The importance of PP2A in the regulation of Akt/PKB activity has long been recognized but the nature of the holoenzyme involved and the mechanisms controlling dephosphorylation are not yet known. We identified IEX-1, an early gene product with proliferative and survival activities, as a specific inhibitor of B56 regulatory subunit-containing PP2A. IEX-1 inhibits B56-PP2A activity by allowing the phosphorylation of B56 by ERK. This leads to sustained ERK activation. IEX-1 has no effect on PP2A containing other B family subunits. Thus, studying IEX-1 contribution to signaling should help the discovery of new pathways controlled by B56-PP2A. By using overexpression and RNA interference, we show here that IEX-1 increases Akt/PKB activity in response to various growth factors by preventing Akt dephosphorylation on both Thr308 and Ser473 residues. PP2A-B56β and γ subunits have the opposite effect and reverse IEX-1-mediated Akt activation. The effect of IEX-1 on Akt is ERK-dependent. Indeed: (i) a IEX-1 mutant deficient in ERK binding had no effect on Akt; (ii) ERK dominant-negative mutants reduced IEX-1-mediated increase in pAkt; (iii) a B56β mutant that cannot be phosphorylated in the ERK/IEX-1 complex showed an enhanced ability to compete with IEX-1. These results identify B56-containing PP2A holoenzymes as Akt phosphatases. They suggest that IEX-1 behaves as a general inhibitor of B56 activity, enabling the control of both ERK and Akt signaling downstream of ERK.

Protein phosphorylation is central to signal transduction by growth factors and the maintenance of cellular homeostasis by controlling many cellular processes such as growth, survival, and differentiation. Deregulation of this activity is a key cause of cancer development. The net activation of a signaling pathway depends on the balance of enzymatic activities between kinases and phosphatases, suggesting that both activation and inactivation signals must be tightly controlled.

**Inhibition of B56-containing Protein Phosphatase 2As by the Early Response Gene IEX-1 Leads to Control of Akt Activity**

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Protein phosphorylation 2A (PP2A)² accounts for the majority of serine/threonine phosphatase activity in the cell (reviewed in Refs. 1 and 2). The predominant form of PP2A is a heterotrimeric holoenzyme, composed of a scaffolding subunit (A), a catalytic subunit (C), and a variable regulatory subunit. The B subunits fall into three major unrelated families, B (PR55), B’ (B56/PR61), and B” (PR72). Each family comprises several isoforms that exhibit significant homology between them. With five different genes, generating at least 8 isoforms named B56 α, β, γ, δ, and ε, the B56 family is the most diverse. Many if not most of the components of signaling cascades are PP2A substrates. However, the function of the individual PP2A subunits in these pathways is only beginning to emerge. Interestingly, recent studies indicate that different PP2A holoenzymes regulate positively or negatively signaling by acting at different levels in a cascade. The best example of this type of regulation is the ERK/MAPK signaling pathway. Indeed, Adams et al. (3) showed that PP2As containing Bo and Bδ are required for activation of the MAPK kinase kinase Raf-1, whereas we demonstrated that the B56 family members shut down the signal by dephosphorylating ERK1 and ERK2 (4). Likewise, the Wnt pathway has been shown to be differently regulated by B56-containing PP2As, which binds to axin and APC (5) and also by PP2As containing the B” subunit acting at the level of Naked cuticle (6). Unraveling the specific contribution of the various PP2A holoenzymes to signaling pathways, as well as the mechanisms regulating their function, is an important step to understand how kinase cascades are regulated and to find new tools to modulate their activities.

The kinase Akt/PKB, a critical component of a pathway controlling growth and survival, is one of the major targets of PP2A enzymes. Akt exerts its function by phosphorylating several proteins involved in cell cycle regulation and apoptosis, including p21Cip/WAF1, BAD, the Forkhead family of transcription factors and glycogen synthase kinase-3 (GSK3) (reviewed in Ref. 7). Activation of Akt occurs upon recruitment to the plasma membrane through binding of its plekstrin homology domain to phosphatidylinositol 3,4,5-triphosphates, which are

