factors are also involved in this process [11]. The progress of this disease is characterized by a transition of the tumor from an estrogen (E_2) responsive to an unresponsive phenotype. At present it is not known whether the unresponsiveness towards E_2 is the cause or the consequence of the loss of the respective E_2 receptors.

A number of human mammary tumor cell lines have been isolated, mostly from pleural effusates [3]; they are used as model systems to investigate molecular mechanisms involved in steroid- and growth factor-induced growth. Mammary tumor cell lines can be divided into two categories; cells containing E_2 receptors (hormone-dependent) and cells without E_2 receptors (hormone-independent). Both cell types have receptors for growth factors such as epidermal growth factor (EGF) [7, 18, 21] and insulin-like growth factor I (IGF-I) [9]. According to recent findings growth of hormone-dependent cells is stimulated by EGF and IGF-I whereas hormone-independent cells are characterized by high growth rates in the absence of growth factors or serum [12].

In order to further characterize the growth response of these cells to E_2 and growth factors, the effect of E_2, EGF, insulin and IGF-I on ribosomal protein S6 kinase was studied in hormone-dependent ZR-75-1 and hormone-independent MDA-MB 231 cells. The activation of the S6 kinase represents a rapid response (within 30 min) to growth factors such as EGF [14, 15]; the subsequent phosphorylation of ribosomal protein S6 is associated in many systems with cellular proliferation [10, 13, 24]. The S6 kinase was found to be activated by growth factors but not by E_2 in human mammary tumor cells. Since this enzyme is also activated by phorbol esters such as TPA [2, 22], it was of interest to study how down regulation of protein kinase C (PKC) activity by TPA affects the response of S6 kinase towards TPA, EGF and IGF-I. Staurosporine, which was found to be a potent inhibitor of S6 kinase activation by growth factors and by TPA, was used to dissociate further the PKC and growth factor mediated activation of S6 kinase.

Results. Effect of estradiol and growth factors on the growth of human mammary tumor cells. The growth of hormone (estrogen) dependent ZR-75-1 cells and the hormone-independent MDA-MB 231 cells was studied in serum-free medium. The phenol red used as a pH indicator in the medium was lowered 10-fold to avoid any estrogenic affect [1]. Under such conditions growth stimulation by E_2 was clearly visible in ZR-75-1 cells (fig. 1 A, bar 3). In contrast, the hormone-independent MDA-MB 231 cells exhibited high growth rates in serum-free medium (fig. 1 B, bar 1) and were not further stimulated by E_2 (fig. 1 B, bar 3). In addition ZR-75-1 cells demonstrated growth stimulation (fig. 1A) by growth factors such as insulin (bar 2), EGF (bar 4) and IGF-I (bar 5) which had no effect on the growth of MDA-MB 231 cells (fig. 1 B).

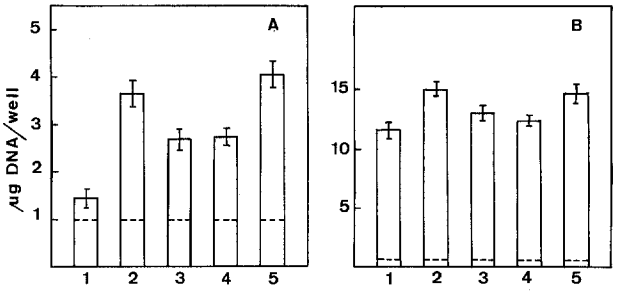

Figure 1. Effect of growth factors and 17β-estradiol (E_2) on the growth of human mammary tumor ZR-75-1 cells (*A*) and MDA-MB 231 cells (*B*) in serum-free medium. Cells were grown as indicated in 'Material and methods'; DNA was determined after 9 days of culture. Dotted lines indicate seeding density at the start of the experiments. 1: control; 2: 5×10^{-7} insulin; 3: 3×10^{-9} M E_2; 4: 10^{-9} M EGF; 5: 10^{-8} M IGF-I.

