Quantification of ARF-GTP in HepG2 by pulldown with GST-GGA3(1-316)

Markus Hafner
LIMES Program Unit Chemical Biology & Medicinal Chemistry, c/o Kekulé Institut für Organische Chemie und Biochemie, University of Bonn

Anton Schmitz
LIMES Program Unit Chemical Biology & Medicinal Chemistry, c/o Kekulé Institut für Organische Chemie und Biochemie, University of Bonn

Michael Famulok
LIMES Program Unit Chemical Biology & Medicinal Chemistry, c/o Kekulé Institut für Organische Chemie und Biochemie, University of Bonn

Method Article

Keywords: ADP ribosylation factors (ARFs), secinH3, cytohesin, GGA3 (Golgi-associated-gamma adaptin ear containing-ARF binding protein 3),

Posted Date: December 15th, 2006

DOI: https://doi.org/10.1038/nprot.2006.412

Abstract

Introduction

G-proteins are small regulatory switches that exist in two states: a GTP-bound “on” state, and a GDP-bound “off” state. Methods for determining the activation state of G-proteins are important for understanding G-protein controlled signaling networks. An important class of G-proteins are ADP-ribosylation factors \(ARFs), which regulate cytoskeletal organization, integrin activation or signalling. Cytohesins are known activators of ARFs which catalyze the exchange of ARF-bound GDP for GTP; thus, cytohesin inhibition leads to ARFs remaining in the GDP-bound “off” state. SecinH3 is a small organic molecule that inhibits the cytohesin class of small \(~47 kDa) GEFs. Its application in HepG2 cells suppresses insulin signaling; in mice it causes hepatic insulin resistance. Here we describe the procedure we used to quantify the amount of activated ARF after stimulation with insulin and incubation with SecinH3, i.e. the amount of ARF loaded with GTP, by pulldown with the N-terminal 316 amino acids of GGA3, a protein that specifically binds to ARF-GTP and not to ARF-GDP.

Reagents

HepG2 cells grown to 75-80% confluence GST-GGA3\(1-316) expressed from a pGEx4T1 and immobilized on glutathione beads SecinH3 \(100 mM in DMSO) D5 \(negative control, 10 mM in DMSO) Insulin \(1.7 mM; Sigma) lysis buffer \(200 mM NaCl, 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 5% glycerol, protease inhibitor mix \(Serva)) anti-ARF6 mAb \(Santa Cruz, sc-7971) standard cell culture material and media standard western-blotting material

Equipment

Standard cell culture equipment Standared western blotting equipment

Procedure

1. 10^7 cells seeded into five 120 mm cell culture dishes and incubated overnight at 37°C, 5% CO_2 in full medium \(EMEM/10% FCS) 2. cells washed twice with PBS and medium exchanged for EMEM w/o FCS 3. grown overnight 4. inhibitors added to dishes and further incubated for 3 hrs at 37°C, 5% CO_2: dishes 1 and 2: 0.2% DMSO; dish 3: SecinH3 12.5 µM; dish 4: SecinH3 6.25 µM; dish 5: D5 25 µM 5. stimulation with 100 nM insulin 10’ at 37°C 6. cells washed twice with ice cold PBS 7. cells harvested with rubber policeman in 1.5 mL lysis buffer 8. incubated 20’ on ice 9. sonicated 7”, 40%, cycle 50 10. centrifuged 10’ at max. speed, 4 °C 11. 1200 µL supernatant incubated with GST-GGA3 beads \(50 µg GST-GGA3\(1-316) per tube) 12. incubated 2 hrs at 4 °C under mild agitation 13. centrifuged for 1’ at 500 x g and supernatant discarded 14. pellets washed 3x with lysis buffer 15. 50 µL Laemmli loading buffer added to pellet and incubated 5’ @ 95 °C 16. proteins separated by 15% SDS-PAGE; loading normalized to protein content in cell lysates 17. blotted on nitrocellulose with semi-dry blotting method 18. nitrocellulose

blocked for 20’ with 5% milk powder in TBS/0.1% Tween-20 19. nitrocellulose washed twice with TBST 20. Anti-ARF6-antibody \(1:100 dilution in 5% BSA/TBST) added and incubated overnight at 4 °C 21. nitrocellulose washed three times with TBST 22. goat-anti-mouse-HRP conjugate \(1:20,000 dilution in 5% milk powder/TBST) added and incubated for one hr at RT 23. nitrocellulose washed with TBST 24. nitrocellulose covered with ECL-solution, incubated 3 min at RT; luminescence visualized with VersaDoc 5000 \(BioRad)

Timing

Approx 3 days