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Immunological Characterization of Avian MAP Kinases: Evidence for Nuclear Localization

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The subcellular distribution and regulation of MAP kinase isoforms in chicken hepatoma DU249 cells was investigated with antibodies directed against peptides patterned after sequences in the mitogen-activated protein (MAP) kinases, sea star p44^{mpk}, and rat p44^{erk1}. MonoQ chromatography of cytosol from these cells afforded the resolution of at least four peaks of myelin basic protein (MBP) phosphotransferase activity, but only one of these (peak II) was stimulated in extracts from phorbol ester-treated cells. A 40- to 41-kDa (p41) doublet on Western blots detected with three different MAP kinase antibodies was coincident with peak II, and it probably corresponded to the avian homolog of p42$^{mapk/erk2}$. Immunofluorescent studies with DU249 cells and chicken embryo fibroblasts revealed that most of the cross-reactive protein with at least two different MAP kinase antibodies was distributed in the nucleus. Subcellular fractionation studies confirmed a predominantly nuclear localization for p41 MAP kinase. Nocodazole arrest of DU249 cells was exploited for the detection of an M-phase-activated MBP kinase that was resolved from p41 MAP kinase by phenyl-Superose chromatography. Western blotting analysis with antibodies for the *cdc*2-encoded protein kinase and p13^{suc1}-agarose binding studies allowed positive identification of this MBP kinase as p34^{cdc2}.

INTRODUCTION

A family of tyrosyl-phosphorylated 42- to 54-kDa protein-seryl/threonyl kinases has been implicated in the signal transduction pathways of a diversity of cytokines (for reviews, see Blenis, 1991; Cobb *et al.*, 1991; Sturgill and Wu, 1991; Pelech and Sanghera, 1992). These mitogen-activated protein (MAP) kinases have also been referred to as microtubule-associated protein-2 (MAP-2) kinases (Ray and Sturgill, 1987; Hoshi *et al.*, 1988; Kyriakis *et al.*, 1991), myelin basic protein kinases (MBP) kinases (Cicirelli *et al.*, 1988; Pelech *et al.*, 1988; Ahn *et al.*, 1990), extracellular-signal regulated kinases (ERKs) (Boulton *et al.*, 1990, 1991a), ribosomal S6 kinase-kinases (RSK-kinases) (Chung *et al.*, 1991a), insulin-stimulated protein kinases (ISPKs) (Gomez and Cohen, 1991), and EGF-receptor threonine (ERT) kinases (Northwood *et al.*, 1991), in part, after the diversity of substrates that have been used for their detection.

At least four MAP kinases have been studied in depth. Three of these, i.e., p42^{mapk} (p42^{erk2}) (Ray and Sturgill, 1987; Hoshi *et al.*, 1988), p44^{erk1} (Boulton *et al.*, 1990, 1991a), and p54 MAP kinase (Kyriakis *et al.*, 1991) were first recognized as activated MAP-2 kinases in growth factor or cycloheximide-treated mammalian cells. p44^{mpk} is a fourth isoform, which was originally shown to be activated near the onset of germinal vesicle breakdown in maturing sea star oocytes (Pelech *et al.*, 1988). In each instance, compelling evidence has been offered for a critical role for the tyrosyl phosphorylation of the MAP kinases for stimulation of their MBP phosphotransferase activities (Anderson *et al.*, 1990; Boulton *et al.*, 1991a; Posada *et al.*, 1991; Sanghera *et al.*, 1991a,b). Comparison of the primary sequences of the 42- to 44-kDa MAP kinases confirmed that they are isoforms with ≥ 80% amino acid identity or greater (Boulton *et al.*, 1990, 1991b; Gotoh *et al.*, 1991a; Her *et al.*, 1991; Posada

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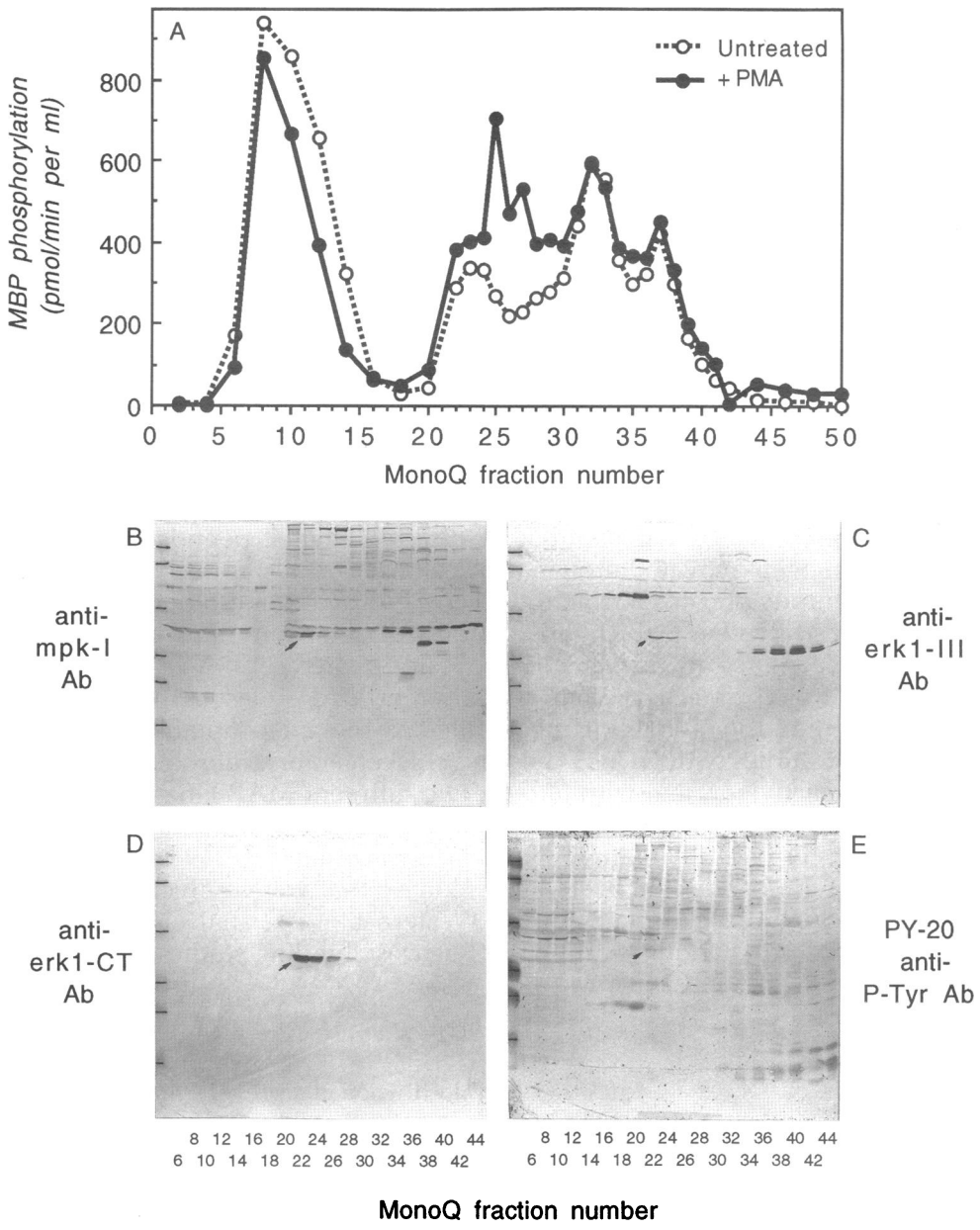

Figure 1. MonoQ chromatography of cytosolic phorbol ester-stimulated MAP kinase from chick DU249 cells. Cytosolic protein (1.0 mg) from untreated cells (○) and cells exposed to 320 nM PMA for 20 min (●) were separately subjected to MonoQ chromatography as described in MATERIALS AND METHODS, and the column fractions were assayed for phosphotransferase activity towards 1 mg MBP/ml (A). Adjacent column fractions were pooled from the MonoQ run with cytosol from the PMA-treated cells and subjected to Western blotting analysis with anti-mpk-I antibodies (B), anti-erk1-III peptide antibodies (C), anti-erk1-CT peptide antibodies (D), and PY-20 antiphosphotyrosine antibody (E). Electrophoretic migrations of the prestained marker proteins phosphorylase b (106K), bovine serum albumin (80K), ovalbumin (50K), carbonic anhydrase (33K), soybean trypsin inhibitor (28K), and lysozyme (19K) are shown in the first lane of each immunoblot. The position of p41 is indicated with a solid arrow. Similar results were obtained in three separate experiments.

et al., 1991; Charest, unpublished data). Enzymological characterisation of highly purified preparations of $p42^{mapk}$, $p44^{mpk}$, and $p44^{erk1}$ from various sources also strongly supports their relatedness as a family (Ray and Sturgill, 1988; Sanghera *et al.*, 1990b; Boulton *et al.*, 1991a; Gotoh *et al.*, 1991b; Rossomando *et al.*, 1991).

