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Epidermal growth factor (EGF) receptor T669 peptide kinase from 3T3-L1 cells is an EGF-stimulated "MAP" kinase

(protein phosphorylation/signal transduction/preadipocyte cell line)

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ABSTRACT **The epidermal growth factor (EGF) receptor is both an activator and a target of growth factor-stimulated kinases involved in cellular signaling. Threonine-669 (T669) of the EGF receptor is phosphorylated in response to a wide variety of growth-modulating agents. MAP kinase is similarly phosphorylated as well as stimulated by growth activators, including EGF. To determine whether a MAP-type kinase is responsible for T669 kinase activity in EGF-stimulated 3T3-L1 cells, we partially purified and characterized the T669 peptide kinase. The results indicate that a MAP kinase phosphorylates the T669 peptide and raise the possibility that this enzyme may participate in a feedback loop, being activated by the EGF receptor and in turn phosphorylating the receptor.**

Signal transduction leading to cell growth is a complex process initiated by the interaction of growth-stimulating factors with specific receptors at the cell surface. One of the major classes of growth factor receptors are the ligand-stimulated tyrosine kinases, exemplified by the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors (1). Once bound to their respective growth factors, these receptors initiate a series of signaling events through tyrosine phosphorylation of interacting proteins, which in turn transmit the signal to the cell nucleus. A major focus of many investigations is establishing the identity of functionally important intermediates and the mechanisms by which they participate in the signaling process. Several putative intermediates that coimmunoprecipitate with the EGF or PDGF receptors have been identified, including phospholipase C-γ (2–4), phosphatidylinositol-3 kinase (5), GTPase-activating protein (6), and Raf (7). Presumably, there are other proteins that complex with these receptors and participate in the signaling process.

The EGF receptor is both an activator and a target of phosphorylation by kinases that are believed to be involved in cellular signaling (8). At least two major sites of phosphorylation on the EGF receptor have been identified: (*i*) threonine-654 (T654), a target of protein kinase C phosphorylation, which mediates inhibition of the EGF-stimulated tyrosine kinase (9, 10); and (*ii*) threonine-669 (T669), the major phosphorylated residue in A-431 human epidermoid carcinoma cells and a residue that is phosphorylated in response to a variety of stimuli including EGF, phorbol esters, and the nonphorbol tumor promoter thapsigargin (11, 12). Although the functional consequence of phosphorylating T669 is not known, this residue is the only major phosphorylated site on the EGF receptor following treatment with thapsigargin, which inactivates the receptor tyrosine kinase through a protein kinase C/T654-independent mechanism (13). Since the kinase that phosphorylates this residue is responsive to a variety of growth signals, it presumably represents one of the functionally activated intermediates in the signaling process.

Here we report that the EGF receptor T669 peptide kinase from 3T3-L1 preadipocytes is a MAP kinase. Originally described as an insulin-stimulated serine/threonine kinase that phosphorylates microtubule-associated protein 2 (MAP-2) (14), MAP kinase has also been shown to phosphorylate myelin basic protein (MBP) (15) and ribosomal protein S6 kinase II *in vitro* (16). A mitogen-activated kinase, MAP kinase integrates a variety of phosphorylating signals in cells (15) and may be the activity associated with pp42 (17), a protein phosphorylated at tyrosine in response to a variety of growth-stimulating factors such as PDGF, phorbol esters, and EGF. Our results suggest that activation of this MAP kinase by the EGF-stimulated receptor leads to direct phosphorylation of EGF receptor by MAP kinase via a feedback loop.

METHODS

Materials and Cells. Murine 3T3-L1 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (GIBCO) in a gassed (5.5% CO_2/94.5% air), humidified atmosphere at 37°C. EGF was purchased from Biomedical Technologies (Stoughton, MA). Monoclonal anti-phosphotyrosine antibody-linked Sepharose beads were a gift from A. R. Frackelton (Brown University and Roger Williams Hospital, Providence, RI). Phosphatase 2A was a gift from M. Mumby (Southwest Medical Center, Houston). *Xenopus* oocyte MAP kinase, partially purified by sequential chromatography on DEAE-cellulose, gel filtration TSK, and phenyl-TSK columns, was a gift from B. Barrett and J. L. Maller (University of Colorado School of Medicine, Denver). Okadaic acid was a gift from H. Fujiki (National Cancer Center Research Institute, Tokyo).

