Src Tyrosine Kinase Mediates Stimulation of Raf-1 and Mitogen-activated Protein Kinase by the Tumor Promoter Thapsigargin

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ABSTRACT

Thapsigargin is a non-phorbol ester-type tumor promoter that elevates the intracellular Ca^{2+} (Ca_{2}^{+}) levels by blocking the microsomal Ca^{2+} ATPase. At present, the consequence of this Ca_{2}^{+} increase and the nature of the tumorigenicity of thapsigargin still remain to be elucidated. Previously, we demonstrated that thapsigargin activates the mitogen-activated protein (MAP) kinase via Ca_{2}^{+} but independently of protein kinase C or Ca_{2}^{+} influx. Here, we show that thapsigargin also rapidly stimulates the Src tyrosine kinase. Transfection of a v-Src gene into a hippocampal cell line (H19-7) renders a constitutive activation of MAP kinase, whereas transfection of a kinase-deficient Src mutant blocks the activation by thapsigargin, suggesting that Src is required for the thapsigargin-induced MAP kinase activation. Cotransfection of a dominant-inhibitory Raf-1 and the v-Src genes into H19-7 cells results in an inhibition of the otherwise constitutively elevated MAP kinase activity, suggesting that Raf-1 is required for the Src-dependent activation of MAP kinase. Similarly, in the LA-90 cells, expression of a temperature-sensitive allele of v-Src constitutively activates Raf-1 and MAP kinase, whereas expression of a dominant-inhibitory Raf-1 mutant abolishes the MAP kinase activation induced by either v-Src or thapsigargin treatment. Together, these results suggest that thapsigargin stimulates MAP kinase signaling via Src and Raf-1. The activation of this Src-MAP kinase pathway suggests a biochemical mechanism for the tumorigenic nature of thapsigargin.

INTRODUCTION

Thapsigargin, originally isolated from the plant Thapsia gargarica L. (Apiaceae), is a non-phorbol ester-type tumor promoter (1). It inhibits the microsomal Ca_{2}^{+} ATPase, causing the Ca_{2}^{+} to leak out of the internal stores and elevating the Ca_{2}^{+} levels (1, 2). The depletion of internal Ca_{2}^{+} stores is then followed by a subsequent "capacitative entry" of extracellular Ca_{2}^{+} through surface channels (3, 4). The effect of thapsigargin on Ca_{2}^{+} mobilization has been studied extensively (3, 5); however, the molecular mechanism for its tumorigenicity remains to be elucidated. Several lines of evidence have suggested that phosphorylation events are essential in relaying thapsigargin action. For example, the transcriptional activation of the glucose-regulated protein GRP78 promoter by thapsigargin required both tyrosine and serine/threonine kinases (6). On the other hand, alkaline phosphatase abolished the activity of thapsigargin-activated calcium influx factors from Jurkat cells to induce rapid chloride current in the oocytes (4).

Previously, our laboratory demonstrated that thapsigargin-induced release of Ca_{2}^{+} activated MAP kinase (also known as extracellular

signal-regulated kinase, or ERK). This activation is independent of protein kinase C or Ca_{2}^{+} influx (2) but requires the presence of Raf-1 (7). The activation of MAP kinase can lead to the specific phosphorylation of EGF receptor on threonine 669 (8, 9) as well as a subsequent stimulation of a membrane-associated phosphatase (10). These events appear to underlie the negative regulation of EGF receptor by both phorbol ester (11) and thapsigargin action (10, 12, 13). Moreover, they suggest that the MAP kinase signaling pathway can play a role in mediating the cellular influence of thapsigargin.

Activation of MAP kinase is a rapid response shared by diverse groups of growth modulators and tumor promoters. Numerous reports have documented a well-conserved signaling pathway for MAP kinase activation that requires a sequential activation of Grb2, Sos, Ras, Raf, and MEKs (reviewed in Ref. 14). It is also known that alternative pathways for activation of MAP kinase exist in parallel (15, 16). Although MAP kinase appears to integrate a network of intracellular signals, it is of importance to identify and distinguish how different extracellular stimuli transfer their signals to MAP kinase.

