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Actions of Rho Family Small G Proteins and p21-Activated Protein Kinases on Mitogen-Activated Protein Kinase Family Members

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The mitogen-activated protein (MAP) kinases are a family of serine/threonine kinases that are regulated by distinct extracellular stimuli. The currently known members include extracellular signal-regulated protein kinase 1 (ERK1), ERK2, the c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPKs), and p38 MAP kinases. We find that overexpression of the Ste20-related enzymes p21-activated kinase 1 (PAK1) and PAK2 in 293 cells is sufficient to activate JNK/SAPK and to a lesser extent p38 MAP kinase but not ERK2. Rat MAP/ERK kinase kinase 1 can stimulate the activity of each of these MAP kinases. Although neither activated Rac nor the PAKs stimulate ERK2 activity, overexpression of either dominant negative Rac2 or the N-terminal regulatory domain of PAK1 inhibits Ras-mediated activation of ERK2, suggesting a permissive role for Rac in the control of the ERK pathway. Furthermore, constitutively active Rac2, Cdc42hs, and RhoA synergize with an activated form of Raf to increase ERK2 activity. These findings reveal a previously unrecognized connection between Rho family small G proteins and the ERK pathway.

To date, three related protein kinase cascades, known as mitogen-activated protein (MAP) kinase modules, that propagate signals received at the plasma membrane to the interior of the cell in mammals have been identified. These cascades have been named according to the final enzyme in each series and are generally known as the extracellular signal-regulated protein kinase (ERK)/MAP kinase pathway, the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway, and the p38 MAP kinase pathway (reviewed in references 5 and 7). The ERK pathway is activated by many growth factors and hormones and is involved in mediating cellular proliferation, transformation, and differentiation. The JNK/SAPK and p38 MAP kinase pathways have been implicated in the protective responses triggered by cytokines and environmental stresses. Each cascade consists of three protein kinases acting in series, a MAP/ERK kinase (MEK) kinase (MEKK), a dual-specificity MEK, and a pleiotropic, proline-directed MAP kinase. Substantial pathway specificity apparently resides in the MEK component, as evidenced by the high degree of selectivity among MAP kinase homologs as substrates (12, 22). The first kinase in the series, the MEKK, is less selective and may recognize MEKs from more than one pathway (46, 47). The last group of kinases in the series, the MAP kinases, have diverse substrates which include transcription factors, cytoskeletal components, and various enzymes (5, 8).

Many of the incoming signals transmitted to these cascades are transduced by the Ras superfamily of small GTP-binding proteins (42). The ERK pathway, for example, is primarily regulated by Ras. In the GTP-bound state, Ras binds the MEKK Raf, thereby recruiting Raf to the plasma membrane (21, 39). Raf is then phosphorylated and becomes activated by an undefined mechanism (9, 44). Once activated, Raf will phosphorylate and activate downstream MEKs. To a much lesser extent, Ras can also activate the JNK/SAPK pathway (16). The JNK/SAPK and p38 MAP kinase pathways appear to be regulated by the related small G proteins Rac and Cdc42hs (6, 27). Rac and Cdc42hs are members of the Rho subfamily of small G proteins (43). Importantly, none of the other Rho-related proteins seem to regulate the JNK/SAPK pathway. The mechanism by which Rac and Cdc42hs regulate the JNK/SAPK pathway is not well understood.

Evidence for the existence of kinases immediately downstream of Rac and Cdc42hs is emerging. Among likely candidates are mammalian homologs of the yeast kinases Ste20 and Ste20-homologous kinase 1 (Shk1) (20, 25, 32). This growing family of enzymes includes three protein kinases known to be activated by binding to GTP-liganded Rac and Cdc42hs. These are rat $p65^{PAK1}$ (p21-activated kinase 1 [PAK1 or PAKα]) (24), a related enzyme, PAK2 (γPAK) (14b, 26, 40), and a third kinase, termed PAK3 (βPAK) (1, 23). Upon binding of GTP-liganded Rac or Cdc42hs to their N termini, these kinases autophosphorylate and become active (24, 26). Although downstream substrates of the PAKs have yet to be identified, their regulatory mechanisms make them likely candidates to control the JNK/SAPK and p38 MAP kinase pathways. Three lines of evidence also support this hypothesis. First, addition of constitutively active PAK1 to *Xenopus* oocyte lysates stimulates the activity of added, recombinant SAPK but not ERK2 (29). Second, expression of the N-terminal regulatory domain of PAK1 can inhibit activation of JNK1 by activated Rac in mammalian cells (27). Third, overexpression of PAK1 was been shown to lead to p38 MAP kinase activation (48).

We have investigated the ability of both PAK1 and PAK2 to activate the known mammalian MAP kinase pathways. We find that overexpression of either PAK1 or PAK2 activates SAPKα and, to a lesser extent, p38 MAP kinase but has no effect on ERK2 activity. The effects of PAKs on the regulation of these kinases are similar to what is observed upon overexpression of either activated Rac2 or rat MEKK1. In addition, although neither activated Rac nor the PAKs activate ERK2, overexpression of either dominant negative Rac2 or the N-terminal

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regulatory domain of PAK1 inhibits Ras-mediated activation of ERK2, suggesting a permissive role for Rac in the control of the ERK pathway. Furthermore, constitutively active Rac, Cdc42hs, and Rho each synergize with active Raf to increase ERK2 activity, revealing a previously unrecognized connection between the Rho subfamily of small G proteins and the ERK pathway.

