Intracellular Calcium Mobilization Induces Immediate Early Gene *pip92* via Src and Mitogen-activated Protein Kinase in Immortalized Hippocampal Cells

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Regulation of intracellular calcium levels plays a central role in cell survival, proliferation, and differentiation. A cell-permeable, tumor-promoting thapsigargin elevates the intracellular calcium levels by inhibiting endoplasmic reticulum Ca^{2+}-ATPase. The Src-tyrosine kinase family is involved in a broad range of cellular responses ranging from cell growth and cytoskeletal rearrangement to differentiation. The immediate early gene *pip92* is induced in neuronal cell death as well as cell growth and differentiation. To resolve the molecular mechanism of cell growth by intracellular calcium mobilization, we have examined the effect of thapsigargin and subsequent intracellular calcium influx on *pip92* expression in immortalized rat hippocampal H19-7 cells. An increase of intracellular calcium ion levels induced by thapsigargin stimulated the expression of *pip92* in H19-7 cells. Transient transfection of the cells with kinase-inactive mitogen-activated protein kinase kinase (MEK) and Src kinase or pretreatment with the chemical MEK inhibitor PD98059 significantly inhibited *pip92* expression induced by thapsigargin. When constitutively active v-Src or MEK was overexpressed, the transcriptional activity of the *pip92* gene was markedly increased. Dominant inhibitory Raf-1 blocked the transcriptional activity of *pip92* induced by thapsigargin. The transcription factor Elk1 is activated during thapsigargin-induced pip92 expression. Taken together, these results suggest that an increase of intracellular calcium ion levels by thapsigargin stimulates the *pip92* expression via Raf-MEK-extracellular signal-regulated protein kinase- as well as Src kinase-dependent signaling pathways.

Research from many laboratories has shown that Ca^{2+} influx through ion channels can regulate gene expression by multiple and diverse mechanisms. Activation of gene expression by Ca^{2+} regulates fundamental biological responses, including cell cycle, hormone secretion, and cell morphology (1). A cell-permeable, tumor-promoting thapsigargin can cause a rise in intracellular Ca^{2+} levels (2).

Src family kinases are involved in a broad range of cellular responses ranging from cell division and cytoskeletal rearrangement in fibroblasts to the differentiation of neuronal PC12 cells (3–5). v-Src is a constitutively active protein-tyrosine kinase that results in cellular transformation, and v-Src mutants deficient in kinase activity do not transform cells, suggesting that phosphorylation of specific cellular targets is important for the transformed phenotype induced by v-Src (6). Many putative substrate proteins have been identified. Ras and Raf have been reported to be required for the transformed phenotype induced by v-Src (7, 8). Although Ras is not a substrate of v-Src, and Raf is phosphorylated on tyrosine, Raf functions downstream of Ras (9).

The immediate early gene *pip92* was cloned from activated T lymphocytes treated with cycloheximide (10) and serum-stimulated BALB/c 3T3 fibroblasts (11). *pip92* is rapidly and transiently induced by stimulation with serum and growth factors in fibroblasts. In addition to cell growth, *pip92* is also induced during neuronal differentiation by differentiating factors (12). Although *pip92* encodes a short-lived, proline-rich protein with no significant sequence similarity to any known protein, little is known about the function of its encoded protein.

Previously it was shown that thapsigargin-induced release of intracellular Ca^{2+} activated mitogen-activated protein kinase $(MAPK)^1$ and extracellular signal-regulated protein kinase (ERK) (13). This activation is independent of protein kinase C or Ca^{2+} influx but requires the presence of Raf-1 (14). Src-tyrosine kinase mediates the stimulation of Raf-1 and ERK by thapsigargin (15). Recently, we have observed that *pip92* is expressed in the mouse brain after a single intraperitoneal injection of excitatory amino acid NMDA (16). Many actions of NMDA are coupled to the influx of extracellular Ca^{2+} mediated directly or indirectly by its receptor present in neurons, and transient changes in intracellular Ca^{2+} levels are known to trigger a number of cellular responses, including change of gene expression (17). Ca^{2+} is known to activate ERK signaling in neuronal PC12 and H19-7 cells (15, 18). On the basis of those findings, it was suggested that the calcium ion and Src kinase could regulate the *pip92* expression. To resolve the mechanism

[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; MEK, MAPK kinase; SRE, serum response element; SRF, serum response factor; GST, glutathione *S*-transferase; HA, hemagglutinin; FGF, fibroblast growth factor.

of thapsigargin-induced cell growth, we analyzed whether *pip92* expression is induced by thapsigargin subsequently followed by intracellular free Ca^{2+} mobilization and whether the activation of ERK and Src kinase is involved. The results indicated that an increase of intracellular calcium ion levels by thapsigargin stimulates *pip92* expression via ERK- as well as Src kinase-dependent signaling pathways, suggesting that *pip92* is likely to play a role in thapsigargin-induced neuronal cell growth.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM), and G418 were purchased from Life Technologies, Inc. Thapsigargin, ionomycin, and PD98059 were purchased from Calbiochem. All other used chemicals were commercial products of analytical grade from Sigma. The *pip92* promoter-chloramphenicol acetyltransferase (CAT) reporter fusion construct (−1281*pip92*/CAT) was provided by L. F. Lau. Plasmids encoding v-Src and kinase-inactive mutant Src were kindly provided by D. Foster, and plasmids for kinase-inactive MAPK kinase (MEK-2A) and constitutively active MEK (MEK-2E) were obtained from G. Johnson. The plasmid encoding a dominant inhibitory Raf-1 kinase (Raf-KR) was given by C. Marshall. Plasmid GST-ElkC, expressing glutathione *S*-transferase fused to the C-terminal peptide (amino acids 307–428) of wild type Elk1 was provided by R. Treismann.

Cell Culture—The rat neuronal hippocampal cell line H19-7 was generated by transduction with the retroviral vectors containing the temperature-sensitive simian virus 40 large T antigen that is functionally active at 33 °C and inactive at 39 °C (19). The cells were cultured at 33 °C in DMEM containing 10% fetal bovine serum and 200 μg/ml G418 to maintain selection pressure on the transduced immortalization vector. When specified, cells were pretreated with 30 μM synthetic MEK inhibitor PD98059 for 30 min before drug stimulation.

