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Mutation of Ha-Ras C Terminus Changes Effector Pathway Utilization*

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In PC12 cells, Ha-Ras modulates multiple effector proteins that induce neuronal differentiation. To regulate these pathways Ha-Ras must be located at the plasma membrane, a process normally requiring attachment of farnesyl and palmitate lipids to the C terminus. Ext61L, a constitutively activated and palmitoylated Ha-Ras that lacks a farnesyl group, induced neurites with more actin cytoskeletal changes and lamellipodia than were induced by farnesylated Ha-Ras61L. Ext61L-triggered neurite outgrowth was prevented easily by co-expressing inhibitory Rho, Cdc42, or p21-activated kinase but required increased amounts of inhibitory Rac. Compared with Ha-Ras61L, Ext61L caused 2-fold greater Rac GTP binding and phosphatidylinositol 3-kinase activity in membranes, a hyperactivation that explained the numerous lamellipodia and ineffectiveness of Rac(N17). In contrast, Ext61L activated B-Raf kinase and ERK phosphorylation more poorly than Ha-Ras61L. Thus, accentuated differentiation by Ext61L apparently results from heightened activation of one Ras effector (phosphatidylinositol 3-kinase) and suboptimal activation of another (B-Raf). This surprising unbalanced effector activation, without changes in the designated Ras effector domain, indicates the Ext61L C-terminal alternations are a new way to influence Ha-Ras-effector utilization and suggest a broader role of the lipidated C terminus in Ha-Ras biological functions.

Ras proteins are monomeric GTP-binding proteins that operate as inducers of signal transduction cascades regulating cell growth and development (1). They cycle between the GDP-bound inactive and the GTP-bound active form. In their active form, Ras proteins interact with and modulate the activity of effector proteins, including Raf kinases, phosphoinositide 3-kinase (PI3-kinase),[1] Ral guanine nucleotide dissociation stimulator, and AF6 (2–7). These proteins initiate multiple signal transduction cascades that must occur cooperatively to produce a full biological response (8). Effector proteins bind to a small region of Ras termed the effector domain (9), whose core is comprised of residues 32–40. This region is also known as Switch I, as its conformation, along with that of an additional Switch II region (residues 61–77), changes substantially when Ras proteins bind GTP (10).

In addition to GDP/GTP cycling, another requirement for Ras activity involves the correct localization of Ras proteins to the inner surface of the plasma membrane. Plasma membrane binding of Ras is critical for its function because, at least in part, this allows Ras to target its effector proteins to the location where they encounter their substrates or can be activated (11). Newly synthesized Ras proteins are partitioned to the cytoplasmic face of the plasma membrane by a series of post-translational lipid modifications of the C terminus of the protein (12). The first lipid to be attached, a farnesyl group, appears to initiate membrane binding. Recent studies indicate that the endoplasmic reticulum is likely to be the site of first contact, followed by further trafficking to the plasma membrane that occurs through as yet unstudied pathways (13). For Ha-Ras, farnesylation of Cys^{186} is followed by the addition of palmitates to two adjacent cysteine residues (Cys^{181} and Cys^{184}). Native Ha-Ras proteins with all C-terminal cysteines and lipids present are >95% membrane-bound at steady state, whereas Ha-Ras proteins that lack cysteines at 181 and 184 and thus have a farnesyl group as the only lipid are >90% cytosolic (14–17). In addition to the lipids' roles in Ha-Ras targeting and stable association with the plasma membrane, the amino acids of the C terminus also appear to be involved in trafficking of Ha-Ras from the endoplasmic reticulum to the cell surface (17).

An important yet unresolved issue is whether the lipids or residues of the C-terminal region make a further contribution to Ha-Ras function by directly supporting interactions with specific effectors. Recent studies show that farnesyl modification of Ha-Ras is important for high affinity interaction with (18, 19) and full kinase activity of Raf-1 (20–22). Farnesylation is also reported to increase *in vitro* binding of Ha-Ras and KRas4B to p110γ, a PI3-kinase catalytic subunit family member (23). Earlier studies employing a constitutively activated yeast Ras2 protein indicated that interaction between Ras2 and adenylyl cyclase is decreased in the absence of the farnesyl lipid modification (24, 25). A possible role in effector interaction for the lipids and C-terminal amino acids of Ras proteins is also implied by recent work reporting that Ha- and K-Ras proteins differ in their ability to activate Raf-1 and PI3-kinase (26, 27). The major differences between the Ha-Ras and KRas4B proteins are confined to their post-translational lipid modifications and their last twenty-five amino acids. Although both proteins are farnesylated, Ha-Ras is modified by palmitates that are attached to cysteines 181 and 184, whereas KRas4B contains no palmitates and has instead a polybasic domain (lysine residues 175–180). Taken together, these data suggest that the C

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[1] The abbreviations used are: PI3-kinase, phosphatidylinositol 3-kinase; HA, hemagglutinin; 4-Ptase, inositol 4-phosphatase; DRM, detergent-resistant membrane; PAGE, polyacrylamide gel electrophoresis; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; PAK, p21-activated kinase; PtdIns, phosphatidylinositol.

terminus of Ras proteins may provide a mechanism (in addition to that of the internal classical effector domain) for influencing effector interactions.

In previous work we had characterized Ext61L, a constitutively activated Ha-Ras protein in which the C-terminal residue of the CAA*X* motif for farnesylation was replaced with six lysines (28). This design prevented attachment of the farnesyl group but retained the natural sites for palmitoylation at the C terminus. The novel lipidation state of Ext61L presented a new way to determine if the absence of a farnesyl group impaired any signaling pathways, in a protein that was acylated and maintained an interaction with membranes through its C terminus. A shift in Ha-Ras function had already been noted with Ext61L. Expression of the protein in PC12 cells caused an unusual morphological differentiation distinct from that induced by Ha-Ras61L with the native C terminus. Importantly, no changes had been made in the effector binding domain of Ext61L, and its GTP binding properties were preserved (28), indicating that these primary requirements for effector interaction were unaltered. Here we describe results that suggest that the accentuated differentiation produced by Ext61L results from heightened activation of one Ras effector (PI-3 kinase) and suboptimal activation of another (B-Raf). These observations lend support to the idea that Ras-effector interactions may be influenced, in addition to the GTP-sensitive switch regions of the protein, by the C-terminal domain.

MATERIALS AND METHODS

Plasmids and Transfections—Construction of Ha-Ras61L and Ext61L in the pcDNA3 vector has been described previously (28). Amounts of plasmid DNA (in brackets) were titrated so that equivalent amounts of Ha-Ras61L (1 μg of DNA), Ext61L (50 ng of DNA), Ha-Ras (1 μg), or ExtWT (1 μg) proteins were produced in the P100/membrane fraction of transfected cells. Plasmids driving expression of Myc- or hemagglutinin (HA)-epitope-tagged versions of the truncated protein Myc-PAK1-(165–205) (3 μg of DNA) (29) or full-length Myc-Cdc42(17N) (1 μg of DNA), Myc-Rac1(17N) (1 μg or 3 μg of DNA), or HA-Rac1 (7 μg of DNA) were kindly provided by Gary Bokoch (La Jolla, CA). Δp85 (1 μg of DNA) and Myc-RhoA(14V) (1 μg of DNA) in the pEXV3 vector have been described elsewhere (30) and were gifts from Gideon Bollag (Richmond, CA) and Lawrence Quilliam (Indianapolis, IN). Wild type B-Raf and inositol 4-phosphatase (4-PTase) in the pcDNA3 vector were gifts from Geoff Clark (Bethesda, MD) and F. Anderson Norris (Ames, IA), respectively. The MEK inhibitor PD98059 and the PI3-kinase inhibitor LY294002 were purchased from Calbiochem and used at a concentration of 50 μM.