Little is known about the direct upstream activators of MAP kinases in signal transduction pathways. It has been found that bacterially expressed recombinant forms of $p42^{mapk}$ (Seger *et al.*, 1991) and $p44^{erk1}$ (Charest, Mordret, Harder, Jirik, and Pelech, unpublished data) undergo weak autophosphorylation in vitro on tyrosyl

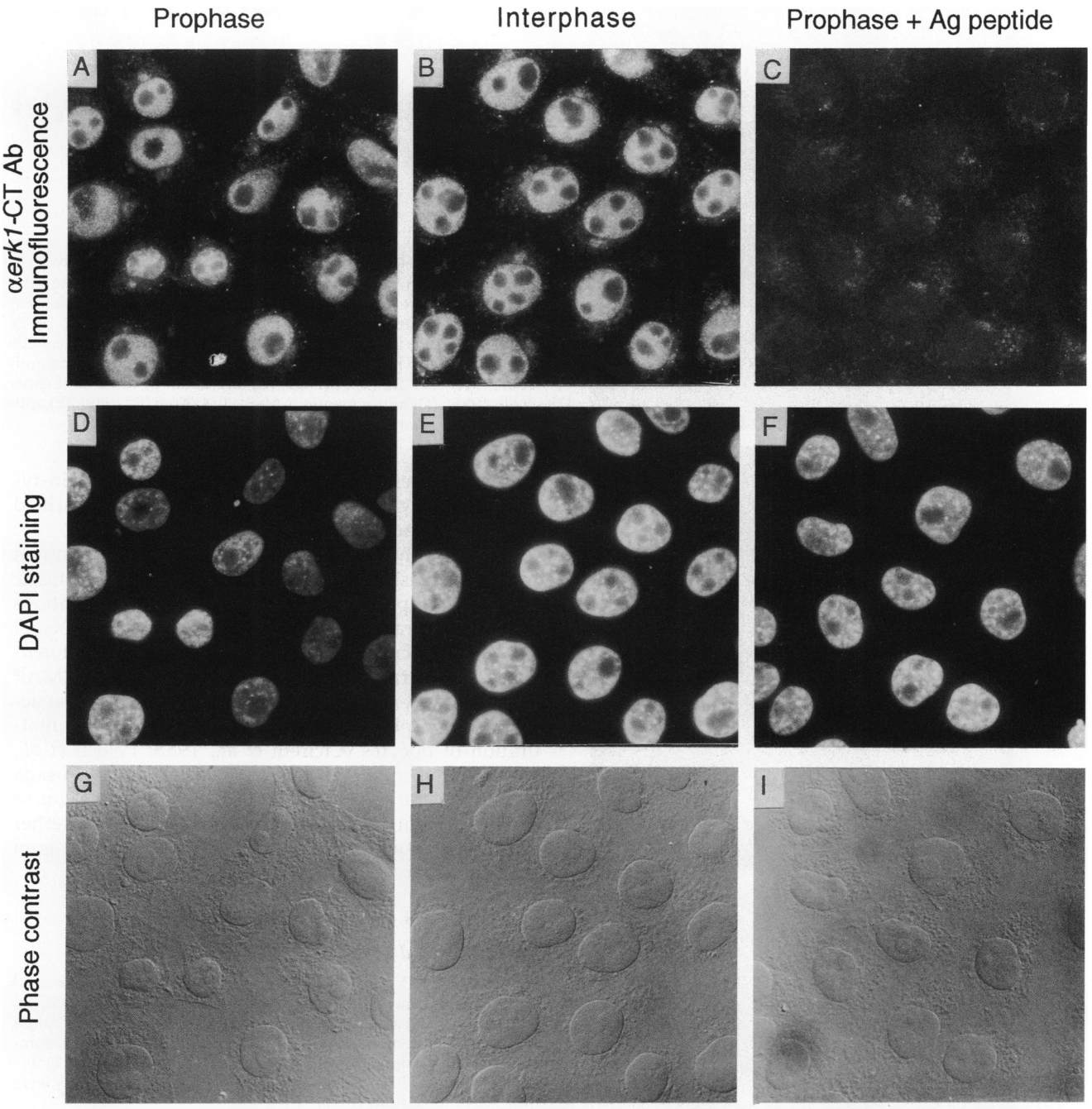

Figure 2. Immunofluorescent analysis of chick DU249 cells with MAP kinase antibody. Exponentially growing cells predominantly in prophase (A, C, D, F, G, and I) or in other stages of interphase (B, E, and H) were fixed with paraformaldehyde dye, permeabilized with Triton X-100 (Nigg *et al.*, 1985). Subsequently they were incubated with affinity-purified antibodies to erk1-CT (A–C). (C) Fixed cells were also incubated in the presence of the antibodies were visualized using the biotin-streptavidin system from Cappel (see MATERIALS AND METHODS). (D–F) Corresponding double staining with the DNA-dye Hoechst 33528. (G–I) Differential interference control (DIC) images. Note that MAP kinases are predominantly visualized in the nucleus.

and threonyl residues, and this was associated with modest increases in their MBP phosphotransferase activities. However, autophosphorylation and $p42^{mapk}$ activation could be dramatically enhanced by an activator protein (Gomez and Cohen, 1991; Seger *et al.*, 1991; Matsuda *et al.*, 1992). Recently, at least two of these activator proteins were demonstrated to be "MAP kinase kinases" (Seger *et al.*, 1992). Furthermore, purified bac-