Determination of DNA Synthesis—DNA synthesis was measured by use of [³H]thymidine incorporation. The H19-7 cells were suspended by trypsinization, counted, and replated into 24-well plates at a density of 30,000 cells/well in media containing 10% fetal bovine serum. The cells were starved in 0.5% fetal bovine serum for 48 h to decrease the cell proliferation and to induce quiescence. Then the media were changed to DMEM plus 10% fetal bovine serum with or without thapsigargin. The cells were incubated with thymidine (1 μCi/well) during the last 4 h. At the end of the incubation, the medium was aspirated, and the cells were washed three times with cold DMEM and then solubilized with Protosol (NEN Life Science Products). Radioactivity was measured with a Beckman scintillation counter.

Measurement of Intracellular Calcium Levels—For Ca^{2+} measurement, Fura-2 loading and fluorescence analyses were carried out as described previously (20). The cells were maintained in DMEM containing 10% fetal bovine serum and plated on a sterile 22 × 22-mm coverslip at a density of 2.5 × 10^5 cells/cm^2. At the confluence of 70–80%, the cells were washed twice with a HEPES-buffered solution (solution A) and loaded with Fura-2 by a 30-min incubation at room temperature in solution A containing 5 μM Fura-2/AM (Molecular Probes, Eugene, OR). Solution A contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4. After the loading, the coverslips were washed once with solution A and assembled to form the bottom of perfusion chamber. The chamber was continuously perfused with either a Ca^{2+}-containing solution (solution A) or a Ca^{2+}-free solution (solution B). Ca^{2+}-free solution B was prepared by replacing CaCl$_2$ with 3 mM EGTA. The osmolarity of all solutions was adjusted to 310 mOsm with the major salts before use. Fluorescence was measured and calibrated using a PTI system (PTI Delta Ram, New Brunswick, NJ) as described previously (20). Fura-2 fluorescence was excited at 355 and 380 nm and calibrated by exposing the cells to solutions containing high and low concentrations of Ca^{2+} and 10 μM ionomycin.

DNA Transfection and CAT Assay—Transient transfection was performed by using LipofectAMINE Plus reagents (Life Technologies) as described by the manufacturer's protocol. Plasmid pCMV-GAL, which contains the *Escherichia coli* β-galactosidase gene driven by the cytomegalovirus, promoter was used as an internal control to determine transfection efficiency. The CAT assay was done with an enzyme-linked immunosorbent assay CAT assay kit (5 Prime → 3 Prime, Inc., Boulder, CO).

RNA Preparations and Northern Blot Analysis—Total cellular RNAs from H19-7 cells were isolated by the single-step extraction procedure using guanidium isothiocyanate as described elsewhere (21). Northern
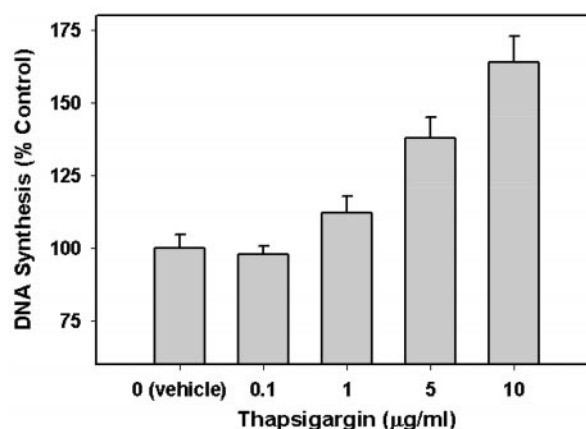

Fig. 1. **Thapsigargin stimulates DNA synthesis in H19-7 cells.** Cells were incubated in DMEM including 10% fetal bovine serum with the indicated concentration of thapsigargin for 24 h. Cell cultures were labeled with [³H]thymidine during the last 4 h of incubation and analyzed for DNA synthesis, as described under "Experimental Procedures." Data are shown as percentage of control (vehicle in media), and all *histograms* represent the mean of three replicates.

blot analysis to measure *pip92* mRNA levels was done as described previously (12).

Src Kinase Assay—Src-tyrosine kinase activity was measured by phosphorylation of rabbit muscle enolase as described previously (15). The samples were resolved on a 12.5% SDS-polyacrylamide gel, and the phosphorylation level of enolase was measured by scintillation counting of excised protein bands.

Immunoprecipitation and Assay of Hemagglutinin (HA)-tagged ERK2—ERK2 kinase activity was measured by immunoprecipitation of the epitope-tagged Erk2, followed by an *in vitro* phosphorylation assay as described previously (12). Transfected cells were stimulated and lysed with solution C, consisting of 20 mM Tris, pH 7.9, 137 mM NaCl, 5 mM Na$_2$EDTA, 10% glycerol, 1% Triton X-100, 0.2 mM *p*-methylsulfonylfluoride, 1 μg/ml aprotinin, 20 μM leupeptin, 1 mM sodium *o*-vanadate, pH 10.0, 1 mM EGTA, 10 mM NaF, 1 mM tetrasodium pyrophosphate, 1 mM β-glycerophosphate, pH 7.4, and 0.1 g/ml *p*-nitrophenylphosphate. The cell lysates were then incubated with protein A-Sepharose coupled with the anti-HA antibody (Santa Cruz Biotechnologies) for 24 h at 4 °C. The immune complexes were washed with lysis buffer and with kinase reaction buffer containing 20 mM HEPES, pH 7.4, 10 mM MgCl$_2$, 1 mM dithiothreitol, 200 mM *o*-vanadate, and 10 mM *p*-nitrophenylphosphate. The assay of immunoprecipitated HA-tagged ERK2 was done using myelin basic protein as a substrate as described previously (12).

Electrophoretic Mobility Shift Analysis—Nuclear extracts were prepared from H19-7 cells as described elsewhere (22). An electrophoretic mobility shift analysis assay was performed as described previously (23).

In Vitro Assay of Elk1 Phosphorylation—*In vitro* Elk1 phosphorylation by using gluthathione-Sepharose 4B beads (Amersham Pharmacia Biotech) was examined as described previously (23).

RESULTS

Thapsigargin Stimulates DNA Synthesis in Rat Hippocampal Neuronal Cells—Immortalized H19-7 cells were generated from rat hippocampal neurons. Initially, we investigated how the stimulation of the H19-7 cells with thapsigargin affects cell growth. Thapsigargin in the range of 0.1–10 μg/ml induced a concentration-dependent increase in DNA synthesis measured by [³H]thymidine incorporation (Fig. 1). Stimulation of the cells with 10 μg/ml thapsigargin for 24 h increased the DNA incorporation ~1.6-fold compared with that of control cells in the absence of thapsigargin. These data indicated that thapsigargin stimulates the proliferation of neuronal H19-7 cells.