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MATERIALS AND METHODS

Plasmids. Rat ERK2 (46) and rat p38 MAP kinase cDNAs were subcloned into the mammalian expression vector pCEP4 with the hemagglutinin (HA) epitope inserted at the N terminus. The rat SAPKα cDNA was subcloned into pCMV5 with an N-terminal HA tag. Human V^{12}Rac2, N^{17}Rac2, V^{12}Cdc42hs, and L^{63}RhoA were subcloned into pCMV5 as was rat MEKK1 (45a). Glycine 12 of wild-type Cdc42hs in pACT2 was mutated to valine by double-stranded, site-directed mutagenesis (Chameleon kit; Stratagene) using the primers 5′-TTGTTGTGGGCGATGTTGCTGTTGGTAAAAC-3′ (Gly to Val) and 5′-GATCCGAATTCGAAGGTCGACAGATCTATGAATCGT-3′ (3′ *Xho*I to *Sal*I in pACT2). The sequence of V^{12}Cdc42hs was confirmed by DNA sequencing. V^{12}H-Ras was contained in pRcCMV (17). A^{15}H-Ras was expressed from pEFmyc (kindly provided by Andrew Thorburn). Wild-type rat PAK2 was subcloned into pCMV5 containing an N-terminal 9E10 c-Myc epitope (pCMV5M). Wild-type rat PAK1 was amplified with Vent polymerase (New England Biolabs), adding 5′ *Eco*RI and 3′ *Xho*I sites. The primers used were 5′-GGAATTCGAATGTCAAATAACGGCTTA-3′ (5′-*Eco*RI) and 5′-CCGCTCGAGGTGATTGTTCTTGGTTGC-3′ (3′-*Xho*I). Correct amplification of wild-type PAK1 was confirmed by DNA sequencing, and the PAK1 cDNA was subcloned into pCMV5M. The isolation of cDNA clones of rat PAK1 and PAK2 (GenBank accession number, U19967) will be described elsewhere (14a). A C-terminal PAK1 deletion mutant containing amino acids 1 to 231 (PAK1 1-231) was amplified by PCR with Vent polymerase, adding 5′ *Eco*RI and 3′ *Xho*I sites. The 3′ primer used was 5′-CCGCTCGAGGGGCGGAGTGGTGTTATT-3′. The 5′ primer was the same as that used for the amplification of wild-type PAK1. Correct amplification of PAK 1 1-231 was confirmed by DNA sequencing, and the cDNA was subcloned into pCMV5. The Raf-1 N terminus (Raf C4B) was subcloned into pCEP4. Constitutively active Raf-1 (Raf BXB) was subcloned into pRcCMV (14). pCMV/βGAL is as described previously (41).

Cell culture and transfection. Human kidney fibroblasts (293 cells) were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (46). Cells (~80% confluent) were transfected with DNA by calcium phosphate coprecipitation (34). Twenty-four hours after transfection the medium was switched to Dulbecco's modified Eagle medium plus 0.5% fetal bovine serum and maintained for an additional 16 to 20 h. In some cases the medium from these cells was transferred to a second set of transfected cells. After being washed once with ice-cold phosphate-buffered saline, the cells were harvested by scraping into 0.5 ml of Triton lysis buffer (19) per 60-mm-diameter dish. Lysates were incubated for 10 min on ice, and insoluble debris was removed by centrifugation at 14,000 × *g* for 10 min at 4°C. Supernatants were used immediately or frozen in liquid N_2 and stored at −80°C.

Immunoprecipitation and kinase assays. To normalize for transfection efficiency among plates, soluble extracts were assayed for β-galactosidase (β-Gal) activity derived from a cotransfected, constitutively transcribed β-Gal expression vector (pCMVβGal) (41). Samples were adjusted to equalize β-Gal activities, and the amount of the expressed, HA-tagged MAP kinase family member was measured by Western blotting (immunoblotting) with mouse anti-HA antibody (BAbCo). Cell lysates adjusted to equalize for anti-HA immunoreactivity were then used for immunoprecipitation of HA-tagged MAP kinase. Extracts were incubated with mouse anti-HA antibody and protein A-Sepharose for 2 h at 4°C. Sepharose beads were then sedimented and washed three times with buffer A (20 mM Tris-HCl [pH 7.5], 1 M NaCl) and then once with buffer B (20 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$). The beads were then resuspended in 40 μl of kinase buffer containing 20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM ATP (4 cpm of [γ^{32}-P]ATP per fmol), and 0.3 mg of either myelin basic protein (MBP), glutathione *S*-transferase (GST)–c-JunΔ(1-221) (16), or GST-ATF2Δ(1-254) per ml (22). HA-ERK2 kinase reaction mixtures were incubated at 30°C for 15 min, while HA-SAPK and HA-p38 kinase reaction mixtures were incubated at 30°C for 30 min. Reactions were stopped by sedimenting the beads and adding 5× Laemmli sample buffer to the supernatants. All experiments were performed at least twice.

RESULTS

Regulation of MAP kinase family members by distinct small G proteins and PAKs. Before investigating the effects of PAKs on the activity of MAP kinase family members, we wished to test MAP kinase regulation by small G proteins in 293 cells. Cells were cotransfected with HA-tagged forms of either ERK2, SAPKα, or p38 MAP kinase and either activated Ras (V^{12}H-Ras) or activated Rac2 (V^{12}Rac2). Thirty-six hours after transfection, tagged MAP kinases were immunoprecipitated from cell lysate volumes containing equal amounts of transfected MAP kinase and the associated kinase activities were determined. As seen in Fig. 1A, expression of V^{12}H-Ras greatly stimulated ERK2 activity (30- to >120-fold). In contrast, expression of V^{12}Rac2 produced an insignificant effect on ERK2 activity. Activated Rac2, on the other hand, was a much better activator of SAPKα than was activated Ras (Fig. 1B). Similarly, V^{12}Rac2 also stimulated p38 MAP kinase activity to a greater extent than did V^{12}H-Ras (Fig. 1C). Expression of activated RhoA (L^{63}RhoA) did not lead to activation of ERK2, SAPKα, or p38 MAP kinase (data not shown). These data are consistent with previously published results for other cell types (6, 27).

