Distinct Protein Phosphatase 2A Heterotrimers Modulate Growth Factor Signaling to Extracellular Signal-regulated Kinases and Akt*

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A key regulator of many kinase cascades, heterotrimeric protein serine/threonine phosphatase 2A (PP2A), is composed of catalytic (C), scaffold (A), and variable regulatory subunits (B, B′, B″ gene families). In neuronal PC12 cells, PP2A acts predominantly as a gatekeeper of extracellular signal-regulated kinase (ERK) activity, as shown by inducible RNA interference of the Aα scaffolding subunit and PP2A inhibition by okadaic acid. Although okadaic acid potentiates Akt/protein kinase B and ERK phosphorylation in response to epidermal, basic fibroblast, or nerve growth factor, silencing of Aα paradoxically has the opposite effect. Epidermal growth factor receptor Tyr phosphorylation was unchanged following Aα knockdown, suggesting that chronic Akt and ERK hyperphosphorylation leads to compensatory down-regulation of signaling molecules upstream of Ras and blunted growth factor response. Inducible exchange of wild-type Aα with a mutant with selective B′ subunit binding deficiency implicated PP2A/B′ heterotrimers as Akt effectors. Conversely, silencing of the B-family regulatory subunits Bo and Bδ led to hyperactivation of ERK stimulated by constitutively active MEK1. In vitro dephosphorylation assays further support a role for Bo and Bδ in targeting the PP2A heterotramer to dephosphorylate and inactivate ERKs. Thus, receptor tyrosine kinase signaling cascades leading to Akt and ERK activation are modulated by PP2A holoenzymes with distinct regulatory properties.

At least 99% of protein phosphorylation in eukaryotic cells occurs on Ser and Thr residues. The greater than 300 protein Ser/Thr kinases in the human genome are opposed by less than 30 protein Ser/Thr phosphatases, of which protein phosphatase 1 and protein phosphatase 2A (PP2A)3 contribute the bulk of activity in most cell types. The notion of Ser/Thr phosphatases as promiscuous and constitutively active enzymes that simply provide the substrates for regulated kinase signaling has been challenged by the discovery of batteries of catalytic subunit-interacting proteins, which impart substrate specificity, subcellular localization, and responsiveness to phosphorylation (1, 2). In the case of PP2A, the predominant holoenzyme is formed by association of a core dimer of catalytic and scaffold subunits with one of a least 12 regulatory subunits in vertebrates. Since vertebrate A and C subunits are each encoded by two genes and since many regulatory subunits are diversified by alternative splicing, several dozen different PP2A heterotrimers are likely to exist in any given cell type. The three unrelated regulatory subunit gene families (B′, B″) are almost certain to utilize different mechanisms to control enzymatic activity and cellular localization of PP2A. The B-family (PR55) of regulatory subunits consists of predicted β-propellers with divergent N-terminal tails that act as subcellular targeting signals (3–5). In addition to interacting with phosphatase substrates (6–12), B′-family (also referred to as B56, PR61) subunits are heavily phosphorylated, which may confer regulation by second messengers (13–16). Members of the B′-family of PP2A subunits (PR72/130, PR59, PR48) feature two calcium binding EF hands with presumed structural rather than regulatory functions (17). Striatin and SG2NA have been referred to as B″ regulatory subunits (18). It may be more appropriate to refer to them as PP2A dimer-associated proteins since their stability does not depend on association with the PP2A core enzyme (17, 19) and since they lack the A subunit binding consensus motif common to B′, B″, and B′′ subunits (20).

A growing body of evidence indicates that PP2A has complex inhibitory and stimulatory effects on hormone and growth factor signaling, in particular the extracellular-signal-regulated (ERK)/mitogen-activated protein kinase (MAPK) cascade (21). PP2A substrates include G-protein-coupled receptors and receptor tyrosine kinases (22–24), receptor-associated proteins (25–28), and all three kinases of the ERK/MAPK cascade core module (Raf (29–32), MAPK/ERK kinase (MEK) (33–35), and ERK (36, 37)).

