An Intracellular Calcium Signal Activates p70 but Not p90 Ribosomal S6 Kinase in Liver Epithelial Cells*

(Received for publication, September 16, 1996)

Lee M. Graves‡§, Yaquin He, John Lambert, Deborah Hunter‡, Xiong Li, and H. Shelton Earp‡

From the Departments of Pharmacology and Medicine and the §Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599-7295

In the rat liver epithelial cell lines GN4 and WB, angiotensin II (Ang II) activates the Gq class of regulatory G-proteins, increasing intracellular calcium, protein kinase C activity, and protein tyrosine phosphorylation. We compared the ability of Ang II and other compounds that increase intracellular calcium (i.e. the calcium ionophore A23187 and thapsigargin) or protein kinase C activity (the phorbol ester 12-O-tetradecanoylphorbol-13-acetate) to activate p70 ribosomal S6 kinase (p70S6K) and p90 ribosomal S6 kinase (p90RSK). In GN4 cells, increasing intracellular calcium stimulated p70S6K activity in a rapamycin- and wortmannin-sensitive manner, but did not affect p90RSK activity. In contrast, 12-O-tetradecanoylphorbol-13-acetate strongly activated p90RSK but only weakly stimulated p70S6K. The ability of calcium to activate p70S6K was confirmed by blocking the A23187-dependent activation through chelation of extracellular calcium with EGTA; the effect of thapsigargin was inhibited by the cell permeant chelator bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM). Similarly, BAPTA-AM prevented the activation of p70S6K by Ang II, suggesting that this signal was largely calcium-dependent. In contrast, the Ang II-dependent activation of mitogen-activated protein kinase and p90RSK was not inhibited but was enhanced by BAPTA-AM. These results show that in GN4 cells, Ang II selectively activates p70S6K through effects on calcium, p90RSK through effects on protein kinase C. The activation of p70S6K by calcium stimuli or Ang II was independent of calmodulin but correlated well with the activation of the recently identified, non-receptor calcium-dependent tyrosine kinase (CADTK)/PYK-2. Both calcium- and Ang II-dependent activation of p70S6K were attenuated by the tyrosine kinase inhibitor genistein, and activation of p70S6K was higher in GN4 than WB cells, correlating with the increased expression and activation of CADTK/PYK-2 in GN4 cells. In summary, these results demonstrate that intracellular calcium selectively activates p70S6K in GN4 cells, consistent with increased CADTK/PYK-2 signaling in these cells.

1 The abbreviations used are: Ang II, angiotensin II; MAPK, mitogen-activated protein kinase; BAPTA-AM, bis-(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester; CADTK, calcium-dependent tyrosine kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; PI-3 kinase, phosphatidylinositol-3 kinase; EGF, epidermal growth factor; TBST, Tris-buffered saline/Tween; PBS, phosphate-buffered saline; PKC, protein kinase C; p70S6K, p70 ribosomal S6 kinase; p90RSK, p90 ribosomal S6 kinase; JNK, c-Jun-N-terminal protein kinase; PAGE, polyacrylamide gel electrophoresis; RIP, radioimmunoprecipitation assay; RAFT, rapamycin and FKBP12 target; FRAP, FKBP-rapamycin-associated protein.

* This study was supported in part by National Institutes of Health Grant GM54010, an American Heart Association (North Carolina Affiliate) grant (to L. M. G.), and an American Cancer Society grant (to H.S.E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. of Pharmacology and Medicine, University of North Carolina, Chapel Hill, NC 27599-7365. Tel.: 919-966-0915; Fax: 919-966-5640; E-mail: lmg@med.unc.edu.

This is an Open Access article under the CC BY license.
Calcium-dependent Activation of p70 S6 Kinase

The p70S6K can be activated by numerous stimuli, including growth factors, inhibitors of protein synthesis (cycloheximide or anisomycin), and, as noted above, hormones such as Ang II (11, 19, 20). Complete activation of p70S6K appears to require multisite phosphorylation by several protein kinases, at least one of which is proline-directed and phosphorylates a sequence(s) in the C-terminal region of the S6 kinase (21–24). Despite the knowledge of specific phosphorylation sites, the activating “S6 kinase kinases” remain to be identified. In addition to the involvement of the rapamycin-sensitive FRAP/RAFT mentioned above, p70S6K is inhibited by wortmannin and LY294002, implicating PI-3 kinase in the signaling to p70S6K (25, 26). These results are further supported by studies involving expression of a constitutively active PI-3 kinase (27). A potential intermediary for PI-3 kinase in p70S6K activation is the serine and threonine kinase PKB, also known as Akt (28, 29), which has been shown to activate p70S6K when co-expressed in cells (30).

The p70S6K is involved in mitogenesis; studies with neutralizing antibodies to p70S6K or the inhibitor rapamycin have shown that this kinase is required for G1 progression (31–33). The ability of p70S6K to phosphorylate the ribosomal S6 protein has been well characterized (34), but the contribution of this event to the regulation of protein synthesis is less clear (reviewed in Ref. 12). Phosphorylation of the S6 protein has been found to enhance the translation of mRNAs containing 5’-poly-pyrimidine tracts (35, 36), and it is likely that p70S6K plays a role in growth-related control of translation. For example, translation of specific messages such as those encoding elongation factor 1α (36) or insulin-like growth factor II (37) appears to be regulated by the S6 pathway; these may be members of a class of proteins required for progression through the G1 phase of the cell cycle.