Superose 12 Gel Filtration. Confluent 100-mm dishes of 3T3-L1 cells either untreated or treated with EGF (200 ng/ml) for 10 min at 37°C were washed at 4°C with Krebs–Ringer bicarbonate with Hepes buffer (pH 7.4), harvested, and homogenized as described by Ray and Sturgill (18). After centrifugation for 5 min in a microcentrifuge, the supernatant was applied at 4°C to a Pharmacia Superose 12 gel filtration column and eluted in buffer A (25 mM Hepes, pH 7.4/2 mM EGTA/0.2 mM phenylmethylsulfonyl fluoride/1 mM dithiothreitol/24 mM β-glycerophosphate) containing 50 mM NaCl. Fractions (0.2 ml) were collected at a flow rate of 0.4 ml/min. The Superose 12 column was calibrated using the

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Abbreviations: BSA, bovine serum albumin; EGF, epidermal growth factor; MAP-2, microtubule-associated protein 2; MBP, myelin basic protein; PDGF, platelet-derived growth factor.

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following proteins: thyroglobulin (669 kDa), apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), transferrin (75 kDa), bovine serum albumin (BSA, 67 kDa), ovalbumin (42 kDa), α-chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa).

Chromatography on Phenyl Sepharose. NIH 3T3-L1 cell extract, prepared as described above, was applied to a DEAE-Sepharose column in buffer A with 25 mM NaCl, and the MAP and T669 kinase activities were eluted with buffer A containing 350 mM NaCl. The eluate was then applied at 4°C to a Pharmacia phenyl-Superose column and washed in buffer A with 250 mM NaCl for 30 min. At fraction 15, linear gradients of 0–60% ethylene glycol and 250–25 mM NaCl were applied for 120 min. Fractions (0.5 ml) were collected at 0.1 ml/min.

Chromatography on Anti-Phosphotyrosine Antibody-Linked Sepharose. Thirty microliters of the partially purified kinase eluted from the phenyl-Superose column was diluted to 300 μl in buffer x [10 mM Tris, pH 7.6/1% (vol/vol) Triton X-100/5 mM EDTA/50 mM NaCl/0.1% BSA] and applied to a 300-μl column of anti-phosphotyrosine (1G2)-Sepharose (19). The kinase was incubated in the column for 2 hr at 4°C. The column was washed with 1.5 ml of buffer X and then 1 ml of buffer X without BSA. The kinase was eluted with 1 mM phenyl phosphate in 1% Triton/10 mM Tris, pH 7.6/5 mM EDTA/50 mM NaCl/0.01% ovalbumin, and 50-μl fractions were collected. As a control, comparable kinase samples were chromatographed on a BSA-Sepharose column. Similar data were obtained when nonspecific IgG-Sepharose was used as a control (I.G.-P. and M.R.R., unpublished results).

Assay of T669 Kinase Activity. The reaction mixture (50 μl), containing 25 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP (50 μCi/nmol; New England Nuclear; 1 μCi = 37 kBq), 50 μg of the T669 peptide, and 5 μl of enzyme from each fraction, was incubated for 30 min at 30°C. The reaction was stopped by the addition of 500 μl of 30% acetic acid. To remove excess ATP, the sample was loaded onto a 2-ml Bio-Rad AG1-X8 column and washed once with 0.5 ml and once with 0.7 ml of 30% acetic acid. The eluates from the ion-exchange column were resolved by HPLC using a Vydac C_{18} reverse-phase column and elution with 0.1% trifluoroacetic acid in 21.5% acetonitrile. Fractions (1 ml) were collected at 1 ml/min. The phosphorylated T669 peptide was eluted in fraction 11.

