Regulation of Extracellular Signal-regulated Kinase Activity by p120 RasGAP Does Not Involve Its Pleckstrin Homology or Calcium-dependent Lipid Binding Domains but Does Require These Domains to Regulate Cell Proliferation

Jackie A. Koehler and Michael F. Moran

Banting and Best Department of Medical Research, Medical Genetics, University of Toronto [J. A. K.], and MDS Proteomics, Inc. [M. F. M.], Toronto, Ontario M9W 7H4, Canada

Abstract
The gene encoding for p120 RasGAP, has been disrupted in mice (M. Henkemeyer et al., Nature (Lond.), 377: 695–701, 1995). In this study, using fibroblasts derived from these mouse embryos (Gap−/−; P. van der Geer et al., Mol. Cell Biol., 17: 1840–1847, 1997), we demonstrate that mitogen-activated protein kinase (MAPK) activation is prolonged after epidermal growth factor (EGF), but not lysophosphatidic acid, stimulation as compared with wild-type cells. Furthermore, these cells exhibited a moderate increase in their proliferative rate and saturation density, as well as a limited ability to form colonies in soft agar. Stable cell lines expressing full-length p120GAP not only restored the ability to down-regulate MAPK after EGF stimulation, but also lowered their saturation densities. Similarly, expression of p120GAP, missing either its pleckstrin homology (PH) or its calcium-dependent lipid binding (CaLB)/C2 domain, restored MAPK down-regulation and retained the ability to associate with p190 RhGAP and to be phosphorylated by v-src but exhibited higher saturation densities similar to Gap−/− cells. Our results, therefore, suggest that p120GAP functions not only by down-regulating the Ras/MAPK pathway after growth factor stimulation but also is important in regulating cell proliferation that involves its PH and CaLB domains.

Introduction
Ras proteins are small guanine nucleotide-binding proteins that control a variety of cellular processes including cell proliferation and differentiation. These small GTPases function as molecular switches, cycling between inactive GDP-bound and biologically active GTP-bound conformations (1). The importance of Ras in growth regulation is exemplified by mutations in Ras that render it constitutively active and that are found in a number of human cancers (2). Ras activation is tightly regulated by the GEFs (3) and the GAPs (1). GEFs promote the release of GDP, thereby allowing GTP to bind to Ras (3). Conversely, GAPs bind to the GTP-bound form of Ras and stimulate its weak intrinsic GTPase activity, thereby accelerating the hydrolysis of GTP to GDP and returning Ras to its inactive conformation (4). Thus, understanding the mechanisms involved in controlling these modulators is necessary to understanding Ras regulation.

It has been well established that Ras is activated on stimulation of tyrosine kinase/growth factor receptors (1). Growth factor stimulation induces a series of SH2/SH3 domain-mediated protein-protein interactions involving the activated receptor; adaptor proteins, such as Grb2 and Shc; and the RasGEF, SOS, which promotes the exchange of GDP for GTP on Ras (5). Activation of Ras results in a conformational change such that it can interact with effector molecules, such as the serine/threonine kinase, Raf (6, 7). Raf then activates the MAPK cascade (8), which ultimately leads to the activation of other signaling proteins including certain transcription factors such as the ternary-complex factor (TCF) Elk-1 (9). GPCRs, which couple to the Gi and Gq subfamily of heterotrimeric G proteins, have also been shown to activate Ras and the Raf/MAPK pathway (10). Although the mechanisms by which GPCRs activate Ras are incomplete, increasing evidence suggests that Gi-mediated Ras activation involves $G_{i_\alpha}$ and intermediate tyrosine kinases to recruit the Grb2/SOS complex to Ras.

RasGAPs bind to the effector domain of activated Ras (Ras-GTP) through a GAP-related catalytic domain, which is both necessary and sufficient for stimulating the GTPase activity of Ras (11). Several Ras-GAPs have been identified in mammalian cells including p120GAP (12), neurofibromin (NF1;...
Ref. 13), p135 SynGAP (14), as well as GAP1\textsuperscript{m} (15) and GAP1\textsuperscript{HP} (16). In addition to their GAP-related catalytic domains, these proteins contain a variety of noncatalytic domains which may regulate their function in response to various extracellular signals.