Ang II also stimulates protein synthesis in a number of cell types (11, 38), an event that may be dependent on calcium (39) and p70S6K (11). Because of our interest in calcium and Ang II signaling, we compared the ability of these stimuli to activate the p70S6K in liver epithelial cells, cells known to have a pro-liferative response to Ang II (1). In addition, Ang II leads to a protein kinase C-independent increase in AP1 binding, as well as stimulation of MAP kinase and c-Jun N-terminal kinase (10) and a calcium-dependent activation of a novel; calcium-dependent tyrosine kinase (CADTK). In this article we describe the finding that agents and hormones that increase intracellular calcium activate p70S6K but not p90S6K. Furthermore, these studies suggest that the initial steps in Ang II action are well correlated with activation of CADTK.

EXPERIMENTAL PROCEDURES

Materials—Human Ang II (DRYVYHHPF) was obtained from Sigma and prepared in 50 mM acetic acid prior to use. The S6 peptide (RLSSSS-LRA) (40) and PKI peptide (TTYADFIASGRTGRNAHHD) (41) were synthesized by Dr. D. Klapper (University of North Carolina, Chapel Hill, NC). Thapsigargin, A23187, bis-(o-aminophenox)-ethane-N,N,N’,N”-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM), 12-O-tetradecanoylphorbol-13-acetate (TPA), calycin A, wortmannin, rapamycin, anisomycin, and calmidazolium were obtained from Biomol and prepared in Me2SO. Calmodulin, leupeptin, and aprotinin were obtained from Sigma. Epidermal growth factor (EGF) was obtained and used as described previously (10). The intact 40 S ribosomal subunit was purified from rat liver by the procedure of Terao and Ogata (42). Cell Culturing and Harvesting—Rat liver epithelial cells (GN4 or WB) were grown at 37°C in Richter’s minimal essential media containing 0.1% bovine serum and supplemented with 10% fetal bovine serum in a humidified 5% CO2 atmosphere as described earlier (3). Cells were serum starved (0.1% fetal bovine serum) for 20–24 h prior to agonist stimulation. Cells were washed twice with phosphate-buffered saline (PBS) and once with Buffer H (50 mM β-glycerophosphate, pH 7.4, 1.5 mM EGTA, 0.16 mM sodium orthovanadate, 1 mM dithiothreitol, 25 μg/ml leupeptin, and 1 μM calmidazolium) from which they were scraped in 0.5 ml of Buffer H. The cells were then sonicated with two 5-s pulses (Fisher MDL 550 Sonic Dismembrator), and the lysates were centrifuged at 100,000 × g (4°C).

p70S6K Assays—The activity of p70S6K in cell lysates was determined as described earlier (43). The 100,000 × g supernatants were assayed for kinase activity by monitoring the phosphorylation of the ribosomal protein S6 (2 μg) in a buffer (20 μl) containing 25 mM β-glycerophosphate (pH 7.4), 1.5 mM EGTA, 0.1 mM NaVO4, 1 mM dithiothreitol, 10 mM MgCl2, 10 μM calmidazolium, 2 μM PKI peptide, and 100 μM (γ-32P)ATP (200 μCi/mmol) for 20 min at 30°C. The reactions were terminated by the addition of SDS-PAGE sample buffer (30 μl), heated, and applied to SDS-PAGE (10% acrylamide). The radioactive band corresponding to the ribosomal S6 protein was identified by staining with Coomassie Blue and autoradiography; the radioactive band was excised from the dried gel and quantitated by liquid scintillation counting. Calmidizolium (10 μM) was included in the assay mixture to prevent the phosphorylation of the ribosomal S6 protein by calcium- and calmodulin-dependent kinases.

Immuno precipitation Assays for p70S6K and p90S6K—In some experiments, p70S6K was assayed by immunoprecipitation of the p70S6K after lysis of the cells in Tris/Triton lysis buffer (20 mM Tris, pH 7.5, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 10 mM calycin A, and 148 μM Na3VO4). The samples were immunoprecipitated as described below and the immunoprecipitate/protein A-agarose beads were washed once with Tris/Triton lysis buffer and twice with PBS (1×). The S6 kinase assays were performed using a modification of the above assay procedure. In these assays, the immunoprecipitate/protein A-agarose beads (20 μl) were assayed in 20 mM Hepes, pH 7.3, 10 mM β-glycerophosphate, 1.5 mM EGTA, 0.1 mM Na3VO4, 1 mM dithiothreitol, 10 mM MgCl2, and 50 μM (γ-32P)ATP (1000 μCi/mmol) plus 250 μM S6 peptide in a final reaction volume of 60 μl. The assay was performed at 30°C for 20 min with constant shaking in an Eppendorf 5416 Thermomixer. The reaction was terminated by the addition of 20 μl of 100 mM EDTA (pH 7.0); the samples were centrifuged for 5 min at 13,000 × g, and the supernatant (∼40 μl) was spotted on Whatman P-81 paper. The papers were washed in 10% phosphoric acid, and the radioactivity incorporated into S6 peptide was determined by liquid scintillation counting. The assays for p90S6K were performed exactly as described above, except that the cell lysates were immunoprecipitated with a rabbit polyclonal antibody developed against the C terminus of p90S6K as described previously (Ref. 44; kindly provided by Dr. Edwin G. Krebs, Department of Pharmacology, University of Washington).