Here, we have begun to dissect the contribution of different PP2A holoenzymes to growth factor signaling in PC12 cells. The net effect of total PP2A silencing or inhibition was ERK and Akt hyperphosphorylation, most likely as a consequence of direct dephosphorylation of these kinases by PP2A. Cautioning against exclusive reliance on silencing approaches, protracted inhibition of PP2A by RNA interference (RNAi) resulted in a compensatory uncoupling of growth factor receptors from their kinase effectors. A combination of mutant Aα subunit exchange, regulatory subunit RNAi, and in vitro dephosphorylation assays indicated that PP2A/B′ heterotrimers regulate Akt, whereas PP2A/Bo and Bδ directly dephosphorylate ERKs.

EXPERIMENTAL PROCEDURES

Cell Culture—Parental PC6-3 cells were cultured (37 °C, 5% CO2) in RPMI 1640 containing 10% horse and 5% fetal bovine serum (both heat-inactivated). Aα-RNAi cell medium was supplemented with 2 μg/ml blasticidin and 200 μg/ml G418 to maintain tetracycline repressor and
inducible short hairpin (sh)RNA constructs, respectively. Medium of Aα-exchange cells additionally included 200 μg/ml hygromycin for selection of the inducible Aα DTP177AAA construct (19). Antibiotics were omitted from cultures seeded for experiments. COS-M6 cells were cultured in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum.

Antibodies—the pan-A (rat monoclonal 6G3) antibody was kindly provided by Gernot Walter (University of California, San Diego). Polyclonal antibodies against Bα and Bβ were a gift from David Virshup (University of Utah), and the polyclonal PR59 antibody was from Egon Ogris (Vienna Biocenter). Commercial sources of antibodies were as follows: FLAG epitope (M2 and its agarose conjugate, Sigma); PP2A catalytic subunit (Pharmingen); total ERK (Santa Cruz Biotechnology, Santa Cruz, CA); phospho-Ser-473 Akt, total Akt, phospho-ERK1/2, phospho-Smad1/5/8, phospho-Tyr-1045 epidermal growth factor (EGF) receptor (Cell Signaling, Beverly, MA); phospho-Tyr (4G10) and total EGF receptor (Upstate Biotechnology, Lake Placid, NY).

Other Reagents—Rat nerve growth factor (NGF, 2.5 S), mouse EGF, and recombinant human fibroblast growth factor-2 (FGF2) were purchased from Upstate Biotechnology, Sigma, and Alomone Labs (Jerusalem, Israel), respectively. Growth factors were stored at −20 °C in lyophilized aliquots and dissolved to 100× in medium prior to use. Okadaic acid and microcystin-LR were purchased from Alexis (Lausanne, Switzerland). Plasmids encoding FLAG-ERK2, Ha-Ras V12, the EGF receptor, and FLAG-Bα/PR59 were donated from Phillip Stork (Vollum Institute), Jeffrey Pessin (State University of New York (SUNY) Stony Brook), Nancy Lill (University of Iowa), and Eric Cohen (University of Montreal), respectively. pFC-MEK1, a plasmid encoding constitutively active MEK1 (S218,222D), was supplied as part of the PathDetect Elk1 trans-reporting system (Stratagene, La Jolla, CA). The coding sequence for rat Bβ was isolated from rat brain RNA by the reverse-transcriptase-PCR (sequence deposited in GenBank™ as AY251278). FLAG epitope tags were added via PCR to the N terminus of Bβ and to the C terminus of Bα and Bβ followed by ligation of PCR products into the pcDNA5/T0 expression vector (Invitrogen).

