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Research Article

C3G mediated suppression of malignant transformation involves activation of PP2A phosphatases at the subcortical actin cytoskeleton

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ABSTRACT

In previous work, we demonstrated that C3G suppresses Ras oncogenic transformation by a mechanism involving inhibition of ERK phosphorylation. Here we present evidences indicating that this suppression mechanism is mediated, at least in part, by serine/threonine phosphatases of the PP2A family. Thus: (i) ectopic expression of C3G or C3GΔCat (mutant lacking the GEF activity) increases specific ERK-associated PP2A phosphatase activities; (ii) C3G and PP2A interact, as demonstrated by immunofluorescence and co-immunoprecipitation experiments; (iii) association between PP2A and MEK or ERK increases in C3G overexpressing cells; (iv) phosphorylated-inactive PP2A level decreases in C3G expressing clones and, most importantly, (v) okadaic acid reverts the inhibitory effect of C3G on ERK phosphorylation. Moreover, C3G interacts with Ksr-1, a scaffold protein of the Ras–ERK pathway that also associates with PP2A. The fraction of C3G involved in transformation suppression is restricted to the subcortical actin cytoskeleton where it interacts with actin. Furthermore, the association between C3G and PP2A remains stable even after cytoskeleton disruption with cytochalasin D, suggesting that the three proteins form a complex at this subcellular compartment. Finally, C3G- and C3GΔCat-mediated inhibition of ERK phosphorylation is reverted by incubation with cytochalasin D. We hypothesize that C3G triggers PP2A activation and binding to MEK and ERK at the subcortical actin cytoskeleton, thus favouring ERK dephosphorylation.

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Introduction

The C3G gene product is a Crk-binding protein, acting as an exchange factor for the Rap and R-Ras proteins [1,2], that plays a crucial role in integrin mediated cell adhesion and migration [3–7]. In several models Crk–C3G complexes participate in ERK activation via Ras- and/or Rap1-dependent mechanisms [8–11], but in others, such as Chinese hamster ovary (CHO) cells, there is an inverse correlation between the presence of a CrkII–C3G functional complex and the ERK activity, which suggests the involvement of C3G in an ERK suppression pathway [12]. In this lane, our laboratory has recently shown in NIH3T3 fibroblasts that C3G suppresses malignant transformation induced by several oncogenes by a process that is independent of its exchange activity but dependent on its SH3-binding domain. The mechanism of suppression of $Hras^{Lys12}$-mediated oncogenic transformation involves inhibition of ERK/MAPK phosphorylation and cyclin A expression, which result in the loss of transformed cells capacity to growth in non-adherent conditions [13,14]. Our data demonstrated that C3G acts at the level of ERK without affecting other steps in the Ras–ERK cascade, suggesting that C3G could operate through the activation of

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cellular phosphatases. In fact, the activity of ERK/MAPK depends on the equilibrium between the activities of the upstream kinases and protein phosphatases. Dephosphorylation of either the threonine or the tyrosine residue is sufficient for inactivation and this can be accomplished by tyrosine-specific phosphatases, serine/threonine phosphatases or by dual specificity (threonine/tyrosine) phosphatases [15,16]. The protein phosphatase 2A (PP2A) family comprises the major serine/threonine phosphatases involved in signal transduction and the regulation of growth and development [17,18]. PP2A family of phosphatases are heterotrimeric holoenzymes consisting of a core dimer composed of a scaffolding/structural (A) subunit and a catalytic (C) subunit. This dimer associates with a variety of regulatory (B) subunits. Thus far, four distinct families of regulatory subunits have been identified: B or PR55, B′ or PR61 (also B56), B″ or PR72, and B‴ or PR93/PR110 [18,19].

Immunoprecipitation studies have revealed that PP2A associates with ERK and MEK [20] as well as with the kinase suppressor of Ras 1 (Ksr-1) [21] and Raf1 [22]. In fact, PP2A phosphatases participate in both positive and negative regulation of ERK/MAPK signaling pathway at multiple entry points [21,23–26] depending on the cell type, its subcellular localization [18] and the holoenzyme composition, being the regulatory B subunits the responsible of the specificity [24,27].

Although C3G in its inactive state shows a cytoplasmic, perinuclear location [13], phosphorylated-activated C3G localizes specifically to the subcortical actin cytoskeleton and Golgi apparatus [28], suggesting a possible function for C3G at these subcellular compartments. In fact, phosphorylation of C3G results in an increase in C3G catalytic activity towards its substrate Rap1, which localizes mainly to the Golgi and lysosomal vesicles [29]. Nevertheless, the location of the Rap1-independent C3G suppressor activity has not been established.

