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Activation of the Mitogen-Activated Protein Kinase Pathway in Triton X-100 Disrupted NIH-3T3 Cells by p21 ras and In Vitro by Plasma Membranes from NIH 3T3 Cells

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We describe a novel Triton-disrupted mammalian cell system wherein the pathways for activation of mitogen-activated protein (MAP) kinases (MAPKs) are capable of direct biochemical manipulation in vitro. MAPKs $p42^{mapk}$ and $p44^{mapk}$ are activated in signal transduction cascade(s) initiated by occupancy of plasma membrane receptors for peptide growth factors, hormones, and neurotransmitters. One likely activation pathway for MAPKs consists of sequential activations of c-ras, c-raf-1, and a protein-tyrosine/threonine kinase, MAP kinase kinase. Triton-disrupted cells retained capacity for activation of the pathway by both peptide growth factors and by addition of GTP-loaded p21 ras^{Val12}. Incubation of disrupted cells with an antibody that neutralized the function of c-ras (Y13-259) abolished receptor-mediated stimulation of MAPK as did acute addition of 200 μM azatyrosine. Activation of the pathway was reconstituted in a cell-free system using high-speed supernatants generated from Triton-disrupted cells together with purified plasma membranes from parental cells and as a heterogeneous system using purified plasma membranes from v-ras–transformed cells. These systems will allow biochemical dissection in vitro of the interaction(s) between c-ras and the MAPK pathway in mammalian cells.

INTRODUCTION

Mitogen-activated protein (MAP) kinases (MAPKs) are proline-sequence-directed Ser/Thr-kinases that require phosphorylation at closely spaced threonine and tyrosine residues for full enzymatic activation (reviewed in Pelech and Sanghera, 1992; Posada and Cooper, 1992). MAPKs participate in protein kinase cascades important for signal transduction in response to many extracellular stimuli. The best characterized MAPKs in mammalian cells are $p42^{mapk}$/ERK2 (Boulton *et al.*, 1991; Her *et al.*, 1991) and $p44^{mapk}$/ERK1 (Boulton *et al.*, 1990), which share amino acid sequence similarity (83% identity), regulatory phosphorylation in a TEY motif (Payne *et al.*, 1991; Rossomando *et al.*, 1991), and optimal substrate specificity for -Pro-X-Ser/Thr-Pro- in peptide/protein substrates (Alvarez *et al.*, 1991; Clark-Lewis *et al.*, 1991). Less is known about p55 MAPK (Kyriakis *et al.*, 1991), which is distinguished by size and substrate specificity and is likely to have distinct intracellular targets and regulation.

$p42^{mapk}$ and $p44^{mapk}$ are activated in vitro by MAP kinase kinase (MAPKK), a protein-tyrosine/threonine kinase (Ahn *et al.*, 1991); MAPKK is also activated by phosphorylation but at serine/threonine residues (Gomez and Cohen, 1991) in response to growth factors and phorbol esters (Rossomando *et al.*, 1992; Seger *et al.*, 1992a). The kinetics of activation of MAPKK suggest that this enzyme activates MAPKs in vivo (Cohen *et al.*, 1992). The primary structures of MAPKK from rat (Wu *et al.*, 1993), mouse (Crews *et al.*, 1992), human T cells (Seger *et al.*, 1992b), and rabbit (Ashworth *et al.*, 1992) have been determined recently by sequencing cloned cDNAs from these species.

Activation of $p42^{mapk}$ and $p44^{mapk}$ by peptide growth factors appears to involve eukaryotic p21 c-ras at an early step. Exogenous introduction of v-ras into some types of intact cells activates the MAPK cascade (Gallego

et al., 1992; Leevers and Marshall, 1992; Pomerance *et al.*, 1992). Dominant negative mutants of p21 ras can be constructed by replacing Ser17 with Asn (Feig and Cooper, 1988). This mutation inhibits GDP-GTP exchange; consequently, the expressed protein cannot be converted normally to the active form and inhibits the action of endogenous c-ras. Inhibition of ras may also be achieved experimentally by overexpression of the GTPase-activating protein (GAP) (Trahey and McCormick, 1987). Expression of p21 ras^{Asn17} in PC12 cells blocked activation of p42/p44 MAPKs by nerve growth factor (Robbins *et al.*, 1992; Thomas *et al.*, 1992; Wood *et al.*, 1992) and phorbol esters (Thomas *et al.*, 1992; Wood *et al.*, 1992). Similarly, overexpression of GAP in NIH 3T3 fibroblasts also blocked activation of p42/p44 MAPKs by both types of stimuli (Nori *et al.*, 1991). In contrast, de Vries-Smits *et al.* (1992) reported that expression of ras^{Asn17} blocked activation of p42/p44 MAPKs in fibroblasts by insulin and platelet-derived growth factor (PDGF) but not activation by phorbol esters. Thus, ras appears to mediate growth factor activation of the MAPK cascade, but the role of protein kinase C is not yet resolved.

Recently, it has been demonstrated that addition of p21 ras · GTP or p21 ras^{Val12} · GTP to crude low-speed extracts prepared from *Xenopus* oocytes, in the presence of an ATP-regenerating system, activated MAPK (Hattori *et al.*, 1992; Shibuya *et al.*, 1992). In the present study, we have examined the involvement of p21 ras on the activation of both MAPKK and MAPK using a mammalian system that is amenable to biochemical manipulation. We find that addition of GTP-loaded p21 ras^{Val12} to Triton-disrupted NIH 3T3 cells rapidly activates both MAPKK and MAPK within 10 min. Anti-ras monoclonal antibody Y13-259, but not Y13-238, prevents activation of MAPK by insulin and PDGF. Furthermore, the MAPK cascade can be reconstituted using 100 000 × *g* supernatants prepared from Triton-disrupted cells and plasma membranes from parental cells or a heterogeneous system using plasma membranes from v-ras–transformed cells.