Assay of MAP Kinase Activity. The reaction mixture (32 μl), containing 50 mM β-glycerophosphate (pH 7.5), 10 mM MgOAc, 1 mM dithiothreitol, 6.4 μg of MBP, 50 μM [γ-^{32}P]ATP (5 μCi/nmol), and 4 μl of enzyme from each fraction, was incubated for 20 min at 30°C. The reaction was stopped by the addition of Laemmli 2× sample buffer, and proteins were resolved by SDS/15% PAGE. Following autoradiography, the bands corresponding to ^{32}P-labeled MBP were cut out for scintillation counting.

Purification of Tyrosine Phosphatase 1B. Recombinant rat brain protein-tyrosine-phosphatase 1 is a truncated form of the enzyme produced by replacing the Lys-323 codon with a stop codon and expressing the construct in *Escherichia coli* behind the bacteriophage T7 promoter (20). The recombinant enzyme, which retained the entire catalytic domain, was purified to homogeneity by chromatography on DEAE-cellulose and thiophosphorylated, reduced, carboxyamidomethylated, and maleylated-lysozyme-Sepharose. Details of the purification and characterization will be published elsewhere.

Tyrosine Phosphatase Treatment. The reaction mixture (75 μl) contained tyrosine phosphatase (25 units/ml; unit = nmol/min), buffer (50 mM Tris·HCl, pH 7.0/0.03 mM EDTA/0.07% BSA/0.003% Nonidet P-40/0.2% 2-mercaptoethanol) (20), and 22 μl of the phenyl-Superose fraction with peak T669 activity. After incubation at 30°C for 40 min, 35 μl of the mixture was added to 35 μl of 2× T669 kinase reaction mixture or 2× MAP kinase reaction mixture (see above) and incubated for an additional 30 min. The Mg^{2+} in these mixtures inactivates the tyrosine phosphatase. Samples were then assayed for T669 kinase activity or MAP kinase activity as described above. Autoradiograms were scanned with a Zeineh soft-laser scanning densitometer to quantitate relative ^{32}P incorporated into MBP.

Serine/Threonine Phosphatase Treatment. The reaction mixture (75 μl) contained phosphatase 2A (1.3×10^{-3} unit/ml; unit = nmol/min), buffer (100 mM Hepes, pH 7.4/10 mM EGTA/2 mM dithiothreitol/0.3% BSA), and 22 μl of the phenyl-Superose fraction with peak T669 activity. After incubation at 30°C for 40 min, 35 μl of the mixture was added to 35 μl of 2× T669 kinase reaction mixture or the MAP kinase reaction mixture (see above) and incubated for an additional 30 min. Okadaic acid was added to these mixtures to inactivate the serine/threonine phosphatase. Samples were then assayed for T669 kinase activity or MAP kinase activity as described above.

RESULTS AND DISCUSSION

T669 kinase activity was assayed in 3T3-L1 cells by using as substrate a synthetic EGF receptor peptide containing T669 (Arg-Glu-Leu-Val-Glu-Pro-Leu-Thr^{669}-Pro-Ser-Gly-Glu-Ala-Pro-Asn-Gln-Ala-Leu-Leu-Arg). Following phosphorylation of the peptide with [γ-^{32}P] ATP and enzyme, samples were passed through an ion exchanger to remove excess ATP, and the phosphorylated peptide was resolved by HPLC on a C_{18} reverse-phase column. Preliminary experiments established that there was no requirement for Ca^{2+} or lipid for activity, and that phosphorylation occurred exclusively on the threonine residue (K.T. and M.R.R., unpublished results). Similar results were obtained by Davis and coworkers (21) for T669 kinase activity in crude cell extracts. Analysis of T669 kinase activity by gel filtration on Superose 12 indicated only a single peak of activity, even in crude extracts of EGF-stimulated 3T3-L1 cells (Fig. 1). In contrast, two peaks of MAP kinase activity were detected using MBP as a substrate. The elution position of the T669 kinase activity corresponded to that of the 42-kDa MAP kinase identified previously as the insulin-stimulated MAP kinase (18). When the T669 kinase activity was chromatographed on DEAE-Sepharose followed by phenyl-Superose, the T669 activity eluted from the DEAE column was contained within the peak of MBP-phosphorylating activity (K.T., I.G.-P., and M.R.R., unpublished data), and the elution profile of the T669 activity on the phenyl-Superose column corresponded exactly to that for MAP kinase (Fig. 2). Phenyl-Superose is a particularly good step for purification, since MAP kinase appears to be unusually hydrophobic and requires ≈40% ethylene glycol for elution. Under our conditions, no T669 kinase or MAP kinase activities were detected in extracts from cells that were not treated with EGF. Thus, on gel filtration, ion-exchange, and hydrophobic chromatographic systems, the T669 and MAP kinases were coeluted. These three chromatographic steps generate highly purified preparations of MAP kinase from murine 3T3-L1 cells in which the only major band visible by silver staining and the only phosphorylated band is the 42-kDa MAP kinase (18).

