Centaurin-α₁ Is an in Vivo Phosphatidylinositol 3,4,5-Trisphosphate-dependent GTPase-activating Protein for ARF6 That Is Involved in Actin Cytoskeleton Organization*

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The ADP-ribosylation factor (ARF) 6 small GTPase regulates vesicle trafficking and cytoskeletal actin reorganization. The GTPase-activating proteins (GAPs) catalyze the formation of inactive ARF6GDP. Centaurin-α₁ contains an ARF-GAP and two pleckstrin homology (PH) domains, which bind the second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP₃). Here, we show that centaurin-α₁ specifically inhibits in vivo GTP loading of ARF6 and redistribution of ARF6 from the endosomal compartment to the plasma membrane, which are indicative of its activation. Centaurin-α₁ also inhibited cortical actin formation in a PIP₃-dependent manner. Moreover, the constitutively active mutant of ARF6, but not that of ARF1, reverses the inhibition of cortical actin formation by centaurin-α₁. An artificially plasma membrane-targeted centaurin-α₁ bypasses the requirement of PIP₃ for its involvement in ARF6 inactivation, suggesting that PIP₃ is required for recruitment of centaurin-α₁ to the plasma membrane but not for its activity. Together, these data suggest that centaurin-α₁ negatively regulates ARF6 activity by functioning as an in vivo PIP₃-dependent ARF6 GAP.

Phosphatidylinositol (PI)³ 3-kinases phosphorylate the 3' position of the inositol ring of PI and its derivatives. The 3-phosphorylated PIs such as PIP₃ function as second messengers in the regulation of many cellular functions, including cell migration and vesicle transport (1). PIP₃ is localized in the cytosolic leaflet of the plasma membrane and acts as a site-specific signal for recruitment and/or activation of cytosolic proteins required for the formation of functional complexes at the plasma membrane. A large number of downstream targets have been identified for this lipid and used to characterize agonist activated PI 3-kinase associated cellular pathways. These include ARF regulators such as cytohesins, ARAP3 (ARF GAP, Rho GAP, ankyrin repeat, PH protein 3) and centaurin-α₁ (2).

ARF family of small GTPases regulate vesicle trafficking by shutting between an inactive GDP- and an active GTP-bound form (3). Among the known six-mammalian ARF isoforms (ARFs 1–6), ARF1 and ARF6 are the most distantly related and the best characterized. ARF1 localizes to the cytosol in GDP-bound form and to the Golgi membrane in GTP-bound form and regulates transport from the Golgi complex. In contrast, ARF6 localizes to endosomes in GDP-bound form and to the plasma membrane in GTP-bound form and regulates transport between these two organelles and cortical actin re-arrangements.

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‡ The abbreviations used are: PI, phosphatidylinositol; PIP₃, PI 3,4,5-trisphosphate; ARF, ADP-ribosylation factor; PH, pleckstrin homology; GE,F, guanine-nucleotide exchange factors; GAP, GTPase-activating proteins; IP₃, inositol 1,3,4,5-tetrakisphosphate; p42IP₃, IP₃-binding 42-kDa protein; PIP₃, BP, PIP₃-binding protein; GFP, green fluorescent protein; RFP, red fluorescent protein; EGF, epidermal growth factor; GGA3, Golgi associated, ARF effector protein 3; GAT domain, GGA and TOM1 proteins; BIG1, befeldin A inhibitory GEF 1; ACAP, ARF GAP, coiled-coil, ankyrin repeats, PH protein; HA, hemagglutinin; DM, double mutant; TRITC, tetra-methylrhodamine isothiocyanate.

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MATERIALS AND METHODS

Plasmids—HA-ARF6/pXS, pEGFP-centaurin-α₁, and its double PH mutant (DM, R149C/R275) constructs were described previously (5, 10). The pEGFP-centaurin-α₁, R49C mutant was generated using specific mutagenic primers and mutagenic mutagenesis according to the manufacturer’s instructions. pEGFP-centaurin-α₁CaAX and its mutants were generated by attaching a C-terminal CAAX motif using PCR
RESULTS AND DISCUSSION