In this report, we focus our investigation on the action of thapsigargin that is related to MAP kinase signaling. We show here that thapsigargin rapidly stimulates the Src tyrosine kinase; the Src kinase activity is required for the thapsigargin-induced activation of Raf-1 and MAP kinase. Because various cellular components, such as cytoskeletal proteins, focal adhesion kinase, and GTPase activating protein, are substrates of Src tyrosine kinase (17), it is conceivable that the stimulation of Src, as well as the subsequent activation of Raf-1 and MAP kinase, may contribute to the molecular basis of the tumorigenicity of thapsigargin.

MATERIALS AND METHODS

EGF (receptor grade) was purchased from Biomedical Technologies (Stoughton, MA). Thapsigargin and MBP were from Sigma Chemical Co. Anti-p21ras monoclonal antibody (Y13—259) and anti-c-Raf-1 antibody were from Santa Cruz Biotechnology, Inc. Anti-Src antibody (A3272) was a gift from Dr. J. Brugge (ARID Pharmaceuticals, Cambridge, MA). Anti-MAP kinase antiserum (Ab283) was developed as described previously (2). The plasmid encoding the kinase-inactive MEK was a gift from Dr. G. Johnson (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO), and plasmids encoding v-Src and kinase-inactive Src were a gift from D. Foster (Hunter College, New York, NY).

Cell Cultures. The H19-7 cells were generated from rat hippocampal neurons (18). The cells were conditionally immortalized by stable transfection with a temperature-sensitive SV40 large T antigen. H19-7 cells were grown at 33°C in DMEM/10% fetal bovine serum and kept under G418 selection throughout the experiments (18, 19). Under these conditions, the cells are undifferentiated and proliferate in response to serum.

The dominant-negative Raf-1 cell line R1 was generated by stably transfecting the LA-90 cells with the mutant Raf vector p301-1 (20). The LA-MNC control cell line was generated by transfecting with the parental vector to p301-1 (pMNC). The LA-90 cells express a temperature-sensitive derivative of Src (20). Experiments with activated Src were performed by shifting the cells to the permissive temperature (33°C) for 10 min; the control experiments were performed at the nonpermissive temperature (39°C; Refs. 7 and 20).

Transient Transfection of H19-7 Hippocampal Cells. The H19-7 cells were transfected with plasmids encoding the hemagglutinin-tagged ERK2, kinase-deficient Src Arg295 (21), v-Src, and dominant-inhibitory Raf p301 and
C4 (7, 20) using the calcium phosphate precipitation method. The Src and Raf-1 constructs were about 4-fold in excess of the epitope-tagged ERK2 construct. A plasmid encoding β-galactosidase driven by a cytomegalovirus promoter was cotransfected to normalize for transfection efficiency. After transfection, the cells were allowed to grow in fresh medium with 10% FBS for 16–24 h. The plates containing the cells from the same transfection were pooled and replated to ensure homogeneous transfection efficiency (approximately 15%). The replated cells were grown in fresh medium with 10% FBS for 20–24 h and subsequently starved for another 20–24 h. The transfected cells were harvested for experiments within 60–72 h.

**Src Kinase Assay.** Src tyrosine kinase activity was measured by phospho-
ylation of rabbit muscle enolase (22, 23). Stimulated and control cells were washed twice with PBS and then lysed in 50 mM Tris, pH 8.0; 0.5% NP-40; 120 mM NaCl; 1 mM EDTA; 20 µg/ml aprotinin; 200 mM Na3VO4; and 0.2 mM PMSF. After removal of debris, the supernatant was incubated with anti-Src antibody (Ab327) for 30 min. Protein A-Sepharose was added for another 30 min. The mixtures were washed in radioimmunoprecipitation assay buffer and once in Tris-buffered saline (20 mM Tris, pH 7.5; 150 mM NaCl). The pellet was resuspended in 10 µl of kinase buffer (20 mM HEPES, pH 7.2; 5 mM MgCl2; and 200 µM Na3VO4) containing 2–5 µg of acid- and heat-treated rabbit muscle enolase. The phosphorylation was initiated by adding 5 µCi [γ-32P]ATP to the mixtures and then incubated at 30°C for 10 min. The reaction was terminated by adding 2× sample buffer and boiling for 2–5 min. The samples were resolved on a 10% SDS-polyacrylamide gel. The level of enolase phosphorylation was measured by scintillation counting of the excised bands.

