

Differential Activation of Mitogen-activated Protein Kinase and S6 Kinase Signaling Pathways by 12-O-Tetradecanoylphorbol-13-acetate (TPA) and Insulin

EVIDENCE FOR INVOLVEMENT OF A TPA-STIMULATED PROTEIN-TYROSINE KINASE*

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AG-18, an inhibitor of protein-tyrosine kinases, was employed to study the role of tyrosine-phosphorylated proteins in insulin- and phorbol ester-induced signaling cascades. When incubated with Chinese hamster ovary cells overexpressing the insulin receptor, AG-18 reversibly inhibited insulin-induced tyrosine phosphorylation of insulin receptor substrate-1, with minimal effects either on receptor autophosphorylation or on phosphorylation of Shc64. Under these conditions, AG-18 inhibited insulin-stimulated phosphorylation of the ribosomal protein S6, while no inhibition of insulin-induced activation of mitogen-activated protein kinase (MAPK) kinase or MAPK was detected. In contrast, 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced activation of MAPK kinase and MAPK and phosphorylation of S6 were inhibited by AG-18. This correlated with inhibition of TPA-stimulated tyrosine phosphorylation of several proteins, the most prominent ones being pp114 and pp120. We conclude that Tyr-phosphorylated insulin receptor substrate-1 is the main upstream regulator of insulin-induced S6 phosphorylation by p70^{s6k}, whereas MAPK signaling seems to be activated in these cells primarily through the adaptor molecule Shc. In contrast, TPA-induced S6 phosphorylation is mediated by the MAPK/p90^{rsk} cascade. A key element of this TPA-stimulated signaling pathway is an AG-18-sensitive protein-tyrosine kinase.

Insulin binding to its receptor leads to complex changes in Ser/Thr phosphorylation of multiple intracellular proteins (1). Enhanced Ser/Thr phosphorylation of the ribosomal protein S6 is one of the early biological responses to insulin and involves two major insulin-stimulated S6 kinases, p90^{rsk} (2) and p70^{s6k} (3). Activation of p90^{rsk} involves formation of a complex between Tyr-phosphorylated forms of two major substrates of the insulin receptor kinase, insulin receptor substrate-1 (IRS-1)¹

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¹ The abbreviations used are: IRS-1, insulin receptor substrate-1; MAP, mitogen-activated protein; MAPK, MAP kinase; MAPKK, MAPK

(4) and Shc (5), with the SH2 domain of Grb2. The insulin signal is further propagated via a sequential activation of m-Sos/Ras (6–9) and the MAPK (extracellular signal-regulated kinase) cascade, including Raf-1 (10), MAPKK (MEK) (11), and MAPK (extracellular signal-regulated kinase) (12) (see Refs. 13 and 14 for review). MAPK then phosphorylates and activates several regulatory proteins (13), including the serine/threonine kinase p90^{rsk} (15), to finally regulate several cellular processes as proliferation, differentiation, and morphology (13, 14).

p70^{s6k} is not activated by MAPK and appears to lie on a separate signaling pathway (16). One of its upstream activators is IRS-1, whose phosphorylation by insulin receptor kinase creates a binding site for the SH2 domains of the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) (17, 18). The association between p85 and IRS-1 results in activation of PI3K (17, 18) via a mechanism independent of the direct activation of PI3K by Ras (19). Activation of PI3K then stimulates p70^{s6k} by an as yet unknown mechanism (20, 21).

A large variety of extracellular signals, aside from insulin, lead to ribosomal S6 phosphorylation. One example is the tumor-promoting phorbol ester (TPA) that activates the Ca²⁺- and phospholipid-dependent protein kinase C (22). Although protein kinase C has been implicated as playing an important role in insulin-induced activation of the MAPK cascade (23), other studies suggest that insulin (24) and insulin-like growth factor I (25) activate the MAPK cascade independent of protein kinase C.

To study the relative contribution of MAPK/p90^{rsk} and PI3K/p70^{s6k} to insulin- and TPA-induced stimulation of S6 phosphorylation, we made use of tyrosinase, synthetic competitive inhibitors of several tyrosine kinases (see Ref. 26 for review) that inhibit insulin receptor kinase activity *in vitro* (27) and block insulin-induced lipogenesis and anti-lipolysis in fat cells (28). Employing Chinese hamster ovary (CHO) cells that overexpress the wild-type insulin receptor gene (CHO.T) (29), we found that AG-18 effectively inhibits insulin-induced IRS-1 phosphorylation as well as S6 kinase activity. On the other hand, insulin induction of the MAPK cascade is not affected by AG-18. The phorbol ester TPA also stimulates S6 kinase activity that is inhibited by AG-18, but unlike the insulin stimulation, this inhibition is correlated with the inhibition of the MAPK cascade. These results implicate a bifurcation in insulin signaling where IRS-1 mediates, to a large extent, S6 phosphorylation via p70^{s6k}, while Shc may mediate a signaling pathway leading to MAPK/p90^{rsk} activation. In contrast, the TPA effect

kinase; PI3K, phosphatidylinositol 3-kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

on S6 phosphorylation seems to be transmitted via the MAPK/p90^{rsk} pathway. Most important, a protein-tyrosine kinase, whose activity is inhibited by AG-18, is one of the elements linking protein kinase C to the MAPK cascade.

