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Involvement of $G_{i/o}$ Proteins in Nerve Growth Factor-Stimulated Phosphorylation and Degradation of Tuberin in PC-12 Cells and Cortical Neurons

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ABSTRACT

Tuberin is a critical translation regulator whose role in nerve growth factor (NGF)-promoted neuronal survival has not been documented. In the present study, we examined the ability of NGF to regulate tuberin in PC-12 cells and primary cortical neurons. Incubation of serum-deprived cells with NGF stimulated tuberin phosphorylation and induced proteosome-mediated tuberin degradation. Inhibition of the phosphatidylinositol-3-kinase (PI3K) by wortmannin or overexpression of the kinase dead Akt mutant completely blocked the NGF-induced tuberin phosphorylation and degradation. It is interesting that the NGF-induced tuberin phosphorylation was partially blocked by pertussis toxin or overexpression of regulators of G protein signaling (regulator of G protein signaling Z1 and Gα-interacting protein), suggesting the participation of $G_{i/o}$ proteins. The use of transducin as a G$\beta\gamma$ scavenger indicated that G$\beta\gamma$ subunits rather than $G\alpha_{i/o}$ acted as the signal transducer. Epidermal growth factor can similarly induce tuberin phosphorylation and degradation via a PI3K/Akt pathway in PC-12 cells, but these responses were insensitive to pertussis toxin treatment. Treatment of PC-12 cells with a specific agonist to the G_i-coupled α_2-adrenoceptor also stimulated tuberin phosphorylation transiently, further demonstrating the involvement of $G_{i/o}$ signaling in tuberin regulation in PC-12 cells. Finally, overexpression of nonphosphorylable tuberin attenuated NGF-promoted survival of PC-12 cells, suggesting that the phosphorylation and degradation of tuberin are important for NGF-promoted cell survival. Together, this study demonstrates the regulatory effect of NGF and $G_{i/o}$ signaling on tuberin.

Tuberin, a product of the tumor suppressor gene tuberous sclerosis complex (TSC) 2, is a critical regulator for translation. It inhibits the mammalian target of rapamycin (mTOR), resulting in inhibition of p70 S6 kinase and activation of eukaryotic initiation factor 4E binding protein 1 (4E-BP1, an inhibitor of translation). Tuberin forms a heterodimer with harmartin (TSC1) to inhibit cell growth and proliferation (Li et al., 2004). In mammalian cells, overexpression of hamartin and tuberin inhibits the phosphorylation of mTOR on Ser^{2448}, which is specifically phosphorylated in response to insulin. Coexpression of both hamartin and tuberin restricts tissue growth and reduces cell size and proliferation. Hamartin-tuberin interaction is regulated by tuberin phosphorylation, and defective phosphorylation of tuberin is associated with the loss of its tumor suppressor activity (Aicher et al., 2001). Because tuberin is highly expressed in normal human brain tissues, it may participate in various neuronal functions.

Neuronal cell survival or death during development is mediated by the integration of a diverse array of signal transduction cascades that are controlled by the availability and acquisition of neurotrophic factors. Neurons deprived of these factors undergo apoptosis, which is an important component of neuronal development. Nerve growth factor (NGF) is the prototypical member of the neurotrophin family, which has multiple effects on susceptible precursor and mature neuronal populations, including antiapoptosis, differentia-

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ABBREVIATIONS: TSC, tuberous sclerosis complex; mTOR, mammalian target of rapamycin; 4E-BP1, eukaryotic initiation factor 4E binding protein 1; NGF, nerve growth factor; GPCR, G protein-coupled receptor; RTK, receptor tyrosine kinase; EGF, epidermal growth factor; Erk, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; PTX, pertussis toxin; RGS, regulator of G protein signaling; GAIP, Gα-interacting protein; UK-14,304, 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine; DMEM, Dulbecco's modified Eagle's medium; E, embryonic day; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; KD, kinase-deficient; WT, wild-type; CA, constitutively active; MEK, mitogen-activated protein kinase kinase; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophynyltio)butadiene; NP, nonphosphorylable; cbl, Casitas B-lineage lymphoma; Gab2, Grb2-associated binder 2.

tion, phenotypic maintenance, and regeneration (Barde, 1989). There is ample evidence to indicate that translation control is a critical process involved in many neurotrophin-mediated neuronal functions in PC-12 cells (Takei et al., 2001). However, the mechanism by which NGF regulates the translational process remains unclear, and the concept of whether NGF can modulate tuberin function has not been explored. Furthermore, it is not clear whether translation control is involved in NGF-regulated neuronal survival.