In order to find out whether growth factors, i.e. EGF and E_2 have different rates of stimulation of DNA synthesis, ZR-75-1 cells were serum-deprived for 3 days as indicated in 'Materials and methods.' Cells were then stimulated with either 10^{-8} M EGF or 10^{-9} M E_2 and incorporation of [^{3}H]-Thymidine into nuclei was measured after 20 h as described in 'Materials and methods'. It was found that controls showed 14.5 ± 3% of labeled nuclei. After 20 h EGF-treated cells showed 74.5 ± 4% and E_2-treated cells showed 68.7 ± 1% labeled nuclei.

Effect of E_2 and EGF on S6 kinase activity and extent of S6 phosphorylation in ZR-75-1 cells. Both E_2 and EGF stimulate the proliferation of ZR-75-1 cells to a similar extent (fig. 1 A). Since a number of mitogens rapidly trigger the activation of S6 kinase and S6 phosphorylation [14, 15, 22], investigations were carried out to see whether treatment with E_2 also leads to such a response. ZR-75-1 cells were grown and serum-deprived for 24 h prior to the addition of either 10^{-8} M E_2 or 10^{-8} M EGF. Stimulations were carried out for 60 min and 120 min, respectively; extracts were subsequently prepared and assayed for S6 kinase activity as described previously [14]. Figure 2 shows an autoradiograph of phosphoproteins separated by SDS gel electrophoresis after incubations of cellular extracts with ribosomes and [^{32}P] ATP as described [14]. The ribosomal protein S6 is designated with an arrow and appears to be more phosphorylated using