EXPERIMENTAL PROCEDURES

Materials—The tyrphostin AG-18 (also denoted as RG50810 (30)) was synthesized as described previously (31). Antibodies against Tyr(P), Shc, and Grb2 were from Affinity (Nottingham, United Kingdom). [γ -³²P]ATP (3000 Ci/mmol, 1 Ci = 37 Bq) was from Amersham (Buckinghamshire, United Kingdom). ATP, poly(Glu,Tyr) (4:1), and myelin basic protein were from Sigma. Wheat germ agglutinin coupled to agarose was from BioMakor, Kiryat Weizmann (Rehovot, Israel). Porcine insulin was from Elanco, Inc.

Cell Cultures—Chinese hamster ovary cells, transfected with a wild-type human insulin receptor gene (CHO.T (29)), were a generous gift of Dr. William J. Rutter (University of California, San Francisco). The cells were grown in F-12 medium as described previously (29).

Purification of Insulin Receptor—Insulin receptors were partially purified from rat liver plasma membranes. The preparation of membranes, solubilization in Triton X-100, and affinity chromatography of insulin receptors on wheat germ agglutinin coupled to agarose were carried out as described previously (32).

Phosphorylation of Exogenous Substrates—The reaction was carried out as described previously (33). Briefly, partially purified insulin receptors (50–200 μ g/ml) were incubated in the presence or absence of 10^{-7} M insulin (in 50 mM Hepes, 0.1% bovine serum albumin, 0.1% Triton X-100, pH 7.6, for 30 min at 22 °C) and the indicated concentrations of AG-18 (dissolved in 10% Me₂SO). Control tubes were incubated with Me₂SO at a final concentration of 1%. Phosphorylation in a final volume of 100 μ l was initiated with 20 μ l of a reaction mixture to yield the following final concentrations: 50 μ M [γ -³²P]ATP, 1 mM CTP, 40 mM magnesium acetate, 0.1% Triton X-100, and 0.2 mg/ml poly(Glu,Tyr) (4:1). Reactions were allowed to proceed for 10 min at 22 °C and were terminated by spotting 80 μ l onto Whatman No. 3MM filter papers that were extensively washed in 10% trichloroacetic acid, rinsed in ethanol, dried, and counted by liquid scintillation in a Betamatic counter.

Treatment of Intact Cells with Tyrphostins—Cells, grown in 60-mm plates, were deprived of serum for 16 h before each experiment. 2 h before treatment, the medium was replaced with fresh medium supplemented with 1 mg/ml bovine serum albumin (Sigma radioimmunoassay-grade). Cells were then incubated with or without tyrphostins for an additional 4 h. Tyrphostins were supplemented as 100 \times concentrated solutions dissolved in 50 mM Hepes, pH 7.6, and 10% Me₂SO. Final Me₂SO concentrations during the incubation did not exceed 0.1%. In some of the experiments, when tyrphostins were present for periods of 16 h, they were added at the time of serum deprivation and were kept throughout the incubations. Following stimulation with the appropriate ligands, cells were washed three times with ice-cold PBS and frozen in liquid nitrogen. Preliminary experiments indicated that this concentration of Me₂SO did not have any adverse effects on the biological activities under study.

Determination of Protein Tyrosine Phosphorylation in Intact Cells—Cell extracts were prepared by the addition of buffer I (50 mM Hepes, 80 mM β -glycerophosphate, 1% Triton X-100, 0.1% SDS, 50 mM NaF, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, and 5 μ g/ml leupeptin, pH 7.6). The extracts were centrifuged for 15 min at 4 °C at 12,000 \times g. Supernatants were mixed with 5 \times concentrated sample buffer (34), resolved by 10% SDS-PAGE under reducing conditions, and transferred to nitrocellulose.

Western Blotting—Samples were boiled in Laemmli sample buffer (34) containing 20 mM dithiothreitol, resolved by SDS-PAGE, and transferred to nitrocellulose. Electrophoretic transfer from the gels to the nitrocellulose papers and incubation with anti-Tyr(P) antibodies were carried out as described previously (33). Detection of bound antibodies was carried out with an ECL kit (Amersham) according to the manufacturer's instructions.