The NH\textsubscript{2}-terminal region of p120\textsuperscript{GAP} contains an SH3 domain flanked by two SH2 domains (12, 17), followed by a PH (18) and a CaLB/C2 domain (19, 20), which mediate interactions with other signaling proteins (17, 21–23), p120\textsuperscript{GAP} complexes with activated EGF and PDGF receptors (24, 25) through its SH2 domains (17, 24, 26), which are believed to be responsible for the translocation of p120\textsuperscript{GAP} from the cytosol to the plasma membrane after growth factor stimulation (23). In addition, its SH2 domains mediate associations with p190, a GAP for the Rho family of small GTPases, and a highly tyrosine phosphorylated protein called p62\textsuperscript{GAP} (23, 27–29). The CaLB domain of p120\textsuperscript{GAP} interacts with negatively charged phospholipids (20) as well as with the Ca\textsuperscript{2+}-dependent phospholipid-binding protein annexin VI (22) and has been shown to be important in the membrane translocation of GAP in response to elevations of intracellular Ca\textsuperscript{2+} (20, 30). Little is known of the function of the PH domain in the regulation of p120\textsuperscript{GAP}. However, increasing evidence suggests that these domains may function in regulating membrane attachment and/or conformational changes mediated by interactions with specific ligands including phospholipids, inositol phosphates, or proteins (31).

Mice homozygous for a null mutation in the gene encoding for p120\textsuperscript{GAP} die by embryonic day 10.5 (32). Analysis of fibroblasts derived from these mouse embryos (Gap\textsuperscript{−/−}) indicated that although the basal levels are similar, in the absence of p120\textsuperscript{GAP} Ras-GTP levels are significantly higher and MAPK activation is prolonged after PDGF stimulation relative to wild-type cells. In addition to the cell migration defects displayed by these cells (33), both the basal and PDGF-induced tyrosine phosphorylation levels of the rhoGAP, p190, are dramatically reduced in the absence of p120\textsuperscript{GAP} (34).

In this study, we show that the duration of MAPK activation is prolonged in cells that lack expression of p120\textsuperscript{GAP} (Gap\textsuperscript{−/−}) after EGF, but not LPA, stimulation as compared with wild-type cells. This suggests that p120\textsuperscript{GAP} is specifically involved in regulating the Raf/MAPK pathway after growth factor stimulation, but that other rasGAP(s) are involved in regulating this pathway in response to GPCRs activation. In addition, Gap\textsuperscript{−/−} cells exhibited a modest increase in their proliferative rate and saturation density as well as in the ability to form small colonies in soft agar. Interestingly, this increase in proliferative potential does not appear to be attributed to aberrantly high levels of MAPK activity in these cells. Full-length p120\textsuperscript{GAP}, stably expressed in Gap\textsuperscript{−/−}, fibroblasts rescued the ability to down-regulate ERK in response to EGF and lowered the saturation density of these cells. By contrast, stable expression of GAP that lacked either the PH or the CaLB/C2 domain exhibited higher saturation densities similar to the Gap\textsuperscript{−/−} parental cells but were able to down-regulate MAPK after EGF stimulation. In addition, these domain-deletion mutants of p120\textsuperscript{GAP} retained the ability to associate with p190 and are tyrosine phosphorylated in the presence of v-src. These findings not only suggest that p120\textsuperscript{GAP} functions by down-regulating the Ras/MAPK pathway in response to growth factor stimulation but also suggest that p120\textsuperscript{GAP} is involved in the regulation of cell proliferation and that the PH and CaLB domains are important in mediating this process.

**Results**

p120\textsuperscript{GAP} Is Required to Down-Regulate the raf/MAPK Pathway in Response to Tryosine Kinase Receptors, but not through GPCRs. It has previously been shown that MAPK activation is prolonged in Gap\textsuperscript{−/−} cells in response to PDGF stimulation (34). Furthermore, LPA has been shown to activate MAPK. This pathway is thought to be mediated through G\textsubscript{pr} subunits and is dependent on Ras (10). We were, therefore, interested in exploring the role of p120\textsuperscript{GAP} in down-regulating MAPK in response to RTKs and to investigate its role in the regulation of MAPK activity in response to heterotrimeric GPCRs. To address these issues, MAPK activation was analyzed in both Gap\textsuperscript{+/+} and Gap\textsuperscript{−/−} cells after treatment with EGF or LPA.

MAPK activation/phosphorylation was examined at various times after stimulation with EGF by immunoblotting for phosphorylated MAPK in lysate samples containing equal amounts of total protein. This phospho-MAPK antibody recognizes specific phosphorylated tyrosine and threonine residues in MAPK that are required for its activation. As shown in Fig. 1, MAPK activity after stimulation with EGF is similar in both cell types (compare Lanes 2 and 5, Fig. 1A), resulting in a 4-fold increase after stimulation for 5 min (Fig. 1C). However, MAPK phosphorylation/activity is prolonged in cells that lack expression of p120\textsuperscript{GAP} (compare Lanes 3 and 6, Fig. 1A), which remained high (~70% of total activation) 40 min after EGF stimulation, compared with Gap\textsuperscript{+/+} cells (~40%; Fig. 1D).

