Cannabinoid Receptor-induced Neurite Outgrowth Is Mediated by Rap1 Activation through Ga<sub>o/i</sub>-triggered Proteasomal Degradation of Rap1GAPII*

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The Ga<sub>o/i</sub>-coupled CB1 cannabinoid receptor induces neurite outgrowth in Neuro-2A cells. The mechanisms of signaling through Ga<sub>o/i</sub> to induce neurite outgrowth were studied. The expression of Ga<sub>o/i</sub> reduces the stability of its direct interactor protein, Rap1GAPII, by targeting it for ubiquitination and proteasomal degradation. This results in the activation of Rap1. Ga<sub>o/i</sub>-induced activation of endogenous Rap1 in Neuro-2A cells is blocked by the proteasomal inhibitor lactacystin. Ga<sub>o/i</sub> stimulates neurite outgrowth that is blocked by the expression of dominant negative Rap1. Expression of Rap1GAPII also blocks the Ga<sub>o/i</sub>-induced neurite outgrowth and treatment with proteasomal inhibitors potentiates this inhibition. The endogenous Ga<sub>o/i</sub>-coupled cannabinoid (CB1) receptor in Neuro-2A cells stimulates the degradation of Rap1GAPII; activation of Rap1 and treatment with pertussis toxin or lactacystin blocks these effects. The CB1 receptor-stimulated neurite outgrowth is blocked by treatment with pertussis toxin, small interfering RNA for Rap, lactacystin, and expression of Rap1GAPII. Thus, the Ga<sub>o/i</sub>-coupled cannabinoid receptor, by regulating the proteasomal degradation of Rap1GAPII, activates Rap1 to induce neurite outgrowth.

The differentiation process in neurons is a complex phenomenon involving multiple changes. These include both changes in electrophysiological characteristics as well changes in morphology characterized by dendritic and axonal outgrowths. A general term “neurite” is used to define these outgrowths which are morphological characteristics of the neuronal differentiation process. Regulation of neurite outgrowth is tightly controlled and many neurotransmitters are induced in this process. Neurite outgrowth in cortical neurons is regulated by D2 dopamine receptors (1). Serotonin 1B receptors are known to enhance neurite outgrowth in thalamic neurons of rodents (2).

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Growth cones from rat cerebellar neurons are guided by the chemoattractant SDF-1, and Xenopus neuron outgrowth is guided by the GABA-B receptors (3). A common feature of all these receptors is that they couple through the Ga<sub>o/i</sub> pathway. It has been known for over a decade that Ga<sub>o/i</sub> can induce neurite outgrowth (4) and that Ga<sub>o/i</sub> is one of the more abundant proteins present in the neuronal growth cones (5). The mechanisms by which the Ga<sub>o/i</sub> signals are transduced have never been clarified. We and others (6, 7) have found that Ga<sub>o</sub> interacts with RapGap and this results in the activation of Rap. The mechanisms by which Ga<sub>o</sub> activates Rap and the consequences of this activation are unknown. To determine both the biological consequences of activation of Ga<sub>o</sub>/Rap pathway and the mechanisms by which this occurs we searched for a cellular system where we could study the biochemical mechanisms within the context of the biological effects. Here we describe how the endogenous cannabinoid receptors in Neuro-2A cells use the Ga<sub>o</sub> pathway to induce neurite outgrowth.

EXPERIMENTAL PROCEDURES

Cell Culture—COS-7 and HEK-293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and Neuro-2A cells were maintained in 44% Dulbecco’s modified Eagle’s medium, 44% F12 media and 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% glucose. FuGENE 6 (Roche Applied Science) was used for the introduction of exogenous DNA into COS-7 cells according to the manufacturer’s instructions. Briefly 100,000 cells were plated per well in a 6-well plate, and 24 h later each well was transfected with 2 μg DNA and 6 μl of FuGENE. For HEK-293T cells, 2 × 10<sup>6</sup> cells were plated on 60-mm dishes and 24 h later transfected with 4 μg of DNA using Lipofectamine 2000® (10 μl). For imaging experiments Neuro-2A cells (5 × 10<sup>4</sup>) were plated in 35-mm Matttek® plates and were transfected using Lipofectamine 2000® the following day. Neuro-2A cells were obtained from ATCC (catalog number CC-131).

Commmunoprecipitation—293T and COS-7 cells were transfected with the indicated constructs using Lipofectamine 2000 (Invitrogen) and FuGENE 6 (Roche Applied Science), respectively. 293T cells were harvested 48 h posttransfection, and immunoprecipitations were done as described previously (6). Samples were separated by SDS-PAGE and immunoblotted with a Ga<sub>o</sub>-specific antibody (Santa Cruz Biotechnology).