Twenty-four hours before transfection, 1×10^5 PC12 cells were plated onto 60-mm tissue culture dishes coated with laminin (10 μg/ml; Life Technologies, Inc.) and grown overnight. These PC12 cells are derived from an early clone of the original isolate (generously provided by J. H. Pate Skene, Duke University), and if continuously subcultured before reaching high cell density, show high transfection efficiency and very low (<1%) spontaneous neurite extension. Transfections were performed using the LipofectAMINE reagent (Life Technologies, Inc.) as described by the manufacturer. For most experiments the total amount of DNA added to each plate was adjusted to 5 μg using empty pcDNA3 vector DNA. For Rac-GTP binding assays a total of 8 μg of DNA (7 μg of Rac1 + 1 μg of a combination of empty vector and Ha-Ras DNA) was added per dish. Quantitation of the percentage of cells bearing neurites was performed on day 2 when neurites could still be accurately assigned to a particular cell body. Pictures of differentiated cells were taken, and biochemical assays were performed on day 4 when the morphological differences between cells expressing Ha-Ras61L and Ext61L were most distinct and when neurites in both types of Ras-transfected cells were well established. The efficiency of transfection was measured by co-transfection of cells with a β-galactosidase expression plasmid (26) and by counting the percent of cells producing an immunofluorescence signal for Ha-Ras (below). Both methods showed that in cultures that were subjected to transfection, 60–70% of the cells expressed the transfected Ha-Ras protein and that 92–95% of these transfected cells expressed neurites. Expression of either cellular (wild type) forms or the activated Myc-RhoA(19N), Myc-Rac1(12V), or Myc-Cdc42(12V) proteins failed to promote PC12 neurite formation (data not shown) showing, as had been reported previously, that expression of these proteins individually is not sufficient to produce the complex response of neural differentiation (31, 32). Immunoblots using monoclonal antibodies to the HA epitope (Babco, MMS-101R) or to the Myc epitope tag (Santa Cruz Biotechnology, 9E10) confirmed that the dominant negative, cellular, and constitutively activated proteins were expressed (see Figs. 2*B* and 3; other data not shown).

Immunofluorescence—PC12 cells were plated on laminin-coated 18-mm coverslips in serum-containing medium. Within 24 h of plating, the cells were transfected as described above and cultured for 4 days. The cells were then washed in phosphate-buffered saline and fixed in fresh 4% paraformaldehyde in 0.1 M phosphate buffer for at least 30 min at room temperature. Cell membranes were permeabilized by incubation of cells in 0.2% Triton X-100, 5% goat serum, and 0.4% bovine serum albumin in phosphate-buffered saline for 20 min at room temperature. To visualize the F-actin cytoskeleton, rhodamine isothiocyanate-phalloidin (Molecular Probes) in phosphate-buffered saline (1 ml of a 1/400 dilution) was added to each coverslip. After a 20-min incubation, coverslips were washed five times in phosphate-buffered saline, and the coverslips were mounted on a glass slide with Vectashield (Vector Laboratories). Detection of cells expressing Ha-Ras was performed by immunofluorescence using monoclonal antibody Y13–238 (Santa Cruz Biotechnology) at a 1/1000 dilution. Fluorescence was detected with a Nikon FXA microscope equipped with a 60× oil objective (1.0 numerical aperture), and images were captured with a Kodak Megaplus 1.4 CCD camera (Kodak Corp.) connected to a Perceptics MegaGrabber framegrabber (Perceptics Corp.) in a Macintosh 8100/80AV computer using NIH Image. Figures were prepared using Adobe Photoshop 4.0 and Macromedia Freehand Version 8.0 for the Macintosh.

Preparation of Subcellular Fractions, Co-immunoprecipitation, and Immunoblotting—Cytosol and crude membrane fractions were separated by hypotonic lysis and high speed centrifugation as described (33). For detection of interaction between Ha-Ras and PI3-kinase, the P100 membrane-containing pellets were dissolved in buffer A (20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.3 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The S100 cytoplasmic fractions were adjusted to the composition of buffer A and Ha-Ras or PI3-kinase immunoprecipitates formed as described (34) with 150 μg of total protein from each fraction, as determined using the DC Protein Assay kit (Bio-Rad). To learn if detergent-resistant membranes (DRMs) were preserved in the chilled 1% Triton X-100 buffer used for immunoprecipitation, any domains were solubilized, and proteins were released by warming the dissolved lysates for 2 min at 37°, followed by normal formation and washing of the immunoprecipitates (35). In addition, the protein concentration in these samples was 150 or 300 μg/ml, far less than the 5 mg/ml needed to retain DRM integrity. To detect interaction between Ha-Ras and B-Raf, P100 membrane pellets were dissolved in buffer B (70 mM β-glycerophosphate (pH 7.2), 100 μM Na_3VO_4, 2 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 1 mM dithiothreitol, 5 μg/ml leupeptin, and 20 μg/ml aprotinin). The S100 fractions were adjusted to the composition of buffer B (36), and 150 μg of total protein from each fraction were used to form either Ha-Ras or B-Raf immunoprecipitates. Ha-Ras, B-Raf, or PI3-kinase proteins were immunoprecipitated using anti-Ha-Ras rat monoclonal antibody (3E4-146, Quality Biotech), anti-p85α rabbit polyclonal serum (06–195, UBI), or anti-B-Raf rabbit polyclonal serum (C19, Santa Cruz Biotechnology). Immunoprecipitates were captured on protein G-agarose beads (Life Technologies, Inc.), washed three times in their respective lysis buffer, and analyzed for co-immunoprecipitating proteins by immunoblotting with a p85α-specific mouse monoclonal antibody (UBI), Ha-Ras mouse monoclonal (3E4–146, Quality Biotech), or B-Raf goat polyclonal antibody (SC166, Santa Cruz Biotechnology). Peroxidase-labeled secondary antibodies (anti-mouse or goat, Pierce) were used with development by ECL (Pierce) using the manufacturer's protocol.

In Vitro Lipid and Protein Kinase Assays—PI3-kinase lipid kinase activity was measured using the *in vitro* assay described by Ref. 34. Anti-p85 immune complexes were prepared from 300 μg of whole cell lysates or from 150 μg (each) of S100 and P100 fractions and incubated with [γ-^{32}P]ATP (10 μCi/reaction, ICN) and phosphatidylinositol (10 μg/reaction, Sigma) for 10 min. The phospholipids were extracted in $CHCl_3$:CH_3OH (1:1) and separated by thin layer chromatography on potassium oxalate-coated silica plates (Analtech) developed in propanol:2 M acetic acid (65:35). Radioactive ^{32}P-phosphatidylinositol-3 phosphate was detected by autoradiography, and the film images were scanned and quantified using the program ImageQuant (Molecular Dynamics). The radioactive phosphatidylinositol-3 phosphate product

was identified on the basis of co-migration with an unlabeled phosphatidylinositol 4-phosphate standard visualized by iodine staining. Duplicate p85 immunoprecipitates were resolved by SDS-PAGE, and p85 was detected by immunoblotting and quantified by scanning to determine the amounts of p85 captured in the immunoprecipitates.

The activity of the endogenous B-Raf kinase was measured with a coupled *in vitro* kinase assay (37). B-Raf immune complexes from whole cell lysates (300 μg) were collected on protein A-conjugated agarose beads and incubated with nonradioactive ATP and purified recombinant MEK protein (100 μg/ml; provided by Dr. Lee Graves, Chapel Hill, NC). After 10 min, recombinant ERK2 (250 μg/ml; provided by Dr. Lee Graves) was added, and after another 10 min, 250 μg/ml myelin basic protein (Fisher) and [γ-^{32}P]ATP (5 μCi/assay) were added. The reactions were finally terminated 10 min later by the addition of 100 mM EDTA; reaction products were spotted onto P-81 phosphocellulose paper (Whatman) and washed in 10% phosphoric acid, and incorporation of ^{32}P into the precipitated myelin basic protein was quantitated by scintillation counting.