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² The abbreviations used are: PP2A, protein phosphatase 2A; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; GSK3, glycogen synthase kinase-3; IEX-1, immediate early response gene X-1; PI3K, phosphoinositide 3-kinase; TPO, thrombopoietin; WT, wild type; HA, hemagglutinin; shRNA, short hairpin RNA; GFP, green fluorescent protein; EPO, erythropoietin; CHO, Chinese hamster ovary; ER, endoplasmic reticulum; FCS, fetal calf serum; PDK1, phosphoinositide-dependent kinase-1.
produced by growth factor-activated phosphoinositide 3-kinase (PI3K). This allows phosphorylation of Akt at two key regulatory residues; Thr<sup>308</sup> in the activation loop and Ser<sup>473</sup> in a C-terminal hydrophobic motif. Phosphorylation of Thr<sup>308</sup> is catalyzed by PDK1 (8, 9); that of Ser<sup>473</sup> is mediated by the mTORC2 complex (10). Turn-off of Akt activity can be mediated by the tumor suppressor phosphatase tensin homologue, which dephosphorylates phosphatidylinositol 3,4,5-triphosphates. Although the importance of PP2A in the regulation of Akt activity has long been recognized (11–15), the nature of the PP2A holoenzyme involved and the mechanisms controlling these dephosphorylation events have not yet been elucidated.

**IEX-1** (immediate early response gene X-1), also known as **IER3, DIF2**, or **Gly96**, is an ubiquitous early response gene product involved in cell proliferation and survival, which is rapidly induced by various growth factors, cytokines, chemical carcinogens, or viral infections (16). Conflicting data have been reported concerning the role of IEX-1 in apoptosis. Indeed, IEX-1 was found to increase apoptosis in response to UV radiation or upon serum deprivation (17, 18) but it also plays a key role in cellular resistance to various apoptotic triggers and contributes to growth factor-mediated survival activities (19–23). These differential effects might be due to the fact that IEX-1 acquires its pro-survival function upon phosphorylation by ERK (19, 23). In addition to this function, we have demonstrated that IEX-1 behaves as an inhibitor of B56-containing PP2A enzymes (4). IEX-1 binds to both B56 family members and to active ERKs, enhances the phosphorylation of B56 by ERK in this complex and triggers the dissociation of the A/C core from the B56 subunit. Inhibition of PP2A-B56 activity by IEX-1 was found to increase ERK signaling in response to various growth factors (4). In the hematopoietic cell line UT7, the specific induction of the IEX-1 protein by thrombopoietin (TPO), correlates with the unique capacity of this cytokine to sustain the ERK signal and is required for this effect (4, 19, 24). IEX-1 has no effect on other B family of PP2As. Thus, studying IEX-1 contribution to signaling cascades should lead to the discovery of new pathways controlled by B56-containing PP2As. The functions of IEX-1 and its induction by various growth factors prompted us to analyze whether it can affect Akt activation. We show here that IEX-1 and PP2A-B56 regulate Akt activity in an opposite manner by controlling the phosphorylation state of both Thr<sup>308</sup> and Ser<sup>473</sup>. These data add to our understanding of both PP2A and Akt regulatory pathways.

**MATERIALS AND METHODS**

**Plasmids**—Plasmids encoding **IEX-1**, **WT**, or deleted of its ERK binding site (**IEX-1-ΔBD**), were described previously (19). IEX-1 was inserted downstream of HA or His tags. pcDNA expression vectors for HA-B55a and 4xHA-B56β were generously provided by Dr. D. Virshup (University of Utah). pcDNA encoding HA-tagged B56γ was given by Dr. N. Nojima (Osaka university). B56β-S368A and B56γ1-S327A were previously characterized (4) and were generated by the QuikChange site-directed mutagenesis kit (Stratagene). The insert was cloned downstream of HA<sub>4</sub>. Mouse ERK1 cDNA was cloned from reverse transcription of NIH3T3 mRNA. Mouse cDNA for ERK2 was a gift from Dr. M. Weber (University of Virginia Cancer Center). ERK1 and ERK2 dominant negative mutants were generated by mutating their Mg<sup>2+</sup> binding pockets (consensus DFG in all kinases). The coding sequences were inserted into pcDNA3 to create plasmids pcDNA3-ERK1-D185A and pcDNA-ERK2-D165A. pcDNA-HA-Elk1 was a gift of R. Hipskind (Montpellier, France). For RNA interference, the target nucleotide sequences and the vectors that drive the expression of small hairpin shRNA for human **IEX-1**, **B56β**, or **B56γ** were described previously (4, 25, 26). Two shRNA targeting different nucleotides of human B56β sequence were used: pSM2c-shB56β-1 and pSM2c-shB56β-2 (Open Biosystems); pMKO-1-shB56γ was a gift of Dr. W. Hahn (Dana Farber Cancer Institute, Boston). The controls used were either sh0 (nucleotides 398–418 in the **IEX-1** sequence), which was found to be ineffective at down-regulating IEX-1 expression, or shGFP (4). The cassette encoding the H1 promoter-shIEX-1 or shRNA-control was introduced in a self-inactivating lentiviral/human immunodeficiency virus vector (TRIPΔU3). This vector also expresses GFP under the EF1α promoter. Production and titration of infectious particles were done as described (24).