Apart from trophic factors, accumulating evidence indicates that agonists acting at G protein-coupled receptors (GPCRs) may play a role in cell survival (Polakiewicz et al., 1998; Dermitzaki et al., 2000). Recent studies have also demonstrated that GRCRs can modulate signals elicited by receptor tyrosine kinases (RTKs) and vice versa (Lowes et al., 2002). GPCRs can stimulate Trk receptor activity to increase the survival of neural cells through the actions of Akt (Lee and Chao, 2001; Rajagopal et al., 2004). Moreover, transactivation of RTKs in response to GPCR signaling has been reported previously (Daub et al., 1996; Luttrell et al., 1999). For example, receptors for epidermal growth factor (EGF), insulin-like growth factor-1, and platelet-derived growth factor can be transactivated through GPCRs to generate proliferative signals via the activation of extracellular signal-regulated kinases (Erks). On the other hand, RTKs are able to regulate GPCR signaling. For instance, NGF can use pertussis toxin (PTX)-sensitive $G_{i/o}$ proteins to carry out its biological effects (Rakhit et al., 2001) and alter lysophosphatidate receptor 1 signaling (Moughal et al. 2004). Apart from the Trk receptor, the platelet-derived growth factor β receptor has been shown to associate with a GPCR that binds sphingosine 1-phosphate in human embryonic kidney 293 cells (Alderton et al. 2001; Waters et al. 2003). It is interesting that the involvement of $G_{i/o}$ signaling has been implicated in NGF-promoted sympathetic neuronal survival (Powell et al., 2002). However, the underlying mechanism by which $G_{i/o}$ proteins regulate NGF-promoted survival and translational control has not been clearly defined.

Therefore, the aim of this study was to investigate the effects of NGF and G protein signaling on the regulation of tuberin in PC-12 cells and primary cortical neurons and the functional role of tuberin in neuronal survival. The PC-12 cell is a well established model of sympathetic neurons and has proven extremely useful in studying signaling pathways involved in neuronal survival and death. We provide evidence that, in serum-free medium, both NGF and EGF can induce phosphorylation and degradation of tuberin via the phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway. However, only NGF can use $G_{i/o}$ proteins to stimulate tuberin phosphorylation in a $G\beta\gamma$-dependent manner. Furthermore, treatment of cells with an agonist of the G_i-coupled α_2-adrenoceptor stimulated tuberin phosphorylation in a PI3K-dependent manner. Finally, our data suggest that the phosphorylation and degradation of tuberin play a role in NGF-regulated neuronal survival. This is the first study that demonstrates the regulatory effect of NGF and $G_{i/o}$ signaling on the activity of tuberin.