Rac GTP/GDP Binding—At 4 days post-transfection, PC12 cells co-expressing HA-tagged Rac1 (7 μg DNA) and either Ha-Ras61L or Ext61L were incubated overnight in medium containing 1% dialyzed calf serum. Cells were then radiolabeled with 0.5–1 mCi/ml ^{32}P inorganic phosphate (NEN Life Science Products) for 4 h in phosphate-free medium containing 1% dialyzed calf serum. Cells were lysed; samples were precleared of Ha-Ras by immunoprecipitation with antibody 3E4–146, and then the HA-Rac1 proteins were isolated by immunoprecipitation, as described above, using an anti-HA antibody (Babco, MMS-101R). GTP and GDP were separated by thin layer chromatography (28) with the following important changes. Cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 20 μg/ml aprotinin, 1 mM EGTA, 1 mM Na_3VO_4. For more quantitative elution of Rac-bound nucleotides it was necessary to heat the samples to 70 °C for 10 min in 20 μl of buffer containing 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 2% (w/v) SDS, 2 mM GDP, and 2 mM GTP.

RESULTS

Ext61L Induces Exaggerated Morphologic Changes in PC12 Cells—PC12 cells respond to the expression of activated Ras proteins by the cessation of growth and the extension of neurites (38–40). This differentiation process is characterized by rearrangements of the actin cytoskeleton at the plasma membrane, leading to the formation of small lamellipodia, growth cones, and the subsequent extension of axon-like processes (41). Previously, the Ext61L protein had been shown to cause morphologic changes in PC12 cells that were easily distinguishable from those caused by Ha-Ras61L, including an increased number and accelerated rate of outgrowth of neuron-like structures and large lamellipodia with ruffles (28). This exaggerated response suggested that the Ext61L protein produced differentiation signals that were either exceptionally strong or that utilized different signaling pathways than Ha-Ras with a native C terminus and lipid modifications.

To examine if the dramatic external morphological changes caused by Ext61L were accompanied by exaggerated changes in the actin filaments that are normally rearranged during neurite outgrowth, the distribution of filamentous actin was examined by immunofluorescence using rhodamine-conjugated phalloidin. Control cells treated with nerve growth factor formed actin-rich neurites after 4 days, with growth cones visible at the ends of the extending neurites (Fig. 1*, NGF panel*). Cell bodies displayed little flattening and low levels of filamentous actin. Similar changes in the actin cytoskeleton accompanied differentiation triggered by expressing Ha-Ras61L (Fig. 1*, 61L panel*). Expression of Ext61L led to more extensive changes in actin structures. Neurites, often longer than 100 μm, developed within 24 h of transfection and at 4 days post-transfection cells exhibited marked somal flattening and very long, thin, actin-rich neurites displaying extensive branching (Fig. 1, *Ext61L 50 μm panel*). At higher magnification, cortical actin and actin-containing microspikes could be seen in the large membrane ruffles, with short perpendicular

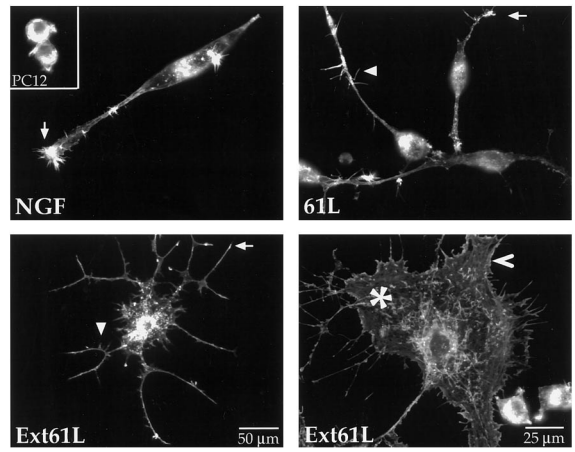

FIG. 1. **Effect of Ext61L on the actin cytoskeleton of PC12 cells.** Fluorescence of rhodamine-conjugated phalloidin was used to visualize polymerized actin in PC12 cells expressing Ha-Ras61L (61L) or Ext61L or treated with 50 ng/ml nerve growth factor for 4 days. Panels labeled PC12, nerve growth factor, or 61L are at the same magnification as the Ext61L panel with the 50-μm scale bar. The *arrows* indicate growth cones; *arrowheads* the microspikes along outgrowths; the *open arrowhead* points toward cortical actin underlying the plasma membrane; and an *asterisk* is placed over a broad ruffle on the flattened cell body. These fluorescent images were typical of cells expressing these constructs, and similar results were recorded in four separate experiments. The scale bar for the higher magnification panel of Ext61L is 25 μm.

filament bundles leading to the edge of the cells and truncated and poorly organized actin filaments throughout the cell interior (Fig. 1*, Ext61L 25 μm panel*)**.** Thus the morphological changes caused by Ext61L involved actin cytoskeletal changes. More importantly, the exaggerated features of the changes caused by Ext61L indicated that Ext61L, although identical to Ha-Ras in its switch I (designated effector domain) and switch II regions, affected the actin cytoskeleton in a distinct way.

Prevention of Neurite Outgrowth by Ext61L Requires Elevated Amounts of Inhibitory Rac1(N17)—Many aspects of actin cytoskeleton regulation, in both fibroblasts and PC12 cells, are controlled by the Rho family of GTPases, which include several Rho, Rac, and Cdc42 proteins (42–44). Each of these proteins has also been reported to function downstream of Ha-Ras and to contribute to the effects of Ha-Ras on the actin cytoskeleton (45–47). The Rac proteins in particular appear to be involved in remodeling of the actin cytoskeleton during formation of ruffles or lamellipodia. The large flattened and ruffled cell bodies of the cells expressing Ext61L suggested that a pathway involving Rac proteins might be strongly activated. Thus more direct studies were undertaken to determine if these Rho family proteins were involved in the unusual changes in the actin cytoskeleton caused by Ext61L.

In PC12 cells, neurite extension has been shown to be reduced when the activity of Rac1, Cdc42, or PAK1 kinase is reduced or when RhoA is activated (31, 32, 48) (see Fig. 6). PC12 cells were therefore co-transfected with DNAs encoding either Ha-Ras61L or Ext61L and DNAs encoding Cdc42(17N), Rac1(17N), PAK-1-(165–205), and RhoA(14V). The length, number and morphology of the resulting neurites were examined 2 days later by phase contrast microscopy, when neurites could still be accurately assigned to a particular cell body. Pictures of differentiated cells were taken on day 4 when the morphological changes caused by Ext61L were most distinct. Because a substantial amount of Ext61L protein is cytosolic, the amounts of the Ras DNAs were carefully titrated to result in expression of equal amounts of each Ras protein in the membranes (P100 fraction) of the PC12 cells (see Fig. 4*C* for example). These amounts of DNA induced ~60% of the cells to

TABLE I
Inhibition of PC12 cell outgrowths

Ras protein expressed[a]	Co-expressed protein or treatment[b]							
	None	RhoA (14V)	Cdc42 (17N)	Pak (165–205)	Rac (17N)[c]	Rac (17N)[d]	LY294002	PD98059
	percentage of cells with outgrowths[e]							
Ha-Ras61L	61 ± 2[f]	25	30	35	32 ± 5	n.d.[g]	34	21
Ext61L	61 ± 1	26	25	27	64 ± 4	35 ± 8	27	16

[a] The percentage of cells that show neurites with empty pcDNA3 vector is <1%.
[b] PC12 cells were co-transfected with plasmids encoding either Ha-Ras61L or Ext61L and, as indicated, Myc-RhoA(14V) (1 μg), Myc-Cdc42(17N) (1 μg), Myc-Pak1(165–205) (3 μg), or Myc-Rac(17N) (1 or 3 μg). Additional cultures were transfected with Ha-Ras plasmids and 5 h later were treated with 50 μM LY294002 or 50 μM PD98059.
[c] Cells transfected with 1 μg of DNA encoding Rac(17N).
[d] Cells transfected with 3 μg of DNA encoding Rac(17N).
[e] Two days later 200–400 cells from each treatment group were counted, and cells with outgrowths longer than twice the diameter of an untransfected PC12 cell (~100 μm) were scored as neurite-bearing.
[f] Numbers represent the average ± S.E. of nine experiments for cells expressing Ext61L, seven for HRas61L, three independent experiments with cells co-expressing Ras and Rac(17N) proteins, and duplicate experiments for all other treatments.
[g] n.d., not determined.

extend outgrowths that at 2 days were at least 100 μm in length (twice the diameter of an untransfected cell). Co-expression of RhoA(14V), Cdc42(17N), or PAK-1-(165–205) reduced the number of cells with outgrowths of this length to ~30% for Ha-Ras61L and ~26% for Ext61L (Table I). This indicated that Ext61L utilized RhoA, Cdc42, and PAK activities to produce neurite outgrowths. In addition, the sensitivity of these pathways to inhibition was roughly similar in cells expressing Ha-Ras61L or Ext61L.