To study whether centaurin-α1 can function as an ARF GAP in vivo, we analyzed the effect of centaurin-α1 on ARF6 localization in EGF-stimulated cells. Previously we have shown that in EGF-stimulated HeLa cells ARF6 re-locates from endosomes to the plasma membrane in a PI 3-kinase-dependent manner, which is indicative of its activation (10). Since centaurin-α1 binds to the PI 3-kinase lipid product, PIP₃, and translocates from the cytosol to the plasma membrane where activated ARF6 localizes, we and others have hypothesized that centaurin-α1 may function as an in vivo GAP for ARF6 (2, 5). To test this hypothesis we have analyzed the ability of centaurin-α1 to affect the intracellular localization of ARF6 in EGF-stimulated cells. For this purpose we have expressed GFP-tagged centaurin-α1 together with HA-tagged ARF6 in HeLa cells and assessed ARF6 localization by immunofluorescence under basal and EGF-stimulated conditions (Fig. 1). As observed previously (10), punctate staining pattern was observed for ARF6 in serum-starved unstimulated HeLa cells (data not shown), and its localization was unaltered when co-expressed with either control GFP or GFP-tagged centaurin-α1, suggesting that centaurin-α1 does not affect ARF6 localization in unstimulated cells. In EGF-stimulated cells, ARF6 localized to the plasma membrane when co-expressed with control GFP protein. However, EGF stimulation of the GFP-centaurin-α1 and HA-ARF6-transfected cells resulted in recruitment of centaurin-α1 to the plasma membrane and inhibition of the re-distribution of ARF6 to the plasma membrane. For this ability centaurin-α1 requires both its catalytic activity and association with the plasma membrane, since its catalytically inactive mutant (R49C), which is able to bind PIP₃, is therefore recruited to the plasma membrane, and the catalytically active double PH mutant (DM), which is unable to bind PIP₃ and hence is not translocated to the plasma membrane, were both incapable of inhibiting EGF-induced ARF6 redistribution. These observations suggest that centaurin-α1 inhibition of the ARF6 activation in EGF-stimulated cells is likely to be mediated by its PIP₃-regulated ARF GAP activity.

It has been shown that EGF-stimulated ARF6 activation results in an increase in cortical actin formation (10). We have therefore used the change in cortical actin formation in EGF-stimulated cells as an assay to study the effect of centaurin-α1 on endogenous ARF6 activation (Fig. 1). In the unstimulated condition, the actin cytoskeleton network in the GFP-tagged centaurin-α1-transfected cells was similar to that in the cells transfected with control GFP, indicating that centaurin-α1 had no effect on the cortical actin network. Upon stimulation with EGF a clear cortical actin formation was observed in GFP expressing control cells but not in GFP-centaurin-α1-expressing cells, indicating that centaurin-α1 prevented cortical actin formation in EGF-stimulated cells. Again this effect was dependent on both plasma membrane recruitment and catalytic activity of centaurin-α1 because neither the catalytically inactive mutant (R49C) nor the double PH mutant of centaurin-α1 (DM) was able to prevent cortical actin formation in EGF-stimulated cells.

We then analyzed whether centaurin-α1 could affect EGF-stimulated cortical actin formation by inhibiting ARF6 activation. To do so we transfected HeLa cells with GFP-centaurin-α1 and the HA-tagged constitutively active mutant of ARF6 (ARF6Q67L) or ARF1 (ARF1Q71L) and subsequently analyzed cortical actin formation upon stimulation with EGF. As shown in Fig. 2, the constitutively active mutant of ARF6, but not that of ARF1, reversed the inhibition of cortical actin formation by centaurin-α1 in EGF-stimulated cells. This result clearly indicates that centaurin-α1 prevents EGF-stimulated cortical actin formation by specifically inhibiting ARF6 activation.

As with other small GTPases, ARFs have also been shown to interact specifically with their effectors such as GGA3 when they are in the active GTP-bound form (3). Recently, Santy and Casanova (12) have made use of this observation and developed a GST-effector pull down assay to study ARF activation in vivo. This assay is useful to correlate the intracellular distribution of ARF with its nucleotide bound status. Using this assay, we performed a biochemical analysis of in vivo ARF activation to...
Further confirm centaurin-α₁ as an in vivo GAP for ARF6. For this purpose, HA-tagged ARF6 was co-expressed with either GFP, GFP-centaurin-α₁ wild type, or GFP-centaurin-α₁ mutants (R49C or DM) in COS cells. Following serum starvation, cells were stimulated with EGF, and activated ARF6 was precipitated from the lysed cells using the GST-GGA3 GAT domain coupled to glutathione-resin. ARF6 activation in EGF-treated cells was inhibited by centaurin-α₁ (Fig. 3A). However, centaurin-α₁ was ineffective as an inhibitor for ARF6 activation when mutated in its catalytic domain (R49C) or the two PH domains (DM) that are required for the membrane association through binding to the PIP₃. This result confirms the observation that centaurin-α₁ inhibits ARF6 activation by functioning as a PIP₃-regulated ARF GAP. We have also assessed biochemically the effect of centaurin-α₁ on ARF1 activation using the in vivo ARF activation assay to confirm the substrate specificity of centaurin-α₁. BIG1, an ARF1 GEF, constitutively activated ARF1, but centaurin-α₁ did not inhibit ARF1 activation by BIG1 in either unstimulated or cells stimulated with EGF (data not shown).