Materials and Methods

Materials. PC-12 cells (CRL-1721) were obtained from American Type Culture Collection (Manassas, VA). The cDNAs of different wild-type and mutant Akt were generous gifts from Dr. Z. G. Wu (Hong Kong University of Science and Technology). The cDNAs encoding wild-type tuberin and nonphosphorylatable tuberin mutant were kindly provided by Dr. J. Q. Cheng (University of South Florida College of Medicine, Tampa, FL). The cDNAs of regulator of G protein signaling (RGS) Z1 and Gα-interacting protein (GAIP) were purchased from Guthrie cDNA Resource Center (Sayre, PA). Recombinant and purified NGF (2.5S) was obtained from R&D Systems (Minneapolis, MN) and Alomone Labs (Jerusalem, Israel), respectively. PTX was purchased from List Biological Laboratories Inc. (Campbell, CA). Wortmannin and EGF were purchased from Calbiochem (San Diego, CA). Various antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA). Cell culture reagents, including LipofectAMINE Plus, were purchased from Invitrogen (Carlsbad, CA). All other chemicals, including UK-14,304, were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Transfection. PC-12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum and 10% (v/v) heat-inactivated horse serum, 50 units/ml penicillin, and 50 μg/ml streptomycin and grown at 37°C in an environment of 7.5% CO_2. Transfection was performed by means of LipofectAMINE Plus reagents as described previously (Lo et al., 2003). Various cDNAs (2 μg/ml) were transfected. After the transfection, PC-12 cells were cultured in normal growth medium supplemented with 0.3 mg/ml G418 (Geneticin). Studies were performed 2 to 3 weeks later with pooled cultures of G418-resistant PC-12 cells. Cells were seeded on six-well plates at a density of 250,000 cells/well and were cultured in the growth medium for 24 h before serum starvation. Where indicated, PTX pretreatment and serum starvation were performed 24 h before the agonist treatment.

Preparation of Primary Cortical Neurons. Cultures of mouse cortical neurons were prepared as described previously (Price and Brewer, 2001). In brief, cortical neuron cultures from embryonic day 18 (E18) mice were dissociated and plated onto polyornithine-laminin (10 μg/ml)-coated 60-mm dishes in Neurobasal/B27 medium at a density of 2×10^6/dish. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. After the cell plating, medium was refreshed daily, and Western blots were performed 6 days later with pooled cultures of E18 cortical neurons.

Western Blotting Analysis. Parental PC-12 cells, G418-resistant stable transfectants, or primary cortical neurons were lysed in 150 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 40 mM NaP_2O_7, 1% Triton X-100, 1 mM dithiothreitol, 200 μM Na_3VO_4, 100 μM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 4 μg/ml aprotinin, and 0.7 μg/ml pepstatin) after drug, inhibitor, or vehicle (dimethyl sulfoxide) treatments and then gently shaken on ice for 30 min. Supernatants were collected by centrifugation at 14,000 rpm for 5 min. Proteins from the supernatants (50 μg) were resolved by 8 or 12% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membrane (Osmonics, Westborough, MA). Phospho-tuberin-Thr^{1462}, total tuberin, and actin were detected by specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The immunoblots were visualized by chemiluminescence with the enhanced chemiluminescence kit from Amersham Biosciences Inc. (Piscataway, NJ). The images detected in X-ray films were quantified by densitometric scanning using the Eagle Eye II still video system (Stratagene, La Jolla, CA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-dephenyl-tetrazolium Bromide (MTT) Colorimetric Assay. Various G418-resistant stable transfectants (10,000 cells/well) in serum-free medium DMEM were added to 96-well plates. These cells were treated with or without 100 ng/ml NGF for 2 days. After replacement of the medium with 0.5 mg/ml MTT in DMEM, cells were returned into the incubator for 4 h. Cells and MTT formazan crystals were then solubilized by trituration in a solubilization buffer (10% SDS in 0.01 M HCl), and the survival profile of the cells was quantified by spectrophotometrically measuring the plate at 570 nm.

Results

NGF Induced the Phosphorylation and Degradation of Tuberin in PC-12 Cells. We first examined the ability of NGF to regulate tuberin phosphorylation and degradation in PC-12 cells. PC-12 cells were treated with 5 or 50 ng/ml NGF for different times (0–30 min), and the phosphorylation and protein levels of tuberin were evaluated. As shown in Fig. 1A, NGF rapidly induced the phosphorylation and decreased the total protein level of tuberin in PC-12 cells. In contrast, NGF had no effect on the protein level of actin. Although 50 ng/ml NGF induced larger extents of tuberin phosphorylation at shorter time points (5–20 min) than 5 ng/ml, tuberin was degraded in both cases at similar rates. The effects of NGF on tuberin phosphorylation and degradation were concentration-dependent with a significant effect observed at 5 ng/ml and reaching a maximal level at 10 to 100 ng/ml (Fig. 1B). Moreover, the proteosome inhibitor MG132 attenuated the NGF-induced tuberin degradation in a dose-dependent manner (Fig. 2). MG132 did not affect the ability of NGF to induce tuberin phosphorylation. These data suggest that NGF down-regulation of tuberin is mediated by a post-translational modification mechanism in which the proteosome pathway is involved.

