Disrupted RabGAP Function of the p85 Subunit of Phosphatidylinositol 3-Kinase Results in Cell Transformation

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Rab proteins regulate vesicle fusion events during the endocytosis, recycling, and degradation of activated receptor tyrosine kinases. The p85α subunit of phosphatidylinositol 3-kinase has GTPase-activating protein activity toward Rab5 and Rab4, an activity severely reduced by a single point mutation (p85-R274A). Expression of p85-R274A resulted in increased platelet-derived growth factor receptor (PDGFR) activation and downstream signaling (Akt and MAPK) and in decreased PDGFR degradation. We now report that the biological consequences of p85-R274A expression cause cellular transformation as determined by the following: aberrant morphological phenotype, loss of contact inhibition, growth in soft agar, and tumor formation in nude mice. Immunohistochemistry shows that the tumors contain activated PDGFR and high levels of activated Akt. Coexpression of a dominant negative Rab5-S34N mutant attenuated these transformed properties. Our results demonstrate that disruption of the RabGAP function of p85α due to a single point mutation (R274A) is sufficient to cause cellular transformation via a phosphatidylinositol 3-kinase-independent mechanism partially reversed by Rab5-S34N expression. This critical new role for p85 in the regulation of Rab function suggests a novel role for p85 in controlling receptor signaling and trafficking through its effects on Rab GTPases.

Rab proteins are small monomeric GTPases important in the regulation of RTK endocytosis, trafficking, and degradation pathways (reviewed in Refs. 6–8). Rab5 regulates vesicle fusion events during the endocytosis of activated RTKs, important for moving the receptor complex from the plasma membrane to the early/sorting endosome. RTKs are believed to continue their downstream signaling during endocytosis. Rab4 regulates vesicle trafficking from the early/sorting endosome back to the plasma membrane to return deactivated RTKs to the cell surface for further rounds of activation and signaling. Some fraction of receptors located in the early/sorting endosome are diverted to the late endosome and lysosome, where they are degraded via a mechanism that requires receptor monoubiquitination (9–13). Because Rab5 and Rab4 regulate the trafficking of activated internalized RTK complexes through the endocytic pathway, they control the location, magnitude, and duration of receptor signaling (14–16).

Recent studies in human disease, including cancer, have suggested that defects in the endocytic pathway can give rise to sustained RTK levels, sustained receptor activation, and downstream signaling driving aberrant cell proliferation, cell survival, and tumorigenesis (1, 17). In particular, defects in receptor monoubiquitination have been linked to cancer (3, 18). A recent report demonstrated that Tpr-Met, a chromosomal translocation product involving the kinase domain of the Met RTK, was not down-regulated efficiently because it was mislocalized to the cytoplasm and lacked the binding site for the Cbl ubiquitin-protein isopeptide ligase (19).

We used a mouse NIH 3T3 fibroblast cell model system to study receptor trafficking of the endogenously expressed PDGFRα. This well-characterized RTK has known downstream signaling pathways, including Ras/MAPK and PI3K/Akt, activated upon PDGF stimulation. We characterized the interaction of the p85α subunit of PI3K with different binding proteins and the functions of these protein complexes in receptor-mediated endocytosis (20, 21). Previously, we reported that the BCR homology domain of p85 has GTPase-activating protein (GAP) activity toward Rab5 and Rab4 (but not Rab11) and enhanced intrinsic Rab GTPase activity by ~1000-fold (20), switching the Rab to an inactive GDP-bound conformation. We generated a p85 mutant lacking RabGAP activity (p85-R274A), yet it retained normal ability to bind to activated PDGFRs as well as to bind to and regulate the p110 catalytic subunit of PI3K (20). NIH 3T3 cells stably expressing p85-R274A were assayed for changes in PDGFR activation, downstream cellular signal-
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ing, and PDGFR degradation (20). The loss of p85 RabGAP activity increased the magnitude and duration of tyrosine-phosphorylated PDGFR and the resulting MAPK and Akt signaling in response to PDGF stimulation. Cells expressing p85-R274A also appeared to have a decreased rate of PDGFR degradation. This suggests that p85 RabGAP activity is important in preventing uncontrolled PDGFR signaling. Moreover, disruption of RabGAP function as a result of p85-R274A expression could have tumorigenic potential because of enhanced PDGFR signaling and diminished PDGFR down-regulation. To test this hypothesis, we have characterized the oncogenic potential of cells expressing the p85-R274A RabGAP mutant, determining changes in cell morphology, proliferation, contact inhibition, and anchorage-independent growth. Cells stably expressing the p85-R274A mutant showed all these characteristics of oncogenic transformation and also formed tumors in nude mice. Coexpression of the dominant negative Rab5-S34N mutant blunted these effects, consistent with the ability of p85-R274A to act at the level of Rab5 function. These results suggest that a subtle point mutation in p85 that disrupts its RabGAP activity is sufficient to convert it into an oncogene and that deregulation of Rab function can lead to cellular transformation.

