Geranylgeranylated RhoB is sufficient to mediate tissue-specific suppression of Akt kinase activity by farnesyltransferase inhibitors

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Abstract Farnesyltransferase inhibitors (FTIs) induce apoptosis by elevating the levels of geranylgeranylated RhoB (RhoB-GG) in cells. However, the mechanism by which RhoB-GG acts is unclear. Here we report that RhoB-GG is sufficient to mediate the suppressive effects of FTIs on the activity of the survival kinase Akt-1 in epithelial cells. This mechanism is tissue-specific insofar as it does not operate in fibroblasts. We discuss how the cell survival functions of RhoB and Akt may be linked biochemically in certain cell types.

Key words: Ras; Rho; Farnesyltransferase inhibitor; Akt; Prk; Signal transduction; Apoptosis

1. Introduction

Farnesyltransferase inhibitors (FTIs) are a novel class of cancer chemotherapeutics in human trials. The development of FTIs was predicated on the discovery that transforming activity of oncogenic Ras requires posttranslational isoprenylation [1]. Subsequent preclinical studies established biological proof-of-principle but raised questions concerning whether Ras alteration was important. In contrast, recent investigations have revealed a crucial role for alteration of the Rho family protein RhoB in the FTI mechanism (reviewed in [2]). Rho proteins regulate actin organization and cell adhesion signaling and RhoB has a non-redundant function in intracellular receptor trafficking of the epidermal growth factor (EGF) receptor [3]. FTI treatment elicits a loss of the farnesylated isoform of RhoB but a gain in the geranylgeranylated isoform of RhoB (termed RhoB-GG), due to modification by geranylgeranyl transferase-I in drug-treated cells [4]. Significantly, the gain of RhoB-GG is both sufficient and necessary to mediate FTI response in malignant cells [5–7]. Thus, FTIs act through a gain-of-function effect related to elevation of RhoB-GG.

How RhoB-GG signals growth inhibition and apoptosis is unclear. In transformed fibroblasts, the proapoptotic activity of FTIs is not mediated by suppression of the important survival kinase Akt-1 [7–9]. However, there are reasons to think that Akt-1 inhibition may be germane to FTI-induced apoptosis by RhoB-GG in other cell types [2]. In this study, we explored the ability of FTIs and RhoB-GG to inhibit the activity of Akt-1 in epithelial cells. We found that Akt-1 activation by oncogenic Ras or EGF could be disrupted by FTI treatment in COS kidney and MCF7 breast epithelial cells, respectively. Significantly, RhoB-GG was sufficient to mediate these effects. The results suggested that FTIs and RhoB-GG inhibit Akt-1 activity in epithelial cell types. A model linking RhoB and Akt signaling is proposed. We suggest that FTIs may have heightened clinical efficacy in malignant settings where Akt is susceptible to suppression by RhoB-GG.

2. Materials and methods

2.1. Plasmid constructions

The following expression vectors have been described: oncogenic v-H-Ras [10]; the engineered geranylgeranylated RhoB isoforms RhoB-GG and RhoB-V14-GG (activated by V14 mutation), and the unprenylated mutant RhoB-S [5]; the dominant inhibitory RhoB mutant RhoB-N19 [11]; the activated RhoA mutant RhoA-V14 [12]; human Akt-1 [8]; and the p110 catalytic subunit of PI3′K [13].

2.2. Tissue culture

COS kidney cells and human MCF7 breast carcinoma cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD, USA) and 50 U/ml penicillin and streptomycin (Fisher, Pittsburgh, PA, USA). COS cells were transfected as follows: 5×10^5 cells were seeded in 60 mm dishes and the next day transfected overnight with 15 μg DNA by a modified calcium phosphate protocol [14]. Empty vector was added where appropriate to normalize the amount of DNA transfected. Twenty-four h after transfection, cells were incubated in serum-free media without or with the addition of LY 294002 for 30 min or FTI L-744,832 for 36 h at the concentrations indicated. Cells were stimulated for 15 min with 100 ng/ml EGF (Life Technologies) before being harvested for extract preparation, kinase assay and Western blotting.

