A Rac1 Effector Site Controlling Mitogenesis through Superoxide Production

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The Rac GTP-binding protein controls signal transduction pathways that are critical for mitogenesis and oncogenesis (1, 2). The biochemical nature of these signaling pathways is presently unknown. Here we report that a region in Rac1 (residues 124–135), previously defined as the insert region (3), is essential for its mitogenic activity. Deletion of this region does not interfere with the ability of Rac1 to induce cytoskeletal changes or to activate the Jun kinase mitogen-activated protein kinase cascade but abrogates Rac1-induced stimulation of DNA synthesis and Rac1-mediated superoxide production in quiescent fibroblasts. Treatment of cells with agents that abolish superoxide generation inhibits specifically the mitogenic effect of Rac1. Our results identify an effector site in Rac1 that is necessary for mitogenic signaling and implicate superoxide generation as a candidate effector pathway of Rac1-dependent cell growth.

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Rac proteins have been shown to play a fundamental role in signaling pathways controlling actin polymerization, transcriptional activation, cellular proliferation, and superoxide generation (1, 4–7). Genetic and biochemical studies have indicated that the signaling activities of Rac are mediated by distinct target molecules (3–10). A number of Rac targets have been identified to date, based on their ability to interact preferentially with the GTP-bound form of Rac. These include the p65 Ser/Thr protein kinase PAK, the Ser/Thr kinase p160 ROCK, a cytoplasmic component of the NADPH oxidase complex p67PHOX, and the Rac-binding protein POR1 (reviewed in Ref. 3). Here we report that a region in Rac1 (residues 124–135), previously defined as the insert region (3), is essential for its mitogenic activity. Deletion of this region does not interfere with the ability of Rac1 to induce cytoskeletal changes or to activate the Jun kinase mitogen-activated protein kinase cascade but abrogates Rac1-induced stimulation of DNA synthesis and Rac1-mediated superoxide production in quiescent fibroblasts. Treatment of cells with agents that abolish superoxide generation inhibits specifically the mitogenic effect of Rac1. Our results identify an effector site in Rac1 that is necessary for mitogenic signaling and implicate superoxide generation as a candidate effector pathway of Rac1-dependent cell growth.

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EXPERIMENTAL PROCEDURES

Microinjection and Immunofluorescence—For microinjection experiments, COS1 or REF-52 cells were plated onto gridded glass coverslips and cultured in DMEM supplemented with FBS (10%). The cells were grown to confluence and then placed in DMEM with 0.5% FBS for 24 h before microinjection. A plasmid mixture containing indicated plasmids in microinjection buffer (50 mM Hepes (pH 7.2), 100 mM KCl, and 5 mM NaPO4) was microinjected into cell nuclei. For monitoring membrane ruffles and protein expression, cells were fixed in 3.7% formaldehyde in PBS for 30 min at room temperature. The coverslips were incubated for 1 h at 37 °C with mouse antibody to T7 epitope (Novagen) in PBS containing albumin (2 mg/ml) and then with a mixture of fluorescein-conjugated goat antibody to mouse monoclonal antibody to mouse immunoglobulin G and rhodamine-labeled phalloidin (0.01 mg/ml) (Molecular Probes). The cells were photographed with a Zeiss Axiphot fluorescence microscope.

DNA Synthesis—For monitoring DNA synthesis, REF-52 cells were plated on gridded coverslips and serum-starved for 24 h. Cells were injected with 2.5 µg/ml of each of the indicated constructs, and BrdUrd (10 µM) was added to the cell culture medium at a 2 h after injection. After 30 h, cells were fixed in acid alcohol (ethanol:water:acetic acid 90:5:5) for 1 h at 20 °C and immunostained with mouse monoclonal antibody to BrdUrd (Sigma) as described (16). At least 100 injected cells were scored in each assay for quantitation. Chemical inhibitors affecting superoxide or other reactive oxidants (300 units/ml superoxide dismutase (Sigma), 20 mM N-acetyl-l-cysteine (Sigma), and 5 µM diphenylene iodonium chloride (Toronto Research) were added immediately after injection.