EXPERIMENTAL PROCEDURES

Plasmids and Cell Culture—The plasmids encoding FLAG-tagged wild-type p85 and p85-R274A have been described (20). The insert for the hemagglutinin (HA) epitope-tagged HA-Rab5-S34N plasmid was generated by PCR amplification of the entire Rab5 coding sequence from the pCMV-Myc-Rab5-HA-Rab5-S34N plasmid. The insert for the hemagglutinin (HA) epitope-tagged wild-type p85 and p85-R274A have been described (20). The insert for the hemagglutinin (HA) epitope-tagged HA-Rab5-S34N plasmid was generated by PCR amplification of the entire Rab5 coding sequence from the pCMV-Myc-Rab5-HA-Rab5-S34N plasmid. The insert for the hemagglutinin (HA) epitope-tagged wild-type p85 and p85-R274A have been described (20).

The EcoRI-digested insert was subcloned into the BglII/EcoRI-digested pUC19 plasmid. The BamHI/EcoRI-digested insert was subcloned into the BglII/EcoRI-digested pUC19 plasmid. The BamHI/EcoRI-digested insert was subcloned into the BglII/EcoRI-digested pUC19 plasmid. The BamHI/EcoRI-digested insert was subcloned into the BglII/EcoRI-digested pUC19 plasmid. The BamHI/EcoRI-digested insert was subcloned into the BglII/EcoRI-digested pUC19 plasmid. The BamHI/EcoRI-digested insert was subcloned into the BglII/EcoRI-digested pUC19 plasmid. The BamHI/EcoRI-digested insert was subcloned into the BglII/EcoRI-digested pUC19 plasmid. The BamHI/EcoRI-digested insert was subcloned into the BglII/EcoRI-digested pUC19 plasmid. The BamHI/EcoRI-digested insert was subcloned into the BglII/EcoRI-digested pUC19 plasmid. The BamHI/EcoRI-digested insert was subcloned into the BglII/EcoRI-digested pUC19 plasmid.

The HA3 vector (23). The resulting plasmid encoded the HA-Rab5-S34N protein containing three copies of the HA tag at the N terminus of dog Rab5-S34N. The DNA encoding HA-Rab5-S34N was excised with HindIII and ligated into similarly digested pCDNA6/V5-HisA (Invitrogen). The resulting plasmid encoded HA-Rab5-S34N with no other Myc or His tags and also encodes blastocidin resistance. The entire HA-Rab5-S34N coding sequence was verified to ensure that it contained only the desired mutation of codon Ser34 to Asn.

Stably transfected NIH 3T3 cells expressing FLAG-p85 (p85) and FLAG-p85-R274A (p85-R274A) were generated and maintained in medium containing G418 (400 μg/ml) as described previously (20). Stable coexpression with HA-Rab5-S34N was achieved by transfection of FLAG-p85-R274A-expressing cells with the plasmid encoding HA-Rab5-S34N and selection in both G418 (400 μg/ml) and blastocidin (5 μg/ml). Clonal isolates were tested for HA-Rab5-S34N expression using an HA immunoblot analysis. Cells were expanded and used for PDGF stimulation time course experiments as done previously (20).