GST Pull-downs—Human Rap1GAPI and Rap1GAPII were cloned by PCR from Jurkat cells and the N terminus of each was subcloned into the pGEX4T2 expression vector (Amersham Biosciences). Bacterially expressed purified protein was bound to glutathione-Sepharose and incubated with 293T cell lysates expressing Ga<sub>o</sub> or Ga<sub>o</sub> subunits followed by SDS-PAGE and immunoblotting with Ga<sub>o</sub> or Ga<sub>o</sub> antibodies (Santa Cruz Biotechnology).

Real-time RT<sup>-</sup>-PCR—Total RNA was isolated from cells using TRIzol reagent (Invitrogen), and real-time RT-PCR was performed on a Ligh...
tyracyl (Roche Applied Science) using the QuantiTeT SYBR Green RT-PCR system (Qiagen) according to manufacturer's instructions. The primers used were as follows: Rap1GAPI, 5′-ATTTACTCTACGTGAGCTGCTG-3′ (forward) and 5′-GCCAAAATCTGTTGTTTG-3′ (reverse); RGS1, 5′-ATGGCGCTGAGGACGGCTG-3′ (forward) and 5′-GAAGCTCGGCTCATCA-3′ (reverse); actin, 5′-GGGACGCTGCTATTGGCC-3′ (forward) and 3′-ACCCACGCTGTTGCAATCA-3′ (reverse). Each reaction contained a 1 μM concentration of each gene-specific primer, reaction mix, and 50 ng of total RNA. The annealing temperature was 55 °C and the extension time was 15 s. Data were analyzed using Lightcycler data analysis software. Crossing points were calculated using the second derivative maximum method. All reactions were done in triplicate and data for Rap1GAPII were normalized against actin RNA levels for each sample.

Ubiquitination Assays—HA-tagged ubiquitin vectors were used to follow the ubiquitination of proteins. COS-7 cells were cotransfected with the indicated plasmid and after 48 h harvested. HepG2 cells were extracted in bacterial lysis buffer containing DTT and protease inhibitors, vector. Rap1GDS-RBD was expressed as a GST fusion protein in bacteria, bacterial expressed GST-RalGDS-RBD fusion protein pre-bound to glutathione beads to detect active GTP-bound form of Rap1. RalGDS-RBD (RalGDS-Rap1 binding domain) assay was performed using the addition of 50 μM of protein-G-agarose. Cell lysates were centrifuged, and RalGDS-RBD was immunoprecipitated with 5 μg of antibody overnight followed by the addition of 15 μg of protein-G-agarose for 4 h. Samples were separated by SDS-PAGE and immunoblotted with an anti-HA antibody (Santa Cruz Biotechnology).

Antibodies—Antibodies specific for Rap1GAPIII and Rap1GAPII were made by immunizing rabbits with a keyhole limpet hemocyanin-conjugated peptide specific for each protein (Sigma-Genosys). The sequence of the immunizing peptide was Rap1GAPII (forward) GPRPRRGSLPA-C. Antibodies were affinity-purified on peptide-conjugated peptide specific for each protein (Sigma-Genosys). The sequence of the immunizing peptide was Rap1GAPII (forward) TARYRHKFLGKHE-C and for Rap1GAPII was N-MAQLRPLVP-PGPRGSLPA-C. Antibodies were affinity-purified on peptide-conjugated columns (Pierce).

Rap Activation Assays—Levels of activated Rap were measured by use of the RapGDS binding domain as described by Carey and Stork (8). The RapGDS-BRD (RALGDS-Rap1 binding domain) assay was performed using bacterial expressed GST-RalGDS-RBD fusion protein pre-bound to glutathione beads to detect active GTP-bound form of Rap1. RapGDS-BRD domain was PCR cloned from Jurkat cDNA and ligated into the pGEX-4T2 vector. RapGDS-BRD was expressed as a GST fusion protein in bacteria, extracted in bacterial lysis buffer containing DTT and protease inhibitors, and then incubated with glutathione beads. RapGDS-BRD-bound beads were incubated with total cell lysates in presence of DTT and protease inhibitors overnight and the immunoprecipitate was tested for the presence of Gαi and Rap1GAP. The FLAG-M2 antibody was used to isolate Gαi from immunoprecipitates (Santa Cruz Biotechnology).

Imaging—Six hours after transfection Neuro-2A cells were serum-starved for 1 h and then transfected with plasmids in order to inhibit proteasomal activity, cells were incubated with 20 μM lactacystin (Sigma, catalog number L-6785) for 2 h prior to imaging. Living cells were imaged with a Zeiss 510 confocal laser-scanning microscope. Multiple (3–5) 0.45-μm Z section slices were obtained for each condition. Representative images are shown. Statistical validation of the image shown is included in the figure legend to Fig. 4. TIFF images were processed with Adobe Photoshop.