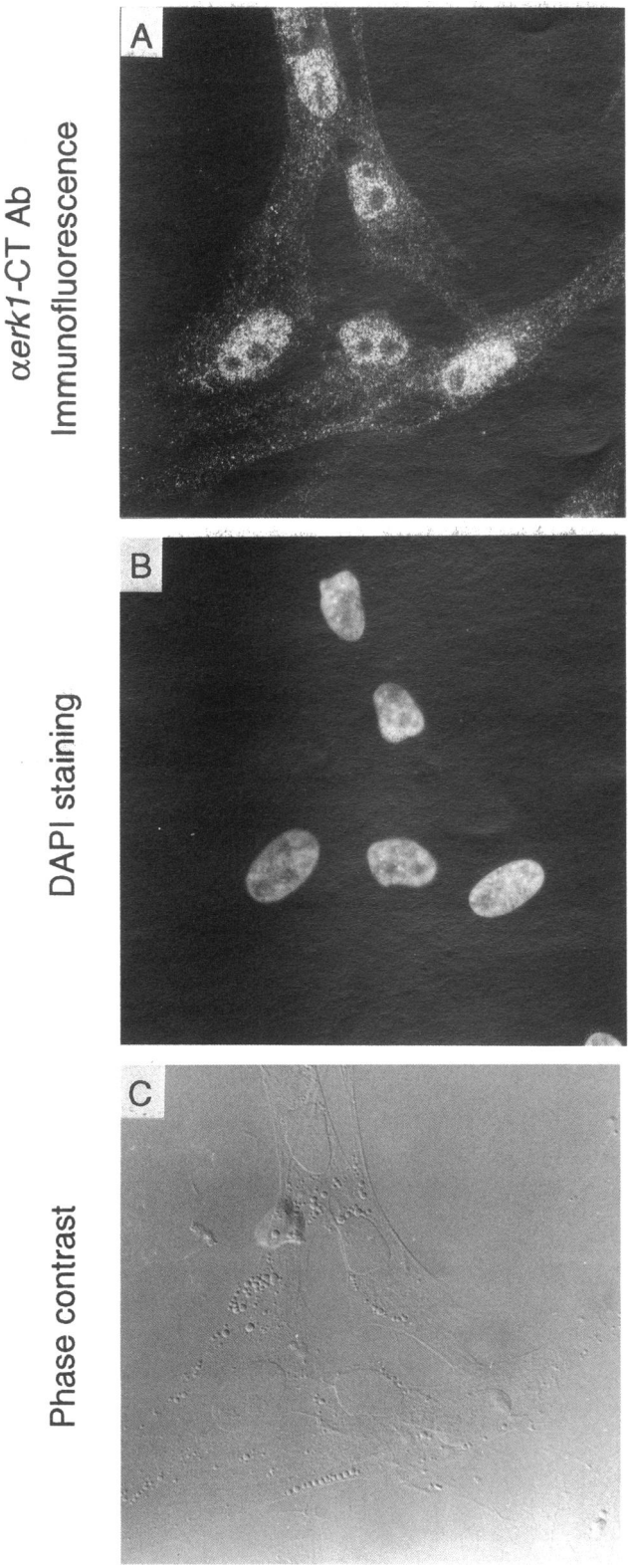

Figure 3. Immunofluorescent analysis of chick embryo fibroblasts with MAP kinase antibody. (A) Primary cell cultures were fixed, permeabilized with Triton X-100 (Nigg *et al.*, 1985) and incubated with affinity-purified antibodies to erk1-CT as described in the legend to Figure 2. (B) Corresponding double staining with the DNA-dye Hoechst 33528. (C) Differential interference control (DIC) image.

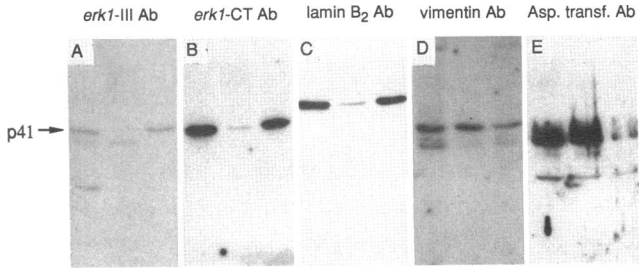

Figure 4. Subcellular fractionation of chick DU249 cells and Western blot analysis with MAP kinase antibodies. Exponentially growing chick DU249 cells were either unfractionated (U) or separated into nuclear (N) and cytoplasmic (C) fractions with enucleation (Krek *et al.*, 1992). Proteins (~2 μg/lane) were separated on a 10% SDS-polyacrylamide gel, blotted onto nitrocellulose, and probed with the following antibodies: (A) erk1-III; (B) erk1-CT; (C) anti-lamin B_2 mAb E3 (Lehner *et al.*, 1986); (D) anti-vimentin mAb Sk 1/4 (Kouklis, 1989); (E) anti-cytoplasmic aspartate aminotransferase (Behra *et al.*, 1981).

ulovirus-expressed murine $p56^{lck}$, a *src*-like protein-tyrosyl kinase, can phosphorylate and activate purified $p44^{mpk}$ (Ettehadieh *et al.*, 1992).

Although the MAP kinases are more widely known for their transient activation when quiescent somatic cells are prompted to re-enter the cell cycle, stimulations of nonproliferating cells such as T lymphocytes (Nel *et al.*, 1990), macrophages (Casillas *et al.*, 1991), and neuronal cells (Stratton *et al.*, 1991) evoke increased MAP kinase activity. Furthermore, MAP kinases are also activated at M-phase during resumption of meiotic maturation of oocytes (Cicirelli *et al.*, 1988; Pelech *et al.*, 1988; Ferrell *et al.*, 1991; Gotoh *et al.*, 1991a,b; Posada *et al.*, 1991; Sanghera *et al.*, 1991a,b). During the course of the present study, we have investigated whether MAP kinases are activated similarly during mitosis in somatic cells.

MATERIALS AND METHODS

Materials and Cells

Bovine brain MBP, nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, and most other reagents were purchased from Sigma (St. Louis, MO). Goat anti-rabbit IgG conjugated to alkaline phosphatase were procured from Bio-Rad (Richmond, CA). Complete and Incomplete Freund's Adjuvant were from GIBCO (Grand Island, NY). [γ-^{32}P]ATP and PY-20 anti-phosphotyrosine monoclonal antibody were from ICN Biomedicals (Irvine, CA).

Cell Culture and Enucleation

Chick embryo fibroblasts were prepared by trypsinization of skins obtained from 11-d-old chicken embryos and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum (FBS), 1% chicken serum, 2% tryptose phosphate broth, and 1% glutamine and antibiotics (100 U/ml each of penicillin and streptomycin). Chicken hepatoma cells (DU249, Langlois *et al.*, 1974) were cultured as reported previously (Nakagawa *et al.*, 1989).

Enucleation of DU249 cells was carried out as described by Krek

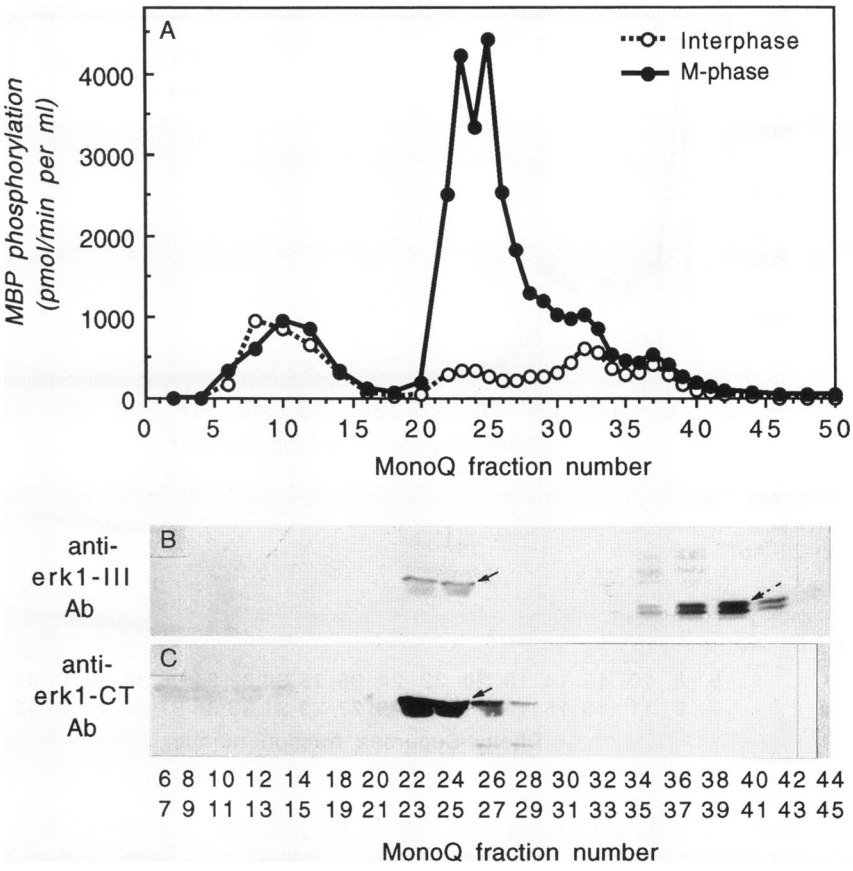

Figure 5. MonoQ chromatography of cytosolic nocodazole-stimulated MBP kinases from chick DU249 cells. Cytosolic protein (1.0 mg) from interphase cells (○) and cells exposed to 300 ng/ml nocodazole for 8 h (●) were subjected to MonoQ chromatography as described in MATERIALS AND METHODS, and the column fractions were assayed for phosphotransferase activity towards 1 mg MBP/ml (A). Adjacent column fractions were pooled from the MonoQ run with cytosol from M-phase cells blocked with nocodazole, and subjected to Western blotting analysis with anti-erk1-III peptide antibodies (B), and anti-erk1-CT peptide antibodies (C). The top and bottom of each box correspond to the electrophoretic migrations of the prestained marker proteins ovalbumin (50K) and carbonic anhydrase (33K), respectively. The positions of the putative 41- and 37-kDa MAP kinases are indicated with solid and broken arrows, respectively. Similar results were obtained in two separate experiments.

et al., (1992). Briefly, cell monolayers were grown in round (18 mm diam) plastic supports that had been cut from tissue culture dishes. Cells were washed twice in prewarmed DMEM (GIBCO) placed cell side down into Corex glass tubes containing 10 ml of prewarmed DMEM (without serum) with cytochalasin B (5 μg/ml) and immediately centrifuged for 8 min at 30–37°C (9500 rpm; in a Sorvall HB 4 rotor). The extent of enucleation was monitored by subsequent staining of DNA with Hoechst dye 33258 (Boehringer Mannheim, Indianapolis, IN). In parallel, cytoplasts as well as the corresponding nuclear pellets were solubilized with 3× sample buffer, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the distribution of the individual proteins was monitored by immunoblotting.