**Cell Cultures**—Chinese hamster ovary cells expressing the erythropoietin (EPO) receptor (CHO-ER) cells were described previously (19). They were cultured in Dulbecco’s minimal essential medium nut Mix F-12 (Ham’s) medium containing 7% fetal calf serum (FCS). HEK-293 and HeLa cells were maintained in Dulbecco’s minimal essential medium supplemented with 10% FCS. UT7 cells (27) were grown in α-minimal essential medium supplemented with 10% FCS and 1 unit/ml EPO (Roche Molecular Biochemicals).

**Transfections and Viral Infections**—CHO-ER and HeLa cells (60–80% confluence) were transiently transfected with 1–2 μg of DNA, using the Lipofectamine Plus reagent (Invitrogen), according to the manufacturer’s instructions. Stable clones of HeLa cells expressing the shRNAs targeting B56γ and B56β were previously described (4). In some experiments, the vectors carrying shRNA were transiently introduced in HeLa cells and the cells were harvested 48 h later. HEK-293 and UT7 cells were incubated with TRIPΔU3-H1-shIEX-1 or control lentiviruses over a period of 36 h. The transduction efficiency, determined by the percentage of GFP positive cells by fluorescence-activated cell sorter analysis, usually reached 98%.

**Western Blotting, Immunoprecipitation, and Kinase Assays**—Akt activation was assessed in total lysates by Western blotting with anti-pAkt-Ser<sup>473</sup> or pAkt-Thr<sup>308</sup> antibodies (Cell Signaling). To follow the kinetics of Akt inactivation, 24 h post-transfection the cells were either treated with the PI3K inhibitor LY294002 or washed 3 times in phosphate-buffered saline and loaded with serum-free medium. The cells were returned to 37°C and harvested at various times. In some experiments, the cells were deprived of serum overnight, and stimulated with EPO or FCS for various times before lysis. For immunoprecipitation experiments, the cells were lysed in 50 mM Tris, pH 7.5, buffer containing 137 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1 mM orthovanadate, 20 mM β-glycerophosphate, 20 mM NaF, 1 mM sodium pyrophosphate, and a protease inhibitor mixture (Roche). Gel electrophoresis, immunoblotting, and immunoprecipitation were carried out as previously described (4). *In vitro* Akt kinase activity was assessed with the non-radioac-
Regulation of Akt Phosphorylation by IEX-1 and B56-PP2As

A

IEX-1: - +
pAkt-T308
pAkt-S473
IEX-1
Akt

B

|                  | control | IEX-1 |
|------------------|---------|-------|
| EPO (10 μ/ml)    | 0 7 15 30| 0 7 15 30|
| pAkt-S473        | 1 1 1 1 1 | 1 1 1 1 1 |
| pAkt-T308        | 1 1 1 1 1 | 1 1 1 1 1 |
| pGSK3            | 1 1 1 1 1 | 1 1 1 1 1 |
| Akt              | 1 1 1 1 1 | 1 1 1 1 1 |

C

|                  | control | IEX-1 |
|------------------|---------|-------|
| EPO (2 μ/ml)     | 0 15 30 | 0 15 30 |
| pAkt-S473        | 1 1 1 1 1 | 1 1 1 1 1 |
| pAkt-T308        | 1 1 1 1 1 | 1 1 1 1 1 |
| pGSK3            | 1 1 1 1 1 | 1 1 1 1 1 |
| Akt              | 1 1 1 1 1 | 1 1 1 1 1 |

RESULTS

IEX-1 Increases Akt Activation—IEX-1 can be induced by serum and various growth factors (4, 16, 19, 24, 28). To determine whether IEX-1 could affect other growth factor-induced signaling pathways in addition to ERK, we measured Akt activity in cells expressing IEX-1. Maximal Akt activation requires phosphorylation at the two activating residues Ser473 and Thr308 (8–10, 29). Thus, CHO cells were transiently transfected with HA-IEX-1 or empty vector and the cells were maintained in serum to activate Akt. Endogenous Akt activation was assessed both by Western blotting with Ser473 and Thr308 phospho-specific Akt antibodies and by immunoprecipitation kinase assays. As shown in Fig. 1A, Akt phosphorylation was greatly enhanced at both Ser473 and Thr308 in cells expressing IEX-1.