To examine the extent of MAPK activation in response to GPCRs, both cell types were stimulated with LPA for various amounts of time. MAPK activation was similar in both cell types in response to LPA stimulation (compare Lanes 2 and 5, Fig. 1B) resulting in a 4- to 5-fold increase after 5 min of treatment (Fig. 1C). However, p120\textsuperscript{GAP} does not appear to be required to down-regulate the MAPK pathway in response to LPA (compare Lanes 3 and 6, Fig. 1B), because this activity returned to basal levels in both cell types by 20 min (Fig. 1D).

Taken together, these results suggest that p120\textsuperscript{GAP} down-regulates the Ras/MAPK pathway in response to RTK activation but not GPCR activation, at least not through the LPA receptor at ligand concentrations used here. Furthermore, these results indicate that p120\textsuperscript{GAP} does not play an essential role in the signaling pathway leading to MAPK activation through GPCR or tyrosine kinase receptor activation of Ras. Gap\textsuperscript{−/−} Cells Exhibit Moderately Higher Proliferative Rates and Saturation Densities in Both High and Low Serum. On culturing Gap\textsuperscript{+/+} and Gap\textsuperscript{−/−} cells, we observed that Gap\textsuperscript{−/−} cells did not appear to be confined to a monolayer (Fig. 2A, right panel). To further investigate this observation, cells were grown in 12 well plates and maintained in either high (10%) or low (0.5%) serum, and viable cells were counted at the designated times after plating. As
Fig. 1. p120GAP is required for the down-regulation of the Ras/MAPK pathway in response to RTK, but not GPCR, activation. Shown are representative phospho-MAPK and MAPK blots of cell lysates after stimulation with EGF (A), or LPA (B) for various amounts of time. GAP+/- cells (Lanes 1–3) and GAP–/- cells (Lanes 4–6) were serum-starved overnight (Lanes 1 and 4) and stimulated for 5 min with either EGF or LPA (Lanes 2 and 5) or analyzed for 40 min (EGF) or for 20 min (LPA) after stimulation (Lanes 3 and 6). All of the experiments were performed using equal amounts of whole cell lysates. Arrows, the migration of ERK1 and ERK2. C, fold activation of ERK2 after treating the cells for 5 min with either EGF or LPA. D, amount of phosphorylation of ERK2 after 40 min (EGF) or 20 min (LPA) as a percentage of the total level of phosphorylation after 5 min of stimulation. Each measurement is the mean phosphorylation level of ERK2 normalized with respect to the level of expression of ERK2 as estimated by phosphorimager quantification. Each measurement represents three independent experiments performed in duplicate. Graphing and SE calculations were performed with SigmaPlot.

shown in Fig. 2B, Gap–/- cells, maintained in high serum, not only had a lower doubling time but also a higher saturation density. Cells grew exponentially by day 4 in high serum, with a calculated population doubling time between days 1 and 4 of ~22 h for Gap–/- cells and of ~25 h for Gap+/+ cells. The saturation densities, in a 12-well tissue culture dish were ~4.6 x 10^5 cells/well for Gap–/- cells and 2.9 x 10^5 cells/well for Gap+/+ cells (Fig. 2B). As with Gap+/+ cells, Gap–/- began to die, as determined by trypan blue staining, shortly after reaching their saturation densities, although the extent of cell death was much more dramatic in Gap–/- cells with prolonged incubation. Gap–/- cells also exhibited a higher growth rate in 0.5% FBS (Fig. 2D). The doubling time in the logarithmic growth phase between days 1 and 9 in low serum was ~42.5 h for Gap–/- cells and ~50.2 h for Gap+/+ cells.

At low cell densities, GAP–/- cells exhibited the adherent and poorly refractile properties of untransformed cells (Fig. 2, A and C, left panel). However, their high saturation densities and their appearance at higher cell densities in both high-(Fig. 2A, right panel) and low-serum conditions (Fig. 2C, right panel) suggest that they may be partially transformed. In particular, these cells tended to condense in localized areas on culturing in low serum beginning at approximately day 11 (Fig. 2D). Thus, it does not appear that the higher growth rate and saturation densities found in cells lacking p120GAP led us to determine whether this was attributable to abnormal levels of MAPK activation. Both Gap–/- and Gap+/- cells were seeded at various dilutions and allowed to grow, in either high or low serum, to the desired cell density. The relative levels of MAPK activation were then determined by immunoblotting for phospho-MAPK.