Thapsigargin and Ionomycin Cause Mobilization of Intracellular Calcium Levels in H19-7 Cells—To examine whether thapsigargin causes a change in intracellular calcium levels, cells were preloaded with Fura-2 and then monitored for changes in intracellular calcium after exposure to thapsigargin in medium containing 1 mM calcium. The time courses of cal-
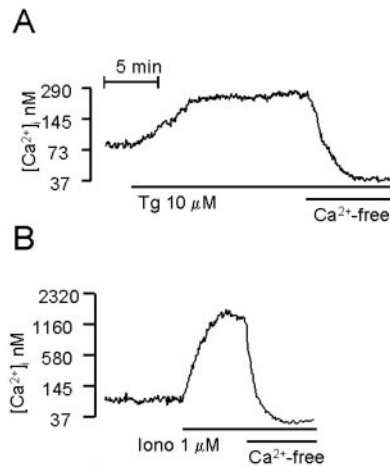
FIG. 2. **Thapsigargin and ionomycin triggered calcium mobilization in H19-7 cells.** The cells were preloaded with Fura-2 and perfused with calcium-containing HEPES-buffered solution A to establish basal Ca^{2+} levels at 33 °C. Subsequently, the cells were treated with 10 μg/ml thapsigargin (*Tg*; *A*) or 1 μM ionomycin (*Iono*; *B*), followed by the addition of Ca^{2+}-free EGTA, as indicated. Each *trace* is from an individual cell in a field of cells and represents a typical response of three independent experiments.

cium mobilization by thapsigargin are shown in Fig. 2. By ~5 min after addition of 10 μg/ml thapsigargin, cytosolic free calcium levels had increased from ~70 to 250 nM, followed by a long plateau. Although there was some variation in the shape of the calcium profile, depending on the particular cell being monitored, in general thapsigargin caused a gradual increase followed by a long plateau phase. Ionomycin also increases the calcium levels by channeling through membranes from a nonspecific source (24). In the same way, addition of 1 μM ionomycin increased the intracellular calcium levels up to ~1500 nM, reaching a peak by 1–2 min. Addition of EGTA to the cells during the plateau phase dropped the calcium down to basal levels.

Induction of pip92 by Intracellular Calcium Mobilization in H19-7 Cells—Next we examined whether intracellular calcium mobilization induced by thapsigargin causes the stimulation of *pip92* expression in H19-7 cells. We first measured the time course of *pip92* mRNA expression by thapsigargin. Northern blot analysis showed that *pip92* is expressed rapidly and transiently within 30–60 min after 10 μg/ml thapsigargin stimulation (Fig. 3*A*) but not with vehicle. Transcriptional activation of the *pip92* gene was examined by using a CAT reporter plasmid linked to a −1281-base pair *pip92* promoter fragment (−1281*pip92*/CAT) transiently expressed in H19-7 cells. The −1281-base pair *pip92* promoter fragment was shown to mediate serum induction in a manner that closely mimics the endogenous gene (25). Treatment of the cells with 10 μg/ml thapsigargin caused rapid stimulation of *pip92* transcriptional activity, which reached a plateau by 1 h as monitored by CAT assay (Fig. 3*B*). Mock-transfected control cells did not activate the *pip92* promoter (data not shown). A similar level of *pip92* mRNA and its transcription were induced with 1 μM calcium ionophore ionomycin, and an additive increase of *pip92* transcription was not observed by the cotreatment with thapsigargin and ionomycin (data not shown).
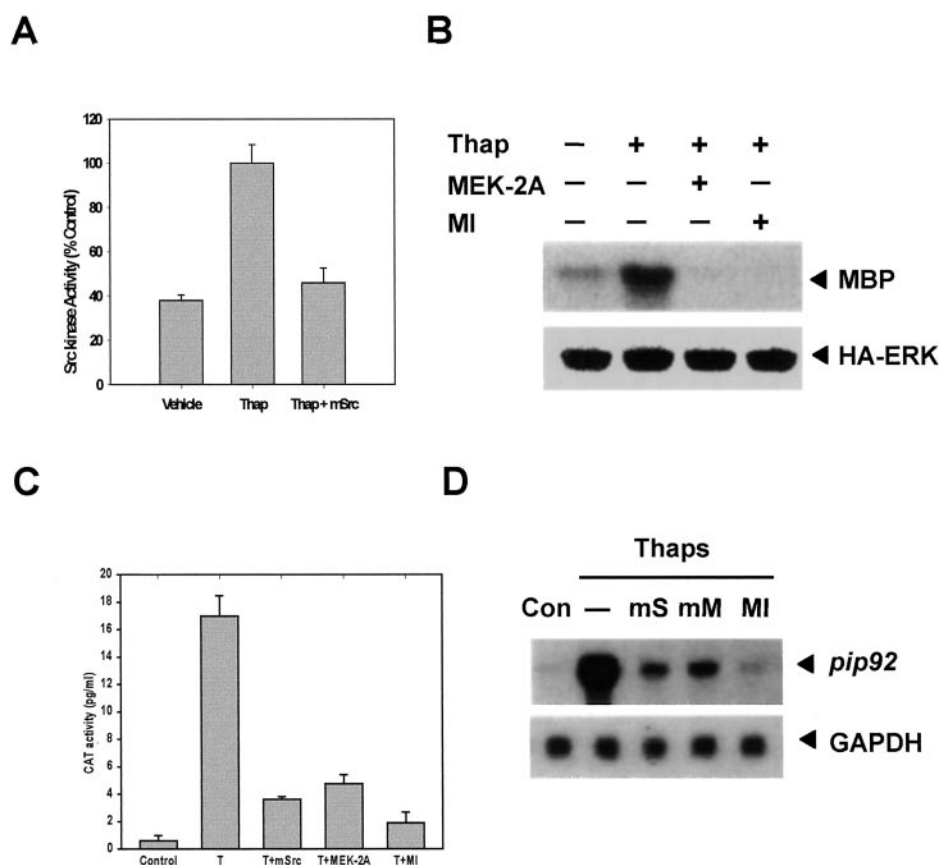
Kinase-inactive Src or MEK Inhibitor Blocks Thapsigargin-induced Activation of pip92 Induction—Many studies demonstrated a role for pp60[SRC]-tyrosine kinase (Src) in Ca^{2+}-induced cell cycle progression and mitogenesis (26–28). To test that Src kinase functions as a mediator for thapsigargin and calcium signaling in H19-7 cells, we assayed the Src kinase activity in response to thapsigargin. Fig. 4*A* shows that thap-
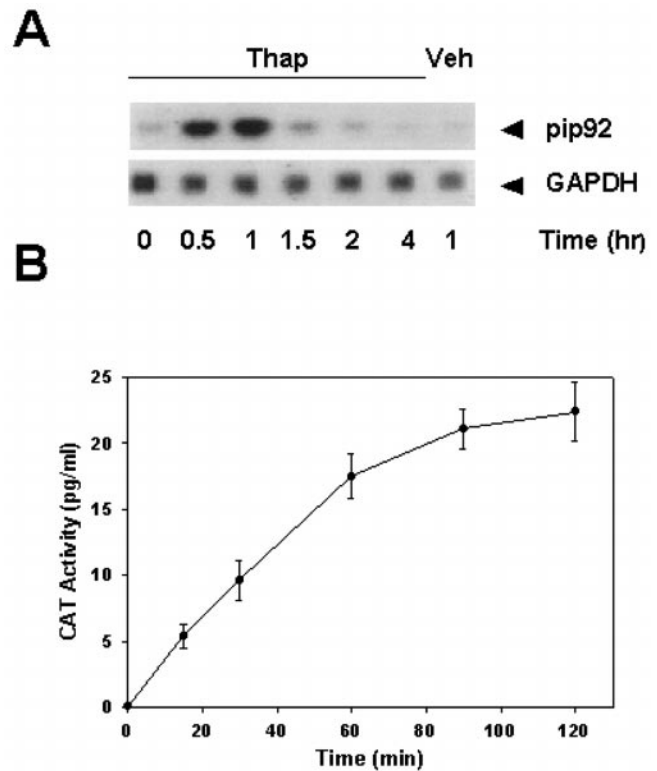
FIG. 3. **Induction of immediate early gene *pip92* by thapsigargin in neuronal H19-7 cells.** *A*, H19-7 cells were treated with 10 μg/ml thapsigargin (*Thap*) or vehicle (*Veh*) at 33 °C for the indicated times. A 10-μg aliquot of total RNA was extracted from cells, applied to each lane, and hybridized to a 125-base pair ^{32}P-labeled *pip92* cDNA fragment. As a control for RNA loading, total RNA was hybridized to ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA probe. *B*, 2 μg of CAT reporter plasmid linked to the −1,281-base pair *pip92* promoter fragment (−1281*pip92*/CAT) was transiently transfected into H19-7 cells. After treatment of the cells with 10 μg/ml thapsigargin, the activity of the expressed CAT enzyme in 40–60 μg of cell lysates was measured at the indicated times. Data are plotted as the mean plus the range of samples from three independent experiments performed in triplicate.