To determine whether IEX-1 could affect Akt activation in response to growth factors, CHO cells expressing the EPO receptor (CHO-ER, 19) were transfected with IEX-1 and analyzed after 3 h starvation in a serum-free medium and stimulation with EPO, under conditions leading to increased ERK activation in the presence of IEX-1 (Ref. 19 and Fig. 1, B and C). No phosphorylation of Akt could be detected in non-stimulated starved cells whether they expressed IEX-1 or not. However, IEX-1 expression resulted in a great increase in EPO-induced Akt phosphorylation on both Ser473 and Thr308, at all time points and doses tested (Fig. 1, A–C). Endogenous Akt phosphorylation was also enhanced and prolonged in UT7 cells stably transfected with IEX-1, as compared with control cells (neo), upon stimulation with TPO (Fig. 1D).

To examine IEX-1-dependent Akt activation further, we measured Akt kinase activity, by using an in vitro kinase assay on its substrate GSK3. Fig. 1E shows that the Akt kinase activity that could be immunoprecipitated from CHO cells expressing IEX-1 was increased, as compared with cells transfected with an empty vector. An increased and sustained in vivo GSK3 phosphorylation that paralleled pAkt levels was also detected in IEX-1-overexpressing cells (Fig. 1B). In addition, the phosho-
rlation levels of several Akt substrates, detected using an anti-
pan phospho-Akt substrate antibody, were greatly increased in
the presence of IEX-1 (Fig. 1B). Thus, expression of IEX-1
increases Akt activity and Akt downstream signaling.

**IEX-1** is an early response gene product that is induced by
various growth factors. Thus Akt activity should be more sus-
tained in response to growth factors triggering IEX-1 expres-
sion. To test this, we made use of the UT7 cell line. Indeed,
whereas both EPO and TPO stimulate Akt activation in this cell
line, only TPO can induce IEX-1 protein expression (Ref. 24
and Fig. 2A). As shown in Fig. 2A, Akt phosphorylation on both
Ser473 and Thr308 was much higher and more sustained in cells
stimulated with optimal doses of TPO, as compared with EPO.
This increase in Akt phosphorylation translated into an
increased Akt activity, as shown by the higher levels of phos-
phorylation of the Akt substrates GSK3 and Foxo3a observed
upon TPO stimulation. Likewise, Western blotting with a pan-
Akt substrate phospho-antibody shows that in TPO-treated,
IEX-1 expressing cells, the global Akt-mediated phosphoryla-
tion is greatly increased, as compared with cells treated with
EPO. Thus, the specific induction of the IEX-1 protein by TPO
in UT7 cells correlates with the unique capacity of this cytokine
to sustain Akt phosphorylation and Akt downstream signaling.

To further confirm the above results, we examined Akt acti-
vation in cells in which TPO-induced IEX-1 expression
was impeded by the presence of IEX-1-specific shRNA. Thus, we
generated UT7 cell lines expressing shRNA for IEX-1. Expres-
sion of endogenous IEX-1 upon TPO stimulation was severely
inhibited in the presence of this shRNA (Fig. 2B, left panel). As
a control, we used cell lines expressing either a shRNA designed
from the IEX-1 nucleotide sequence (sh0, see “Materials and
Methods”) but that was found unable to down-regulate endog-
ogenous IEX-1 levels (Fig. 2B), or a shRNA targeting GFP. As
shown in Fig. 2B (right panel), TPO-mediated phosphorylation
of both Akt and its downstream substrate GSK3 was greatly
reduced in shRNA-IEX-1 expressing cells, as compared with
control cells. This effect was particularly striking after 1.5 and
3 h TPO treatments, time points at which IEX-1 is maximally
induced by the cytokine. The decreased Akt activation upon
down-regulation of IEX-1 expression is not specific to TPO
signaling or to the particular cell context of UT7 cells, as shown
by the impaired serum-induced Akt phosphorylation at both
Ser473 and Thr308 in HEK293 expressing IEX-1-shRNA (Fig.
2C). In these cells, basal expression of IEX-1 was observed but
its level increased following serum stimulation. These results
show that the induction of IEX-1 by various growth factors
plays an important role in their capacity to control Akt activity.

**IEX-1 Prevents Akt Dephosphorylation**—We next deter-
mined by which mechanism IEX-1 increased Akt activity. The
marked ability of IEX-1 to sustain Akt signal at long stimulation
time points (Fig. 1) suggested that IEX-1 may prevent Akt inac-
tivation rather than activate upstream kinases. To test this pos-
sibility, CHO-ER cells, transfected or not with IEX-1, were first
subjected to a growth factor pulse with an optimal dose of Epo
IEX-1 reduces the rate of Akt dephosphorylation rather than delaying Akt dephosphorylation on both Ser473 and Thr308. A similar effect was observed with B56 regulatory subunits containing PP2A phosphatases. By contrast, in cells expressing IEX-1, although the basal Akt activation after optimal EPO stimulation was unchanged, the rate of disappearance of pAkt upon addition of LY294002 was greatly delayed (Fig. 3A). This shows that Akt is rapidly dephosphorylated at its two activating residues by endogenous active phosphatases. By contrast, in cells expressing IEX-1, although the basal Akt activation after optimal EPO stimulation was unchanged, the rate of disappearance of pAkt upon addition of LY294002 was greatly delayed (Fig. 3A). This suggests that IEX-1 reduces the rate of Akt dephosphorylation rather than increases its activation.