p70S6K Immunoprecipitation and Immunoblotting—For immunode tection of p70S6K, cells were washed as described above and lysed in RIPA buffer containing 2% Triton (pH 7.4), 10 mM glyc erol, 0.1% SDS, 0.5% deoxycholate, 1.0% Triton X-100, and 2.0 mM EDTA plus 10 mM calcium A and 0.5 mM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 13,000 × g for 10 min (4°C). The samples were immunoprecipitated using a rabbit polyclonal antibody developed against a C-terminal peptide (QAPFMISKREPHLRMLN) of the p70S6K (kindly provided by Dr. J. Weiel, Glaxo-Welcome). Immunoprecipitation was facilitated by the addition of protein A-agarose (20 μl packed beads), and the immunoprecipitates were washed three times with RIPA buffer prior to addition of SDS-PAGE sample buffer. Samples were applied to SDS-PAGE and blotted to polyvinylidene difluoride, and the immunoprecipitated p70S6K was detected by incubating blots with the C-terminal p70S6K antibody (diluted 1:2500 in TBST). The immunoblot was incubated with goat anti-rabbit alkaline phosphatase-conjugated antibodies (diluted 1:5000 in TBST), and the color was developed according to the manufacturer’s procedure (Promega).

Measurement of CADTK Tyrosine Autophosphorylation—The activity of CADTK was assayed by determining the amount of tyrosine autophosphorylation by a modification of the procedure described earlier (41). Briefly, the cells were washed twice with PBS and either treated with RIPA or Triton lysis buffer as described above for the S6 kinase immunoblottting experiments. The CADTK was immunoprecipitated with a rabbit polyclonal antibody developed against a glutathione S-trans ferase-CADTK fusion protein (5). The CADTK immunoprecipitates were washed three times with RIPA or Triton lysis buffer and applied to SDS-PAGE (8% acrylamide). After electrophoresis the pro-
Calcium Activates p70^S6K but Not p90^RSK—Incubation of GN4 rat liver epithelial cells with compounds that increase intracellular calcium (i.e. the ionophore A23187, thapsigargin (which releases calcium from internal stores; Ref. 45), or Ang II), stimulates intracellular protein tyrosine phosphorylation (2). To investigate the influence of calcium-stimulated tyrosine phosphorylation on “downstream” kinase signaling pathways, we examined the ability of calcium to activate the ribosomal S6 kinase p70^S6K. Incubation of GN4 cells with 1 mM A23187 or 2 mM thapsigargin increased S6 kinase activity as measured by the phosphorylation of the 40 S ribosomal S6 protein (Fig. 1A). A typical response to these stimuli was a 2–3-fold increase over basal levels, similar to that produced by EGF (See Fig. 3). The calcium-stimulated increase in S6 kinase activity was inhibited by the calcium chelators EGTA and BAPTA-AM. Preincubation with EGTA prevented activation of p70^S6K by A23187; the cell-permeant chelator BAPTA-AM (BAPTA) inhibited both the thapsigargin- and ionophore-stimulated S6 kinase activity (Fig. 1A). These results indicate that for these agonists the increase in p70^S6K activity is calcium-dependent, and with thapsigargin, the effect is primarily dependent on intracellular calcium.

In addition to the increased the phosphorylation of the ribosomal S6 protein, calcium activated p70^S6K as demonstrated by SDS-PAGE and immunoblotting analysis (Fig. 1B). In these experiments, p70^S6K was immunoprecipitated from cell lysates and immunoblotted for p70^S6K as described under “Experimental Procedures.” Four immunoreactive bands corresponding to the multiple phosphorylated forms of p70^S6K were detected (46). In all experiments, increased p70^S6K activity (as determined by S6 protein phosphorylation) correlated with an upward mobility shift of both the slowest and fastest migrating immunoreactive bands on SDS-PAGE. The increased upward mobility shift of p70^S6K produced by A23187, or thapsigargin was prevented by preincubation with calcium chelators (Fig. 1B). BAPTA preincubiation reduced the mobility shift of p70^S6K from untreated and A23187- or thapsigargin-treated cells, demonstrating that BAPTA affected both the stimulated and basal S6 kinase activity. In contrast, EGTA inhibited only the A23187-stimulated S6 kinase mobility shift, similar to the results obtained by assaying S6 protein phosphorylation (Fig. 1, A and B).

Like thapsigargin, Ang II rapidly increases intracellular calcium and tyrosine phosphorylation in rat liver epithelial cells (2). As shown in Fig. 2, incubation of GN4 cells with Ang II rapidly stimulated p70^S6K activity comparable with that observed with thapsigargin. The increase in p70^S6K activity was apparent as early as 2–5 min after Ang II addition, with the peak of S6 phosphorylation occurring after 10–20 min. The effect of Ang II and thapsigargin was sustained for more than 60 min (data not shown). Similar results were found by analyzing immunoblots of p70^S6K, Ang II, thapsigargin, and A23187 stimulated a time-dependent increase in the p70^S6K mobility shift on SDS-PAGE, which paralleled the increase in S6 protein phosphorylation (see Fig. 4A).