As expected, the levels of MAPK phosphorylation/activation were higher in both cell types that were actively proliferating in 10% FBS (subconfluent cells: Lanes 1, 4, 7, and 10, Fig. 3A). Whereas this decreased once the cells reached confluence (Lanes 2, 5, 8, and 11, Fig. 3A) or 2 days later (Lanes 3, 6, 9, and 12, Fig. 3A). Although ERK2 activation is similar in both cell types at various cell densities (compare Lanes 7–9 with 10–12, Fig. 3A), we found that the levels of ERK1 activation in Gap–/- cells were generally higher at all cell densities compared with Gap+/- cells (compare Lanes 1–3 with 4–6, Fig. 3A). This is attributable to the lower levels of ERK1 expression (~1.75-fold less) in Gap–/- cells observed throughout this experiment (compare Lanes 13–15 with 16–18, Fig. 3A). Nonetheless, the same trend was found in that the levels of ERK1 activation decreased on reaching higher densities. In contrast to that found with cells maintained in high (10%) serum, the levels of MAPK activation were comparable between the two cell types at all cell densities when maintained in low (0.5%) serum (Fig. 3B). However, at higher cell densities the level of MAPK activation was found to be slightly higher in Gap–/- cells (compare Lane 4 with Lanes 5–6 and Lane 10 with Lanes 11–12, Fig. 3B). Thus, it does not appear that the higher growth rate and saturation densities, found with cells lacking expression of p120GAP, are attributable to abnormally high levels of MAPK activation, especially at higher cell densities. These results also suggest that p120GAP may not be essential in down-regulating the Ras/MAPK pathway or that other mechanisms are involved in regulating MAPK activation at higher cell densities.

Gap–/- Cells Exhibit Anchorage-independent Growth. The higher saturation densities seen with Gap–/- cells prompted us to examine the ability of these cells to form colonies in soft agar. When Gap+/+ cells were embedded in 0.33% agar and cultured, the majority of cells remained as single cells with the exception of two small colonies that formed by 5 weeks (one on two separate plates seeded with 1 x 10^4 cells; Fig. 4A). During the same time period, Gap–/- cells formed a number of small/medium-sized colonies (~0.2% of the cells seeded; Fig. 4B). Generally, these colonies were much smaller than those of Src 3T3 cells (Fig. 4C). The number of colonies was also considerably less than that seen with Src 3T3 cells (~0.2% versus ~1.6% of the cells seeded; Fig. 4D). Finally, Src 3T3 colonies began to appear 2 weeks after culturing, whereas Gap–/- colonies did not become visible until 4–5 weeks. Thus, Gap–/- cells were
able to form colonies in soft agar; however, the efficiency of colony formation was considerably less than the v-src-transformed cell line.

**PH- and CaLB/C2-deleted GAP Stable Cell Lines Exhibit Higher Saturation Densities.** To determine whether these growth-enhancing properties were caused by the lack of p120GAP and to address the functional role of the PH and CaLB/C2 domains in the regulation of p120 GAP, stable cell lines expressing wild-type GAP (WT) or mutants lacking either the PH (ΔPH) or CaLB/C2 (ΔC) domains were made in Gap+/− fibroblasts. Despite numerous attempts to generate these cell lines, only a few clones of each could be isolated. Furthermore, all of the clones exhibited low expression of wild-type GAP (Fig. 5A, Lanes 1–3 and 15–16) or the mutants (ΔPH, Lanes 7–10; ΔC, Lanes 11–14) compared with the Gap+/− parental cell line (Lane 4), as shown in Fig. 5A. Despite the fact that expression levels were low, the wild-type expressing clones had densities comparable with Gap+/− cells 2–3 days after reaching confluence. In contrast, the stable cell line using vector alone (pcDNA3), and most clones expressing either PH- or CaLB/C2-deleted GAP, had similar higher cell densities compared with the Gap−/− parental cells (Fig. 5B). Although we did observe some variability among the wild-type cell lines with regard to their expression level and saturation density (e.g., WT12 and WT1f), it is possible that there are certain levels of GAP that are required to rescue this phenotype and that, within one of these windows of expression, further rescue is not possible until the next “threshold” level is reached. Nonetheless, re-expression of GAP in all five of the wild-type clones reduced the saturation density of these cells, whereas three of the four PH- and CaLB-deleted clones did not. The morphologies of some of these stable cell lines are shown in Fig. 6. At low cell densities, these cell lines had morphologies that were similar to both parental cell lines (compare Fig. 6 with Fig. 2A, left panels). We did not observe any obvious differences in cell size between Gap+/− and Gap−/− cells or between the different stable cell lines, with the exception of the PH-deleted GAP clone, PH20, and wild-type clone, WT35f, which appeared somewhat larger on examination under the microscope when the cells were counted (data not shown) and displayed more spreading (Fig. 6, left panels) than did the other clones, which may account for their lower saturation densities. This suggests
that the higher saturation densities observed are reflecting differences in their proliferation rather than cell size. At higher cell densities, clones expressing full-length GAP (WT) displayed a morphology similar to Gap$^{-/-}$ cells (compare Fig. 2A with Fig. 6B, right panels), whereas the clone expressing vector alone (pcDNA3), as well as clones expressing PH-deleted GAP (ΔPH) or CaLB/C2-deleted GAP (ΔC) generally exhibited a stratified morphology, similar to Gap$^{-/-}$ cells (compare Fig. 2A with Fig. 6, A, C, and D, right panels). Taken together, these findings suggest that the PH and CaLB domains may be important for p120GAP to regulate proliferation.