NGF-Induced Phosphorylation and Degradation of Tuberin Are Dependent on the PI3K/Akt Pathway. Increasing evidence indicates that tuberin is a physiological substrate of Akt (Dan et al., 2002). A number of studies have demonstrated that there are several potential Akt phosphorylation sites in tuberin, but not in hamartin (for review, see Li et al., 2004). To investigate the requirement of the PI3K/Akt pathway in NGF-induced tuberin regulation in PC-12 cells, we used a specific PI3K inhibitor, wortmannin. Our results demonstrated that both the NGF-induced tuberin phosphorylation and degradation

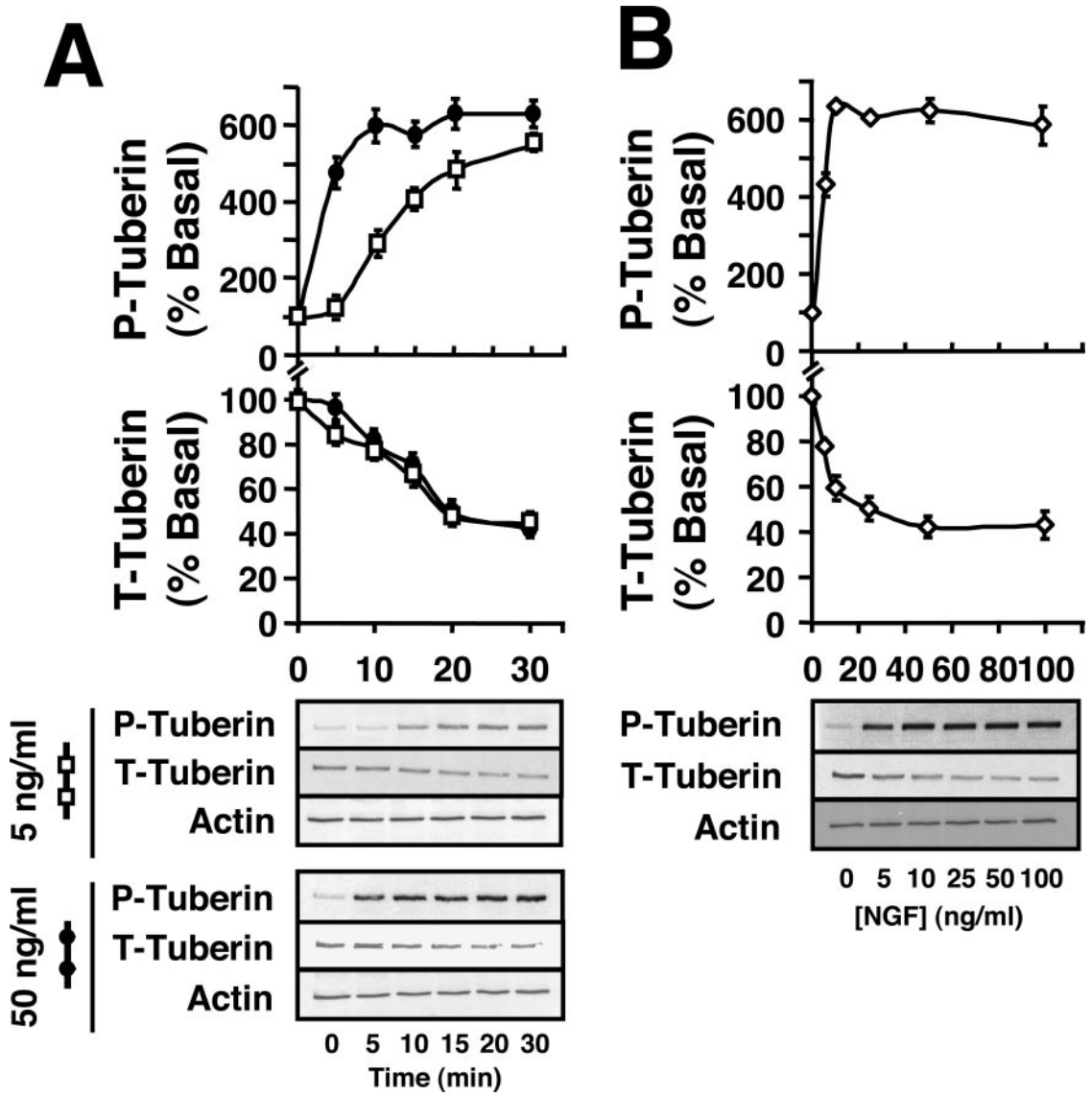

Fig. 1. NGF induced the phosphorylation and decreased the total protein level of tuberin in serum-starved PC-12 cells in a time- and dose-dependent manner. PC-12 cells were treated with 5 or 50 ng/ml NGF for various times (A) or with different concentrations of NGF for 10 min (B). Tuberin phosphorylation (P-tuberin) and total protein levels (T-tuberin) were determined by Western blot analysis. The immunoblots shown represent one of three sets; two other sets yielded similar results. The bands detected in X-ray films were quantified, and the value of untreated basal control was set at 100%. Values shown represent the mean ± S.E. from three separate sets of immunoblots.

were significantly inhibited by treatment of PC-12 cells with 100 nM wortmannin before the NGF challenge (Fig. 3A). To investigate the involvement of the PI3K downstream effector Akt in NGF-induced tuberin regulation, a kinase-deficient Akt mutant (Akt-KD) was used. After G418 selection, the transfected PC-12 cells were treated with or without NGF. Overexpression of Akt-KD, but not wild-type Akt (Akt-WT), blocked the NGF-induced tuberin phosphorylation (Fig. 3B). NGF also failed to induce tuberin degradation in