Immunoblot Analysis—Immunoblot analyses have been described previously (24) and used the following antibodies from Santa Cruz Biotechnology: PDGFR (catalog no. sc-432), glyceraldehyde-3-phosphate dehydrogenase (catalog no. sc-25778), pTyr (catalog no. sc-508), and HA (catalog no. sc-7392). The additional antibodies used were as follows: pMAPK (pThr202/pTyr204, Cell Signaling, catalog no. 9101), MAPK (BD Transduction Laboratories, catalog no. M12320), pAkt (pSer473, New England Biolabs, catalog no. 9271), Akt (New England Biolabs, catalog no. 9272), and FLAG (Sigma, catalog no. F3165). All antibodies were used according to the supplier's instructions and visualized using chemiluminescence. Gel electrophoresis and immunoblotting were carried out simultaneously for the different cell lines. Blots were probed and exposed to film together with the same exposure shown for each. Blots shown are representative of a typical result from at least three independent experiments.

Cell Morphology, Contact Inhibition, and Anchorage-independent Growth—The morphology pictures were taken under phase contrast using a Coolpix 990 digital camera (Nikon) using an Eclipse TE300 microscope (Nikon). The total magnification was ×400. For the contact inhibition assay, cells (2.5 × 104) were seeded on a 10-cm plate and allowed to grow for 14 days. The medium was changed every 1–2 days. The plates of cells were fixed with methanol and stained with Giemsa stain (Sigma). Photographs of the entire plates were taken with a Gel Doc 2000 (Bio-Rad). The results shown are typical for three independent experiments. For the anchorage-independent growth assay, the cells were grown in soft agar as described previously (24). Cells (5 × 103) were suspended in soft agar in 6-cm dishes and allowed to grow. An additional ~1 ml of soft agar medium was layered on top after 7 days. Photographs were taken as described for the cell morphology experiments above after 20 days of growth. The colonies formed are reported as the number of colonies/5 × 103 cells seeded on each plate. Three independent experiments were carried out, and the mean ± S.D. was plotted. A one-way analysis of variance was carried out using Prism software (version 4, GraphPad Software Inc., San Diego, CA) to determine the statistical significance of the differences observed. The p values for the statistically different results are indicated.

Proliferation Assay—Cell proliferation was assayed using the Cell Titer 96 non-radioactive proliferation kit (Promega) according to the manufacturer's instructions. The cells were serum-starved in 0.5% fetal bovine serum-containing medium for 24 h before being plated at 5 × 103 cells/well in a 96-well plate. The cells were tested for growth over 3 days in medium containing either fetal bovine serum (2%) or PDGF-BB (50 ng/ml). Each experiment was done with duplicate samples in each of four independent experiments. The results were plotted using Prism software, and the S.E. is indicated with error bars.

Tumor Formation and Immunohistochemistry—Male Swiss nude mice were injected subcutaneously with 2.5 × 106 cells (either NIH 3T3 or p85-R274A) in the right flank as described (25). Tumor development was monitored every 2–3 days by measuring the length and width of the tumor. The tumor volume was determined by the equation V = 4/3πlength×width². The average tumor volume was plotted using Prism software, and the S.E. is indicated with error bars. Mice were handled according to acceptable care guidelines approved by the University of Saskatchewan animal care committee (protocol no. 19970017). Tumors were excised and paraffin-embedded, and the hematoxylin-eosin staining was performed as described (25). The
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p85-R274A induces a transformed phenotype in NIH 3T3 cells—NIH 3T3 cell lines stably expressing FLAG-tagged wild-type p85 and a p85 mutant with a single point mutation (Arg274 to Ala) in the BCR homology domain (p85-R274A) that disrupts its RabGAP activity were used (Fig. 1A) (20). The expression levels of wild-type p85 and p85-R274A in the clonal cell lines studied were similar as determined using immunoblot analysis (Fig. 1B). Glycerinaldehyde-3-phosphate dehydrogenase is shown as a loading control (Fig. 1B). Cells overexpressing wild-type p85 and untransfected control NIH 3T3 cells both displayed a flattened morphological phenotype by bright-field microscopy, whereas the p85-R274A-expressing cells appeared more dense and rounded (Fig. 1C). In addition, p85-R274A-expressing cells spontaneously formed foci on subconfluent plates under normal growth conditions, suggesting the loss of growth arrest via cell contact inhibition (data not shown). These observations, coupled with our previous report of enhanced and sustained PDGFR activation and downstream signaling (20), prompted us to further investigate the tumorigenic potential of these cells.