MAPK Reporter Assays—The PathDetect Elk1 trans-reporting system was modified for the dual-luciferase assay (Promega, Madison, WI) to quantify ERK activation according to the manufacturers’ instructions. PC6-3 cells were plated at 100,000–150,000 cells/well in 24-well plates and transfected 24 h later in triplicate using Lipofectamine 2000 (BD Biosciences). Aα-RNAi and Aα-exchange cells were transfected with 0.5 μg/well reporter plasmid mix (by mass: 92.5% pFR-Luc, 5% pFA2-Elk1, 2.5% pRL-SV40) and 0.5–2 ng/well activator plasmid (Ha-Ras V12, MEK1 S218,222D) or pcDNA3.1 empty vector. Doxycycline (1 μg/ml) or 0.1% ethanol vehicle was added at the same time. PC6-3 cells subjected to transient RNAi were transfected with 0.25 μg/well pSUPER-based plasmids (38) expressing shRNAs, 0.25 μg/well reporter plasmid mix, and 0.5 ng/well MEK1 S218,222D. After 3 days, cells were preincubated for 1 h in medium with one-tenth original serum concentration ±300 nM okadaic acid (OA) followed by 5–6 h of stimulation with growth factors as indicated. Cultures were lysed and subjected to dual-luciferase assays using a Berthold Sirius tube luminometer. Photinus and Renilla luciferase activity ratios were expressed relative to basal conditions without ERK activator plasmids or growth factor stimulation.

Quantitative Immunoblotting with Phospho-specific Antibodies—Aα-RNAi or Aα-exchange cells were seeded at 150,000 cell/well in 24-well plates, in some experiments transfected with EGF receptor plasmids, and treated ±doxycycline (Dox) for 3 days. Cells were serum-starved ±300 nM OA for at least 2 h prior to adding growth factors at staggered times. After washing with phosphate-buffered saline, cells were harvested in SDS sample buffer supplemented with 1 mM EDTA and 1 μM microcystin-LR, and lysates were probe tip-sonicated to shear the DNA. Protein concentrations were determined by a dot blot assay and normalized prior to SDS-polyacrylamide electrophoresis and electrophoretic transfer of samples to nitrocellulose membranes. Enhanced chemiluminescence (SuperSignal, Pierce) images were captured using a Kodak Imager 440, and band intensities were quantified with the ImageJ software gel analyzer plug-in. Phospho-specific antibody signals were divided by total protein antibody signals to control for loading differences. All signal intensities scaled linearly with the amount of lysate loaded.
RNAi of Bα and Bβ—Double-stranded oligonucleotides encoding shRNAs were ligated into the H1 promoter-driven pSUPER plasmid as described (38). The following sequences were targeted for RNAi: Bα3, 5′-AATCCAGTCTCATAGCAGAGG-3′; Bα4, 5′-AAGTG-GCAAGCGAAAGAAAGA-3′; Bβ1, 5′-AACAGAATGCTGCTCTTTTTC-3′; Bβ2, 5′-AACACTCGGAGGGATGTTACA-3′.

[32P]ERK2 Dephosphorylation Assays—2 × 10⁶ COS-M6 cells were plated into a 100-mm dish and cotransfected 24 h later with 1.2 μg of MEK1 S218,222D plasmid using Lipofectamine 2000. After 3 days, cells were metabolically labeled for 3 h with 0.6 mCi/ml [32P] orthophosphate (ICN, Irvine, CA) in phosphate-free Dulbecco’s modified Eagle’s medium containing 1% dialyzed fetal bovine serum. Cells were rinsed once with phosphate-buffered saline and lysed in 1.5 ml of immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol, 1 mM β-glycerophosphate, 1 mM Na₂VO₃, 1 mM Na₄P₂O₇, 1 μM microcystin-LR, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM benzamidine). Debris was pelleted (20,000 × g, 15 min), and 50 μl of FLAG-ERK2 was immunoprecipitated from the cleared lysate using FLAG antibody (M2)-conjugated agarose (3 h at 4 °C). Beads were washed once with 2 ml of immunoprecipitation buffer and four times with 2 ml of immunoprecipitation buffer lacking phosphatase inhibitors. PP2A preparations were adjusted for equal catalytic subunit content after quantitative immunoblotting. For phosphatase assays, 50 μCi/ml [32P] orthophosphate was added to dissociate FLAG-tagged substrate and phosphatase from the antibody beads for more efficient dephosphorylation. Reactions were started by the addition of 20 μl of diluted [32P] ERK2 substrate to 5 μl of PP2A holoenzyme preparation, incubated for 30 min at 30 °C with intermittent agitation on an Eppendorf shaking incubator and then terminated by the addition of trichloroacetic acid to a final concentration of 20% (w/v). Following centrifugation at 22,000 × g, acid-soluble [32P] orthophosphate was quantified by liquid scintillation counting.
Percent ERK2 dephosphorylation was calculated after subtraction of counts from a "blank" reaction containing, instead of PP2A, immunoprecipitates from a mock transfection.

