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Signaling from G Protein-coupled Receptors to c-Jun Kinase Involves $\beta\gamma$ Subunits of Heterotrimeric G Proteins Acting on a Ras and Rac1-dependent Pathway*

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Stimulation of a variety of cell surface receptors enhances the enzymatic activity of mitogen-activated protein kinases (MAPKs). MAPKs have been classified in three subfamilies: extracellular signal-regulated kinases (ERKs), stress-activated protein kinases or c-Jun NH_2-terminal kinases (SAPKs/JNKs), and p38 kinase. Whereas the pathway linking cell surface receptors to ERKs has been partially elucidated, the mechanism of activation of JNKs is still poorly understood. Recently, we have shown that stimulation of G protein-coupled receptors can effectively induce JNK in NIH 3T3 cells (Coso, O. A., Chiariello, M., Kalinec, G., Kyriakis, J. M., Woodgett, J., and Gutkind, J. S. (1995) *J. Biol. Chem.* 270, 5620–5624). In the present study, we have used the transient expression in COS-7 cells of m1 and m2 muscarinic receptors (mAChRs) as a model system to study the signaling pathway linking G protein-coupled receptors to JNK. We show that stimulation of either muscarinic receptor subtype leads to JNK activation; however, this effect was not mimicked by expression of activated forms of α_s, α_{i2}, α_q, or α_{13} G protein α subunits. In contrast, overexpression of G$\beta\gamma$ subunits potently induced JNK activity. Furthermore, we show that signaling from m1 and m2 mAChRs to JNK involves $\beta\gamma$ subunits of heterotrimeric G proteins, acting on a Ras and Rac1-dependent pathway.

Stimulation of a variety of cell surface receptors causes a rapid elevation of the enzymatic activity of a family of closely related serine-threonine kinases, known as mitogen-activated protein kinases (MAPKs)[1] (2). The function of MAPKs is to convert extracellular stimuli to intracellular signals which, in turn, control the expression of genes that are essential for many cellular processes, including cell growth and differentiation (3). MAPKs have been classified in three subfamilies: extracellular signal regulated kinases (ERKs), including ERK1 and ERK2, also known as $p44^{mapk}$ and $p42^{mapk}$, respectively; stress-activated protein kinases (SAPKs), also termed c-Jun NH_2-terminal kinases (JNKs); and p38 kinase (2). ERKs phosphorylate and regulate the activity of certain enzymes, including phospholipase A_2 and $p90^{rsk}$, and nuclear proteins, such as the ternary complex factor $p62^{TCF}$ or Elk-1 (4). The latter represents a critical event in controlling the expression of several genes, including c-*fos* (5). JNKs phosphorylate the amino-terminal transactivating domain of c-Jun and ATF2 (6), thereby increasing their transcriptional activity. p38 is the homologue of the *Saccharomyces cerevisiae* HOG1 gene, and its function is still unknown, although recently available information suggests that p38 might play a critical role in the inflammatory response (7).

Recent findings have helped to unveil the pathway linking cell surface receptors to ERKs (3). In contrast, the mechanism of activation of JNKs is still poorly understood. JNKs were shown to be activated by a variety of stimuli distinct from those that elevate the enzymatic activity of ERKs, including protein synthesis inhibitors, heat shock, changes in osmolarity, and ultraviolet irradiation (6, 8). JNKs can be also activated by agents acting on cell surface receptors, such as tumor necrosis factor-α, interleukin-1, or epidermal growth factor (8). Furthermore, available evidence suggest that whereas Ras controls the activation of ERKs, members of the Rho family of small GTP-binding proteins, Rac1 and Cdc42, regulate the activity of JNKs (9).

Recently, we have shown that stimulation of certain G protein-coupled receptors expressed in NIH 3T3 cells can effectively induce JNK activity, however, following a time course clearly distinct from that of ERK activation (1). In the present study, we have used the transient expression in COS-7 cells of m1 and m2 muscarinic receptors (mAChRs) as a model system to study the biochemical route connecting G protein-coupled receptors to an epitope-tagged JNK. We present evidence that signaling from m1 and m2 mAChRs to JNK involves $\beta\gamma$ subunits of heterotrimeric G proteins, acting on a Ras- and Rac1-dependent pathway.