MAP kinase purified by DEAE-cellulose and phenyl-Superose from EGF-stimulated 3T3-L1 cells contains a single tyrosine-phosphorylated protein as detected by anti-phosphotyrosine antibodies and ^{32}P-labeling on one- and two-dimensional electrophoretograms (17). To determine whether the T669 kinase, like MAP kinase, was tyrosine-phosphorylated, two approaches were used. (*i*) The DEAE/phenyl-Superose-purified T669 kinase (see Fig. 2) was resolved by SDS/PAGE, electroeluted onto nitrocellulose, and

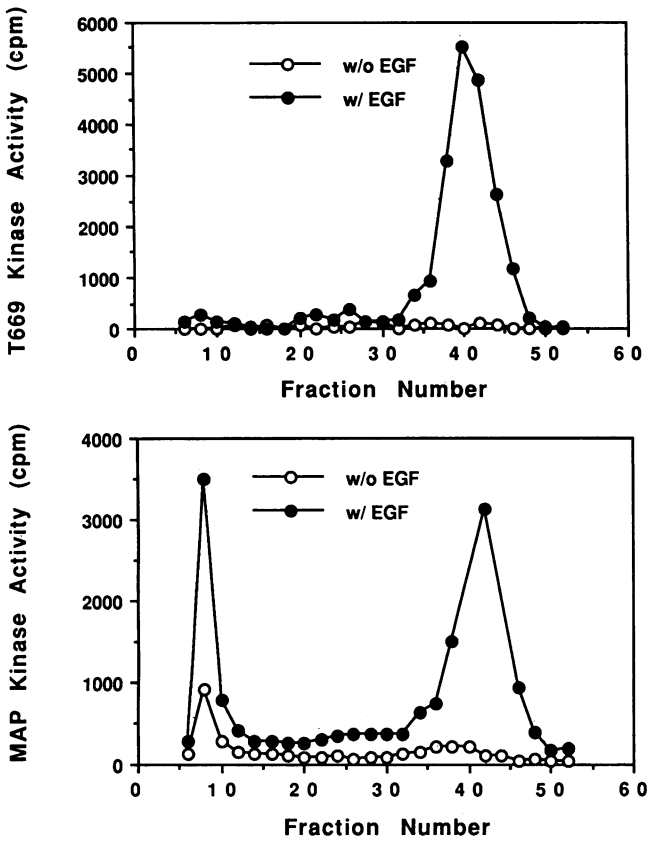

FIG. 1. Cochromatography of T669 kinase and MAP kinase on a Superose 12 gel filtration column. Five confluent 100-mm dishes of 3T3-L1 cells either untreated (○) or treated with EGF (200 ng/ml) for 10 min at 37°C (●) were solubilized and the extracts resolved by gel filtration. (*Upper*) T669 kinase activity. (*Lower*) MAP kinase activity.

then probed with 1G2 anti-phosphotyrosine antibody (19). Only a single band was observed in the 42-kDa region of the gel (I.G.-P., K.T., and M.R.R., unpublished results), consistent with previous reports for MAP kinase (17). (*ii*) The phenyl-Superose eluate was chromatographed on a 1G2 anti-phosphotyrosine-Sepharose column. The selectivity of this column for phosphotyrosine residues has been demonstrated (19). Thirty-three percent of the T669 kinase activity and 32% of the MAP kinase activity were retained by the column and specifically eluted with 1 mM phenyl phosphate (Fig. 3). In contrast, no T669 or MAP kinase activities were retained by a BSA-Sepharose column that was run concurrently (Fig. 3). These results indicate that the T669 kinase is tyrosine-phosphorylated.