RESULTS

PP2A Modulates the MAPK Cascade at the Level of ERK—Inducible knockdown of αα, the principal PP2A scaffold subunit, promotes apoptosis in PC-3 cells, a subline of neuronal PC12 pheochromocytoma cells. The loss of viability of αα-RNAi cells starts 4 days after induction by Dox and is associated with attenuated Akt phosphorylation in response to EGF treatment (19). Viability, as well as growth factor responsiveness, are rescued by constitutive or inducible expression of a subunit (73% knockdown).

To further explore the effect of αα silencing on growth factor signaling, ERK/MAPK reporter assays were carried out in αα-RNAi cells treated ±Dox for 3 days to induce shRNA expression, which results in 20–25% inhibition of PP2A activity (19). Cultures were also treated for 5 h with 300 nM okadaic acid to acutely inhibit PP2A. At this concentration, okadaic acid is a selective inhibitor of PP2A, and possibly, the related PP4, PP5, and PP6 catalytic subunits in intact cells (39, 40). Okadaic acid increased MAPK reporter activation dramatically (170 ± 41-fold, n = 3), whereas αα silencing had a more modest, but still significant, effect (2.7 ± 0.3-fold, n = 3; Fig. 1A). Similar effects were observed by immunoblotting with an antibody specific for ERK1/2 dually phosphorylated on Thr and Tyr residues in the activation loop (Fig. 1A).

To determine the level at which PP2A inhibits the MAPK cascade, cells were transfected with constitutively active Ras (oncogenic Ha-Ras V12) or MEK1 (S218,222D). Plasmid concentrations were titrated to achieve 100–150-fold reporter activation over empty vector transfected cells. Okadaic acid and αα silencing stimulated ERK activity further, to about 600- and 400-fold above basal, respectively (Fig. 1B). Importantly, Ras V12 and MEK1 S218,222D-induced reporter activations were equivalently augmented by disabling the phosphatase. The most parsimonious interpretation of these results is that PP2A inhibits the ERK/MAPK cascade predominantly at the level of the terminal kinases, the ERKs.

PP2A Silencing Attenuates, whereas PP2A Inhibition Promotes Growth Factor-dependent Akt and ERK Phosphorylation—To further investigate the loss of EGF responsiveness following αα silencing (19), αα-RNAi cells were pretreated with okadaic acid or Dox as before followed by stimulation with various growth factors. EGF stimulation led to transient activation of Akt (detected with a phospho-Ser-473 specific antibody) and ERK, both peaking at 5 min (Fig. 2A). Okadaic acid potentiated basal as well as EGF-stimulated Akt and ERK phosphorylation, whereas αα silencing attenuated phosphorylation of both kinases. Opposite effects of PP2A inhibition and knockdown were also observed on FGF2- and NGF-stimulated Akt and ERK phosphorylation (Fig. 2B and not shown). On the other hand, phosphorylation of the transcription factors Smad1/5/8 following stimulation of bone morphogenetic protein-2 Ser/Thr kinase receptors was unaffected by either manipulation of PP2A, demonstrating specificity for receptor tyrosine kinase pathways (Fig. 2C). ERK/MAPK reporter assays confirmed that although okadaic acid stimulated growth factor-dependent transcriptional activity by 4–20-fold, αα knockdown resulted in a paradoxical loss of responsiveness to EGF, FGF2, and NGF (~50%, Fig. 2D). This blunted reporter activation was observed over a wide range of EGF and NGF concentrations (Fig. 2, E and F).

A detailed time course of NGF-dependent Akt and ERK phosphorylation was examined in ±Dox-treated αα-RNAi cells (representative immunoblot in Fig. 3A, quantification of three independent experiments in Fig. 3, B and C). Aα knockdown decreased peak ERK and Akt phosphorylation somewhat and had a dramatic effect on the sustained phase of NGF signaling.