MATERIALS AND METHODS

Materials

The vectors for expression of c-Ha-*ras* and v-Ha-*ras* in *Escherichia coli* (Gibbs *et al.*, 1984) were given to us by Dr. J. Gibbs (Merck, West Point, PA). v-ras NIH 3T3 cells and NIH 3T3 HIR3.5 cells were generously provided by Drs. L. Feig (Tufts University School of Medicine, Boston, MA) and Johnathan Whittaker (Department of Medicine, State University of New York at Stony Brook, Stony Brook, NY). Azatyrosine was provided by Dr. Kwen-Jen Chang (Burroughs Wellcome, Research Triangle Park, NC). Reagent-grade Triton X-100, creatine kinase, and creatine phosphate were obtained from Sigma-Aldrich (St. Louis, MO). Recombinant PDGF was purchased from Amgen (Thousand Oaks, CA). Recombinant human insulin was manufactured by Eli Lilly (Indianapolis, IN).

Antibodies

Hybridomas for Y13-259 and Y13-238 monoclonal antibodies (Furth *et al.*, 1982) were obtained from the American Type Culture Collection (Rockville, MD). Hybridoma cells were grown in serum-free medium for antibody production. Antibodies were purified to homogeneity by protein-G affinity chromatography and equilibrated with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.5, 150 mM NaCl.

Cell Culture

Cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal calf serum (GIBCO, Grand Island, NY) in an atmosphere of 10% CO_2 before use.

Preparation of Triton-disrupted Cells

Procedures used were modified from protocols developed by G. Romero (unpublished data). Cells were washed twice with Krebs-Ringer HEPES buffer (Ray and Sturgill, 1987) and serum starved for 16 h in DMEM. Medium was aspirated from 10 plates (100 mm) of confluent cells. Cells were washed and then scraped into phosphate-buffered saline and pooled. The pooled cells were isolated as a cell pellet and transferred to a conical 1.5-ml microfuge tube. The volume of the cell pellet (500–1000 μl) was estimated by comparison to quantities of buffer in an equivalent tube. The cells were resuspended in an equal volume of chilled buffer, 10 mM HEPES, pH 7.4 at 4°C, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 50 μg/ml leupeptin, 50 μg/ml pepstatin, 1 mM benzamidine/HCl, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5% (vol/vol) Triton X-100, by gentle agitation. The resuspended cells were disrupted using a Tissue Tearor (model 985-370, Fisher Scientific, Pittsburgh, PA) at a setting of one for 5 s. Equal portions (200–300 μl) of Triton-disrupted cells were distributed into tubes for the various experimental manipulations. Triton-disrupted cells were placed on ice (1 h) before use. Comparable activation of MAPKK and MAPK by agonists occurred with either immediate (20 min) or delayed (1 h) use.

Purification of ras and GTP Loading

p21 ras^{Val12} (and also p21 c-ras) was expressed as a fusion protein in *E. coli* containing plasmid pUCEJ, essentially as described in Farnsworth *et al.* (1991). The expressed protein was purified to near-homogeneity (>90% pure) by successive Mono Q and Superose 12 (Pharmacia LKB, Piscataway, NJ) chromatographies, concentrated, and stored in portions at −70°C.

The p21 ras^{Val12} (and p21 c-ras) protein (16 μg) was loaded with GTP in a final volume of 5 μl, containing 12.5 mM tris-(hydroxymethyl)aminomethane (Tris)/HCl (pH 7.5 at 4°C), 2.5 mM GTP, 5 mM EDTA, 0.1 mM EGTA, for 60 min on ice. Mock samples for control additions (see below) were provided by omitting all components but the p21 ras^{Val12} protein.

Purification of Plasma Membranes from NIH 3T3 Fibroblasts

Plasma membranes were prepared as described (Luttrell *et al.*, 1990) except for the following modifications. Overconfluent NIH 3T3 cells transformed with v-ras (10 plates, 100 mm) were washed and scraped into 50 ml of phosphate-buffered saline. The cells were pelleted (1000 × *g*, 5 min), and the cell pellet was resuspended and homogenized in 5 ml of 50 mM HEPES, pH 7.6 at 4°C, 8.6% (wt/vol) sucrose, 10 mM EDTA, 10 mM EGTA, 1 mM PMSF, 40 μg/ml leupeptin, 0.4 mg/ml egg-white trypsin inhibitor, 40 μg/ml pepstatin (buffer A), by 15 strokes in a tight-fitting Dounce homogenizer. The homogenate was layered onto 7 ml of buffer A containing 39% (wt/vol) sucrose and spun at 31 000 × *g* for 30 min in an SW41 rotor (Beckman, Palo Alto, CA). The plasma membrane fraction was collected from the top of the 39% sucrose layer, diluted 1:5 with buffer A (minus sucrose), and respun at 40 000 × *g* for 60 min to pellet the plasma membranes. The pellet was resuspended in buffer A (minus sucrose) to 3–4 mg/

ml total protein, aliquoted, frozen in liquid nitrogen, and stored at −70°C.

In Vitro Stimulation with Agonists and Chromatography

Immediately before stimulation with agonists, additions (5 μl total) were made to each portion of Triton-disrupted cells to provide an ATP-regenerating system consisting of (final concentrations) 5 mM creatine phosphate, 100 μM ATP, and 80 μg/ml creatine kinase. Cells were incubated with or without an agonist (PDGF [50 nM], insulin [1 μM], 75 ng/μl p21 ras^{Val12} [final concentrations]) at 37°C for 10 min with intermittent mixing. Control cells received mock samples omitting p21 ras^{Val12}. Portions of a stock solution of azatyrosine (2 mM in phosphate-buffered saline) or vehicle (control) were added to yield a final concentration of 200 μM azatyrosine where indicated. After 10 min of stimulation, each sample was made 25 mM EDTA, 5 mM EGTA, 25 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM Na_3VO_4 by addition of 10 μl of a concentrated stock, followed by centrifugation for 5 min at 14 000 × *g*. The protein concentrations of the resulting supernatants were measured, and 1 mg of protein from each condition was subjected to Mono Q (HR5/5) anion-exchange chromatography for separation of MAPKK and MAPK activities essentially as described in Dent *et al.* (1992). The flow rate was 0.5 ml/min and 1.0 ml fractions were collected. The NaCl gradient for elution is shown in each figure.