In cardiac myocytes, Ang II and calcium have been reported to stimulate the activity of MAPK and the ribosomal S6 kinase p90^RSK (47). Although Ang II activated MAPK in GN4 cells by
a PKC-dependent mechanism, thapsigargin does not stimulate MAPK in these cells (5, 10). We therefore compared the ability of thapsigargin and EGF to activate p70\(^{S6K}\) and p90\(^{RSK}\) in GN4 cells. As expected, both agonists stimulated p70\(^{S6K}\) (Fig. 3A), but only EGF stimulated p90\(^{RSK}\) in GN4 cells (Fig. 3B). The kinase assay on p90\(^{RSK}\) immunocomplexes using the S6 peptide (RRLSSLRA) as a substrate also demonstrated that A23187 did not activate p90\(^{RSK}\), whereas Ang II potently activated this enzyme, as previously reported (10) (data not shown).

**Calcium-dependent p70\(^{S6K}\) Activation Is Inhibited by Wortmannin and Rapamycin**—To further rule out the possibility that p90\(^{RSK}\) was contributing to the phosphorylation of the S6 protein in cell lysates and to investigate the mechanism by which calcium stimulates p70\(^{S6K}\) we examined the effect of compounds known to inhibit p70\(^{S6K}\) activation. Rapamycin has been previously shown to inhibit p70\(^{S6K}\) without affecting p90\(^{RSK}\) activity (14, 15). As shown in (Fig. 4A), incubation of cells with 10 nM rapamycin completely inhibited the Ang II- and calcium ionophore (A23187)-stimulated increase in p70\(^{S6K}\) S6 peptide kinase activity. Similarly, rapamycin blocked the thapsigargin-dependent activation of p70\(^{S6K}\); (unstimulated, 35.7 ± 0.4 pmol/min/ml; thapsigargin, 94.3 ± 7.1 pmol/min/ml; thapsigargin and rapamycin, 23.9 ± 0.2 pmol/min/ml). Rapamycin also blocked Ang II-, A23187-, and thapsigargin-dependent p70\(^{S6K}\) activation, as assessed by the gel mobility immunoblotting (Fig. 4B). These results demonstrate that Ang II and calcium stimulate p70\(^{S6K}\) through a rapamycin-sensitive pathway and confirm that p70\(^{S6K}\) and not p90\(^{RSK}\) is the major ribosomal S6 kinase activated by calcium stimuli in GN4 cells.

Studies with the inhibitor wortmannin and constitutively active forms of PI-3 kinase have demonstrated a role for this enzyme in the regulation of p70\(^{S6K}\) (25–27). To determine whether calcium stimulated p70\(^{S6K}\) through a PI-3-kinase-dependent pathway, the effect of wortmannin on p70\(^{S6K}\) activity was examined. As shown in Fig. 4B, incubation of GN4 cells with wortmannin (50 nM) inhibited the mobility shift of p70\(^{S6K}\) stimulated by Ang II, A23187, or thapsigargin. The inhibition

**Fig. 3.** Thapsigargin activates p70\(^{S6K}\), but not p90\(^{RSK}\). Serum-starved GN4 cells were incubated with 2 \(\mu\)M thapsigargin (Thaps) or 6.6 nM EGF for the lengths of time indicated. The cells were washed two times with PBS and harvested in Tris/Triton lysis buffer as described under “Experimental Procedures.” The lysate from each 10-cm dish was immunoprecipitated with antibodies specific for p70\(^{S6K}\) (A) or p90\(^{RSK}\) (B) and assayed for S6 peptide kinase activity as described above. The S6 peptide activity is plotted as the amount of activity (pmol/min/ml), and the results represent the mean ± S.E. (bars) of duplicate samples. Data plotted are representative of \(n = 3\) experiments.

**Fig. 4.** Rapamycin and wortmannin inhibit the activation of p70\(^{S6K}\) by calcium agonists. A, GN4 cells were incubated with 0.1% Me\(_2\)SO (carrier for rapamycin) or rapamycin (Rap, 10 nM, 15 min) prior to the addition of A23187 (1 \(\mu\)M) or Ang II (Ag II, 1 \(\mu\)M). Cell lysates were prepared as described earlier, and the S6 kinase activity is plotted as radioactivity (cpm, \(^{32}\)P) incorporated into the ribosomal S6 protein. The results represent the mean ± S.E. (bars) of duplicate samples. Data plotted are representative of \(n = 3\) experiments. B, GN4 cells were incubated with 1 \(\mu\)M Ang II, 2 \(\mu\)M thapsigargin or 1 \(\mu\)M A23187 for the lengths of time indicated. In some experiments, cells were incubated with 10 nM rapamycin (R) or 50 nM wortmannin (W) for 15 min prior to the addition of agonist. The p70\(^{S6K}\) was immunoprecipitated and detected by immunoblotting as described in the legend to Fig. 1.
of p70S6K by wortmannin occurred at low concentrations (10–50 nM), consistent with the effects of this compound on PI-3 kinase (48). Similar inhibitory effects of wortmannin were found on the calcium-dependent activation of S6 kinase, as determined by S6 protein phosphorylation (data not shown). Thus, this calcium-stimulated signaling pathway appears to be mediated through both a PI-3 kinase and FRAP/RAFT-dependent pathway.