Superoxide Production—COS1 cells were plated onto glass coverslips, grown to confluence, and then either microinjected or transfected with the indicated expression vectors. For microinjection, cells were injected with 50 µg/ml of expression plasmids. After 10 h, the cells were incubated with either 300 units/ml SOD or vehicle (DMEM). The medium was then replaced with DMEM containing 0.5% nitro blue tetrazolium (Sigma) with or without SOD. The cells were incubated for 1 h at 37 °C before being fixed and stained with anti-T7 monoclonal antibodies. For quantitation, COS1 cells on coverslips were placed in 10-cm tissue culture dishes and transfected with 10 µg of the indicated expression vector using the CaPO4 method. pCMV-GFP was used as a negative control. After 12 h of incubation with the DNA-CaPO4 precipitate, cells were washed three times with PBS and allowed to recover in DMEM containing 5% fetal calf serum for 12 h. The serum containing medium was then replaced with DMEM for an additional 12 h. The coverslips were incubated in 0.5% NBT in DMEM for 1 h. Cells were stained using either anti-Raf (Transduction Laboratories) or T7 monoclonal antibodies, and those that expressed the exogenous protein were then scored for the presence of the reduced form of NBT, blue formazan. Measurement of intracellular reactive oxygen species generation by the DCFDA loading method was carried out as described (17).

Jun Kinase (JNK) Activity—For monitoring JNK activation, cells were cotransfected with 10 µg of FLAG-tagged JNK1 and 10 µg of expression vectors containing no insert, RacV12, or RacV12.A18s using
the CaPO₄ method. After a 12-h incubation with the DNA-CaPO₄ precipitates, cells were incubated in medium containing FBS (5%) for 6 h and then incubated for 12 h in serum-free medium. JNK1 immunocomplex kinase assay was carried out as described (8). The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. Fold activation was determined using the Storm 860 PhosphorImager in combination with Image Quant v1.1 software (Molecular Dynamics).

Expression Plasmids—Plasmid pCGT, which is derived from pCGN with a replacement of the HA epitope by the T7 epitope, was used as a mammalian expression vector to express the various Rac mutants. pCGT RacV12.H40 and pCGT RacV12.L37 were created as described (8) and ligated into the XhoI-BamHI sites of pCGT. pCGT RacV12,ΔIns was created as described (3) except that the Cysⁱ¹⁹ to Ser mutation originally made to increase protein stability was not incorporated. The CMV-GFP vector was constructed by fusing the cytomegalovirus promoter to the GFP reporter.

RESULTS AND DISCUSSION

To investigate the significance of the insert region for the biological effects of Rac, we examined the signaling activities of a mutant form of activated Rac (RacV12) containing a complete deletion of the insert region between residues 124–135 (RacV12,ΔIns). Earlier microinjection studies have established that Rac-mediated changes in the actin cytoskeleton lead to the formation of membrane ruffles (4). Therefore, the role of the insert region in Rac-induced actin polymerization was examined by microinjection of serum-starved COS1 cells with expression plasmids encoding T7 epitope-tagged versions of Rac mutants. We found that RacV12,ΔIns was as effective as RacV12 in inducing actin polymerization and membrane ruffling, as judged by filamentous actin staining with rhodamine phalloidin (Fig. 1a). Immunofluorescence staining of the injected cells confirmed that both Rac mutants were expressed to the same extent and displayed an overall similar subcellular distribution pattern (not shown). These results indicate that the insert region is not necessary for effector interactions that control actin cytoskeleton rearrangements. It has been shown that Rac can stimulate kinase cascades leading to the activation of the JNK mitogen-activated protein kinase cascade (5, 6). This activation is presumably mediated by the activation of the Rac target PAK (18–20). To test the Rac insert region mutant for its ability to activate the JNK mitogen-activated protein kinase cascade, COS1 cells were cotransfected with expression plasmids encoding the Rac mutants and a plasmid encoding FLAG-tagged version of JNK1. JNK activity was assayed by immunocomplex kinase assay using glutathione S-transferase-Jun as the substrate and visualized by autoradiography. Expression of JNK1 and Rac mutants was determined by Western blot analysis with polyclonal antibodies to JNK and monoclonal antibodies to T7. c, relative percentages of BrdUrd incorporation in cells expressing Rac-V12,ΔIns were scored per condition in each experiment. 100% maximum corresponds to 52 ± 14% of the total injected cells.