Assays

MAPKK was measured in a single-stage assay by its ability to stimulate the myelin basic protein (MBP)-phosphotransferase activity of recombinant $p42^{mapk}$ essentially as described in Wu *et al.* (1992). MAPK was measured after partial purification by Mono Q chromatography (see legends) to resolve MAPK from other MBP kinases. Assays comprised 10 μl sample, with or without 0.1 μg recombinant $p42^{mapk}$, were initiated by addition of 30 μl of buffer containing MBP substrate (0.5 mg/ml final) and γ^{32}P-ATP (0.15 mM final, 5000 cpm/pmol), and incubated at 30°C as described previously (Dent *et al.*, 1992; Wu *et al.*, 1992). Assays were terminated after 50 min (120 min in Figure 5) by aliquoting 30 μl of reaction mixture onto P81 paper followed by immersion in 180 mM phosphoric acid. Filter papers were washed and counted in scintillant. Results shown are the average of values (differing by <15%) determined from two to six experiments performed on different days with different cellular preparations.

Microscopy and Photography

Suspensions of NIH 3T3 cells that had been disrupted in Triton X-100 were diluted as for kinase assays except using one volume of 3% (wt/vol) formaldehyde, 2% (wt/vol) glutaraldehyde in 200 mM Na cacodylate, pH 7.4. After fixing for 2.5 h at 4°C, the suspension was gently pelleted and postfixed overnight in 1% (wt/vol) OsO_4 in 0.1 M Na cacodylate. The pellets were washed in 0.15 M NaCl (3 × 15 min), bisected longitudinally, and stained with 0.5% (wt/vol) magnesium uranyl acetate in saline. Pellets were then dehydrated in acetone followed by propylene oxide and embedded in Spurr's resin using standard procedures. After polymerization for 48 h at 65°C, sections (<1000 Å) were cut, stained with 10% (wt/vol) uranyl acetate in methanol and 0.25% lead citrate, and observed and photographed using a Jeol 100C electron microscope (Jeol USA, Peabody, MA).

RESULTS

Activation of MAPKK by p21 ras·GTP in Triton-disrupted cells

Characterization of the earliest steps in the MAP kinase cascade in mammalian cells would be greatly facilitated by development of broken cell systems amenable to biochemical manipulation and fractionation. Generation of semiintact cells by gentle homogenization in the presence of Triton X-100 was explored as a method likely to preserve membrane-dependent responses.

$p42^{mapk}$ is specifically phosphorylated and activated by MAPKK (Seger *et al.*, 1992a). Consequently, MAPKK activity can be assayed in crude lysates by measuring the increase in MAPK activity (assayed as MBP-phosphotransferase activity) in the presence versus the absence of the unphosphorylated B form of recombinant $p42^{mapk}$ (Wu *et al.*, 1991). Addition of p21 ras^{Val12}·GTP to Triton-disrupted NIH 3T3 cells in the presence of an ATP-regenerating system rapidly stimulated MAPKK activity (Figure 1). Near maximal stimulation (6-fold) was observed at the earliest time examined (3 min). The

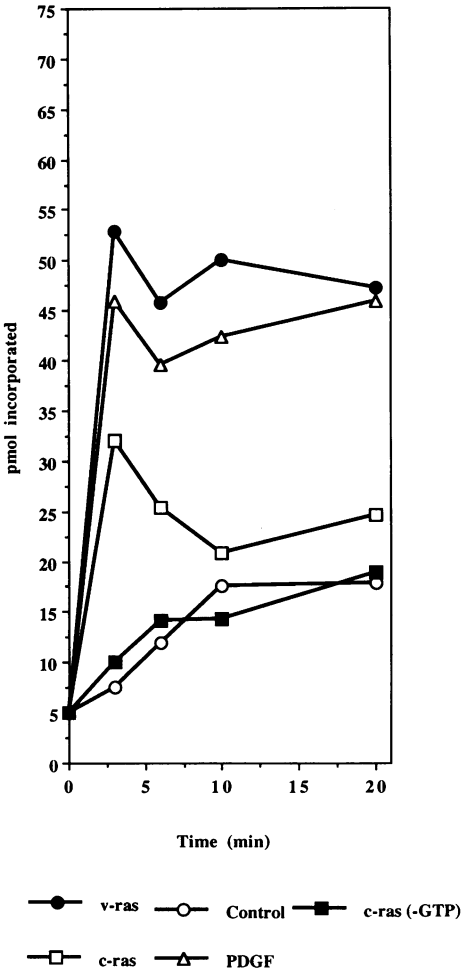

Figure 1. Time course of MAPKK activation by p21 ras^{Val12}, PDGF, GTP-loaded p21 c-ras, and unloaded p21 c-ras in Triton-disrupted NIH 3T3 cells. Triton-disrupted cells were stimulated with either GTP-loaded p21 ras^{Val12} (●), PDGF (△), GTP-loaded p21 c-ras (□), GDP-loaded p21 c-ras (■), or mock sample (○), as described in MATERIALS AND METHODS. At the times indicated, an aliquot from each condition was removed (30 μl), placed on ice, and the kinase reactions quenched as described in MATERIALS AND METHODS. After completion of the time course, each aliquot was spun at 14 000 × *g* for 5 min and 10 μl of the supernatant was diluted 1000-fold in MAPKK assay buffer (Wu *et al.*, 1992) and the MAPKK activity was determined.

time course of activation by p21 ras^{Val12} was virtually identical to that elicited by PDGF and was as rapid as activation in intact cells. In contrast, addition of an equivalent amount of GTP-loaded p21 c-ras caused only a partial activation of MAPKK, which was completely absent when GTP loading was not performed. A time-dependent stimulation (3.5-fold over 20 min) of MAPKK activity occurred in these preparations, independent of any agonist additions. However, this constituted a small percentage of the stimulated MAPKK activity by agonists (15% at 3 min, 35% at 20 min). The persistence of MAPKK activity in the p21 ras^{Val12}- and PDGF-stimulated samples is in contrast to a number of cell lines where MAPKK activity is transient (Gomez and Cohen, 1991; Rossamando *et al.*, 1992). This was in part due to the near linear increase of MAPKK basal activity in control samples. The ATP-regenerating system was one factor required to sustain the agonist-independent MAPKK activity; other reasons for the elevated MAPKK activity in this system have yet to be discovered.