We then tested whether PAK1 and PAK2 were able to activate MAP kinase family members. If the PAKs are downstream effectors of Rac, then their expression might be expected to produce effects similar to those caused by overexpression of activated Rac. As seen in Fig. 1A, overexpression of either wild-type PAK1 or wild-type PAK2 had little effect on ERK2 activity. Coexpression of V^{12}Rac2 with PAK1 or PAK2 also did not lead to ERK2 activation. In contrast, expression of either wild-type PAK1 or wild-type PAK2 significantly increased the kinase activity of cotransfected SAPKα (16- or 12-fold, respectively [Fig. 1B]). This activation does not occur with catalytically defective PAK1 (D-406 to A mutant) (not shown) but does occur in the absence of additional stimuli, suggesting that a minimal level of signaling in these pathways must occur in resting 293 cells. Activation of SAPKα by PAKs was consistently greater than the activation caused by V^{12}H-Ras, but less than that observed with V^{12}Rac2. As shown in Fig. 1B, coexpression of V^{12}Rac2 with PAK1 or PAK2 did not increase the level of SAPKα activity to more than that observed with V^{12}Rac2 alone. This was the case even when submaximal amounts of V^{12}Rac2 were transfected (data not shown). Expression of either PAK1 or PAK2 alone also led to a smaller but reproducible increase in p38 MAP kinase activity. As with SAPKα, p38 MAP kinase activity stimulated by PAKs was less than that observed with V^{12}Rac2 but greater than that caused by V^{12}H-Ras (Fig. 1C). Coexpression of PAK1 with V^{12}Rac2 resulted in a slightly increased level of p38 MAP kinase activity compared with that caused by V^{12}Rac2 or PAK1 expression alone. In 293 cells, none of these molecules activated p38 MAP kinase by more than 10-fold when GST-ATF2 was used as a substrate.

Expression of rat MEKK1 activates both SAPKα and p38 MAP kinase. Previously, MEKK1 has been reported to activate the JNK/SAPK pathway (28, 47). As a potential downstream mediator of PAKs we tested its effects on SAPKα and p38 MAP kinase activity in 293 cells. This rat MEKK1 clone encodes a protein kinase of approximately 195 kDa that includes 2.5 kb of 5′ coding sequence not included in previously published MEKK1 clones (19, 45). As expected, expression of rat MEKK1 caused a large increase in SAPKα activity (54-fold [Fig. 2A]). Anisomycin, a known activator of the JNK/SAPK pathway (10), weakly activated SAPKα in the absence of MEKK1 (fourfold) and had no further effect on SAPKα activity in the presence of MEKK1. The expression of rat MEKK1 also led to a significant activation of p38 MAP kinase activity (fourfold [Fig. 2B]). Rat MEKK1 was consistently as effective an activator of p38 MAP kinase as V^{12}Rac2. The weak effect on p38 MAP kinase may result from the limited ability of

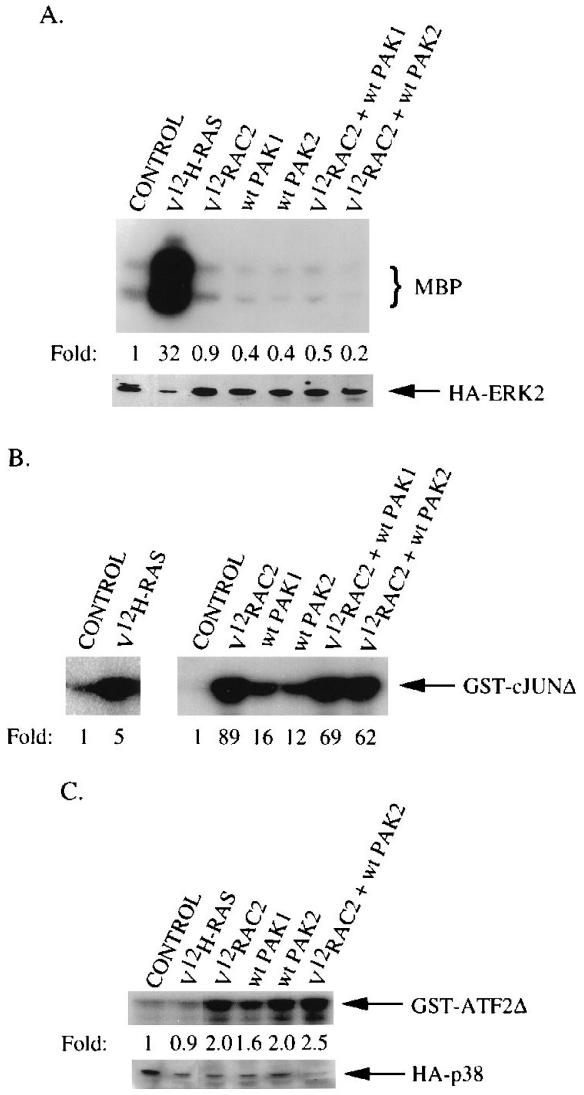

FIG. 1. Activation of MAP kinase pathways by cotransfected PAK1 and PAK2. 293 cells were cotransfected with either HA-ERK2 (A), HA-SAPKα (B), or HA-p38 MAP kinase (C) and the plasmids encoding the proteins shown. Fold refers to the fold increase in substrate phosphorylation over that occurring in the control lane. Below the MBP and GST-ATF2Δ kinase assays in panels A and C are Western blots for expressed HA-ERK2 and HA-p38 MAP kinase, respectively. The control used is the pCMV5 plasmid lacking a cDNA insert. The plasmids and amounts transfected are as follows: pCEP4/HA-ERK2, pCMV5/HA-SAPKα, and pCEP4/HA-p38 MAP kinase, 7.0 μg each; pCMV5, 7.0 μg; pRcCmv/V^{12}H-Ras, 1.0 μg; pCMV5/V^{12}Rac2, 2.0 μg; pCMV5M/PAK1, 7.0 μg; and pCMV5M/PAK2, 7.0 μg. Representative autoradiographs are shown in each panel. wt, wild type.

