Rac-dependent Anti-apoptotic Signaling by the Insulin Receptor Cytoplasmic Domain*

(Received for publication, March 29, 1999, and in revised form, July 28, 1999)

Jason E. Boehm‡‡, Oleg V. Chaika‡, and Robert E. Lewis‡¶**

From the ‡Eppley Institute for Research in Cancer and Allied Diseases, ¶Department of Biochemistry and Molecular Biology, and §Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska 68198-6805

Mutations in the cytoplasmic domain of the insulin receptor that block the ability of the receptor to stimulate glucose uptake do not block the receptor’s ability to inhibit apoptosis (Boehm, J. E., Chaika, O. V., and Lewis, R. E. (1998) J. Biol. Chem. 273, 7169–7176). To characterize this survival pathway we used a chimeric receptor (CSF1R/IR) consisting of the ligand-binding domain of the colony-stimulating factor-1 receptor spliced to the cytoplasmic domain of the insulin receptor and a mutated version of the chimeric receptor containing a 12-amino acid deletion of the juxtamembrane domain (CSF1R/IRA960). In addition to the inhibition of apoptosis, activation of either the CSF1R/IR or the CSF1R/IRΔ960 rapidly induced membrane ruffling in Rat1 fibroblasts. The small GTPase Rac mediates membrane ruffling. Activated and dominant-inhibitory mutants of Rac and other small GTPases were expressed in Rat1 fibroblasts to examine a potential link between the intracellular pathways that induce membrane ruffling and promote cell survival. The anti-apoptotic action of the CSF1R/IRΔ960 was reversed by dominant-inhibitory RacN17, but not by RasN17 or Cdc42N17. Activated RacV12, but not RasD12 or Cdc42V12, promoted cell survival in the absence of insulin. These data implicate Rac as a mediator of an unique anti-apoptotic signaling pathway activated by the insulin receptor cytoplasmic domain.

The cellular receptor for insulin controls diverse intracellular pathways that regulate metabolism, growth, and development. One conventional approach for identifying the molecular components that constitute these different pathways has been the selective mutagenesis of the receptor’s cytoplasmic domain in an effort to discretely dissociate one biological action of insulin from another (1–7). These observations have led to the suggestion that different regions of the insulin receptor cytoplasmic domain play distinct roles in modulating the biological effects of insulin.

The insulin and IGF I receptors are potent inhibitors of apoptosis (6, 8, 9). Recent studies with a CSF1R/IR suggest that the insulin receptor cytoplasmic domain activates intracellular signaling pathways that are independent of its ability to phosphorylate IRS proteins and Shc (5, 6). The mutated CSF1R/IRΔ960 lacks the ability to phosphorylate the receptor substrates IRS-1 and Shc. Coincident with the loss of receptor substrate phosphorylation, the Δ960 deletion also blocks the activation of ERK MAP kinases, the activation of PI 3-kinase, and the stimulation of glucose transport. However, the Δ960 deletion does not prevent the receptor from inhibiting apoptosis (6).

Previous studies of anti-apoptotic signaling by growth factor receptors have identified PI 3-kinase and the serine/threonine kinase Akt as part of the mechanism that promotes cell survival (10–13). The insulin and IGF I receptors activate PI 3-kinase and, subsequently, Akt through their ability to phosphorylate IRS proteins at sites that facilitate interaction of these receptor substrates with the noncatalytic subunit of PI 3-kinase, p85 (14–16). These observations suggested that alternative pathways could contribute to the insulin receptor’s anti-apoptotic activity.

To identify additional mediators of anti-apoptotic signaling activated by the insulin receptor cytoplasmic domain, other biological effects of the CSF1R/IRΔ960 were investigated. We observed that the CSF1R/IRΔ960 rapidly induced membrane ruffling in a manner dependent on the small GTPase Rac. A contribution of Rac to anti-apoptotic signaling by the chimeric receptor was indicated by the observation that dominant-negative RacN17 blocked survival signals from the CSF1R/IRΔ960. Furthermore, activated RacV12, but not activated RasD12 or Cdc42V12, was sufficient to promote survival in the absence of growth factor stimulation. These results implicate Rac as a mediator of an additional anti-apoptotic signaling pathway activated by the insulin receptor cytoplasmic domain in a manner independent of IRS phosphorylation.