To confirm activation of the MAPK pathway by p21 ras^{Val12}, MAPKK activity and the MBP-phosphotransferase activity of MAPK were measured in fractions from Mono Q chromatography of lysates from Triton-disrupted cells that had been incubated with or without p21 ras^{Val12} · GTP for 10 min (Figure 2). Under the chromatography conditions used, MAPKK was not adsorbed and eluted in fractions 2–5, and the p42/p44 MAPKs were unresolved and eluted together in fractions 10–14 (Figure 2). Elution of both isoforms of MAPK in fractions 10–14 was confirmed by Western blotting. Incubation with p21 ras^{Val12} increased MAPKK 2.5-fold and MAPK 2.8-fold in comparison with activities in corresponding fractions from control cells.

Addition of p21 ras^{Val12} with or without prior GTP-loading was similarly efficacious, implying that significant GTP levels for exchange are also maintained by the ATP-regenerating system. In contrast, stimulation by p21 c-ras appeared to require GTP loading (Figure 1). Consistent with assays of extracts (Figure 1), appreciable MAPKK and MAPK activities were detected in the absence of agonist effectors, exceeding basal activities present in equivalent numbers of serum-starved cells (Figure 2). Because effects of peptide growth factor and p21 ras^{Val12} were observed in Triton-disrupted cells, additional experiments were conducted to characterize them.

Structure of Triton-disrupted Cells

The observed stimulation of MAPKK and MAPK by both receptor binding and exogenous p21 ras^{Val12} addition led us to examine the extent of dispersion of the disrupted cells morphologically. Shearing cells in the presence of 0.25% Triton would be expected to disrupt the plasma membrane, and the extent of solubilization of cellular components is likely to depend on the ratio of detergent mass to cell number. By light microscopy,

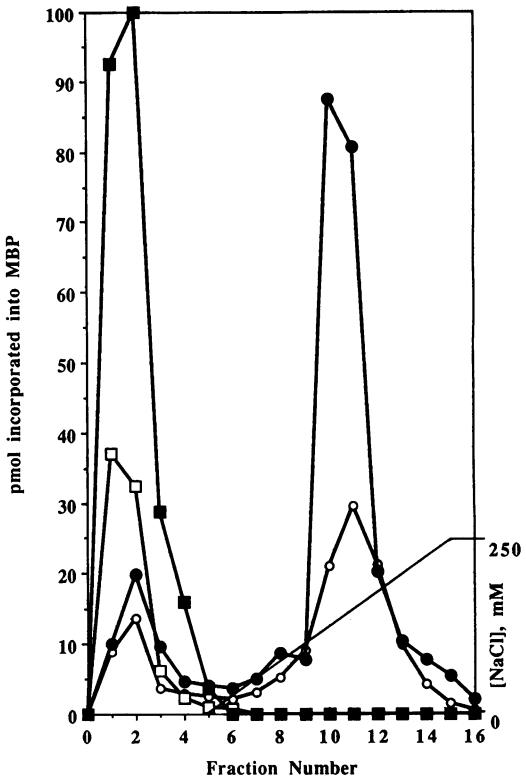

Figure 2. MAPKK and MAPK activities in fractions from Mono Q chromatography of lysates from Triton-disrupted NIH 3T3 cells that had been stimulated by the addition of GTP-loaded p21 ras^{Val12}. Triton-disrupted cells were stimulated with GTP-loaded p21 ras^{Val12} (closed symbols) or mock controls (open symbols), and, after incubation and centrifugation, portions of the supernatant (1 mg total protein) were subjected to Mono Q chromatography (see MATERIALS AND METHODS). MAPKK (■, □); MAPK (●, ○); right ordinate, NaCl gradient. The data represent the average of four separate experiments (stimulation in the range 2.0- to 3.0-fold).

cellular profiles were generally visible using differential interference contrast optics, although variable amounts of residual cellular matrix surrounded the swollen nuclei. There was a background of fine particulates suggesting large-scale release but not solubilization of cellular organelles. All nuclei could be stained by Trypan blue. These observations were borne out by electron microscopic examination of cell suspensions that were identically incubated as samples used for kinase analysis before fixation and preparation for microscopy (Figure 3). As can be seen in the micrographs, plasma membranes are intermittently disrupted (or solubilized) in large segments. No cell examined had an intact plasma membrane. Cellular organelles and cytoskeletal elements are substantially retained within cell profiles but also appeared dispersed beyond cellular boundaries. Separate Western blotting studies indicate that 20–40% of the insulin receptors in NIH 3T3 HIR3.5 cells were solubilized by Triton-disruption and not sedimentable after centrifugation at 100 000 × *g* for 15 min. Accordingly, although there is partial solubilization of mem-