sigargin activates Src-tyrosine kinase 2.5-fold. Transfection of kinase-deficient Src mutant resulted in the inhibition of thapsigargin-induced Src kinase activity to the basal level in H19-7 cells, suggesting that thapsigargin may use Src kinase for downstream signaling events. To test whether MAP kinase is also a downstream target of thapsigargin-induced calcium mobilization, the cells were transiently transfected with a kinase-inactive MEK mutant (MEK-2A), which encodes a protein with 2 alanine residues substituted at the sites of the activating serine residues, together with an HA epitope-tagged ERK2. The addition of thapsigargin resulted in the significant increase of ERK activity, measured by using an epitope-tagged ERK2-immune complex kinase assay (Fig. 4*B*), and kinase-deficient MEK-2A completely blocked the thapsigargin-induced HA-ERK2 activity. To further verify the role of MEK-ERK activation by thapsigargin, we measured the ERK2 activity by thapsigargin after pretreating the cells with 30 μM MEK inhibitor PD98059 for 30 min. Previously we have shown that activation of ERK by fibroblast growth factor (FGF) is completely blocked by 30 μM PD98059 in H19-7 cells (29). Addition of MEK inhibitor also significantly blocked the thapsigargin-induced HA-ERK2 activity. In all samples, ERK2 enzymes were present at the same levels. These results suggest that calcium mobilization by thapsigargin activates both ERK and Src kinase in H19-7 cells.

To test whether *pip92* is activated via the activation of Src

A

B

C

D

FIG. 4. **Dominant-negative Src kinase mutant, kinase-inactive MEK, or chemical MEK inhibitor blocks thapsigargin-induced** ***pip92*** **expression.** *A*, where specified, H19-7 cells were transfected with 10 μg of kinase-deficient Src mutant plasmid (*mSrc*) or parental vector (control). The cells were stimulated with 10 μg/ml thapsigargin (*Thap*) or vehicle for 1 h, and then Src kinase activity was measured as the phosphorylation of rabbit muscle enolase. *B*, the cells were transfected with 2.5 μg of an HA-tagged ERK2 plasmid (*HA-ERK*) or together with 10 μg of a kinase-inactive MEK mutant (*MEK-2A*), as indicated. After transfection, the cells were stimulated with 10 μg/ml thapsigargin (*Thap*) for 1 h. Where indicated, cells were pretreated with 30 μM MEK inhibitor (*MI*) PD98059 for 30 min before cell harvest. The HA-tagged ERK2 was assayed for kinase activity by using myelin basic protein (*MBP*) as a substrate. As a control for equal protein loading, HA-tagged ERK2 levels were measured by Western analysis using anti-HA antibodies. *C*, where indicated, 2 μg of −1281*pip92*/CAT plasmid DNA was transiently cotransfected into H19-7 cells with 10 μg of a kinase-inactive Src (*mSrc*) or MEK mutant (*MEK-2A*). After H19-7 cells were either untreated or pretreated with 30 μM MEK inhibitor (*MI*) PD98059 for 30 min, the cells were stimulated with vehicle (*Control*) or 10 μg/ml thapsigargin (*T*) for 1 h. The transcriptional activity of *pip92* was measured by CAT assay. Data are plotted as the mean plus the range of samples from two independent experiments performed in duplicate. *D*, where indicated, the cells were transiently transfected with 10 μg of a kinase-inactive Src (*mS*) or MEK-2A mutant (*mM*). Where specified, H19-7 cells were pretreated with 30 μM MEK inhibitor (*MI*) PD98059 for 30 min. The cells were then stimulated with 10 μg/ml thapsigargin (*Thaps*) or vehicle (*Con*) for 1 h, and Northern blot analysis of *pip92* mRNA was done. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

kinase and ERK in response to thapsigargin, the H19-7 cells were transiently transfected with the combination of −1281*pip92*/CAT and a kinase-inactive Src or MEK-2A, and transcriptional activity of *pip92* by thapsigargin was measured. As shown in Fig. 4*C*, thapsigargin-induced activation of the *pip92* promoter was significantly blocked by the Src kinase mutant. In the presence of kinase-inactive MEK-2A, transcriptional activation of *pip92* by thapsigargin was also significantly decreased (Fig. 4*C*). In an effect similar to that of MEK-2A, pretreatment of the cells with the MEK inhibitor remarkably suppressed *pip92* expression by thapsigargin. The induction of *pip92* mRNA levels by thapsigargin was also greatly reduced by mutant Src, MEK-2A, or PD98059, measured by Northern blot analysis (Fig. 4*D*), suggesting that the regulation of the episomally expressed *pip92* reporter does reflect mRNA expression from the endogenous gene. Overall these results indicated that the activation of Src kinase and ERK is necessary for thapsigargin-induced activation of *pip92*.