**Epitope-tagged ERK2 Immune Complex Kinase Assay.** The experiment was performed according to a modified procedure as described previously (24). The transfected cells were stimulated and lysed with 0.5 ml of ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.5; 1% (w/v) Triton X-100; 40 mM β-glycerophosphate; 100 mM NaCl; 50 mM NaF; 2 mM EDTA; 200 µM sodium orthovanadate; and 0.2 µg/ml bovine serum albumin. Insoluble materials were removed by centrifugation at 14,000 rpm for 10 min at 4°C. The cell lysates were then incubated with 25 µl of protein A precoupled with the antibody 12CA5 specific to the hemagglutinin epitope (Babco, Emeryville, CA) for 16 h at 4°C. The immune complexes were washed four times with the lysis buffer and once with the kinase buffer containing 20 mM HEPES, pH 7.4; 10 mM MgCl2; 1 mM DTT; 200 µM Na3VO4 and 10 mM p-nitrophenyl phosphate.

The immune-complex kinase activity was measured by phosphorylation of the MBP. Briefly, the immune complexes were resuspended in a final volume of 40 ml of kinase buffer with 0.25 mg/ml MBP and 50 mM ATP (5 µCi [γ-32P]ATP). The phosphorylation reaction was initiated by adding the ATP, and samples were incubated at 30°C for 20 min. The reactions were terminated by adding 20 ml of 2× Laemmli sample buffer. Samples were resolved on a 12% SDS gel. An autoradiogram was developed from the dried gel. The MBP bands were excised from the gel, and radioactivity was counted by liquid scintillation.

**Raf-1 Immune Complex Kinase Assay.** Raf-1 kinase activity was ana-
ylyzed by an immune complex kinase assay (25). Briefly, cells were washed twice with ice-cold PBS and lysed in 10 mM Tris-HCl, pH 7.4; 1% Triton X-100; 1 mM EDTA, 150 mM NaCl; 50 mM NaF; 0.1% BSA; 20 µg/ml aprotinin; 200 mM Na3VO4; and 0.2 mM PMSF (lysis buffer). The cell debris and nuclei were removed by centrifugation, and the supernatants were incubated with anti-c-Raf antibody (Santa Cruz Biotechnology, Inc.) for 1.5 h at 4°C. Protein A-Sepharose was added for another 30 min. Then, the mixtures were diluted with an equal volume of lysis buffer plus 10% sucrose and centrifuged for 20 min at 2500 rpm. The pellet was washed twice with lysis buffer containing 20 mM HEPES, pH 7.4; 10 mM MgCl2; 1 mM DTT; 200 µM Na3VO4 and 10 mM p-nitrophenyl phosphate.

The immuno-complex kinase assay was performed in a final volume of 40 ml of MBP buffer with 0.25 mg/ml MBP and 50 mM ATP (5 µCi [γ-32P]ATP). The phosphorylation reaction was initiated by adding the ATP, and samples were incubated at 30°C for 20 min. The reactions were terminated by adding 20 ml of 2× Laemmli sample buffer. Samples were resolved on a 12% SDS gel. An autoradiogram was developed from the dried gel. The MBP bands were excised from the gel, and radioactivity was counted by liquid scintillation.

**Fig. 1.** A, thapsigargin activates Src tyrosine kinase in H19—7 cells. Confluent H19—7 cells were serum-starved for 48 h in DMEM before treatment with 1 µg/ml thapsigargin or 10 ng/ml EGF for various times as indicated. The kinase activity of Src was measured as phosphorylation of rabbit muscle enolase by the Src-immune complex precipitated with a specific antibody, Ab327, as described in “Materials and Methods.” Radioactivity of phosphorylated enolase was quantitated by scintillation counting of the excised enolase bands.

B, thapsigargin activates Src tyrosine kinase in H19—7 cells. Confluent H19—7 cells were serum-starved for 48 h in DMEM before treatment with 1 µg/ml thapsigargin or 10 ng/ml EGF for various times as indicated. The kinase activity of Src was measured as phosphorylation of rabbit muscle enolase by the Src-immune complex precipitated with a specific antibody, Ab327, as described in “Materials and Methods.” Radioactivity of phosphorylated enolase was quantitated by scintillation counting of the excised enolase bands.