**PH- and CaLB/C2-deleted GAP Stable Cell Lines Are Able to Activate and Down-Regulate MAPK in Response to EGF.** To investigate whether the phenotype observed with PH- and C2-deleted GAP stable cell lines was caused by an inability to inactivate Ras, we tested Ras activity indirectly by monitoring the ability of these mutant stable cell lines to down-regulate MAPK in response to EGF stimulation. Again, equal amounts of cell lysates were analyzed at various times after EGF stimulation by immunoblotting for phospho-MAPK. MAPK activation was similar in all of the stable cell lines after stimulation with EGF, resulting in a 6- to 9-fold increase in MAPK phosphorylation (Fig. 7A). Again, this activity remained high (−60−70% of total activation observed after 5 min of stimulation) in Gap$^{-/-}$ cells as well as in the cell line stably transfected with the empty pcDNA3 vector, 40 min after EGF simulation, as compared with Gap$^{-/-}$ cells (−30%; Fig. 7B). Whereas, the stable cell lines expressing full-length GAP or either of the GAP deletion mutants were able to down-regulate MAPK activity to similar levels (−30−40%) as compared with Gap$^{-/-}$ cells in response to EGF.

These results demonstrate that the PH and CaLB domains are not required for GAP to be activated in response to EGF stimulation. Although we have not tested this directly, the ability of the domain deletion mutants to down-regulate MAPK after EGF stimulation suggests that the deletion of the PH or CaLB domains does not interfere with the ability of
p120GAP to inactivate Ras or to associate with activated growth factor receptors. **PH- and CaLB/C2-deleted Mutants of GAP Are Able to Associate with p190 (rhoGAP) and Are Phosphorylated by v-src.** To rule out the possibility that deletion of the PH or CaLB domain compromises the ability of GAP to associate with known interacting proteins, we wished to address whether these GAP deletion mutants retained the ability to interact with p190. In addition, p190 associates with GAP in the presence and absence of p190 tyrosine phosphorylation (35), which raises the possibility that domains other than its SH2 domains could be involved in the phosphotyrosine-independent association with p190. We were specifically interested in the association with p190 because it is a GAP for the Rho family of GTPases that regulate cytoskeleton organization (27) and may, therefore, contribute to the phenotype observed with the mutant GAP stable cell lines. As shown in Fig. 8B, WT GAP and either of the domain-deleted GAP mutants were able to interact with p190 when cotransfected with v-src in HEK 293 cells (Fig. 8B, Lanes 2–5). However, in the absence of v-src, the CaLB/C2-deleted mutant of GAP did not bind p190 as efficiently as WT GAP or the PH-deleted mutant of GAP (Fig. 8B, Lanes 6–9). In addition, all of the GAP constructs were tyrosine phosphorylated when cotransfected with v-src (Fig. 8A, Lanes 2–5). These observations indicate that neither the PH nor the CaLB domain is necessary for the association between GAP and p190 in the presence of v-src and that deletion of either of these domains does not inhibit the tyrosine phosphorylation of GAP by v-src. However, these results do not rule out the possibility that the lack of the PH or CaLB domain may affect other phosphorylations or may have a more subtle effect on the association with p190, such as altered affinity or localization.

**Discussion**

To address the function of p120GAP in RTK and GPCR signaling, we used fibroblasts derived from mouse embryos with a null mutation in the gene encoding for p120GAP, and we analyzed MAPK activation in response to EGF and LPA. We observed that both EGF and LPA led to the activation of MAPK in either Gap+/− or Gap−/− cells. Furthermore, in the absence of p120GAP, MAPK activation is prolonged after EGF stimulation. This is consistent with a previous study that suggested that p120GAP is specifically activated and functions to down-regulate the Ras/MAPK pathway in response to PDGF stimulation but is not required for mitogenic signaling (34).

Our results also indicate that p120GAP is not required for the down-regulation of the Raf/MAPK pathway in response to LPA. This was a somewhat surprising result. LPA receptors are known to couple to three different G proteins (Gi, Gq, and G12; Refs. 36, 37). Activation of Gi induces activation of Ras and MAPK mediated by Gαi, and protein kinases, whereas Gq activation initiates phospholipase Cg activation, resulting in the production of diacylglycerol (DAG) and the release of Ins(1,4,5)P3, which in turn elevates intracellular calcium. Because p120GAP contains a PH domain implicated in binding to Gαi subunits (38, 39) and a CaLB/C2 domain, which is responsible for the Ca2+–induced membrane localization of p120GAP (20), we expected MAPK activation to be prolonged in cells that lack p120GAP expression. Our results could be explained by the activation of other rasGAPs. Gap1 family members, including Gap1tm and Gap1IR/IRBP, contain two C2 domains as well as a PH and adjacent Btk motif (15, 16). In addition, Gα12 directly binds to and stimulates the activity of Gap1tm (40). Thus, other rasGAPs, such as Gap1 family members, which contain a PH and an adjacent Btk motif in Gα12 binding, may be involved in the inactivation of Ras and the MAPK pathway in response to certain GPCRs such as the LPA receptor. However, we are not excluding the possibility that p120GAP may be involved in other aspects of LPA-mediated signaling.