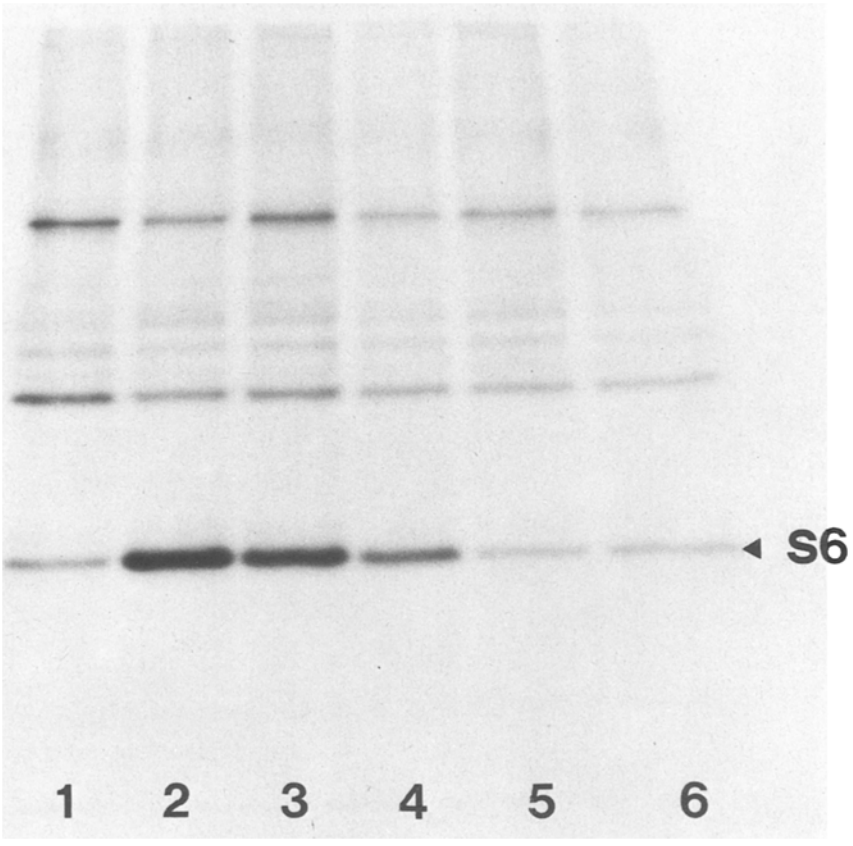

Figure 2. S6 kinase activity in cell-free extracts from EGF- or E_2-treated ZR-75-1 cells. Autoradiograph of a SDS gel. The 40 s ribosomal subunits from *A. Salina* were incubated with [^{32}P] ATP and cell extracts as described in 'Material and methods'; phosphoproteins of the respective extracts were separated on a 15% SDS-polyacrylamide gel. The arrow designates 40 s ribosomal protein S6. Track 1: serum-starved cells; tracks 2 and 3: cells stimulated 30 and 60 min with 10^{-8} M EGF; track 4: cells stimulated 120 min with EGF; tracks 5 and 6: cells stimulated for 60 and 120 min with 10^{-8} M E_2, respectively.

extracts from EGF-treated cells (fig. 2, tracks 2–4) as compared to control cells (track 1). S6 kinase activity of EGF-treated cells is maximal at 30 min (track 2) and decreases with increasing incubation time of EGF (tracks 3 and 4). In contrast, cell extracts treated for 60 or 120 min with E_2 (tracks 5 and 6) exhibited no increase in S6 kinase activity above control levels (track 1).

In 3T3 cells S6 kinase activation by EGF is followed by increased S6 phosphorylation [15]. In order to investigate if E_2 could lead to increased S6 phosphorylation, possibly via a protein kinase different from the EGF-activated S6 kinase, the extent of S6 phosphorylation was measured in E_2-treated ZR-75-1 cells. Ribosomes were isolated from serum-deprived cells (controls) and from cells stimulated either with EGF or E_2 for 60 min and 120 min. Ribosomal proteins were analyzed by a 2-dimensional gel system, where S6 has a different mobility depending on its phosphorylation state. Ribosomes from control cells show S6 in is unphosphorylated from (fig. 3A); EGF treatment of ribosomes for 60 or 120 min leads to the appearance of phosphorylated S6 derivatives which migrate more slowly in both dimensions of electrophoresis (fig. 3B, D). In contrast, ribosomes incubated with E_2 exhibited a S6 form in the same native position as the controls (fig. 3C, E); also prolonged E_2 incubations up to 6 h led to the same result (data not shown).

Activation of S6 kinase by EGF, Insulin, IGF-I and TPA in ZR-75-1 cells; effects of long-term treatment with TPA. Phorbol esters such as TPA bind to protein kinase C (PKC) and lead to its translocation to membranes followed by down regulation of PKC activity in human mammary tumor cells [8, 19]. Also profound effects have been found on the growth and the morphology of these cells by TPA [8]. Therefore, we investigated the effect of TPA on S6 kinase activation; especially in view of the down regulation of PKC activity and its influence on the S6 kinase activation by growth factors.

An 8-fold increase of S6 kinase above control levels was observed when ZR-75-1 cells were exposed to 3×10^{-7} M TPA for 30 min (fig. 4, bar 4). However, when cells were treated for 48 h with 3×10^{-7} TPA and then rechallenged for 30 min with TPA, no increase in S6 kinase activity was observed (fig. 4, lane 9). According to these results S6 kinase activation by TPA does not occur after the down regulation of PKC activity. S6 kinase activity of cells kept for 48 h in serum-free medium and subsequently stimulated for 30 min with 10^{-8} EGF (fig. 4, bar 2), 10^{-8} M insulin (fig. 4, bar 3) and 10^{-8} M IGF-I (fig. 4, bar 5) led always to a 6–7-fold increase of S6 kinase activity. Whereas restimulation of S6 kinase in cells which were kept for 48 h in the presence of 3×10^{-7} M TPA with TPA was ineffective (fig. 4, bar 9), treatment with EGF (bar 7), insulin (bar 8) and IGF-I (bar 10) led to a similar increase in S6 kinase activity as in cells not pretreated with TPA. Therefore, these growth factors are able to activate S6 kinase also in the absence of PKC activity.