To confirm these results, CHO cells were stimulated with FCS and Akt phosphorylation was measured after various times following arrest of Akt activation induced by FCS starvation. As above with the addition of LY294002, removal of the stimulus resulted in a rapid decrease in Akt phosphorylation (Fig. 3B). IEX-1 expression greatly delayed this response, as shown by the presence of pAkt up to 1 h following stimulus removal. Quantification experiments showed that the half-life for the pAkt-Ser473 signal increased from 12 ± 4 min in control cells to 59 ± 15 min (mean ± S.E., n = 7) in IEX-1-expressing cells. That of pAkt-Thr308 varied from 16 ± 3 to 60 ± 11 min (n = 4) in control and IEX-1-transfected cells, respectively. Thus, altogether, these results indicate that IEX-1 increases Akt activity by reducing its rate of dephosphorylation on both Ser473 and Thr308.

**IEX-1 InhibitsAkt Dephosphorylation by PP2As Containing the B56 Regulatory B Subunits**—PP2A is the main phosphatase known to act on both Ser473 and Thr308 (11–15). We previously found that IEX-1 increases ERK activation by preventing its dephosphorylation specifically by PP2A holoenzymes containing the B56 family of B regulatory subunits (4). The kinetics of Akt phosphorylation and dephosphorylation upon growth factor starvation were measured after various times (A) or with 10% FCS for 10 min (B). Akt phosphorylation was followed after serum starvation. As shown in Fig. 4A, expression of B56β strongly reduced the capacity of IEX-1 to prolong Akt phosphorylation on both Ser473 and Thr308. A similar effect was observed with B56γ (Fig. 4B). In contrast, neither B family member Bα (Fig. 4A) nor Bβ (Fig. 4C) could compete with IEX-1 to induce extension of Akt phosphorylation. This suggests that the ability of IEX-1 to delay Akt dephosphorylation on both Thr308 and Ser473 is related to its capacity to inhibit specifically B56- but not B-containing PP2A phosphatases.
To confirm these data, we next wished to examine whether Akt phosphorylation can indeed be regulated by the family of B56-containing PP2As. To study the effect of B subunits on Akt dephosphorylation specifically without interference with a possible effect on its upstream activation, CHO cells transfected with either B or B56 subunits were pulse-stimulated in the presence of FCS or EPO as above and the phosphorylation of Akt was measured at various time intervals following growth factor starvation. Fig. 5A shows that expression of the B56 subunit B56γ/H9252 accelerated the disappearance of both Akt-Ser473 and -Thr308 phosphorylated forms, after activation stopped. Given that the transfection efficiency in CHO cells reaches about 60%, these data reveal that Akt phosphorylation was almost abolished in cells expressing the B56 subunits. In contrast, as above with IEX-1, Akt phosphorylation was not reduced in the presence of Bα (Fig. 5B).

Conversely, we used RNA interference to down-regulate the expression of B56 members. shRNA targeting specifically either B56β or B56γ were chosen according to described sequences previously validated by us and/or others (4, 25, 26). Their ability to affect in a specific manner the expression of their target, either B56β or γ, without affecting other B56 members and to down-regulate the corresponding endogenous B56 species is illustrated in Fig. 5C. Expression of either shB56β or shB56γ in HeLa cells resulted in a strong increase in the levels of Akt phosphorylation observed 48 h after transfection in cells grown in serum, as compared with control cells transfected with shGFP (Fig. 5D, left panel). To test the effect of down-regulating B56 expression directly on Akt dephosphorylation, HeLa cells stably expressing shB56β or shB56γ were starved overnight, stimulated with serum for 5 min, and starved again. Akt phosphorylation was determined at various times after serum removal. Quantification of pAkt is expressed relative to the levels of phosphorylation at their zero time point after normalization to Akt levels.