Indirect Immunofluorescence

Indirect immunofluorescent experiments were performed essentially as described (Nigg *et al.*, 1985; Riabowol *et al.*, 1989). Briefly, cells were grown on coverslips for 48 h and fixed with 3% paraformaldehyde and 2% sucrose, for 5 min. They were then rinsed with phosphate-buffered saline (PBS), permeabilized for 5 min with cold 0.5% Triton X-100 in PBS, rinsed again with PBS, and incubated for 1 h at room temperature with affinity purified anti-MAP kinase antibodies (final titre of 1:4000 in PBS). Subsequently, cells were washed with PBS and incubated for 30 min with anti-rabbit IgGs coupled to streptoavidin (1:50 in PBS). After several washes with PBS, cells were further incubated for 30 min with biotinylated IgGs coupled to FITC.

Hoechst dye 33258 (Boehringer Mannheim) was used at a dilution of 1:1000 from a 5 mg/ml stock solution. Coverslips were mounted in 90% glycerol, 10% tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 9.0, and were viewed at a 40× magnification, with a Reichert-Jung Polyvar fluorescence microscope.

Subcellular Fractionation and Column Chromatography

Chick DU249 cells were grown to ~70% confluency before they were activated with 320 nM PMA for 20 min. Mitotic cells were obtained by mechanical shake off, after addition of 300 ng/ml nocodazole for 8 h. Cells were washed twice with ice-cold PBS and immediately frozen in liquid nitrogen. Subsequently, the thawed cells were sonicated in 0.5 ml of buffer containing 20 mM Mops (pH 7.2), 60 mM sodium β-D glycerol phosphate, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM EDTA, 1 mM sodium orthovanadate, 50 μg/ml leupeptin, 0.5% aprotinin, and 1 mM phenylmethanesulfonyl fluoride. Samples were centrifuged at 100 000 × *g* for 1 h at 4°C to obtain a cytosolic fraction. The supernatants were quickly frozen and stored at −70°C in aliquots.

For MonoQ chromatographies, ~1 mg cytosolic protein was loaded onto a MonoQ (Pharmacia) (1 ml) column equilibrated in column buffer A (25 mM β-glycerol phosphate, 10 mM Mops, pH 7.2, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM sodium orthovanadate), at a flow rate of 1 ml/min. The column was developed with a 10 ml linear 0–0.8 M NaCl gradient in buffer A at 1 ml/min with a fast protein liquid chromatography (FPLC) system (Pharmacia LKB Biotechnology, Piscataway, NJ), and 250-μl fractions were collected.

For polylysine-agarose chromatographies, ~1 mg cytosolic protein was applied to a polylysine-agarose (2 ml) column equilibrated in buffer B (12.5 mM Mops, pH 7.2, 12.5 mM β-glycerol phosphate, 5 mM EGTA, 7.5 mM $MgCl_2$, 0.05 mM NaF, 1 mM dithiothreitol), at a flow rate of 1 ml/min. The column was developed with a 20 ml linear 0–0.8 M NaCl gradient in buffer B at 1 ml/min with a Pharmacia FPLC system, and 250-μl fractions were collected.

For phenyl-Superose chromatographies, ~1 mg cytosolic protein was loaded onto a phenyl-Superose (1 ml) column equilibrated in

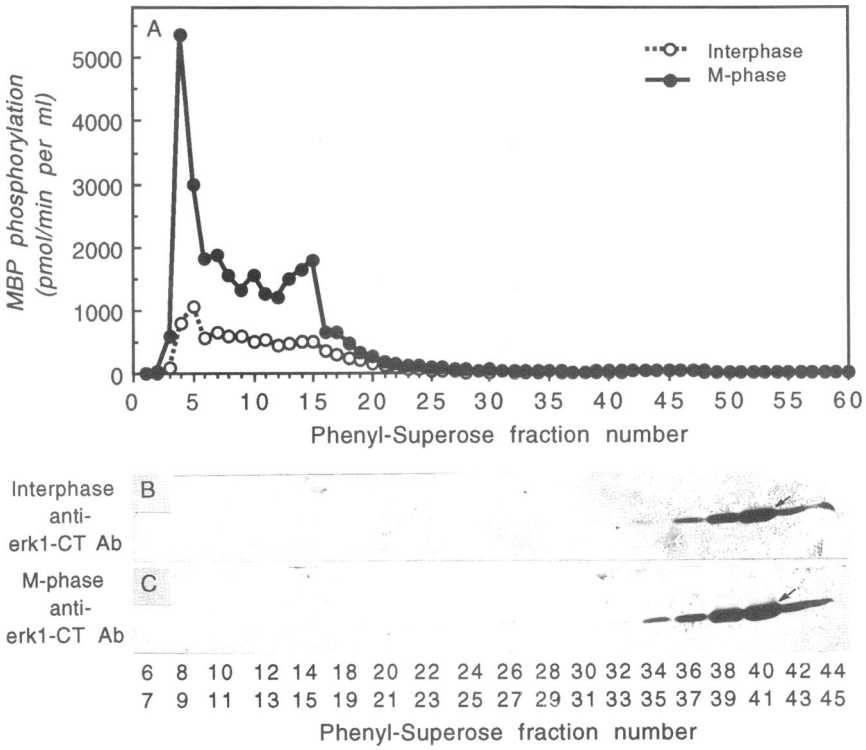

Figure 6. Phenyl-Superose chromatography of cytosolic nocodazole-stimulated MBP kinases from chick DU249 cells. Cytosolic protein (1.0 mg) from interphase cells (○) and cells exposed to 300 ng/ml nocodazole for 8 h (●) was subjected to phenyl-Superose chromatography as described in MATERIALS AND METHODS, and the column fractions were assayed for phosphotransferase activity toward 1 mg MBP/ml (A). Adjacent column fractions were pooled from phenyl-Superose runs of extracts from interphase cells (B) and M-phase cells blocked with nocodazole (C), and subjected to Western blotting analysis with anti-erk1-CT peptide antibodies. The top and bottom of each box correspond to ~75 and ~35 kDa, respectively. The position of the putative 41-kDa MAP kinase is indicated with a solid arrow. Similar results were obtained in two separate experiments.

buffer B containing 250 mM NaCl, at a flow rate of 0.2 ml/min. The column was developed with a 15 ml linear 0–60% ethylene glycol, 250–25 mM decreasing NaCl gradient in buffer B at 0.2 ml/min with a Pharmacia FPLC system, and 250-μl fractions were collected.

Binding to $p13^{suc1}$-agarose beads was assessed by incubating 0.25 ml of each MonoQ column fractions with $p13^{suc1}$-agarose beads for 30 min in an ice bath. Subsequently, the beads were pelleted by centrifugation in a microfuge and the supernatant removed. The pellet was washed in bead buffer (buffer B containing 0.2% Nonidet P-40 and 250 mM NaCl) and then resuspended in buffer B before measurement of protein kinase activity.

Kinase and Protein Assays

MBP phosphorylating activity was assayed as described previously (Sanghera *et al.*, 1991a). With bovine serum albumin as a standard, the protein concentrations of the chick cell extracts were determined by the method of Bradford (1976).

Electrophoresis

SDS-PAGE was performed on 1.5-mm thick gels with the buffer system described by Laemmli (1970). An 11% separating gel and a 4% stacking gel were used. Samples were boiled for 5 min in the presence of 5× concentrated SDS-sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 0.01% bromophenol blue, 10% mercaptoethanol, and 20% glycerol) and electrophoresed for 17 h at 10 mA.