MAP kinase has an additional property that exemplifies its role as a "switch" kinase: it integrates phosphorylation signals from both tyrosine and threonine/serine kinases and, in order to be active, must be phosphorylated at both tyrosine and threonine residues (15). Thus, MAP kinase can be selectively deactivated by dephosphorylation of phosphotyrosine residues with a tyrosine phosphatase or by dephosphorylation of phosphothreonine residues with a threonine phosphatase. In several independent experiments, treatment of the phenyl-Superose eluates with recombinant rat protein-tyrosine-phosphatase 1B (20) inhibited the T669 and MAP kinase activities by 21–80% of their original activities (Table

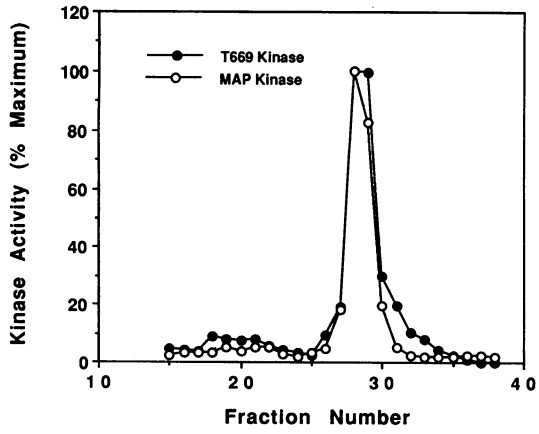

FIG. 2. Cochromatography of T669 kinase (●) and MAP kinase (○) on a phenyl-Superose column. NIH 3T3-L1 cell extracts, prepared as in Fig. 1, were applied sequentially to a DEAE-Sepharose column and phenyl-Superose column. The maximum incorporation of ^{32}P into T669 was 4603 cpm (fraction 28) and into MBP was 24,253 cpm (fraction 28). No T669 kinase activity was detected in the flow-through fractions. A similar profile was observed when extracts from insulin-treated cells were used (data not shown).