2.3. Retroviral-mediated gene transfer

RhoB-GG or control genes were introduced into MCF7 human breast carcinoma cells using the murine retrovirus expression vector MSCVpac [15]. The amphotropic packaging cell line PA317 was transfected essentially as described [16] with 20 μg MSCVpac vectors using a modified calcium phosphate protocol [14]. Media supernatants containing recombinant retroviruses were harvested 48 h after transfection. Cells were refed and media harvested 8 h later and mixed with the first round to maximize viral yield. Supernatants were clarified through a 0.45 μm filter (Millipore) and supplemented with 4 μg/ml polybrene (Sigma). MCF7 cells seeded in 100 mm dishes were incubated with 4 ml supernatant and then supplemented after several h with 4 ml growth media. Stably infected cell populations were selected the next day by 72 h incubation in growth media containing 2 μg/ml puromycin (Sigma). Cells were then incubated overnight in media lacking serum before stimulation for 15 min with 100 ng/ml EGF and extract preparation.

2.4. Akt kinase assay

Cells were lysed in extraction buffer containing 20 mM Tris–HCl (pH 7.5), 137 mM NaCl, 15% glycerol, 1% NP40, 2 mM PMSF, 2 μg/

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ml aprotinin and leupeptin, 2 mM benzamidine, 20 mM sodium fluoride, 10 mM sodium pyrophosphate and 1 mM sodium vanadate. Cell lysate proteins were quantitated by Bradford assay and aliquoted for Western analysis and Akt-1 kinase activity. in vitro kinase assays were performed as follows. Lysates were subjected to immunoprecipitation in NP40 buffer [17] with anti-HA monoclonal antibody 12CA5 (Roche Boehringer Biochemicals) or anti-Akt-1 (Santa Cruz Biotechnology; cat. No. sc1618) in the presence of 30 μl protein A-Sepharose beads for 4 h at 4°C. The immunoprecipitate was washed once in NP40 buffer and once in reaction buffer (below). Kinase reactions included 10 μCi γ-^{32}P ATP (NEN) and 4 μM unlabelled ATP in a 25 μl reaction buffer composed of 20 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT and 4 μg histone H2B (Sigma) as substrate. The reaction was incubated for 30 min at room temperature and then fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Relative incorporation of radioactivity was determined by the autoradiography of dried polyacrylamide gels.

2.5. Western analysis

Cells were washed in cold phosphate-buffered saline and lysed in 1% NP40 lysis buffer. Cellular protein was quantitated by the Bradford assay and equivalent amounts of cellular proteins were fractionated by SDS–PAGE. Gels were analyzed by standard Western blotting methods using 1 μg/ml anti-HA antibody 12CA5 or anti-Akt-1 antibody. Detection of the primary antibody was carried out using an ECM system for detection of murine antibody (Amersham).

3. Results

To determine whether FTI treatment influenced Akt-1 kinase activity in epithelial cells, we performed a set of transient expression assays in COS cells where Akt-1 was activated by the co-expression of oncogenic v-H-Ras or by treatment with EGF. Briefly, cells were transfected with various expression plasmids and 24 h later incubated overnight in serum-free medium in the presence or absence of the FTI L-744,832 [18]. The Akt-1 gene product used in this study was tagged at its N-terminus with the influenza virus hemagglutinin (HA) epitope recognized by the monoclonal antibody 12CA5 [19]. Akt-1 was immunoprecipitated from cell extracts with 12CA5 and its activity was measured by an in vitro kinase assay using histone H2B as substrate. Equal amounts of cell extract proteins were subjected to Western analysis with 12CA5 to confirm that the same amount of Akt-1 was added to each kinase reaction.

FTI was sufficient to inhibit Akt-1 activation by v-H-Ras in a dose-dependent manner (see Fig. 1). Under the conditions of the assay, v-H-Ras activated Akt-1 kinase activity several-fold. FTI treatment reversed this effect but did not suppress kinase activity below the basal level present in the absence of v-H-Ras (data not shown). Thus, FTI treatment inhibited activation due to co-expression of v-H-Ras but not the intrinsic or background activity displayed by Akt-1 in immunoprecipitates from COS cells.