C, thapsigargin activates Src tyrosine kinase in H19—7 cells. Confluent H19—7 cells were serum-starved for 48 h in DMEM before treatment with 1 µg/ml thapsigargin or 10 ng/ml EGF for various times as indicated. The kinase activity of Src was measured as phosphorylation of rabbit muscle enolase by the Src-immune complex precipitated with a specific antibody, Ab327, as described in “Materials and Methods.” Radioactivity of phosphorylated enolase was quantitated by scintillation counting of the excised enolase bands.
THAPSIGARGIN ACTIVATES Src, Raf-1, AND MAP KINASE

A

![Western Blot](image1)

B

![Western Blot](image2)

C

![Western Blot](image3)

D

![Western Blot](image4)

Fig. 2. A, thapsigargin (Thaps) activates Raf-1 in H19–7 cells. Cells were grown and stimulated with thapsigargin or EGF as in Fig. 1. The kinase activity of Raf-1 was measured by Raf-1 immune complex phosphorylation assay using a recombinant polyhistidine-tagged, kinase-inactive MEK as substrate as described in “Materials and Methods.” Inset, a representative autoradiogram. B, forskolin (FSK) pretreatment inhibits thapsigargin- and EGF-induced activation of Raf-1. H19–7 cells were treated as indicated: 1 μM thapsigargin (TG) for 5 min; 10 nM EGF for 5 min; FSK + TG or FSK + EGF (10 μM) pretreated for 15 min followed by thapsigargin or EGF for 5 min. Raf-1 activity was analyzed as in A. C and D, dominant-inhibitory Raf-1 blocks thapsigargin but not EGF-induced MAP kinase activity. H19–7 cells were transfected with 4 μg of a hemagglutinin-tagged Raf-1 expression vector. After transfection, the cells were crown. Pooled, replated, and serum-starved (10 mM) pretreated for 15 min followed by thapsigargin or EGF for 5 rain. Raf-1 activity phosphorylation by the ERK2 immune complex as in Fig. 1.

RESULTS

Thapsigargin Activates Src Tyrosine Kinase in H19–7 Hippocampal Cells. To test the possibility that the tyrosine kinase pp60Src functions as a mediator for thapsigargin/Ca²⁺ signaling, we directly analyzed Src tyrosine kinase activity in response to thapsigargin in H19–7 cells. The Src tyrosine kinase activity was measured by immune complex assay using acid-treated rabbit muscle enolase as substrate (22, 23). Fig. 1A shows that thapsigargin stimulates Src approximately 2-fold within 5 min, and the kinase activity is maintained for at least 30 min without significant attenuation. The EGF also elicits a quick induction of Src activity to a extent similar to thapsigargin. This result suggests that thapsigargin may use Src for downstream signaling events.

Kinase-deficient Src Blocks Thapsigargin-induced Activation of MAP Kinase. We have shown that thapsigargin can activate MAP kinase in human foreskin (HSP) fibroblasts, A431, LA-90, and H19–7 cells (2, 7, 19). To test whether Src is required for mediating the thapsigargin-induced activation of MAP kinase, we transiently transfected the H19–7 cells with a kinase-deficient Src mutant (21)
passing Raf-1 to activate MAP kinase. These results are also in agreement with our previous observations regarding Raf-1-dependent activation of MAP kinase. Interestingly, the EGF-induced ERK2 was only slightly inhibited by the kinase-deficient Src (Fig. 3B). This result indicates that Src is necessary for thapsigargin-dependent activation of MAP kinase. Interestingly, the EGF-induced ERK2 was only slightly inhibited by the kinase-deficient Src (Fig. 1C), suggesting that Src is not crucial for EGF-activated MAP kinase signaling.

