Activity of Rap1 Is Regulated by Bombesin, Cell Adhesion, and Cell Density in NIH3T3 Fibroblasts*

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Rap1 and Ras are homologous GTPases that are implicated in cell proliferation and differentiation. At present, little is known about the regulation of Rap1 activity. Using a recently developed assay with activation-specific probes, we found increased activity of endogenous Rap1 in NIH3T3 cells after stimulation with the neuropeptide growth factor bombesin in a concentration- and time-dependent manner. The activity of endogenous Ras was unaffected. Analysis of putative effectors showed no activation of c-Raf-1 or B-Raf after bombesin stimulation. However, MAPK/Erk-phosphorylation and the proliferation rate was increased. In addition, Rap1 was activated during cell adhesion to coated and uncoated tissue culture plates, as well as in response to various mitogens. Surprisingly, the basal Rap1 activity was observed to be cell density-dependent, with low levels when cells were reaching confluency. The results suggest that Rap1 acts as an important mediator of mitogenic signals distinct to Ras activity.

Small GTPases of the Ras family are involved in the growth control of various cell types. They switch from a GDP-loaded, inactive state to a GTP-bound, active state. This switch is controlled by the action of guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (reviewed in Ref. 1). Constitutively active Ras is a very potent oncogene, and Ras is frequently found to be mutated in many human cancers. The closely related GTPase Rap1 was originally discovered as a transformation suppressor of Ki-Ras (Krev-1 (2)). It shares about 50% homology to Ras and has a similar effector domain.

Rap1 can bind Ras effectors but does not activate them, leading to the model that Rap1 is antagonizing Ras function by trapping Ras effectors in an inactive state (1). cAMP-dependent protein kinase PKA activates Rap1 by phosphorylation on Ser-179 (3), a possible reason for the down-regulation of the MAPK/Erk pathway (4–6).

In contrast, Rap1 and cAMP induce proliferation and growth in Swiss 3T3 cells (4, 7), suggesting that Rap1 also can mimic Ras function in some cases (1). Recent studies in PC12 cells have shown that B-Raf, but not c-Raf-1, can be activated by Rap1 (6, 8–10). The mechanism involves PKA-mediated Rap1 activation (11) and complex formation between Crk and C3G (10), a guanine-nucleotide exchange factor for Rap1, finally leading to sustained activation of the MAPK pathway.

Based on the knowledge about GTPase effector molecules, activation-specific probes for Ras and Rap have recently been developed (12–15). These constructs preferentially recognize and precipitate GTP-loaded forms of specific GTPases. This allows determination of the activity of endogenous Ras and Rap1 without radioactive in vivo labeling.

Bombesin belongs to a group of mitogenic neuropeptides (16). It has been described as an autocrine growth factor in human small cell lung cancer (Ref. 17 and reviewed in Ref. 18). Bombesin is a tetradecapeptide and binds to Gq-coupled seven-transmembrane receptors. In 3T3 cells, which were often used as a model system, it induces DNA synthesis and proliferation (16). Different signal transduction pathways have been suggested to mediate bombesin effects (18–20). However, the role of small GTPases is unclear (21, 22).

Thus, we set out to analyze whether bombesin can promote GTP loading of Ras and Rap1. Following treatment of NIH3T3 cells with bombesin, TPA, or the tyrosine phosphatase inhibitor vanadate, activation of endogenous Rap1 was detected, whereas Ras activity was not significantly increased. We did not observe an activation of c-Raf-1 or B-Raf by bombesin. A variety of other stimuli, as well as cell adhesion after replating, were also found to activate Rap1. Conversely, the basal Rap1 activity was down-regulated when cells were grown at high density, possibly implicating Rap1 in contact inhibition and quiescence.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained from Sigma except for PDGF-BB (Life Technologies, Inc.), poly-β-lysine (Boehringer Mannheim), and TPA (Calbiochem).

Cell Culture—NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% FCS, seeded at 7 × 10^5/cm^2 and starved for 20–24 h with 0.5% FCS prior to stimulation. Adhesion experiments were performed as described by Vuori et al. (24). Briefly, NIH3T3 cells were starved, trypsinized, and treated with trypsin inhibitor. After washing with Dulbecco’s modified Eagle’s medium containing 0.5% bovine serum albumin, cells were held in suspension for 40 min and then allowed to adhere for 45 min after replating on tissue culture dishes (Starstedt) coated as indicated or further kept in suspension for 20 min.

Activation Assays for p21 GTPases—Activation assays for Rap1 and Ras were performed as recently described in detail (13, 15). Cells were washed twice with chilled HEPES-buffered saline (25 mM HEPES, pH 7.5, 150 mM NaCl) and lysed in Mg^{2+}-containing lysis buffer (MLB; 25 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM sodium vanadate, 10 μg/ml aprotinin, 0.5 μg/ml leupeptin). After lysis for 15 min and clarification at 16,000 × g for 20 min, the protein concentra-
Rap1 Activation in NIH3T3 Cells

RESULTS AND DISCUSSION

Using recently developed nonradioactive GTPase activation assays (12–15), we tested whether the neuropeptide growth factor bombyxin can activate endogenous Rap1 or Ras in NIH3T3 cells. Rap1 was rapidly activated, peaking at 3 min but maintained for more than 90 min (Fig. 1A). A concentration of 6 nM was found to be sufficient for Rap1 activation. In contrast, Ras is not significantly activated above basal levels by bombyxin (Fig. 1B). This is in agreement with previous findings on endogenous Ras by Mitchell and co-workers (22), although others have reported reduced bombyxin-mediated DNA synthesis following microinjection of neutralizing anti-Ras antibodies (21).