The diminished responsiveness of growth factor signaling cascades following αα-RNAi not only contrasted with the effects of pharmacological PP2A inhibition but also with the increase in basal and constitutively active Ras/MEK1-dependent ERK activity seen after αα knockdown (Fig. 1). It was therefore most likely that the blunted growth factor response reflects a cellular adaptation to a chronic decrease in PP2A activity, presumably mediated by feedback of the disinhibited kinases.
Aa-RNAi Uncouples EGF Receptor Activation from Downstream Signaling—To pinpoint the site of this compensatory response to Aa silencing, the activation state of the EGF receptor was assessed. Low expression levels (<30,000 receptors/cell in parental PC12 cells (41)) precluded an analysis of the endogenous EGF receptor. Instead, Aa-RNAi cells were transfected with EGF receptor plasmids at a concentration that preserved the ligand dependence of Tyr phosphorylation. Although Akt and ERK stimulation by EGF was blunted as before, Aa knockdown had no effect on expression levels of the EGF receptor, total Tyr phosphorylation, or phosphorylation of Tyr-1045, an auto-phosphorylation site that recruits the ubiquitin-protein isopeptide ligase c-Cbl (42) (Fig. 4). Thus, Aa silencing attenuated growth factor-dependent ERK and Akt phosphorylation downstream of the EGF receptor. Since Aa silencing stimulated oncogenic Ras-dependent ERK activation (Fig. 1B), compensatory uncoupling may occur at the level of receptor tyrosine kinase adaptor proteins or guanylate exchange factors.

B'-family Regulatory Subunits Mediate Akt Modulation by PP2A—The observation that PP2A keeps both Akt and ERK signaling in check raised the question whether different PP2A regulatory subunits might be involved. If so, cells would have the ability to throttle the output of both kinase cascades independently. Aa-exchange PC6-3 cell lines were previously generated in which Dox treatment silences the endogenous Aa subunit, concomitant with expression of an RNAi-resistant Aa subunit mutant defective in binding to select regulatory subunit families (19). In Aa(+/-)-exchange cells, wild-type Aa is replaced with the Aa DTP177AAA mutant, which binds to B and B' but not B' subunits (19, 43) (Fig. 5A). Quantitative Aa subunit exchange is evident not only as the disappearance of wild-type Aa and appearance of the epitope-tagged, slightly larger mutant but also as the selective loss of B'α and B'β protein after 3 days in Dox (Fig. 5B) because monomeric B' (and B) family subunits are unstable in cells (5, 19, 44, 45). The loss of B'-family subunits was not accompanied by detectable changes in the levels of catalytic or other regulatory subunits (Fig. 5B) and appearance of the epitope-tagged, slightly larger mutant but also as the selective loss of B'α and B'β protein after 3 days in Dox (Fig. 5B) because monomeric B' (and B) family subunits are unstable in cells (5, 19, 44, 45). The loss of B'-family subunits was not accompanied by detectable changes in the levels of catalytic or other regulatory subunits (Fig. 5B) because monomeric B' (and B) family subunits are unstable in cells (5, 19, 44, 45).

In contrast to Aa silencing, selective removal of PP2A/B' heterotrimmers had no effect on growth factor-dependent ERK phosphorylation (Fig. 5C). However, indistinguishable from Aa silencing, Aα(+/-)-exchange attenuated Akt phosphorylation in response to EGF, FGF2, and NGF (Fig. 5, C and D). ERK/MAPK reporter assays corroborated the lack of involvement of PP2A/B' holoenzymes in this kinase cascade, whereas cells retained sensitivity to okadaic acid (Fig. 5E). Following the same reasoning as before, blunted growth factor-activation of Akt due to PP2A/B' removal was interpreted as overcompensation of basal Akt.
hyperphosphorylation. These results therefore implicate PP2A/B family
holoenzymes in the modulation of Akt, but not ERK1/2, in PC6-3 cells.