Neurite Outgrowth Assays—Neuro-2A cells were plated in 6-well dishes. For dominant negative experiments, cells were cotransfected with dominant negative mutant of Rap1 or control bicistronic vector, IRES (Clontech BD Sciences) containing GFP or GFP-Gαo. For siRNA experiments, HPP-grade siRNA for Rap1 (DNA target sequence AAGCAATGGAGTAGATTGGC with dTdToverhangs) was synthesized by Qiagen. Cells were transfected with siRNA for Rap1 or control oligonucleotides for 4 h with Qiagen TransMessenger transfection reagent. Four hours after adding siRNA, cells were washed with 1× PBS and then transfected with bicistronic vector containing GFP or GFP-Gαo. Twenty-four hours posttransfection GFP-positive cells were counted for neurite outgrowth. The cells displaying neurite outgrowth were those that had cellular projections of length two times greater than the cell diameter. In some experiments randomly chosen regions of the plate containing 100 cells were scored under a phase contrast microscope (Nikon TMS). All assays were done in triplicate and in two or three independent experiments, and the indicated values are represented as mean ± S.E.

For studies examining the effect of agonist, pertussis toxin, or lactacystin, ~1–5 × 10⁶ cells were plated in 6-well plates and treated with 15 ng/ml pertussis toxin for 16 h or with 20 μM lactacystin (proteasomal inhibitor) or 10 mM SR141716A (CB1 receptor antagonist) for 2 h followed by treatment with 10 μM HU-210 for 16 h. Neurite outgrowth was quantitated as described above. All assays were done in triplicate and data represented as mean ± S.E.

RESULTS

Cannabinoid CB1 Receptor-induced Neurite Outgrowth in Neuro-2A Cells through Gαi/o and Rap—The mouse neuroblastoma cell Neuro-2A can be induced to differentiate with outgrowth of neurites under a variety of stimuli including retinoic acid, the ganglioside GM1, low serum (9), or inhibition of protein kinase C (10). We initially screened the Neuro-2A cells for endogenous Gαi/o-coupled receptors and found that these cells express the cannabinoid CB1 receptor. Treatment of serum-starved Neuro-2A cells with the CB1 receptor agonist HU-210 resulted in a significant increase in neurite outgrowth in a concentration dependent manner. Addition of the antagonist SR 141716A inhibited CB1 receptor agonist-induced neurite outgrowth (Fig. 1A). To establish that the CB1 receptor effects were due to coupling to a Gαi/o pathway we treated the cells with pertussis toxin, a blocker of signaling through this pathway, and tested for the effects of the CB1 receptor agonist. Pretreatment with pertussis toxin fully blocked the CB1 receptor-induced neurite outgrowth (Fig. 1B). Micrographs of control cells and cells with HU-210-induced neurites are shown in Fig. 1, C and D. Since the effect of pertussis toxin indicates that the Gαi/o pathway was involved, we tested whether transfection with Gαi/o-induced neurite outgrowth in Neuro-2A cells. In addition, Gαi/o activates Rap (6, 7), we also tested whether the Gαi/o effect was blocked by dominant negative Rap (Rap1 DN). As shown in the experiment in Fig. 1E, Gαi/o induced neurite outgrowth, and this was blocked by cotransfection with Rap1-DN. Gαi/o interacts with Rap1GAPII and Rap1GAPII preferentially bound Gαi/o. Initially, we had cloned Rap1GAP from a chick dorsal root ganglion library that had been used for the yeast two-hybrid screen. Subsequently we cloned human Rap1GAP from a chick dorsal root ganglion library that had been used for the yeast two-hybrid screen. Initially, we had cloned Rap1GAP from a chick dorsal root ganglion library that had been used for the yeast two-hybrid screen. Subsequently we cloned human Rap1GAP from a chick dorsal root ganglion library that had been used for the yeast two-hybrid screen. Subsequently we cloned human Rap1GAP from a chick dorsal root ganglion library that had been used for the yeast two-hybrid screen. Subsequently we cloned human Rap1GAP from a chick dorsal root ganglion library that had been used for the yeast two-hybrid screen.
themselves, both CFP-Rap1GAPI and CFP-Rap1GAPII showed diffuse cytosolic fluorescence with negatively imaged organelles (Fig. 2C, panels i and iii). Expression of Gαo led to the selective accumulation of CFP-tagged Rap1GAPII to perinuclear vesicles (please compare Fig. 2C, panel ii versus panel iv) indicating a functional effect of Gαo on Rap1GAPII. However, we were unable to detect any measurable FRET between YFP-tagged Gαo and CFP-tagged Rap1GAPII (data not shown).