EXPERIMENTAL PROCEDURES

Plasmids and Constructs—cDNAs encoding Myc-tagged RacV12, RacC17, Cdc42V12, Cdc42N17, Ha-RasD12, and Ha-RasV12 were gifts from A. Hall (London). Each cDNA was cloned into the EcoRI site of the retroviral vector pSRa. Recombinant retroviruses were made by transfection into a packaging cell line (17). cDNAs encoding RacV12 and RacC17 containing a T7 epitope tag were gifts of D. Bar-Sagi (Stony Brook, NY). The CSF1R/IR and CSF1R/IRΔ960 were constructed previously (5).

Cell Lines—Rat1 mycER fibroblasts (18) (G. Evan, London) were

* This work was supported in part by Grants R01-RCA-0733 from the American Cancer Society, DK52809 from the National Institutes of Health, 99-24 from the Nebraska Department of Health and Human Services, and P30 CA36727 from the National Cancer Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by Training Grant CA09476 from the National Cancer Institute.

** To whom correspondence should be addressed: Eppley Cancer Institute, University of Nebraska Medical Center, 98855 Nebraska Medical Center, Omaha, NE 68198-6805. Tel.: 402-559-8290; Fax: 402-559-4651; E-mail: rlewis@unmc.edu.

1 The abbreviations used are: IGF, insulin-like growth factor; IRS, insulin receptor substrate; CSF-1, colony-stimulating factor-1; CSF1R/IR, colony-stimulating factor-1 receptor/insulin receptor chimera; CSF1R/IRΔ960, receptor chimera containing a 12-amino acid deletion in the cytoplasmic juxtamembrane region; PI, phosphatidylinositol; GFP, green fluorescent protein; MAP, mitogen-activated protein; ERK, extracellular regulated kinase; TRITC, tetramethylrhodamine B isothiocyanate.

28632 This paper is available on line at http://www.jbc.org
transfected with the CSF1R/IR or the CSF1R/IR\textsuperscript{960} with LipofectAMINE (Life Technologies, Inc.). Stable lines expressing each chimeric receptor were isolated as described previously (5) by selection with G418 followed by fluorescence-activated cell sorting using an antibody to the ligand binding domain of the human CSF-1 receptor. For the expression of activated and dominant-negative forms of Ras, Rac, and Cdc42, Rat1\textit{mycER} cells were infected at a multiplicity of infection of at least 5 with ecotropic retroviruses encoding each Myc-tagged cDNA and selected with G418 (400 \mu g/ml).

**Analysis of Akt**—Cells expressing the CSF1R/IR or the CSF1R/IR\textsuperscript{960} (5 \times 10^5 cells) were treated with or without 10 nM CSF-1 or 100 nM insulin for 10 min, lysed, and the phosphorylation state of Akt was determined by Western blot with PhosphoPlus Akt (Ser\textsuperscript{473}) antibodies (New England Biolabs). Akt kinase activity was determined as described previously (19) by the phosphorylation of histone H2B in immunoprecipitates of Akt from treated and untreated cells.

**Microinjection and Immunofluorescence**—Rat\textit{mycER} (5 \times 10^4) cells expressing the CSF1R/IR or the CSF1R/IR\textsuperscript{960} were plated onto grid-lined glass coverslips (Belco), incubated in serum-free Dulbecco's modified Eagle's medium for 24 h, and injected as described previously for REF-52 fibroblasts (20) with 50 ng/ml pEGFP or pEFGFP. Cells were treated with 100 nM tamoxifen, 10 nM CSF-1, or 1 \mu M insulin as indicated, and cells expressing GFP were observed every 2 h for a total of 20 h. The amount of apoptosis among injected cells was quantified. Cell death caused by microinjection was determined by the injection of pEFGFP alone and subtracted from the amount of death induced by each experimental condition. Microinjected cells expressing GFP were evaluated within the microscopic field for each condition. Each condition was repeated at least five times.

