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Rap1

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Gene symbols: Rap1a, Rap1b

1. General Function

Rap1 (Ras-proximate-1) is a small GTP-binding protein of Mr 21,000, which belongs to the Ras family. Rap1 was identified in 1989 as a suppressor structurally similar to Ras (ca. 50 % homology) that reversed the phenotype of NIH3T3 transformed by Kirsten sarcoma virus and was referred to as Krev-1 (27, 38), though nowadays several studies indicate that Rap1 can act in a Ras-independent manner (42). Rap1 is also referred to in early literature as *smg* p21, when it was identified as a small GTP binding protein of 21 KDa purified from the cytosol fraction of human platelets (26, 37). There are two isoforms of Rap1, Rap1a and Rap1b, which are 95 % identical at the amino acid sequence (51). Some biological actions of Rap1 have been associated binding to and, thereby localizing two Rac GEFs VAV1 and TIAM1 to the sites of cell spreading, by regulating cadherin-mediated cell-cell contacts, and by interacting with and regulating myosin II, which is a constituent of the cytoskeleton (19). Rap1 has been implicated in adhesion-dependent signals during leukocyte migration and with a specific isoform (32) and some cells, such as B cells and platelets, express primarily one isoform, Rap1b (10, 28). Both isoforms are geranylgeranylated at the carboxyl-terminal Cys residue, which allows Rap1 to attach to biological membranes. In addition, they have clustered polybasic amino acids, which interact with the polar head groups of the acidic phospholipids contributing to membrane association (25, 51).

Rap1 is ubiquitously expressed and well known for its role in cell proliferation, differentiation, polarity as well as integrin-mediated cell adhesion and cadherin-mediated cell junction formation (6). In a variety of cell types, Rap1 regulates cell spreading by mediating the functions of integrins, by extravasation. CD31, which is an important integrin adhesion amplifier, is able to activate Rap1, and thereby, stimulate T lymphocyte adhesion to intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) (43). Rap1 regulates the ERK cascade in different manners depending on the cell type. In

HEK293 and NIH 3T3 cells Rap1 inhibits Ras-induced ERK activation via sequestration of Raf-1 (46) while in Rat-1 cells Rap1 does not affect Ras-induced ERK activation (60). In other cell types, unlike Ras, Rap1 activates the ERK pathway through direct association with B-Raf (49, 53, 59).

In addition to Rap1's role in normal cells, Rap1 activation has been implicated in the reduction of pancreatic tumor cell proliferation. In pancreatic cancer Panc1 and MiaPaCa cell lines Rap1 has been activated by forskolin, a direct adenylate cyclase stimulator, and a reduction of cell growth has been observed in Panc1 cells independently on Ras/Raf/MEK/ERK pathway (35).

Rap1, like other small G proteins, cycles between an inactive GDP-bound and an active GTP-bound forms and this cycle is regulated by two groups of regulatory proteins: guanine nucleotide exchange factors (GEFs), which induce dissociation of GDP from Rap1 followed by binding of GTP, and GTPase-activating proteins (GAPs), which convert active GTP-bound to the inactive GDP-bound form. The activation of Rap1 by GEFs is induced by second messengers, including calcium, diacylglycerol (DAG), phospholipase Cγ (PLCγ) and cAMP (51, 59). The Rap1GEF activated by an increase in cAMP is the *Exchange protein activating cAMP (Epac)* family which is constituted by two isoforms, Epac1 and Epac2 (14, 24). An increase in calcium and DAG levels induce *CalDAG-GEF* activation which is another family of Rap1GEF constituted by four isoforms, CalDAG-GEF I, II, III and IV; only CalDAG-GEF I and III are able to activate Rap1 (56). All CalDAG-GEFs have a CD25 domain, which is necessary for the GEF activity, as well as calcium- and DAG-binding domains. *C3G* was the first RapGEF identified with a characteristic catalytic region consisting of a CDC25 homology domain, a Ras exchange motif (REM) and a proline-rich sequences which bind to the SH3 domain of the adaptor proteins Crk and Grb2 (40). Another GEF for Rap1 is *PDZ-GEF,* which contains Ras association, and Ras-GEF domains, as well as a carboxyl-terminal motif for binding to PDZ domains (31)*. DOCK4* is an atypical GEF, which has GEF activity for both Rap and Rac (55). The finding that Rap1 could be activated by stimulation of a number of membrane receptors broadened its possible roles in signal transduction.

