Tumor-related Alternatively Spliced Rac1b Is Not Regulated by Rho-GDP Dissociation Inhibitors and Exhibits Selective Downstream Signaling*

Paulo Matos‡§, John G. Collard‡§, and Peter Jordan‡§

From the ‡Centro de Genética Humana, Instituto Nacional de Saúde “Dr. Ricardo Jorge,” Avenida Padre Cruz, 1649-016 Lisboa, Portugal and §Division of Cell Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Rac1 is a member of the Rho family of small GTPases, which control signaling pathways that regulate actin cytoskeletal dynamics and gene transcription. Rac1 is activated by guanine nucleotide exchange factors and inactivated by GTPase-activating proteins. In addition, Rho-GDP dissociation inhibitors (Rho-GDIs) can inhibit Rac1 by sequestering it in the cytoplasm. We have found previously that colorectal tumors express an alternatively spliced variant, Rac1b, containing 19 additional amino acids following the switch II region. Here we characterized the regulation and downstream signaling of Rac1b. Although little Rac1b protein is expressed in cells, the amount of activated Rac1b protein often exceeds that of activated Rac1, suggesting that Rac1b contributes significantly to the downstream signaling of Rac in cells. The regulation of both Rac1 and Rac1b activities is dependent on guanine nucleotide exchange factors and GTPase-activating proteins, but the difference in their activation is mainly determined by the inability of Rac1b to interact with Rho-GDI. As a consequence, most Rac1b remains bound to the plasma membrane and is not sequestered by Rho-GDI in the cytoplasm. Unlike Rac1, activated Rac1b is unable to induce lamellipodia formation and is unable to bind and activate p21-activated protein kinase nor activate the downstream protein kinase JNK. However, both Rac1 and Rac1b are able to activate NFκB to the same extent. These data suggest that alternative splicing of Rac1 leads to a highly active Rac variant that differs in regulation and downstream signaling.

This work was supported in part by Fundação para a Ciência e Tecnologia, Portugal, Grant POCI 2976/99. 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 Ph.D. Fellowship BD 21381/99 and an EMBO short term fellowship.
|| To whom correspondence should be addressed. Tel.: 351-21-7519380; Fax: 351-21-7526410; E-mail: peter.jordan@insa.min-saude.pt.

1 The abbreviations used are: GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; GDI, guanine nucleotide dissociation inhibitor; GFP, green fluorescent protein; GST, glutathione S-transferase; JNK, Jun N-terminal protein kinase; NFκB, nuclear factor-κB; PAK, p21-activated protein kinase; HA, hemagglutinin; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline.

Received for publication, July 28, 2003, and in revised form, September 17, 2003
Published, JBC Papers in Press, September 23, 2003, DOI 10.1074/jbc.M308215200

Experimental Procedures

Cell Culture and Transfection—Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (HT29, SW480, ZR75.1, HeLa) or 10% bovine serum (NIH 3T3) (Invitrogen) and regularly checked for mycoplasma infection. For transfections, cells at 50–75% confluence were transfected using LipofectAMINE Plus (Invitrogen), according to the manufacturer’s instructions, and cells treated with 10 μg/mL of DEAE-dextran (Invitrogen) to mediate transfection. After transfection, cells were cultured in fresh medium for 24 h. For cotransfections, cells were transiently transfected with equal amounts of pCMV-HA-Rac1 or pCMV-HA-Rac1b expression vectors and pCMV-HA-Pak1, pCMV-HA-Pak2, or pCMV-HA-Pak3 expression vectors.
Regulation and Properties of Alternatively Spliced Rac1b

Endogenous Rac1b Is a Highly Activated Variant—Rac1b is an endogenous splice variant of Rac1 (27). In order to study the physiological role of Rac1b, we raised antibodies against the 19-amino acid insert that distinguishes human Rac1b from Rac1. Western blot analysis showed that these antibodies specifically recognize Rac1b but not Rac1 in mouse 3T3 fibroblasts overexpressing both cDNAs (Fig. 1A). With these antibodies several tumor cell lines as well as mouse fibroblasts were subsequently screened for the presence of endogenous Rac1b protein by Western blotting. As shown in Fig. 1B, expression of endogenous Rac1b by both reverse transcriptase-PCR and Western blotting was specifically found in HT29 (colon adenocarcinoma), ZR75.1 (breast carcinoma), and HeLa (cervix carcinomas), whereas SW480 (colon adenocarcinoma) and 3T3 fibroblasts expressed Rac1 only. Commercially available anti-Rac1 antibodies recognized both Rac1 and Rac1b equally well (see Fig. 1A). This allowed us to optimize electrophoretic separation of both Rac1 and Rac1b and to compare the endogenous levels of both proteins. As shown in Fig. 1B, in all three Rac1b positive cell lines the level of Rac1b protein is low compared with that of Rac1. By densitometric analysis of Western blots, we estimated a 20-fold excess of Rac1 over Rac1b protein in HT29 cells.