Although Gap−/− cells exhibited the poorly refractile morphology of nontransformed cells at low cell densities, our observations suggest that these cells may be partially transformed. We have observed that these cells not only exhibit a moderately higher proliferative rate and saturation density relative to Gap+/− cells, in both high and low serum conditions, but they also display enhanced colony forming ability in soft agar albeit with a much lower efficiency than Src-transformed fibroblasts. Furthermore, these enhanced proliferative properties did not appear to be due to aberrantly high levels of activated MAPK in these cells. These phenotypic characteristics are similar to those observed with stable...
cell lines that express only the SH2–3-2 region of p120GAP (41) or activated Rho/Rac family members (42–44) that exhibit the proliferative, but not the morphological, aspects of Ras transformation. However, this increase in proliferative potential was less than that of Ras-transformed cells (42, 43).

Although this study is based on one Gap/H11001 and Gap/H11002 cell line, reexpression of full-length GAP in Gap/H11002 cells not only rescued the prolonged activation of MAPK after EGF stimulation, but also reduced the saturation density of these cells, which demonstrated that these phenotypes are attributable to the lack of p120GAP. In addition, the Gap−/− cell line behaves similarly to the Gap+/+ clone in that its basal level of MAPK activation, duration of MAPK activity in response to LPA, and modulation of MAPK activity under different growth conditions (i.e., 10% and 0.5% FBS) were comparable, demonstrating that many other aspects of these cells examined in this study are not altered.

Contact-induced growth inhibition is a characteristic feature of normal cells grown in a monolayer, although the molecular events underlying this process, or how fibroblasts communicate these changes, is not fully understood. Ras-GAP activity is higher in cell extracts from confluent Balb 3T3

Fig. 6. Morphology of representative stable cell lines maintained in 10% FBS. Vector (pcDNA3; A), WT (B), ΔPH (C), and ΔCaLB/C2 (D) stable cell lines. Right panels, cells at low density. Left panels, cells at high density.
or NIH 3T3 fibroblasts than those from subconfluent cells (45, 46), indicating tight regulation of Ras modulators. Furthermore, increased levels of PI3-K, an effector of Ras, and its product PI(3,4,5)P3, have been observed in density-arrested fibroblasts (47). In this study, we found that stable cell lines expressing PH- or CaLB/C2-deleted GAP mutants had higher saturation densities similar to that of Gap\(^{+/+}\) cells. The reason for this is unclear at present. Our results suggest that these domain-deletion GAP mutants are functional in the sense that they are able to down-regulate the MAPK pathway in response to EGF stimulation, to associate with p190, and to become tyrosine phosphorylated in the presence of v-src. It is also unlikely that the higher saturation densities exhibited by these cells are caused by their low levels of expression because the WT stable clones examined had similar expression levels, yet displayed growth characteristics similar to those of Gap\(^{+/+}\) cells. Perhaps on cell contact, certain signaling events recruit p120GAP to the plasma membrane to access Ras or to regulate Rho family GTPases. As previously mentioned, PI3-K and the PI3-K lipid product PI(3,4,5)P3 are higher in contact-inhibited cells. Although, by sequence alignment analysis, the PH domain of p120GAP was not predicted to be able to bind PI3-K lipid products with high affinity (48), a dot-blot assay revealed that the PH domain of p120GAP is able to bind a variety of phospholipids, including PI(4,5)P2 and PI3-K lipid products (49). In addition, the CaLB/C2 domain is responsible for the Ca\(^{2+}\)-induced membrane localization of p120GAP (20, 30) and associates with the Ca\(^{2+}\)-dependent phospholipid binding protein, annexin VI (22). Alternatively, we have observed that both the PH and the CaLB domains are required for p120GAP to associate with the membrane-associated protein RACK1 (50). The higher saturation densities observed with the PH- and CaLB/C2-deleted GAP mutants may reflect the inability of these mutants to respond to signals induced by contact and/or by their inability to stably interact with an associating protein such as RACK1, annexin VI, or other novel targets.
The comparable levels of activated MAPK between Gap\(^{-/-}\) and Gap\(^{+/+}\) cells at various cell densities, together with the phenotypic similarities between Gap\(^{-/-}\) and cells expressing activated Rho or Rac, suggest that the proliferative characteristics exhibited by Gap\(^{-/-}\) cells or the domain deletion mutant clones may be attributable to another Ras-mediated event or that p120\(^{GAP}\) may be acting independently of Ras to control cell proliferation. With the evidence suggesting a connection between p120\(^{GAP}\) and independently of Ras to control cell proliferation. With the phenotypic similarities between Gap\(^{-/-}\) cells (34), it is intriguing to speculate that the higher saturation densities observed may be attributable to the deregulation of the Rho family GTPases. This may be attributable to the inability of these GAP mutants, and/or associating protein(s) such as p190, to localize properly in response to signals that are generated on cell contact or that their mislocalization leads to their adhesion properties to become compromised. In this respect, it is interesting to note that p120\(^{GAP}\)-deficient cells have previously been observed to lose cell-cell contact on stimulation with PDGF (34). Taken together, our data suggest that p120\(^{GAP}\) may play an important role in controlling cell growth, perhaps by its involvement in cell-cell or cell-substrate adhesion and that the PH and CaLB domains are important in mediating this process.