Production of Kinase Antibodies

Synthetic peptides were selected on the basis of the amino acid sequences of rat *erk1* subdomain III (*erk1*-III, PFEHQTYCQRTLREIQILLGFRHENVIGIRDILRAP-GGC) and its C-terminus (*erk1*-CT, CGG-PFTFDMELDDLPKERLKELIFQETARFQPGAPEAP) (Boulton *et al.*, 1990); the ATP-binding site of sea star $p44^{mpk}$ (*mpk*-I, GLAYIGEGAYGMVC) (Posada *et al.*, 1991); subdomain III of human *cdc2* (PSTAIRE, EGVPSTAIREGVLKE) (Lee and Nurse, 1987); and the C-terminus of chicken *cdc2* (serum R6, Krek and Nigg, 1989). The New Zealand White rabbits were immunized subcutaneously at four sites with ~500 μg of KLH-coupled peptide emulsified in complete Freund's adjuvant (1 ml final volume). Rabbits were subsequently boosted every 4 wk intramuscularly at two sites with 500 μg of KLH-coupled peptide emulsified in incomplete Freund's adjuvant. Ear bleeding was performed 2 wk after each boost. The blood was permitted to clot at 37°C for 30 min and then incubated at 4°C overnight to allow the clot to contract. The antiserum was removed and stored at −20°C. The thawed antiserum were later loaded on the appropriate peptide-agarose column, and the anti-peptide antibodies were eluted from the resin in a solution 0.1 M glycine, pH 2.5, which was subsequently neutralized with saturated Tris. Antibody titers were estimated by standard enzyme-linked immunosorbent assay (ELISA) techniques. The erk1-III, erk1-CT, and PSTAIRE antibodies are now commercially available through Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-$p44^{mpk}$ antibodies, affinity purified on a $p44^{mpk}$-agarose column, were prepared as described (Sanghera *et al.*, 1991a).

Immunoblotting

Column chromatography fractions of DU249 cell cytosol were subjected to SDS-PAGE, and subsequently the separating gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 5 min and then sandwiched with a nitrocellulose membrane. Proteins were then transferred for 3 h at 300 mA. Subsequently, the nitrocellulose membrane was blocked with TBS (Tris-buffered saline) containing 5% skim milk or 5% BSA (for phosphotyrosine blots) for 2 h at room temperature. The membrane was washed twice with TBS containing 0.05% Tween 20 (TTBS) for 5 min before incubation with rabbit polyclonal anti-antibodies for MAP and *cdc2* kinases (in 1% skim milk-TTBS; 1:1000 dilution) or mouse monoclonal anti-phosphotyrosine antibody (in 1% BSA-TTBS; 1:1000 dilution) overnight at room temperature. The next day, the membrane was washed twice with TTBS before incubation with the second antibody (goat anti-

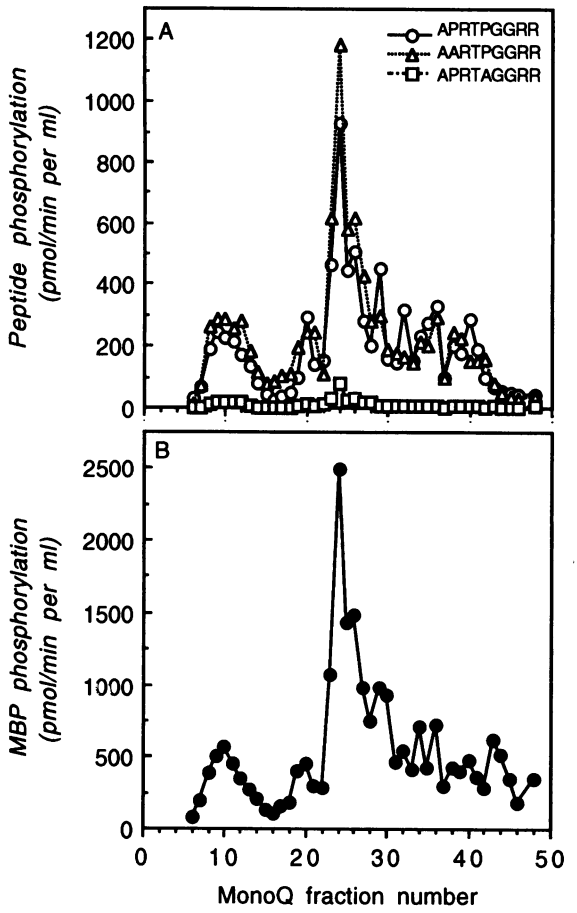

Figure 7. MonoQ chromatography of cytosolic nocodazole-stimulated MBP peptide kinases from chick DU249 cells. Cytosolic protein (1.0 mg) from cells exposed to 300 ng/ml nocodazole for 8 h was subjected to MonoQ chromatography as described in MATERIALS AND METHODS. Column fractions were assayed for phosphotransferase activity towards 2 mM concentrations of the synthetic peptides: APRTPGGRR (○), AARTPGGRR (△), and APRTAGGRR (□) (A), and 1 mg MBP/ml (●) (B). Similar results were obtained in two separate experiments.

rabbit IgG or goat anti-mouse IgG coupled to alkaline phosphatase in 1% skim milk-TTBS or 1% BSA-TTBS, respectively; 1:3000 dilution) for 2 h at room temperature. The membrane was rinsed with 2 washes of TTBS, followed by 1 wash with TBS before incubation with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium color development solution (mixture of 3% nitro blue tetrazolium in 1 ml 70% dimethylformamide and 1.5% 5-bromo-4-chloro-3-indolyl phosphate in 1 ml 100% dimethylformamide before being added to 100 ml of 0.1 M $NaHCO_3$, 10 mM $MgCl_2$, pH 9.8). The color was developed in 5 min to 4 h, and the reaction was stopped by rinsing membrane in a large volume of water.

RESULTS

Isoforms of Avian MAP Kinases

Immunological studies with mammalian cells have implied the existence of a family of MAP kinases (Boulton and Cobb, 1991; Rossomando *et al.*, 1991). In preliminary experiments, we employed a panel of MAP kinase antibodies to probe for avian MAP kinases in MonoQ fractionated cytosolic extracts from proliferating chick DU249 cells (Figure 1). Two of the polyclonal antibodies, which were raised in rabbits against synthetic peptides modeled after the kinase catalytic subdomain I region of sea star $p44^{mpk}$ (anti-mpk-I) and the C-terminus of rat $p44^{erk1}$ (anti-erk1-CT), weakly immunoreacted with a 41- to 42-kDa protein doublet that eluted just after the wash through fractions from the MonoQ column (i.e., fractions 6–15) (Figure 1, B and D). (The 41- to 42-kDa doublet is more evident with anti-erk1-CT antibody in Figure 5C.) These MonoQ fractions also contained the major peak of MBP phosphotransferase activity in these interphase cells (Figure 1A). The two aforementioned anti-peptide antibodies also strongly reacted with a 40- to 41-kDa protein (p41) doublet in MonoQ fractions 22–27, which was approximately coincident with the second peak of MBP phosphotransferase activity. p41 was by far the most prominent protein evident with anti-erk1-CT antibody in the entire Western blot of the various MonoQ fractions. Although barely visible in Figure 1C (see also Figure 5B), p41 could be detected with a third polyclonal anti-peptide antibody based on the kinase subdomain III region of rat $p44^{erk1}$ (anti-erk1-III). The MonoQ elution behavior of p41 and its size on SDS-polyacrylamide gels as visualized with anti-erk1-CT antibody were consistent with its assignment as the avian homologue of $p42^{mapk}$ (Ettenhadieh *et al.*, 1992; Okuda *et al.*, 1992). This isoform is known be activated in mammalian cells that are exposed briefly to phorbol ester tumor promoters (Rossomando *et al.*, 1991). After MonoQ chromatography of extracts from DU249 cells that had been pretreated for 20 min with 320 nM phorbol myristate-13-acetate (PMA), a stimulated peak of MBP phosphotransferase activity indeed coeluted with p41 (Figure 1A). Western blot analysis of the MonoQ fractions from PMA-treated cells with the antiphosphotyrosine monoclonal antibody PY-20 did reveal a tyrosyl phosphorylated protein that comigrated with p41 (Figure 1E).