Because the expression level is not always indicative for the active, functional pool of a protein, we analyzed the activation state of Rac1 and Rac1b in these cells by GST-PK-Crib double pull-down assays (Fig. 1B). Surprisingly, a significant amount of active Rac1b was pulled down from all Rac1b-expressing cell lines (see Fig. 1B). In order to confirm that the pull-down assay specifically isolated activated Rac1b, we transfected Myc-tagged wild type as well as active Q61L and inactive T17N mutants of Rac1b into cells and tested their activation state by pull-down assays. As shown in Fig. 1C, wild type Rac1b and the active Q61L mutant of Rac1b were clearly pulled down in this assay, whereas the inactive mutant T17N had no affinity for the GST-PK-CD probe. This indicates that Rac1b binds PK-CRIB in the active GTP-bound state. Interestingly, as shown in Fig. 1B, active Rac1b can exceed the amount of active Rac1 in cells. In HT29 colon cells, for example, we found three times more active Rac1b than Rac1, as estimated by Western blotting. Thus, although Rac1b protein is present in small amounts in cells, its contribution to the pool of active Rac in vivo can be very significant. Apparently, alternative splicing of Rac1 yields a highly activated variant of Rac1 that can lead to a significant increase in the total amount of activated Rac in cells.

Activation of Rac1b in Vivo Requires GEFs—We next examined the mechanism underlying the high activation level of Rac1b. Previous in vitro studies have indicated that bacterially expressed Rac1b is predominantly in the active, GTP-bound form (24). We therefore wondered whether the activation of Rac1b in vivo was independent of endogenous GEFs. To study this, HT29 cells that express endogenous Rac1 and Rac1b (see Fig. 1B) were incubated for 15 min with different concentrations of either wortmannin or LY294002 to inhibit phosphatidylinositol 3-kinase, or genistein to inhibit tyrosine phosphorylation. Both pathways have been reported previously to mediate activation of endogenous Rac-GEFs such as Tiam1 (15, 30) or Vav (31–32), respectively. As shown in Fig. 2A, both inhibitors reduced the activation of Rac1b as well as of Rac1b in a dose-dependent manner. These data suggest that the activa-
tions of Rac1 and Rac1b are both dependent on GEFs. Indeed, we found that the Rac activator Tiam1 (33) is able to activate both Rac1 and Rac1b (not shown) and is able to bind Rac1 and Rac1b to the same extent (Fig. 2B). These data indicate that Rac1b is able to associate with GEFs leading to its activation, similarly as found for Rac1.

Bcr-GAP Down-regulates Rac1 as Well as Rac1b—Because GAPs contribute to the activation state of Rho-like GTPases, by the capacity to stimulate their intrinsic GTPase activity, we determined whether Rac1b differs from Rac1 with respect to the down-regulation by GAPs. Recombinant Rac1b has been found to possess a reduced intrinsic GTPase activity when compared with Rac1; however, the addition of GAP protein strongly accelerated the GTPase reaction of both variants.3 In order to confirm in vivo that GAP-stimulated GTP hydrolysis affects Rac1 as well as Rac1b, full-length Bcr-GAP was co-transfected into SW480 cells together with tagged Myc-Rac1 and Myc-Rac1b. With increasing amounts of Bcr, we found a corresponding decrease in the activities of endogenous Rac1 as well as of exogenous Myc-Rac1 and Myc-Rac1b proteins (Fig. 3). Thus, both Rac1 and Rac1b are able to associate with Bcr and as a consequence are down-regulated because of the acceleration of the intrinsic GTPase activity of both proteins.

3 R. Ahmadian, personal communication.
Rac1b Is Resistant to Down-regulation by Rho-GDI—Subsequently we analyzed the effect of Rho-GDI on the activation state of Rac1 and Rac1b. Rho-GDI binds and masks the hydrophobic C-terminal region of Rac, the same region that is responsible for targeting Rac to the plasma membrane (35). Thus, Rho-GDI maintains Rac in the cytoplasm and must dissociate to allow Rac to translocate to the membrane and interact with membrane-associated activators (36). When SW480 cells were co-transfected with increasing amounts of Rho-GDI, activation of both Myc-Rac1 and endogenous Rac1 was completely prevented. In contrast, the activation state of Myc-Rac1b remained unaffected (Fig. 4A). Apparently, Rho-GDI is unable to...
down-regulate the activation of Rac1b. To support this observation, Rho-GDI was overexpressed in HT29 cells or HeLa cells, which contain endogenous levels of both Rac1 and Rac1b (see Fig. 1B). Upon introduction of Rho-GDI, endogenous active Rac1b could still be isolated from these cells, whereas endogenous active Rac1 was no longer detectable (Fig. 4B, shown for HT29). These data suggest that Rho-GDI somehow is unable to down-regulate the activation state of Rac1b.