Constitutively Active Src Kinase and MEK Increase Basal pip92 Expression in Neuronal H19-7 Cells—v-Src is a constitutively active tyrosine kinase. To test whether Src kinase is sufficient for mediating thapsigargin-induced activation of

MAP kinase and *pip92* expression, v-Src was transfected into H19-7 cells together with −1281*pip92*/CAT or HA-ERK2 plasmid. Expression of constitutive v-Src kinase in H19-7 cells resulted in a significant increase in *pip92* transcriptional activity (Fig. 5) and activation of ERK in a constitutive manner, which is comparable with that by thapsigargin (Fig. 6*B*). In a similar way, transient transfection of the constitutively active MEK mutant (MEK-2E), which encodes a protein with 2 glutamic acid residues substituted at the sites of the activating serine residues, markedly induced *pip92* transcription without thapsigargin treatment (Fig. 5). It was previously shown that expression of MEK-2E is effective at generating constitutively active MAPK, and the activity is comparable with that induced by growth factor stimulation of the cells (30). Pretreatment of the cells with 30 μM MEK inhibitor also blocks the *pip92* expression induced by v-Src or MEK-2E treatment (Fig. 5). Previously, it was also shown that dominant negative Src blocks thapsigargin-induced activation of ERK (15). Taken together, these results confirmed that ERK activation is necessary for the Src kinase-induced *pip92* activation.

Dominant Inhibitory Raf-1 Mutant Blocks Thapsigargin-induced pip92 Expression—To further characterize the effect of

FIG. 5. **Effect of constitutively active MEK and Src kinase on *pip92* expression in H19-7 cells.** Where indicated, 2 μg of −1281*pip92*/CAT plasmid DNA was transiently transfected into H19-7 cells with 5 μg of a parental vector (*Control*), constitutively active Src (*v-Src*) or MEK mutant (*MEK-2E*). When specified, cells were treated with 30 μM PD98059 for 30 min (*MI*), and the transcriptional activity of *pip92* was measured by CAT assay. Data are plotted as the mean plus the range of samples from two independent experiments performed in duplicate.
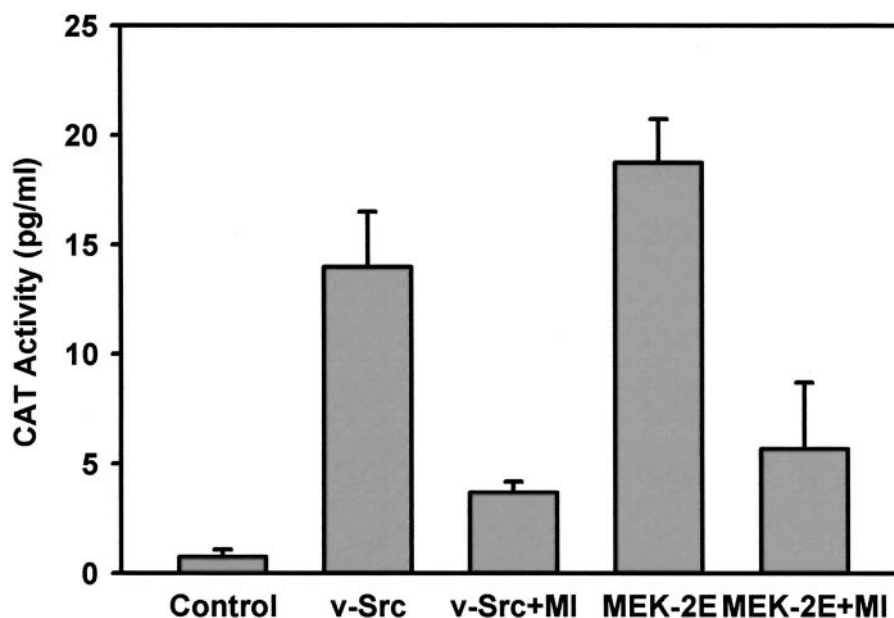
Raf-1 activity on the thapsigargin-induced signaling pathway leading to the induction of *pip92*, we transiently transfected the cells with the kinase-inactive Raf-1 mutant (Raf-KR) and measured the induction of *pip92* transcriptional activity by thapsigargin. As shown in Fig. 6*A*, the dominant negative Raf-1 mutant suppressed *pip92* induction by thapsigargin significantly. In a similar way, when the cells were cotransfected with constitutively active v-Src and kinase-deficient Raf-KR, the *pip92* activation was blocked remarkably. To further determine the requirement of Raf-1 for thapsigargin-induced MAP kinase signaling, we transiently transfected the H19-7 cells with Raf-KR with an HA-tagged ERK2 plasmid. As analyzed by an epitope-tagged ERK2 immune complex assay (Fig. 6*B*), the dominant negative Raf-KR mutant blocks the epitope-tagged ERK2 activity induced by thapsigargin and v-Src kinase. The data indicate that Raf-1 kinase is required for thapsigargin-induced MAP kinase activation and *pip92* expression.