FIG. 1. Effects of RacV12 and RacV12,ΔIns on membrane ruffling, JNK activation, and DNA synthesis. a, serum-starved COS1 cells were coinjected with expression plasmids encoding the indicated T7 epitope-tagged Rac mutants or with CMV-GFP as a vector control (Vect., 50 μg/ml). b, RacV12-dependent induction of membrane ruffles was measured by fluorescence microscopy (Fig. 1a). a, RacV12,ΔIns expression plasmids. JNK activity was measured by immunocomplex kinase assays with glutathione S-transferase-Jun as the substrate and visualized by autoradiography. Expression of JNK1 and Rac mutants was determined by Western blot analysis with polyclonal antibodies to JNK and monoclonal antibodies to T7. c, relative percentages of BrdUrd incorporation in cells expressing Rac-V12,ΔIns were scored per condition in each experiment. 100% maximum corresponds to 52 ± 14% of the total injected cells.

Because the Rac insert region has been implicated in the activation of NADPH oxidase in phagocytic cells (3, 15), we investigated the relationship between Rac-mediated superoxide production and mitogenesis in nonphagocytic cells. COS1 cells were transfected with Rac mutants expression plasmids, and superoxide production was examined using the NBT reduction assay (21). This assay involves the incubation of cells with NBT, which when reduced forms an insoluble purple precipitate, and has been used to demonstrate a superoxide-generating NADPH oxidase system in fibroblasts (22). Cells expressing RacV12 were positive for NBT staining, as detected by bright field microscopy (Fig. 2a). RacV12-dependent NBT reduction was inhibited by the incubation of transfected cell with SOD, indicating that NBT staining is because of Rac-induced superoxide production (Fig. 2a). This finding is consistent with earlier reports implicating Rac in the regulation of intracellular reactive oxygen species production in...
FIG. 2. Analysis of superoxide production using the NBT reduction assay. a, serum-starved COS1 cells were injected with expression vectors for pCGT-RacV12. Cells were then incubated for 10 h to allow the protein to express followed by a 1-h incubation in 0.5% NBT containing DMEM. In the lower panels, cells were incubated in 300 units/ml SOD (bovine erythrocyte source) 1 h previous to the addition of NBT. Top, cells expressing T7-tagged RacV12 as determined by indirect immunofluorescence using an anti-T7 monoclonal antibody. Middle, bright field micrographs of the corresponding fields showing the result of NBT reduction. Bottom, phase contrast micrographs of the corresponding fields.

b, quantification of superoxide production. COS1 cells transfected with the indicated constructs were incubated with NBT as above. Cells expressing the proteins as determined by immunofluorescence or autofluorescence in the case of GFP were scored for the presence of blue formazan precipitate. Values correspond to the means of three independent experiments, and the error bars represent the standard deviations. At least 100 cells were scored per condition in each experiment.

It should be noted that the NBT reduction assay was not sufficiently sensitive to detect Rac-mediated superoxide production in REF-52 cells. Another method for the detection of increase in intracellular reactive oxygen species involves the loading of cells with the fluorophore DCFDA, which fluoresces upon interaction with H$_2$O$_2$ (17). Using this method, we were able to detect an increase in DCFDA fluorescence in cells injected with RacV12. In contrast, no increase in DCFDA fluorescence was detected in cells injected with RacV12ΔIns (not shown). Thus the results obtained in REF-52 cells and COS1 cells with respect to the relative abilities of Rac mutants to stimulate the production of reactive oxygen species are qualitatively similar. However, because the DCFDA loading method does not permit a quantitative analysis in which protein expression and production of reactive oxygen species can be correlated on a per cell basis, we have used the NBT reduction assay in the subsequent experiments. As illustrated in Fig. 2b, virtually all cells expressing RacV12 were positive for NBT staining. The frequency of NBT-stained cells was reduced to nearly background levels in cells expressing RacV12ΔIns, indicating that this mutant failed to induce superoxide production. Likewise, Raf-CAAX was also deficient in superoxide production. These observations together with the finding that RacV12ΔIns lacks mitogenic activity suggest a role for superoxide production in mediating the effects of Rac on cell proliferation.