Protein kinase A (PKA), which is activated by cAMP formation, participates in Rap1 phosphorylation in certain cell types, including neutrophils, platelets, fibroblasts, thyroids, and enteroendocrine cells (41, 52). Phosphorylation appears to regulate both Rap1 activation as well as Rap1 membrane association. In platelets where Rap1 is one of the most abundant phosphoproteins, PKA-catalyzed phosphorylation at Ser^{179} affects Rap1 membrane binding and makes Rap1 sensitive to the action of Rap1GEF to stimulate its GDP/GTP change reaction, but does not by itself affect GDP/GTP binding or intrinsic GTPase activity (18). Ser^{179} belongs to the hydrophobic acid polybasic region of Rap1, which is essential for Rap1 to bind to membrane. Since PKA phosphorylates Rap1 downstream of this polybasic region, PKA reduces Rap1 membrane-binding affinity. However, the role of PKA-dependent phosphorylation of Rap1 can not be generalized since in thyroid follicular cells the phosphorylation of Rap1 does not affect its perinuclear subcellular localization (44). In mouse pancreatic acini PKA does not seem to be required for Rap1 activation and translocation since the PKA inhibitor H-89 does not either block forskolin-induced Rap1 activation or affect Rap1 membrane binding (45). Recently, a mass spectroscopic study has shown that when PKA phosphorylates Rap1b on Ser^{179}, it produces a conformational switch in active Rap1, which affects allosterically the effector domain (15). Active Rap1 activates multiple downstream effectors including RapL, RalGDS, Afadin, Arap3, Nore1B, RIAM, Raf-1,

and B-Raf (42). *RapL* is a major effector of Rap1 in immune cells (23). *RIAM* (Rap1-GTP-interacting adaptor molecule) links Rap1 to integrin activation and interacts with profilin and Ena/VASP proteins which regulate actin dynamics (30). In some cells, Rap1 is activated by C3G and stimulates ERK pathway via interaction with *B-Raf* (58).

Several actin modulating proteins have been directly regulated by Rap1 (40). Recently, *RA-RhoGAP*, an inhibitor of RhoA activation, has demonstrated to be a Rap1 effector (1).

Rap1 interacts with other small GTP-binding proteins including Rac, which is involved in the regulation of actin cytoskeleton dynamics (36). In certain cell types upon Rap1 activation, Rac1 is activated contributing to cell movement and spreading (2, 50). In MCF-7 human breast epithelial cells IQGAP, an effector of Rac1 (29), has mostly interacted with the active form of Rap1 and reduced Rap1 activation induced by cAMP, resulting in a decrease in cell adhesion (20). In PC12 cells, active Ras regulates Epac2 function and thereby Epac2-mediated activation of Rap1 (34). In addition to its role as Rap1GEF, Epac2 has been shown to exert physiological function in pancreatic β cells, where participates in cAMP-regulated insulin granule exocytosis (16, 22).

2. Specific function in the pancreas

In mouse pancreatic acini Rap1A and Rap1B have been identified by RT-PCR. Moreover, western-blotting demonstrates the presence of Rap1 protein but the antibody is not able to distinguish between both isoforms (45). Rap1 is activated following stimulation of acini with CCK, carbachol, and VIP as shown using a pull-down assay (45). Several second messengers are able to activate Rap1; calcium ionophore A-23187, phorbol ester, forskolin, 8-bromo-cAMP, and the Epac-selective cAMP analog 8-pCPT-2'-*O*-Me-cAMP all induce an increase in GTP-Rap1 levels. Using RT-PCR two Rap1GEFs have been found in mouse pancreatic acini: Epac1 and CalDAG-GEF III. The presence of other Rap1GEFs such as C3G and PDZ-GEF has not been evaluated. Epac1 protein is present in the zymogen granule area likely associated with Rap1 as shown using western-blotting and immunocytochemistry. Rap1 is present on zymogen granule membranes, as shown by mass spectrometry, immunohistochemistry and Western-blotting (8). Immnunohistochemistry localization in mouse and rat acini is shown in Fig. 1 and Fig. 2.