EXPERIMENTAL PROCEDURES

Cell Lines and Transfection—COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% of fetal bovine serum. Cells were transfected by the DEAE-dextran technique, adjusting the total amount of DNA to 5–10 μg/plate with vector DNA (pcDNA3; Invitrogen), when necessary (9).

Expression Plasmids—Expression plasmids for an epitope-tagged JNK (pcDNA3-HA-JNK), for m1 and m2 mAChRs, for the dominant-negative mutants of the small GTP-binding proteins Ras, RhoA, Rac1, and Cdc42, as well as expression plasmids for β_1 and γ_2 subunits of heterotrimeric G proteins, or the isoprenylation defective mutant of γ_2, γ_2*, have been described (9, 10). Plasmids expressing GTPase-deficient, constitutively activated forms of representative α subunits of heterotrimeric G proteins have been described previously. In each case, a critical glutamine residue in the G3 region of $G\alpha_s$ (codon 227), $G\alpha_{i2}$ (codon 205), $G\alpha_q$ (codon 209), and $G\alpha_{13}$ (codon 226) was replaced for leucine (QL mutants) (10, 11). An expression plasmid for a chimeric molecule between the extracellular and transmembrane domains of CD8 fused to the COOH-terminal domain of βARK (pcDNA-CD8-βARK) has been described recently (11).

JNK Assay and Western Blots—JNK activity in lysates from COS-7

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK (or SAPK), c-Jun NH_2-terminal kinase (or stress-activated protein kinase).

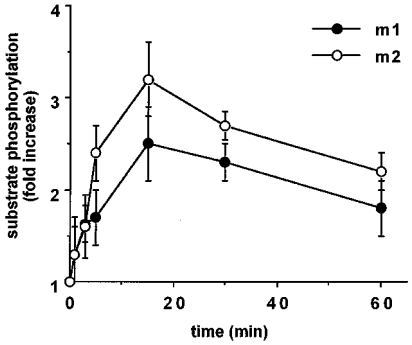

FIG. 1. **The cholinergic agonist carbachol induces JNK (SAPK) activity in COS-7 cells transfected with expression plasmids for m1 or m2 muscarinic receptors.** COS-7 cells were transfected with expression plasmids for m1 or m2 mAChRs (1 μg/plate) together with a plasmid expressing an epitope-tagged JNK (pcDNA3-HA-JNK, 1 μg/plate), as indicated. Cultures were stimulated by addition of carbachol (10 μM) for the indicated time, cells were lysed, and JNK activity was determined in the HA immunoprecipitates as described under "Experimental Procedures." Data represent the mean ± S.E. of three independent experiments, expressed as -fold increase in JNK activity with respect to nonstimulated cells.

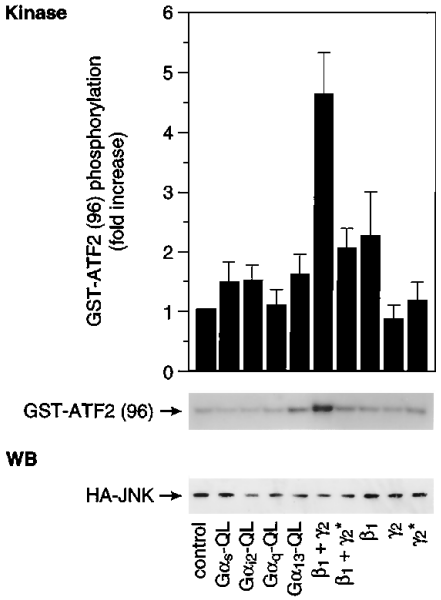

FIG. 2. **Overexpression of $\beta\gamma$ subunits of heterotrimeric G proteins results in stimulation of JNK activity.** COS-7 cells were transfected with pcDNA3-HA-JNK (1 μg/plate) together with pcDNA3 vector (control) or with an expression vector carrying cDNAs for the activated (*QL*) forms of the α subunits of G_s, G_{i2}, G_q, and G_{13}, or expressing β_1, γ_2, or γ_2* G protein subunits, alone or in combination, as indicated (2 μg/plate in each case). Kinase reactions and Western blot (*WB*) analysis were performed in anti-HA immunoprecipitates from the corresponding lysates, as described under "Experimental Procedures." Autoradiograms correspond to representative experiments. ^{32}P-Labeled products as well as specific bands detected by the anti-HA antibody are indicated with an *arrow*. Data represent the mean ± S.E. of four to five independent experiments, expressed as -fold increase with respect to vector-transfected cells (control).