**RESULTS AND DISCUSSION**

Previous studies have shown that mutations in the CSF1R/IR can dissociate its ability to phosphorylate important intracellular substrates from certain receptor-stimulated biological effects (5, 6). A 12-amino acid deletion (\Delta 960) blocks the ability of the CSF1R/IR to phosphorylate IRS-1 and Shc, activate the MAP kinases ERK1 and ERK2, activate PI 3-kinase associated with IRS-1, and stimulate glucose transport (5, 6). Despite the uncoupling of these intracellular effectors from the receptor, the CSF1R/IR\textsuperscript{960} still retains its ability to stimulate adipocyte differentiation (5) and protect cells against apoptosis (6). However, inhibition of apoptosis and induction of differentiation are assayed over much longer periods of time (hours to days) than the amount of time (minutes) required to detect biological events that are inhibited by the \Delta 960 mutation. This fact prevented us from excluding the possibility that small amounts of IRS or Shc phosphorylation could result from prolonged activation of the CSF1R/IR\textsuperscript{960} to inhibit apoptosis or stimulate differentiation. We, therefore, looked for biological effects of insulin that could be mimicked by activation of the CSF1R/IR and occurred over the same rapid time course as the phosphorylation of insulin receptor substrates and the stimulation of glucose uptake.

Insulin and insulin-like growth factors rapidly induce membrane ruffling (lamellipodia) in cells (21, 22). To investigate the effect of the \Delta 960 deletion on the ability of the chimeric receptor to stimulate membrane ruffling, Rat\textit{mycER} fibroblasts stably expressing the CSF1R/IR, the CSF1R/IR\textsuperscript{960}, or transfected with the expression vector alone were treated with or without 10 nM CSF-1 or 100 nM insulin for 2 min. The cells were fixed immediately, and polymerized actin was detected with TRITC-labeled phalloidin. Fluorescence microscopy revealed
that CSF-1 and insulin rapidly induced lamellipodia in cells expressing either the CSF1R/IR or CSF1R/IRΔ960 (Fig. 1).

However, in control cells only insulin was able to induce membrane ruffling (Fig. 1). The ability of the CSF1R/IRΔ960 to rapidly stimulate membrane ruffling suggested that the induction of this cytoskeletal rearrangement was not dependent upon IRS and Shc phosphorylation. Lamellipodia formation is dependent upon the activation of the small GTPase Rac (23). To investigate the role of Rac in lamellipodia formation by the CSF1R/IRΔ960, dominant-inhibitory RacN17 was microinjected into Rat1mycER fibroblasts expressing the CSF1R/IRΔ960, and the cells were treated with CSF-1. Expression of RacN17 blocked the ability of CSF-1 to induce membrane ruffling in microinjected cells, but not in adjacent, un.injected cells (data not shown).

We observed previously that the Δ960 mutation blocks the ability of the CSF1R/IR to stimulate PI 3-kinase activity associated with tyrosine phosphorylated proteins, including IRS-1 (6). Phosphorylation of the 3' position of PI is thought to be a key event in the activation of guanine nucleotide exchange factors for Rac (24, 25). Binding of PI3,4,5P3 to the pleckstrin homology domain of Akt is also critical to the activation of the serine/threonine kinase (26–29). To confirm that the CSF1R/IRΔ960 was not activating known PI 3-kinase signaling pathways, the ability of cells expressing the CSF1R/IR or the CSF1R/IRΔ960 to activate Akt was examined. Insulin (100 nM) stimulated the phosphorylation of Akt in cells expressing the intact CSF1R/IR but not from cells expressing the CSF1R/IRΔ960 (Fig. 2C). These data indicate the activation of Akt by the insulin receptor kinase is dependent upon IRS-associated PI 3-kinase activity but that the rapid, Rac-dependent induction of lamellipodia by the CSF1R/IRΔ960 is independent of the activation of PI 3-kinase. Alternatively, a second, distinct PI 3-kinase pathway may also be stimulated independent of IRS proteins by the insulin receptor kinase to activate Rac.