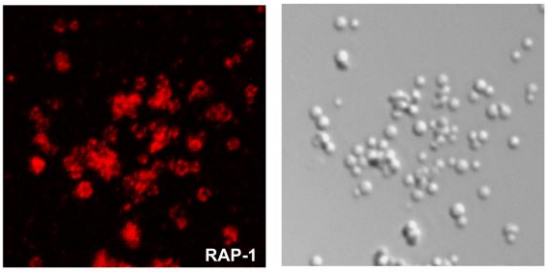

Figure 1. Immunostaining of purified isolated rat zymogen granules for Rap1. Zymogen granules were purified using Percoll gradient procedure and allowed to attach to a glass slide. Rap1 localization was demonstrated by confocal microscopy using rabbit anti-Rap1 antibody from Santa Cruz *(red)*. Zymogen granule fluorescence image is paired with the corresponding Nomarski image (Reproduced from Reference 8).

With respect to its role in pancreatic exocrine function, Rap1 has been reported to be required for pancreatic amylase secretion (45). The overexpression of Rap1GAP, a protein involved in the inhibition of Rap1 activation, decreases pancreatic amylase secretion induced by carbachol and cholecystokinin (CCK) as well as stimulators of cAMP pathway (45). A proposed model for Rap1-mediated response on pancreatic amylase secretion is shown in Fig. 3. Rap1 activation is not required for CCK-induced calcium mobilization since the expression of Rap1GAP in pancreatic acini does not affect the response to CCK (45). The participation of Rap1 in exocrine secretion has also been shown in other tissues and cell types such as parotid glands and acrosomes. Rap1 is present in secretory granules of parotid glands (11, 21, 48). It translocates from

the membrane to the cytosol upon stimulation with the β-adrenergic agonist isoproterenol, and this event occurs in parallel to an increase in amylase secretion (12). In the acrosome, unlike in Although in some tissues, Rap1 translocation from the membrane to the cytosol is correlated to Rap1 activation, in mouse pancreatic acini Rap1 does not translocate upon stimulation with pancreatic acini, Rap1, which is activated by Epac1, induces intracellular calcium mobilization to achieve exocytosis (7).

different secretagogues (45).