Gαo Degradation of Rap1GAPII Activates Rap and Induces Neurite Outgrowth—During our coexpression studies when we immunoblotted for both Gαo and Rap1GAPII we consistently found that expression of Gαo appeared to reduce the levels of Rap1GAPII proteins. We had found this to be true in both 293T cells and COS-7 cells. To explore the reasons for this we expressed Rap1GAPI, Rap1GAPII, and the N-terminal truncated RapGAP in control, WT Gαo, and activated Gαo expressing cells. We found that coexpression of Gαo and Rap1GAPII resulted in a significant decrease in the levels of Rap1GAPII (Fig. 3A). The decrease in levels of Rap1GAPII appears to be specific for members of the Gαo family, since coexpression of Gαq or Gαi did not affect the levels of Rap1GAPII (Fig. 3B). Under conditions where the Rap1GAPII protein levels were decreased, we did not find any changes in the Rap1GAPII mRNA levels as assayed by real-time PCR (Fig. 3C). Treatment of Rap1GAPII-expressing cells with inhibitors of various proteases indicated that the proteasomal inhibitors (MG-132, lactacystin, and PSI) blocked the disappearance of Rap1GAPII indicating that decrease in Rap1GAPII protein levels is due to proteasomal degradation (Fig. 3D). Such proteasomal degradation of RapGAP has been observed in thyroid cells (13). Since RapGAP may be ubiquitinated for targeting for proteasomal degradation, and since ubiquitination is often a regulated process, we determined if the Rap1GAPII ubiquitination was affected by Gαo. For this we expressed FLAG-tagged Rap1GAPIII and HA-tagged ubiquitin in the absence and presence of Gαo. The presence of Gαo increased the ubiquitination of Rap1GAPII (Fig. 3E). Although Rap1GAPIII

FIG. 1. CB1 receptor induces neurite outgrowth in Neuro-2A cells. A, Neuro-2A cells were serum-starved and treated with the indicated concentrations of the CB1 receptor agonist (CB1R-Ag, HU-210 (Hu)) with or without excess (10 μM) antagonist SR141716A. Cells with neurites at least twice the diameter of the cell body were scored 16 h later. B, Neuro-2A cells were treated overnight with pertussis toxin (PT) (15 ng/ml) before the addition of CB1 receptor agonist. Neurite outgrowth was scored 16 h later (C and D). Representative confocal pictures of control and HU-210-treated Neuro2A cells are shown. Bars represent 10 μm. E, Neuro-2A cells transfected with control or dominant negative Rap1 (Rap1-DN) with and without Gαo were scored for neurite outgrowth. For A, B, and E values are mean ± S.E. of triplicate determinations.
G\textsubscript{onq}-induced Proteasomal Degradation of Rap1GAPII

is ubiquitinated, the extent of ubiquitination appears to be far less than that of a well known ubiquitinated protein, Traf2 (Fig. 3F). Taken together these data indicate that G\textsubscript{onq} promotes the ubiquitination of Rap1GAPII and thus targets it for proteasomal degradation.

These experiments raised two questions: can the effect of G\textsubscript{onq} be observed on native Rap1GAPII, and does the degradation of Rap1GAPII stimulate Rap activity? To address these issues we transfected G\textsubscript{onq} into Neuro-2A cells and tested for its effects on the levels of Rap1GAPII and on the activation state of Rap in the presence and absence of proteasomal inhibitors. When G\textsubscript{onq} was expressed in Neuro-2A cells, there was a decrease in the endogenous level of Rap1GAPII, and this decrease could be blocked by treating the cells with MG132 (Fig. 4A). We also determined the activation state of Rap in cells transfected with G\textsubscript{onq}, in the presence and absence of proteasomal inhibitor. G\textsubscript{onq} stimulated the activation of Rap-1, and this effect was blocked by treating the cell with a proteasomal inhibitor (Fig. 4B).