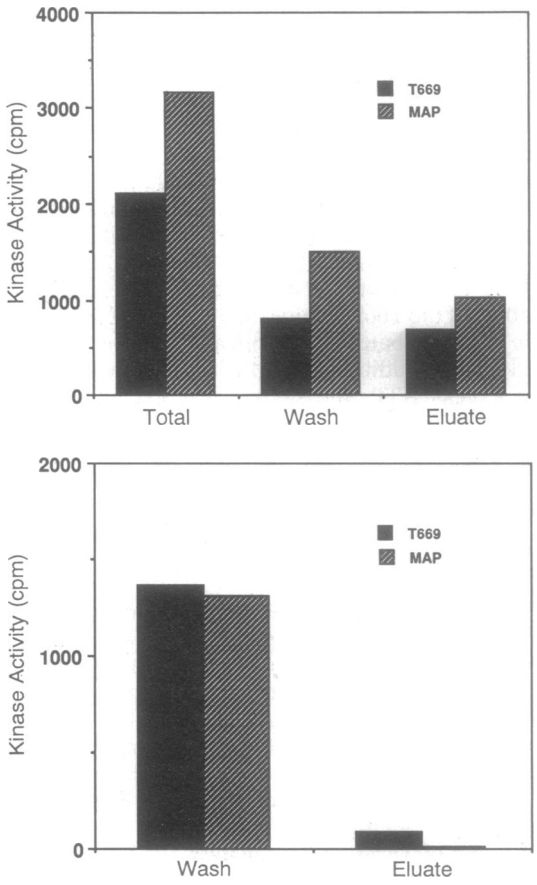

FIG. 3. Cochromatography of T669 kinase and MAP kinase on an anti-phosphotyrosine column. (*Upper*) The partially purified kinase eluted from the phenyl-Superose column was applied to an anti-phosphotyrosine-Sepharose column. Samples (10 μl) of each wash fraction (nos. 1–6) and eluate fraction (nos. 1–6) were assayed for T669 (solid columns) and MBP (hatched columns) activity. The summed activities of the wash fractions and of the eluate fractions were compared to the total precolumn activity, which was 0.02 pmol of phosphate per 30 min for T669 and 0.29 pmol per 30 min for MBP. (*Lower*) The partially purified kinase eluted from the phenyl-Superose column was applied to a BSA-Sepharose column. Samples (10 μl) of wash fractions 1, 3, and 5, and eluate fractions 1, 3, and 5, were assayed for T669 (solid columns) and MBP (hatched columns) activity. The summed activity of these wash fractions and these eluate fractions represents about half of the total precolumn activity. These data are representative of two independent experiments.

Table 1. Inhibition of T669 kinase and MAP kinase activities by tyrosine or serine/threonine phosphatases

Treatment	MBP activity, % control	T669 activity, % control
None	100	100
Phosphatase 1B		
Experiment 1	75	79
Experiment 2	49	37
Experiment 3	20	21
Phosphatase 2A		
Experiment 1	62	57
Experiment 2	50	63
Experiment 3	46	43
Experiment 4	27	0

Samples were treated and assayed as described in the legend of Fig. 4.

1). Similarly, treatment of the phenyl-Superose eluate with phosphatase 2A (22), a serine/threonine phosphatase, inhibited the T669 and MAP kinases by 37–100% (Table 1). The degree of inactivation by the phosphatases varied depending upon conditions of the assay such as incubation time and activity of the phosphatase preparations. Vanadate, a selective inhibitor of tyrosine phosphatases, blocked the inhibition of kinase activity by phosphatase 1B as shown in a representative experiment (Fig. 4). Inclusion of okadaic acid, a selective inhibitor of phosphatase 2A (23), similarly counteracted the action of the serine/threonine phosphatase (Fig. 4). These results confirm that the T669 kinase must be tyrosine- as well as serine/threonine-phosphorylated and shares with MAP kinase the properties of a switch kinase.

To determine whether the ability of MAP kinase to phosphorylate T669 has been evolutionarily conserved, we tested a highly purified preparation of MAP kinase from *Xenopus* oocytes (24). With the same assay described in the legend to Fig. 1 [with the exception that bovine MAP-2 (0.1 mg/ml) was used as substrate for MAP kinase instead of MBP (0.2 mg/ml)], 19,683 cpm was incorporated into T669 (specific activity, 0.18 pmol of phosphate per 20 min for 5 μl of enzyme) and 2700 cpm was incorporated into MAP-2 (specific activity, 0.24 pmol per 20 min for 5 μl of enzyme). The apparent discrepancy between the oocyte and murine MAP kinase activities toward the different substrates is presumably due to the higher specific activity of the oocyte kinase and the fact that MBP is a more effective substrate for the enzyme than MAP-2 under the assay conditions used (ref. 15; K.T. and M.R.R., unpublished results). These results indicate that the oocyte MAP kinase is at least as active in phosphorylating T669 as the partially purified MAP kinase from 3T3-L1 cells and that the T669 peptide and MAP-2 are phosphorylated to a comparable extent by the oocyte MAP kinase.

The T669 peptide contains only a single phosphorylatable threonine residue, in the sequence Pro-Leu-Thr-Pro. Although we have not found a site with this precise sequence in other substrates of MAP kinase such as ribosomal S6 kinase II, MAP-2, and MBP, there are closely related sequences that may represent potential phosphorylation sites for MAP kinase. For example, mouse MBP contains the sequence Pro-Arg-Thr^{97}-Pro (24, 25). Once the substrate specificity has been more fully defined, it may be possible to use the consensus sequence to identify other physiologically important substrates of MAP kinase.