Angiotensin II Activates p70S6K in a Calcium-dependent Manner—Ang II stimulates an inositol phosphate-mediated increase in intracellular calcium (reviewed in Ref. 49), and since our results demonstrated that calcium stimulates p70S6K activity, we examined whether the activation by Ang II was calcium-dependent. Incubation of GN4 cells with the calcium chelator BAPTA inhibited the Ang II-stimulated S6 kinase activity in a dose-dependent manner, with >90% inhibition occurring at 50 µM BAPTA (Fig. 5A). Like the results found with S6 kinase activity, BAPTA inhibited the Ang II-stimulated p70S6K mobility shift (Fig. 5B) at concentrations similar to those required to inhibit the activation by A23187 or thapsigargin (Fig. 1, A and B). Chelation of extracellular calcium with EGTA (5 mM) only minimally affected the Ang II-stimulated S6 kinase activity, further supporting the thesis that intracellular calcium was required for the activation of p70S6K (Fig. 5B). The calcium-dependent activation of p70S6K occurred independently of calmodulin, since the calmodulin inhibitor calmidizolium (50 µM) did not inhibit this event. Similarly, neither calmidizolium nor W-7 prevented the autophosphorylation of CADTK, although calmidizolium (15 µM) inhibited the Ang II-dependent activation of the myosin light chain peptide kinase activity, a known calmodulin-dependent process (data not shown). In comparison, preincubation of GN4 cells with BAPTA did not inhibit but significantly stimulated the activation of p90RSK by Ang II in a dose-dependent manner (Fig. 5C). Furthermore, incubation with BAPTA alone (50 µM) increased the basal level of p90RSK to levels equivalent to that of Ang II stimulation in these cells. Similar to the results obtained with p70S6K, BAPTA pretreatment potently enhanced the basal and Ang II-stimulated level of MAPK activity (data not shown).

PKC Activates p90RSK but Not p70S6K in GN4 Cells—Since Ang II increases diacylglycerol formation and PKC activity in GN4 cells, we investigated whether the activation of p70S6K by Ang II was also PKC-dependent. Incubation of cells with phorbol ester (TPA, 100 nM) for 20 min did not stimulate p70S6K activity in these cells, although p90RSK was stimulated by TPA (100 nM, 5 min) (Fig. 6, A and B). To further investigate the involvement of PKC, PKC activity was down-regulated by chronic incubation with TPA (1 µM, 24 h). The activation of p70S6K by Ang II was not inhibited by this treatment, indicating that PKC did not play a substantial role in regulating p70S6K in these cells (Fig. 6A). In comparison, this treatment effectively eliminated the activation of p90RSK by TPA as expected (Fig. 6B).

The Activation of p70S6K by Calcium Requires a Tyrosine Kinase—In epithelial cells, the Ang II- and calcium-stimulated increase in protein tyrosine phosphorylation is inhibited by the tyrosine kinase inhibitor genistein (3, 51). GN4 cells were briefly incubated with genistein (10–200 µM) to test the ability of this compound to prevent the activation of p70S6K by Ang II or calcium stimuli. As shown in Fig. 7A, genistein inhibited the Ang II-stimulated p70S6K activity in a dose-dependent manner, suggesting the involvement of a tyrosine kinase in the calcium and Ang II signaling to p70S6K. Similarly, genistein inhibited the activation of p70S6K by thapsigargin (data not shown).

Recently, we have purified the major calcium- and Ang II-stimulated tyrosine kinase (CADTK) from GN4 rat liver epi-

---

**Fig. 5. Angiotensin II activates p70S6K in a calcium-dependent manner.** A, p70S6K activity. Serum-starved GN4 cells were incubated with 1 µM Ang II for 20 min; in some experiments cells were incubated with the indicated concentrations of BAPTA for 15 min prior to the addition of Ang II (AgII). Cell lysates were prepared, and the p70S6K was immunoprecipitated and assayed as described earlier. The p70S6K activity is plotted as pmol/min/ml, and the results represent the mean ± S.E. (bars) of duplicate samples. B, serum-starved GN4 cells were incubated with MeSO (carrier), 5 mM EGTA (E), or 50 µM BAPTA (B) for 15 min prior to the addition of Ang II (1 µM) for the amount of time indicated; p70S6K was analyzed for activity by SDS-PAGE immunoblotting as described earlier. C, p90RSK activity. Cells were treated exactly as described in A, and the p90RSK was immunoprecipitated and assayed as described earlier. The p90RSK activity is plotted as pmol/min/ml, and the results represent the mean ± S.E. (bars) of duplicate samples. Data plotted are representative of n = 3 experiments. Cont, control.
thelial cells (4), cloned the DNA, and raised specific antisera to the protein (5). We compared the ability of genistein to inhibit the activity of CADTK by assaying the tyrosine autophosphorylation of CADTK, as described under “Experimental Procedures.” The amount of tyrosine autophosphorylation of this kinase appears to be proportional to the enzymatic activity.2 As shown in Fig. 7B, incubating GN4 cells with concentrations of genistein from 50 to 400 μM led to a progressive decrease in the Ang II-stimulated CADTK tyrosine autophosphorylation. Similar concentrations of genistein were required to inhibit the tyrosine autophosphorylation of the EGF receptor in these cells. In comparison, the calcium-dependent tyrosine phosphorylation of an exogenous substrate (e.g. paxillin, a 68–70-kDa protein) tyrosine-phosphorylated by CADTK was inhibited by even lower concentrations of genistein (100–200 μM) than was the CADTK autophosphorylation.3 The results with genistein implicate a tyrosine kinase in the activation of p70S6K by calcium or Ang II, suggesting a role for CADTK.