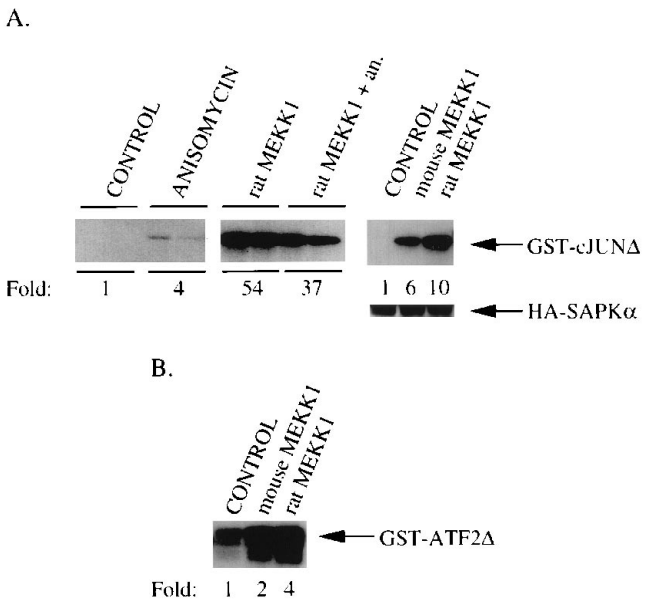

FIG. 2. Activation of SAPKα and p38 MAP kinase activities by MEKK1. 293 cells were transfected with the same quantities of plasmids as described in the legend to Fig. 1. The control used is the pCMV5 plasmid lacking a cDNA insert. (A) Activation of transfected HA-SAPKα by anisomycin (an.) (10 μg/ml; 30 min), cotransfected rat MEKK1, or mouse MEKK1. Control, anisomycin, and rat MEKK1 lanes are shown in duplicate. GST–c-Jun is used as a substrate. The lower right-hand portion of the figure shows a Western blot for immunoprecipitated HA-SAPKα. (B) Activation of HA-p38 MAP kinase by cotransfected mouse MEKK1 and rat MEKK1. GST-ATF2Δ is used as a substrate.

MEK4 (also known as SEK and JNK kinase), an MEKK1 substrate, to phosphorylate and activate p38 MAP kinase in vivo (11).

Expression of dominant negative forms of PAK1 and Rac2 interferes with V^{12}H-Ras-stimulated ERK activity. Because neither PAK1 nor PAK2 stimulated ERK2 activity, we expected that overexpression of the N-terminal regulatory domain of PAK1 (PAK1 1-231) would also have no effect on ERK2 activation. Surprisingly, coexpression of PAK1 1-231 reproducibly inhibited ERK2 activation by V^{12}H-Ras (up to 33% in three experiments) (Fig. 3). Full-length PAK1 did not cause this effect (not shown). The degree of inhibition was similar to that achieved by coexpression of dominant negative Rac2 (N^{17}Rac2). As anticipated, coexpression of the N terminus of Raf-1 (Raf C4B) with V^{12}H-Ras also reduced ERK2 activation (40%) (Fig. 3). Coexpression of PAK1 1-231 with Raf C4B reduced ERK2 activation by V^{12}H-Ras by more than 75% (Fig. 3), a much greater effect than was observed with either protein alone. Because expression of the N terminus of PAK1 should presumably inhibit signaling by binding to the upstream Rac and Cdc42hs (24, 26), these data suggest that the activation of ERK2 caused by overexpression of V^{12}H-Ras may involve other small G proteins. This idea is supported by the finding that N^{17}Rac2 expression can also inhibit ERK2 activation by V^{12}H-Ras. A comparable mutant of RhoA (N^{19}RhoA) was less effective than N^{17}Rac2 (not shown).

Constitutively active forms of Rac2, Cdc42hs, and RhoA synergize with constitutively active Raf to activate ERK2. To examine this further, we tested the ability of Rac to cooperate with Raf-1 to activate ERK2. Expression of Raf BXB, an amino-terminal truncation mutant of Raf-1 that is constitutively active in many cell types (15, 44), stimulated ERK2 activity approximately ninefold (Fig. 4A). Coexpression of V^{12}Rac2 with Raf BXB, however, greatly enhanced ERK2 activation (70-fold in Fig. 4A and 118-fold in Fig. 4B). This represents an eightfold or greater increase in ERK2 activation over that achieved with Raf BXB expression alone. Similar results were observed with V^{12}Rac2 and a Raf BXB-CAAX (43a) construct that targets Raf to the plasma membrane (data not shown). We then tested whether expression of the related small G proteins Cdc42hs and RhoA also potentiated Raf BXB-mediated ERK2 activation. As expected, expression of activated Cdc42hs (V^{12}Cdc42hs in Fig. 4B) or activated RhoA (L^{63}RhoA) (not shown) alone did not increase ERK2 activity. However, coexpression of either V^{12}Cdc42hs or L^{63}RhoA and

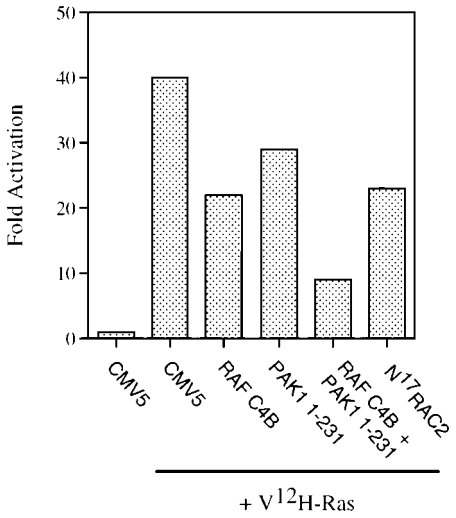

FIG. 3. Inhibition of V^{12}H-Ras-mediated ERK2 activation by N^{17}Rac2, the PAK1 N terminus, and the amino terminus of Raf-1. 293 cells were transfected with HA-ERK2 and either empty vector (pCMV5) or the vectors containing the cDNAs shown. HA-ERK2 activity was measured by MBP immunocomplex assays. Fold activation refers to the fold increase in MBP kinase activity over that in the control lane (first bar [CMV5]). V^{12}H-Ras was cotransfected with the remaining lanes (remaining five bars). Seven micrograms of Raf C4B and PAK1 1-231 and 2 μg of N^{17}Rac2 were transfected. The quantities of the other plasmids used were as described in the legend to Fig. 1.