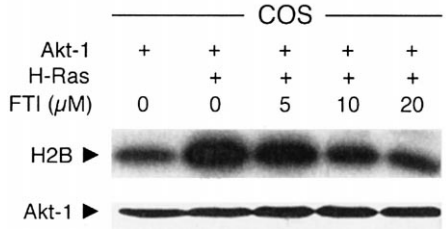

Fig. 1. FT inhibition suppresses Akt-1 activation by oncogenic H-Ras in COS epithelial cells. COS cells were transfected with 5 μg of each of the expression vectors noted. The day after transfection the growth media was removed and replaced with media containing 0.1% fetal bovine serum. Where indicated, FTI L-744,832 was added to cells at this time to the concentration noted. Cell extracts were prepared and analyzed for Akt-1 expression by Western analysis and for Akt-1 activity by in vitro kinase assay, using histone H2B as substrate.

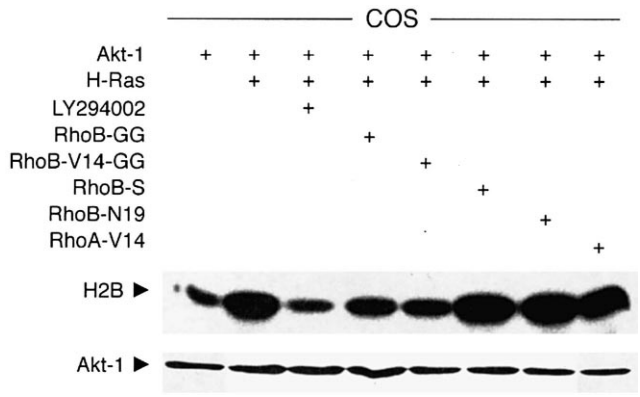

Fig. 2. RhoB-GG suppresses activation of Akt-1 by Ras in COS epithelial cells. Cells were transfected and processed as above except that RhoB-GG co-expression rather than FTI treatment was tested for inhibition of Akt-1 activity.

To determine whether RhoB-GG was sufficient to mediate the effect of FTIs, we performed a similar set of experiments except that vectors expressing RhoB-GG or control genes were co-transfected with activated v-H-Ras and Akt-1 and the FTI treatment was omitted. In these experiments, we also used the PI3′K inhibitor LY294002 as a positive control for Akt-1 inhibition. As before, v-H-Ras activated Akt-1 several-fold and treatment with 5 μM LY294002 was sufficient to fully reverse this effect. Similarly, cotransfection of RhoB-GG was sufficient to suppress v-H-Ras-dependent activation of Akt-1 kinase activity almost as effectively as LY294002 (see Fig. 2). This level of suppression was not accentuated by an activated isoform of RhoB-V14-GG, suggesting that like FTI treatment RhoB-GG suppressed activation to basal level but not beyond. Geranylgeranylation was crucial because an unprenylated mutant (RhoB-S) was inactive. A dominant inhibitory mutant (RhoB-N19) also lacked activity, arguing that suppression was related to GTP binding and therefore interaction with effector molecules (rather than Rho guanine nucleotide exchange factors). Lastly, the effect was highly specific to RhoB-GG, insofar as the closely related but differently localized RhoA protein did not affect Akt-1 activation, even when activated by mutation (RhoA-V14). Western analysis

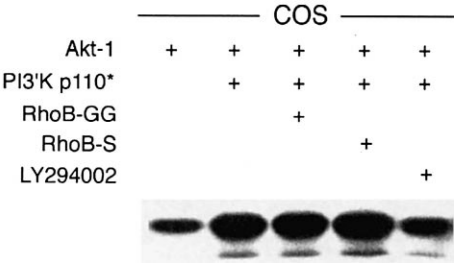

Fig. 3. RhoB-GG does not suppress activation of Akt-1 by PI3′K. COS cells were transfected and processed as above except that Akt-1 was activated by overexpression of the p110 catalytic subunit of PI3′K.

