TAP isolation of Ras protein complexes from mammalian cells

Transfection:
Transfect 5x10^8 NIH 3T3 cells with TAP-Ras fusion constructs using Lipofectamine (Invitrogen).

Perform all steps in a laminar flow hood, with gloves pulled over clamped disposable sleeves. Use only unused pipets, pipet tips, dishes and disposable columns and clean all pipettors and equipment thoroughly with 70% ethanol before use.

Cell lysis
Wash cells 3x with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). Remove last traces of PBS. Freeze dishes at -80°C overnight. Lyse cells:
Scrape cells into 1 ml lysis buffer* + 1 mM EGTA. Keep on ice 30 min.
Remove insoluble material by centrifugation at 14000xg for 10 min at 4°C.

Binding to Ni^{++} beads
Transfer 400 µl 1:1 Ni^{++}-charged sepharose beads to a 10ml Bio-Rad disposable filtered column, wash with 5 ml lysis buffer + 40 mM imidazole.
Cap bottom of column, add cell lysate, cap the top, and incubate 1 hr at 4°C in constant rotation.
Remove cap and drain column.
Wash beads with 30 ml lysis buffer + 40 mM imidazole.

Elution from Ni^{++} beads with imidazole
Elute in 5 ml lysis buffer + 400 mM imidazole + 2 mM CaCl_2.

Binding to calmodulin resin
In a fresh column, add 100 µl 1:1 calmodulin beads. Wash with 5 ml lysis buffer + 400 mM imidazole + 2 mM CaCl_2.
Cap columns. Add Ni^{++} eluates to columns and cover. Incubate 1 hr 4°C in constant rotation.
Wash columns with 5 ml lysis buffer + 400 mM imidazole + 2 mM CaCl_2.
Wash with 5 ml lysis buffer + 400 mM imidazole + 2 mM CaCl_2 with no detergent.

Elution from calmodulin columns
Elute columns in 200 µl lysis buffer + 4 mM EGTA with no detergent.
Send snap-frozen eluates for LC/MS analysis.

* Lysis buffer: 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM MgCl_2, 0.5% NP-40, plus freshly added protease inhibitor cocktail (Roche) and 10 µM GTP.
TAP-Ras constructs

<table>
<thead>
<tr>
<th>TAP Tag</th>
<th>Full-length R-Ras</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-His tag</td>
<td>1 PP 46 74 193 PP</td>
</tr>
<tr>
<td>HHHHHH</td>
<td>SW1 SW2</td>
</tr>
<tr>
<td>Protein A repeats</td>
<td>CP CVLL</td>
</tr>
<tr>
<td>TEV</td>
<td></td>
</tr>
<tr>
<td>CBD</td>
<td>Hypervariable region</td>
</tr>
</tbody>
</table>

CBD = Calmodulin-binding domain
PP = Proline-rich sequence
SW1/2 = switch1/2
TEV = TEV protease cleavage site
* = palmitoylation site
¥ = methylation and geranylgeranylation site

http://www.cellmigration.org/resource/discovery/discovery_resources.shtml#protocols
Ras-TAP Purification Strategy

Transfect 5x10^8 NIH 3T3 mouse fibroblasts

1st step enrichment

Lyse cells in NP-40 buffer

Ni^{2+}-coated beads

Cellular proteins

Elute with imidazole

2nd step purification

Plus 2 mM Ca^{2+}

Calmodulin-coated beads

3rd step purification

Elute with 10 mM EGTA

LC/MS/MS analysis

NIH 3T3 mouse fibroblasts

TAP-Ras

Binding partners

TAP-Ras

Residual non-specific proteins

http://www.cellmigration.org/resource/discovery/discovery_resources.shtml#protocols
N-terminal TAP-tagged GTPase constructs

nTAP: pEF4 expression construct containing the N-terminal TAP tag. Coding sequences were cloned in frame for generation of a fusion construct using BamH1 (5’).

nTAP-RRas(G38V)**
nTAP-RRas(T43N)**
nTAP-HRas(G12V)**
nTAP-HRas(T17N)
nTAP-Rap1a(G12V)
nTAP-Rap1a(T17N)
nTAP-KRas(G12V)
nTAP-KRas(T17N)

These constructs can obtained from Lawrence Goldfinger (lgoldfinger@ucsd.edu) or Mark Ginsberg (mhginsberg@ucsd.edu).

http://www.cellmigration.org/resource/discovery/discovery_resources.shtml#protocols
Confirmation that TAP-Ras fusion constructs are functional in vivo.

To confirm that full-length Ras constructs maintain Ras functions in vivo in the presence of a TAP fusion, we analyzed the ability of TAP fusions of distinct Ras isotypes to modulate integrin activation, as previously observed (Oertli et al., 2000). Expression of activated H-Ras(G12V) suppresses integrin activation in CHO cells bearing chimeric integrins αIIbα6β3β1, which are constitutively active, as measured by cell labeling with the ligand-mimetic antibody, PAC1, a generous gift of Dr. S. Shattil (Shattil et al., 1985;Hughes et al., 1997). Activated R-Ras reverses integrin suppression, and an additional mutation in the effector loop (G38VD64A) does not disrupt this function of R-Ras. However, an alternate mutation of the same residue (G38VD64E) blocks the ability of R-Ras to reverse suppression of integrin activation by activated H-Ras (Oertli et al., 2000). As shown below, TAP fusion to H-Ras and R-Ras did not alter the abilities of these GTPases to modulate integrin activation in cells.

Integrin activation was measured in CHO cells expressing constitutively active chimeric integrins. nTAP-H-Ras(G12V) suppresses integrin activation, similar to untagged H-Ras(G12V) (Oertli et al., 2000). Activated R-Ras(G38V) with or without a TAP tag fusion or PEA-15 (control) rescues H-Ras-mediated suppression, as does an effector loop double mutant nTAP-R-Ras(G38VD64A). nTAP-R-Ras(T43N) (dominant negative) and nTAP-R-Ras(G38VD64E) do not rescue suppression, consistent with previous observations (Oertli et al., 2000).

** These constructs have been confirmed for functionality by the integrin activation assay.

Reference List

http://www.cellmigration.org/resource/discovery/discovery_resources.shtml#protocols

Oertli, B., Han, J., Marte, B.M., Sethi, T., Downward, J., Ginsberg, M., and Hughes, P.E. (2000). The effector loop and prenylation site of R-Ras are involved in the regulation of integrin function [In Process Citation]. Oncogene 19, 4961-4969.