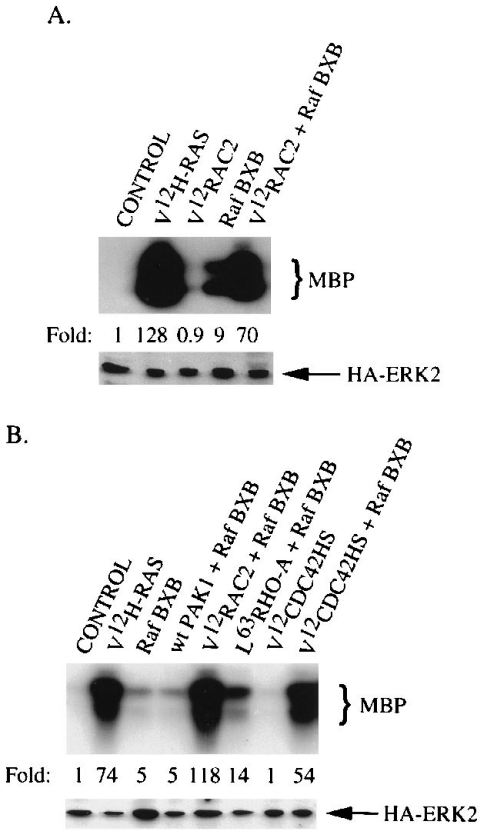

FIG. 4. Potentiation of Raf BXB-mediated activation of ERK2 by activated Rac2, Cdc42hs, and RhoA. Shown is the kinase activity of immunoprecipitated HA-ERK2 with MBP as a substrate. Pictured below each panel is a Western blot of expressed HA-ERK2. Seven micrograms of Raf BXB was transfected. The quantities of the other plasmids were as described in previous figure legends. (A) Synergism between V^{12}Rac2 and Raf BXB in mediating ERK2 activation. (B) Activated Cdc42hs and, to a lesser extent, activated RhoA synergize with Raf BXB to activate ERK2.

Raf BXB did lead to a synergistic activation of ERK2 (11- and 3-fold more, respectively, than with Raf BXB alone [Fig. 4B]). V^{12}Cdc42hs was almost as effective as V^{12}Rac2 in synergizing with Raf BXB to activate ERK2, while L^{63}RhoA was reproducibly less effective. Since both Rac and Cdc42hs are potential activators of PAK1 in vivo, we tested whether wild-type PAK1 and Raf BXB would also synergize to activate ERK2. We found that PAK1 did not significantly increase Raf BXB-mediated ERK2 activation (Fig. 4B).

To test the possibility that activated forms of Rac or Cdc42hs might potentiate Raf BXB activation of ERK2 through an autocrine mechanism, we collected conditioned medium from cells transfected with Raf BXB, V^{12}Rac2, V^{12}Cdc42hs, and L^{63}RhoA or combinations thereof and measured the stimulation of ERK2 activity in a second group of cells exposed to the medium. The conditioned medium from transfected cells did not increase ERK2 activity more than 2.5-fold regardless of the vectors transfected into the cells from which it had been harvested (Fig. 5). In comparison, epidermal growth factor caused an 8.5-fold increase in ERK2 activity.

A less direct experiment was also performed to examine the requirement for Ras in the potentiation of ERK2 activation by Raf BXB and V^{12}Rac2. An autocrine loop that results in an increase in a Raf-dependent activation of ERK2 might be expected to work through Ras. Therefore, Raf BXB and V^{12}Rac2, V^{12}Cdc42hs, or L^{63}RhoA were cotransfected with the interfering Ras mutant, A^{15}H-Ras (4). Coexpression of A^{15}H-Ras had no effect on the synergistic activation of ERK2 by Raf BXB plus V^{12}Rac2 but reproducibly decreased the effect of Raf BXB plus V^{12}Cdc42hs on ERK2 an average of 70% (data not shown). ERK2 stimulation by Raf BXB plus V^{12}Cdc42hs in the presence of A^{15}H-Ras was reduced to approximately the same as that by Raf BXB alone. Coexpression of A^{15}H-Ras with L^{63}RhoA plus Raf BXB only minimally affected ERK2 activity (data not shown).

DISCUSSION

Our results show that wild-type forms of both PAK1 and PAK2 activate SAPKα and to a lesser extent p38 MAP kinase in 293 cells. We also find that rat MEKK1 activates both pathways, contrary to previously published results (11). However, none of our findings address possible interactions among small G proteins, PAKs, and MEKK1 in causing stimulation of SAPKα or p38. The effects of Rac, PAK, and MEKK1 on p38 MAP kinase are much less than their effects on SAPK. Earlier studies with inhibitory mutants have been used to suggest that these molecules act in sequence (27). Because the mechanisms of action of the inhibitory mutants have not been defined, conclusions from such studies must be withheld until other experimental approaches can be used to confirm or refute a sequential model. Lastly, we show that expression of either dominant negative Rac2 or the PAK1 regulatory domain can inhibit V^{12}H-Ras-stimulated ERK2 activation to a degree comparable to that observed with expression of the Raf-1 amino terminus and that coexpression of the PAK1 and Raf-1 N termini largely abolishes Ras-mediated ERK2 activation. Consistent with these results, expression of either activated Rac2 or activated Cdc42hs greatly potentiates Raf BXB-mediated ERK2 activation.