S6 Kinase Assay—The 40 S ribosomal subunit of *Artemia salina* was prepared as described previously (35). CHO.T cells, grown in 60-mm plates, were incubated for 16 h in serum-free medium in the absence or presence of AG-18. Insulin (0.1 μ M) or TPA (0.4 μ g/ml) was added to the cells, and incubation was continued for an additional 10–60 min as indicated. Following treatment, cells were lysed in 1 ml of buffer II (50 mM Hepes, pH 7.6, 30 mM β -glycerophosphate, 15 mM MgCl₂, 10 mM EGTA, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 25 μ g/ml aprotinin) by freezing and thawing (three times). The

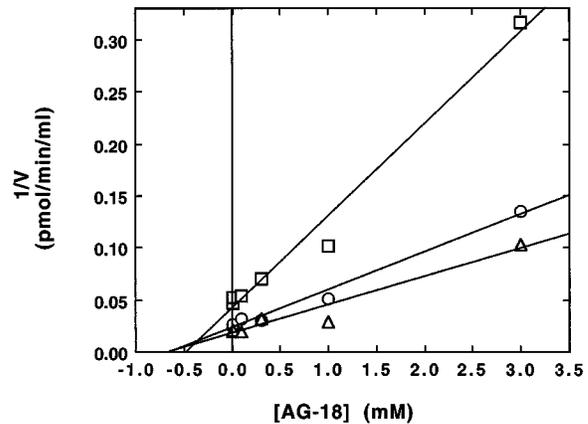


FIG. 1. Inhibition of insulin receptor kinase-catalyzed phosphorylation of poly(Glu,Tyr) (4:1) by AG-18. Phosphorylation was carried out as described under "Experimental Procedures" with 0.2 (\square), 0.5 (\circ), and 1.0 (\triangle) mg/ml poly(Glu,Tyr) (4:1) and in the presence of the indicated concentrations of AG-18. The results are presented as Dixon plots. Each point is an average of duplicate measurements that did not vary by >10%.

extracts were centrifuged at 12,000 \times g for 15 min at 4 °C, and the supernatants were assayed for S6 kinase activity using the 40 S ribosomal protein as substrate (35).

Determination of MAPK and MAPKK Activities—Determination of these activities was carried out as described previously (36). Briefly, cells were harvested in buffer H (50 mM β -glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM sodium vanadate, 1 mM benzamide, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 2 μ g/ml pepstatin A) and disrupted by 2 \times 7-s sonication (50 watts) on ice, followed by centrifugation at 20,000 \times g for 15 min at 4 °C. The supernatant contained the cytosolic extracts to be examined. All subsequent steps were performed at 4 °C. Cytosolic extracts so obtained (0.5 ml) were fractionated on 0.35-ml DEAE-cellulose minicolumns. The flow-through and wash in 0.02 M NaCl in buffer A (50 mM β -glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mM sodium vanadate) were collected and measured for MAPKK activity toward extracellular signal-regulated kinase-2 in a double coupled assay (36). Elution with 0.75 ml of buffer A (including 0.22 M NaCl) contained >85% of MAPK activity measured against myelin basic protein as described (36).

Intracellular ATP Content—The intracellular ATP content was determined as described (37). Briefly, CHO.T cells, grown in 10-cm plates, were incubated for 2–16 h in serum-free medium in the absence or presence of AG-18 (0–200 mM). Following treatment, cells were lysed in 1 ml of buffer, and the ATP content of the 12,000 \times g supernatant was determined by the luciferin/luciferase assay (37). The chemiluminescence was monitored using a Lumac 3M Luminometer (Model M2010A). Results are the means of duplicate measurements that did not vary by >5%. Each experiment was carried out at least three times with essentially similar results.

RESULTS

Effects of AG-18 on Insulin Receptor Kinase Activity in Vitro and Protein Tyrosine Phosphorylation in Intact Cells—It has been previously shown that several tyrphostins inhibit the kinase activity of the insulin receptor *in vitro*. Among various tyrphostins studied in the present as well as previous work (27), AG-18 was found to be the most potent inhibitor. Although AG-18 (0–150 μ M) failed to inhibit the autophosphorylation of the insulin receptor kinase under our *in vitro* assay conditions (data not shown), it inhibited insulin receptor kinase activity toward exogenous substrates with a K_i of 0.5 mM (Fig. 1) and was therefore employed to study the effects of tyrphostins on insulin- and TPA-induced tyrosine phosphorylations and biological responses in CHO.T cells.