cells transfected with an expression vector for an epitope-tagged JNK (pcDNA3-HA-JNK) was determined upon immunoprecipitation with the anti-HA-specific monoclonal antibody 12CA5 (Babco) using bacterially expressed GST-ATF2(96) fusion protein as a substrate, as described previously (9). The products of the kinase reactions were fractionated in SDS-12% polyacrylamide electrophoresis gels, and radioactivity incorporated into GST-ATF2(96) was determined with the use of a PhosphorImager system (Molecular Dynamics). Parallel immunoprecipitates were analyzed by Western blotting after SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with the anti-HA monoclonal antibody. Immunocomplexes were visualized by enhanced chemiluminescence detection (Amersham Corp.) using goat anti-mouse serum coupled to horseradish peroxidase as a secondary antibody (Cappel).

RESULTS AND DISCUSSION

In order to study the mechanism controlling the activation of JNK by m1 and m2 G protein-coupled receptors, we coexpressed these receptors together with an epitope-tagged JNK (HA-JNK) in COS-7 cells. We observed that when cotransfected with the HA-JNK cDNA, both m1 and m2 were expressed at similar high levels (data not shown), and the epitope-tagged JNK was efficiently expressed and readily detectable upon immunoprecipitation with the anti-HA monoclonal antibody (9). In cells expressing either muscarinic receptor, the cholinergic agonist carbachol induced an increase in JNK activity, as judged by its *in vitro* phosphorylating activity using GST-ATF2(96) as a substrate (Fig. 1). Whether mediated by m1 or m2 receptors, induction of GST-ATF2(96) phosphorylation showed a peak at approximately 15 min after stimulation, being of 2–4-fold higher than the control with a slow decrease at later times (Fig. 1). Thus, JNK is activated by either m1 or m2 G protein-coupled receptors when expressed in COS-7 cells. Whereas m1 receptors are typical of those coupled through G proteins of the G_q family to phospholipase C activation, m2 is known to couple through G_i to a number of effector pathways, including to the inhibition of adenylyl cyclases (12). Interestingly, both m1 and m2 mAChRs appear to activate JNK irrespective of their G protein-coupling specificity.

As an approach to investigate which G proteins mediate the activation of JNK, we took advantage of the observation that the expression of GTPase-deficient, mutationally activated forms of G protein α subunits can activate effector pathways by obviating the need for receptor stimulation (13). Thus, we coexpressed the epitope-tagged JNK together with GTPase-deficient mutants for $G\alpha_s$, $G\alpha_{i2}$, $G\alpha_q$, and $G\alpha_{13}$, which are representative members for each of the four $G\alpha$ subunit families (14). Expression of each G protein α subunit, when transfected into COS-7 cells, could be demonstrated by immunoblotting with subtype-specific antibodies (data not shown; Refs. 11 and 12). However, these activated mutants enhanced JNK activity only to a very limited extent, either when each $G\alpha$ subunit was expressed alone (Fig. 2) (15) or in all possible combinations (data not shown). Thus, α subunits of heterotrimeric G proteins might not mediate JNK activation by G protein-coupled receptors.

When activated, receptors linked to G proteins catalyze the replacement of GDP by GTP-bound to the α subunit and induce the dissociation of α-GTP from $\beta\gamma$ dimers. Although the α subunits were thought to be solely responsible for coupling receptors to second-messenger-generating systems, recent work has established a critical role for $\beta\gamma$ dimers in signal transduction (10, 14). Thus, the failure of mutationally activated G protein α subunits to elevate JNK activity prompted us to explore whether $\beta\gamma$ dimers participate in signaling to JNK. We observed that, when cotransfected, $\beta_1\gamma_2$ subunits induce a remarkable increase in the phosphorylating activity of the epitope-tagged JNK, although expression of the HA-JNK was similar for each transfected cell population (Fig. 2). Similar results were obtained when $\beta_2\gamma_2$ subunits were expressed (data not shown). In contrast, JNK was poorly activated when coexpressed with β_1 or γ_2 alone or when cotransfected with β_1 and an altered form of the γ_2 subunit, designated γ_2*, that lacks an isoprenylation signal and fails to associate to the plasma membrane (10) (Fig. 2). We conclude that membrane-bound $\beta\gamma$ subunits of heterotrimeric G proteins, but not $G\alpha$ subunits, can effectively stimulate JNK activity in COS-7 cells.