Next, we determined whether we could observe the proteasome sensitivity of G\textsubscript{onq} activation of Rap in live cells. For this, cells were transfected with GFP-tagged Rap1GAPII and have been previously used to measure Rap activation in intact cells (14). Neuro-2A cells were transfected with the fluorescent probe without or with G\textsubscript{onq} or as control) and then treated without or with lactacystin. The cells were imaged by confocal microscopy. The expression of G\textsubscript{onq}, but not G\textsubscript{on}, led to a strong accumulation of fluorescence in the perinuclear vesicles (Fig. 4C, panel ii versus panel iv). This indicates that Rap1 is being activated in these vesicles. When the cells were treated with the proteasomal inhibitor lactacystin, the G\textsubscript{onq}-induced accumulation of fluorescence was not observed (Fig. 4C, panel ii versus panel v) indicating that Rap1 activation by G\textsubscript{onq} is dependent on proteasomal activity in live cells. These results indicate that in Neuro-2A cells G\textsubscript{onq} induces activation of Rap through the degradation of Rap1GAPII. If this is true, and since Rap is downstream of G\textsubscript{onq} (Fig. 1D) in the induction of neurite outgrowth, expression of excess Rap1GAPII in the presence of a proteasomal inhibitor should block G\textsubscript{onq} induced neurite outgrowth. To test this prediction we expressed G\textsubscript{onq} in the presence of submaximal concentrations of Rap1GAPII and Rap1GAPII. This resulted in about a 50% inhibition of G\textsubscript{onq} induced neurite outgrowth. This would be expected since both RapGAPs should effectively reduce the activation of Rap1. However, the experiments in Figs. 1–3 predict that inhibiting the proteasome should selectively enhance the effect of Rap1GAPII. When the Rap1GAPII expressing cells were treated with MG-132 no further significant inhibition of neurite outgrowth was observed. However, when Rap1GAPII-expressing cells were treated with MG-132, there was a marked increase in the inhibition of neurite outgrowth with a close to complete blockade of G\textsubscript{onq}-induced neurite outgrowth (Fig. 4D).

Taken together the experiments in Fig. 4 indicate that G\textsubscript{onq} by inducing the degradation of Rap1GAPII activates Rap to induce neurite outgrowth.

Cannabinoid receptor activity, through the G\textsubscript{onq} pathway, stimulates the degradation of Rap1GAPII, the activation of Rap, and neurite outgrowth.

We next determined whether the G\textsubscript{onq}-Rap1GAPII-Rap1 pathway for the regulation of neurite outgrowth could be regulated in a receptor-dependent manner. We also tested whether the pathway and the biological effects could be observed in a completely native setting. Treatment of serum-starved Neuro-2A cells with the CB1 receptor agonist resulted in a time-dependent activation of Rap1. Activation is substantial (~2-fold) within 1 h and maximal by 2 h. If the ligand is present, the activation persists for 16 h (Fig. 5A). These results suggest that the CB1 receptor can activate Rap relatively fast. Since the CB1 receptor is known to couple through the G\textsubscript{onq} pathway, we tested whether receptor stimulation of Rap1 activation is blocked when the cells are treated with pertussis toxin. The CB1 receptor agonist did not stimulate Rap1 activation or the degradation of Rap1GAPII in cells treated with pertussis toxin (Fig. 5B, left panels). Since the coupling between G\textsubscript{onq} and Rap is due to the degradation of Rap1GAPII we...
tested whether proteasomal inhibitor treatment blocked receptor activation of Rap. When CB1 receptor agonist was added to lactacystin-treated cells, the degradation of Rap1GAPII and activation of Rap was blocked. (Fig. 5B, right panels). We next tested the role of Rap in CB1 receptor-induced neurite outgrowth. For this, cells were transfected with Rap1 specific siRNA or control siRNA. The siRNA for Rap1 inhibited receptor regulated neurite outgrowth (Fig. 5C). Since both Goαi and Rap are downstream of the CB1 receptor for neurite outgrowth, and given the connectivity between Goαi and Rap1, both proteasomal inhibitors and Rap1GAPII would be predicted to block CB1-receptor induced neurite outgrowth. The experi-