As shown in Fig. 2 (A and B) and consistent with previous studies (38, 39), incubation of CHO.T cells with insulin induced tyrosine phosphorylation of two major proteins: the 95-kDa β -subunit of the insulin receptor (insulin receptor kinase) and

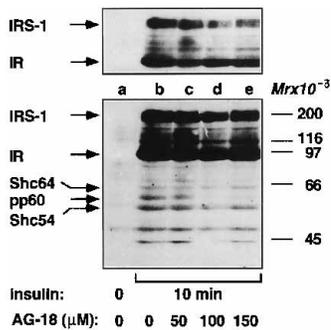


FIG. 2. Inhibition of protein tyrosine phosphorylation in CHO.T cells by AG-18. Confluent CHO.T cells were incubated for 16 h in serum-free F-12 medium with the indicated concentrations of AG-18. At the end of incubation, buffer or 0.1 μM insulin was added for a 10-min incubation period. Supernatants, prepared from the cell extracts, were resolved by 10% SDS-PAGE and immunoblotted with anti-Tyr(P) antibodies. The upper part of B, where the insulin receptor (IR) and IRS-1 are overexposed, is reproduced at a lower exposure in A.

one of its major cellular targets, IRS-1 (4, 18, 38, 40, 41). Additional proteins that underwent enhanced Tyr phosphorylation in response to insulin were pp60/pp62 (42–44) and two (out of the three) isoforms of Shc (Shc64 and Shc54), whose identity was verified by immunoprecipitation from cell extracts with Shc antibodies (data not shown).

Incubation of the cells for 16 h with increasing concentrations of AG-18 resulted in a dose-dependent inhibition of insulin-stimulated phosphorylation of several proteins (Fig. 2, A and B). This inhibition could not be attributed to an inhibitory effect of AG-18 on insulin binding since incubation of CHO.T cells with this drug had no effect on the number of insulin receptors expressed on the cell surface nor did it affect the affinity of insulin for its receptors in these cells (data not shown). AG-18 inhibited insulin-induced Tyr phosphorylation of IRS-1, with a half-maximal effect at $\sim 50 \mu\text{M}$. Inhibition of pp60/pp62 phosphorylation was also readily detected (Fig. 2, A and B). In contrast, autophosphorylation of pp95, the β -subunit of the insulin receptor, as well as Tyr phosphorylation of Shc64 were largely unaffected, whereas phosphorylation of Shc54 was only partially inhibited. Failure of AG-18 to inhibit Shc phosphorylation was also reflected by the inability of the drug to inhibit insulin-induced complex formation of Shc and Grb2. This was demonstrated when insulin-treated cells were preincubated for 16 h either in the presence or absence of 150 μM AG-18. Under these conditions, similar amounts of Grb2 (2.3 versus 1.9% of the total) were found in Shc immunoprecipitates.

Interestingly, AG-18 was a more potent inhibitor when applied to intact cells (Fig. 2) compared with its inhibitory effects on substrate phosphorylation of the insulin receptor kinase in a cell-free system (Fig. 1). These differences could be accounted for by AG-18 accumulation within the cell, making its effective intracellular concentration higher than that applied extracellularly. Alternatively, the native conformation of the insulin receptor kinase maintained *in vivo* could be more susceptible to the inhibitory effects of the drug.

Effect of AG-18 on Intracellular ATP Content—Compounds with structural similarity to AG-18, such as SF 6847, could act as inhibitors of oxidative phosphorylation (45). To rule out the possibility that the inhibitory effects of AG-18 on Tyr phosphorylation are simply due to a marked reduction in intracellular energy charge, its effects on intracellular ATP content were studied. Incubation of CHO.T cells for 16 h in the presence of increasing concentrations of AG-18 (0–200 μM) did not reduce the intracellular ATP content. The latter corresponded to 67.5 and 56.8 nmol of ATP/mg of protein in CHO.T cells incubated in the absence or presence of 200 μM AG-18, respectively.

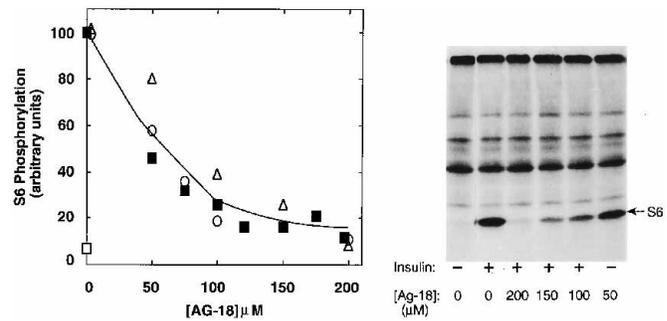


FIG. 3. Effect of AG-18 on insulin-induced phosphorylation of ribosomal protein S6. Left, confluent CHO.T cells were incubated for 16 h in serum-free medium with the indicated concentrations of AG-18. At the end of incubation, buffer (\square) or 0.1 μM insulin was added for 10 (\blacksquare), 20 (\circ), or 60 (\triangle) min. Cells were then washed three times with ice-cold PBS and frozen in liquid nitrogen. Cell extracts were prepared, and S6 kinase activity was determined. The intensity of the band corresponding to S6 was quantitated by densitometry and is presented in arbitrary units. Right, an autoradiogram of the effects of AG-18 on S6 phosphorylation following a 60-min incubation with insulin is presented.