We next explored a role for $\beta\gamma$ complexes in JNK stimulation by mAChRs. To approach this question, we employed a chi-

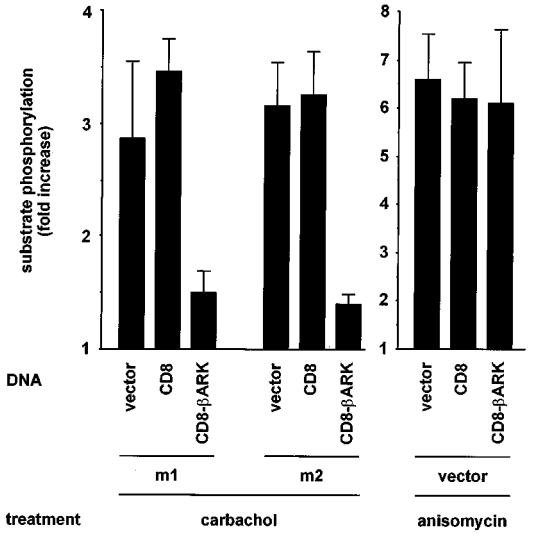

FIG. 3. **Effect of a $\beta\gamma$ scavenging protein in JNK activation.** pcDNA3-HA-JNK was cotransfected with expression plasmids for m1 or m2 mAChRs (1 μg/plate), and β_1 and γ_2 subunits (2 μg per plate), together with vector alone (vector) or plasmids expressing the CD8 receptor or a chimeric molecule CD8-βARK, as indicated. Cells were stimulated with carbachol (10 μM) or anisomycin (10 μg/ml) for 15 min or left untreated. Quantitation of JNK activity present in anti-HA immunoprecipitates was performed as in Fig. 1. Data represent the mean ± S.E. of three independent experiments, expressed as -fold increase with respect to unstimulated cells.

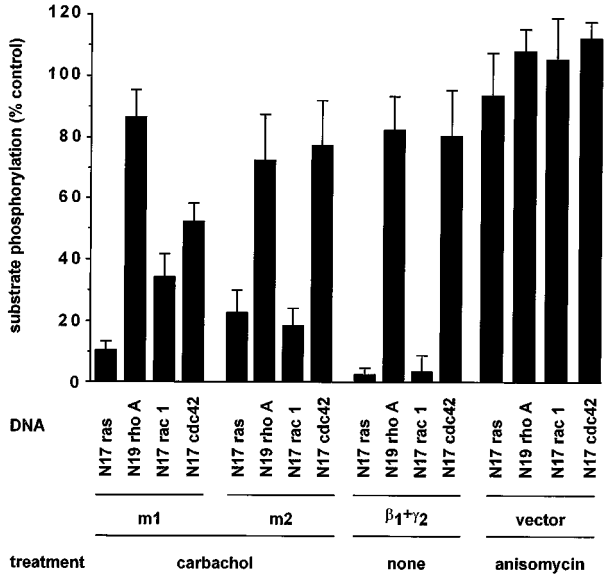

FIG. 4. **Effect of dominant-negative mutants for Ras, RhoA, Rac1, and Cdc42 on JNK activation.** COS-7 cells were transfected with pcDNA3-HA-JNK and expression plasmids for m1 and m2 receptors (1 μg/plate) or for β_1 and γ_2 G protein subunits (2 μg/plate), together with either pcDNA3 (vector) without insert or carrying cDNAs for N17Ras, N19RhoA, N17Rac1, or N17Cdc42 (1 μg/plate), and cells were left untreated or stimulated with carbachol (10 μM) or anisomycin (10 μg/ml) for 15 min, as indicated. Cell lysates were processed as in Fig. 1. Data represent the mean ± S.E. of four to five independent experiments, expressed as percent of activation with respect to the corresponding vector cotransfected control (100%).