The Stimulation of p70S6K Correlates with Increased CADTK Activity—To further investigate whether CADTK was involved in the regulation of p70S6K, we compared the activation of p70S6K in cells known to exhibit different levels of Ang II- and thapsigargin-dependent CADTK activation. Previously we showed that transformed GN4 rat liver epithelial cells exhibit approximately 3–4 times more Ang II-stimulated tyrosine phosphorylation than the parental cell type from which it was derived (WB) (3). We have recently confirmed that GN4 cells exhibit approximately 5 times more Ang II-dependent activation and 2–3-fold more CADTK protein than WB cells (5).

2 L. Xiong, unpublished observations.

3 L. Xiong, unpublished data.
exposure to these compounds. Similarly, thapsigargin stimulated a similar 2–3-fold higher increase in S6 kinase activity in GN4 cells (data not shown).

To eliminate the possibility that the enhanced calcium-dependent activation was simply due to increased expression of the p70S6K in GN4 cells, the amount of p70S6K activity and protein was compared in the two cell lines by immunoblotting and activity assays as described earlier. As determined by immunoblotting, the amount of the p70S6K protein and the nuclear form of this kinase (p85S6K) (33) was comparable in the two cell types (Fig. 8B). Importantly, in GN4 cells, Ang II, thapsigargin, and A23187 stimulated a pronounced mobility shift of both the p70 and p85 S6 kinases; by contrast, much less mobility shift was observed in WB cells, in agreement with the S6 kinase activity assays. Furthermore, the protein synthesis inhibitor anisomycin (20) strongly stimulated the mobility shift (Fig. 8B) and activity of p70S6K (and p85S6K) equivalently in WB and GN4 cells (Fig. 8C). These results demonstrate that differences in p70S6K or p85S6K expression do not account for the enhanced activation by calcium in GN4 cells. Instead these results suggest that the increased expression of CADTK in GN4 cells facilitates the activation of p70S6K by Ang II or calcium stimuli.

**DISCUSSION**

Ang II has numerous effects on rat liver epithelial cells. It is a weak mitogen, a response that is initiated via the AT1 receptor, transduced by Gq protein stimulation of phospholipase C, and effected by serine, threonine, and tyrosine phosphorylation (1). Elevating intracellular calcium with thapsigargin or ionophore (A23187) mimics the Ang II-stimulated increase in protein tyrosine phosphorylation in GN4 cells (2), and our laboratory has recently isolated a likely candidate for the calcium-stimulated entity that regulates tyrosine phosphorylation, a novel CADTK. Incubating GN4 cells with Ang II or agonists that raise intracellular calcium (i.e., thapsigargin and A23187) activates CADTK (4), whereas incubation with the calcium chelator BAPTA-AM inhibits activation of this enzyme. The effect of calcium on CADTK is indirect; adding calcium or calcium and calmodulin to cell lysates does not activate CADTK, and thus the mechanism by which calcium and other signals regulate this enzyme remains to be established.

In addition to activating PKC, the calcium- and calmodulin-dependent protein kinase, and CADTK in GN4 cells, Ang II also activates MAPK and JNK and increases AP-1 binding (the latter can be accomplished in a PKC-independent manner) (10). We now demonstrate Ang II and calcium-dependent activation of p70S6K. The challenge is to discern which of these multiple Ang II-dependent pathways are downstream of PKC, calcium, and calmodulin, CADTK, or even G-protein βγ subunits.

We began this process by purifying CADTK; peptide and cDNA cloning has identified this enzyme as the rat homologue of a novel human nonreceptor tyrosine kinase, PYK-2 (4–6). Using PC12 cells, PYK-2 was shown to be activated by elevating intracellular calcium or PKC activity. In these cells, PYK-2 stimulation increased MAPK activity, providing a potential mechanism for calcium-dependent regulation of MAPK in these and other cells (6, 52). In contrast, our studies in rat epithelial cells demonstrate that a calcium signal (i.e., thapsigargin and A23187) does not significantly activate MAPK and that Ang II-dependent MAPK activation is primarily a PKC-dependent process. Instead, Ang II and a calcium signal (thapsigargin) substantially activate JNK in a calcium-dependent manner (10). In fact, the Ang II effect is PKC-independent and is amplified in cells depleted of PKC. JNK activation by Ang II and thapsigargin correlates with increased expression and tyrosine autophosphorylation of CADTK in our rat liver epithelial cells. We have shown that the chemically transformed GN4 cell line expresses more CADTK than the parental cell line (WB), and the activation of CADTK by Ang II is approximately 5-fold greater in confluent GN4 cells when compared with WB cells. Activation of JNK by Ang II and thapsigargin is also 5-fold higher in GN4 cells, whereas the total JNK activity induced by stress (e.g., anisomycin) is equal in WB and GN4 cells. Thus in GN4 cells, CADTK is not involved in MAPK activation but is likely to be a mediator of signaling to JNK.
Because some of the stimuli known to activate JNK also increase p70S6K activity in other cell types, (i.e. Ang II (11) and anisomycin (20)), we compared the ability of a calcium signal to regulate p70S6K in epithelial cells. In this study we find that like JNK (10), p70S6K is regulated by agonists that raise intracellular calcium, whereas neither p90RSK nor MAPK is affected by the calcium-activated pathway. This is contrasted to results in PC12 cells (6, 52) and suggests that the ability of calcium to regulate these signaling pathways is cell type-specific.