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FIGURE 5. B56-containing PP2As dephosphorylate Akt. A and B, the B56- but not the B-family of PP2A dephosphorylates Akt. CHO cells were transfected with 1 μg of 4xHA-B56β (A) or HA-Bα (B), stimulated with EPO for 5 min and then washed. Akt phosphorylation was determined at the indicated times after starvation. C and D, down-regulation of B56 subunits leads to increased Akt phosphorylation. C, specificity of the various shRNA. CHO cells (left panel) or HeLa cells (right panel) were transfected with vector expressing shRNA for B56β (shB56β-1 and/or shB56β-2) or B56γ, alone or together with either HA-B56β or HA-B56γ. The expression of the B regulatory subunits was assessed by Western blotting with anti-HA or anti-pan-B56γ or anti-B56β antibodies, as indicated. D, increased Akt phosphorylation in shB56-expressing cells. Left panel, HeLa cells were transfected with vectors expressing B56β, B56γ, or control (GFP) shRNAs and Akt phosphorylation was analyzed after 48 h of culture in the presence of serum. Right panel, HeLa cells expressing the indicated shRNA were starved overnight, stimulated with serum for 5 min, and starved again. Akt phosphorylation was determined at various times after serum removal. Quantification of pAkt is expressed relative to the levels of phosphorylation at their zero time point after normalization to Akt levels.

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Regulation of Akt Phosphorylation by IEX-1 and B56-PP2As

The inhibitory effect of IEX-1 on PP2A-mediated dephosphorylation of Akt is ERK dependent. A, IEX-1 does not interact with Akt. CHO cells were transfected with His-IEX-1 and the presence of Akt and ERK was analyzed in anti-His immunoprecipitates. B, the ability of IEX-1 to increase Akt activity requires the ERK binding motif on IEX-1. CHO-ER cells were transfected with pcDNA or vectors encoding HA-IEX-1, WT, or ΔBD. After EPO stimulation (5 min at 37 °C), the cells were deprived of growth factors and analyzed for Akt phosphorylation at various times. C, the ability of IEX-1 to increase Akt activity requires ERK kinase activity. CHO cells were transfected with a mixture of ERK1 and ERK2 (0.25 μg each), either WT or kinase-dead (ERK-AFG), along with empty pcDNA, and 0.5 μg of HA-Ekl1 (left panel) or IEX-1 (right panel). The phosphorylation of Elk1 or endogenous Akt was assessed 24 h after transfection in cells grown in serum. D, the ability of IEX-1 to increase Akt activity involves ERK-induced B56 phosphorylation. CHO cells were transfected with 0.5 μg of IEX-1 together with 0.5 μg of the indicated B56 forms and assayed for Akt phosphorylation as above. Quantifications are shown after normalization to the total level of Akt.

Inhibition of PP2A-induced Akt Dephosphorylation by IEX-1 Is Dependent on ERK—To explore the mechanism by which IEX-1 prevents the activity of B56-containing PP2As on pAkt, we first examined whether IEX-1 could interact with Akt. Thus, IEX-1 was immunoprecipitated from cells co-transfected with His-tagged IEX-1 and HA-Akt. In agreement with our previous data (19), anti-His antibodies could co-precipitate His-IEX-1 and endogenous active pERK from CHO lysates. In contrast, no HA-Akt, or its phosphorylated species, could be detected in these precipitates (Fig. 6A). Likewise, IEX-1 was not found in anti-Akt precipitates (data not shown). These results suggest that IEX-1 most probably does not act by competing with PP2A for Akt binding.

We previously demonstrated that IEX-1 inhibits B56-containing PP2A activity by a mechanism involving ERK-mediated phosphorylation of the B56 subunits (4). Therefore, if the inhibition of Akt dephosphorylation involves the same mechanism, one would expect this effect to be dependent on ERK activity. To test this possibility we first examined the effect of an IEX-1 protein mutated in its ERK binding site (ΔBD-IEX-1). This mutant has completely lost the ability to bind pERK and to inhibit B56-PP2A activity (4, 19). As shown in Fig. 6B, by contrast with the WT protein, ΔBD-IEX-1 could not enhance Akt activation or extend the duration of its phosphorylation upon starvation. This shows that IEX-1-mediated Akt phosphorylation is dependent on its ability to interact with ERK.

To confirm these results, we expressed IEX-1 together with ERK1 and ERK2 kinase-dead proteins or their wild-type counterparts as a control. ERK1/2-AFG forms were created by mutation of the Mg\(^{2+}\) binding site. This mutation destroys the kinase activity of ERKs but preserves their phosphorylation by MEK within the activation loop, so that they behave as dominant negative mutants on the activity of endogenous ERKs. Indeed, as shown in Fig. 6C (left panel), expression of ERK1-AFG and ERK2-AFG led to a decreased phosphorylation of the ERK substrate Elk1, as shown both by Western blotting with anti-phospho-specific Elk1 antibodies and the disappearance of the upper Elk1-shifted band. Expression of dominant negative ERK proteins had no effect on the basal Akt phosphorylation obtained in the absence of IEX-1. This shows that Akt activation is independent of ERK activity. However, it reduced the capacity of IEX-1 to enhance Akt phosphorylation at both Thr\(^{308}\) and Ser\(^{473}\) residues (Fig. 6C, right panel). In four independent experiments, the levels of pAkt-Ser\(^{473}\) reached in IEX-1 expressing cells were decreased by 53.5 ± 7.5%, upon transfection with ERK-AFG, as compared with ERK-WT. These results indicate that the capacity of IEX-1 to protect Akt phosphorylation requires ERK kinase activity.