p85-R274A-expressing cells show a loss of contact inhibition and anchorage-independent growth—An important characteristic of transformation frequently observed in cell monolayers is the loss of contact inhibition. All cell lines were tested for loss of contact inhibition by growing the cells past 100% confluence and determining whether cells continued to grow and form foci. Cells were plated and grown for 14 days with

FIGURE 1. Altered morphological appearance of NIH 3T3 cells expressing p85-R274A. A, the domain structure of the FLAG-tagged p85 proteins stably expressed in the NIH 3T3 cells is shown. Wild-type FLAG-tagged p85 is the full-length protein, and the FLAG-p85-R274A mutant is also full-length but has a single point mutation changing Arg274 to Ala and severely compromising its RabGAP activity. B, cell lysates (40 μg of protein) from the indicated cell lines were resolved by SDS-PAGE and transferred to nitrocellulose. Duplicate immunoblots were probed with the indicated antibodies and visualized using chemiluminescence. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. C, the morphology of control NIH 3T3 cells and stable p85- and p85-R274A-expressing cell lines is shown. The images are phase contrast, taken at ×400 magnification.

FIGURE 2. p85-R274A-expressing cells lack contact inhibition and anchorage dependence for growth, indicative of transformed cells. A, the indicated cell lines were plated (2.5 × 10^5 cells/10-cm plate), and after 14 days of growth, the cells were stained with Giemsa and viewed for focus formation. A typical result from one of three independent experiments is shown. B, cells (5 × 10^5) were plated in soft agar and grown for 20 days. A typical field of view is shown. The mean ± S.D. of the number of colonies formed per 5 × 10^4 cells plated from three independent experiments is shown. **, p < 0.001 compared with the NIH 3T3 control.

- p85-R274A
- FLAG
- GAPDH
- B

follows the primary antibodies were used for immunohistochemistry: pPDGFR (pTyr^1577, Santa Cruz Biotechnology, catalog no. sc-12907R; 1/50 dilution), PDGFR (Santa Cruz Biotechnology, catalog no. sc-432; 1/100), pAkt (pThr^308, Santa Cruz Biotechnology, catalog no. sc-16646R; 1/400), Akt (Cell Signaling, catalog no. 9272; 1/20), pMAPK (pThr^202/pTyr^204, Cell Signaling, catalog no. 9101; 1/400), MAPK (BD Transduction Laboratories, catalog no. M12320; 1/100), and FLAG (Sigma, catalog no. F3165). The labeled antigens were visualized with the DAKO autostainer using an EnVision + detection kit according to the manufacturer’s directions using the appropriate secondary antibodies (EnVision + horseradish peroxidase, DAKO, catalog no. K4011 or K4007). Samples were counterstained with hematoxylin (Richard-Allan Scientific). Photos were taken with a Coolpix 990 digital camera using an Eclipse E400 microscope (Nikon) at a total magnification of ×100 unless otherwise indicated.

