

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

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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 MgCl₂, 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

Standard western blotting equipment

Procedure

1. 10^7 cells seeded into five 120 mm cell culture dishes and incubated overnight at 37°C, 5% CO₂ 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% CO₂: 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

Inhibition of cytohesins by SecinH3 leads to hepatic insulin resistance

by Hafner, M. et al.

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