In the B56-IEX-1-pERK complex, B56 subunits are phosphorylated by ERK at a Ser/Pro consensus ERK phosphorylation site that is conserved in all B56 members (Ser\(^{368}\) in B56β). Mutation of the serine to alanine at this site leads to a B56 species whose activity is resistant toward inhibition by IEX-1 (4). As shown in Fig. 6D, at a dose where the WT-B56β protein could only reverse partially IEX-1-mediated increase in Akt phosphorylation, the B56β-S368A completely abolished this effect. Altogether these results indicate that IEX-1-mediated ERK-dependent inhibition of B56-containing PP2As is responsible for its ability to control both ERK and Akt activation.

DISCUSSION

Given the importance of the Akt signaling pathway on many cellular functions and its frequent deregulation in cancer, considerable efforts have been made in the past few years to identify new regulators of its activity. Nonetheless, in contrast to the flood of information regarding the activation mechanism of Akt downstream of PI3K, we have a more limited understanding...
about how Akt activity is turned off. In this work we identify two new actors that influence the duration of Akt activity by regulating directly its dephosphorylation in an opposite fashion: (i) the PP2A-containing B56-regulatory subunits that rapidly and specifically dephosphorylate Akt; and (ii) the early gene IEX-1, which prolongs its activity by preventing this step. The importance of this regulatory pathway to modulate the amplitude and duration of Akt signaling is illustrated by the reduced or increased phosphorylation of Akt and of its downstream substrates occurring upon down-regulation of IEX-1 or B56 subunits expression, respectively.

Several proteins that regulate positively Akt activity have been described. JIP1 (30) and Ft1 (32) tether Akt to PDK1 and increase its phosphorylation, whereas TCL1, by binding to the Akt plekstrin homology domain, augments directly Akt kinase activity and transport without affecting its phosphorylation (33). In contrast to these mechanisms, we found that IEX-1 acts by preventing Akt dephosphorylation on its activating residues. IEX-1 could prolong Akt phosphorylation on both Thr308 and Ser473 after a short pulse stimulation by growth factors; it reduced by 4–5-fold the rate of decay of Akt-phosphorylated forms induced upon inhibition of upstream kinases by serum starvation or addition of LY294002. This effect of IEX-1 on Akt is similar to that found on ERK (4, 19) suggesting that it may be related to its capacity to inhibit the activity of the B56 family of PP2As. Indeed, expression or down-regulation of B56β or γ subunits regulated the decay of Akt phosphorylation in a fashion opposite to that of IEX-1. Both subunits could also reverse the IEX-1 effect on Akt. Just as IEX-1, Hsp90 prevents Akt dephosphorylation by PP2A (13). However, whereas Hsp90 binds directly to Akt and prevents its access to the phosphatase, we could not detect Akt in anti-IEX-1 immunoprecipitates, indicating that the effect of IEX-1 on Akt is indirect and relies only on its ability to inhibit the phosphatase activity of B56-containing PP2As. These results indicate that the action of IEX-1 on PP2A is not limited to steric interference that would occur only within the B56-IEGF-1 ERK complex. They show that IEX-1 is a more general inhibitor of PP2A-B56 activity than expected. The indirect effect of IEX-1 protecting both ERK and Akt from phosphatase action is reminiscent of that recently described for caveolin-1, a protein that inhibits both PP1 and PP2A through scaffolding interactions (31). Whether these different Akt regulatory mechanisms coexist in a given cellular context remains to be determined.

One of the important results of this study is the identification of PP2As containing B56-regulatory subunits as the holoenzymes controlling Akt dephosphorylation. Indeed, in different cell types, overexpression of B56β and γ-family members members accelerated the dephosphorylation of pAkt, whereas their down-regulation by shRNA increased Akt phosphorylation in serum-cultured cells and delayed Akt dephosphorylation upon starvation. By contrast, expression of the B-family members Bo, Bβ (Fig. 4B), or Br (data not shown) had no effect. The ability of IEX-1, which was found to bind to and inhibit specifically B56- but not B-containing PP2As, to increase the half-life of Akt phosphorylation further argues in favor of a role of B56 but not of B holoenzymes in Akt inactivation. Previous studies have shown that PDK1, the upstream Akt Thr308 kinase, may also be a substrate of PP2A (15, 31). We have not tested directly the effect of B56-containing PP2As on PDK1 activity. However, the ability of B56 subunits to increase the rate of disappearance of Akt phosphorylation after addition of the PI3K inhibitor strongly suggests that B56-PP2A holoenzymes affect Akt activity by increasing Akt dephosphorylation directly rather than by preventing its phosphorylation by PDK1. This is also supported by the ability of B56 subunits to regulate Ser473 phosphorylation.