The major immunoreactive protein with the anti-erk1-III antibody on whole Western blots of the MonoQ fractions of cytosol from DU249 cells was a 36- to 37-kDa doublet (p37) that eluted in fractions 37–42 (Figure 1C). This protein eluted in the right shoulder of the fourth MBP phosphotransferase activity peak from MonoQ, but there was no evidence for any changes in the behavior of p37 on Western blots nor enhancement of MBP kinase activity after PMA treatment. A similar size protein that cofractionated with p37 on MonoQ was strongly detected with the anti-mpk-I antibody (Figure 1B) but not with anti-erk1-CT antibody (Figure 1D) nor with PY-20 antibody (Figure 1E). Although several other proteins could be detected with the anti-mpk-I antibody on Western blots of the MonoQ fractions, their specific assignment as MAP kinases as op-

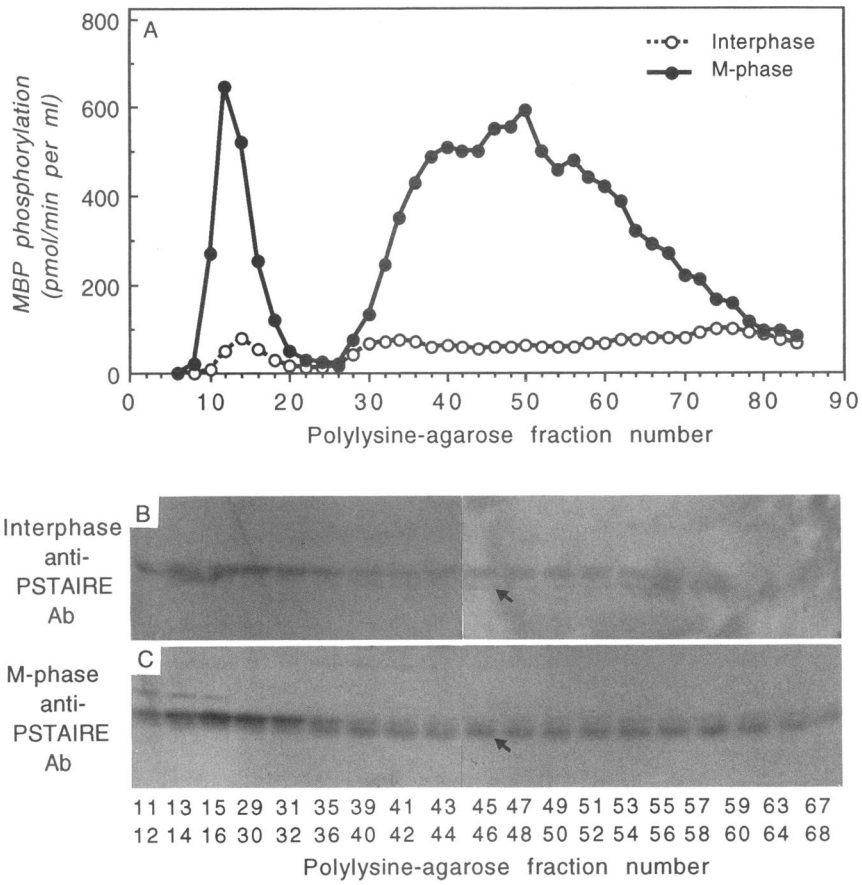

Figure 8. Polylysine-agarose chromatography of cytosolic nocodazole-stimulated MBP kinases from chick DU249 cells. Cytosolic protein (1.0 mg) from interphase cells (○) and cells exposed to 300 ng/ml nocodazole for 8 h (●) was subjected to polylysine-agarose chromatography as described in MATERIALS AND METHODS, and the column fractions were assayed for phosphotransferase activity toward 1 mg MBP/ml (A). Adjacent column fractions were pooled from polylysine-agarose runs of extracts from interphase cells (B) and M-phase cells blocked with nocodazole (C) and subjected to Western blotting analysis with anti-PSTAIRE peptide antibodies. There was an increase in the relative background on these immunoblots due to the dilution of the predominant antigen over so many fractions. The top and bottom of each box correspond to ~45 and ~25 kDa, respectively. The expected position of the dephosphorylated form of $p34^{cdc2}$ is indicated with a solid arrow. Similar results were obtained in two separate experiments.

posed to other protein kinases is equivocal, because several of the residues in subdomain I, on which the production of anti-mpk-I antibody was based, are highly conserved in protein kinases (Hanks *et al.*, 1988).

Subcellular Distribution of MAP Kinases

The anti-erk1-CT antibody was found to be highly selective for p41 in DU249 cell cytosol (Figure 1). Therefore, it was feasible to exploit this immunological reagent to explore the subcellular distribution of the putative 41-kDa MAP kinase in DU249 cells in immunocytochemistry studies (Figure 2). This antibody yielded somewhat similar immunofluorescent staining patterns for DU249 cells in primarily prophase (Figure 2A) and at various stages of interphase (Figure 2B). By far most of the immunoreactive protein was located in the nucleus of these cells, as revealed by DNA staining with Hoechst dye 33258 (Figure 2, D–F). In control experiments, where the cells were incubated without primary antibodies or in the presence of 0.5 mg/ml erk1-CT peptide (Figure 2C), the nuclear staining was abolished. A nuclear distribution for MAP kinases was also evident for primary cultures of chick embryo fibroblasts, although cytoplasmic enzyme was also discernable (Figure 3). Intense nuclear staining was also observed in DU249 cells and chick embryo fibroblasts that were probed with affinity-purified rabbit antibodies raised against purified sea star $p44^{mpk}$ and the anti-erk1-III antibody.

To confirm that the 41-kDa MAP kinase was located in the nuclei of DU249 cells, a second strategy was adopted in which cells were enucleated by cytochalasin B treatment and subsequent centrifugation. On the one hand, most of the p41 immunoreactive protein with both anti-erk1-III and anti-erk1-CT antibodies distributed with the nuclei-enriched material, as did lamin B2, which served as positive control for a nuclear protein (Figure 4). However, some p41 immunoreactivity with both antibodies was evident in the cytoplasts, consistent with the Western analysis of MonoQ-fractionated DU249 cell cytosol in Figure 1, C and D. On the other hand, p37 that was revealed with anti-erk1-III antibody was only found in the nuclei-depleted cytoplasts, as was aspartate aminotransferase, which functioned as a positive control for a cytosolic enzyme (Figure 4).

Characterization of an M-Phase-Activated MBP Kinase

Previous studies have documented the marked activation of sea star $p44^{mpk}$ and *Xenopus* $p42^{mapk}$ at M-phase