Fig. 3. Goαi-induced proteasomal degradation of Rap1GAPII. A, COS-7 cells were cotransfected with the indicated plasmids, and total cell lysates were immunoblotted with Rap1GAPII (top panel), Goαi (middle panel), or actin (bottom panel) antibodies. B, COS-7 cells were cotransfected with a Rap1GAPII expression plasmid, pEGFP-C1, and various Go subunit plasmids. Total cell lysates were immunoblotted with Rap1GAPII (top panel) and EGFP (bottom panel) antibodies. C, COS-7 cells were cotransfected with the indicated plasmids (Goαi and/or Rap1GAPII) in duplicate. One set was harvested for total cellular protein while the other was harvested for total RNA. Total cell lysates were immunoblotted with antibodies for Rap1GAPII (top panel), Goαi (middle panel), or actin (bottom panel). The higher molecular weight band seen in all lanes in the Goαi immunoblot is a cross-reactive protein. Real-time RT-PCR was performed on total RNA isolated from each condition. Rap1GAPII mRNA levels were normalized to actin mRNA levels for each sample. The relative RNA level for Rap1GAPII is shown. D, COS-7 cells were transfected with vector only (lane 1, negative control), a Rap1GAPII expression plasmid only (lane 2), or co-transfected with Rap1GAPII and Goαi expression vectors (lanes 3–10). Thirty-six hours after transfection cells were incubated with dimethyl sulfoxide (DMSO) (lanes 2 and 3) or with the following inhibitors for 12 h: MG132 (10 μM), lactacystin (10 μM), PSI (10 μM), ALLM (25 μM), E64d (25 μM), monensin (10 μM), or ammonium chloride (10 mM). Total cell lysates were harvested and immunoblotted with antibodies for Rap1GAPII (top panel) or Goαi and actin (bottom panel). E, COS-7 cells were cotransfected with the indicated expression plasmids, and 40 h later cells were treated with dimethyl sulfoxide or MG132 (10 μM) for 6 h. Total cell lysates were immunoprecipitated with M2-FLAG antibody followed by immunoblotting with an anti-HA antibody to detect ubiquitin conjugates as indicated (top panel). Total cell lysates were immunoprecipitated with an antibody to Goαi (bottom panel). WB, Western blot. F, 293T cells were cotransfected with the indicated expression plasmids. Both Rap1GAPII and Traf were individually tagged with the FLAG epitope. The indicated concentrations of Rap1GAPII vector and 1 μg of Traf vector were used. 40 h later cells were treated with MG132 (10 μM) for 6 h. Total cell lysates were immunoprecipitated (IP) with M2-FLAG antibody followed by immunoblotting with an anti-HA antibody to detect ubiquitin conjugates, which are indicated (top panel). Total cell lysates were immunoprecipitated with an antibody to Goαi (bottom panel). WB, Western blot.
ments in Fig. 5, D and E, indicate that as predicted, treatment of cells with lactacystin or transfection with excess Rap1GAPII blocks receptor induced neurite outgrowth. These results indicate that in a fully native system the CB1 receptor through Gαo promotes the degradation of Rap1GAPII to activate Rap and induce neurite outgrowth.

**DISCUSSION**

Typically, signaling through heterotrimeric G protein pathways involves information transfer through non-covalent protein-protein interactions or through protein phosphorylation/dephosphorylation reactions. Such regulation is often sufficient for acute signal transmission and for short term integration between signaling pathways. However, these mechanisms do not readily allow for a signaling network to propagate or integrate signals across time scales. The mechanism that we describe here allows for such capability. The regulated degradation of RapGAP occurs in a time scale of 1–2 h in two native systems (Neuro-2A cells and rat hippocampal slices).

\(^2\) J. D. Jordan and R. Iyengar, unpublished observations.

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**Fig. 4.** Gαo activates Rap1 in a proteasome-dependent manner and induces neurite outgrowth though Rap1GAPII. A. Neuro-2A cells were transfected with WT Gαo or control vector. MG132 (10 μM) or dimethyl sulfoxide was added to the cells and incubated for 4 h. The cells were lysed, and the lysates were subjected to immunoblot analysis using antibodies for Rap1GAPII, Gαo, and β-actin. B. Neuro-2A cells transfected with or without Gαo and treated with or without MG-132 were harvested, and GST-RalGDS-RBD pull-down assays were performed. GTP-bound Rap1 was detected by immunoblotting with a Rap-1-specific antibody. Total cell lysates were immunoblotted with a Rap1-specific antibody and Gαo-specific antibody (Sigma). C. Gαo activates Rap1 on perinuclear vesicles in living cells. Neuro-2A cells were cotransfected with GFP-RalGDS-RBD and either vector (panel i), Gαo (panels ii and iii), or Gαq (panel iv). Cells were treated with either vehicle (panels i, ii, and iv) or 20 μM lactacystin (panels iii and v) as indicated and imaged by laser scanning microscopy. The accumulation of fluorescence in the second panel (panel ii) is indicated by arrows. Scale bars indicate 10 μm. To obtain a quantitative estimate of the number of cells that displayed Gαo-induced activation of Rap1, we counted fields of 50 cells for perinuclear accumulation of fluorescence in three independent experiments. For control (reporter transfected) 7/151 showed Rap1 activation; for Gαo transfected 52/152, and for Gαq transfected and treated with lactacystin 17/152. Using one-tail Student’s t test we found that the differences between control cells and cells transfected with Gαo as well as between Gαo transfected cells treated with and without lactacystin were highly significant (p < 0.001). D. Neuro-2A cells expressing the indicated constructs (Gαo, Rap1GAPI, or Rap1GAPII) were treated with 0.1% dimethyl sulfoxide (gray) or 2 μM MG132 in 0.1% dimethyl sulfoxide (black) for 16 h prior to counting. The dashed line represents the basal neurite outgrowth. Values are mean ± S.E. (n = 3).
heterologous sensitization. Signal flow, through both heterotrimeric and small G protein pathways, is regulated by the activity of the corresponding GAP proteins. From a systems design perspective it would be advantageous to regulate the levels of these crucial regulators and thus set a threshold for signal flow through these pathways. The Gαo/i-mediated targeting of Rap1GAPII for proteasomal degradation indicates that such a mechanism, for signal flow as well as integration through threshold control, exists. Thus, a relatively brief activation of the Gαo/i-coupled receptor and the consequent activation of the heterotrimeric G protein Gα subunit could result in a prolonged activation of the small GTPase downstream of the heterotrimeric G protein. Duration and magnitude of activation of the small G protein often play a defining role in the conversion of biochemical signals into biological effects. A classical example is the persistent activation of Ras and the triggering of proliferation. Here it appears that Rap1 plays a crucial role in the Gαo/i pathway-induced neurite outgrowth. The mechanism that we elucidate in this study would allow Gαo/i to activate Rap1 for prolonged periods of time. These periods would be largely defined by the rates of degradation and synthesis of Rap1GAPII in cells where this isoform predominates, as is the case with Neuro-2A cells. Thus we would like to propose that signal dependent targeting of regulators for degradation by the proteasome may be one mechanism by which short duration signals in G protein pathways may be converted to long duration downstream signals.