Thapsigargin Activates Raf-1 in H19-7 Hippocampal Cells. Previously, we reported that Raf-1 is involved in thapsigargin-induced MAP kinase activation in the LA-90 cell line (7). Therefore, to further characterize the thapsigargin-induced signaling pathway, we also examined the Raf-1 activity in H19-7 cells. As shown in Fig. 2A, a 2-fold activation by thapsigargin is observed in 5 min, whereas EGF induces a nearly 5-fold activation of Raf-1 activity toward phosphorylation of a recombinant MEK (25). The level of thapsigargin-induced Raf-1 activation is consistent with that of MAP kinase activation by thapsigargin in all cell types we have tested previously. In addition, the Raf-1 activity induced by either thapsigargin or EGF is abolished by pretreatment with forskolin (Fig. 2B), indicating that the Raf-1 activity is sensitive to cAMP inhibition. Pretreatment of the H19-7 hippocampal cells with dibutyryl cAMP and forskolin also completely blocks the activation of MAP kinase induced by thapsigargin (data not shown), suggesting that in the H19-7 hippocampal cells, the MAP kinase signaling pathway is antagonized by cAMP as in several other cell types (26).

Dominant-Inhibitory Raf-1 Blocks Thapsigargin-induced Activation of MAP Kinase. To further determine the requirement of Raf-1 for thapsigargin-induced MAP kinase signaling, we transiently transfected the H19-7 cells with a dominant-negative Raf-1 p301-1 mutant (7, 20) together with a hemagglutinin-tagged ERK2 plasmid. As analyzed by an epitope-tagged ERK2 immune complex assay (Fig. 2C), the dominant-inhibitory Raf-1 blocks the epitope-tagged ERK2 activity induced by thapsigargin but fails to inhibit the ERK2 activation induced by EGF. The data indicate that Raf-1 is required for thapsigargin-induced MAP kinase activation in H19-7 cells, whereas EGF may use an alternative pathway by-passing Raf-1 to activate MAP kinase. These results are also in agreement with our previous observations regarding Raf-1-dependent and -independent mechanisms for MAP kinase activation in the LA-90 cells (7).

Dominant-Inhibitory Raf-1 Blocks Src-dependent Activation of MAP Kinase. To further determine whether Raf-1 is required for mediating Src-dependent activation of MAP kinase, we performed various combinations of transient transfection in H19-7 cells with an oncogenic v-Src, a dominant-inhibitory Raf-1 (C4), and the epitope-tagged ERK2 constructs. As shown in Fig. 3, transfection with the v-Src allele induces an activation of epitope-tagged ERK2 activity (approximately 3-fold). In contrast, cotransfection of the v-Src with the dominant-inhibitory Raf-1 substantially blocks the otherwise constitutively activated ERK2 activity.

Another line of evidence indicating Src requires Raf-1 for MAP kinase activation comes from studies with the LA-90 cells. The BALB/c 3T3-derived LA-90 cells contain a temperature-sensitive Src. The cells are cultured at the nonpermissive temperature for Src activity (39°C) and can be shifted to the permissive temperature (33°C) to activate the endogenous Src kinase. Two derivatives of LA-90 cells were then established (7, 18): R1, which contains an expression vector for dominant-inhibitory Raf-1 (p301-1), and MNC, which contains the expression vector alone as a control. As shown in Fig. 4A, incubating the LA-90 cells at 33°C for 10 min to activate the endogenous Src induced a significant Raf-1 activation in the MNC control cells (Fig. 4A), indicating that Src activation can lead to Raf-1 activation.

Together with an epitope-tagged ERK2 as described in "Materials and Methods." Using epitope-tagged ERK2-immune complex kinase assays, we found that in the presence of the kinase-deficient Src, thapsigargin-induced activation of ERK2 was substantially blocked (Fig. 1B). This result indicates that Src is necessary for thapsigargin-dependent activation of MAP kinase. Interestingly, the EGF-induced ERK2 was only slightly inhibited by the kinase-deficient Src (Fig. 1C), suggesting that Src is not crucial for EGF-activated MAP kinase signaling.