Here we show a novel function of C3G as phosphatase activator. Furthermore, the inhibitory effect of C3G on ERK phosphorylation seems to be mediated by phospho-ERK specific PP2A phosphatases. In addition, the C3G suppression function is located at the subcortical actin cytoskeleton, where C3G and PP2A interact. We propose that C3G suppressor activity of Ras-mediated transformation is located at the subcortical actin cytoskeleton and involves the inactivation of the Ras–ERK pathway at the level of ERK by specific PP2A holoenzymes.

Materials and methods

Cell cultures

In this work we used mass cultures of NIH3T3 cells stably transfected with pLTR2, pLTR2C3G (containing the complete human C3G cDNA) or pLTR2C3GΔCat (a C3G mutant devoid of its catalytic domain but retaining the transformation suppression function) alone or in combination with pMEXneoHras^{Lys12} [13,14]. For simplification, we will refer to these constructs in the text as pLTR2, C3G, C3GΔCat and Hras^{Lys12}, respectively.

Antibodies and organelle markers

For immunoblotting and immunoprecipitation studies we used: anti-C3G (C-19), anti-C3G (H-300), anti-Actin (C-11), anti-MEK1 (C-18), anti-ERK1 (K-23) and anti-Ksr-1 (C-19) from Santa Cruz Biotechnologies (Santa Cruz, CA, USA); anti-phospho-p44/42 MAP Kinase (Thr202/Tyr204) (anti-phospho-ERK1/2) and anti-phospho-MEK1/2 (Ser217/221) from Cell Signaling Technology, Inc. (Beverly, MA, USA); anti-PP2A, C subunit, clone 1D6 from Millipore-Upstate (Billerica, MA); anti-phospho-PP2A (Y307) from Epitomics, Inc. (Burlingame, CA, USA).

For immunofluorescence studies we used as primary antibodies: anti-PP2A, C subunit, clone 1D6 and anti-C3G 1008 [13]. As secondary antibodies we used: Cy3 anti-rabbit, FITC anti-mouse and Cy3 anti-mouse from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). For detection of cytoplasmic organelles we used: Oregon Green® 514 phalloidin from Invitrogen-Molecular Probes (Carlsbad, CA, USA) and anti-GM130 from BD Biosciences (San Jose, CA, USA).

Immunoblotting

Whole cell lysates were prepared by extracting with Cell Lysis Buffer purchased from Cell Signaling Technology, Inc. (#9803) or with RIPA buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100 (or NP-40), 0.1% Na deoxycholate, 0.1% SDS) supplemented with 1 mM PMSF, 10 μg/ml Aprotinin and 10 μg/ml Leupeptin. The cell debris was removed by spinning at 10,000×*g* for 10 min at 4 °C.

Immunoprecipitation

Immunoprecipitation was performed essentially as described [30]. Briefly, 500 μg of protein from whole cell lysates were incubated with the antibody for 2 h. After this, GammaBind G Sepharose beads (Amersham Biosciences, Uppsala, Sweden) were added, and the incubation was continued for one more hour. The beads with the immunocomplexes were pelleted and washed with 1× Cell Lysis Buffer (see above) three times and either boiled with 1× sample buffer for immunoblotting or processed for kinase assay.

Immunofluorescence

Culture plates (60 mm) containing 2–3 coverslips were covered with 1 μg/ml poly-lysine (Sigma-Aldrich Corp., St. Louis, MO, USA) during 15 min, water washed, dried under hood for 2 h and seeded with 30,000 cells/plate. After overnight growth and treatment with agonists and inhibitors, cells were washed twice with cold PBS and fixed with freshly prepared 3.7% formaldehyde during 15 min, washed again three more times in PBS and permeated with 0.1% Triton in PBS for additional 10 min. After blocking in 1% BSA/PBS during 15 min cells were incubated with primary antibody, diluted in same blocking solution, for 1 h followed by incubation in the dark with fluorescent fluorochrome-labelled specific secondary antibody. Finally, after three washes in PBS, coverslips were mounted over the slides with Mowiol 4-40 (Calbiochem, EMD Biosciences, San Diego, CA, USA) or, in the case of organelle staining, incubated with the specific marker and then mounted. Fluorescent images were taken with confocal microscope Zeiss LSM510 using software LSM510 2.8. Cy3 is excited with ^{2}He/^{10}Ne laser (emission 543 and 633 nm). FITC and Oregon Green® 514 phalloidin are excited with ^{18}Ar laser (emission 458, 488 or 514.5 nm).

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