Our data do not prove but are consistent with the notion that Rac or Cdc42hs activates SAPK by first activating PAKs, as

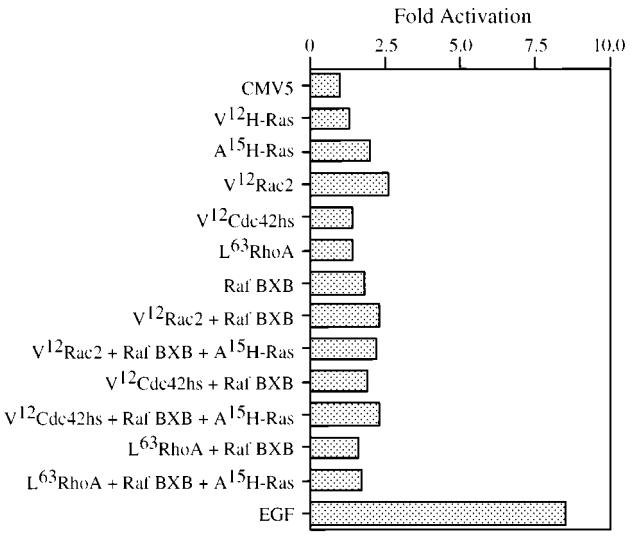

FIG. 5. Stimulation of HA-ERK2 activity by conditioned media. 293 cells were transfected with the plasmids shown on the left. Eighteen hours after transfection, the media were changed to Dulbecco's modified Eagle medium plus 0.5% fetal bovine serum. Twenty hours after this, conditioned media were collected. Separate cells transfected with HA-ERK2 alone were then exposed to the conditioned media for 5 min before being harvested as described previously. Epidermal growth factor stimulation (final concentration, 100 ng/ml) was for 5 min. Shown are results from a representative experiment. Fold activation refers to the increase in HA-ERK2 activity over that obtained with media from cells transfected with empty vector alone.

suggested previously (27). The degree of SAPKα activation caused by overexpression of the PAKs was consistently less than that produced by overexpression of V^{12}Rac2 or MEKK1 but greater than that produced by activated Ras or anisomycin. Such differences in the degree of SAPKα activation stimulated by Rac and PAK could be merely due to the fact that an activated Rac mutant was used, whereas the PAKs expressed were wild type. These differences could also reflect a necessary interaction of Rac with other signaling molecules that would not be activated by overexpression of PAKs. In this context, overexpression of a small G protein such as Rac, which clearly communicates with other small G proteins, nucleotide exchange factors, and linker molecules (3, 25, 37, 43), might be expected to stimulate SAPKα and p38 MAP kinase activities through multiple pathways, only a subset of which may include PAK1 and PAK2. The exact mechanisms of such cross-communication, as well as the proteins involved, remain to be determined.

Other Ste20-related kinases which have also been reported to activate the stress pathways have been cloned. One example is the mixed-lineage family of kinases, of which GC kinase and MLK are typical (30). These kinases each contain an amino-terminal kinase domain which is similar to those of PAKs (35 to 40% identity) as well as a carboxyl-terminal extension different from those of other Ste20-related kinases. It has recently been suggested that they contain G protein binding sites similar to that of PAK (2), indicating that their mode of regulation may be similar.

Previously, MEKK1 has been reported to activate the JNK/SAPK pathway. We find that in 293 cells, rat MEKK1 can activate not only SAPKα but also p38 MAP kinase. In fact, MEKK1 activates p38 as well as or better than Rac or Cdc42hs. This was unanticipated because it was reported previously that Rac could activate p38 MAP kinase (11) although MEKK1 could not (48). This apparent discrepancy could have several explanations, one of which may be the different cell types used in these studies. It may also be accounted for by differences in the MEKK1 clones that were expressed. The MEKK1 cDNA used here (45) contains 2.5 kb of 5′ coding sequence not found in the MEKK1 sequence previously published (19). This upstream coding sequence contains putative SH3-binding and pleckstrin homology domains that may be required for optimum signaling.

The inhibition of Ras-mediated ERK2 activation by dominant negative Rac and the PAK N terminus probably reflects cross talk between MAP kinase pathways that have been previously viewed as independent. Because our data show that interfering with Rac-associated signaling alters Ras-mediated ERK2 activation, we conclude that these pathways are tightly connected. Given its in vitro binding specificity (24, 26), the PAK1 N terminus is likely to function by complexing with endogenous Rac and Cdc42hs. By analogy to N^{17}H-Ras, N^{17}Rac2 may titrate Rac-associated guanine nucleotide exchange factors, thereby preventing enhanced exchange on endogenous Rac (13, 33). In either case, normal Rac function would be blocked. The similar effects of PAK1 1-231 and N^{17}Rac2 on Ras-mediated ERK2 activity suggest that Ras-mediated activation of ERK2 requires normal Rac function. Because Rac does not activate ERK2, its role is likely indirect and may be due to an interaction with undefined effector molecules common to both small G proteins. It is tempting to speculate that molecules such as p190 Rho GTPase-activating protein, which itself is known to interact with both Ras and Rac, would serve as the link between Ras and Rac (35, 36).

It is also possible that Rac affects ERK2 activation through Raf. The finding that activated forms of Rac and Cdc42hs both synergize with Raf BXB or Raf BXB-CAAX to activate ERK2 suggests such a connection. The connection is probably due to an intracellular interaction, as experimental results are inconsistent with an autocrine mechanism. Upon binding to GTP-bound Ras, wild-type Raf-1 is activated by posttranslational mechanisms that include phosphorylation on defined residues which are included in the Raf BXB truncation mutant (9, 44). One possibility is that the phosphorylation of these sites might be controlled by Rac- and Cdc42hs-dependent pathways, perhaps directly by PAKs. MEK is a less likely site for the positive effects of Rac because its activity is modestly decreased following phosphorylation by PAK in vitro (14c). Our current hypothesis is that Ras sends two signals to the ERK pathway that are required for activation, one which targets Raf to the plasma membrane and a second which activates Raf and may also be Ras independent under certain circumstances. This hypothesis is supported by the observation that the activity of Raf BXB localized to the plasma membrane by addition of a C-terminal farnesylation signal can be further increased with growth factor stimulation (21). Rac and other Rho family members may be involved in transducing the second of these signals. Such a mechanism is analogous to the regulation of the *Schizosaccharomyces pombe* MEKK, byr2, by Ras. A direct effect is exerted by the binding of Ras to byr2, with a second, indirect input from Ras being routed through Cdc42 and the *S. pombe* PAK homolog Shk1 (14d, 25).