In contrast to the sensitivity of Ext61L-triggered outgrowths to the inhibitory Rho, Cdc42, and PAK proteins, the ability of an inhibitory Rac(N17) to decrease responses of Ext61L-expressing cells was much more limited. At an amount of Rac(N17) expression that strongly suppressed formation of neurites by Ha-Ras61L (Fig. 2, *inset*), cells expressing Ext61L showed no decrease in the rate of neurite elongation or in the number of cells with neurite outgrowths (Table I and Fig. 2*B*). However, the neurites on cells co-expressing Ext61L and Rac(17N) did display a subtle change in morphology; processes were smooth, had only a single growth cone and no branching, and showed fewer ruffles and lamellipodia (Fig. 2*B*). 3-Fold more Rac(17N) DNA was needed to cause clear inhibition of outgrowths from cells co-expressing Ext61L (Fig. 2*C*). This DNA concentration caused production of ~3-fold more Rac(17N) protein (data not shown). To verify that this depressed outgrowth response was not caused by toxicity from a high amount of Rac(17N), the amount of Rac(17N) DNA was held constant and the amount of Ext61L DNA was decreased 5-fold. This protocol also inhibited neurite outgrowth. Thus both the previous observation of numerous lamellipodia and this poor response of Ext61L-induced neurites to Rac(17N) suggested that the Rac proteins might be hyperactivated in the cells expressing Ext61L.

Ext61L Induces a Sustained Increase in GTP-bound Rac1—The possibility of excessive stimulation of the Rac pathway was examined by comparing GTP binding of Rac in cells expressing Ext61L or Ha-Ras61L. PC12 cells were transfected with either Ha-Ras61L or Ext61L, and, to obtain sufficient amounts of Rac for this direct analysis, an epitope-tagged, cellular form of Rac1 (HA-Rac1). This cellular form of Rac would replicate the sensitivity of the endogenous Rac proteins to Ras-mediated effects on guanine nucleotide exchange factors and GTPase-activating proteins. HA-Rac1 caused no effects on formation of outgrowths when expressed in concert with Ras proteins (data not shown). At 4 days post-transfection, when neurites in both types of Ras-transfected cells were well established, the cells were labeled with $^{32}P_i$. To prevent the co-expressed Ras proteins from contributing to the nucleotides to be detected, the Ha-Ras proteins were first removed by immunoprecipitation, then anti-HA

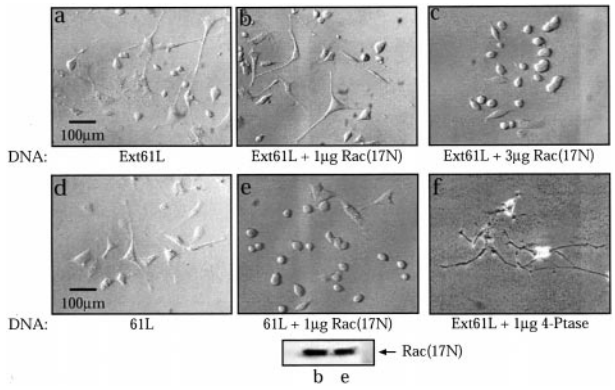

FIG. 2. **Effects of Rac1(17N) on formation of Ext61L-induced outgrowths.** PC12 cells were co-transfected with plasmids encoding Ext61L (*a*, *b*, *c*, and *f*) or Ha-Ras61L (*d* and *e*) and the indicated amounts of Myc-Rac1(N17) (*b*, *c,* and *e*) or 4-Ptase (*f*). After 4 days cells were imaged using phase-contrast microscopy. Expression of Rac(17N) did not alter the expression of the Ras proteins. The *inset* is of an anti-Myc immunoblot showing that similar amounts of Myc-Rac(N17) protein were expressed in cells in *b* (Ext61L) and *e* (61L).

immunoprecipitates were formed, and the radioactive nucleotides bound to the HA-tagged Rac1 were analyzed. Anti-Ha-Ras immunoblots performed on HA-immunoprecipitates prepared from replicate, nonradioactive plates confirmed that no Ha-Ras was present in the Rac1 samples (data not shown). In undifferentiated cells in which only HA-Rac1 had been introduced, the basal amount of GTP-bound HA-Rac1 averaged 8 ± 2% (Fig. 3). Co-expression of Ha-Ras61L caused a modest increase in Rac GTP binding, 1.3-fold, to 10 ± 0.7%. This increase in Rac GTP binding was similar to the level attained during the transient stimulation of Rac GTP binding by PDGF that had been reported previously (49). Increasing the amount of Ha-Ras61L DNA transfected did not further increase HA-Rac1 GTP binding (data not shown), suggesting that with Ha-Ras61L, cellular regulatory mechanisms were able to limit Rac activation to levels only slightly above those in control cells. However, Ext61L stimulated HA-Rac1 more strongly, resulting in a 2.1-fold increase in GTP-bound HA-Rac1 (17 ± 0.8%) over the Mock-transfected cells and a 1.7-fold increase over that in the Ha-Ras61L-transfected cells. Thus even after 4 days, expression of the Ext61L protein continued to support increased GTP binding to Rac1. This sustained activation of Rac quite likely contributes to the formation of the large lamellipodia and other dramatic changes in the PC12 cell actin cytoskeleton.

Ext61L-mediated Neurite Outgrowth Requires PI3-Kinase

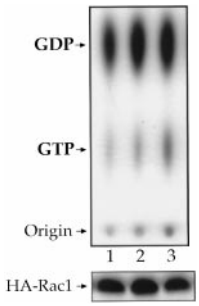

FIG. 3. **Identification of nucleotide bound to HA-Rac1 proteins.** PC12 cells were transfected with HA-Rac1 and either Ha-Ras61L or Ext61L, and 4 days later they were incubated with $^{32}P_i$ for 4 h. The HA-Rac1 protein was immunoprecipitated, and bound nucleotides were eluted, separated by thin-layer chromatography, and detected by autoradiography. The amounts of GTP were quantified by phosphoimager analysis. The averages and S.D., from four independent experiments, of the portion of HA-Rac1 that bound GTP were 8 ± 2% in cells without Ras (*lane 1*), 10 ± 0.7% in cells co-expressing Ha-Ras61L (*lane 2*), and 17 ± 0.8% in cells expressing Ext61L (*lane 3*). An immunoblot (*lower panel*) of replicate plates verified that similar amounts of the HA-Rac1 were present in all immunoprecipitates.