meric molecule combining the extracellular and transmembrane domain of CD8 fused to the carboxyl-terminal domain of βARK, which includes the $\beta\gamma$-binding region (11). This chimeric molecule expresses the CD8 antigen at the cell surface, localizing the βARK carboxyl-terminal domain to the inner face of the plasma membrane. The CD8-βARK chimera is expected to bind and sequester free $\beta\gamma$ complexes when dissociated from Gα subunits upon receptor stimulation, thus blocking $\beta\gamma$-dependent pathways (11). As shown in Fig. 3, coexpression of CD8-βARK with the m1 or m2 mAChRs nearly abolished the activation of JNK in response to carbachol, while CD8 alone had no demonstrable effect. In contrast, JNK activation by stress-inducing agents such as anisomycin (9) was unaffected by CD8-βARK, demonstrating the specificity of this approach (Fig. 3). Taken together, these findings strongly suggest that signaling from m1 and m2 mAChRs to JNK is mediated by $\beta\gamma$ subunits of heterotrimeric G proteins.

We have recently shown that the Rho-related small GTP-binding proteins Rac1 and Cdc42 are integral components of signaling pathways linking certain cell surface receptors to JNK (9). Thus, we set out to investigate whether these small GTPases mediate JNK activation by G protein-coupled receptors, using as a tool the expression of dominant-negative mutants for Ras, RhoA, Rac1, and Cdc42. None of these dominant-inhibitory small GTP-binding proteins affected the JNK activation in response to anisomycin (Fig. 4). In contrast, as shown in Fig. 4, coexpression of N17Ras and N17Rac1 prevented JNK activation by either mAChRs, or when induced by $\beta\gamma$ complexes, N17Cdc42 diminished JNK stimulation by m1 mAChRs, and the dominant-negative form of RhoA did not display any significant effect. Similarly, we have recently shown that JNK activation by EGF in COS-7 cells is also blocked by N17Ras, N17Rac1, and N17Cdc42 (9). Thus, these findings support a role for Ras and Rac1 or Cdc42 in linking both tyrosine kinase and G protein-coupled receptors to the JNK pathway.

Although Ras controls the activity of ERKs, recent available evidence suggests that Rac1 and/or Cdc42 regulate JNK activation (9). Thus, distinct small GTP-binding proteins appear to link cell surface receptors with independent signaling pathways leading to the activation of each member of the MAPK superfamily. In this study, we present evidence supporting a role for $\beta\gamma$ subunits of heterotrimeric G proteins in communicating G protein-coupled receptors with the JNK pathway, acting on a Ras- and Rac1-dependent biochemical route. Furthermore, we and others have previously shown that $\beta\gamma$ complexes link this class of cell surface receptors to ERKs, in this case acting through Ras (10, 16). Thus, taking these findings together we can postulate that $\beta\gamma$ heterodimers provide a link between heterotrimeric G proteins and small GTP-binding proteins.

The molecular basis for this interaction is still poorly defined (16). However, it is strikingly similar to that of the pathway linking the G protein-coupled pheromone receptors to MAPK-related enzymes in the budding yeast *S. cerevisiae*. In this case, extracellular ligands (α or a factors) activate pheromone receptors which, in turn, induce the dissociation of a heterotrimeric G protein into α (GPA1) and $\beta\gamma$ (Ste4, Ste18) subunits (see Ref. 17 for review). Free $\beta\gamma$ dimers then activate a serine-threonine kinase (Ste20), initiating activity from a linear cascade of kinases, including sequentially Ste11, Ste7, and the yeast MAPK homologues Fus3 and Kss1. Extensive search for molecules linking yeast $\beta\gamma$ complexes to Ste20 has led to the recent discovery that Cdc42 participates in Ste20 activation (18), and that Ste4 might directly bind and activate Cdc24, a nucleotide exchange factor for Cdc42 (19). Available data suggest that additional proteins might also be involved, including a protein designated Ste5, which plays a role as a platform or scaffold recruiting Ste11, Ste7, Kss1/Fus3 (20), and another small GTP-binding protein, the Ras homologue Rsr1 (21). The latter also appears to play an important role by binding Cdc24 and, probably, by positioning this guanine nucleotide exchange factor within the cell, thus allowing its interaction with Cdc42 and its targets (21). These observations represent an interesting ex-