PIP₃ is involved not only in the recruitment of cytosolic proteins to the plasma membrane but also in activation of some of the membrane recruited proteins (1). To ascertain whether PIP₃ is required just for the membrane recruitment of centaurin-α₁ or for both the membrane recruitment and activation of centaurin-α₁, we generated a plasma membrane targeted version of GFP-tagged centaurin-α₁ wild type and its mutants (R49C and DM) by attaching the CAAX motif (which constitutes a site that is post-translationally modified by prenylation) of K-Ras to the C terminus of centaurin-α₁ (GFP-centaurin-α₁CAAX). We then analyzed the effect of these constructs on the constitutive redistribution of ARF6 to the plasma membrane, indicative of its activation. We have previously shown that the cytohesin 2 GEF constitutively redistributes ARF6 to the plasma membrane when it is targeted to the plasma membrane by attaching the CAAX motif to its C-terminal end (10). As illustrated in Fig. 4, GFP-centaurin-α₁CAAX mainly localized to the plasma membrane and inhibited the constitutive redistribution of ARF6 to the plasma membrane by cytohesin 2CAAX. The ability of centaurin-α₁CAAX to prevent constitutive re-distribution of ARF6 was dependent on the ARF6 GAP activity, since the GFP-centaurin-α₁CAAX (R49C) mutant was ineffective in prevention of ARF6 re-distribution. However, centaurin-α₁CAAX does not require the functional PIP₃ binding PH domains for this purpose because GFP-centaurin-α₁CAAX (DM) was as effective as the wild type in inhibiting ARF6 redistribution. These data suggest that centaurin-α₁ requires PIP₃ for recruitment to the plasma membrane but not for its activity. We have confirmed this observation further by analyzing the effect of centaurin-α₁CAAX and its mutants on in vivo GTP loading of ARF6 (Fig. 5). Centaurin-α₁CAAX wild type and its PH domains mutant (DM) prevented the activation of ARF6 by cytohesin 2CAAX. However, the catalytically inactive mutant of centaurin-α₁CAAX was unable to prevent the constitutive activation of ARF6. We found no increase in the constitutive redistribution or GTP loading of ARF6 by EGF stimulation, suggesting maximal activation of ARF6 by cytohesin 2CAAX in unstimulated cells. Consistent with this, we also found out that the effect of the CAAX constructs of centaurin-α₁ on the constitutive redistribution and GTP loading of ARF6 in unstimulated cells was identical to that in EGF-stimulated cells (data not shown).

Activation of the Ras family of small GTPases leads to biological responses whereas the ARF family of GTPases requires not only activation but also inactivation to elicit cellular responses (3). Since ARFs have undetectable levels of intrinsic GTP binding and hydrolysis, they are totally dependent on extrinsic GEFs for GTP binding and GAPs for GTP hydrolysis. Therefore ARF activation and inactivation require the presence of both GEF and GAP activity in the same membrane compartment. Previously, we have shown that ARF GEFs such as cytohesin 2 recruit to the plasma membrane via PIP₃ binding, thereby activating ARF6 (10). We demonstrate in this report that centaurin-α₁ recruitment to the plasma membrane in agonist-stimulated cells leads to an inhibition of ARF6 activation, as assayed by exogenously expressed ARF6 redistribution and GTP loading and endogenous ARF6-dependent rearrangement of the actin cytoskeleton. Moreover, the constitutively active mutant of ARF6 (ARF6Q67L), but not that of ARF1, prevented inhibition of the actin cytoskeleton rearrangements by centaurin-α₁ in agonist-stimulated cells. Together, these results indicate that centaurin-α₁ specifically acts as GAP for ARF6 in
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**Fig. 4.** Effect of membrane-targeted centaurin-α, and its mutants on ARF6 activation by cytohesin 2. HeLa cells were transiently transfected with the indicated expression vectors. After 48 h, the cells were serum-starved, fixed, immunostained with an anti-HA antibody, and imaged using a confocal microscope. Images are representative of 90–95 transfected cells from three experiments.

The other ARF GAPs that show ARF6 substrate specificity are ARAP3, ACAP (ARF GAP, guaied-coil, gkyrin repeats, PH protein) family and GIT (G protein-coupled receptor kinase interactor) family proteins (13–15). ARAP3 has recently been purified using a PIP3 affinity column from pig neutrophil cytosol and its human homologue subsequently cloned. ARAP3 is so called because of its ability to act as a GAP for ARF as well as Rho, thereby providing the potential link between ARF6 and Rho signaling.

ARAP3 binds PIP3 in vitro through its N-terminal PH domain and translocates to the plasma membrane in intact cells in a PI 3-kinase and PH domain-dependent manner. Moreover, PIP3 stimulates its ARF6 GAP activity in vitro.