Activity—There are several guanine nucleotide exchange factors that have been reported to be able to stimulate Rac GTP binding (50, 51), but it is not clear which of these might be the relevant guanine nucleotide exchange factor for neurite outgrowth in PC12 cells. Importantly, each of these guanine nucleotide exchange factors contain a pleckstrin homology domain. Recent reports imply that binding of phosphoinositides (PtdIns-3,4-P_2 or PtdIns-3,4,5-P_3) to the pleckstrin homology domain may influence membrane binding or activity of some guanine nucleotide exchange factors (45). To learn if PtdIns-3,4-P_2 was needed for production of the unusual morphology caused by Ext61L, expression of a Type I 4-Ptase was used to deplete this second messenger by removing the phosphate at the 4 position of PtdIns-3,4-P_2, producing the inactive PtdIns-3-P (52, 53). Although 4-PTase did not, on its own, cause differentiation of the PC12 cells (data not shown), co-expression of 4-Ptase with Ext61L caused a moderate reduction in the number of cells that had outgrowths. This reduction was substantial, given that 4-Ptase does not hydrolyze PtdIns-3,4,5-P_3, so this other active phosphoinositide was still present. More notably, in those cells that did have outgrowths, the cell bodies no longer displayed the distinctive lamellipodia, multiple neurites, or branching that characterize Ext61L-mediated differentiation (Fig. 2*F*). Thus, by limiting PtdIns-3,4-P_2 accumulation the exaggerated morphology caused by Ext61L was suppressed and came to resemble more closely the response caused by Ha-Ras61L. This result indicated that phosphoinositides play a role in the abnormal outgrowths caused by Ext61L.

One likely source for these phosphoinositides could certainly be a PI3-kinase, as p110α or p110β isoforms of PI3-kinase catalytic subunits of this enzyme have been identified as Ras effector proteins (54). However, in T lymphocytes and human neutrophils, a second, PI3-kinase-independent pathway linking Ras to Rac activation has been reported (55, 56). Inhibitors of PI3-kinase were used to explore if a PI3-kinase supplied the phosphoinositols used for Ext61L-induced process formation. The PI3-kinase inhibitor LY294002 effectively decreased neurite formation induced by expression of Ext61L (Table I). Similar results were obtained with wortmannin (data not shown). In addition, co-expression of Δp85, a putative dominant negative deletion mutant of the regulatory p85 subunit of PI3-kinase, also effectively suppressed Ext61L-mediated neurite formation (data not shown). These data indicated that neurite outgrowth induced by Ext61L was PI3-kinase-dependent.

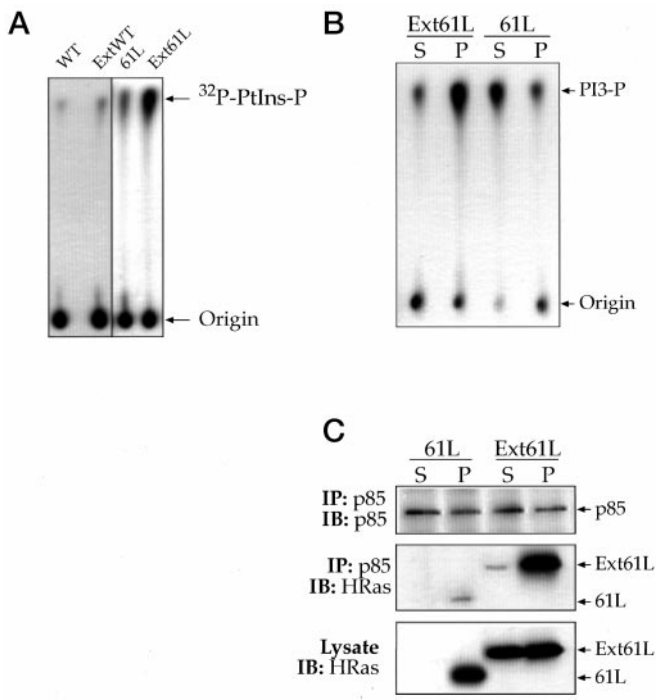

FIG. 4. **PI3-kinase is more active in cells expressing Ext61L.** *A,* whole cell lysates (300 μg of total protein) prepared from PC12 cells expressing cellular Ha-Ras (*WT*), the cellular form of ExtRas (*ExtWT*), Ha-Ras61L (*61L), or Ext61L* were immunoprecipitated using an anti-p85 polyclonal antibody. PI3-kinase activity associated with the immunoprecipitates was measured with an *in vitro* lipid kinase assay using phosphatidylinositol as substrate. The phosphorylated products of the reactions were resolved by TLC, visualized by autoradiography, and quantified by phosphoimager analysis. The *arrow* indicates the radioactive ^{32}P-phosphatidylinositol-3 phosphate (*^{32}P-PtIns-P*). *B,* cytosolic (*S*) and membrane (*P*) fractions were prepared 4 days after transfection from PC12 cells expressing Ha-Ras61L or Ext61L. Anti-p85 immunoprecipitates were formed from 150 μg of protein from each fraction, and activity of the captured PI3-kinase was assessed with an *in vitro* lipid kinase assay. The phosphorylated products were resolved by TLC, visualized by autoradiography, and quantitated by phosphoimager analysis. Ha-Ras and p85 immunoblots of duplicate samples confirmed that equivalent amounts of Ha-Ras and p85 protein were present in the P fractions of the precipitates. An average 30 ± 4% of the total PI3-kinase activity in PC12 cells expressing Ha-Ras61L was membrane-associated, whereas 82 ± 6% of the total PI3-kinase activity in cells expressing Ext61L was membrane-associated. These values represent the average and S.D. of four independent experiments. *C,* cytosolic (*S*) and particulate (*P*) fractions (150 μg of total protein) were prepared from PC12 cells expressing Ha-Ras61L or Ext61L. PI3-kinase immunoprecipitates were formed using an anti-p85 polyclonal antibody and resolved by SDS-PAGE, and p85 (*upper panel*) or Ha-Ras (*middle panel*) was detected by immunoblotting. Separate aliquots of each fraction were taken before immunoprecipitation; acetone precipitated and amounts of Ha-Ras proteins present in the fractions were detected by immunoblotting (*lower panel*). *IP* denotes immunoprecipitation, and *IB* denotes immunoblot.

From all of these results it thus appeared that Ext61L strongly stimulated production of phosphoinositides that led to activation of Rac GTP binding.

Ext61L Activates PI3-Kinase More Strongly than Does Ha-Ras61L—A reasonable hypothesis for how Ext61L might enhance production of 3′-phosphoinositides was through increased binding to or activation of the p110 (α or β) catalytic subunits of PI3-kinase. To test this possibility, the activation states of the endogenous PI3-kinase in PC12 cells expressing Ext61L or Ha-Ras61L were compared. Although experiments to isolate p85 or p110 proteins in an Ha-Ras immunoprecipitate were attempted, the small amount of p85 or p110 that could be captured proved to be insufficient to clearly distinguish PI3-kinase subunits from nonspecific proteins by immunoblot de-

tection. Therefore, immunoprecipitation of p85 (using antibody that allowed co-capture of p110α/p110β subunits) was used to isolate PI3-kinase and obtain a comprehensive picture of the activity of total p110, whether associated with Ha-Ras or not. In cells expressing Ext61L, the total kinase activity of p110 was ~2-fold (1.76 ± 0.5, n = 3) higher than in cells expressing equivalent amounts of Ha-Ras61L (Fig. 4*A*). A similar ~2-fold higher total PI3-kinase activity was also found in cells assayed at 24 h after transfection (data not shown). To test if this increase in p110 activity was dependent upon GTP binding of the Ext61L protein, a largely GDP-bound cellular version, Ext-WT, was examined (28). Total p110 PI3-kinase activity in cells expressing ExtWT was roughly one-third (0.3 ± 0.01, n = 3) of that in cells expressing Ext61L (Fig. 4*A*), indicating that Ext61L GTP binding contributed to the activation of PI3-kinase. However, only membrane-bound Ras is thought to be competent for PI3-kinase activation, and unlike fully membrane-bound Ha-Ras61L with the native lipid modifications, there is a substantial amount of cytosolic, GTP-bound Ext61L (28; see Fig. 4*C*). Therefore a second set of assays was performed to learn if the increase in total PI3-kinase activity resulted from enhanced activation in primarily one location, or in both cytosolic and membrane fractions. In cells expressing Ha-Ras61L, the majority of the PI3-kinase activity was found in the cytosolic fraction (70 ± 4%; Fig. 4*B*). In contrast, in cells expressing Ext61L, more than three-fourths of the PI3-kinase activity was membrane-associated (82 ± 6%; Fig. 4*B*). Cytosolic PI3-kinase activity was definitely not enhanced and instead appeared to be diminished, whereas the activity of PI3-kinase in the membrane fraction was elevated ~2-fold compared with its activity in membranes from Ha-Ras61L-expressing cells. Thus the increase in total PI3-kinase activity caused by expression of Ext61L resulted primarily from increased amount or activity of PI3-kinase that was associated with membranes. The increase in membrane-located PI3-kinase activity could therefore produce 3′-phosphoinositides that might lead to activation of Rac and other cytoskeletal proteins that contribute to actin rearrangements during differentiation of PC12 cells.