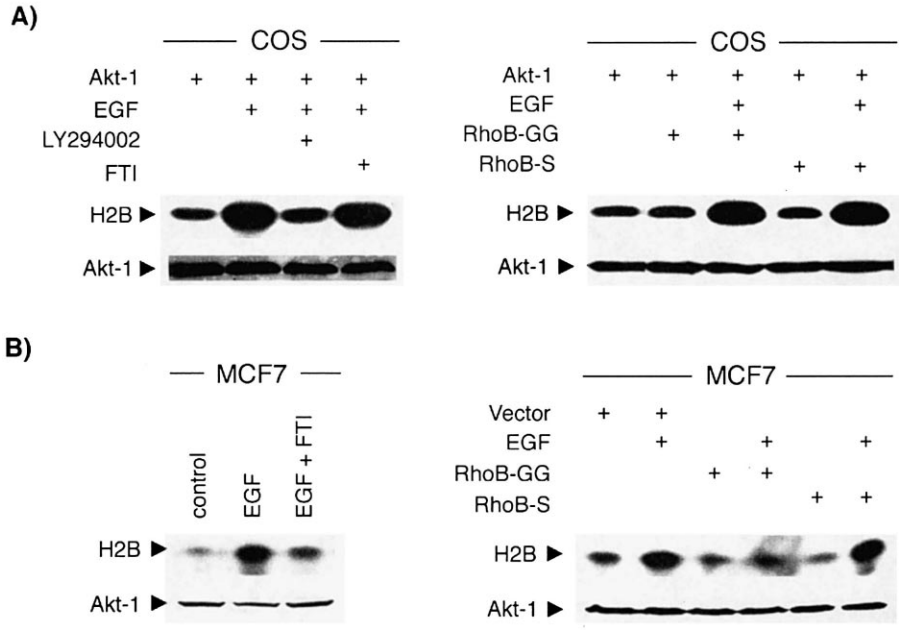

Fig. 4. FTI or RhoB-GG suppress activation of endogenous Akt-1 by EGF in MCF7 carcinoma cells. Cells were infected with amphotropic retroviral vectors as noted in Section 2 and processed as above for Akt-1 kinase activity and Western analysis. Briefly, cells were deprived of serum and treated where indicated with 10 μM L-744,832. Twenty-four h later cells were harvested or stimulated for 15 min with 100 ng/ml EGF before harvest. A: FTI and RhoB-GG do not affect activation of Akt-1 by EGF in transiently transfected COS cells. B: RhoB-GG is sufficient to mediate FTI suppression of endogenous Akt-1 when the latter is activated by EGF in MCF7 breast carcinoma cells.

confirmed that similar amounts of Akt-1 protein were analyzed. We concluded that RhoB-GG was sufficient to mediate FTI suppression of Akt-1 activation by oncogenic H-Ras.

To determine whether the suppressive effects of RhoB-GG were due to interference with Akt-1 recruitment to membranes, we tested whether RhoB-GG affected Akt-1 activation by an activated isoform of the p110 catalytic subunit of PI3′K [13]. This constitutive activity of this isoform mimicked the constitutive activation of PI3′K that occurs in cells transfected with v-H-Ras. As expected, Akt-1 activity was simulated several-fold by p110. However, in this case, RhoB-GG did not suppress Akt-1 activity, similar to the inactive mutant RhoB-S (see Fig. 3). In contrast, cell treatment with 5 μM LY294002 fully suppressed p110-mediated activation of Akt-1. We concluded that RhoB-GG suppressed activation of Akt-1 through a mechanism(s) other than blocking its recruitment to cell membranes.

In COS expression trials where Akt-1 was activated by 15 min treatment with 100 ng/ml EGF, instead of by co-expression of v-H-Ras, we observed that neither FTI treatment nor ectopic RhoB-GG had any effect. Treatment of COS cells with EGF led to a several-fold activation of exogenous Akt-1 activity, similar to v-H-Ras. However, while LY294002 inhibited Akt-1 activation by EGF, neither FTI nor RhoB-GG were effectual in blocking this activity (see Fig. 4A). A similar lack of effect by each was also apparent in tests where Akt-1 was stimulated by treatment with platelet-derived or insulin-like growth factors (data not shown).