Fig. 3. Apoptosis in cells stably expressing activated or dominant-inhibitory small G proteins. A, Rat1mycER cells stably expressing RacV12, Cdc42V12, RasN17, or vector only (VO) were treated with or without 100 nM tamoxifen and with or without 1 μM insulin. After 20 h of treatment at 37 °C, at least 300 cells were counted for each treatment and the percentage of apoptotic cells was calculated. The expression of the small G protein in each cell line is indicated by the Western blots above each graph. B, Rat1mycER cells (circles) or Rat1mycER cells expressing RacV12 (squares) were treated with closed symbols or without open symbols 100 nM tamoxifen to induce apoptosis. The cells were observed for 18 h using time-lapse video microscopy. The percentage of apoptotic cells was calculated every 2 h. C, Rat1mycER cells stably expressing Cdc42N17, or RasN17, were treated as described for A, and the percentage of apoptotic cells was calculated. The results are presented as the mean ± S.D. of three or more independent experiments. D, Rat1mycER cells expressing Cdc42N17 were treated with 100 nM tamoxifen (closed squares) or 100 nM tamoxifen and 1 μM insulin (open squares). The cells were observed, and the percentage of apoptotic cells was determined as described in B. Statistical significance was determined by a Student's t test; p < 0.01 for a–e, (A) and a–d (C) in comparison with control cells treated with tamoxifen alone. Asterisks indicate the statistical difference (p < 0.01) in the level of apoptosis between Rat1mycER cells treated with tamoxifen and tamoxifen-treated Rat1mycER cells expressing RacV12 (B) or in the level of apoptosis of Rat1mycER cells stably expressing Cdc42N17 treated with tamoxifen or tamoxifen and insulin (D).
The expression of dominant-negative Cdc42V17 or RasN17 in Rat1mycER cells had no effect on tamoxifen-induced apoptosis and did not alter insulin's ability to inhibit tamoxifen-induced apoptosis (Figs. 3, C and D). We were unable to create cell lines stably expressing RacN17. As a second approach toward determining whether Rac contributed to survival signaling, plasmids encoding dominant-negative RacN17 were microinjected into Rat1mycER fibroblasts expressing the CSF1R/IR960 (Fig. 4). Apoptosis in untreated cells was 11.5%, whereas tamoxifen treatment alone elevated the level of apoptosis to 36.3%. Tamoxifen treatment in combination with insulin or CSF-1 significantly reduced the amount of apoptosis to 18.9% and 20%, respectively. Untreated cells injected with an expression plasmid encoding RacN17 showed a level of apoptosis comparable with untreated cells or cells treated with tamoxifen plus insulin or CSF-1. Tamoxifen increased apoptosis in cells injected with plasmids encoding RacN17 to the same extent as control injected cells treated with tamoxifen, which demonstrated that dominant-inhibitory RacN17 has no protective effect on the cells. However, the anti-apoptotic effects of insulin and CSF-1 were lost in cells injected with RacN17 (Fig. 4).

The data demonstrate that receptor signaling to Rac is independent of the phosphorylation of IRS-1 and Shc, as a deletion in the juxtamembrane domain prevented the chimeric CSF1R/IR (6) and the intact insulin receptor (1, 30–32) from interacting with and phosphorylating these important signaling intermediates. Ras (33), Cdc42, (34) and PI 3-kinase (35) are considered to be activators of Rac. However, CSF1R/IR activation of Ras-regulated pathways is inhibited by the Δ960 mutation (6), and neither dominant-inhibitory RasN17 nor Cdc42V17 blocked the ability of the CSF1R/IR960 to induce membrane ruffling (data not shown) or survival (Fig. 3). PI 3-kinase associated with IRS-1 is activated by the CSF1R/IR, but is not activated by the CSF1R/IRΔ960 (6). These previous observations and the data presented here suggest the existence of a novel intracellular signaling pathway used by the insulin receptor kinase to induce membrane ruffling and to transmit intracellular signals promoting cell survival.