Activation of S6 kinase by EGF and TPA in ZR-75-1 cells; effect of staurosporine. So far no potent inhibitors of S6

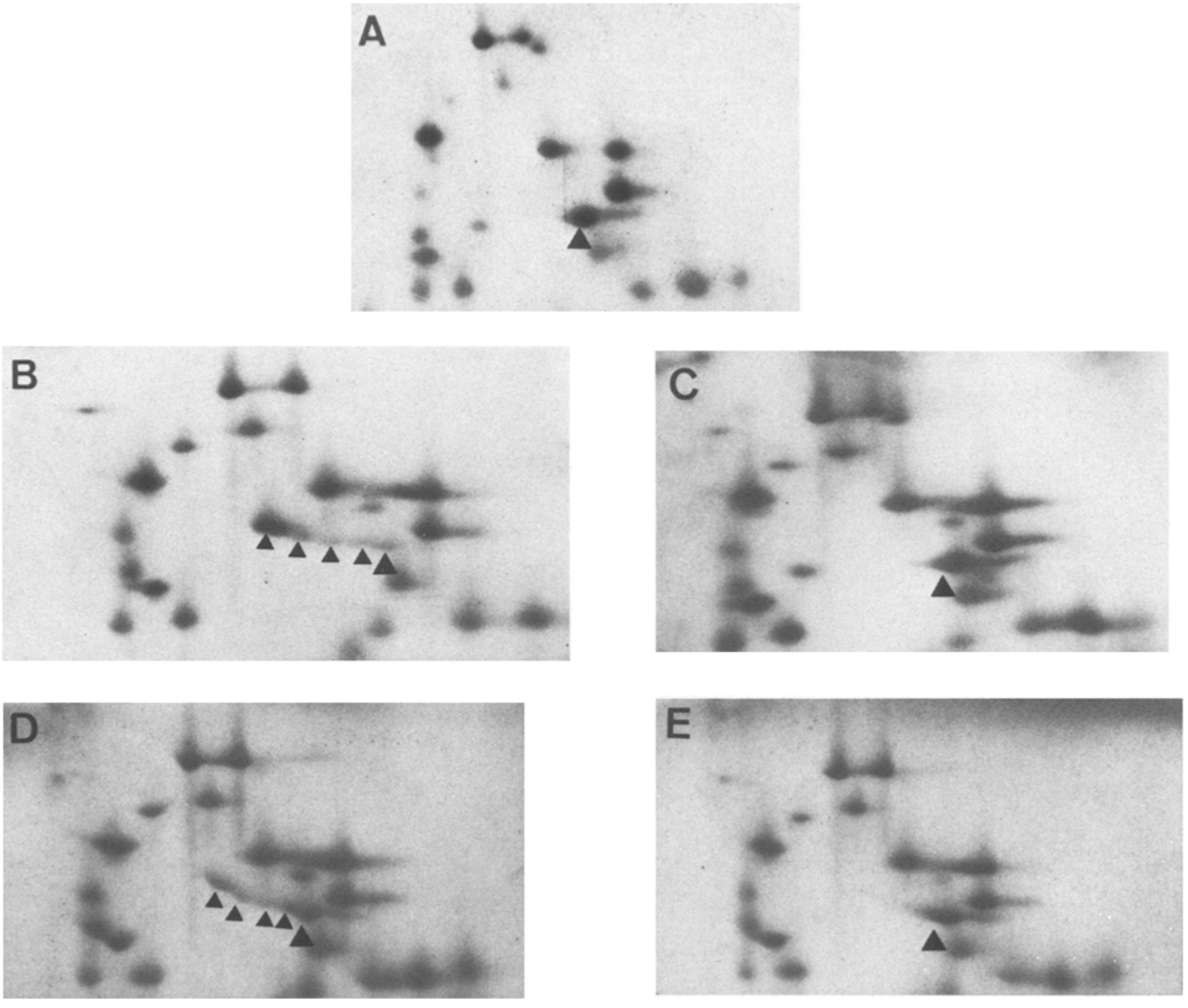

Figure 3. Stimulation of S6 phosphorylation in ZR-75 cells by EGF or E_2. Coomassie Blue staining of ribosomal proteins after 2 dimensional electrophoresis. Serum-starved cells were stimulated with either 10^{-8} M EGF or 10^{-8} M E_2. Ribosomal proteins were isolated and analyzed as described in 'Material and methods'. Control (*A*), 60 min EGF (*B*), 60 min E_2 (*C*), 120 min EGF (D), 120 min E_2 (*E*).

kinase activation by growth factors have been described. Since staurosporine is a good protein kinase inhibitor at low (nanomolar to micromolar) concentrations in vitro[23], we investigated whether this compound is also able to block activation of S6 kinase by EGF. Serum-deprived ZR-75-1 cells were incubated for 30 min with 10^{-8} M EGF in the presence of 10^{-10} M staurosporine. The drug was found to be an effective inhibitor of S6 kinase activation by EGF, with an IC_{50} of about 10^{-7} M (fig. 5, triangles). Subsequently we studied whether the same dose-dependent inhibition could be found during S6 kinase activation by TPA, since the results obtained with TPA-desensitized cells (fig. 4) indicated a different pathway probably mediated by PKC. When cells were treated for 30 min with 10^{-7} M TPA in the presence of