We therefore determined whether the extra 19 amino acids in Rac1b could physically interfere with binding of Rho-GDI. From the available crystal structures of Rho-GDI complexed with Rac or Cdc42 (36–37), it appears that 19 additional amino acids following the switch II region might disturb the binding to the C-terminal sandwich domain of Rho-GDI. To address this question, we transfected HA-Rho-GDI together with either Myc-Rac1 or Myc-Rac1b into SW480 cells and immunoprecipitated with either anti-Myc or anti-HA antibodies. Indeed, Rho-GDI could only be co-immunoprecipitated with Rac1 but not with Rac1b (Fig. 5A), and vice versa the immunoprecipitation of HA-Rho-GDI contained Myc-Rac1 but not Myc-Rac1b (data not shown). From these studies we concluded that Rho-GDI is unable to interact with Rac1b, which explains its inability to down-regulate Rac1b activity. The impaired binding of Rac1b to Rho-GDI might thus contribute to the high activation status of Rac1b found in cells.

Besides inhibition of nucleotide exchange, Rho-GDI can also retain Rho GTPases in an inactive cytosolic complex. We therefore analyzed the intracellular localization of Rac1 and Rac1b in HT29 cells following overexpression of Rho-GDI. As shown in Fig. 5, Rac1 localized to the plasma membrane at sites of cell-cell contact but became cytoplasmic following co-expression of Rho-GDI, consistent with studies reported previously (38–39). In contrast, Rac1b localized at the plasma membrane but remained at the plasma membrane, even in the presence of high amounts of Rho-GDI (Fig. 5D). These data are consistent with the inability of Rho-GDI to associate with Rac1b and illustrate in vivo that Rho-GDI is unable to bind Rac1b. The impaired Rho-GDI binding will favor a more permanent localization of Rac1b at the plasma membrane, where it can be continuously activated by membrane-localized GEFs.

Rac1b Differs in Downstream Signaling—We next asked whether the high activation status that was found for endogenous Rac1b in vivo could lead to increased Rac-mediated downstream signaling. Hallmark features of activated Rac1 are the
induction of lamellipodia formation, as well as the activation of the protein kinases PAK and JNK (1). As described for fibroblasts, overexpression of Rac1-Q61L in SW480 colon carcinoma cells induced lamellipodia symmetrically along the entire cell surface (Fig. 6A). Surprisingly, expression of wild type Rac1b or Rac1b-Q61L did not lead to significant induction of lamellipodia in SW480 cells, although the protein was localized at the plasma membrane, preferentially at sites in contact to neighboring cells (Fig. 6A). Apparently, Rac1b is unable to induce cytoskeletal changes similar to those found with activated Rac1.

In order to further investigate Rac-mediated activation of downstream protein kinases, the active Q61L and inactive T17N mutants of both Rac1 and Rac1b were co-expressed in SW480 cells with either PAK1 or JNK. Subsequently, cell lysates were analyzed by Western blotting using antibodies specific for the activated, phosphorylated kinases. Activated Rac1 stimulated PAK (Fig. 6B) and JNK (Fig. 6C) activation, whereas the dominant negative mutant Rac1-N17 did not, consistent with previous observations with these Rac mutants (7, 8). In contrast, neither of the Rac1b mutants was able to induce any meaningful activation of these kinases (Fig. 6, B and C). Thus in contrast to Rac1, Rac1b is unable to activate PAK and to stimulate the JNK pathway. The inability of active Rac1b to activate PAK reflects impaired interaction because only active Rac1 was able to co-immunoprecipitate full-length PAK (Fig. 6D).

Interestingly, analysis of other Rac-mediated signaling pathways revealed that both activated Rac1 and Rac1b are able to stimulate the NFκB pathway. The transcription factor NFκB becomes active following phosphorylation and subsequent degradation of its cytosolic inhibitor IκB (40). As shown in Fig. 7A, expression of both Rac1-Q61L as well as Rac1b-Q61L induced IκB phosphorylation, whereas the inactive N17 mutants of both Rac variants did not. In addition, expression of both Rac1-Q61L as well as Rac1b-Q61L induced translocation of the DNA-binding subunit NFκB-p65 (RelA) from the cytosol into the cell nucleus (Fig. 7B). These data suggest that Rac1b is able to stimulate only a restricted subset of downstream signaling pathways that are mediated by Rac1.