To further test this idea, we examined the effects of various agents that interfere with superoxide production on Rac-induced mitogenesis. Treatment of RacV12 expressing cells with the specific flavoprotein inhibitor diphenylene iodonium (DPI) and SOD had no appreciable effect on Rac-induced membrane ruffling or JNK activation. The anti-oxidant N-acetyl cysteine (NAC) had no effect on Rac-induced membrane ruffling but moderately inhibited Rac-induced JNK activation. In addition, these inhibitors had no effect on the levels of expression of Rac or JNK as determined by Western blot analysis (not shown). In contrast, NAC,

FIG. 3. Effect of superoxide inhibitors on Rac-induced membrane ruffling, JNK activation, and DNA synthesis. a, quiescent REF-52 cells were microinjected with T7 epitope-tagged RacV12 (50 μg/ml). 6 h after injection, chemical inhibitors were added to the culture medium (20 μM DPI, 300 units/ml SOD, 20 mM NAC). After 1 h, cells were stained with anti-T7 monoclonal antibodies to visualize protein expression and membrane ruffles. Identical results were obtained when inhibitors were added immediately after injection for 16 h. b, COS1 cells were transfected as in Fig. 1b; however, the indicated chemical inhibitors (5 μM DPI, 300 units/ml SOD, 20 mM NAC) were added either 16, 5, or 1 h prior to cell lysis for JNK assay. Fold activation was determined using a Storm 860 PhosphorImager. Results are from a single representative experiment. Experiments were repeated twice with similar results. c, quiescent REF-52 cells were microinjected with RacV12 plus Raf-CAAX (black bars) or treated with 20% FCS (gray bars), and relative percentages of BrdUrd positive cells were determined after 30 h. Controls correspond to cells injected with vector alone (Vect.) or serum-starved cells. Inhibitors were added to the culture medium immediately after injection or at the time of serum addition. Values correspond to the means of three independent experiments, and the error bars represent the standard deviations. At least 100 cells were scored per condition in each experiment. 100% maximum corresponds to 47 ± 12% of total injected cells and to 52 ± 8% of serum-stimulated cells.

FIG. 4. Production of superoxide by Rac mutants. a, COS1 cells were transfected with 10 μg of the indicated construct, and NBT reduction was scored as in Fig. 2b. Values correspond to the means of three independent experiments, and the error bars represent the standard deviations. At least 100 cells were scored per condition in each experiment. b, schematic illustration of the effector pathways activated by different Rac mutants and their relative contribution to Rac-induced mitogenesis.
DPI, and SOD blocked the stimulation of DNA synthesis induced by coinjection of RacV12 and Raf-CAAX (Fig. 3c). All inhibitors were used at the minimal concentration, which results in the complete inhibition of Rac-induced superoxide production, as determined by DCFDA fluorescence. Furthermore, at these concentrations, DPI and SOD had no effect on serum-induced DNA synthesis (Fig. 3c). In contrast, NAC induced a 70% inhibition of DNA synthesis, indicating that this agent exerts a more general inhibitory effect on cell growth. We conclude that Rac-mediated superoxide production is specifically required for its growth promoting effects. Significantly, DPI exerted an inhibitory effect on DNA synthesis if added at intervals up to 10 h after injection. Beyond this interval DPI addition had no effect on the ability of RacV12 and Raf-CAAX to stimulate DNA synthesis. In REF-52 cells, the $G_1$ phase of the cell cycle is approximately 18 h long (not shown). Thus it appears that production of reactive oxygen species is required up to mid $G_1$ phase.

Using effector-binding loop mutants of Rac, it has been recently demonstrated that the activation of PAK and JNK are not required for Rac-induced mitogenesis (8–10). On the other hand, a correlation was observed between Rac-induced actin polymerization and cell cycle progression (8, 9). Our finding that RacV12,ΔIns retained the ability to induce membrane ruffling but was no longer able to stimulate DNA synthesis indicates that Rac-induced actin polymerization is not sufficient for its growth promoting activity. This result is consistent with a recent report suggesting that membrane ruffling is not sufficient for the full transforming activity of Rac (10). Utilizing the NBT reduction assay, the capacity of the Rac effector-binding loop mutants RacV12,L37 and RacV12,H40 to stimulate superoxide production was tested. The RacV12,L37 mutant activates JNK but is defective in inducing actin polymerization, whereas the RacV12,C40 induces actin polymerization but is defective in JNK activation (Fig. 4b and Ref. 8). Both mutants were as effective as RacV12 in inducing superoxide production (Fig. 4a). However, as shown previously, only the RacV12,H40 is functional in promoting cell proliferation and transformation (8). Therefore, although necessary, superoxide production is not sufficient for the mitogenic activity of Rac. Together, these observations suggest that both Rac-induced actin polymerization and superoxide production are required for Rac-controlled cell proliferation (Fig. 4b). It remains to be determined whether additional Rac-mediated signals contribute to its mitogenic activity.