staurosporine (fig. 5, circles), no significant inhibitory effect was observed below 10^{-9} M staurosporine; a result similar to that with EGF activation. However, TPA-induced S6 kinase activation was inhibited down to basal levels in the presence of 10^{-7} M staurosporine in contrast to only about 50% inhibition of EGF activation at this dose. Basal S6 kinase activity levels (without growth factors) were also inhibited in a dose-dependent manner by staurosporine, with a complete inhibition (no incorporation of ^{32}P into S6) at 10^{-6} M (data not shown).

S6 kinase activity of the hormone-independent mammary tumor cell line MDA-MB 231. Growth rates of MDA-MB 231 cells are high in serum-free medium and cannot be increased by EGF, insulin or IGF-I (fig. 1 B) These cells contain receptors for both insulin[17] and EGF.[18, 21] Therefore, we investigated the effect of these factors, as well as of IGF-I on the S6 kinase activity. None of these growth factors increased S6 kinase activity (fig. 6, bars 1–4). This finding is not due to the absence of the enzyme, since it can still be activated by orthovanade (fig. 6, bar 6) and by fetal calf serum (fig. 6, bar 5). Thus in the case of MDA-MB 231 cells the inability of some growth factors to stimulate proliferation is already expressed at the level of S6 kinase activation, which is an early post receptor response to growth factors.