To corroborate evidence of Akt-1 suppression and address the caveats associated with the COS transient expression assay, we investigated the susceptibility of endogenous Akt-1 in MCF7 breast carcinoma cells, which respond to RhoB-GG by apoptosis [6]. Briefly, serum-deprived MCF7 cells were stimulated as before by a 15 min treatment with 100 ng/ml EGF following overnight treatment with FTI or transfection with RhoB-GG or control vectors. Extracts were prepared in a similar manner to COS cells and processed by in vitro kinase assay as before. We observed stimulation of endogenous Akt-1 activity by EGF and suppression by FTI or RhoB-GG but not the inactive RhoB-S mutant (see Fig. 4B). Panc-1 human pancreatic carcinoma cells have been reported to be susceptible to FTI-induced apoptosis [20], and we observed similar suppression of EGF-stimulated Akt-1 activity by RhoB-GG in Panc-1 cells (data not shown). We concluded that RhoB-GG was sufficient to mediate suppression of Akt-1 when activated by EGF in human cells.

4. Discussion

This study defines a mechanism through which FTI can inhibit activation of the survival kinase Akt-1 in epithelial cells. This mechanism is tissue-specific insofar as it does not operate in transformed fibroblasts [7–9]. RhoB-GG is sufficient to mediate apoptosis by FTI treatment in MCF7 carcinoma cells [6]. The findings of this study establish that RhoB-GG is also sufficient to mediate Akt-1 suppression by FTI. Our results are consistent with a report that FTI treatment can suppress activation of the related Akt-2 kinase in ovarian and pancreatic carcinomas [20]. The ability of RhoB-GG to mediate FTI effects suggests how FTI may affect Akt-2 in these epithelial tumor cell types.

We observed differential susceptibility of Akt-1 in COS cells when Akt-1 was activated by oncogenic Ras versus EGF treatment. This difference did not reflect a deficit in the ability to block Akt-1 when it is stimulated by EGF, because both FTI and RhoB-GG could suppress EGF-stimulated activation of Akt-1 in MCF7 or Panc1 carcinoma cells. Thus, it seems likely that a variation in the configuration of Akt signaling

pathways regulated by EGF in COS and MCF7 cells accounts for the difference in RhoB-GG susceptibility. This variation may be useful to pinpoint the mechanism by which RhoB-GG regulates Akt-1 activity. One question is whether isoprenylation type influences the ability to suppress Akt-1 activity. Farnesylated RhoB is eliminated by FTI treatment and therefore not a player in the FTI response. However, it would be of interest to compare the effects of isoprenylation on Akt-1 activity using a RhoB mutant made recently that is strictly farnesylated in cells [21].

Akt-1 is activated in several steps, the first of which does not appear to be affected by RhoB-GG. Recruitment of PI3′K to activated receptors leads to an increase in the level of phosphatidylinositol 3,4-phosphate in membranes, causing recruitment of proteins such as Akt-1 and Pdk1 that contain Pleckstrin homology (PH) domains, which bind to the bisphosphate inositol headgroup on the lipid. This step was not susceptible to RhoB-GG inhibition, insofar as RhoB-GG was unable to block Akt-1 activation by a constitutively activated isoform of the p110 catalytic subunit of PI3′K. The second step in Akt-1 activation is phosphorylation by Pdk1, and possibly other kinases, which are stimulated by upstream signals that are largely uncharacterized. Later steps in Akt-1 activation involve additional protein–protein interactions and nuclear translocation [22,23]. By inference RhoB-GG must act at one or more of these steps. For proper trafficking of the EGF receptor RhoB must recruit Prk to early endosomal membranes [3,24]. Interestingly, Prk was recently reported to associate with and regulate the substrate specificity of Pdk1, promoting the latter to phosphorylate the so-called Pdk2 activation site (S473) on Akt-1 [25]. RhoB-GG does not localize to the appropriate endosomal compartment in FTI-treated cells [26], so one possibility is that RhoB-GG influence Akt activation by affecting Prk-dependent control of Pdk1 localization and/or activity [2].