Although many papers have reported the dephosphorylation of Akt by PP2A, very few of them have documented the involvement of specific regulatory subunits in this action. Data in support of a role of B56-containing PP2As in Akt regulation came from studies on the transforming ability of SV40 small t, which was shown to be both dependent on Akt activation (34) and partially mimicked by down-regulation of B56γ (26, 35). Our results confirm the role of B56γ in Akt dephosphorylation. However, by contrast with Chen et al. (35) we found that dephosphorylation of Akt does not seem to be linked solely to the B56γ member. Indeed, expression or down-regulation of either B56γ or B56β subunits is sufficient to modulate pAkt levels. Although we have not been able to test all B56 subunits, these results suggest that at least B56γ and B56β enzymes have redundant effects on Akt activity. The preferential participation of one B56 family member to Akt regulation may depend on the cell context and on the level of expression of a given subunit and/or its subcellular localization.

Other studies have suggested that PP2As containing the B-family subunits could be involved in Akt regulation. Bo was found in the caveolin-1 complex with PP2A-C and PP1. However, caveolin-1 alters both PDK1 and Akt activities and the role of the Bo subunit in this action was not evaluated (31). Beaulieu et al. (36) showed that β-arrestin2 promotes dephosphorylation of Akt by forming a complex with Akt, PP2A-C, and Bo. Thus, whereas we found that B56-containing PP2A can dephosphorylate Akt activated by various growth factors, the action of Bo-containing PP2A might be restricted to dopaminergic neurotransmission or other pathways coupled to β-arrestin2.

Recently, Newton and colleagues (37) identified PHLPP, a phosphatase from the PP1IC family, as a new Akt phosphatase acting mostly on Ser473. Based on a greater sensitivity of Thr308 than of Ser473 phosphorylation to the PP2A inhibitor okadaic acid, they suggested that PHLPP would be the key phosphatase for Ser473, whereas PP2A would regulate specifically the inactivation of Akt at Thr308. By contrast, our results show that both Ser473 and Thr308 are sensitive to expression of B56 subunits and are similarly regulated by IEX-1, indicating that both residues can be substrates for B56-containing PP2As. This is in agreement with many other reports showing, both in vitro and in vivo, that PP2A effectively dephosphorylates the two activating residues of Akt (12–14). Phosphorylation of Thr308 by PDK1 is necessary to activate Akt and this activity is augmented about 5–10-fold through Ser473 phosphorylation by the mTORC2 complex (10, 29). This suggests that PP2A may function as the primary Akt phosphatase. Dephosphorylation of both residues by PP2A-B56 holoenzymes and inhibition of this effect by IEX-1 may provide a
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rapid and complete turn-on and off mechanism to regulate Akt activity. As for activation, the extent of dephosphorylation and therefore inactivation of Akt by PP2A and PHLPP may be regulated separately, creating a modulatory network of regulation that may lead to a fine tuning of Akt signal.

We previously demonstrated that IEX-1-mediated inhibition of B56-containing PP2As results from its ability to allow B56 phosphorylation by active ERK in a ternary B56±IEX-1±pERK complex. By using ERK kinase-inactive dominant negative mutants, IEX-1 proteins unable to bind ERK, as well as the B56 species mutated in their major ERK phosphorylation site (4), we found that IEX-1 capacity to regulate Akt phosphorylation requires ERK activity. This suggests that Akt regulation by IEX-1 lies downstream of ERK activation. ERK and Akt have been shown to cooperate for activation of various common substrates (38–40). Several cross-talk mechanisms between these two signaling pathways have also been described. Notably, depending on the differentiation state, Akt-mediated phosphorylation of Raf-1 can lead to inhibition of ERK activity. As for activation, the extent of dephosphorylation could lead to chronic inhibition of B56-containing PP2A activity in tumor cells and favor both ERK and Akt activation. B56-containing PP2As regulate also several oncoproteins such as c-Myc (49), Bcl2 (50), and β-catenin (5). Whether IEX-1 contributes to tumor formation and inhibits other B56-controlled signaling pathways downstream of ERK activation will need to be determined.

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