Activation of Transcription Factor Elk1 during Thapsigargin-induced pip92 Expression—Previous studies have shown that serum response element (SRE) present in the pip92 promoter can interact with recombinant serum response factor (SRF) and Elk1 proteins forming a tertiary complex (16, 25). Elk1 can be phosphorylated by ERK, and it is critical for transcriptional activation of *pip92* and c-*fos*. Next we examined whether Elk1 can be phosphorylated by thapsigargin. After cells were stimulated with 10 μg/ml thapsigargin, nuclear extracts and cell lysates were prepared. Electrophoretic mobility shift analysis using *pip92* SRE oligonucleotide and anti-phospho-Elk1 antibodies showed that endogenous Elk1 does not bind to the pip92 SRE element without stimulation and is activated by thapsigargin (Fig. 7*A*). The cell lysates were incubated in the presence of radiolabeled ATP with the wild-type GST-Elk1 C terminus (GST-ElkC) prebound to Sepharose 4B beads. As shown in Fig. 7*B*, wild-type GST-ElkC was phosphorylated by extracts from the cells stimulated with thapsigargin. To demonstrate that the phosphorylation of GST-ElkC by nuclear extracts from thapsigargin-treated cells is specifically mediated by ERK and Src kinase, the effect of the inhibition of ERK or Src kinase activity was examined. Pretreatment of the cells with PD98059 before stimulation or removal of ERK by the incubation of cell lysates with agarose-ERK-immunoglobulin after stimulation resulted in a complete block of GST-ElkC phosphorylation by cell lysates. Similarly, transfection of kinase-inactive MEK and the Src mutant remarkably inhibited

GST-ElkC phosphorylation by thapsigargin-induced cell lysates. These results implied that Src kinase and ERK mediate thapsigargin-induced activation of pip92 via the phosphorylation of Elk1.

DISCUSSION

Thapsigargin, a non-phorbol ester-type tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Although the mechanism of tumor promotion by thapsigargin is not well understood, treatment of mouse NIH 3T3 fibroblasts with thapsigargin induced rapid expression of the c-*fos* and c-*jun* protooncogenes (31). Furthermore, thapsigargin could synergize with another tumor promoter, phorbol 12-myristate 13-acetate, to induce c-*fos* but not c-*jun*. The stress-inducible glucose-regulated proteins, a class of calcium binding molecular chaperones localized in the endoplasmic reticulum, have been implicated in the development of thapsigargin-induced tumorigenicity (32). In addition, the present study suggests that Elk1-dependent activation of pip92 might contribute the tumorigenesis of thapsigargin in neuronal cells.

It has been well documented that thapsigargin and ionomycin mobilize Ca^{2+} through distinct mechanisms, thapsigargin by inhibiting microsomal Ca^{2+}-ATPases and ionomycin by channeling calcium through the membrane from nonspecific sources (33). On the basis of the finding that both thapsigargin and ionomycin are able to induce *pip92* expression, and there was no synergistic effect by the coaddition of the two agents (Fig. 2), Ca^{2+} appears to be a common mediator for the *pip92* induction. Although the two drugs produced a 6–7-fold differential increase of intracellular calcium levels, similar levels of *pip92* induction were observed, suggesting that a slight increase of intracellular calcium mobilization by thapsigargin is likely to be sufficient to induce *pip92*.

Previously we demonstrated that pip92 is expressed rapidly and transiently during NMDA-induced cell death in H19-7 cells (16). In the present study we have shown that pip92 is also expressed during thapsigargin-induced cell proliferation. Both processes are commonly mediated by the increase of intracellular Ca^{2+} influx. Many of the same immediate early genes such as c-*fos* and *pip92* are induced in a variety of biological contexts as diverse as mitogenic responses and the cellular response to cytotoxic stimuli. It will be interesting to investigate the molecular mechanism of variable biological responses

A

B
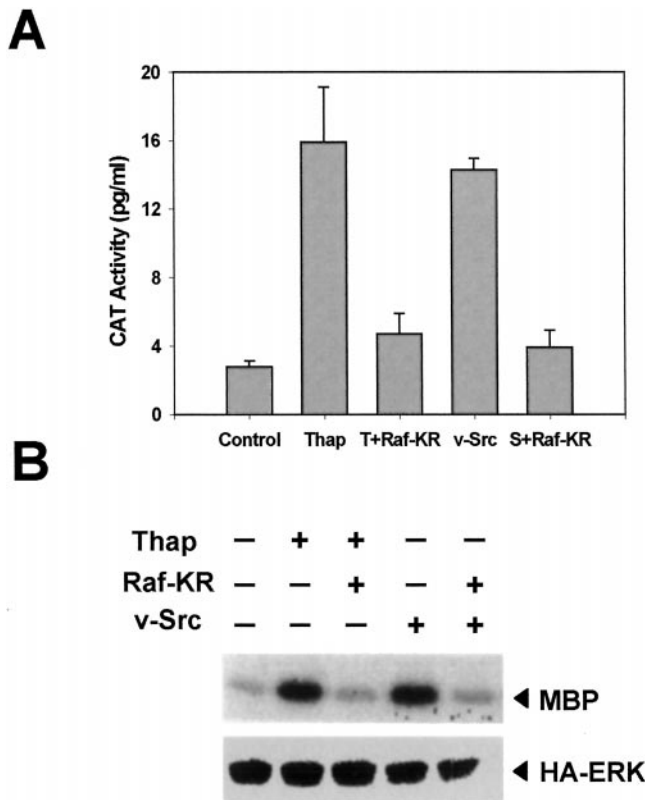
FIG. 6. **Transcriptional activation of *pip92* by thapsigargin and v-Src is inhibited by dominant-inhibitory Raf-1.** *A*, two micrograms of −1281*pip92*/CAT reporter plasmids or with 10 μg of a kinase-inactive Raf mutant (*T+Raf-KR*) were transiently transfected into H19-7 cells, as indicated, and the cells were stimulated with vehicle (*Control*) or 10 μg/ml thapsigargin (*Thap*) for 1 h. Where specified, 2 μg of −1281*pip92*/CAT plasmid was transiently transfected into the cells with 10 μg of oncogenic v-Src or a combination with 5 μg of Raf-KR (*S+Raf-KR*). The transcriptional activity of *pip92* was measured at 33 °C as described under "Experimental Procedures." Data are plotted as the percentage of *pip92* transcriptional activity by thapsigargin and represent the mean plus the range of samples from two independent experiments. *B*, the cells were transiently transfected with 1 μg of HA-ERK2 plasmid with or without 5 μg of kinase-inactive Raf (*Raf-KR*) and/or constitutively active src (*v-Src*) plasmid. After transfection, the cells were stimulated with 10 μg/ml thapsigargin (*Thap*) for 1 h, and the kinase activity of HA-ERK2 was measured as described in Fig. 3*B*. As a control for equal protein loading, HA-tagged ERK2 levels were measured by Western analysis using anti-HA antibodies. *MBP*, myelin basic protein.

resulting from the use of genes of which products are used in many contexts. The mechanism may be triggered as a consequence of differential quantitation of different ligands or different kinetics of the induction of immediate early genes and their products. Because Ca^{2+} signals are short-lived when compared with alterations in differentiated gene expression, it is generally considered that genes coding for short-lived transcription factors (*e.g.* c-*fos* and c-*jun*) are the immediate targets of Ca^{2+} signaling (34, 35). On the basis of the previous finding that Pip92 is selectively expressed in the nucleus as a fusion with green fluorescent protein (23), Pip92 seems to be a second messenger, for example, as a transcription factor, rather than a direct effector of a variety of cell responses, such as cell growth, differentiation, and transformation. We are currently investigating the functional roles of the Pip92 protein in the nucleus.