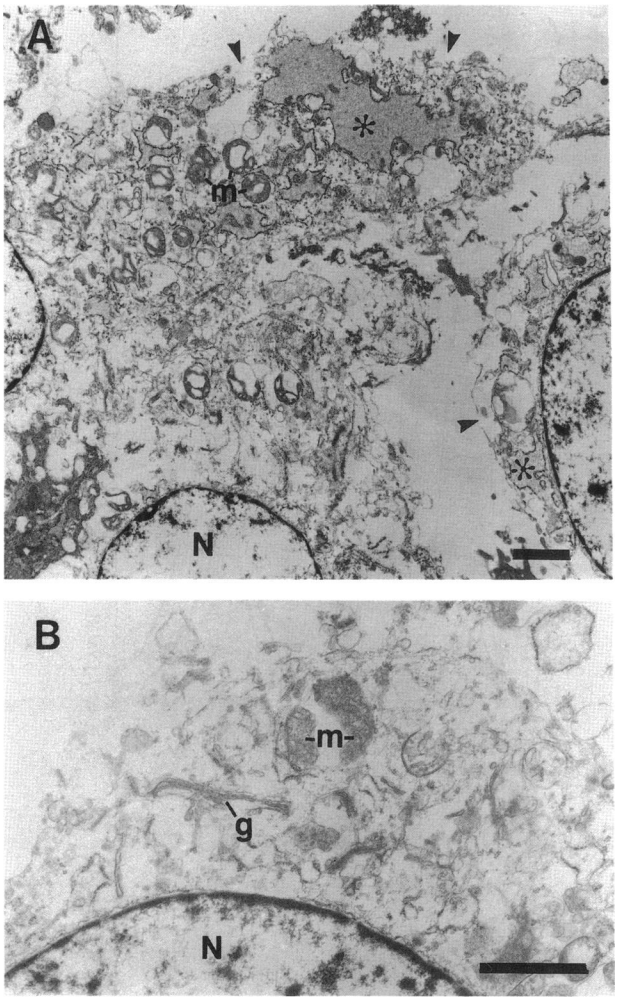

Figure 3. Electron micrographs showing the appearance of NIH 3T3 cells after Triton-disruption as used for in vitro activation of the MAP kinase pathway. At low magnification (A), boundaries of individual cells are recognizable, yet it is clear that extensive segments of plasma membranes and underlying cellular contents are missing (arrows). Nuclei with peripheral heterochromatin are largely retained as are several patches of rough endoplasmic reticulum with interual content (*) and mitochondria (m). At higher magnification (B), the focal plasma membrane disruptions are clear, and extensive extraction of cytoplasmic contents leaves a residual cytoskeleton and associated organelles. N, nucleus; G, stacked Golgi cisternae; m, mitochondria. Bars in A and B, 1 μm.

brane components, the general appearance is semiintact after Triton treatment under the conditions used.

ras-*dependent Activation of the MAP Kinase Cascade in Triton-disrupted Cells by PDGF and Insulin*

The ability of growth factors to stimulate the MAP kinase pathway in Triton-disrupted cells was examined utilizing NIH 3T3 cells and NIH 3T3 cells overexpressing the human insulin receptor (NIH 3T3 HIR3.5) (Figure 4). To this end, MAPKK activity and the MBP-phosphotransferase activity of MAPK were measured in chromatographic fractions from Mono Q chromatography of lysates from agonist-stimulated or control reactions (Figure 4, A and C).

Incubation of Triton-disrupted NIH 3T3 cells for 10 min with PDGF (Figure 4A) increased MAPKK activity 2.7-fold and MAPK activity 2.7-fold. Activation of the latter was accompanied by tyrosine phosphorylation of p42/p44 MAPKs assessed by Western blotting across the fractions. Similarly, incubation of Triton-disrupted NIH 3T3 cells overexpressing the insulin receptor for 10 min with insulin (Figure 4C) increased MAPKK activity 2.6-fold and MAPK activity 2.4-fold. Thus, these primary agonists activate the MAP kinase pathway in the disrupted cells.

Notably, the Triton-disrupted cells have also enabled examination of ras involvement in the MAPK pathway in some detail. Monoclonal antibodies Y13-259 and Y13-238 are well-characterized anti-ras antibodies (Furth *et al.*, 1982). Antibody Y13-259 neutralizes p21 *ras* function by binding to an epitope in close proximity to the putative effector domain, in part by inhibiting GDP exchange (Sigal *et al.*, 1986; Hattori *et al.*, 1987). Microinjection of antibody Y13-259 into NIH 3T3 cells blocks subsequent mitogenic responses to serum and growth factors (Stacey *et al.*, 1988). Microinjection of antibody Y13-259 also reverses transformation caused by v-*src* but, significantly, not transformation by v-*raf*. Although antibody Y13-238 also binds specifically to p21 ras, it does not inhibit p21 ras function when microinjected into cells.

The role of p21 ras in activation of MAPKK and MAPK by growth factors was tested using the anti-ras antibodies (Figure 4, B and D). Triton-disrupted NIH 3T3 cells or NIH 3T3 HIR3.5 cells were incubated on ice together with either antibody Y13-259 or antibody Y13-238 and subsequently stimulated with either PDGF (Figure 4B) or insulin (Figure 4D), respectively. Antibody Y13-259 markedly decreased activation of MAPKK and MAPK by PDGF (Figure 4B, >85%) and insulin (Figure 4D, >90%). Inhibition of PDGF and insulin responses by antibody Y13-259 was specifically due to neutralization of p21 ras function because antibody Y13-238 had no apparent effect on either the PDGF (Figure 4B) or insulin (Figure 4D) stimulated responses.

Effect of Azatyrosine

Azatyrosine is an analogue of tyrosine that was first identified as an agent that caused reversion of v-ras–transformed cells to an apparently normal phenotype (Shindo-Okada *et al.*, 1989). Because azatyrosine has been shown to block activation of MAPK and $p34^{cdc2}$ and maturation of *Xenopus* oocytes (Chung *et al.*, 1991; Campa *et al.*, 1992), we investigated its ability to inhibit the MAPK pathway in this system. Addition of 200 μM (final concentration) azatyrosine acutely with PDGF (10-min incubations) abolished the ability of the growth factor to stimulate both MAPKK and MAPK.