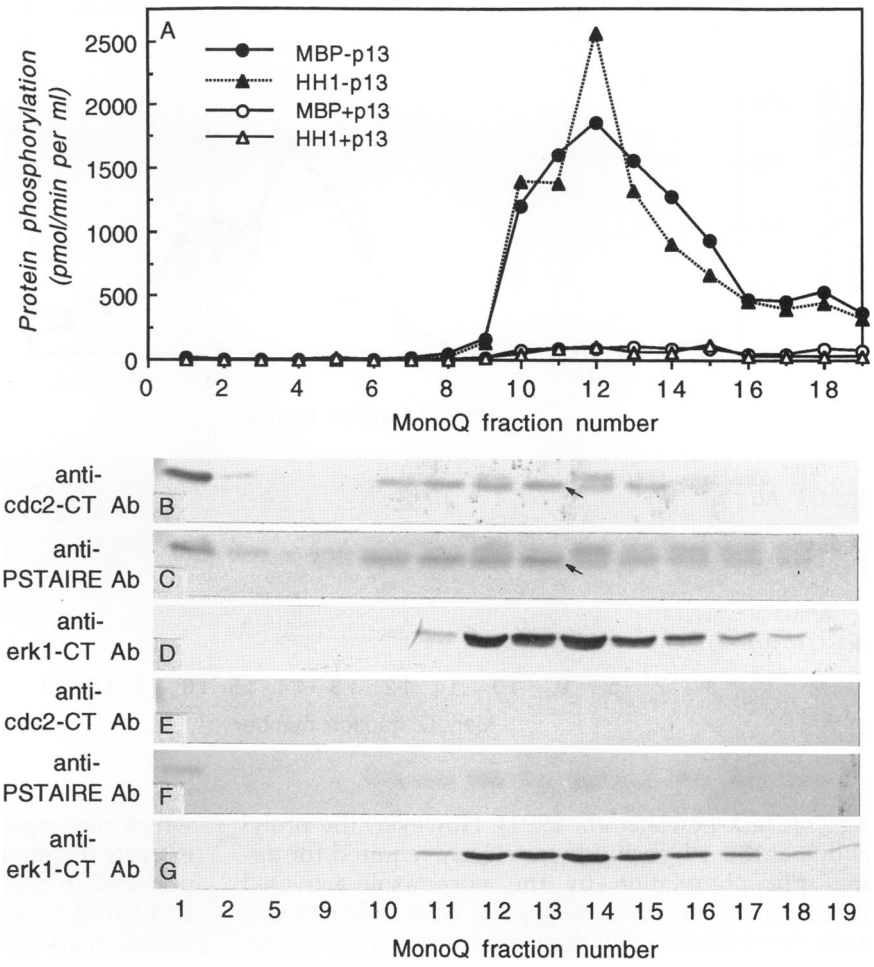

Figure 9. $p13^{suc1}$ depletion of nocodazole-stimulated MBP kinase from MonoQ fractions of chick DU249 cells. Cytosolic protein (1.0 mg) from cells exposed to 300 ng/ml nocodazole for 8 h was subjected to MonoQ chromatography as described in MATERIALS AND METHODS, except that 0.5-ml fractions were collected. Subsequently, 0.25 ml of each column fraction was incubated with $p13^{suc1}$-agarose beads for 30 min. The column fractions were assayed for phosphorylating activity toward 1 mg MBP/ml (○, ●) and 1 mg histone H1/ml (△, ▲) before (●, ▲) and after incubation with $p13^{suc1}$-agarose (○, △) (A). The MonoQ column fractions before (B–D) and after incubation with $p13^{suc1}$-agarose (E–G) were subjected to Western blotting analysis with anti-cdc2-CT antibodies (B and E), anti-PSTAIRE antibodies (C and F) and anti-erk1-CT antibodies (D and G). The top and bottom of B, C, E, and F correspond to the electrophoretic migrations of the prestained marker proteins carbonic anhydrase (33K) and soybean trypsin inhibitor (28K), respectively. The position of $p34^{cdc2}$ is indicated with a solid arrow. (D and G) Region of the immunoblots between ~45K and 35K. Similar results were obtained in two separate experiments.

during meiotic maturation of oocytes (Cicirelli *et al.*, 1988; Pelech *et al.*, 1988; Ferrell *et al.*, 1991; Gotoh *et al.*, 1991a,b; Posada *et al.*, 1991; Sanghera *et al.*, 1990, 1991a,b). To investigate the possible activation of MAP kinases during mitotic M-phase in somatic cells, nocodazole was used to block DU249 cells in early M-phase. After MonoQ chromatography of cytosolic extracts from nocodazole-arrested cells as compared with interphase cells, MAP kinase assays revealed a >10-fold stimulation of an MBP phosphotransferase activity in column fractions that contained p41 (detected with anti-erk1-CT) (Figure 5).

Despite the correlation between nocodazole-stimulated MBP kinase activity and p41 immunoreactivity on MonoQ (Figure 5), these events were separable by phenyl-Superose chromatography (Figure 6). In this instance, p41 bound extremely tightly to phenyl-Superose, as has been reported for $p42^{mapk}$ from mammalian cells (Rossomando *et al.*, 1991). By contrast, most of the nocodazole-stimulated MBP phosphotransferase activity was not retained by the phenyl-Superose column or interacted weakly. Another finding that tended to discount p41 as the nocodazole-stimulated MBP kinase was the lack of immunoreactivity of this protein after MonoQ and phenyl-Superose chromatographies with the anti-phosphotyrosine antibody PY-20. $p42^{mapk}$, $p44^{erk1}$, and $p44^{mpk}$ are characteristically activated as a consequence of tyrosyl phosphorylation and a stimulation of this magnitude is usually accompanied by strong immunoreactivity with anti-phosphotyrosine antibody (Anderson *et al.*, 1990; Boulton *et al.*, 1991a; Posada *et al.*, 1991; Sanghera *et al.*, 1991a,b).

We have recently demonstrated with $p44^{mpk}$ and synthetic peptides patterned after the Thr-97 phosphorylation site in MBP the importance of prolyl residues at the −2 and +1 positions for substrate recognition for phosphorylation by MAP kinases at the 0 position (Clark-Lewis *et al.*, 1991). When the MonoQ fractions from nocodazole-treated DU249 cells were assayed with a synthetic peptide, i.e., APRTPGGRR, which is an efficient substrate for $p44^{mpk}$, an identical kinase activity profile to that obtained with MBP was observed (Figure 7). A peptide analogue, i.e., AARTPGGRR, in which the prolyl residue at the −2 position was replaced with an alanyl residue was an equally efficient substrate (Figure 7A), even though it was poorly phosphorylated by

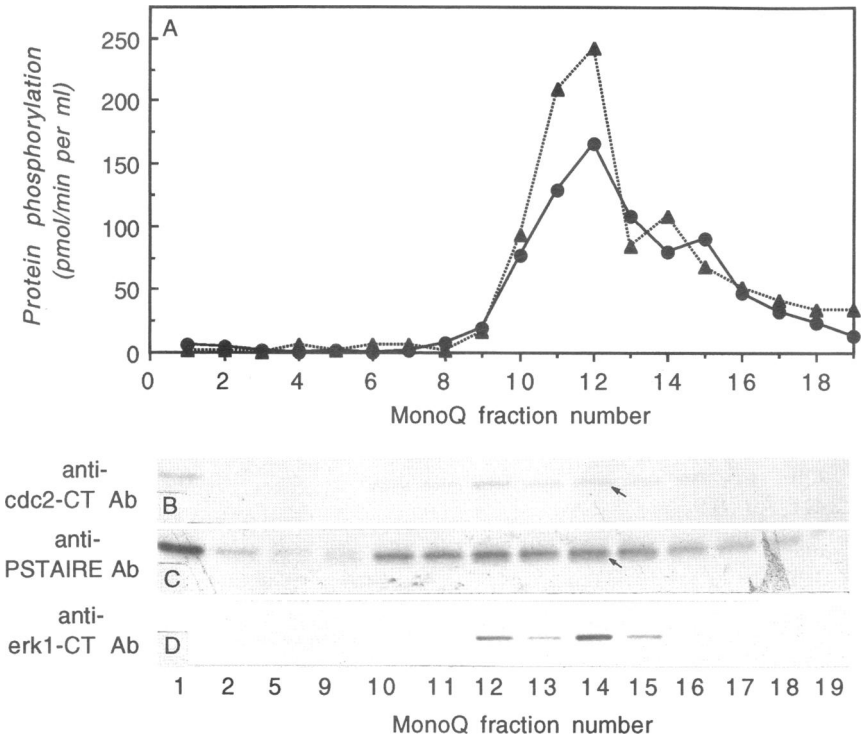

Figure 10. p13^{suc1} Binding of nocodazole-stimulated MBP kinase from MonoQ fractions of chick DU249 cells. Two hundred and fifty-microliter aliquots of the MonoQ column fractions shown in Figure 9A were incubated with p13^{suc1}-agarose beads for 30 min. The p13^{suc1}-agarose beads were suspended and assayed for phosphorylating activity toward 1 mg MBP/ml (●) and 1 mg histone H1/ml (▲) (A). Western blotting was performed with anti-cdc2-CT antibodies (B), anti-PSTAIRE antibodies (C) and anti-erk1-CT antibodies (D). The top and bottom of B and C correspond to the electrophoretic migrations of the prestained marker proteins carbonic anhydrase (33K) and soybean trypsin inhibitor (28K), respectively. The position of p34^{cdc2} is indicated with a solid arrow. (D) region of the immunoblots between ~45K and 35K. Similar results were obtained in two separate experiments.

p44^{mpk} (Clark-Lewis *et al.*, 1991). However, the prolyl residue at the +1 position was clearly required for optimal phosphorylation by the nocodazole-activated protein kinase, because the peptide APRTAGGRR was barely phosphorylated in these experiments (Figure 7A). Analysis of the major MBP phosphotransferase activity peaks after chromatography of the nocodazole-treated cell extracts on phenyl-Superose and polylysine-agarose (see below) with the MBP peptide analogues similarly established the importance of the prolyl residue at the +1 position and the dispensibility of the prolyl residue at the −2 position.