Signal transmission through regulation of GAP activity assumes the presence of a constitutively active guanine nucleotide exchange factor that would turn on the small GTPase. In

FIG. 5. CB1-receptor triggers neurite outgrowth in Neuro-2A cells through Rap1 by stimulating Rap1GAPII degradation. Neuro-2A cells grown to 50% confluence were starved overnight in serum-free medium. A, cells were stimulated with the CB1 receptor agonist, HU-210 (CB1R-Ag), for the indicated time periods. Cells were harvested in lysis buffer containing multiple protease inhibitors. Rap1GTP was pulled down using Rap-RBD and immunoblotted using polyclonal anti-Rap1 antibody. Cell lysates were also used for determination of total Rap1 by immunoblotting. B, Neuro-2A cells were pretreated either with pertussis toxin (PT) at 15 ng/ml overnight or lactacystin (Lact) at 20 μM for 2 h, and then cells were stimulated with CB1R-Ag for 2 h. Cells were harvested in lysis buffer containing protease inhibitors. Rap-1-GTP was pulled down using Rap-RBD and immunoblotted using polyclonal anti-Rap1 antibody. Cell lysates were also used for determination of total Rap1 by immunoblotting. RapGAPII was first immunoprecipitated with rabbit polyclonal anti-RapGAPII antibody and then detected by immunoblotting with goat antiRapGAP antibody (Santa Cruz Biotechnology). C, control. D, Neuro-2A cells transfected with the control non-silencing siRNA or Rap1 siRNA were stimulated with different concentrations of HU-210. Data represent mean ± S.E. (n = 3–5). D, serum-starved cells were treated with 20 μM lactacystin for 2 h prior to the addition of CB1-R agonist. Cells were scored for neurite outgrowth 16 h later. E, cells were transfected with or without 0.1 μg Rap1GAPII cDNA, cultured in serum-free medium, and stimulated with CB1R-Ag (1 μM HU-210) for 16 h and scored for neurite outgrowth.
likely that the Go subunit in GDP bound form could play a role in regulating signal flow prior to re-association with Gβγ and returning to the resting state. Further experiments are needed to unequivocally establish such a scheme; however, our observations are supportive of a mechanism for signal flow from heterotrimeric to small GTPases as is described in the model in Fig. 6. We do not as yet understand the mechanisms by which Go promotes the ubiquitination of Rap1GAPII. The simplest model would involve the presentation of Rap1GAPII to an E3 ligase. We have not as yet been able to identify the E3 ligase for Rap1GAPII and experiments to define the interactions and relationships between Go, Rap1GAPII, and the E3 ligase await this identification.

There are some differences in our studies as compared with previous studies. Mochizuki et al. (7) found that Rap1GAPII bound more avidly to the activated form Go as compared with WT Go. We do not understand the basis for the different observations. However, that study was conducted only with exogenously expressed components and the observed interactions could be related to context of the pathway created by the exogenous expression of multiple components including a high level of receptors. Rap1GAPI has also been reported to interact with Go, although it has not been specified if this interaction occurs with Rap1GAPI or Rap1GAPII (18).