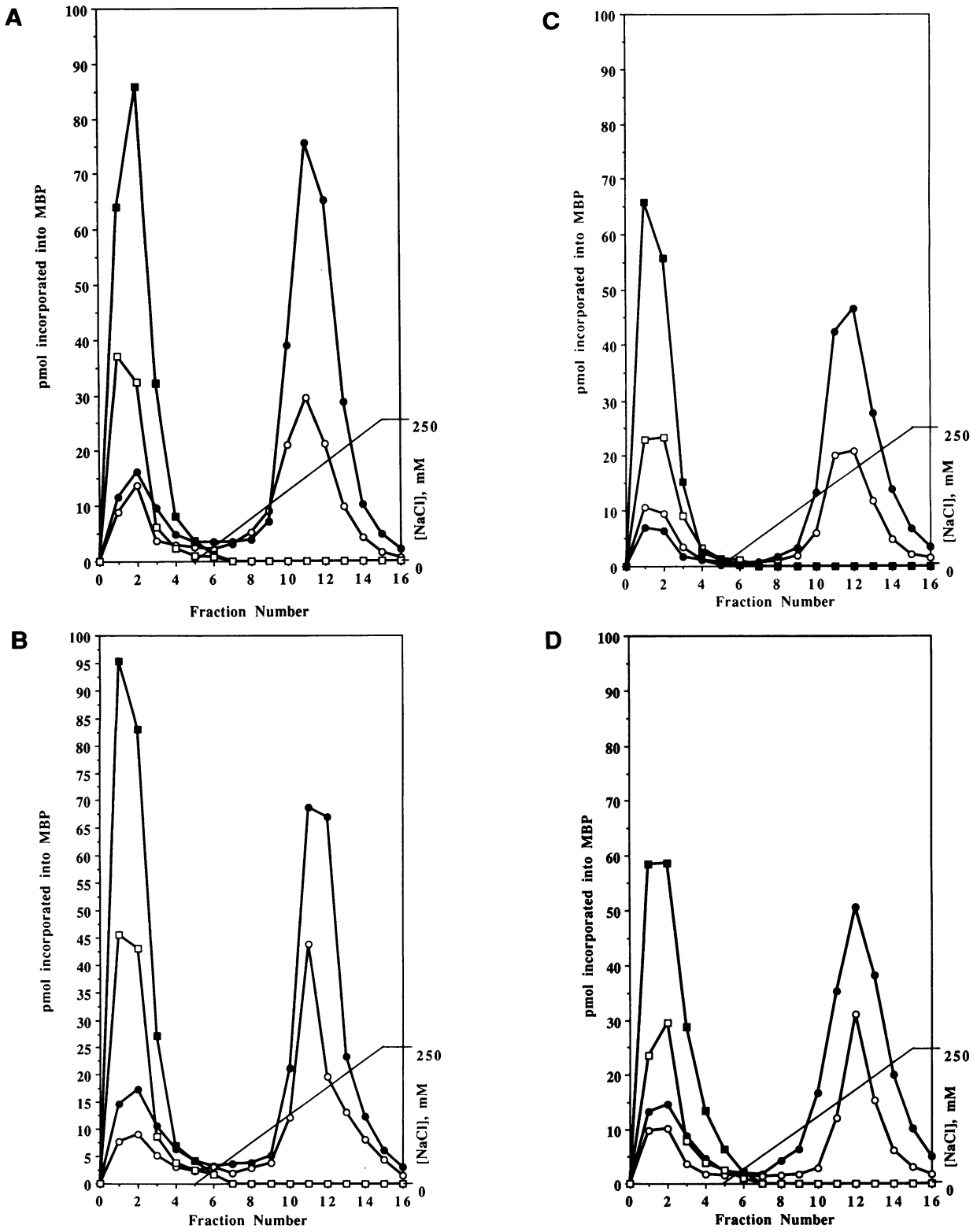

Figure 4. Activation of MAPKK and MAPK in Triton-disrupted NIH 3T3 and NIH 3T3 HIR3.5 cells by PDGF and insulin, respectively, and abrogation by anti-ras antibody Y13-259. (A and C) Triton-disrupted cells were stimulated (closed symbols) with PDGF (A) or insulin (C) or not stimulated (vehicle only; open symbols) and processed by Mono Q chromatography and by assay of fractions for MAPKK (■, □) and MAPK (●, ○) activities (see MATERIALS AND METHODS and Figure 2). (B and D) Triton-disrupted cells were incubated with 50 μg of either anti-ras antibody Y13-238 (closed symbols) or Y13-259 (open symbols) for the 1 h in which the disrupted cells were left on ice. These cells were then incubated with either PDGF (B) or insulin (D) and processed to determine the MAPKK (squares) and MAPK (circles) activities, as above (stimulation in the range 2.0- to 3.0-fold).

Reconstitution of the MAP Kinase Cascade in a Mammalian Cell-Free System with Parental Plasma Membranes and as a Heterogeneous System with Plasma Membranes from v-ras–transformed Cells

p21 ras is normally found in the plasma membrane by immunolocalization studies, anchored to the membrane or to a membrane-associated protein (Gibbs and Marshall, 1989). Efficient membrane localization of p21 ras requires farnesylation at a CAAX motif together with palmitoylation or, alternatively in the case of Ki-ras-4B, a polybasic region (Hancock *et al.*, 1989; Itoh *et al.*, 1993). C-terminal farnesylation at CAAX is required for cellular transformation by oncogenic p21 ras^{Val12} (Kato *et al.*, 1992) and interference by ras^{Asn17} (Feig and Cooper, 1988). The ratio of active GTP bound to inactive GDP bound p21 ras appears to be controlled in vivo by the relative activities of guanine nucleotide exchange proteins and GAPs (Li *et al.*, 1992; Zhang *et al.*, 1992; Medema *et al.*, 1993). ras GAP has also been proposed to be a downstream effector mediating signal transduction from ras (Medema *et al.*, 1993).

After removal of membranes from Triton-disrupted NIH 3T3 cells by centrifugation at 100 000 × *g*, activation of either MAPKK (Figure 5A) or MAPK (Figure 5B) by p21 ras^{Val12}·GTP in the resulting supernatant was reduced by >75% in comparison with an unspun control. Similarly, PDGF and insulin failed to stimulate the MAP kinase pathway in equivalent portions of this supernatant. Thus, activation of MAPKK and MAPK by p21 ras^{Val12}·GTP may require either target and/or accessory factors present in the particulate fraction.

MAPK is constitutively activated in v-ras–transformed NIH 3T3 cells (Gallego *et al.*, 1992) (data not shown). By inference, necessary activating components may be present in plasma membranes from these cells. To test this hypothesis, plasma membrane fractions were isolated from v-ras–transformed NIH 3T3 cells and parental cells by sucrose-density centrifugation. Addition of plasma membranes from v-ras–transformed cells to the 100 000 × *g* supernatant caused activation of MAPKK (Figure 5A) and MAPK (Figure 5B). Addition of GTP-loaded plasma membranes from parental cells also caused activation of MAPKK (Figure 5C) and MAPK (Figure 5D); however when GTP loading was omitted, no stimulation in activity was observed (Figure 5, C and D). Omission of an ATP-regenerating system resulted in little or no stimulation of either MAPKK or MAPK activities.

Stimulation of MBP kinase activity was not due to MBP kinase activity(ies) associated with the plasma membranes for several reasons. First, control assays for total MBP kinase activity associated with the plasma membranes were performed and were insignificant in comparison with the activities measured. Second, the membranes were removed by pelleting before Mono Q chromatography for analysis. Finally, the assay of MAPKK measures the *stimulated* MBP kinase from assays performed in the presence and absence of recombinant $p42^{mapk}$.