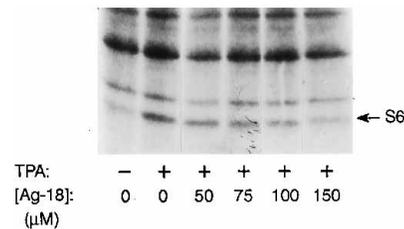


FIG. 4. Effect of AG-18 on TPA-induced phosphorylation of S6. Confluent CHO.T cells were incubated for 16 h in serum-free medium with the indicated concentrations of AG-18. At the end of incubation, buffer or 0.4 $\mu\text{g/ml}$ TPA was added. Following a 30-min incubation, cells were washed three times with ice-cold PBS and frozen in liquid nitrogen. S6 phosphorylation was determined as described under "Experimental Procedures."

Inhibition of Insulin- and TPA-induced S6 Phosphorylation by AG-18—Insulin induces S6 phosphorylation in a time-dependent manner. A maximal effect is attained by 10 min and is persistent for at least 1 h (data not shown). As shown in Fig. 3 (left), a 16-h incubation of CHO.T cells with increasing concentrations of AG-18 yielded a dose-dependent inhibition of insulin-stimulated phosphorylation of S6. A similar extent of inhibition was obtained whether the cells were stimulated with insulin for 10, 20, or 60 min. The effects of AG-18 were rather specific (Fig. 3, right) since only insulin-mediated phosphorylation was inhibited, whereas insulin-independent phosphorylation of several other proteins remained unaffected. Half-maximal inhibition of S6 phosphorylation required incubation with 50 μM AG-18, a concentration that was similar to that required for half-maximal inhibition of IRS-1 phosphorylation, while nearly maximal inhibition was obtained at 200 μM . S6 phosphorylation could also be induced upon stimulation of the cells with TPA (Fig. 4). Here again, the stimulatory effect of TPA was inhibited upon preincubation of the cells with increasing concentrations of AG-18, with a half-maximal effect being obtained at 75 μM .

Effects of AG-18 on Insulin- and TPA-induced Activation of the MAPK Cascade—Phosphorylation of the ribosomal protein S6 is mediated by at least two different protein kinases known as p90^{rsk} and p70^{s6k} (2). Since p90^{rsk} is activated by MAPKs (10, 15, 16, 46, 47), we studied the effects of AG-18 on insulin- and TPA-stimulated MAPKs. Consistent with previous studies (48), insulin added to CHO.T cells markedly enhanced MAPK activity; however, we failed to detect significant inhibition of

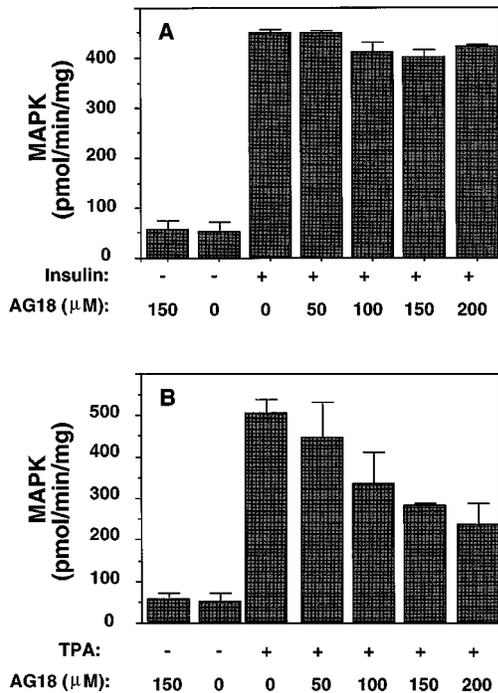


FIG. 5. Effect of AG-18 on insulin- and TPA-induced activation of MAPK. Confluent CHO.T cells were incubated for 16 h in serum-free medium with the indicated concentrations of AG-18. At the end of incubation, 0.1 μ M insulin (A) or 0.4 μ g/ml TPA (B) was added for a 10- or 30-min incubation period. Cells were then washed three times with ice-cold PBS; cell extracts were prepared; and MAPK activity was assayed as described under "Experimental Procedures."

this activity when the cells were preincubated with AG-18 (Fig. 5A). In contrast, AG-18 at 100 μ M inhibited by 50% the maximal stimulatory effect of TPA on MAPK activity (Fig. 5B). This concentration is similar to that required to inhibit TPA-stimulated phosphorylation of S6 by 50%. Interestingly, even in the presence of 200 μ M AG-18, no more than 50% of the TPA-stimulated MAPK activity was inhibited. This suggests the presence of both AG-18-sensitive and AG-18-insensitive pathways leading to MAPK activation upon TPA stimulation. Similar results were obtained when we studied the activity of MAPKK, the dual specificity Tyr/Thr kinase that phosphorylates and activates MAPK (11, 13). While TPA-stimulated MAPKK was partially inhibited by AG-18 (Fig. 6B), no such inhibitory effect was observed in insulin-stimulated cells (Fig. 6A).