Materials and Methods

Construction of Mammalian Expression Vectors. The cDNAs encoding KT3-tagged full-length GAP and C2-deleted GAP were subcloned from pCMV1 into the SV40-based pECE mammalian expression vector (20). PCR was used to delete sequences encoding the PH domain (residues 477–576 of GAP) from pECE GAP KT3 using the primer pairs 5′-ATGGTTCCATGGGAAGATTTC-3′ and 5′-CATTCGTCTGTGGCATGC-3′, and 5′-AACATTGTTAATTACGGAA-3′, 5′-CATTCTCGTCTGGCATGC-3′. These PCR products were combined followed by a second round of PCR using the oligonucleotides 5′-ATGGTTCCATGGGAAGATTCC-3′ and 5′-AAACATTGTTAATTATCGGA-3′, 5′-CATTCTCGTCTGGCATGC-3′. The final PCR product was then digested with the restriction enzymes AflII and EcoRV and subcloned into pECE GAP KT3, previously digested with the same restriction enzymes. The pECE plasmids encoding KT3-tagged full-length (WT) GAP and ΔPHGAP were digested with ScaI and Smal and treated with Klenow to create blunt ends. The cDNAs were then subcloned into the pcDNA3.1zeo(+) plasmid (Invitrogen) that was previously digested with EcoRI and treated with Klenow. To obtain pcDNA3.1zeo ΔCGAP KT3, a WT fragment from pLXSN WT GAP KT3, previously made in our lab, was replaced with a ΔC fragment by digestion with BglII and EspI to obtain pLXSN ΔCGAP KT3. The cDNA encoding KT3-tagged ΔCGAP was then subcloned from the pLXSN plasmid into pcDNA3.1zeo(+) by digestion with EcoRI. All of the constructs were verified by DNA sequencing.

Cell Lines and Transfections. Gap\(^{-/-}\) and Gap\(^{+/+}\) mouse embryonic fibroblasts, kindly provided by Tony Pawson, Mt. Sinai Hospital, Toronto, Canada (34), were grown in DMEM containing 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml), and were maintained at 37°C in a 5% CO\(_2\) incubator. HEK 293 cells were maintained as above with the addition of 10 μg nonessential amino acids and 4.5 g/liter l-glutamine (Life Technologies, Inc.). Stable cell lines expressing full-length p120\(^{GAP}\) or mutants of GAP in which the PH or CaLB/C2 domains were deleted were maintained as above with the addition of 600 μg/ml zeocin (Invitrogen). S7a and Src 3T3 cells were maintained in DMEM containing 5% calf serum (CS).

To produce stable cell lines in Gap\(^{-/-}\) fibroblasts, Gap\(^{+/+}\) fibroblasts were transfected with 8 μg of pcDNA3 zeo plasmids using Lipofectamine (Life Technologies, Inc.) according to the manufacturer’s instructions. Approximately 48 h after transfection, the cells were split 1:4 into DMEM containing 10% FBS and 600 μg/ml zeocin. The cells were maintained for 2–3 weeks replacing the selective media every 3–4 days until individual colonies appeared. Colonies were then picked using cloning cylinders and were transferred to 6-well plates. When the cells neared confluence, they were split 1:10, and the remaining cells were screened for expression of GAP by immunoblot analysis using the monoclonal GAP antibody (B4F8; Santa Cruz Biotechnology). HEK 293 cells were transfected with 8 μg of pcDNA3 zeo or the GAP constructs as mentioned above and pECE v-src using the calcium-phosphate precipitation method.