RESULTS

- p85-R274A

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**FIGURE 3. Reduced growth factor requirements for cells expressing p85-R274A (and to a lesser extent, p85) compared with control NIH 3T3 cells.**

On each indicated day, the amount of cell proliferation was measured with the addition of tetrazolium dye for 4 h. The formazan produced was measured at an absorbance (Abs) of 570 nm. The mean of four independent experiments, each with duplicate samples ± S.E., is plotted. Where no error bars are visible, they are smaller than the size of the symbols on the graph. A, cell proliferation in reduced 2% fetal bovine serum (FBS) medium over 3 days. B, cell proliferation in serum-free medium containing PDGF (50 ng/ml) over 3 days. C, the rate of cell proliferation in fetal bovine serum (2%), plotted as the change in absorbance per day from data in A. The linear fit of rate lines is NIH 3T3 (R² = 0.2312; poor fit because cells are likely dying), p85 (R² = 0.9928), and p85-R274A (R² = 0.9987). D, the rate of cell proliferation in PDGF (50 ng/ml), plotted as the change in absorbance per day from data in B. The linear fit of rate lines is NIH 3T3 (R² = 0.9965), p85 (R² = 1.0000), and p85-R274A (R² = 0.9995).

Regular medium changes. Focus formation was visualized by Giemsa staining (Fig. 2A). The p85-R274A-expressing cell lines developed numerous foci, in contrast to the untransfected control NIH 3T3 and p85-expressing cells, which did not.

Another important property in tumor formation is the ability of cells to grow without attachment. To measure the ability of cells to grow without attachment, cells were seeded into soft agar. After 20 days, cells were visualized microscopically and counted. The p85-R274A-expressing cell lines developed large colonies in soft agar, whereas untransfected control cells and p85-expressing cells did not (Fig. 2B). These results demonstrate that p85-R274A expression is sufficient to cause cells to lose both contact inhibition and to display anchorage independence for growth, both hallmarks of cellular transformation.

We confirmed these results using different clonal cell lines expressing p85-R274A, suggesting that the effects observed were the result of lost p85 RabGAP function. Subsequent experiments were carried out using p85-R274A clone 1 (Fig. 1B).

p85-R274A-expressing Cells Show Reduced Growth Factor Dependence—Transformed cells typically grow better than normal cells under conditions in which exogenous growth and survival factors are limiting. To determine whether there was a difference in the growth properties of cells defective in p85 RabGAP function, cells were grown in low serum medium (2%) or serum-free medium supplemented only with PDGF (50 ng/ml). Both wild-type p85- and p85-R274A-expressing cell lines proliferated better in 2% serum (Fig. 3A) and 50 ng/ml PDGF (Fig. 3B) compared with untransfected control NIH 3T3 cells. This was determined using a non-radioactive cell proliferation assay in which viable cell number was measured. Some portion of this effect appears to be due to the contribution of increased p85 expression (wild-type or R274A); however, the p85-R274A-expressing cells consistently showed greater rates of cell proliferation (Fig. 3, C and D), especially under serum-free medium + PDGF conditions. This is consistent with the transformed phenotype of p85-R274A-expressing cells and with sustained activation of MAPK and Akt pathways in response to PDGF reported previously (20). Because these pathways drive cell proliferation and promote cell survival, this could explain the enhanced growth rates we observed under these serum-depleted conditions.

**p85-R274A Cells Form Tumors in Nude Mice**—To assay the tumorigenic potential of the p85-R274A cells, we determined whether these cells form tumors in nude mice. We injected four nude mice subcutaneously with either 2.5 × 10⁶ untransfected control NIH 3T3 or p85-R274A-expressing cells and monitored tumor development (Fig. 4A). After 16 days, mice injected with p85-R274A-expressing cells started to form tumors that rapidly grew in volume (Fig. 4, A and B). All mice injected with p85-R274A-expressing NIH 3T3 cells developed tumors, whereas none of the mice injected with control cells developed tumors. Nude mice injected with p85-expressing NIH 3T3 cells also did not form tumors (data not shown). Tumors from p85-R274A mice were excised, paraffin-embedded, sectioned, and stained with hematoxylin-eosin (Fig. 4C). The tumor is seen infiltrating the muscle (Fig. 4C, top). The staining also showed that the tumors had a spindle cell pattern of a fibrosarcoma, consistent with a tumor of fibroblast origin (Fig. 4C, middle). The tumors were found to be highly proliferative, invasive, and well vascularized with 50–60 mitotic events per 10 high-powered microscopic fields (Fig. 4C, bottom).