Superoxide generation has been frequently implicated in the control of normal cell growth and the promotion of malignant transformation (24–27). Our findings indicate that Rac-induced superoxide production is a critical mediator of mitogenic signaling. The molecular identification of the cellular targets of Rac-mediated superoxide generation should provide insights into the mechanisms linking reactive oxygen species and growth control.

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REFERENCES
1. Qui, R.-G., Chen, J., Kirn, D., McCormick, F. & Symons, M. (1995) Nature 374, 457–459
2. Olson, M., Ashworth, A. & Hall, A. (1995) Science 268, 1270–1272
3. Freeman, J., Abo, A. & Lambeth, J. D. (1996) J. Biol. Chem. 271, 19784–19801
4. Ridley, A. J., Paterson, H. F., Johnston, C., Diekmann, D. & Hall, A. (1992) Cell 70, 401–410
5. Coso, O. & Gutfnick, J. S. (1995) Cell 81, 1137–1146
6. Minden, A., Lin, A., Clare, F.-X., Abo, A. & Karin, M. (1995) Cell 81, 1147–1157
7. Abo, A., Pick, E., Hall, A., Totty, N., Teahan, C. & Segal, A. (1991) Nature 353, 668–670
8. Joneson, T., McDonough, M., Bar-Sagi, D. & VanAelst, L. (1996) Science 274, 1374–1376
9. Lamarche, N., Tapon, N., Stowers, L., Burbelo, P., Aspenstrom, P., Bridges, T., Chant, J. & Hall, A. (1996) Cell 87, 519–529
10. Westwick, J., Lambert, Q., Clark, G., Semons, M., Aelst, L. V., Pestell, R. & Der, C. (1997) Mol. Cell Biol. 17, 1324–1335
11. Tapon, N. & Hall, A. (1997) Curr. Opin. Cell Biol. 9, 86–92
12. Diekmann, D., Nohes, C., Burbelo, P., Abo, A. & Hall, A. (1995) EMBO J. 14, 5297–5305
13. Kwong, C., Adams, A. & Leto, T. (1995) J. Biol. Chem. 270, 19868–19872
14. Nisimoto, Y., Freeman, J., Motahebi, A., Hirshberg, M. & Lambeth, J. D. (1997) J. Biol. Chem. 272, 18834–18841
15. Gili, J. & Pick, E. (1995) J. Biol. Chem. 270, 29079–29082
16. Joneson, T., White, M., Wigler, M., Bar-Sagi, D. (1996) Science 271, 180–182
17. Sundareshan, M., Yu, Z.-Y., Perrans, V. J., Sulciner, D. J., Gutkind, J. S., Iranis, K., Goldschmidt-Clermont, P. J. & Finkel, T. (1996) Biochem. J. 318, 379–382
18. Bagrodia, S., Derijard, B., Davis, R. & Cerione, R. (1995) J. Biol. Chem. 270, 27995–27998
19. Brown, J., Stowers, L., Baer, M., Trejo, J., Coughlin, S. & Chant, J. (1996) Curr. Biol. 6, 598–605
20. Zhang, S., Han, J., Sells, M. A., Chernoff, J., Knaus, U. G., Ulevitch, R. J. & Bokoch, G. M. (1995) J. Biol. Chem. 270, 23934–23936
21. Maly, F.-E., Nakamura, M., Gauchat, J.-F., Urwyler, A., Walker, C., Dahinden, C., Cross, A., Jones, O. & DeWeck, A. (1989) J. Immunol. 142, 1260–1267
22. Mei, T., Cross, A., Hancock, J., Kaup, F. & Jones, O. (1991) Biochem. J. 275, 241–245
23. Sulciner, D., Iranis, K., Yu, Z., Ferrans, V., Goldschmidt-Clermont, P. J. & Finkel, T. (1996) Mol. Cell Biol. 16, 1115–1121
24. Burdon, R. & Gill, V. (1993) Free Radical Res. Commun. 19, 203–213
25. Burdon, R. (1995) Free Radical Res. Med. 18, 775–784
26. Iranis, K., Xia, Y., Zweier, J., Solbott, S., Der, C., Fearon, E., Sundareshan, M., Finkel, T. & Goldschmidt-Clermont, P. J. (1997) Science 275, 1649–1652
27. Oberley, T., Schultz, J., Li, N. & Oberley, L. (1995) Free Radical Res. Med. 19, 53–56