**Fig. 3.** Constitutive activation of MAP kinase by v-Src is inhibited by dominant-inhibitory Raf-1. H19-7 cells were transfected with 4 μg of epitope-tagged ERK2 plasmid (control), a combination of 4 μg of epitope-tagged ERK2 and 12 μg of v-Src (vSrc), or a combination of 4 μg epitope-tagged ERK2, 12 μg of v-Src, and 12 μg of a dominant-inhibitory Raf-1 mutant C4 (vSrc+Raf-). Calf thymus DNA was used to equalize the total DNA for transfection. A plasmid encoding β-galactosidase was also cotransfected for normalization. Cells were harvested after 40 h of serum starvation. The epitope-tagged ERK2 was immunoprecipitated and analyzed for kinase activity as in Fig. 1.

**Fig. 4.** Effects of temperature-sensitive Src on Raf-1 and MAP kinase activities in LA-90 cells. LA-90 (MNC, control; R1, dominant-inhibitory Raf-1 mutant) cells were grown and maintained at 39°C (nonpermissive temperature) as described in "Materials and Methods." To activate the temperature-sensitive Src cell plates were shifted from 39°C to 33°C for 10 min. A, activation of Src leads to activation of Raf-1. MEK phosphorylation by the Raf-1 immune complex precipitated from MNC (33°C) and R1 (33°C) cells at 39°C (nonpermissive) or 33°C (permissive). The level of phosphorylation was measured by scintillation counting of the MEK band excised from the gel. The fold increase was calculated after subtracting the background cpm. B, Raf-1 is required for thapsigargin- and Src-dependent activation of MAP kinase. MNC and R1 cells were incubated at 39°C or 33°C for 10 min without treatment, or treated with 1 μg/ml thapsigargin (TG) or 10 nm EGFr at 39°C for 5 min. Cell extracts were analyzed by Western blotting. Activation of ERK1 and ERK2 is identified by the characteristic bandshifts.
calcium/calmodulin-dependent protein kinase IV has recently been shown to interact with a calcium/calmodulin-dependent protein kinase IV remains to be elucidated. A recent report also suggested that Src may directly interact with Raf-1, and the association may increase the autophosphorylation of Raf-1 (33). Because we have previously observed a Raf-1-dependent activation of MAP kinase by thapsigargin, it was of interest to determine the involvement of Raf-1 in Src-induced MAP kinase activation. The studies with the dominant-inhibitory Raf-1 mutant (Figs. 3 and 4) strongly suggest that Src can be an upstream activator for Raf-1, and Raf-1 may be required for the Src-dependent activation of MAP kinase.

In addition, the thapsigargin-induced activation of Raf-1 is sensitive to cAMP-inhibition (Fig. 2). This phenomenon is similar to other cell types that are also sensitive to cAMP-inhibition (26) but represents a different signaling mechanism from that observed in PC-12 cells in which cAMP activates MAP kinase (34). This cAMP inhibition of thapsigargin-induced Raf-1 and MAP kinase activity, together with the result that a kinase-deficient, dominant-inhibitory Raf-1 mutant blocks thapsigargin-induced MAP kinase activation, strongly indicates that Raf is essential for the thapsigargin-dependent action. In the case of EGF-induced MAP kinase activation, neither Src or Raf-1 appear to be absolutely required (Figs. 1 and 2). It is likely that different upstream MAP kinase activators also mediate EGF action, as observed previously for other growth factors (26, 35), in contrast to the more stringent requirements for the thapsigargin-induced pathway.

Thapsigargin-elicited calcium transients can exert multiple effects and may use various routes for signaling (36, 37). There are a number of different signaling intermediates, including Ca^{2+}/calmodulin dependent protein kinases, the classic protein kinase C family, phosphatases (e.g., calcineurin), proteases (e.g., calpain), ion channels, and various Ca^{2+}-binding proteins that require Ca^{2+} for their physiological function. To assess the involvement of these intermediates, we have tested various inhibitors for the Ca^{2+}-dependent kinases, phosphatases, or proteases, but we found no inhibitory effect toward thapsigargin-induced MAP kinase activation (data not shown).

Our results presented here, showing that Src mediates thapsigargin-induced activation of Raf-1 and MAP kinase, provide a possible biochemical basis for the tumorigenicity of thapsigargin. Because many other Ca^{2+}-induced cellular responses may also use a similar mechanism, continued elucidation of the mechanism by which thapsigargin/Ca^{2+} activates Src should provide more insights into oncogenic signaling pathways.

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