**DISCUSSION**

The data described in this paper demonstrate that alternative splicing of the small GTPase Rac1 profoundly alters its regulation and downstream signaling properties. This difference is due an extra domain of 19 amino acids resulting from inclusion of exon 3b into the Rac1 mRNA. It is interesting to note that this alternative splicing event is specific for the human RAC1 gene because we did not find any homologous, additional exons in the human RAC2, RAC3, or CDC42 gene structures.

We found that alternative splicing affects the regulation of Rac1b activity. The inclusion of the extra domain prevents the binding to Rho-GDI, one of the regulators of Rac activity. As a consequence, Rac1b is unable to cycle between the plasma membrane and the cytoplasm. It has been shown that Rho-GDI binds Rac1 and masks the hydrophobic C-terminal region that is responsible for targeting Rac to the plasma membrane (36). Thus, Rho-GDI maintains Rac in the cytoplasm and must dissociate to allow Rac to translocate to the membrane and to interact with membrane-associated activators (36). By *in vitro* studies, it has been demonstrated that release of Rho-GDI is required for translocation of Rac1 to plasma membranes and for the subsequent interaction with exchange factors, such as Tiam1 (41). In addition, integrins induce spatially controlled Rac-effector coupling *in vivo* by directing Rac1 to membranes and dissociating it from Rho-GDI (42). Because Rac1b is unable to interact with Rho-GDI, it is more persistently localized at the plasma membrane. This leaves the Rac1b variant in a preferred spatial position to become activated. The lack of binding to Rho-GDI, and the increased GTP-GDP cycling rate of recombinant Rac1b as demonstrated *in vitro* (24) most likely explain the high activation state found for endogenous Rac1b in cells. Even at low amounts of endogenous Rac1b protein in
cells, a considerable portion is present in an activated state and may contribute to specific Rac-mediated downstream signaling.

Our in vivo data also demonstrate that Rac1b retained the ability to cycle between the GDP- and GTP-bound form and rather exists in a high steady-state activation level. Reconstituent GAP protein (24). In addition, we found that the Bcr-down regulates Rac1 and Rac1b in vivo equally well. Moreover, inhibition of the activity of cellular GEFs revealed that the activation of Rac1b depends on continuous action of GEFs. More specifically, the Rac activator Tiam1 associates with both Rac1 and Rac1b and is able to activate both proteins. All these data strongly suggest that the activity of Rac1b can be regulated by GEFs and GAPs and that Rac1b does not represent a constitutive activated splice variant of Rac1.

Intriguingly, the extra 19 amino acids in Rac1b appear to confer selectivity in downstream Rac signaling properties. In cultured cells, we found that activated Rac1b could neither stimulate formation of lamellipodia or activation of the protein kinase PKA nor the JNK pathway. In this respect it should be noted that the PAK-CRIB domain used in the pull-down assays binds equally to Rac1 and Rac1b, whereas full-length PKA does not significantly associate with Rac1b (Fig. 6D). Previous work has indicated that CRIB domain-containing fragments of effectors such as WASP or PAK are necessary for specific binding to Rac1 (11). This might suggest that the activation of Rac1b in cells promotes only specific downstream signaling pathways. Many downstream signaling pathways of Rac have been described that are independent of the ability of Rac1 to induce cytoskeletal changes or to activate the Jun kinase mitogen-activated protein kinase cascade (44–48). These pathways often involve transcriptional regulation of genes. So far, the expression of Rac1b has been found in breast and colon tumors. This might indicate that this splice variant somehow facilitates the growth or survival of these epithelial tumors. Currently, we are studying which of the many Rac-specific downstream signaling pathways are activated by Rac1b and which of those pathways could play a role in the formation or progression of epithelial tumors.

Acknowledgments—We thank Jean Paul ten Klooster for help with the pull-down assays; Celia Carvalho for help with the LSM510; and our colleagues Ignacio Rubio, Christian Gesaphe, and Lee Price for helpful discussions. We acknowledge the following for providing plasmids used in this study: Drs. J. Morris, Kings College, London, UK (pCMV-FLAG-JNK, pCMV-FLAG-MKK4); J. Chernoff, Philadelphia (pCMV6-Myo-PAK1); V. Braga, Imperial College, London, UK (pGEX-2T-Rho-GD1a); N. Hotchin, University of Birmingham, UK (pCDNA3-GFP-Rac1); M. Karin, San Diego (pCDNA3-1bB); C. Paya, Rochester, NY (pCDNA3-HA-RelA); and R. Khosravi-Far, Beth Israel Deaconess Medical Center, Boston (pCMV5-Bcr-GAP).