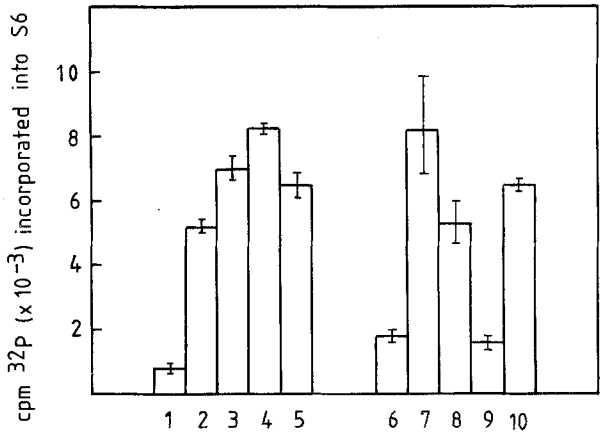

Figure 4. Activation of S6 kinase by growth factors and TPA in ZR-75 cells: long-term effect of TPA. Cells were kept either in serum-free medium (1–5) or in the presence of 3×10^{-7} M TPA (6–10) for 48 h. S6 kinase activity was measured in extracts obtained from unstimulated cells (1, 6) or cells stimulated for 30 min with 10^{-8} M EGF (2, 7), 10^{-8} M Insulin (3, 8), 3×10^{-7} M TPA (4, 9) and 10^{-8} M IGF-I (5, 10). Results are means of duplicates from a typical experiment and are expressed as cpm [^{32}P] incorporated into S6; 8×10^3 cmp correspond to 19 pmol phosphate/min/mg protein.

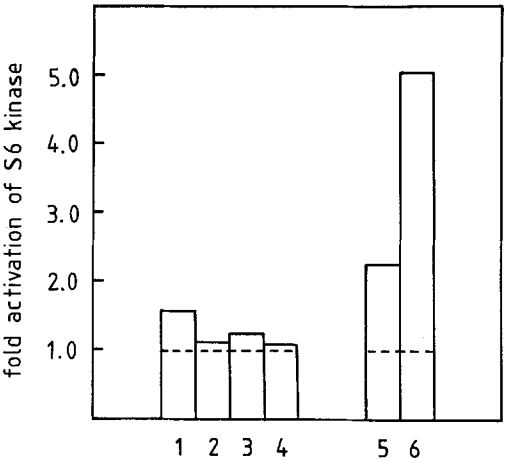

Figure 6. Effect of growth factors on the S6 kinase activity in MDA-MB 231 cells. Serum-deprived cells were incubated for 30 min with either 10^{-8} M EGF (1), 10^{-8} M insulin (2), 3×10^{-7} M TPA (3), 10^{-8} M IGF-I (4), 10% fetal calf serum (5) and 1 mM Na orthovanadate (6). S6 kinase activity was measured as described in 'Materials and methods'. Results (means of duplicates differing less than 10%) are expressed as fold activation above controls. Controls (1.0-fold activation) correspond to 6.7 pmol phosphate incorporated into S6/min/mg protein.

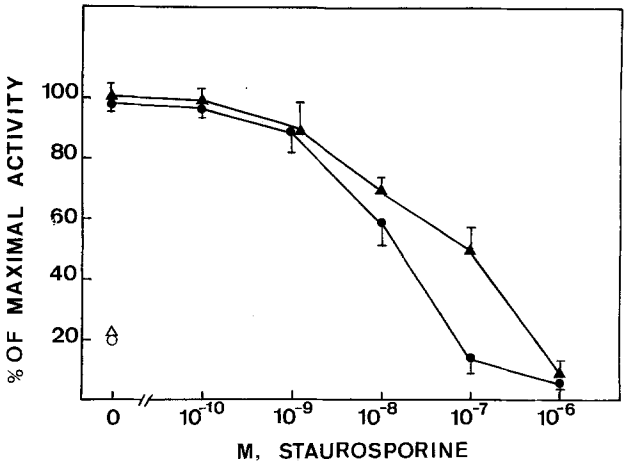

Figure 5. Effect of staurosporine on the S6 kinase activation. Serum deprived cells were incubated for 30 min either with 10^{-8} M EGF (closed triangles) or 3×10^{-7} M TPA (closed circles) in the presence of increasing concentrations of staurosporine. S6 kinase activity was measured in extracts as described in 'Materials and methods'. Results are means of 3 independent experiments differing less than 10% and are expressed in % of maximal activity. Open symbols show basal activity in the absence of EGF or TPA.