Immunoprecipitation and Western Blot Analysis. Two days after transfection, HEK 293 cells were washed twice with ice-cold Ca\(^{2+}\)-free PBS and lysed in phospholipase C lysis buffer [50 mM HEPES (pH 7.0), 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM EDTA, 1.5 mM MgCl\(_2\), 100 mM NaF, 10 mM NaPPi, 1 mM Na\(_2\)VO\(_3\), 10 μg/ml aprotinin, and 0.5 mM aminoethylbenzene sulfonic acid] and clarified by centrifugation. Lysates were then preincubated by incubation with mouse IgG-goat antianti-mouse agarose (Sigma Chemical Co.). p120\(^{GAP}\) was immunoprecipitated from preincubated lysates by the addition of 1 μg of monoclonal anti-KT3 ascites fluid for 1.5 h, followed by the addition of 20 μl of goat antianti-mouse agarose, and mixed by inversion for an additional hour at 4°C. As a positive control, p120\(^{GAP}\) was immunoprecipitated from S7a preincubated lysates by the addition of 1 μg of polyclonal GAP antibody as described previously (23). Immune complexes were then collected by centrifugation, washed with lysis buffer A [20 mM Tris-HCl (pH 8.0), 1% NP40, 100 mM NaCl, 1 mM Na\(_2\)VO\(_3\)], and boiled in 3× Laemmli sample buffer [187.5 mM Tris-HCl (pH 6.8), 30% glycerol, 6% (w/v) SDS, 0.015% bromphenol blue, and 15% (w/v) β-mercaptoethanol].

Samples were resolved by SDS-PAGE and transferred onto nitrocellulose using the semi-dry method. The blots were then incubated with blocking buffer (TBS, 0.1% Tween 20, 5% BSA) for 1 h at room temperature, followed by incubation with specific antisera in blocking buffer for 1 h at room temperature or overnight at 4°C. The filters were washed twice in TBS/0.1% Tween 20 (TBST) and then once in TBS and then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The membranes were washed again, followed by chemiluminescence detection (Pierce Super Signal) on Kodak Blue XB-1 film. MAPK and phospho-MAPK blots were quantified using the phosphorimager (Bio-Rad GS-250, CH screen, molecular
analysed and used as previously described (23). Goat antiserum and antirabbit IgG-horseradish peroxidase secondary antibodies were purchased from Bio-Rad.

**Cell Counting.** For growth curves, 1 x 10⁴ or 5 x 10³ GAP⁻ /− cells were seeded and grown in 12-well dishes in either 10% or 0.5% FBS, respectively. Viable cells were then counted at the designated time points. Stable cell lines were grown and maintained in 10% FBS and counted 2–3 days after reaching confluence. Trypsinized and resuspended cells were then mixed with 10–20% trypan blue stain [0.5% (w/v) trypan blue, 0.85% (w/v) NaCl, 10 mM Tris-HCl (pH 7.2)] and were counted using a hemocytometer.

**Analysis of MAP Kinase Activation.** To analyze MAPK activation at different stages of confluence, GAP⁻ /− or GAP⁻ /− cells were plated in 6-well dishes at various dilutions and allowed to grow until they reached the desired density. To analyze MAPK activation after stimulation, cells, at approxi- mately 95% confluence, were serum-starved overnight in media lacking serum. The following day, cells were stimulated with either 50 ng/ml EGF (Upstate) or 20 μM LPA (Sigma Chemical Co.) for 5, 20 (LPA), or 40 (EGF) min. The cells were then washed twice with PBS and lysed in 150 μl I x sample buffer [2% (w/v) SDS, 10% (w/v) glycerol, and 62.5 mM Tris-HCl (pH 6.8)]. The cells were then collected into microfuge tubes, boiled for 5 min, and briefly sonicated to shear genomic DNA. The protein concentration was then determined using the Bio-Rad DC protein assay. After the protein assay, β-mercaptoethanol and bromophenol blue were added to the samples to a final concentration of 5% (w/v) and 0.005% (v/v) respectively. Approximately 10 μg of total protein was subjected to SDS-PAGE and immunoblot analysis. After immunoblotting with MAPK (New England Biolabs Inc.), the blots were stripped by washing in water for 5 min, followed by 0.2 N NaOH for 10 min and then water again for another 5 min. The stripped blots were then incubated in BSA blocking buffer for 1 h and probed for phospho- MAPK (New England Biolabs Inc.) overnight at 4°C. All of the blots were quantified using a phosphorimagery (Bio-Rad).

**Growth in Soft Agar.** The experiment was performed as described previously (54). Each cell dilution (1 x 10⁵, 1 x 10⁴, 1 x 10³) was performed in triplicate. The plates were incubated at 37°C in a 5% CO₂ humidified incubator and fed once a week by the addition of 0.5 ml of DMEM and 10% FBS.

**References**