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References

[1] Gibbs, J., Oliff, A. and Kohl, N.E. (1994) Cell 77, 175–178.
[2] Prendergast, G.C. (2000) Curr. Opin. Cell Biol. 12, 166–173.
[3] Gampel, A., Parker, P.J. and Mellor, H. (1999) Curr. Biol. 9, 955–958.
[4] Lebowitz, P., Casey, P.J., Prendergast, G.C. and Thissen, J. (1997) J. Biol. Chem. 272, 15591–15594.
[5] Du, W., Lebowitz, P. and Prendergast, G.C. (1999) Mol. Cell. Biol. 19, 1831–1840.
[6] Du, W. and Prendergast, G.C. (1999) Cancer Res. 59, 5924–5928.
[7] Liu, A.-X., Du, W., Liu, J.-P., Jessell, T.M. and Prendergast, G.C. (2000) Mol. Cell. Biol. 16, 6105–6113.
[8] Du, W., Liu, A. and Prendergast, G.C. (1999) Cancer Res. 59, 4808–4812.
[9] Law, B.K., Norgaard, P., Gnudi, L., Kahn, B.B., Poulson, H.S. and Moses, H.L. (1999) J. Biol. Chem. 274, 4743–4748.
[10] Kohl, N.E. et al. (1993) Science 260, 1934–1937.
[11] Prendergast, G.C., Khosravi-Far, R., Solski, P., Kurzawa, H., Lebowitz, P. and Der, C.J. (1995) Oncogene 10, 2289–2296.
[12] Lebowitz, P., Du, W. and Prendergast, G.C. (1997) J. Biol. Chem. 272, 16093–16096.
[13] Klippel, A., Reinhard, C., Kavanaugh, W.M., Apell, G., Escobedo, M.A. and Williams, L.T. (1996) Mol. Cell. Biol. 16, 4117–4127.
[14] Chen, C. and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752.
[15] Hawley, R.G., Lieu, F.H., Fong, A.Z. and Hawley, T.S. (1994) Gene Ther. 1, 136–138.
[16] Pear, W., Nolan, G., Scott, M. and Baltimore, D. (1993) Proc. Natl. Acad. Sci. USA 90, 8392–8396.
[17] Harlow, E. and Lane, D. (1988) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[18] Kohl, N.E. et al. (1995) Nat. Med. 1, 792–797.
[19] Niman, H.L., Houghten, R.A., Walker, L.E., Reisfeld, R.A., Wilson, I.A., Hogle, J.M. and Lerner, R.A. (1983) Proc. Natl. Acad. Sci. USA 80, 4949–4953.
[20] Jiang, K., Coppola, D., Crespo, N.C., Nicosia, S.V., Hamilton, A.D., Sebti, S.M. and Cheng, J.Q. (2000) Mol. Cell. Biol. 20, 139–148.
[21] Chen, Z., Sun, J., Pradines, A., Favre, G., Adnane, J. and Sebti, S.M. (2000) J. Biol. Chem. 275, 17974–17978.
[22] Pekarsky, Y. et al. (2000) Proc. Natl. Acad. Sci. USA 97, 3028–3033.
[23] Chan, T.O., Rittenhouse, S.E. and Tsichlis, P.N. (1999) Ann. Rev. Biochem. 68, 965–1014.
[24] Mellor, J., Flynn, P., Nobes, C.D., Hall, A. and Parker, P.J. (1998) J. Biol. Chem. 273, 4811–4814.
[25] Balendran, A., Casamayor, A., Deak, M., Paterson, A., Gaffney, P., Currie, R., Downes, C.P. and Alessi, D.R. (1999) Curr. Biol. 9, 393–404.
[26] Lebowitz, P.F., Davide, J.P. and Prendergast, G.C. (1995) Mol. Cell. Biol. 15, 6613–6622.