Bα and Bβ Regulatory Subunits Target PP2A to Modulate ERK Activity—The single B-family subunit of Drosophila (twins/aar) was implicated as an inhibitor of the ERK/MAPK cascade (45). Since Bα and Bβ are the predominant B-family regulatory subunits expressed by undifferentiated PC6-3 cells (46), their contribution to ERK regulation was tested by RNAi. Two shRNAs driven by the H1 promoter (38) targeting each of the two gene products were found to effectively and specifically silence expression of transiently transfected FLAG-tagged Bα and Bβ constructs, with an order of shRNA potency of α4>α2 and δ1>δ2 (Fig. 6A). Knockdown of endogenous B-family subunits was confirmed with an antibody that does not discriminate between these highly homologous isoforms (not shown). To specifically explore PP2A action on the terminal kinases in the ERK/MAPK module, ERKs were directly activated by transfecting PC6-3 cells with constitutively active MEK1, titrating plasmid levels to achieve ~30-fold reporter activation in the presence of a control plasmid that expresses a nonsense shRNA. Both Bα and Bβ-directed shRNAs potently silenced ERK reporter activation over control shRNA transfected cells, with Bα-directed shRNAs effecting greater stimulation than Bβ-directed shRNAs (Fig. 6B), which may reflect difference in endogenous protein expression levels. Within each shRNA set, the shRNAs that produced better silencing (α4, δ1) also led to higher reporter activation. The data thus point to Bα- and Bβ-containing PP2A holoenzymes as ERK inhibitors in PC6-3 cells.

PP2A/Bα and PP2A/Bβ Are Specific ERK Phosphatases—The PP2A core dimer effectively dephosphorylates the activation loop Thr of ERK2 in vitro, resulting in a 10~100-fold drop in activity (36, 47). However, indirect actions of PP2A/Bα and Bβ are also plausible and could involve, for instance, dephosphorylation-dependent activation of one of the ERK-specific tyrosine or dual-specificity phosphatases (36, 48). This issue was explored by in vitro phosphatase assays. Different PP2A heterotrimers were immunosolated from COS-M6 cells transiently transfected with FLAG-tagged regulatory subunits (Bα, Bβ, B′β, B′/PR72 (4)). The substrate, FLAG-tagged ERK2, was isolated from COS-M6 cells coexpressing constitutively active MEK1 after metabolic labeling with 32PO4−. The purity of the 32P/ERK2 preparation is illustrated in Fig. 7A. Incubation with purified PP2A/Bα holoenzymes decreased both 32P labeling and phospho-ERK immunoreactivity to a level that may reflect mostly the singly Tyr-phosphorylated species. When dephosphorylation was quantified as the release of 32PO4−, PP2A holoenzymes containing Bα and Bβ were found to catalyze ERK dephosphorylation much more avidly than PP2A/B′β, PP2A/B′/PR72 displayed intermediate activity toward radiolabeled ERK2. These data are consistent with direct dephosphorylation and inactivation of the ERKs by PP2A/Bα and Bβ and possibly B′/PR72.

DISCUSSION

Many, if not most, components of growth factor signaling cascades are PP2A substrates (for review, see Ref. 21). In particular, evidence abounds for both positive and negative regulation of ERK/MAPK signaling by PP2A at multiple levels (45, 46, 49~52). This report employed two novel approaches, Aα subunit silencing and Aα-exchange, in addition to pharmacological inhibition to arrive at three main conclusions regarding regulation of growth factor signaling by specific PP2A holoenzymes. First, the net effect of PP2A inhibition and silencing was increased Akt and ERK/MAPK activity; the latter was shown to occur at the level of ERK itself. Second, chronic PP2A inhibition by Aα silencing promoted uncoupling of growth factor receptors from Akt and ERK, presumably as an adaptive response to hyperphosphorylation of the kinases. Third, two unrelated PP2A regulatory subunit families modulate Akt and ERK signaling. ERK modulation in PC6-3 cells likely involve direct dephosphorylation by PP2A/Bα and Bβ.
types, although recent findings challenge the perception of RNAi as a tool with scalpel-like precision (53). Rescue experiments with wild-type and mutant Aα cDNAs demonstrate the specificity of Aα silencing (Ref. 19 and this report). However, growth factor signaling was altered in opposite directions in Aα-silenced and acutely PP2A-inhibited cells. Because both okadaic acid and Aα silencing specifically inhibited ERK in epistasis experiments, we consider it most likely that the more protracted inhibition of PP2A by RNAi triggers a compensatory downregulation of signaling molecules between receptor tyrosine kinases and Ras-family small G proteins.