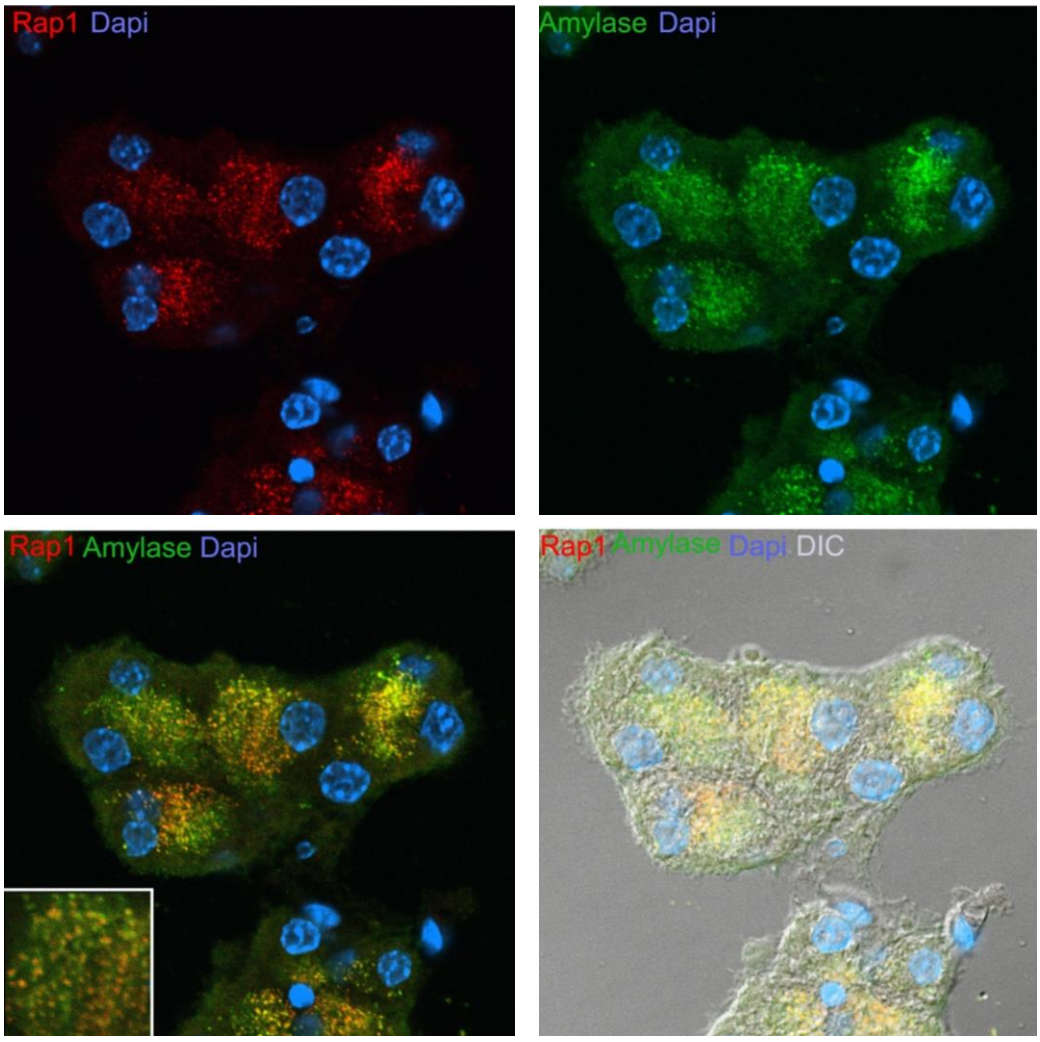

Figure 2. Confocal immunostaining of isolated mouse pancreatic acini for Rap1. Isolated mouse pancreatic acini were prepared and fixed with paraformaldehyde. Cryostat sections were mounted on SuperFrost Plus slides (Fischer). The sections were incubated with the following primary antibodies: polyclonal rabbit anti-Rap1 (Santa Cruz Biotechnology) (1:200) *(red),* polyclonal sheep anti-human salivary amylase (U.S. Biological) (1:100 to 1:200) *(green)*. The sections were then incubated with secondary antibodies Cy3-conjugated donkey anti-rabbit IgG (1:200) or fluorescein-conjugated anti-sheep IgG (1;200) (Jackson Immunoresearch Laboratories, Inc). Prolong Gold with 4,6-diamidino-2-phenylindole (DAPI) (*blue*) was added to mounting medium to counterstain nuclei. Digitized images were collected with an Olympus Fluoview 500 confocal microscope. Rap1 is localized on zymogen granules in close proximity to amylase. The figure was kindly provided by Dr. Stephen Ernst, The University of Michigan.

While Rap1 has been involved in ERK activation in diverse cells as previously mention, in mouse pancreatic acini CCK-induced ERK phosphorylation (13) has not been affected by Rap1 activation since the expression of Rap1GAP does not modify the response to CCK (unpublished data).
Recently, a study has shown that Rap1, which is activated by PKCα, mediates integrin-induced aggregation in platelets (17). In pancreatic acini high concentrations CCK induces acini aggregation, an effect which seems to be mediated by PKC (unpublished data). Although CCK induces Rap1 activation in mouse pancreatic acini, the participation of Rap1 in aggregation is unlikely since the expression of Rap1GAP does not affect the induction of aggregation by CCK (unpublished data).
To date, the effectors involved in the pancreatic exocytotic response to Rap1, as well as the ability of Rap1 to interact with other small G proteins have not been studied.