To understand more of how Ext61L might be enhancing PI3-kinase activity, the interaction between the p85-p110 PI3-kinase complex and Ras proteins was examined. A modest amount of Ha-Ras61L could be detected in p85 immunoprecipitates prepared from membrane fractions (Fig. 4*C, middle panel*). Because Ha-Ras61L is fully membrane-bound, no protein was detected in the soluble fraction. In cells expressing the Ext61L protein, which is ~60% cytoplasmic, a small amount of Ext61L interacted with the p85 in the cytosolic fraction. However, the amount of Ext61L in the p85 immunoprecipitate from the membrane fraction was 6–10-fold greater than the amount of Ha-Ras61L that bound to p85 in the membrane sample (Fig. 4*C, middle panel*). This increase in PI3-kinase-Ext61L interaction occurred despite the nearly equal amounts of Ext61L and Ha-Ras61L proteins that were available in the P100 samples (Fig. 4*C, bottom panel*). The enhanced interaction did not appear to reflect differential isolation of Ext61L in DRMs that might have been present in the p85 immunoprecipitates, as warming the Triton X-100-containing, resuspended P100 fractions for 2 min at 37 °C to solubilize DRMs and release proteins prior to formation of the immunoprecipitates (35) did not diminish the enhanced amount of Ext61L found in the p85 sample (data not shown). Thus, much more Ext61L than Ha-Ras61L interacted with PI3-kinase, and increased binding was, as with the lipid kinase activity, predominant in the membrane fraction. The sum of all these results suggested that the unusual structure or lipidation of the C terminus of Ext61L, in the absence of any mutation in the effector domain, caused hyperactivation of a PI3-kinase/Rac pathway that contributed to an exaggerated and abnormal program of neuronal differentiation in PC12 cells.

Ext61L Binding to B-Raf Is Increased but Activation Is Decreased—However, the Rac pathway is not solely responsible for differentiation. Cooperation from other pathways is also required for Ras-mediated PC12 cell differentiation. To learn if the novel structure of Ext61L allowed it to activate all Ras effector proteins, or if it selectively stimulated PI3-kinase, the effect of Ext61L on the activity of the B-Raf/MEK/ERK pathway was examined. The compound PD98059 was used to inhibit the activation of MEK (57) and phosphorylation of its targets, ERK1 and ERK2, monitored by immunoblotting. Treatment of PC12 cells with PD98059 almost completely blocked activation of ERKs caused by expression of either Ext61L or Ha-Ras61L (data not shown). In addition, PD98059 blocked Ext61L-induced differentiation of PC12 cells as effectively as it blocked Ha-Ras61L-induced outgrowths (Table I). Thus differentiation induced by Ext61L required MEK activity and appeared to be as sensitive to loss of its activity as Ha-Ras61L.

To more directly assess interaction of Ext61L and an actual effector, endogenous B-Raf was isolated by immunoprecipitation and assayed for the co-immunoprecipitation of Ext61L by immunoblotting. B-Raf immunoprecipitates contained 6–10-fold more Ext61L than Ha-Ras61L and these complexes were found specifically in membrane-containing fractions (Fig. 5*A*). A similar dramatic increase in B-Raf-Ext61L complex formation was observed when a cDNA was used to overexpress B-Raf in PC12 cells co-expressing Ext61L or Ha-Ras61L (data not shown). Thus B-Raf could bind much more Ext61L than Ha-Ras61L, although both proteins bound GTP to the same extent (28). This result was similar to what had been observed for interaction between PI3-kinase and Ext61L and indicated that both effectors formed complexes with Ext61L remarkably well.

Overexpression of B-Raf was then used to ask the reciprocal question of whether the nonfarnesylated Ext61L could bind more B-Raf than could fully lipid-modified Ha-Ras61L. Ras immunoprecipitates were prepared and probed with B-Raf antibodies. In Ha-Ras61L immunoprecipitates from membrane fractions, detectable amounts of the co-expressed exogenous B-Raf protein were observed. However, much more B-Raf was present in Ext61L immunoprecipitates from membrane fractions (Fig. 5*B*), although equivalent amounts of membrane-associated Ha-Ras61L and Ext61L were available (data not shown). This result showed that Ext61L, even though it lacked the farnesyl moiety, could still bind B-Raf.

However, full activation of Raf kinase activity requires much more than simple binding between the designated Ras effector domain and the Ras binding domain of Raf (21). To determine whether increased association between B-Raf and Ext61L was translated into increased activation of B-Raf, endogenous B-Raf was captured by immunoprecipitation and its kinase activity measured using an *in vitro* assay. In cells expressing Ha-Ras61L, B-Raf activity was stimulated 4-fold over basal levels (Fig. 5*C*). In contrast, B-Raf activity in cells expressing Ext61L, although elevated (2-fold), was one-half that of the B-Raf activity in Ha-Ras61L-expressing cells (Fig. 5*C*). Similar results (~4-fold increase in B-Raf activity with Ha-Ras61L but only ~2-fold increase with Ext61L) were found in assays performed 1 day after transfection of the cells (data not shown). Thus, despite the obvious increase in binding between B-Raf and Ext61L, this interaction produced little B-Raf activation.

Because ERK1 and ERK2 play key roles in Ras signaling and can further indicate B-Raf activity, Ext61L effects on ERK phosphorylation in the intact cell were examined. Although