The role of intracellular calcium and Ang II (through its effects on intracellular calcium) in stimulating p70S6K has not been studied extensively. In one article, A23187 weakly activated p70S6K in Swiss 3T3 fibroblasts (53), a cell line that does not appear to contain CADTK.2 Earlier, the work of Meier et al. (54) reported the activation of an S6 kinase in kidney epithelial cells in response to calcium stimuli (A23187), although the identity of this kinase was not established. It is clear that in this study we were measuring the calcium-dependent activation of p70S6K in cell lysates. First, although the cell lysate assay could detect p90RSK phosphorylation of the 40 S ribosomes, we have used a specific immune complex assay to show that there is little if any thapsigargin-dependent p90RSK activation. Second, all our assay data have been confirmed by immunoblot analysis of the p70S6K mobility shift, an assay that highly correlates with the activation. Third, rapamycin, which inhibits p70S6K but not p90RSK activation, abolished the Ang II- or thapsigargin-dependent activation of 40 S ribosome phosphorylation in cell lysates.

Importantly, our results suggest that most of the Ang II-dependent activation of p70S6K is calcium-dependent. Preincubation with BAPTA prevented the Ang II-dependent activation of p70S6K but not p90RSK. Instead, p90RSK and MAPK were substantially activated by BAPTA, alone or in the presence of Ang II. At this point, the mechanism of the BAPTA effect is unclear; we can only speculate that calcium may regulate one or more phosphatases in the MAPK cascade or that BAPTA has a non-calcium-dependent effect on the MAPK pathway. We also cannot exclude that additional G-protein-coupled signals, e.g. βγ subunit or other G-protein-dependent responses, contribute to the stimulation of p70S6K by Ang II. Recently the activation of p70S6K by receptors coupled to G, has been described, supporting a potential role for βγ subunits in the regulation of p70S6K (55).

In this study we also found that agonists that increase intracellular calcium (A23187, thapsigargin, and Ang II) in GN4 cells activate p70S6K following a rapid stimulation of CADTK. The cell-permeable chelator BAPTA-AM inhibited the activation of both CADTK and p70S6K by thapsigargin, A23187, or Ang II, consistent with a role for CADTK upstream of p70S6K in these cells. In further support of a role for CADTK, the calcium and Ang II-dependent activation of p70S6K was prevented by genistein at concentrations similar to those required to inhibit the thapsigargin-dependent JNK activation and the thapsigargin- and Ang II-dependent tyrosine phosphorylation of CADTK substrates (e.g. paxillin) in vivo.2 Other findings linking CADTK to the p70S6K pathway come from a comparison of p70S6K activity in WB and GN4 cells. The GN4 cell line expresses 2–3-fold greater CADTK and has 5-fold greater Ang II-dependent CADTK and JNK activation (5). GN4 cells also exhibit 2–3-fold greater p70S6K activation in response to Ang II and thapsigargin. The failure of p70S6K to match the 5-fold difference in CADTK and JNK activation between GN4 and WB cells may well stem from the complex pathway to p70S6K, which appears to involve regulation of at least two upstream kinase pathways (see below).

One possibility is that a calcium signal may be activating PI-3 kinase, since low concentrations ofwortmannin (50 nm) inhibited the activation of p70S6K by A23187, thapsigargin, or Ang II. Numerous studies have suggested that PI-3 kinase is an upstream mediator of p70S6K activity (reviewed in Ref. 12), although much of the evidence for PI-3 kinase (including our study) has been obtained with the inhibitor wortmannin (48), the selectivity of which has been recently questioned (56). With the caveat of specificity, the activation of CADTK by Ang II or thapsigargin is not inhibited by wortmannin,2 suggesting that PI-3 kinase is downstream or unrelated to the calcium-dependent activation of CADTK. Wortmannin also does not inhibit JNK activation, another pathway linked to the regulation of CADTK in GN4 cells.2 Thus, if CADTK is a common activator of both p70S6K and JNK in GN4 cells, as our results suggest, at some point the signaling pathways diverge such that a wortmannin-sensitive step is specific to the p70S6K pathway.

Our results are also consistent with the calcium-dependent activation of another limb of the p70S6K pathway, the one that activates the rapamycin-sensitive kinase, i.e. FRAP/RAFT. Rapamycin inhibits the activity of the FRAP/RAFT kinases by binding to its cognate binding protein, FKBP12, thus inhibiting p70S6K stimulation (16). However, like the PI-3 kinases, the role of FRAP/RAFT in regulating p70S6K is poorly understood, largely because substrates for these kinases remain to be identified. Again the calcium-dependent pathway to JNK and p70S6K is divergent in GN4 cells, since rapamycin blocks Ang II- and thapsigargin-dependent p70S6K activation but does not affect the stimulation of CADTK or JNK by these agonists.2

In addition to increasing intracellular calcium, Ang II also stimulates PKC activity (1, 5). Despite the fact that PKC has been shown to activate p70S6K in other cell types (57), our results do not support a role for PKC in the regulation of p70S6K in GN4 cells. Although Ang II stimulated p70S6K in a manner similar to thapsigargin or A23187, TPA alone did not increase p70S6K activity in GN4 cells, although MAPK and p90RSK were activated as expected.