An immunohistochemical analysis of the p85-R274A tumors demonstrated high levels of FLAG-p85-R274A expression in tumor tissue. We also detected substantial PDGFR expression in tumor tissue (Fig. 5C), and some PDGFR was activated, as assessed using the phosphospecific antibody to Tyr457 within the receptor kinase domain, indicative of its activation state (pPDGFR pTyr457) (Fig. 5B). We noted minimal activation of MAPK (pMAPK pThr202/pTyr204) (Fig. 5, E and F) but high levels of Akt activation (pAkt pThr308) (Fig. 5, H–I) in tumor tissue, two distinct downstream signaling targets of activated PDGFR. Thus, in vivo as in cell culture, the PDGFR and its downstream MAPK and Akt signaling pathways are activated in RabGAP-defective p85-R274A-expressing cells. As controls, tumor sections were probed with secondary antibodies alone to demonstrate an absence of nonspecific staining (Fig. 5, D and G). These results clearly demonstrate the physiological consequence of p85-R274A expression is tumor formation with activated PDGFR signaling, especially via the Akt cell survival pathway.

**Enhanced PDGFR Signaling and Cell Transformation of p85-R274A Are Attenuated by Rab5-S34N**—Expression of the RabGAP-defective p85-R274A mutant resulted in enhanced...
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PDGFR activation, downstream signaling primarily to the MAPK pathway (20), and cellular transformation. We showed previously in vitro that p85-R274A mutation compromises intrinsic p85 RabGAP activity toward Rab5, suggesting that expression of p85-R274A may alter the regulation of Rab proteins in cells. Thus, expression of p85-R274A may displace endogenous p85 and maintain both Rab5 and Rab4 in their active GTP-bound state, promoting rapid PDGFR endocytosis (Rab5-GTP) and/or recycling (Rab4-GTP) (Fig. 6A).

Receptor tyrosine kinase signaling to the Akt pathway is thought to originate from receptor activation of the PI3K/Akt pathway at the plasma membrane. In contrast, Ras/MAPK signaling has been proposed to emanate from intracellular signaling endosomes and thus continue during endocytosis and trafficking of the receptor to the early/sorting endosome (26–34). By blocking PDGFR endocytosis using a Rab5 dominant negative mutant (Rab5-S34N), we expected to primarily block MAPK signaling to a greater extent than Akt signaling. In addition, expression of Rab5-S34N would act upstream of the p85-R274A RabGAP defect and therefore be sufficient to counteract the proposed rapid endocytosis and recycling of activated PDGFR (Fig. 6A). To test this model, we determined whether coexpression of a dominant negative Rab5-S34N mutant, with preferential binding to GDP, would block the effects of p85-R274A expression on PDGFR activation, signaling, and cell transformation.

After 60 min of PDGF stimulation, PDGFR remained activated and highly expressed in p85-R274A-expressing cells, whereas coexpression of Rab5-S34N with p85-R274A caused reduced PDGFR activation and receptor levels (Fig. 6B). Activation of downstream targets including MAPK and Akt was also evaluated upon coexpression of Rab5-S34N, assessed using phosphospecific antibodies to their activated forms (Fig. 6C). Coexpression with Rab5-S34N was sufficient to reduce pMAPK levels in p85-R274A-expressing cells as our model predicted. These results indicate that expression of a dominant negative Rab5-S34N mutant can block the ability of p85-R274A expression to cause sustained PDGFR activation, expression, and signaling.