Previous studies have shown that Ras and Rac, activated Raf and Rac, as well activated Raf and RhoA cooperate to transform cells (18, 31). Our findings suggest that components of the MAP kinase pathway, such as Raf, could be a focal point of their interaction. Further studies will be required to define the mechanism of this interaction.

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ADDENDUM

PAK has now been shown to activate JNK/SAPK in Cos cells (38).

REFERENCES

1. **Bagrodia, S., S. J. Taylor, C. L. Creasy, J. Chernoff, and R. A. Cerione.** 1995. Identification of a mouse $p21^{Cdc42/Rac}$ activated kinase. J. Biol. Chem. **270:** 22731–22737.
2. **Burbelo, P. D., D. Drechsel, and A. Hall.** 1995. A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. J. Biol. Chem. **270:**29071–29074.
3. **Cantor, S. B., T. Urano, and L. A. Feig.** 1995. Identification and characterization of Ral-binding protein 1, a potential downstream target of Ral GTPases. Mol. Cell. Biol. **15:**4578–4584.
4. **Chen, S. Y., S. Y. Huff, C. C. Lai, C. J. Der, and S. Powers.** 1994. Ras-15A protein shares highly similar dominant-negative biological properties with Ras-17N and forms a stable, guanine-nucleotide resistant complex with CDC25 exchange factor. Oncogene **9:**2691–2698.
5. **Cobb, M. H., and E. Goldsmith.** 1995. How MAP kinases are regulated. J. Biol. Chem. **270:**14843–14846.
6. **Coso, O. A., M. Chiariello, J.-C. Yu, H. Teramoto, P. Crespo, N. Xu, T. Miki, and J. S. Gutkind.** 1995. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell **81:**1137–1146.
7. **Davis, R.** 1994. MAPKS: new JNK expands the group. Trends Biochem. Sci. **19:**470–473.
8. **Davis, R. J.** 1993. The mitogen-activated protein kinase signal transduction pathway. J. Biol. Chem. **268:**14553–14556.
9. **Dent, P., T. Jelinek, D. K. Morrison, M. J. Weber, and T. W. Sturgill.** 1995. Reversal of Raf-1 activation by purified and membrane-associated protein phosphatases. Science **268:**1902–1906.
10. **Dérijard, B., M. Hibi, I.-H. Wu, T. Barrett, B. Su, T. Deng, M. Karin, and R. J. Davis.** 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell **76:**1025–1037.
11. **Dérijard, B., J. Raingeaud, T. Barrett, I. Wu, J. Han, R. J. Ulevitch, and R. J. Davis.** 1995. Independent human MAP kinase signal transduction pathways defined by MEK and MKK isoforms. Science **267:**682–685.
12. **English, J. M., C. A. Vanderbilt, S. Xu, S. Marcus, and M. H. Cobb.** 1995. Isolation of MEK5 and differential expression of alternatively spliced forms. J. Biol. Chem. **270:**28897–28902.
13. **Feig, L. A.** 1994. Guanine-nucleotide exchange factors: a family of positive regulators of Ras and related GTPases. Curr. Opin. Cell Biol. **6:**204–211.
14. **Frost, J. A., A. S. Alberts, E. Sontag, K. Guan, M. C. Mumby, and J. R. Feramisco.** 1994. Simian virus 40 small t antigen cooperates with mitogen-activated kinases to stimulate AP-1 activity. Mol. Cell. Biol. **14:**6244–6252.

14a. **Frost, J. A., M. R. Hutchison, and M. H. Cobb.** Unpublished data.

14b. **Frost, J. A., M. R. Hutchison, S. Marcus, and M. H. Cobb.** Unpublished data.

14c. **Frost, J. A., S. Xu, A. Dang, S. J. Mansour, N. G. Ahn, and M. H. Cobb.** Unpublished data.

14d. **Gilbreth, M., P. Yang, J. A. Frost, A. Polverino, M. H. Cobb, and S. Marcus.** Submitted for publication.