AG-18 Inhibits TPA-stimulated Protein Tyrosine Phosphorylation—Since AG-18 is thought to be a selective inhibitor of protein-tyrosine kinases (26), the above results suggest the involvement of a TPA-activated and an AG-18-inhibited protein-tyrosine kinase in mediating the activation of the MAPK cascade. To directly address this possibility, the effects of TPA and AG-18 on protein tyrosine phosphorylation were evaluated. Incubation of CHO.T cells with TPA resulted in a time-dependent enhancement of tyrosine phosphorylation of several proteins, the most prominent ones having molecular masses of 114 and 120 kDa (Fig. 7, upper). Tyrosine phosphorylation of these proteins was inhibited in a dose-dependent manner by AG-18 *in vivo*. Furthermore, there was a good correlation between the concentrations of AG-18 required to inhibit TPA-induced tyrosine phosphorylation (of pp114 and pp120), activation of the MAPK cascade, and phosphorylation of ribosomal S6 protein (Fig. 7, lower). These results are compatible with a model in which protein-tyrosine kinases medi-

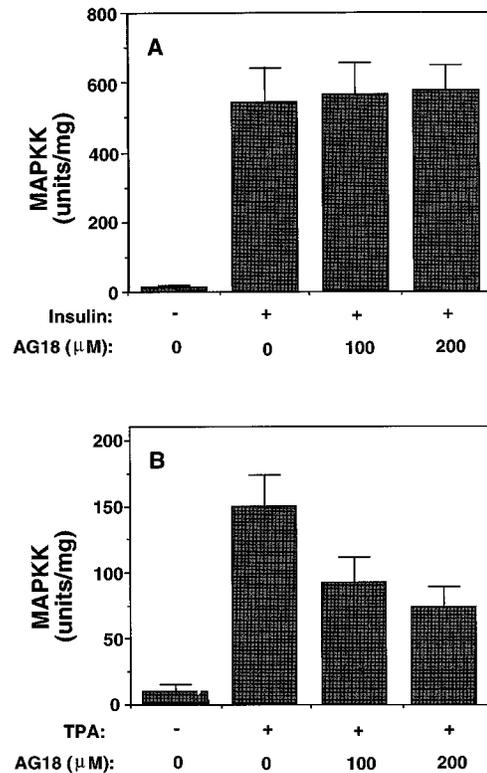


FIG. 6. Effect of AG-18 on insulin- and TPA-induced activation of MAPKK. Confluent CHO.T cells were incubated for 16 h in serum-free medium with the indicated concentrations of AG-18. At the end of incubation, 0.1 μ M insulin (A) or 0.4 μ g/ml TPA (B) was added for a 10- or 30-min incubation period. Cells were then washed three times with ice-cold PBS; cell extracts were prepared; and MAPKK activity was assayed as described under "Experimental Procedures."

ate at least part of the effects of TPA on MAPK activation and S6 phosphorylation.

DISCUSSION

A protein-tyrosine kinase inhibitor from the tyrphostin family (AG-18) was used to distinguish between the pathways leading to the activation of p70^{S6k} and the activation of the MAPK/p90^{RSK} cascade. AG-18 effectively inhibits insulin-induced activation of S6 kinase while having no inhibitory effect on insulin-induced activation of the MAPK cascade. These results indicate that activation of MAPK *per se* is not sufficient for stimulation of S6 phosphorylation and suggest that insulin-induced activation of S6 kinase(s) may occur through an alternative pathway. In this respect, our results complement studies demonstrating that MAPK activation by the insulin receptor is not required for insulin-induced metabolic processes such as glucose transport or glycogen synthase in 3T3-L1 adipocytes (49).