Previously we have shown that both FGF and Raf activate MAPK-independent kinases that can stimulate Elk1 phosphorylation and *pip92* transcription (12). An increase of intracellular calcium levels by thapsigargin or ionomycin caused a

A

B

C
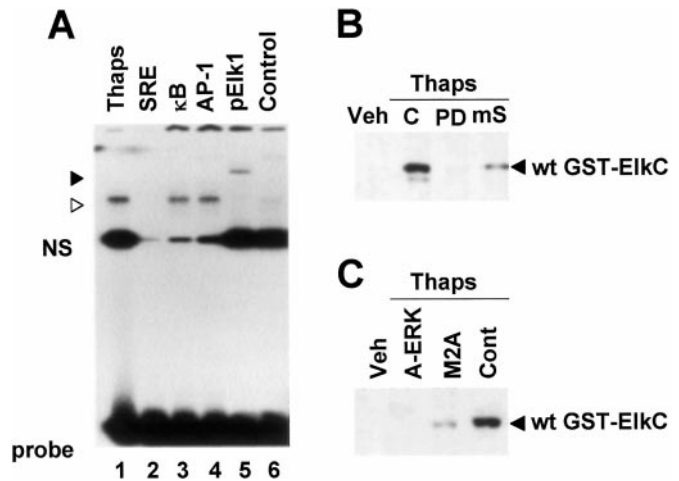
FIG. 7. **Activation of endogenous Elk1 by thapsigargin and inhibition of *in vitro* Elk1 phosphorylation by kinase-inactive Src kinase and ERK.** *A*, nuclear extracts from control H19-7 cells or cells stimulated with 10 μg/ml thapsigargin (*Thaps*) were incubated with end-labeled SRE oligonucleotide, and the resulting protein-DNA complexes were resolved by nondenaturing polyacrylamide gel electrophoresis. For Elk1 supershift analysis, after binding, 0.5 μg of phospho-Elk1 antibodies were added, and the reaction mixture was incubated at 4 °C for 2 h before gel electrophoresis. In *lanes 2–4*, 100-fold excesses of various oligonucleotides (*SRE*, *κB*, and *AP-1*) were included as competitors, as indicated above each *lane*. In *lane 5*, antibodies against phospho-Elk1 (*pElk1*) were included. One SRE-protein complex and a supershifted SRE-phospho-Elk1 binding complex are denoted are denoted by *open* and *filled arrows*, respectively. *Lane 6*, control cells without stimulation. Nonspecific complex (*NS*) and free probe (*probe*) are indicated on the *left*. *B* and *C*, where specified, 5 μg DNA of a kinase-inactive Src mutant (*mS*) or MEK-2A (*M2A*) was transiently transfected into H19-7 cells. Where indicated, the cells were pretreated with 30 μM PD98059 for 30 min (*PD*), followed by the stimulation with 10 μg/ml thapsigargin (*Thaps*). With the cell extracts, prepared from vehicle-treated (*Veh*), thapsigargin-stimulated cells or incubated with agarose-ERK-antibody (*A-ERK*) after stimulation, containing 50–60 μg of proteins and 75 μl of Sepharose 4B beads, *in vitro* Elk1 phosphorylation was performed as described under "Experimental Procedures."

rapid and transient increase of *pip92* gene expression, and the signals for intracellular calcium mobilization are transmitted through the activation of Src kinase- and Raf-MEK-ERK-dependent signaling pathways in H19-7 cells. Elk1 becomes a transcriptional activator to induce pip92 in response to thapsigargin. However, because of a previous finding that kinase-inactive Src kinase did not completely block the activation of ERK by FGF and neuronal differentiation by Raf-1 activation (29), Src-tyrosine kinases appear to be differentially activated via distinct signaling pathways by FGF and thapsigargin in H19-7 cells.

Many studies demonstrated a role for Src kinase in intracellular calcium signaling. For example, functional calcium-sensing receptors in rat fibroblasts are required for activation of Src kinase and mitogen-activated protein kinase in response to extracellular calcium (36). The Src kinase pathway is involved in Ca^{2+}-dependent pancreatic exocytosis (37), and angiotension II-induced phosphorylation of p130Cas by Src family tyrosine kinases is dependent on intracellular calcium and protein kinase C (38). Considerable evidence also supports the role of Src family kinases in the stimulation of ERK. Many G protein-coupled receptors initiate Ras-dependent activation of the ERK cascade by inducing the tyrosine phosphorylation of proteins that serve as scaffolds for the plasma membrane recruitment of Ras guanine exchange factors. Activation of Src kinase by the α-thrombine (39), lysophosphatidic acid (40), angiotension II (41), *N*-formyl peptide chemoattractant (42), α2A-adrenergic (39, 40), and M1 muscarinic receptors (41) has been reported.

A number of studies have suggested that Src cooperates with

Ras to activate MAP kinase in some signaling events. For example, activation of Src family kinases and Ras is required for activation of ERKs by angiotension II in smooth muscle cells (43). Oxidative stress, such as H_2O_2, activates ERKs through Src kinase in cultured cardiac myocytes of neonatal rats (44). However, conflicting results also have been reported to indicate that both can activate independent pathways in certain instances. In fibroblast NIH3T3 cells, Myc, but not Fos or Jun, was able to rescue the inhibition of platelet-derived growth factor-stimulated DNA synthesis by dominant negative Src, whereas Fos and Jun, but not Myc, rescued the cell growth block given by dominant negative Ras (45). Src appears to activate a Ras- and Raf-independent pathway during the differentiation of H19-7 cells by FGF (29). Studies using dominant inhibitory Raf-1 mutants also indicate that Src can be an upstream activator for Raf-1, and Raf-1 may be required for the Src-dependent activation of MAP kinase (15).