To test whether this would also revert the transformed phenotype of p85-R274A expression, the biological effect of Rab5-S34N expression on cell morphology (Fig. 7A), contact inhibition (Fig. 7B), and anchorage-independent growth properties (Fig. 7C) of p85-R274A-expressing cells was determined. Coexpression of Rab5-S34N was capable of reverting the transformed cell morphology of p85-R274A-expressing cells to a more flattened, normal appearance (Fig. 7A). Results from both tumorigenic assays showed that expression of Rab5-S34N had a dominant negative effect to block the oncogenic properties of p85-R274A expression, reducing the number of foci as well as the size and number of colonies in soft agar (Fig. 7, B and C). The ability of Rab5-S34N expression to revert the transformed phenotype of p85-R274A-expressing cells supports the postulate that Rab5 acts upstream of the p85-R274A RabGAP defect.

DISCUSSION

Many types of tumors have increased RTK levels that are thought to drive aberrant cell proliferation and cell survival (1, 35, 36). In some cases, high RTK expression is known to be the result of gene amplification or deregulation of receptor transcription (35). A newly emerging cause for increased RTK levels has recently been suggested to result from defects in receptor-mediated endocytosis and degradation pathways (1). We have shown previously that p85a has intrinsic GAP activity regulating several small monomeric G proteins, including Rab5 and Rab4 (20). These Rab GTPases play critical roles in down-regulation and degradation of activated growth factor receptors via regulation of vesicle fusion during receptor endocytosis and recycling. We have also shown that expression of the RabGAP-
deficient p85-R274A mutant substantially decreases the rate of PDGFR degradation upon PDGF stimulation. Furthermore, these receptors are highly tyrosine-phosphorylated and cause sustained activation of MAPK and Akt signaling pathways, known to promote cell division and survival (20). The p85-R274A mutant protein was shown to retain its ability to bind to activated PDGFRs and to associate with the p110 catalytic subunit of PI3K, resulting in normal levels of PDGF-stimulated PI3K activity (20). Thus, the RabGAP-deficient p85-R274A exerts its biological effects through a PI3K-independent process. We have subsequently shown that p85-R274A-expressing cells have several characteristics indicative of deregulated growth, including loss of contact inhibition for growth, growth independent of attachment, and the ability to form tumors in nude mice. Thus, p85-R274A-expressing cells possess the hallmarks of transformed cells, and we further demonstrated that a dominant negative Rab5-S34N mutant could block p85-R274A-induced transformed properties.

Two other Rab5 GAPs, PRC17 and Tre2, are known oncogenes. Overexpression of wild-type PRC17 or mutated non-functional Tre2 transforms cells (37), suggesting a role for GAPs in cancer. There is a small but growing number of reports that Rab proteins and other regulators of endocytosis can be deregulated in cancers. There are several endocytic proteins (v-Cbl, Hip1, and Tsg101) that are known to be transforming when their expression is altered or they are mutated (reviewed in Refs. 17 and 38).

There is also evidence that expression of Rab proteins themselves is altered in cancers. Rab20 is expressed in pancreatic cancer (39), and although the function of Rab20 is not well characterized, it is known to localize near the Golgi body and apical plasma membranes, suggesting that it may regulate trafficking between these two membranes (39, 40). The rab32 gene is hypermethylated in gastric and endometrial adenocarcinomas (41). The Rab32 protein is involved in mitochondrial fission and anchoring protein kinase A to membranes (42). Members of the Rab11 family, which are involved in endocytic recycling pathways, are overexpressed in cancers of the skin, ovary, and breast (43, 44). This provides further evidence that alterations in receptor endocytosis and/or recycling pathways may contribute to human cancers. These data suggest that there may be a novel class of oncogenes that increase receptor levels and enhance cellular signaling by modulating the endocytosis, recycling, and degradation of growth factor receptors.