More complex, alternative explanations of the discrepant effects of Aα silencing and okadaic acid on growth factor responses are also possible and could involve minor PP2A holoenzymes or other okadaic acid-sensitive phosphatases (PP4, PP5, and PP6). One scenario, for instance, requires an okadaic acid-insensitive form of PP2A with a strong stimulatory effect upstream of Ras that exceeds ERK inhibition by a second, okadaic acid-sensitive PP2A holoenzyme.

It is likely that the more parsimonious explanation, feedback adaptation to kinase hyperactivity, applies to long term perturbation of many signaling cascades. Therefore, our experiments underscore the importance of combining RNAi with more acute manipulations to infer the cellular function of signal transduction enzymes.

Akt Modulation by PP2A/B′B′——The five vertebrate B′-family subunits (B′α–e) are established regulators of Wnt/β-catenin signaling (6, 54–57) but have not previously been linked to receptor tyrosine kinase signaling. Predicted to adopt an α-helical repeat structure, the conserved middle two-thirds of B′-family subunits interact with the PP2A core dimer (20), whereas the more divergent N and C termini contain multiple phosphorylation sites, which have been shown to either inhibit or stimulate PP2A holoenzyme activity (14–16). Here, replacing the endogenous Aα subunit with a mutant that does not interact with B′-family subunits selectively impaired Akt phosphorylation. Since okadaic acid resulted in Akt hyperphosphorylation, the blunted growth factor response following knockdown of B′-family subunits probably reflected a cellular adaptation, just like the blunted Akt and ERK activation following Aα knockdown. Because ERK phosphorylation was unaffected by B′ silencing, adaptation appears to occur after the divergence of ERK/MAPK and Akt signaling cascades. We additionally speculate that within the B′-family, Akt modulation may be mediated by one or more of the cytosolic (B′α/B′e), as opposed to the mostly nuclear (B′γ/B′ε) gene products.

PP2A is the major phosphatase targeting Akt in vitro (58), and several reports have documented coimmunoprecipitation of the two enzymes (59–61). It is thus likely that Akt modulation involves direct dephosphorylation by PP2A-containing B′-family subunits, although more complex scenarios are certainly possible.

ERK Modulation by PP2A/Bα and Bβ——B-family PP2A subunits participate in both positive and negative regulation of the ERK/MAPK cascade. In both vertebrate and invertebrate systems, ERK stimulation by B-family subunits occurs downstream of Ras and upstream or at the level of Raf and may, among other mechanisms, involve dephosphorylation of inhibitory phosphorylation sites on Raf (29–32, 49, 51). In the PC6-3 cell line used in the present study, overexpression of By, a neuron-specific B′-family regulatory induced during NGF-mediated differentiation, activates ERK/MAPK signaling at the level of the B-Raf isoform (46). Inhibition of ERK/MAPK signaling by the singe Drosophila B-family subunit was demonstrated using RNAi, although the site of PP2A action was not established (45). The present data demonstrate that although PP2A may have multiple entry points into the MAPK cascade, direct inhibition of ERKs predominated in PC6-3 cells. Silencing of either By or Bβ, the two B′-family subunits expressed in dividing, chromaffin-like PC6-3 cells, disinhibited ERK activation by constitutively active MEK1. In vitro phosphatase assays show that Bα and Bβ, but not B′β, mediate direct dephosphorylation of ERK2. Since a PP2A heterotrimer containing the predominantly nuclear B′β/PR72 subunit (17) had intermediate ERK2 phosphatase activity, it is possible that B′ subunit-containing PP2A holoenzymes complete the phosphorylation cycle of ERKs that have translocated into the nucleus.

In summary, dedicated PP2A heterotrimers with unique regulatory potentials control different aspects of growth factor signal transduction. Whether and how cells take advantage of this complexity remains to be explored.

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