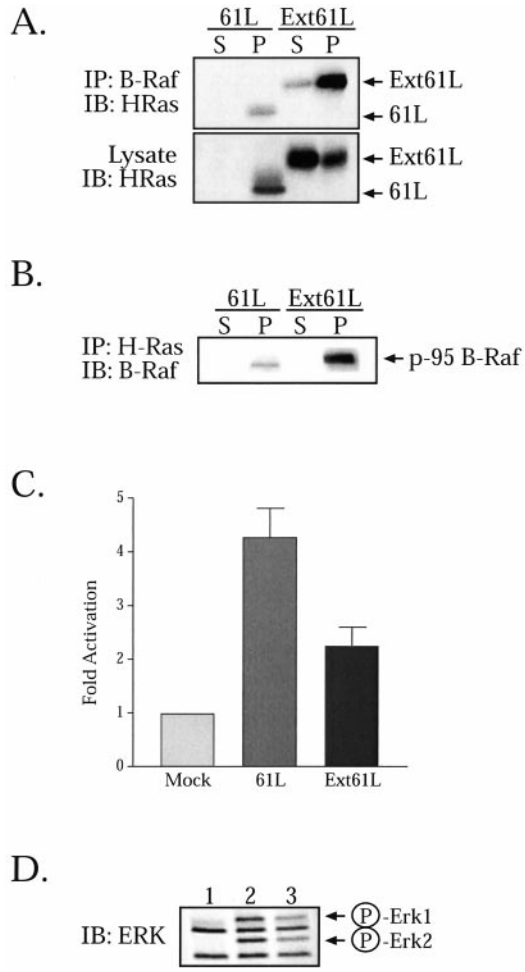

FIG. 5. **Ha-Ras61L expression causes greater activation of B-Raf than Ext61L.** *A,* cytosolic (*S*) and particulate (*P*) fractions (150 μg of total protein) were prepared from PC12 cells expressing Ha-Ras61L (*61L*)or Ext61L proteins. The endogenous B-Raf was immunoprecipitated and resolved by SDS-PAGE, and any associated Ha-Ras protein was detected with Ha-Ras antibody. Separate aliquots of each fraction were taken before immunoprecipitation and acetone precipitated, and amounts of Ha-Ras proteins present in the fractions were detected by immunoblotting. *IP* denotes immunoprecipitation, and *IB* denotes immunoblot. *B,* cytosolic (*S*) and particulate (*P*) fractions (150 μg of protein from each fraction) were prepared from PC12 cells transfected with a plasmid encoding p95 B-Raf along with either Ha-Ras61L or Ext61L DNAs. Four days later the cells were lysed; Ha-Ras protein immunoprecipitates were formed and resolved by SDS-PAGE, and any associated B-Raf protein was detected with B-Raf antibody. *C,* whole cell lysates (300 μg of total protein) were prepared 4 days post-transfection from PC12 cells expressing Ha-Ras61L or Ext61L. B-Raf immunoprecipitates were formed, and coupled immune complex kinase assays were performed utilizing recombinant Mek1 and ERK2 proteins and [γ-^{32}P]ATP to ultimately label myelin basic protein as a substrate. The radioactivity incorporated into myelin basic protein was quantified by scintillation counting. B-Raf activity in mock-transfected cells was set to 1.00. In cells expressing Ha-Ras61L, B-Raf activity averaged 3.95 $\pm$ 1.4-fold higher; in cells expressing Ext61L, B-Raf activity averaged 2.28 $\pm$ 0.58-fold higher than mock-transfected cells. These values represent the average and S.D. of four independent experiments. A representative B-Raf activity in Ha-Ras61L transfected cells was 16,014 cpm. *D,* whole cell lysates (300 μg of total protein) were prepared from PC12 cells that were mock-transfected (*lane 1*) or cells expressing Ha-Ras61L (*61L*) (*lane 2*) or Ext61L (*lane 3*) proteins 4 days after transfection and displayed by SDS-PAGE. ERK1 and ERK2 proteins were detected by immunoblotting. ERK activation is indicated by the amount of phosphorylated ERK (*P-ERK*). *IB* denotes immunoblot.

Ha-Ras61L expression caused robust phosphorylation of both ERK1 and ERK2, Ext61L expression produced a noticeably smaller increase (Fig. 5*D*). The poor ERK response suggests either that Ext61L does not efficiently activate the B-Raf/MEK/ERK pathway within cells or that other effects of Ext61L down-regulate this pathway. The poor stimulation of B-Raf activity by Ext61L was very different from its hyperactivation of PI3-kinase activity and demonstrates that effects of Ext61L vary between effectors, leading to unbalanced use that may further contribute to its unusual activity.

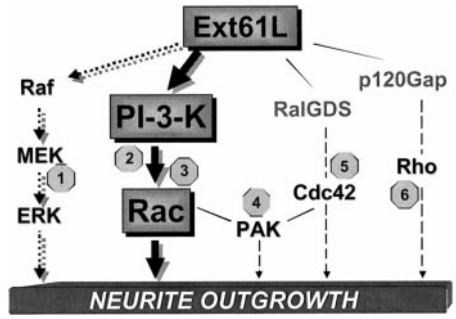

FIG. 6. **Schematic signaling pathways of Ext61L in PC12 cell neurite outgrowth.** Several of the Ras effectors proteins and some of the pathways through which they are proposed to contribute to neurite outgrowth are depicted. Not all effectors, proposed pathways, or connections are shown. The hierarchy of interactions between the Rho family GTPases reported in fibroblasts may not occur in neural cells. The octagonal "stop signs" indicate positions where pathways were inhibited by the following compounds or proteins: *1,* PD98059 inhibitor of MEK; *2,* LY294002 inhibitor of PI3-kinase; *3,* Rac(17N); *4,* PAK1-(165–205); *5,* Cdc42(17N); *6,* RhoA(14V).

DISCUSSION

Activation of a PI3-Kinase Pathway—Ext61L appears to define a new class of PI3-kinase selective Ha-Ras protein in which C-terminal alterations in Ha-Ras can lead to an enhancement of the activity of this effector protein and its targets (Fig. 6). The data presented here also provide support for a model in which binding of effector proteins to Ras-GTP, in addition to a required and nondiscriminating interaction with the classical Ras effector domain, is influenced by the lipidated C-terminal domain.

The hyperactivation of a PI3-kinase/Rac pathway found in these studies is likely to play a key role in the prominent, actin-based morphological changes observed in PC12 cells that express Ext61L. Expression of either cellular (wild type) forms or the activated Myc-RhoA(19N), Myc-Rac1(12V), or Myc-Cdc42(12V) proteins failed to promote PC12 neurite formation,[2] as had been reported previously (31, 32). Preliminary studies also indicated that co-expression of Ha-Ras61L with a constitutively active form of PI3-kinase (p110-CAA*X*) could produce many, but not all, of the morphological events seen with Ext61L.[2] Thus multiple pathways must participate cooperatively to produce the coupled and complex program of cessation of cell cycling and neurite outgrowth that produce differentiation of PC12 cells. Future efforts will require determining how other effector pathways, such as Cdc42 and Rho, contribute to the unusual cytoskeletal changes caused by Ext61L.

There are two ways that seem most likely to explain how Ext61L may enhance PI3-kinase signaling. Ext61L may be distributed in the plasma membrane in a location that favors PI3-kinase activity once the enzyme is bound to Ha-Ras through normal means. This model emphasizes the traditional role of the C terminus in lipid bilayer interactions. However, the multiple lysines and lack of a farnesyl group in Ext61L may also alter the conformation of the C terminus, particularly when it is in contact with the bilayer. These unique C-terminal features of Ext61L may allow it to bind PI3-kinase more avidly.

[2] M. A. Booden and J. E. Buss, unpublished results.

This model focuses on a new, more direct role for the C terminus in protein interaction that is separate from, but could easily cooperate with, its use in membrane binding.

Contribution of Membrane Binding to Ext61L Function—Ext61L differs from the many other C-terminal mutants of Ha-Ras. Most C-terminal alterations have caused mislocalization to internal membranes (17) or prevented membrane binding entirely. Such C-terminal mutant proteins display poor biological activity. The results presented here indicate that the alterations in the lipidation and C terminus of Ext61L, rather than being detrimental to function, actually enhance the ability of the protein to cause differentiation of PC12 cells, as shown by the multiple and branched neurite outgrowths and the large lamellipodia. A distinctive aspect of Ext61L is the large portion (60%) of the protein that is present in the cytosol. This cytoplasmic protein lacks lipids but retains the six additional lysines and, having the activating 61L mutation, is GTP-bound (28). However, neither PI3-kinase nor B-Raf interacts well with this protein (see Figs. 4*C* and 5*A*). This result indicates that the lysines in the Ext61L protein cannot by themselves be responsible for the enhanced effector binding and that soluble Ext61L likely contributes little to the signals produced by Ext61L. More importantly, these data suggest that these two effectors can discriminate between cytosolic and membrane-bound Ext61L, even when this Ha-Ras protein is in a GTP-bound form. The results therefore indicate that effector binding can be influenced by more than the classical effector domain or activation state of Ha-Ras and point out that lipid modification and/or membrane binding of the C-terminal domain are crucial for this regulation.