Discussion. 17 β-estradiol (E_2), EGF and IGF-I stimulate growth of ZR-75-1 cells (fig. 1 A). Insulin, which is known to stimulate DNA synthesis in some cell types, probably acts through IGF-I receptors at high concentrations as used in our study. In contrast to growth factor-activated DNA synthesis, E_2-stimulated proliferation is not well characterized. In mammary tumor cells E_2 increases the production of growth factors such as the EGF-homologous α-TGF and IGF-I[4–6], and subsequently autocrine growth has been proposed[5]. In this case, E_2 should also trigger molecular responses characteristic for growth factors. When activation of S6 kinase, a typical response to many growth factors[2, 14, 15, 22], was studied, it was found in contrast to EGF and IGF-I that E_2 did not increase S6 kinase activity (fig. 2, fig. 4) and S6 phosphorylation (fig. 3). Previously it has been shown that S6 kinase activation by EGF and α-TGF in ZR-75-1 cells exhibited a dose response curve similar to that for EGF (α-TGF) stimulated growth[16]. Therefore S6 kinase activation should be noticeable at concentrations effective in stimulating growth. However, E_2 by-passes the activation of S6 kinase and S6 phosphorylation. Consequently these results do not support the hypothesis that E_2 stimulates the growth of ZR-75-1 cells via the production of α-TGF. Since all these experiments were performed using sparse, subconfluent cultures, one cannot exclude the possibility that in addition to a direct stimulation of DNA synthesis by E_2, the α-TGF induced by E_2 may cause an additional growth stimulation in dense cultures. We have observed in serum-deprived ZR-75-1 cells a similar increase in DNA synthesis after 20 h of stimulation with EGF or E_2, by measuring [^{3}H] thymidine incorporation into nuclei (in both cases about 70% of the nuclei were labeled). It would be of interest to know whether E_2-induced α-TGF production during this first round of DNA synthesis is sufficient to account for the effect of E_2. Unfortunately the growth factor measurements in E_2-conditioned media are often performed after longer periods of cell growth[4–6].

In this context it is of interest to note that hormone-independent mammary tumor cell lines also produce α-TGF and IFG-I[4–6]. However, our results obtained in MDA-MB 231 cells demonstrate clearly no growth response by EGF and IGF-I (fig. 1 B) and also no S6 kinase activation by these growth factors (fig. 6). It appears that despite the presence of EGF and IGF-I receptors, these receptors are inactive in terms of mediating S6 kinase activation and/or growth. The fact that Na orthovanadate and to a lesser extent also fetal calf serum activate S kinase (fig. 6, bars 5, 6) indicates that the lack of response seen with EGF, insulin and IGF-I is not due to the absence of S6 kinase in MDA-MB-231 cells. Therefore the growth factor production of hormone-independent mammary tumor cells may play a role in paracrine rather than autocrine growth stimulation.

Since EGF and IGF-I stimulated the S6 kinase activity of ZR-75-1 cells (fig. 4), we investigated whether the effects of these growth factors are mediated by protein kinase C (PKC). It was found that down regulation of PKC activity

by long-term treatment (48 h) with TPA[8, 19] blocked the activation of S6 kinase by TPA (fig. 4). In contrast, EGF and IGF-I stimulated S6 kinase also in the absence of PKC activity (fig. 4). Recently it has been shown by ion-exchange column fractionation of extracts obtained from cells stimulated with TPA[2, 22], EGF[15], serum[2] and insulin[22] that S6 kinase is a distinct protein kinase different from PKC. However, in the case of TPA, PKC activity is necessary to activate S6 kinase (fig. 4)[2, 22].

The mechanisms by which growth factors or TPA activate S6 kinase are unknown. Phosphorylation may play a role in its activation because phosphatase activity inhibits the enzyme[15]. In the case of EGF, phosphorylation by the EGF receptor tyrosine kinase could activate the enzyme, whereas TPA action may be mediated via phosphorylation by PKC. Purification of S6 kinase and antibody studies will be necessary to correlate the phosphorylation state of the enzyme with its activation. In this study we attempted in intact cells to dissociate TPA activation of S6 kinase from EGF activation by the use of staurosporine, a protein kinase inhibitor. Staurosporine was found to be an effective inhibitor of activation of S6 kinase by EGF with an IC_{50} of about 10^{-7} when added to ZR-75-1 cells. Staurosporine also inhibited TPA activation of S6 kinase, however with a different IC_{50} close to 10^{-8} M (fig. 5). These results support the hypothesis that different pathways are involved in EGF- and TPA mediated activation of S6 kinase. Studies are in progress to investigate the effect of staurosporine on EGF and IGF-I stimulated growth of ZR-75-1 cells.