The requirement of a prolyl residue at the +1 position is also a characteristic of cyclin-dependent protein kinases such as cdc2-encoded protein kinase (p34^{cdc2}), which is known to be activated in nocodazole-blocked HeLa cells (Draetta and Beach, 1988). The nocodazole-stimulated MBP phosphotransferase activity could be resolved by polylysine-agarose chromatography into a narrow peak that weakly bound to this resin and a very broad peak that interacted more tightly (Figure 8A). To test whether the major MBP kinase corresponded to a cyclin-dependent protein kinase, the polylysine-agarose column fractions were immunoblotted with anti-PSTAIRE antibody, which recognizes a sequence of residues in the catalytic subdomain III region that is highly conserved in cyclin-dependent kinases (Lee and Nurse, 1987). In these experiments, coincident with the broad major MBP kinase peak, the anti-PSTAIRE antibody detected a 33- to 34-kDa protein doublet, for which the upper band predominated in the interphase extracts (Figure 8B) and the lower band was more pronounced in the M-phase extracts (Figure 8C). Such a band shift to a lower apparent molecular weight is a typical marker of the tyrosyl dephosphorylation and activation of p34^{cdc2} (Draetta and Beach, 1988).

To further test whether the nocodazole-activated MBP kinase was the avian homologue of p34^{cdc2} in the DU249 cells, the MonoQ fractions in a repeat of the experiment shown in Figure 5 were assayed with histone H1, a routinely used substrate for p34^{cdc2}. As illustrated in Figure 9A, histone H1 appeared to be an equally effective substrate for the activated MBP kinase. Moreover, the MonoQ fractions with peak of histone H1/MBP phosphotransferase activity featured a 33 kDa immunoreactive protein that was detectable with antisera raised against a peptide based on the C-terminus of avian p34^{cdc2} (cdc2-CT) (Krek and Nigg, 1989), as well as by the anti-PSTAIRE antibody (Figure 9, B and C). Incubation of the MonoQ fractions with p13^{suc1}-agarose beads, which exhibit a high affinity for p34^{cdc2}, resulted in a depletion of the histone H1/MBP phosphotransferase activity (Figure 9A) and immunoreactivity to the p34^{cdc2}-specific antibodies (Figure 9, E and F). The bulk of the immunoreactivity to the anti-erk1-CT antibody in the MonoQ fractions was not removed by the p13^{suc1}-agarose beads (Figure 9, D and G). Although only about a tenth of the starting histone H1/MBP phosphotransferase activity was recovered on the p13^{suc1}-agarose beads (Figure 10A), they contained nearly all of the im-

munoreactive 33-kDa protein with the PSTAIRE and cdc2-CT antibodies (Figure 10, B and C). By contrast, there was relatively little p41 detectable with the erk1-CT antibody, and it did not coincide with the major peak of histone H1 kinase activity (Figure 10D). We concluded that the major nocodazole-activated MBP kinase in the chick DU249 cells was none other than $p34^{cdc2}$.

DISCUSSION

In this study, we sought to characterize the major MAP kinases in an avian cell line. One of these, i.e., p41, was extremely reminiscent of $p42^{mapk}$ with respect to its immunoreactivity with MAP kinase antibodies and size on SDS-polyacrylamide gels, its MonoQ and phenyl-Superose chromatographic behavior, and its sensitivity to activation as a consequence of cell exposure to the phorbol ester PMA. Another potential MAP kinase of 41- to 42-kDa eluted in the MonoQ wash through fractions of DU249 cell cytosol and immunoreacted with several distinct MAP kinase antibodies. This putative MAP kinase has not previously been reported, although we have detected it near the MonoQ wash through fractions of cytosolic extracts from numerous mammalian cells including rat neurons, macrophages, B- and T-cells, and human A431 cells (unpublished data). As yet, we have not identified a stimulus that leads to enhancement of its MBP phosphotransferase activity in any model system. A third potential MAP kinase, i.e. p37, was detected with at least one MAP kinase antibody and bound much more tightly to MonoQ than p41, but it was not activated after phorbol ester-treatment of DU249 cells. In view of the limited characterization of p37, its assignment as a MAP kinase remains equivocal. We have no strong evidence from chick DU249 cells for the 44 kDa MAP kinase(s) detected in mammalian cells (Boulton and Cobb, 1991; Rossomando *et al.*, 1991), which eluted with and/or slightly after $p42^{mapk}$ on MonoQ.

As part of the characterization of avian MAP kinases, we investigated their subcellular distribution. Through immunofluorescent staining analysis with three different MAP kinase antibodies and enucleation experiments with cytochalasin B, it was revealed that p41 was predominently nuclear in its distribution, but somewhat excluded from structures that resembled nucleoli (Figures 2–4). Similar observations have also been independently made by Blenis and his colleagues with HeLa cells (Chen *et al.*, 1992). In this regard, it is most significant that several potentially physiological substrates for MAP kinases are known to be nuclear. For example, the *rsk*-encoded S6 kinase, which is phosphorylated and activated by various MAP kinases (Sturgill *et al.*, 1988; Chung *et al.*, 1991b), also localizes to the nucleus (Blenis, 1991). Several oncogene-encoded transcription factors including *c-jun* (Alvarez *et al.*, 1991; Pulverer *et al.*, 1991), *c-myc* (Alvarez *et al.*, 1991), and *c-myb* (Luscher and Pelech, unpublished data) are in vitro substrates of MAP kinases. Finally, $p44^{mpk}$ and $p42^{mapk}$ have been shown to phosphorylate nuclear lamin B_2 at Ser-16, and this facilitates disassembly of lamin polymers in vitro (Peter *et al.*, 1992).

A strong case can be advanced for a role for MAP kinases in the regulation of M-phase events in the meiotic maturation of echinoderm and amphibian oocytes (Cicirelli *et al.*, 1988; Pelech *et al.*, 1988). By contrast, so far there is no clear evidence that MAP kinases are modulated during mitotic M-phase, and reports of MAP kinase activation appear to be restricted to cells found in G_0 to G_1 transition. After fertilization of *Xenopus* eggs, $p42^{mapk}$ is rapidly tyrosyl dephosphorylated concomitant with loss of its MBP phosphotransferase activity, and neither appear to be recovered at M-phase in the subsequent mitotic cell divisions (Ferrell *et al.*, 1991; Posada *et al.*, 1991). A previous report (Gotoh *et al.*, 1991b) of reactivation of the MBP phosphotransferase activity of $p42^{mapk}$ during the first mitotic M-phase in fertilized *Xenopus* eggs can now be interpreted in light of the results of the present study to be instead due to stimulation of $p34^{cdc2}$. Indeed, MBP was almost as effective as a substrate for $p34^{cdc2}$ as was histone H1 (Figures 9 and 10).

We have exploited nocodazole treatment of DU249 cells to facilitate their arrest at the beginning of M-phase. This protocol results in a marked stimulation of the histone H1 phosphotransferase activity of $p34^{cdc2}$ in mammalian cells (Draetta and Beach, 1988). $p34^{cdc2}$ is activated concomitantly or slightly before MAP kinases in maturing sea star and frog oocytes (Cicirelli *et al.*, 1988; Pelech *et al.*, 1988). However, we failed to detect any activation of MAP kinase in nocodazole-blocked DU249, even though it was clearly present in the nucleus of these cells. We cannot eliminate the possibility that MAP kinases participate at an earlier or latter stage in M-phase, just outside the nocodazole arrest point. In consideration of the very transitory activation of MAP kinases that accompany mitogenic stimulation of quiescent cells (Pelech *et al.*, 1988, Sturgill and Wu, 1991), M-phase modulation of MAP kinases could be equally brief and technically difficult to detect.

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