Within the plasma membrane, Ha-Ras may further partition into glycolipid-enriched membrane subdomains ("rafts"). It is suspected that both of the lipids attached to Ha-Ras play a significant role in this lipid bilayer distribution, but their individual contributions remain unresolved (58–60). The presence of an isoprenoid is reported to inhibit such partitioning, whereas palmitoylation encourages targeting to rafts. The effect of the combination of palmitates and lysine residues in Ext61L is not known. Our current co-immunoprecipitation results suggest that enhanced interactions of Ext61L and PI3-kinase can still be detected even under conditions in which rafts (DRMs) are solubilized (35). We have previously shown that Ext61L is successfully targeted to the plasma membrane (28), but additional studies will be needed to learn if the novel lipidation of Ext61L causes it to partition between rafts and the general lipid bilayer differently from fully lipidated Ha-Ras. An abnormal distribution might contribute to Ext61L's strong activation of PI3-kinase by either improving access of p110 to Ext61L-GTP or by enhanced proximity of Ras-bound p110 to its phosphatidylinositol substrates (61). In either case, the increased production of such lipid products will activate a variety of pleckstrin homology domain-containing proteins that can regulate the actin cytoskeleton, as shown here (45, 62), and others, such as members of the Akt/protein kinase B family that stimulate pathways that regulate cell survival (62–64). The dramatic increase in co-immunoprecipitation of Ext61L by both PI3-kinase and B-Raf clearly indicates that the novel C-terminal structure of Ext61L has a substantial influence on interactions with these two effectors. These data are compatible with a mechanism involving change in access of effectors to Ras. However, although the amount of Ext61L that interacted with PI3-kinase was >5-fold more than with Ha-Ras61L, a similar increase was also seen with B-Raf and was thus nonselective and cannot explain the differential activation of these effectors. Whereas the current studies do not resolve these issues, the use of Ext61L should facilitate characterization of how membrane distribution, engagement, and activation of these two effectors are interrelated.

Involvement of the Ext61L C Terminus in Effector Interaction—The additional argument can be made that changes in Ext61L membrane binding simply augment more direct changes in the interaction between Ext61L and p110 or B-Raf. Ext61L is exceptional in that it unbalances effector utilization without introducing mutations in the effector domain or affecting GTP binding. Previous studies seeking to correlate binding of individual Ras effectors with biological activity have exploited Ras proteins with mutations in the classical effector domain (residues 32–40). These individual point mutations in the effector domain, although remarkably able to prevent binding of the targeted effector, also weaken the activation of the remaining effectors (65), and the cumulative biological result is impaired Ras function. The retention of effector domain structure in Ext61L can be inferred by the sensitivity of Ext61L function to such mutations in the classical effector domain; a Ext61L(Y40C) mutant no longer causes neurite outgrowth.[3] In contrast to the effector domain mutants, at least 2 cellular responses mediated by Ext61L remain robust. The transforming activity of Ext61L in NIH 3T3 cells is equivalent to that of Ha-Ras61L (28). In PC12 cells Ext61L is exceptionally potent, and even the cellular version, ExtWT, can cause outgrowth of neurites (28). However, ExtWT activates PI3-kinase much less well than Ext61L in PC12 cells and shows an appropriate lack of transforming activity in NIH 3T3 cells. This indicates that although the C-terminal extension imparts several novel properties to Ext61L, GTP binding enhances Ext61L signaling and remains an important component of the signaling mechanism. The current findings thus indicate that Ext61L unbalances effector utilization and activates biological signaling through a unique mechanism that differs from that caused by the effector domain mutants.

Because the classical effector domain of Ext61L appears unchanged, the unique C-terminal structure of Ext61L appears to be the source of increased interaction between Ext61L and the two effectors PI3-kinase and B-Raf. As noted above, the multiple lysines cannot be solely responsible for this enhanced effector binding, as they are present on the cytosolic protein that binds both B-Raf and PI3-kinase poorly, although they are likely to contribute to binding in the membrane form of Ext61L. These results indicate that enhanced interaction with PI3-kinase requires juxtaposition of the C terminus of Ext61L to the membrane. This raises the possibility that when the C terminus of Ext61L (and perhaps that of native Ha-Ras as well) interacts with the lipid bilayer, this domain might adopt a definable tertiary structure important for effector interactions. The possibility of a nonrandom structure of the membrane binding domain of Ha-Ras has not been examined previously. This lack of attention comes, in part, because although the three-dimensional structures of several forms of Ha-Ras have been solved, these structures are missing the last ~20 amino acids and provide no information on the placement of the C terminus or its lipids. Such information may now be obtainable using the recently reported success of bioorganic synthesis of lipidated Ha-Ras proteins (66). Importantly, the data from Ext61L suggest that at least two effector proteins may sense C-terminal conformation. This model further implies that lipid modifications of Ha-Ras might play a direct role in imposing or contributing to this structure. Such a prospect would broaden the job description of lipids beyond targeting and partitioning of Ha-Ras in the membrane. Our work with Ext61L and a second, nonfarnesylated Ha-Ras with C-terminal palmitates

[3] M. A. Booden and J. E. Buss, unpublished data.

show that palmitates can physically support partial Ha-Ras membrane binding, despite their dynamic turnover in the cell (28, 67). Those results support the possibility that in native Ha-Ras the farnesyl group could be free for roles besides membrane tethering.

Is a Farnesyl Group Involved in Effector Activation?—The exceptional activation of PI3-kinase by Ext61L implies that stimulation of this lipid kinase by Ha-Ras can occur even if the Ras protein lacks farnesyl. The increased binding of PI3-kinase to Ext61L, perhaps in conjunction with improved access to membrane phosphatidylinositols, appears sufficient to explain this enhancement. B-Raf can also bind Ext61L quite well, implying that a farnesyl group is not a requirement for binding of this second effector.

However, the most unexpected feature of Ext61L signaling was that the increased physical interaction between Ext61L and B-Raf was not translated into increased B-Raf activity. *In vitro* assays detected moderate B-Raf activity in cells expressing Ext61L, and in intact cells the MEK/ERK pathway was still functional, and its activity was required, as shown by inhibition of neurite outgrowth with PD98059. However, neither B-Raf nor its pathways were stimulated by Ext61L as well as by native Ha-Ras61L. Such impairment could arise indirectly from the hyperactivation of the PI3-kinase. The phospholipid-stimulated kinase Akt has recently been reported to phosphorylate and inhibit Raf-1 (68).

A second intriguing possibility is that B-Raf is poorly activated because Ext61L lacks a farnesyl. Recent studies have demonstrated that full activation of Raf-1 requires interaction of the Raf-1 cysteine-rich domain with Ras (22, 69). The Raf-cysteine-rich domain interaction with Ha-Ras is distinct from the interaction with the classical effector domain and independent of the guanine nucleotide state of Ha-Ras and seems to require C-terminal post-translational lipid modification of Ha-Ras. Additional reports indicate that there is a structural conformation present in the prenylated form of Ha-Ras that is needed to induce full activity of Raf-1 and B-Raf (19, 69). The suboptimal activation of B-Raf by a nonfarnesylated Ext61L tends to support this model. It is not yet clear if this "structure" is the farnesyl group itself needed for a direct physical interaction with the Raf protein or if it is a conformation of the broader C-terminal domain that is induced by the presence of a farnesyl group (and palmitate) and their interaction with the lipid bilayer. Because Ext61L maintains an interaction with membranes through the C-terminal domain, this nonfarnesylated protein provides an excellent tool to study the contributions of a farnesyl group to Ras-effector interactions and the effects of its absence in the physiological context of the intact cell.

To study the role of the C terminus in Ha-Ras signaling, both membrane binding and activity must be retained. In contrast to the poor or even absent activity seen with previous C-terminal mutants, Ext61L displays membrane binding and strong biological function and thus provides the first available model for testing the roles of the farnesyl group and palmitates in the setting of the intact cell. The unbalanced effector utilization induced by Ext61L shows that changes in the lipidation and C-terminal residues of Ha-Ras can also be used to manipulate signaling of PI3-kinase and B-Raf. Using further alterations of the C terminus it may be possible to develop biologically functional, pathway-selective Ras proteins. It may eventually be possible to use the C terminus or mimics of its structure to interfere with specific, oncogenic Ras signaling pathways.

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