Although ACAPs and the GIT family ARF GAPs have also shown to act as GAPs for ARF6, it is not yet known whether these proteins bind PIP3 or the other PI 3-kinase lipid products or if they are regulated by PI 3-kinase in vivo. Moreover, the importance of PIP3 for either recruitment, or recruitment and activation of these ARF GAPs, including ARAP3, in vivo is unknown. Here we have addressed not only the importance of centaurin-α1 in regulation of ARF6 signaling but also how PIP3 regulates centaurin-α1 activity in intact cells. In conclusion, we have shown here that the PIP3-dependent plasma membrane association of centaurin-α1 induces its catalytic activity. This, coupled with the plasma membrane recruitment of the cytosine family of ARF GEFs via PIP3 binding, provides a mechanism whereby agonist stimulated PI 3-kinase dynamically regulates the activation/inactivation cycle of ARF6 at the plasma membrane.

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**REFERENCES**

1. Vanhaesebroeck, B., Leeners, S. J., Ahmadi, K., Timms, J., Kato, R., Driscoll, P. C., Woscholski, R., Parker, P. J., and Waterfield, M. D. (2001). Annu. Rev. Biochem. 70, 535–560

2. Hawad, M. A., Follarin, N., Martin, R., and Jackson, T. R. (2002). Biol. Res. 35, 247–265

3. Randazzo, P. A., Nie, Z., Miura, K., and Hsu, V. W. (2000). Science’s STKE http://stke.sciencemag.org/cgi/content/full/OC_sigtans;2000/58/e1

4. Donaldson, J. G. (2003) J. Biol. Chem. 278, 41573–41576

5. Cullen, P. J., and Venkateswarlu, K. (1999) Biochem. Soc. Trans. 27, 683–689

6. Hammond-Odie, L., Jackson, T. R., Proft, A. A., Blader, I. J., Tuck, C. W., Prestwich, G. D., and Theibert, A. B. (1996) J. Biol. Chem. 271, 18859–18868

7. Tanaka, K., Imajoh-Ohmi, S., Sawada, T., Shirai, R., Hashimoto, Y., Iwasaki, S., Kasai, K., Kanamoto, Y., Shirai, T., Terada, Y., Kimura, K., Nagata, S., and Fuki, Y. (1997) Eur. J. Biochem. 245, 512–519

8. Venkateswarlu, K., Oatey, P. B., Tavare, J. M., Jackson, T. R., and Cullen, P. J. (1999) Biochem. J. 340, 359–363

9. Venkateswarlu, K., and Cullen, P. J. (1999) Biochem. Biophys. Res. Commun. 262, 237–244

10. Venkateswarlu, K., and Cullen, P. J. (2000) Biochem. J. 345, 719–724

11. Venkateswarlu, K. (2003) J. Biol. Chem. 278, 43460–43469

12. Sanyt, L. C., and Casanova, J. E. (2001) J. Cell Biol. 154, 599–610

13. Krugmann, S., Anderson, K. E., Ridley, S. H., Risso, N., McGregor, A., Cosedell, J., Davidson, K., Equinoa, A., Ellson, C. D., Lipp, P., Manufova, M., Kristakos, N., Painter, G., Thruning, J. W., Cooper, M. A., Lim, Z-Y., Holmes, A. B., Dove, S. K., Michell, R. H., Grewal, A., Nazarian, A., Erdjument-Bromage, H., Tempot, P., Stephens, L. R., and Hawkins, P. T. (2002) Mol. Cell 9, 95–108

14. Jackson, T. R., Brown, F. D., Nie, Z., Miura, K., Foroni, L., Sun, J., Hsu, V. W., Donaldson, J. G., and Randazzo, P. A. (2000) J. Cell Biol. 151, 627–638

15. Vitale, N., Patton, W. A., Moss, J., Vaughan, M., Lefkowitz, R. J., and Fremont, R. M. (2000) J. Biol. Chem. 275, 13901–13906

**FIG. 5.** Biochemical analysis of the effect of membrane-targeted centaurin-α1 and its mutants on ARF6 activation by cytohesin 2. COS cells were transiently transfected with the indicated expression plasmids. ARF6 H11001 was precipitated from the cell lysates with GST-GGA3 GAT. The precipitates and the cell lysates were immunoblotted (II) with an anti-HA antibody to determine ARF6 GTP and total ARF6, respectively. The cell lysates were also immunoblotted with anti-GFP and anti-RFP antibodies to demonstrate the relative level of expression of the indicated GFP- and RFP-tagged proteins, respectively. II, quantification of data obtained from three similar experiments. Data are the means ± S.E.