Material and methods. Materials. EGF was from Collaborative Research, Lexington/MA (USA); IGF-I was from Amersham; Insulin was from Sigma, Staurosporine was a gift from Dr A. Matter, Ciba-Geigy, Basel.

Methods. Growth experiments. Cells (ZR-75-1 and MDA-MB 231, American Type Culture Collection) were grown in 24 well plates in serum-free improved minimal essential medium, zinc option (IMEM-20) complemented with fatty-acid-free bovine serum albumin (1 mg/ml), iron-free transferrin (5 µg/ml), insulin (5 ng/ml), sodium selenite (10 ng/ml), Hepes (15 mM, pH 7.3), gentamycin (5 µg/ml). After trypzinization of cells, protease activity was blocked by addition of soybean trypsin inhibitor (Sigma, type I-S, 10 µg/ml) and cells were washed twice in serum-free medium. Cells were then seeded in 24 well plates coated with collagen type IV from human placenta (Sigma) in the above medium. Media were changed every 3 days. After 9 days of growth in the presence of E_2 or the indicated growth factors cell numbers and DNA content were determined as described[12].

For nuclear labeling, ZR-75-1 cells were seeded out serum-free on collagen-treated 35 mm petri dishes at low densities (40,000 cells/cm^2) and kept serum-free for 3 days. Then the fresh medium with BSA and Hepes was added containing 1 µCi/ml [Methyl-^{3}H]-thymidine (Amersham) as well as 10^{-8}/EGF or 10^{-9} M E_2. After 20 h the cells were fixed by dropwise addition of Carnoy's fixative (25% acetic acid/75% ethanol) until equal volumes of medium and fixative were reached. After 5 min, the solution was discarded and fresh, undiluted fixative was added for another 10 min. Then the fixative was removed and cells air-dried. The cell layers were then first coated with a gelatine layer (50 mg gelatine + 5 mg $KCrSO_4$ dissolved in 10 ml water at 50 °C) and then after drying coated with Ilford L4 emulsion dissolved before with water and gelatine solution in a 1:1:1 ratio. After an exposure of 24 h, the films were developed with Neutol (Agfa) and fixed with Hypam (Ilford). Nuclei were stained with hematoxylin and then the percentage of labeled nuclei determined by counting at least 500 nuclei per dish.

S6 kinase assays. Cells were grown in 100 mm plates in Richters medium supplemented with 1 µg/ml insulin and 5% Fetal Calf Serum (FCS) up to a density of 5×10^6 cells. Cultures were then shifted overnight to Richters medium without insulin and FCS, cells were treated with E_2 or growth factors as indicated in the legends to figures and cellular extracts were prepared as described before [14]. The 40s ribosomal subunits used as substrate were prepared from *Artemia salina* cysts as described[16]. Phosphorylation assays, SDS electrophoresis and autoradiography were performed as indicated before[14].

S6 kinase activity was quantified by cutting out the S6 band from the dried SDS gels, followed by liquid scintillation counting.

Two-dimensional gel electrophoresis of ribosomal proteins. Ribosomes were isolated from serum-deprived cells that had been treated with E_2 or EGF as indicated and two-dimensional gel electrophoresis of ribosomal proteins was performed as described[15, 16]. Proteins were stained with Coomassie Blue.

Acknowledgments. This study was funded in part by the Swiss National Sience Foundation Number 3.344-0.86 and the Roche Research Foundation (I.N.). The excellent technical assistance of Mrs I. Lützelschwab and E. Silber is appreciated.

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