To demonstrate definitively that activation of the MAPK pathway by the plasma membranes from NIH 3T3 cells was due to the presence of ras, additional experiments were performed using the monoclonal anti-ras antibodies. Incubation of the plasma membranes with the neutralizing antibody Y13-259 abrogated the ability of the membranes to activate the MAPK cascade. Antibody Y13-238 had no discernable effect on activity (Figure 5). Activation of the cascade in these cell-free systems therefore requires either GTP-loaded c-ras or v-ras and possibly a plasma membrane environment. Thus, we can reconstitute a mammalian cell-free system with GTP-loaded parental plasma membranes or create a heterogeneous system using v-ras–transformed plasma membranes, both of which activate the MAPK cascade in vitro.

DISCUSSION

We have developed a novel Triton-disrupted mammalian cell system that is amenable to biochemical manipulation and have studied the role of p21 ras in MAP kinase activation. As in crude lysates from *Xenopus* oocytes (Hattori *et al.*, 1992; Shibuya *et al.*, 1992), addition of exogenous p21 ras^{Val12}·GTP to Triton-disrupted NIH 3T3 cells, in the presence of an ATP-regenerating system, increased the activity of both MAPKK and MAPK. Activation was reconstituted by addition of purified plasma membranes from GTP-loaded parental cells or in a heterogeneous system with plasma membranes from v-ras–transformed cells.

Considerable evidence exists supporting a requirement for farnesylation of p21 ras for cell transformation (Casey *et al.*, 1989; Hancock *et al.*, 1989) and at least some interactions in vitro with guanine nucleotide exchange proteins (Horiuchi *et al.*, 1992). As bacterially produced p21 ras is not posttranslationally modified, the results obtained in Figures 1 and 2 would appear to be in conflict. However, rapid activation of the MAP kinase pathway by bacterially produced p21 ras has also been observed in the *Xenopus* system (Hattori *et al.*, 1992; Shibuya *et al.*, 1992) and by scrape-loading Swiss 3T3 fibroblasts (Leevers and Marshall, 1992). It is possible that a portion of the added p21 ras, added in excess, is processed to confer activity in these complex systems. Modification would have to be fast and efficacious to account for the observed kinetics. In our system, the added detergent could also conceivably be capable of mimicking in some way the posttranslational modification of ras.

Responses to agonists were preserved in Triton-treated cells and enabled studies of the effects of anti-ras antibody Y13-259. Antibody Y13-259, but not Y13-238, blocked activation of MAPKK and MAPK by insulin and PDGF. These results correlate precisely with results obtained by expression of a dominant-negative

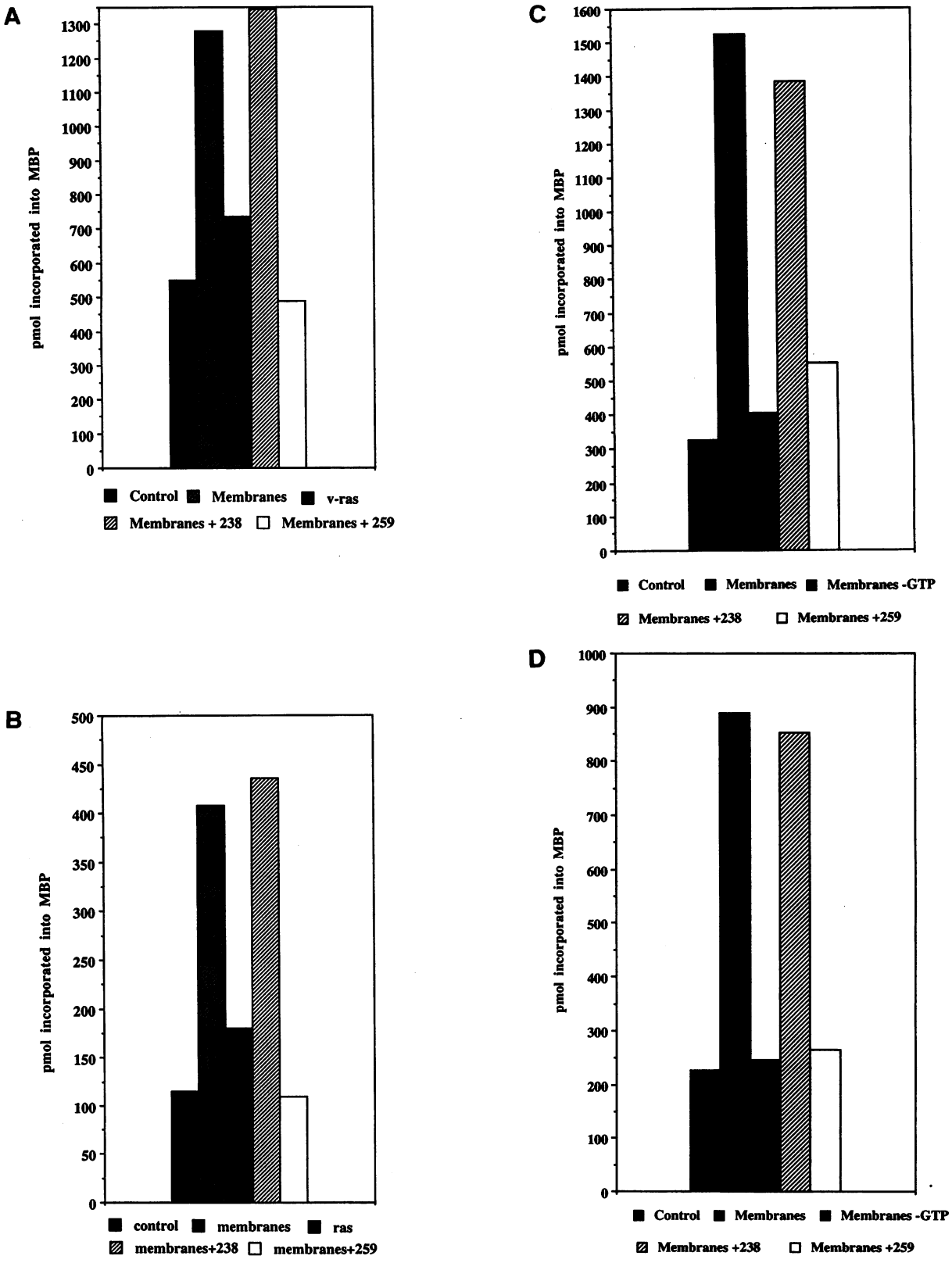

Figure 5. Activation of MAPKK and MAPK in a cell-free system by purified plasma membranes from v-ras–transformed (A and B) and parental NIH 3T3 cells (C and D). Triton-disrupted cells were centrifuged at 14 000 × *g* for 10 min, and the supernatants respun at 100 000 × *g* for 15 min. The resulting supernatants were respun again in an identical manner. Plasma membrane fractions (200 μg) were pelleted (100 000