The results presented here indicate that the EGF receptor T669 peptide kinase in 3T3-L1 preadipocytes is an enzyme sharing all the major characteristics of MAP kinase. We cannot rule out the possibility that there are other MAP kinases that are activated by EGF and of similar molecular weight. Recent cloning of MAP kinase has revealed that there are at least two members of the MAP kinase family (26). In

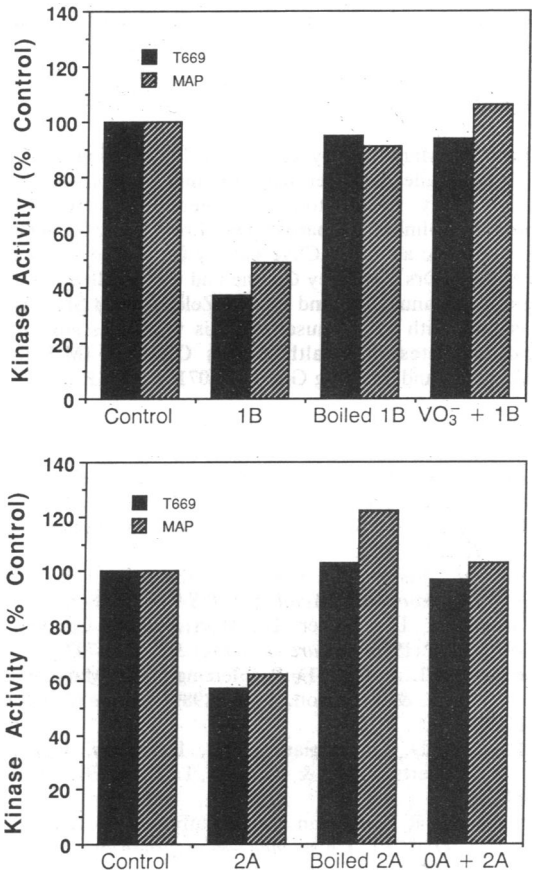

FIG. 4. Phosphatase inhibition of T669 kinase and MAP kinase activities. MAP kinase activity was partially purified by DEAE-cellulose and phenyl-Superose as described (18). Extract from NIH 3T3-L1 cells treated with 100 nM EGF for 10 min prior to lysis was applied to a DEAE-cellulose column and eluted with a NaCl gradient. The fractions containing MAP kinase activity were then chromatographed on phenyl-Superose as in Fig. 2, yielding a similar elution pattern with specific activities of 0.006 pmol of phosphate per 30 min for T669 and 0.068 pmol per 30 min for MBP in the peak fractions. (*Upper*) Treatment of T669 kinase by tyrosine phosphatase 1B. The tyrosine phosphatase reaction mixtures contained aliquots of phenyl-Superose fractions with peak T669 activity and either no addition (Control), phosphatase 1B (1B), phosphatase 1B that had been boiled for 5 min (Boiled 1B), or phosphatase 1B with 0.1 mM vanadate (VO_3^- + 1B). Results are expressed as percent of control kinase activity (0.005 pmol of phosphate incorporated into T669 in 30 min). (*Lower*) Treatment of T669 kinase by serine/threonine phosphatase 2A. The serine/threonine phosphatase reaction mixtures contained aliquots of phenyl-Superose fractions with peak T669 activity and either no addition (Control), phosphatase 2A (2A), phosphatase 2A that had been boiled for 5 min (Boiled 2A), or phosphatase 2A with 1 μM okadaic acid (OA + 2A). The activities of the control samples were 0.003 pmol of phosphate per 30 min for T669 and 0.03 pmol per 30 min for MBP. The results presented in this figure are representative of at least three independent experiments.

addition, two EGF-stimulated MBP-phosphorylating activities from Swiss 3T3 cells have recently been resolved by Mono Q chromatography, but they differ in apparent molecular weight as determined by gel filtration (27). Although only one tyrosine-phosphorylated protein was detected by Sturgill and colleagues (17) in their DEAE/phenyl-Superose-purified MAP kinase preparations, their two-dimensional gels do not rule out the possibility that there are multiple MAP kinases with similar isoelectric points and molecular weights. For example, there could be multiple mitogen-activated kinases with different substrate specificities. In either case, the T669 kinase from 3T3-L1 preadipocytes has properties that identify it as the 42-kDa enzyme that has been termed MAP kinase

(17, 18), and this MAP kinase is the only detectable activity in these cells that phosphorylates the T669 peptide of the EGF receptor. These results suggest that a MAP kinase phosphorylates the T669 site of the EGF receptor *in vivo*.

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