15. **Heidecker, G., M. Huleihel, J. L. Cleveland, W. Kolch, T. W. Beck, P. Lloyd, T. Pawson, and U. R. Rapp.** 1990. Mutational activation of c-*raf*-1 and definition of the minimal transforming sequence. Mol. Cell. Biol. **10:**2503–2512.
16. **Hibi, M., A. Lin, T. Smeal, A. Minden, and M. Karin.** 1993. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev. **7:**2135–2148.
17. **James, G. L., M. S. Brown, M. H. Cobb, and J. L. Goldstein.** 1994. Benzodiazepine peptidomimetic interrupts the MAP kinase activation pathway in Ras-transformed Rat-1 cells, but not in untransformed cells. J. Biol. Chem. **269:**27705–27714.
18. **Khosravi-Far, R., P. A. Solski, G. J. Clark, M. S. Kinch, and C. J. Der.** 1995. Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation. Mol. Cell. Biol. **15:**6443–6453.
19. **Lange-Carter, C. A., C. M. Pleiman, A. M. Gardner, K. J. Blumer, and G. L. Johnson.** 1993. A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science **260:**315–319.
20. **Leberer, E., D. Dignard, D. Harcus, D. Y. Thomas, and M. Whiteway.** 1992. The protein kinase homologue Ste20p is required to link the yeast pheromone response G-protein βτ subunits to downstream signalling components. EMBO J. **11:**4815–4824.
21. **Leevers, S. J., H. F. Paterson, and C. J. Marshall.** 1994. Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. Nature (London) **369:**411–414.
22. **Lin, A., A. Minden, H. Martinetto, F.-X. Claret, C. Lange-Carter, F. Mercurio, G. Johnson, and M. Karin.** 1995. Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. Science **268:**286–290.
23. **Manser, E., C. Chong, Z.-S. Zhao, T. Leung, G. Michael, C. Hall, and L. Lim.** 1995. Molecular cloning of a new member of the p21-Cdc42/Rac-activated kinase (PAK) family. J. Biol. Chem. **270:**25070–25078.
24. **Manser, E., T. Leung, H. Salihuddin, Z.-S. Zhao, and L. Lim.** 1994. A brain serine/threonine protein kinase activated by Cdc42 and Rac1. Nature (London) **367:**40–46.
25. **Marcus, S., A. Polverino, E. Chang, D. Robbins, M. H. Cobb, and M. H. Wigler.** 1995. Shk1, a homolog of the *Saccharomyces cerevisiae* Ste20 and mammalian $p65^{PAK}$ protein kinases, is a component of a Ras/Cdc42 signaling module in fission yeast *Schizosaccharomyces pombe*. Proc. Natl. Acad. Sci. USA **92:**6180–6184.
26. **Martin, G. A., G. Bollag, F. McCormick, and A. Abo.** 1995. A novel serine kinase activated by Rac1/CDC42Hs-dependent autophosphorylation is related to PAK65 and STE20. EMBO J. **14:**1970–1978.
27. **Minden, A., A. Lin, F.-X. Claret, A. Abo, and M. Karin.** 1995. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. Cell **81:**1147–1157.
28. **Minden, A., A. Lin, M. McMahon, C. Lange-Carter, B. Dérijard, R. J. Davis, G. L. Johnson, and M. Karin.** 1994. Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. Science **266:**1719–1723.
29. **Polverino, A., J. Frost, P. Yang, M. Hutchison, A. M. Neiman, M. H. Cobb, and S. Marcus.** 1995. Activation of MAPK cascades by p21-activated protein kinases in cell-free extracts of *Xenopus* oocytes. J. Biol. Chem. **270:**26067–26070.
30. **Pombo, C. M., J. H. Kehrl, I. Sánchez, P. Katz, J. Avruch, L. I. Zon, J. R. Woodgett, T. Force, and J. M. Kyriakis.** 1995. Activation of the SAPK pathway by the human *STE20* homologue germinal centre kinase. Nature (London) **377:**750–754.
31. **Qiu, R.-G., J. Chen, D. Kirn, F. McCormick, and M. Symons.** 1995. An essential role for Rac in Ras transformation. Nature (London) **374:**457–459.
32. **Ramer, S. W., and R. W. Davis.** 1993. A dominant truncation allele identifies a gene, *STE20*, that encodes a putative protein kinase necessary for mating in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **90:**452–456.
33. **Ridley, A. J., H. F. Paterson, C. L. Johnson, D. Diekmann, and A. Hall.** 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell **70:**401–410.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Settleman, J., C. F. Albright, L. C. Foster, and R. A. Weinberg.** 1992. Association between GTPase activators for Rho and Ras families. Nature (London) **359:**153–154.
36. **Settleman, J., V. Narasimhan, L. C. Foster, and R. A. Weinberg.** 1992. Molecular cloning of cDNAs encoding the GAP-associated protein p190: implications for a signaling pathway from Ras to the nucleus. Cell **69:**539–549.
37. **Shou, C., C. L. Farnsworth, B. G. Neel, and L. A. Feig.** 1992. Molecular cloning of cDNAs encoding a guanine-nucleotide-releasing factor for Ras p21. Nature (London) **358:**351–354.
38. **Shubha, B., B. Dérijard, R. J. Davis, and R. A. Cerione.** 1995. Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. J. Biol. Chem. **270:**27998.
39. **Stokoe, D., S. G. MacDonald, K. Cadwallader, M. Symons, and J. F. Hancock.** 1994. Activation of Raf as a result of recruitment to plasma membrane. Science **264:**1463–1467.
40. **Teo, M., E. Manser, and L. Lim.** 1995. Identification and molecular cloning of a $p21^{cdc42/rac1}$-activated serine/threonine kinase that is rapidly activated by thrombin in platelets. J. Biol. Chem. **270:**26690–26697.
41. **Thorburn, J., J. A. Frost, and A. Thorburn.** 1994. Mitogen-activated protein kinases mediate changes in gene expression, but not cytoskeletal organization associated with cardiac muscle cell hypertrophy. J. Cell Biol. **126:**1565–1572.
42. **Valencia, A., P. Chardin, A. Wittinghofer, and C. Sander.** 1991. The *ras*

protein family: evolutionary tree and role of conserved amino acids. Biochemistry **30:**4637–4648.

43. **Vojtek, A. B., and J. A. Cooper.** 1995. Rho family members: activators of MAP kinase cascades. Cell **82:**527–529.

43a. **Whitehurst, C., and T. Geppert.** Unpublished data.

44. **Whitehurst, C. E., H. Owaki, J. T. Bruder, U. R. Rapp, and T. D. Geppert.** 1995. The MEK kinase activity of the catalytic domain of RAF-1 is regulated independently of Ras binding in t cells. J. Biol. Chem. **270:**5594–5599.

45. **Xu, S., D. J. Robbins, L. B. Christerson, J. M. English, C. A. Vanderbilt, and M. H. Cobb.** Cloning of Rat MEKK1 cDNA reveals an endogenous membrane-associated 195 kDa protein with a large regulatory domain. Proc. Natl. Acad. Sci. USA, in press.

45a. **Xu, S., D. J. Robbins, L. B. Christerson, J. M. English, C. A. Vanderbilt, and M. H. Cobb.** Submitted for publication.

46. **Xu, S., D. Robbins, J. Frost, A. Dang, C. Lange-Carter, and M. H. Cobb.** 1995. MEKK1 phosphorylates MEK1 and MEK2 but does not cause activation of MAP kinase. Proc. Natl. Acad. Sci. USA **92:**6808–6812.

47. **Yan, M., T. Dal, J. C. Deak, J. M. Kyriakis, L. I. Zon, J. R. Woodgett, and D. J. Templeton.** 1994. Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. Nature (London) **372:**798–800.

48. **Zhang, S., J. Han, M. A. Sells, J. Chernoff, U. G. Knaus, R. J. Ulevitch, and G. M. Bokoch.** 1995. Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. J. Biol. Chem. **270:** 23934–23936.