ample of convergence between Ras-like (Rsr1) and Rho-like (Cdc42) biochemical routes and might also provide a clue regarding the pathway connecting mammalian $\beta\gamma$ to JNK. Based upon these observations in yeast and our present findings, we can hypothesize that upon receptor stimulation $\beta\gamma$ dimers might recruit a yet to be identified guanine nucleotide exchange factor for Rac1 and/or Cdc42 and that Ras functioning may be necessary for the effective activation of these GTP-binding proteins and the consequent stimulation of JNK. We conclude that our present study might represent a biologically relevant example of a signal transduction pathway extraordinarily conserved from yeast to mammals and might provide an attractive model to elucidate the nature of those molecules linking heterotrimeric G proteins to small GTP-binding proteins.

REFERENCES

1. Coso, O. A., Chiariello, M., Kalinec, G., Kyriakis, J. M., Woodgett, J., and Gutkind, J. S. (1995) *J. Biol. Chem.* **270,** 5620–5624
2. Cano, E., and Mahadevan, L. C. (1995) *Trends Biochem. Sci.* **20,** 117–122
3. Marshall, C. J. (1995) *Cell* **80,** 179–185
4. Davis, R. J. (1993) *J. Biol. Chem.* **268,** 14553–14556
5. Hill, C. S., and Treisman, R. (1995) *Cell* **80,** 199–211
6. Gupta, S., Campbell, D., Derijard, B., and Davis, R. J. (1995) *Science* **267,** 389–393
7. Lee, J. C., Laydon, J. T., Mc Donnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., Landvatter, S. W., Strickler, J. E., McLaughlin, M. M., Siemens, I. R., Fisher, S. M., Livi, G. P., White, J. R., Adams, J. L., and Young, P. R.. (1994) *Nature* **372,** 739–746
8. Kyriakis, J., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E., Ahmad, M., Avruch, J., and Woodgett, J. (1994) *Nature* **369,** 156–160
9. Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J. S. (1995) *Cell* **81,** 1137–1146
10. Crespo, P., Xu, N., Simonds, W. F., and Gutkind, J. S. (1994) *Nature* **369,** 418–420
11. Crespo, P., Cachero, T. G., Xu, N., and Gutkind, J. S. (1995) *J. Biol. Chem.* **270,** 25259–25265
12. Ashkenazi, A., Peralta, E. G., Winslow, J. W., Ramachandran, J., and Capon, D. J. (1989) *Trends. Pharmacol. Sci.* (suppl.) 16–22
13. Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56,** 615–649
14. Neer, E. (1995) *Cell* **80,** 249–257
15. Prasad, M. V., Dermott, J. M., Heasley, L. E., Johnson, G. L., and Dhanasekaran, N. (1995) *J. Biol. Chem.* **270,** 18655–18659
16. van Biesen, T., Hawes, B. E., Luttrell, D. K., Krueger, K. M., Touhara, K., Porfiri, E., Sakaue, M., Luttrell, L. M., and Lefkowitz, R. J. (1995) *Nature* **376,** 781–784
17. Bardwell, L., Cook, J. G., Inouye, C. J., and Thorner, J. (1994) *Dev. Biol.* **166,** 363–379
18. Simon, M. N., De Virgilio, C., Souza, B., Pringle, J. R., Abo, A., and Reed, S. (1995) *Nature* **376,** 702–705
19. Zhao, Z., Leung, T., Manser, E., and Lim, L. (1995) *Mol. Cell. Biol.* **15,** 5246–5257
20. Whiteway, M. S., Wu, C., Leeuv, T., Clark, K., Fourest-Lieuvin, A., Thomas, D. Y., and Leberer, E. (1995) *Science* **269,** 1572–1575
21. Zheng, Y., Bender, A., and Cerione, R. A. (1995) *J. Biol. Chem.* **270,** 626–630