Inhibition of S6 phosphorylation by AG-18 largely parallels the inhibitory effects of AG-18 on insulin-induced phosphorylation of IRS-1 and is compatible with the notion that IRS-1 phosphorylation mediates many insulin responses (41, 50, 51), including the stimulation of S6 phosphorylation. The latter presumably involves activation of PI3K (17, 18) and subsequent activation of p70^{S6k} (20, 21, 52), which occurs independent of activation of p21^{ras} and the MAPK cascade (53). Conversely, we have shown that inhibition of IRS-1 and S6 phosphorylation occurs without inhibition of the MAPK cascade. Although we cannot rule out the possibility that AG-18 fails to inhibit phosphorylation of IRS-1 at Tyr^{S95}, which is part of the Grb2-binding site (54), our findings support the view that

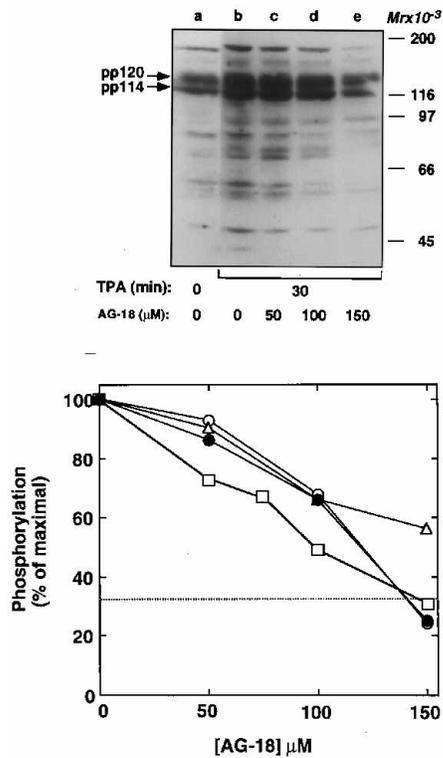
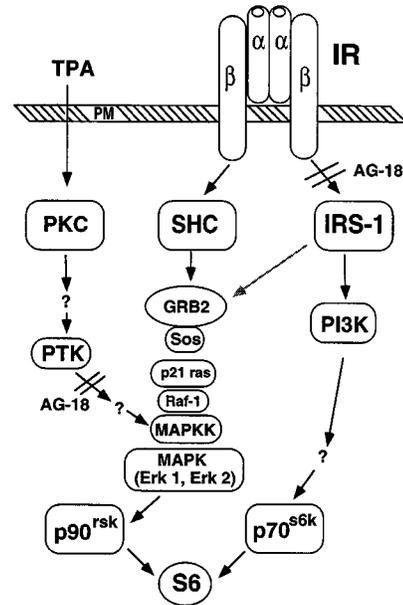


FIG. 7. Effect of AG-18 on TPA-induced protein tyrosine phosphorylation. Upper, confluent CHO.T cells were incubated for 16 h in serum-free medium with the indicated concentrations of AG-18. At the end of incubation, 0.4 μg/ml TPA was added for a 30-min incubation period. Cells were then washed three times with ice-cold PBS, and cell extracts were prepared, resolved by 10% SDS-PAGE, and immunoblotted with anti-Tyr(P) antibodies. Lower, the intensity of the bands corresponding to pp120 (○) and pp114 (●) was quantitated by densitometry, and the percent inhibition induced by AG-18 was calculated. The effects of AG-18 on TPA-induced S6 phosphorylation (■) and MAPK activity (□) are also presented for comparison.

there are alternative pathways for insulin-induced activation of the MAPK cascade, independent of IRS-1 (55, 56). This conclusion is supported by the observation that association of Grb2/Sos with IRS-1 plays little if any role in MAPK activation in L6 myoblasts (57). A likely candidate to stimulate the MAPK cascade is Shc, which serves as a downstream effector of the insulin receptor (5) and acts to activate the Ras/MAPK pathway (8, 55, 58–60). Indeed, AG-18 failed to inhibit insulin-induced Tyr phosphorylation of Shc64, and phosphorylation of Shc54 was only partially inhibited. Similarly, AG-18 failed to inhibit insulin-induced complex formation between Shc and Grb2. Hence, although our results clearly support the involvement of Shc in MAPK stimulation, different Shc isoforms might play different roles in insulin signal transduction, and further studies are required to address this possibility.

Activation of p90^{rsk} as a result of Shc phosphorylation, together with activation of the MAPK cascade, could account for the residual S6 phosphorylation observed in the presence of 150 μM AG-18 in insulin-treated cells. The fact that this residual S6 phosphorylation is rather low (~20%) suggests, however, that the predominant mode of insulin-activated S6 phosphorylation (at least in CHO cells) occurs through the IRS-1/PI3K/p70^{s6k} signaling pathway. Taken together, our findings are consistent with a model (Scheme 1) in which IRS-1 mediates insulin-induced activation of p70^{s6k}, whereas the signals leading to the activation of the MAPK/p90^{rsk} cascade are transmitted via the adaptor molecule Shc.