ras mutant or GAP, which in a number of different cell types has been shown to block the activation of the MAPK cascade (Nori *et al.*, 1991; Robbins *et al.*, 1992; Thomas *et al.*, 1992; Wood *et al.*, 1992). In these studies, expression of a dominant-negative mutant or GAP, even during "transient" expression, occurs over many hours (>16 h) and might cause blockade of the MAPK response by an unexpected alteration within the cell that is not directly due to the expressed protein. In contrast, the experiments herein using disrupted cells and a neutralizing anti-ras antibody were performed over only 70 min, including 1 h on ice, and are controlled for nonspecific effects. Thus, the ability to demonstrate a requirement for p21 ras for activation of MAPK in response to insulin and PDGF by two independent methods and methodologies establishes the following conclusion unequivocally. p21 ras function is required for activation of MAPK in NIH 3T3 cells in response to insulin and PDGF.

Acute addition of azatyrosine during the 10-min incubation also blocked activation of the pathway by PDGF. This result implies a direct effect of azatyrosine on component(s) in the pathway, because the length of incubation would appear to preclude incorporation of azatyrosine into protein.

Recently c-raf-1 and v-raf have been shown to phosphorylate and activate MAPKK (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992), and activation of c-raf-1 is also blocked by expression of the dominant negative mutant of ras (Troppmair *et al.*, 1992; Wood *et al.*, 1992). Because the same sets of agonists stimulate both c-raf-1 (assessed by mobility shift during sodium dodecyl sulfate-polyacrylamide gel electrophoresis and increased autophosphorylation in immunoprecipitates) and MAPKK (Heidecker *et al.*, 1992), c-raf-1 is thought to be one physiological activator of MAPKK in vivo. c-raf-1 contains a C-terminal kinase domain and a regulatory N-terminal domain (Bruder *et al.*, 1992). The overall structure of c-raf-1 is similar to protein kinase C, including the presence of a conserved -C-X_2-C-X_{7-13}-C-X_2-C- motif, required for phorbol ester binding in protein kinase C (Ono *et al.*, 1989; Heidecker *et al.*, 1992). Significantly, expression of the regulatory N-terminal domain prevents activation of endogenous c-raf-1 (Bruder *et al.*, 1992). These observations have raised the possibility that c-raf-1 may be allosterically activated.

Herein, we have demonstrated that antibody Y13-259 blocked the ability of the plasma membranes from v-ras–transformed cells to activate the pathway. Furthermore, parental plasma membranes loaded with GTP, but not unloaded, also caused activation. Our results imply that either ras generates a message/lipid that turns over rapidly, which may interact with target(s), or that ras directly interacts with its target(s), both possibilities requiring active GTP-loaded ras for maintenance.

Recently, Itoh *et al.* (1993) demonstrated that in contrast to results with extracts of *Xenopus* eggs prepared by low-speed centrifugation (Hattori *et al.*, 1992; Shibuya *et al.*, 1992), nonfarnesylated p21 ras failed to significantly activate MAPK in membrane-free extracts prepared by repeated high-speed centrifugations. Similarly, we found that bacterially expressed p21 ras^{Val12} did not activate MAPKK or MAPK in 100 000 × *g* supernatants. Itoh *et al.* (1993) infer from their data that the farnesylation of p21 ras is required for activation of MAPK via a cytoplasmic target and does not require localization in a membrane. This clearly implies that farnesylation of ras has another major function apart from membrane localization.

Farnesylation is apparently required for critical protein–protein interaction(s) of ras in vivo. It is not clear whether this permits ras to interact with one or with several potential proteins. Two candidates are readily apparent. The farnesyl group may be required for p21 ras to interact with GAP, which may then transduce the signal to c-raf-1, activating the cascade. A more intriguing possibility is that the N-terminal lipid binding/regulatory domain of c-raf-1 may be capable of directly interacting with the farnesyl group of ras (provided ras is loaded with GTP). The latter possibility is consistent with the demonstrated ability of constructs of raf containing the putative lipid-binding cysteine motif to confer the dominant-negative phenotype (Bruder *et al.*, 1992), the absolute requirement for farnesylation for ras function (Kato *et al.*, 1992), and the ability of farnesylated ras to activate the MAPK pathway in the absence of a membrane environment (Itoh *et al.*, 1993). Furthermore, Moodie *et al.* (1993) have recently demonstrated that bacterial nonfarnesylated ras linked to beads is capable of binding a portion of the raf present in a brain cytosolic extract but only when liganded to a GTP analogue.

The demonstration in this article that the MAPK pathway is activated by agonists in Triton-disrupted and cell-free systems should enable further biochemical dissection of the pathway in mammalian cells and the

× *g*, 15 min) and resuspended in 20 μl of 20 mM Tris/HCl, pH 7.5 at 4°C, 10 mM EDTA. Resuspended plasma membranes (50 μg) were incubated on ice for 1 h either alone or with 20 μg of anti-ras antibodies Y13-238 or Y13-259. The v-ras/c-ras in the membranes from each condition was then loaded with GTP or not loaded (see MATERIALS AND METHODS). High-speed supernatant (200 μl) and an ATP-regenerating system were added simultaneously to treated plasma membranes from each condition and incubated for 10 min at 30°C before quenching. Samples were chromatographed and assayed as described in MATERIALS AND METHODS and Figure 2. Each bar represents the number of pmol ^{32}P incorporated into MBP from each chromatography by MAPKK (A) and by MAPK (B) for membranes from v-ras–transformed cells. Each bar in C and D represents the number of pmol ^{32}P incorporated into MBP from each chromatography by MAPKK (C) and MAPK (D) for membranes from parental cells. The data shown are representative of two separate experiments.

precise nature of the interaction between ras, GAP, and raf to be elucidated.

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