REFERENCES

1. Hardingham, G. E., and Bading, H. (1999) *Microsc. Res. Tech.* **46,** 348–355
2. DiGiovanni, J. (1992) *Pharmacol. Ther.* **54,** 63–128
3. Kremer, N. E., D'Arcangelo, G., Thomas, S. M., DeMarco, M., Brugge, J. S., and Halegoua, S. (1991) *J. Cell Biol.* **115,** 809–819
4. Thomas, S. M., Soriano, P., and Imamoto, A. (1995) *Nature* **376,** 267–271
5. Twamley-Stein, G. M., Pepperkok, R., Ansorge, W., and Courtneidge, S. A. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 7696–7700
6. Snyder, M. A., Bishop, J. M., McGrath, J. P., and Levinson, A. D. (1985) *Mol. Cell. Biol.* **5,** 1772–1779
7. Jiang, H., Lu, Z., Luo, J. Q., Wolfman, A., and Foster, D. A. (1995) *J. Biol. Chem.* **270,** 6006–6009
8. Qureshi, S. A., Joseph, C. K., Gupta, R., Hendrickson, M., Song, J., Bruder, J., Rapp, U., and Foster, D. A. (1993) *Biochem. Biophys. Res. Commun.* **192,** 969–975
9. Alexandropoulos, K., Qureshi, S. A., Bruder, J., Rapp, U. R., and Foster, D. A. (1992) *Cell Growth Differ.* **3,** 731–737
10. Coleclough, C., Kuhn, L., and Lefkovits, I. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 1753–1757
11. Charles, C. H., Simske, J. S., O'Brien, T. P., and Lau, L. F. (1990) *Mol. Cell. Biol.* **10,** 6769–6774
12. Chung, K. C., Gomes, I., Wang, D., Lau, L. F., and Rosner, M. R. (1998) *Mol. Cell. Biol.* **18,** 2272–2281
13. Chao, T. S., Byron, K. L., Lee, K. M., Villereal, M., and Rosner, M. R. (1992) *J. Biol. Chem.* **267,** 19876–19883
14. Chao, T. S., Foster, D. A., Rapp, U., and Rosner, M. R. (1994) *J. Biol. Chem.* **269,** 7337–7341
15. Chao, T. S., Abe, M., Herschenson, M. B., Gomes, I., and Rosner, M. R. (1997) *Cancer Res.* **57,** 3168–3173
16. Chung, K. C., Shin, S. W., Yoo, M., Lee, M. Y., Lee, H. W., Choe, B. K., and Ahn, Y. S. (2000) *J. Neurochem.* **75,** 9–17
17. Bading, H., Hardingham, G. E., Johnson, C. M., and Chawla, S. (1997) *Biochem. Biophys. Res. Commun.* **236,** 541–543
18. Egea, J., Espinet, C., and Comella, J. X. (1999) *J. Biol. Chem.* **274,** 75–85
19. Eves, E. M., Tucker, M. S., Roback, J. D., Downen, M., Rosner, M. R., and Wainer, B. H. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 4373–4377
20. Lee, M. G., Zeng, W., and Muallem, S. (1997) *J. Biol. Chem.* **272,** 32951–32955
21. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
22. Xu, J., Wu, Y., He, L., Yang, Y., Moore, S. A., and Hsu, C. Y. (1997) *Biochem. Biophys. Res. Commun.* **235,** 394–397
23. Chung, K. C., Kim, S. M., Rhang, S., Lau, L. F., Gomes, I., and Ahn, Y. S. (2000) *Eur. J. Biochem.* **267,** 4676–4684
24. Kauffman, R. F., Taylor, R. W., and Pfeiffer, D. R. (1980) *J. Biol. Chem.* **255,** 2735–2739
25. Latinkic, B. V., and Lau, L. F. (1994) *J. Biol. Chem.* **269,** 23163–23170
26. Broome, M. A., and Hunter, T. (1996) *J. Biol. Chem.* **271,** 16798–16806
27. Erpel, T., Alonso, G., Roche, S., and Courtneidge, S. A. (1996) *J. Biol. Chem.* **271,** 16807–16812
28. Hille, B. (1992) *Ionic Channels of Excitable Membranes*, 2nd Ed., Sinauer Associates, Sunderland, MA
29. Kuo, W. L., Chung, K. C., and Rosner, M. R. (1997) *Mol. Cell. Biol.* **17,** 4633–4643
30. Kuo, W. L., Abe, M., Rhee, J., Eves, E. M., McCarthy, S. A., Yan, M., Templeton, D. J., McMahon, M., and Rosner, M. R. (1996) *Mol. Cell. Biol.* **16,** 1458–1470
31. Schonthal, A., Sugarman, J., Brown, J. H., Hanley, M. R., and Feramisco, J. R. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 7096–7100
32. Gazit, G., Lu, J., and Lee, A. S. (1999) *Breast Cancer Res. Treat.* **54,** 135–146
33. Mintz, E., and Guillain, F. (1997) *Biochim. Biophys. Acta* **1318,** 52–70
34. Santella, L., and Carafoli, E. (1997) *FASEB J.* **11,** 1091–1109
35. van Haasteren, G., Li, S., Muda, M., Susini, S., and Schlegel, W. (1999) *J. Recept. Signal Transduct. Res.* **19,** 481–492
36. McNeil, S. E., Hobson, S. A., Nipper, V., and Rodland, K. D. (1998) *J. Biol. Chem.* **273,** 1114–1120
37. Tsunoda, Y., Yoshida, H., Africa, L., Steil, G. J., and Owyang, C. (1996) *Biochem. Biophys. Res. Commun.* **227,** 876–884
38. Sayeski, P. P., Ali, M. S., Harp, J. B., Marrero, M. B., and Bernstein, K. E. (1998) *Circ. Res.* **82,** 1279–1288
39. Chen, Y. H., Pouyssegur, J., Courtneidge, S. A., and Van Obberghen-Schilling, E. (1994) *J. Biol. Chem.* **269,** 27372–27377
40. Luttrell, L. M., Hawes, B. E., van Biesen, T., Luttrell, D. K., Lansing, T. J., and Refkowitz, R. J. (1996) *J. Biol. Chem.* **271,** 19443–19450
41. Ishida, M., Marrero, M. B., Schieffer, B., Ishida, T., Bernstein, K. E., and Berk, B. C. (1995) *Circ. Res.* **77,** 1053–1059
42. Ptasznik, A., Traynor-Kaplan, A., and Bokoch, G. M. (1995) *J. Biol. Chem.* **270,** 19969–19973
43. Schieffer, B., Paxton, W. G., Chai, Q., Marrero, M. B., and Bernstein, K. E. (1996) *J. Biol. Chem.* **271,** 10329–10333
44. Aikawa, R., Komuro, I., Yamazaki, T., Zou, Y., Kudoh, S., Tanaka, M., Shiojima, I., Hiroi, Y., and Yazaki, Y. (1997) *J. Clin. Invest.* **100,** 1813–1821
45. Barone, M. V., and Courtneidge, S. A. (1995) *Nature* **378,** 509–512