Because many pathways induced by growth-promoting agents seem to converge at small GTPases (1), other stimuli were also tested. Treatment with the tyrosine-phosphatase inhibitor vanadate as well as the protein kinase C-activating phorbol ester TPA led to activation of Rap1. No significant Ras activation was observed in NIH3T3 cells following these stimuli, although vanadate resulted in a mobility shift of the Ras protein (Ref. 23 and data not shown). Therefore the mechanisms of Ras and Rap1 activation can vary greatly in different cell types.

Another putative activator of Rap1, the nonhydrolyzable 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) in combination with the phosphodiesterase-inhibitor isobutylmethylxanthine activated Rap1 in NIH3T3 cells (Fig. 2B), similar to the recent findings in PC12 cells (9, 11) and fibroblasts (3). In addition, Rap1 was also stimulated by PDGF, FCS, and LPA (Fig. 2B). Disruption of the cytoskeleton with cytochalasin D did not inhibit the bombyxin-mediated Rap1 activation, indicating that an intact F-actin network is not always a prerequisite for bombyxin-signalizing (20). Several pathways appear to lead from receptors to Rap1 activation, and detailed analysis of these pathways remains to be done.

Induction of proliferation by bombyxin is accompanied by increased MAPK activity (19, 22). To analyze possible links between Rap1 activation and the MAPK pathway, the activity of endogenous Raf kinases was determined. In contrast to recent findings in PC12 cells (10), we did not observe increased B-Raf activity after treatment with bombyxin or LPA (Fig. 3A), whereas an approximately 3-fold activation of both c- and B-Raf after FCS/TPA treatment was found. Bombyxin and LPA also failed to increase c-Raf-1 kinase activity, supporting earlier studies (22). However, using an anti-phospho-ERK antibody, ERK activation (Fig. 3B) and induction of cell growth and proliferation were observed after bombyxin treatment (data not shown). Thus, in NIH3T3 cells stimulated with bombyxin, effectors other than c- and B-Raf mediate Rap1 activation, as previously proposed (5).

At present, the only known GEF acting on Rap1 is C3G (Crk SH3-binding GEF) (1). Because C3G is recruited to signal transduction complexes when cells adhere after replating on certain substrates (24), we tested whether Rap1 is activated during adhesion. With NIH3T3 cells kept in suspension, a low basal Rap1 activity was seen. Rap1 was considerably activated when cells were replated on fibronectin-coated, poly-L-lysine-coated or uncoated tissue culture plates (Fig. 4). Additionally, the basal Ras activity decreased when cells were allowed to
It was therefore tempting to speculate that B-Raf is an important activator of the MAPK pathway in bombesin-stimulated NIH3T3 cells. Although we could show that NIH3T3 cells contain at least some p94B-Raf, both B-Raf and c-Raf-1 were not activated following treatment with bombesin or LPA (Fig. 3). This demonstrates that the activation of B-Raf is not a general response to GTP loading of endogenous Rap1. There may be other Rap1 effectors and Erk activators that are critical in NIH3T3 cells (1, 5).

It is striking that many of the stimuli we found to activate Rap1 have previously been reported to induce tyrosine phosphorylation of the Crk-associated substrate p310Cas (20, 24). After bombesin stimulation, several pathways are triggered (18–22). Beside Ca2+ influx and protein kinase C activation, which can affect Rap1 as well (15, 18, 20), tyrosine phosphorylation of focal adhesion kinase (16, 19) and p130Cas occurs, which can affect Rap1 as well (15, 18, 20). Other mitogens like LPA, phorbol ester, and PDGF (20) or integrin engagement (24) can also induce complex formation of other Rap1 effectors and Erk activators that are critical in NIH3T3 cells (1, 5).

In summary, we have shown that endogenous Rap1 is activated by various mitogens and mediates signals from receptor tyrosine kinases, G-protein coupled receptors, and so far unidentified receptors sensing adhesion or cell density. Depending on the stimuli, a differential activation of Rap1 and Ras could be observed, suggesting that in some cases, for instance after bombesin stimulation, Rap1 rather than Ras may promote growth. Indeed, it was described previously that Rap1, as well as its activator PKA, can act as an inducer of proliferation in certain 3T3 cell lines (4, 7). In agreement with our results, other groups also found a Rap1 activation after endothelin stimulation, another neuropeptide growth factor that signals through Gαi-coupled receptors.2

There is growing evidence for a signaling pathway from PKA (11) and Crk (10) through Rap1 to B-Raf, resulting in sustained Erk activation and finally differentiation of PC12 cells (6, 8, 9). It was therefore tempting to speculate that B-Raf is an important activator of the MAPK pathway in bombesin-stimulated NIH3T3 cells. Although we could show that NIH3T3 cells contain at least some p94B-Raf, both B-Raf and c-Raf-1 were not activated following treatment with bombesin or LPA (Fig. 3). This demonstrates that the activation of B-Raf is not a general response to GTP loading of endogenous Rap1. There may be other Rap1 effectors and Erk activators that are critical in NIH3T3 cells (1, 5).

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