The observations in this study highlight the multifaceted regulatory capability of the Go family subunits, in contrast to Go and Go family members. Go subunits in addition to inhibiting adenyl cyclase (19) and stimulating STAT3 through c-Src (20) and regulating neuronal function through GRIN (21) now appear capable of activating and modulating signal flow through the Rap1 pathway. It is interesting that in neuronal systems the Go pathway can often synergize or positively affect the cAMP pathway. This often occurs through Gβγ regulation of adenyl cyclase 2 (22). Our present observations suggest that it can also occur through Go subunits, since Gα-mediated degradation of RapGAP can enhance or synergize cAMP-mediated Rap activation. Thus even well studied pathways such as the classical heterotrimeric pathway may as yet hold surprises and the complexity of upstream regulatory mechanisms within signaling networks continues to grow. Such complexity may have important biological functions. Recent studies indicate that the sequential activation of Cdc42 and Rap plays an important role in determining which neurites become axons (23). CB1 receptors, like D2-dopamine receptors, are often presynaptic receptors that modulate transmitter release. It is possible that they are also present in growing axons and thus contribute to the activation of Rap to specify polarity during hippocampal development. Further studies are needed to determine whether such mechanisms are operative during the development of the hippocampus.

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REFERENCES

1. Reinoso, B. S., Undie, A. S., and Levitt, P. (1996) J. Neurosci. Res. 43, 439–453
2. Lotto, B., Upton, L., Price, D. J., and Gaspar, P. (1999) Neurosci. Lett. 269, 87–90
3. Xiang, Y., Li, Y., Zhang, Z., Cui, K., Wang, S., Yuan, X. B., Wu, C. P., Poo, M. M., and Duan, S. (2002) J. Neurosci. Res. 75, 127–134
4. Strittmatter, S. M., Fishman, M. C., and Zhu, X. P. (1994). J. Neurosci. 14, 2207–2208
5. Strittmatter, S. M., and Fishman, M. C. (1991) BioEssays 13, 127–134
6. Jordan, J. D., Carey, K. D., Stork, P. J., and Iyengar, R. (1999) J. Biol. Chem. 274, 21507–21510
7. Mochizuki, N., Oiba, Y., Kiyokawa, E., Kurata, T., Murakami, T., Ozaki, T., Kitabatake, A., Nagashima, K., and Matsuda, M. (1999) Nature 400, 891–894
8. Carey, K. D., and Stork, P. J. S. (2002) Methods Enzymol. 345, 397–404
9. Wu, G., Fang, Y., Lu, Z.-H., and Leeden, R. W. (1998) J. Neurocytol. 27, 1–14
10. Minana, M.-D., Felipe, V., and Grisolia, S. (1999) Proc. Natl. Acad. Sci. U. S. A. 87, 4335–4339
11. Siderovski, D. P., Diverse-Pierluissi, M., and De Vries, L. (1999) Trends Biochem. Sci. 24, 340–341
12. Kimple, R. J., Kimple, M. E., Betts, L., Sondek, J., and Siderovski, D. P. (2002) Nature 416, 878–881
13. Tsygankova, O. M., Feshchenko, E., Klein, P. S., and Meinkoth, J. L. (2002) J. Biol. Chem. 277, 5501–5507
14. Bivona, T. G., Wiener, H. H., Ahearn, I. M., Silletti, J., Chiu, V. K., and Philips, M. R. (2004) J. Cell Biol. 164, 461–470
15. Bos, J. L., de Rooij, J., and Reedquist, K. A. (2001) Nat. Rev. Mol. Cell 2, 369–377
16. Bhalla, U. S., Ram, P. T., and Iyengar, R. (2002) Science 297, 1018–1023
17. Takesono, A., Cismowski, M. J., Elbas, C., Bernard, M., Chung, P., Hazard, S., III, Duzic, E., and Lanier, S. M. J. (1999) J. Biol. Chem. 274, 33262–33265
18. Casey, P. J., and Meng, J. W. (2002) J. Biol. Chem. 277, 43417–43424
19. Taussig, R., Iniguez-Lluhi, J. A., and Gilman, A. G. (1993) Science 261, 218–221
20. Ram, P. T., and Iyengar, R. (2001) Oncogene 20, 1601–1606
21. Chen, L. T., Gilman, A. G., and Kozasa, T. (1999) J. Biol. Chem. 274, 26931–26938
22. Tang, W. J., and Gilman, A. G. (1991) Science 254, 1500–1503
23. Schwamborn, J. C., and Puschel, A. W. (2004) Nat. Neurosci. 7, 923–929
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