This is the first description of a subtle point mutation in p85α capable of transforming cells into tumor cells by perturbing the Rab-mediated regulation of PDGFR activation, signaling, and down-regulation. To date, there are relatively few studies that have looked for p85α mutations in human cancers or other diseases (45–49). Truncation of p85α because of a premature stop codon (p65; amino acids 1–571) or a frameshift mutation between these two membranes (39, 40). The rab32 gene is hypermethylated in gastric and endometrial adenocarcinomas (41). The Rab32 protein is involved in mitochondrial fission and anchoring protein kinase A to membranes (42). Members of the Rab11 family, which are involved in endocytic recycling pathways, are overexpressed in cancers of the skin, ovary, and breast (43, 44). This provides further evidence that alterations in receptor endocytosis and/or recycling pathways may contribute to human cancers. These data suggest that there may be a novel class of oncogenes that increase receptor levels and enhance cellular signaling by modulating the endocytosis, recycling, and degradation of growth factor receptors.

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4 M. D. Chamberlain, S. F. Poland, and D. H. Anderson, unpublished data.

**FIGURE 5. Tumors express high levels of FLAG-p85-R274A, some activated PDGFR, and substantial levels of activated Akt.** Immunohistochemical analysis of the tumor sections from nude mice injected with FLAG-p85-R274A-expressing cells was carried out by probing with the indicated primary antibodies, followed by secondary antibodies linked to horseradish peroxidase. Adjacent tumor sections probed with different antibodies are shown to allow comparisons of expression of the indicated proteins. Controls are shown in which the primary antibody has been omitted. Total magnification for the images was ×100.
(p76; amino acids 1–636) has been identified in murine (46) and human (45) lymphoma cells, respectively. The C-terminal region missing in these p85α mutant proteins includes all or part of the C-terminal SH2 domain and results in deregulation of the PI3K p110 subunit. Two mutational screens of p85α have also been reported, focusing exclusively on exons encoding the C-terminal portion of the protein in cancer cells (47, 48). Exons 12–15 (amino acids 476–724) were screened for mutations in 12 colon and 2 ovarian cancer cell lines (48). Four defects (three colon and one ovarian) were identified: one with an early stop codon after Asp<sup>605</sup> and three containing small in-frame deletions (ΔLeu<sup>570</sup>–Gln<sup>572</sup>, ΔLeu<sup>570</sup>–Asp<sup>578</sup>, and ΔMet<sup>582</sup>–Asp<sup>605</sup>). These deletions are located near a negative regulatory phosphorylation site (Ser<sup>608</sup>) within p85α and were suggested to cause the observed increased PI3K activity (48). Exons 11–15 (amino acids 454–724) of p85α were screened for mutations in glioblastoma cells, and one contained a stop codon after Gln<sup>579</sup>, again resulting in a truncated p85α protein and increased PI3K activity (47). A study of diabetic patients reported one patient with a mutation in p85α (R409Q) causing impaired insulin-stimulated PI3K activity and insulin resistance, whereas a common p85α sequence variant (M326I) had no effect (49). Our new evidence indicates that a single point mutation (R274A) within the N-terminal RabGAP (also known as the BCR homology) domain of p85α is sufficient for cell transformation. Therefore, a more rigorous analysis of p85α for mutations in human cancers should be undertaken to determine whether similar p85α mutations that compromise its RabGAP activity are present.

The newly discovered ability of p85α to regulate Rab5 and Rab4 GTPases allows it to control receptor trafficking events during receptor-mediated endocytosis and degradation. A single point mutation in p85α (p85-R274A) renders it RabGAP-defective, and cellular expression of this mutant causes high levels of PDGFR activation and signaling resulting in cell transformation. The p85-R274A mutation does not alter its ability to bind and regulate PI3K p110 activity in response to PDGF stimulation (20), suggesting that the effects of this mutation are independent of PI3K activity. These results suggest multiple regulatory functions for the p85α protein in addition to its...
established role in downstream PI3K p110 signaling. The ability of p85α to regulate Rab-mediated endocytosis and receptor trafficking allows it to control the location, magnitude, and duration of PDGFR signaling. Disruption of the temporal control of the GTPase cycles of Rab5 and Rab4 by expression of p85-R274A increases the magnitude and duration of PDGFR signaling, resulting in cell transformation.

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