TPA also increases CADTK activity in these cells, albeit less potently than Ang II and more slowly than thapsigargin. These results present a paradox, since our studies suggest that CADTK is upstream of p70S6K, and yet TPA does not activate p70S6K. We have found that a brief (5 min) TPA pretreatment inhibited the activation of p70S6K by Ang II or thapsigargin by approximately 40% in GN4 cells.4 Similarly, an even more profound inhibitory effect of PKC has been found on the activation of JNK by calcium stimuli or Ang II in GN4 cells.2 Taken together, these studies demonstrate that PKC alone is incapable of activating p70S6K and may inhibit the downstream calcium-dependent signals to both JNK and p70S6K in GN4 cells.

In summary, we have demonstrated a calcium-dependent pathway that results in substantial activation of p70S6K. This calcium-dependent pathway has characteristics similar to the calcium-dependent pathway to JNK that we have previously studied in this cell line (5, 10). Intriguingly, this calcium-dependent pathway does not activate MAPK or p90RSK; instead these enzymes are activated in PKC-dependent manner. Considerable circumstantial evidence points to CADTK as an initial mediator of the calcium signal to both JNK and p70S6K. However, the pathway to these enzymes clearly diverges at some point such that the calcium-dependent activation of JNK is insensitive to wortmannin and rapamycin, whereas the activation of p70S6K is completely inhibited by these compounds. The exact signaling downstream from CADTK and, in fact, unequivocal proof of CADTK involvement must await further studies.

4 L. M. Graves, unpublished observations.
Acknowledgments—We acknowledge the excellent technical assistance of Ruth Dy and Rumei Li.

REFERENCES

1. Huckle, W. R. & Earp, H. S. (1994) Proc. Growth Factor Res. 5, 177–194
2. Huckle, W. R., Prokop, C. A., Dy, R. C., Herman, B. & Earp, H. S. (1990) Mol. Cell. Biol. 10, 6290–6298
3. Huckle, W. R., Dy, R. C. & Earp, H. S. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8837–8841
4. Earp, H. S., Dy, R. W., Dawson, T. L., Li, X., Graves, L. M. & Dy, R. (1995) J. Biol. Chem. 270, 28440–28447
5. Yu, H., Li, X., Marchetto, G., Dy, R., Hunter, D., Dawson, T., Wilm, M., Anderegg, R., Graves, L. M. & Earp, H. S. (1996) J. Biol. Chem. 271, in press
6. Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Flowman, G. D., Rudy, B. & Schlessinger, J. (1995) Nature 376, 737–745
7. Sasaki, H., Nagura, K., Ishino, M., Tobioka, H., Kotani, K. & Sasaki, T. (1995) J. Biol. Chem. 270, 21206–21219
8. Avraham, S., London, R., Ota, S., Higegawara, D., Li, J., Jiang, S., Passtor, L. M., White, R. A., Groepman, J. E. & Avraham, H. (1995) J. Biol. Chem. 270, 27742–27751
9. Duff, J. L., Berk, B. C. & Corson, M. A. (1995) Biochem. Biophys. Res. Commun. 198, 257–264
10. Zohn, I. E., Yu, H., Li, X., Marchetto, G., Dy, R., Hunter, D., Dawson, T., Wilm, M., Anderegg, R., Graves, L. M. & Earp, H. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 585–590
11. Terasa, N., Franklin, R. A., Lucas, J. J., Bader, H., Waterfield, M. D. & Panayotou, G. (1996) Trends Biochem. Sci. 21, 513–520
12. Proud, C. G. (1996) Trends Biochem. Sci. 21, 513–520
13. Ballou, L. M., Luther, H. & Thomas, G. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6160–6168
14. Ballou, L. M., Siegmann, M. & Thomas, G. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 80–85
15. Sadoshima, J., Qiu, Z., Morgan, J. P. & Izumo, S. (1995) J. Biol. Chem. 270, 987–990
16. Pelech, S. L., Olwin, B. B. & Krebs, E. G. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5968–5972
17. Walsh, D. A. & Glass, D. B. (1991) Methods Enzymol. 201, 304–316
18. Dasso, M. & Opperman, R. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 80–85
19. Wahl, M. C., Friend, S. H., Bridges, J. K. & Beach, D. (1995) Cell 81, 1–15
20. Gietzen, K., Sadorf, I. & Bader, H. (1982) Biochem. Biophys. Res. Commun. 97, 303–316
21. Gardner, J., Cheng, J. Q., Testa, J. R. & Tsichlis, P. N. (1993) Oncogene 8, 745–754
22. Bellacosa, A., Frank, T. F., Gonzalez, P. M. E., Datta, K., Taguchi, T., Gardner, J., Cheng, J. Q., Testa, J. R. & Tsichlis, P. N. (1993) Oncogene 8, 745–754
23. Ballou, L. M., Siegmann, M. & Thomas, G. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 6160–6168
24. Pelech, S. L., Olwin, B. B. & Krebs, E. G. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5968–5972