Tyrphostins were previously shown to inhibit insulin-stimulated lipogenesis in fat cells, while they failed to inhibit the



SCHEME 1. Tentative model illustrating the signaling pathways induced by insulin and TPA that stimulate S6 phosphorylation. Many proteins known to take part in other aspects of insulin and TPA-mediated responses were eliminated for simplicity. The expected sites of action of AG-18 are indicated. PM, plasma membrane; IR, insulin receptor; PKC, protein kinase C; PTK, protein-tyrosine kinase, Erk, extracellular signal-regulated kinase.

anti-lipolytic effect of the hormone (28). These differences could be accounted for by the different potency of tyrphostins to inhibit phosphorylation of insulin receptor kinase substrates that could mediate these processes (IRS-1 and Shc). Accordingly, we suggest that IRS-1 is more prone to inhibition by these competitive inhibitors because it is less abundant and/or has a lower affinity toward insulin receptor kinase when compared with other insulin receptor substrates (e.g. Shc) that mediate activation of the MAPK cascade. This assumption is supported by recent findings (56), where cells expressing insulin receptor mutants (of Tyr autophosphorylation sites within the kinase region) maintained insulin-induced phosphorylation of Shc, whereas phosphorylation of IRS-1 was largely reduced. Alternatively, some of the insulin-induced Tyr-phosphorylated proteins (e.g. Shc64) could serve as substrates for intermediary protein-tyrosine kinases rather than as substrates for the insulin receptor kinase itself. These intermediary protein-tyrosine kinases could undergo activation upon insulin receptor autophosphorylation, which is not inhibited by AG-18 (see above). Activation could involve, for example, binding of SH2 domains of these putative intermediary protein-tyrosine kinases to unique Tyr(P) residues within the cytoplasmic portion of the insulin receptor.

A different picture emerges when the effects of TPA on S6 phosphorylation are studied. A good concordance exists between the inhibitory effects of AG-18 on TPA-induced MAPKK and MAPK activity and S6 phosphorylation, which suggests that protein kinase C induces S6 phosphorylation preferentially through the MAPK signaling pathway. Moreover, the difference in the effects of AG-18 on insulin- versus TPA-activated MAPK suggests that, in these cells, insulin-induced activation of the MAPK cascade occurs via a protein kinase C-independent pathway. Hence, p70^{s6k} appears to be mainly responsible for insulin-induced S6 phosphorylation (20), while p90^{rsk} could mediate the effects of TPA (61).

Since AG-18 is a very poor inhibitor of Ser/Thr protein kinases, including protein kinase C (62), the inhibitory effects of AG-18 suggest that one or more AG-18-sensitive protein-

tyrosine kinases mediate the effects of protein kinase C on the MAPK pathway and S6 phosphorylation. This conclusion is supported by the facts that (i) TPA induces protein tyrosine phosphorylation in CHO.T cells, and (ii) AG-18 inhibits both TPA-stimulated tyrosine phosphorylation and TPA-stimulated MAPK activity with a similar dose-response curve. Although we cannot rule out the possibility that insulin and protein kinase C activate different isoforms of the dual specificity MAPKK, our results are most consistent with the hypothesis that the TPA-activated protein-tyrosine kinase presumably differs from the dual specificity kinase, MAPKK. This conclusion is primarily based on the fact that insulin-induced activation of MAPKK is insensitive to the presence of AG-18.

Several studies implicate protein kinase C, the direct effector of TPA, as a mediator of protein tyrosine phosphorylation events. In rat basophilic leukemia cells, Tyr phosphorylation of a 110-kDa protein occurs secondary to calcium influx and protein kinase C activation (63). Activation of protein kinase C and/or the induction of calcium influx was implicated in immunoglobulin E receptor-induced Tyr phosphorylation of focal adhesion-associated tyrosine kinase (pp125^{FAK}) in fibronectin-adherent rat basophilic leukemia cells (64, 65). Similarly, protein kinase C was shown to mediate carbachol-stimulated tyrosine phosphorylation in human SH-SY5Y neuroblastoma cells (66). Our results suggest that a protein kinase C-stimulated protein-tyrosine kinase should be present upstream of MAPKK in the protein kinase C signaling pathway, leading to the activation of p90^{sk}.

The nature of the TPA-activated protein-tyrosine kinase is presently unknown, but among its potential substrates, we find pp114 and pp120, whose inhibited phosphorylation correlates with inhibition of MAPK activity. Hence, we can formulate a tentative signaling cascade (Scheme 1) in which a TPA-activated protein-tyrosine kinase stimulates the common Grb2/Sos and the Ras signaling pathway (8, 55, 58–60) and in such a way leads to activation of MAPK and S6 phosphorylation (13). Further studies are required, however, to figure out the role of pp114/pp120 and to determine whether the TPA-activated protein-tyrosine kinase indeed utilizes the Grb2/Sos/Ras signaling elements to induce activation of the MAPK cascade.

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