REFERENCES

1. Hall, A. (1998) Science 279, 509–513
2. Fukata, M., and Kaibuchi, K. (2001) Nat. Rev. Mol. Cell Biol. 2, 887–897
3. Suhai, E., and Marshall, C. J. (2002) Nat. Rev. Cancer 2, 153–142
4. Malliri, A., van der Kamen, E. A., Clerk, K., van der Valk, M., Michels, F., and Collard, J. G. (2002) Nature 41, 867–871
5. Manser, E., Leung, T., Salihuddin, H., Zhao, Z. S., and Lim, L. (1994) Nature 367, 40–46
6. Miki, H., Yamaguchi, H., Suetsugu, S., and Takenawa, T. (2000) Nature 408, 732–735
7. Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Goto, K. J. (1999) J. Cell Biol 141, 1137–1146
8. Minden, A., Lin, A., Claret, F. X., Abo, A., and Karin, M. (1995) Cell 81, 1147–1157
9. Poonia, R., Montaner, S., Saniger, L., Sanchez-Perez, I., Bravo, R., and Lecal, J. C. (1997) Genes Dev. 11, 463–475
10. Joyce, D., Bouzhazah, B., Fu, M., Albanese, C. D’Amico, M., Steer, J., Klein, J. U., Lee, R. J., Segall, J. E., Westwick, J. K., Der, C. J., and Pestell, R. G. (1997) J. Cell Biol. 137, 1385–1398
11. Ridley, A. J., Comoglio, P. M., and Hall, A. (1995) Mol. Cell. Biol. 15, 1110–1122
12. Takahashi, K., Sasaki, T., Kotani, H., Nishioka, H., and Takay, Y. (1997) J. Cell Biol. 139, 1047–1059
13. Kraus, V. M., Machesky, L. M., Hall, A., and Hotchin, N. A. (1997) J. Cell Biol. 137, 1421–1431
14. Klemke, R. L., Long, J., Molander, R., Brooks, P. C., Vupri, K., and Cherehes, D. A. (1998) J. Cell Biol. 140, 961–972
15. Sander, E. E., van Delft, S., ten Klooster, J. P., Resel, T., van der Kamen, R. A., Michels, F., and Collard, J. G. (1999) J. Cell Biol. 147, 1099–1102
16. Zondag, G. C. M., Evers, E. E., ten Klooster, J. P., Janssen, L., van der Kamen, R. A., and Collard, J. G. (2000) J. Cell Biol. 149, 775–781
17. Hopkins, A. M., Walsh, S. V., Verklade, P., Boquet, P., and Nuratt, A. (2003) J. Cell Sci. 116, 725–742
18. Festa, J. G., Just, L., and Kainz, B. (1999) Int. J. Cancer 81, 682–687
19. Schaezler, A., Frechhel, D., Knaus, U., Dehne, K., Gerhard, M., Graeff, H., Harbeck, N., Schmitt, M., and Lengyl, E. (2000) Oncogene 19, 3013–3020
20. Rieth, S., Vieth, P., Camens, J., Boud, C., Chevillard, S., and de Gunzburg, J. (2001) J. Biol. Chem. 276, 137, 734–738
21. Gidlea, J. J., Seraj, M. J., Oxford, G., Harding, M. A., Hampton, G. M., Moskaluk, C. A., Frierson, H. F., Conaway, M. R, and Theodorescu, D. (2002) Cancer Res. 62, 6418–6423
22. Jordan, P., Brazio, R., Boavida, M. G., Gesapa, C., and Castrue, E. (1999) Oncogene 18, 6835–6839
23. Matos, P., Kung, J., Marques, B., Beck, S., Verissimo, F., Gesapa, C., Boavida, M. G., Scherer, S. W., and Jordan, P. (2000) Biochem. Biophys. Res. Commun. 271, 741–751
24. Akhtiar, N., Hudson, K. R., and Hotchin, N. A. (2000) Cell Adh. Commun. 7, 456–470
25. Han, J., Luby-Phelps, K., Dais, B., Shu, X., Xia, Y., Mosteller, R. D., Krishna, U. M., Falck, J. R., and Boedtker, H. (1998) Science 271, 273–278
26. Crespo, P., Schuebel, K. E., Ostrom, A. A., Hahn, K. M., and Theodorescu, D. (1998) Oncogene 17, 1270–1289
27. Deaconess Medical Center, Boston (pCMV5-Bcr-GAP).