The PI 3-kinase-dependent activation of Akt and the subsequent phosphorylation of the pro-apoptotic protein Bad (12), caspase 9 (36), and the forkhead transcription factor FKHRl1 (37) have been identified previously as elements of a growth factor-mediated survival mechanism. The data presented here suggest that growth factors with the ability to activate the small GTPase Rac may stimulate additional pathways to inhibit apoptosis. Rac is an effector of multiple signaling pathways (38), any one of which may contribute to the inhibition of apoptosis. Activated Rac promotes survival in BaF3 cells in a manner that is inhibited by the p38 kinase inhibitor SB203580 (39). Rac can also activate the NADPH burst oxidase (40), which, in turn, can activate the anti-apoptotic transcription factor NFκB (41, 42). This latter possibility suggests an additional survival pathway dependent on new gene transcription. In this regard, it is interesting to note that a novel anti-apoptotic signaling pathway was recently described for the IGF I receptor that is sensitive to the RNA polymerase II inhibitor α-amanitin (19). The identification of Rac as a transitional element in these biological events should provide a valuable point of reference for the identification and ordering of additional signaling intermediates that regulate cytoskeletal changes and survival by the insulin receptor kinase.

Acknowledgments—We express our appreciation to A. Hall, D. Bar-Sagi, and G. Evan for reagents; to Genetics Institute (Cambridge, MA.) for the invaluable gift of recombinant human CSF-1; and to R. Cotter for preliminary experiments that led to these studies.

REFERENCES

1. White, M. F., Livingston, J. N., Backer, J. M., Lauris, V., Ullrich, A., and Kahn, C. R. (1988) Cell 54, 641–649
2. Maegawa, H., McClain, D. A., Friedenberg, G., Olefsky, J. M., Napier, M., Lipari, T., Dull, T. J., Lee, J., and Ullrich, A. (1988) J. Biol. Chem. 263, 8912–8917
3. Theis, R. S., Ullrich, A., and McClain, D. A. (1989) J. Biol. Chem. 264, 12820–12825
4. Yonezawa, K., Ando, A., Kaburagi, Y., Yamamoto-Honda, R., Kitamura, T., Hara, K., Nakafuku, M., Okabayashi, Y., Kaso, K., Kasuga, M. (1994) J. Biol. Chem. 269, 4634–4640
5. Chaika, O. V., Chaika, N., Volle, D. J., Wilden, P. A., Pirruccello, S. J., and Lewis, R. E. (1997) J. Biol. Chem. 272, 11968–11974
6. Boehm, J. E., Chaika, O. V., and Lewis, R. E. (1998) J. Biol. Chem. 273, 28635
Inhibition of Apoptosis

7169–7176

7. Chaika, O. V., Chaika, N., Volle, D. J., Hayashi, H., Ebina, Y., Wang, I. M., Pierce, J. H., and Lewis, R. E. (1999) J. Biol. Chem. 274, 12075–12080

8. Harrington, E. A., Bennett, M. R., Fanidi, A., and Evan, G. I. (1994) EMBO J. 13, 3286–3295

9. Baserga, R., Hongo, A., Rubini, M., Prisco, M., and Valentini, B. (1997) Biochim. Biophys. Acta. Cancer 1332, F105–F126

10. Hossenlopp, P., Seurin, D., Segovia-Quinson, B., Hardouin, S., and Binoux, M. (1986) Anal. Biochem. 154, 138–143

11. Dudek, H., Datta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R. J., Cooper, G. M., Segal, R. A., Kaplan, D. R., and Greenberg, M. E. (1997) Science 275, 661–665

12. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H. A., Gotoh, Y., and Masters, S., Cooper, G. M., Segal, R. A., Kaplan, D. R., and Greenberg, M. E. (1997) Cell 91, 231–241

13. Kauffman-Zeh, A., Rodriguez-Viciana, P., Ulrich, E., Gilbert, C., Coffer, P., Downward, J., and Evan, G. (1997) Nature 385, 544–548

14. Shoelson, S. E., Chatterjee, S., Chaudhuri, M., and White, M. F. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2027–2031