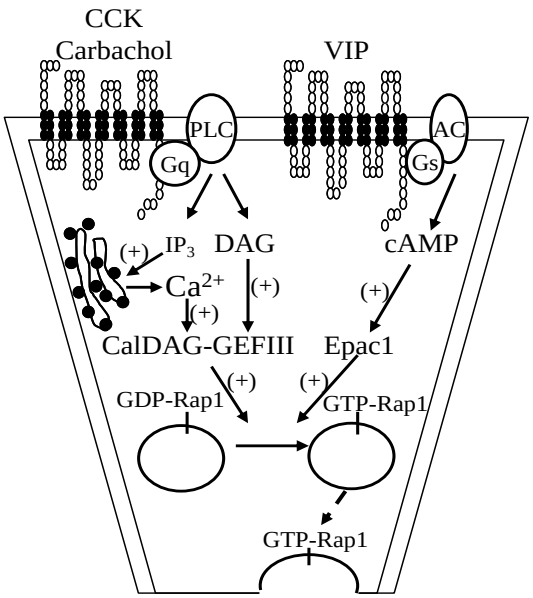

Figure 3. Activation of Rap1 after the stimulation by secretagogues CCK, carbachol and VIP in mouse pancreatic acini. Different second messengers including DAG, calcium and cAMP generated from the activation of CCK, muscarinic and VPAC receptors are able to induce Rap1 activation via either CalDAG-GEF or Epac1, and thereby regulate amylase secretion in mouse pancreatic acinar cells (From Reference 45).

3. Tools for study of Rap1

a. cDNA

cDNA clones for human wild-type, constitutively active and dominant negative Rap1A in pcDNA 3.1 are available from UMR cDNA Resource center, Missouri University of Science and Technology (www.cdna.org). A plasmid coding for GST-RalGDS-Rap1 binding domain (RBD) is available from this lab.

b. Antibodies

Rabit polyclonal antibody raised against a peptide mapping near the C-terminus of Rap1 of human origin from Santa Cruz Biotechnology (# sc-65) has been used to identify Rap1 by western-blotting and immunohistochemistry in pancreatic acini (8, 45). We have also successfully used a mouse monoclonal Rap1 antibody from BD Transduction Laboratories (# 610195).
Specific antibodies against Rap1a and Rap1b have been generated by immunization of rabbits with peptides derived from the C-terminus of the Rap1a and Rap1b proteins (28), though they have not been used by us on pancreas.
There are several antibodies raised against Rap1GEFs and Rap1GAP as well as Rap1 effectors available: rabbit polyclonal Rap1GAP antibody from Santa Cruz Biotechnology (# sc-28189), polyclonal C3G and PDZ-GEFs antibodies from Bethyl Laboratories. These two last antibodies have not been checked by us on pancreas. Polyclonal Epac antibodies are available which are discussed under that molecule.

c. Viruses

Sindbis virus coding for constitutively active Rap1B (V12) and dominant negative Rap1B (N17) have been described (3). An adenovirus expressing Rap1GAP has been prepared by Dr. Patrick Casey of Duke University (54) and is available from us with permission of Dr. Casey.

d. Mice Models

The followed knock-out and transgenic mice have been developed:

- Conditional knockout of *rap1a/rap1b* in forebrain (39).
- transgenic mice expressing active Rap1A within the T cell lineage (47).
- Rap1a and Rap1b null mice (33, 57). Some Rap1a -/- embryos have died in utero (33). Rap1b -/- mice have shown an increase in embryonic lethality and evidence of prolonged bleeding time and hemorrhage in liver, brain, and abdominal cavity (9).

e) Assay of the active state of Rap1 has been carried out by use of a pull-down assay using GST-RalGDS (45). We carry out this assay using GST-RalGDS prepared in our lab (see Methods). A commercial kit, StressXpress Rap1 activation kit, is now available from Stressgen Bioreagents (# EKS-455).

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