15. Backer, J. M., Myers, M. G., Jr., Shoelson, S. E., Chin, D. J., Sun, X.-J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E. Y., Schlessinger, J., and White, M. F. (1992) EMBO J. 11, 3469–3479

16. Myers, M. G., Jr., Backer, J. M., Sun, X. J., Shoelson, S., Hu, P., Schlessinger, J., Youkim, M., Schaffhausen, B., and White, M. F. (1992) Proc. Natl. Acad. Sci. U. S. A. 90, 10350–10354

17. Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8392–8396

18. Evan, G. I., Wyllie, A. H., Gilbert, C. S., Littlewood, T. D., Land, H., Brooks, M., Waters, C. M., Penn, L. Z., and Hancock, D. C. (1992) Cell 69, 119–128

19. Kulik, G., and Weber, M. J. (1998) J. Biol. Chem. 273, 7473–7478

20. Klippel, A., Kavanaugh, W. M., Pot, D., and Williams, L. T. (1997) Mol. Cell. Biol. 17, 338–344

21. Roussos, O., Nijhawan, D., and Reed, J. C. (1997) Cell 86, 887–898

22. Kotani, K., Yonezawa, K., Ueda, H., Kitamura, Y., Sakaue, H., Ando, A., Chavanieu, A., Calas, B., Grigorescu, F., Nishiyama, M., Waterfield, M. D., and Kasuga, M. (1994) EMBO J. 13, 2313–2321

23. Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992) Cell 70, 401–410

24. Han, J. W., Luby-Phelps, K., Das, B., Shu, X. D., Xie, Y., Mosteller, R. D., Krishna, U. M., Falek, J. R., White, M. A., and Broek, D. (1998) Science 279, 558–560

25. Nimnaul, A. S., Yatsula, B. A., and Bar-Sagi, D. (1998) Science 279, 560–563

26. Franke, T. F., Yang, S.-I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) Cell 81, 727–736

27. Klippel, A., Ravagnan, W. M., Pot, D., and Williams, L. T. (1997) Mol. Cell. Biol. 17, 338–344

28. Franke, T. F., Kaplan, D. R., Cantley, L. C., and Toker, A. (1997) Science 273, 665–668

29. Frech, M., Andjelkovic, M., Ingleby, E., Reddy, K. K., Falek, J. R., and Hemmings, B. A. (1997) J. Biol. Chem. 272, 8474–8481

30. Backer, J. M., Schroeder, G. G., Cahill, D. A., Ulrich, A., Siddle, K., and White, M. F. (1991) Biochemistry 30, 6366–6372

31. Gustafson, T. A., He, W., Craparo, A., Schaub, C. D., and O’Neill, T. J. (1995) Mol. Cell. Biol. 15, 2500–2508

32. O’Neill, T. J., Craparo, A., and Gustafson, T. A. (1994) Mol. Cell. Biol. 14, 6433–6442

33. Joneson, T., White, M. A., Wigler, M. H., and Bar-Sagi, D. (1996) Science 271, 810–812

34. Nubes, C. D., and Hall, A. (1995) Cell 81, 53–62

35. Rodriguez-Viciana, P., Warne, P. H., Khwaja, A., Marte, B. M., Pappin, D., Das, P., Waterfield, M. D., Ridley, A., and Downward, J. (1997) Cell 89, 457–467

36. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) Science 282, 1318–1321

37. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Joo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) Cell 96, 857–868

38. Tapon, N., and Hall, A. (1997) Curr. Opin. Cell Biol. 9, 86–92

39. Nishida, K., Kaziro, Y., and Sato, T. (1999) Oncogene 18, 407–415

40. Abo, A., Pick, E., Hall, A., Totty, N., Teahan, C. G., and Segal, A. W. (1991) Nature 353, 668–670

41. Perona, R., Mentaner, S., Saniger, L., Sanchez-Perez, I., Bravo, R., and Lacal, J. C. (1997) Genes Dev. 11, 463–475

42. Sulciner, D. J., Irani, K., Yu, Z. X., Ferrans, V. J., Golde, H.-G. Schmitt-Clermont, P., and Finkel, T. (1997) Mol. Cell. Biol. 16, 7115–7121