Akt-KD-expressing cells. We then examined a constitutively active Akt construct (Akt-CA) for its function in tuberin phosphorylation in the transfected PC-12 cells. Compared with the transfectants overexpressing Akt-WT, expression of Akt-CA induced a significant increase of tuberin phosphorylation (Fig. 3C). The protein level of tuberin was also reduced in cells expressing Akt-CA. In contrast, pretreatment of an MEK1/2 inhibitor (U0126), at concentrations sufficient to block NGF-induced Erk1/2 activation, did not affect the NGF-induced tuberin regulation (data not shown). These results indicated that NGF stimulates tuberin phosphorylation and degradation via the PI3K/Akt pathway, but not the Erk pathway.

NGF Used $G_{i/o}$ Proteins to Stimulate Tuberin Phosphorylation and Degradation in a $G\beta\gamma$-Dependent Manner. A previous study has implicated the involvement of $G_{i/o}$ signaling in NGF-stimulated sympathetic neuronal survival (Powell et al., 2002). To determine whether $G_{i/o}$ signaling is also involved in NGF-induced tuberin regulation, PC-12 cells were pretreated with PTX (100 ng/ml; 24 h) to ADP-ribosylate and inactivate $G_{i/o}$ proteins (Fig. 4A). At a low concentration of NGF (5 ng/ml), NGF-induced tuberin phosphorylation became apparent after 10 min. PTX partially inhibited the NGF-induced phosphorylation of tuberin at 10 min, but not at 15 min when the induction of tuberin phosphorylation reached near maximal activity. At a high concentration of NGF (50 ng/ml), PTX significantly reduced the NGF-dependent phosphorylation of tuberin at 5 min. However, PTX had no significant inhibitory effect on NGF-induced tuberin phosphorylation at or greater than 10 min. Because the NGF purified from mouse submaxillary gland may be contaminated with GRCR ligands, we repeated the experiments by using recombinant NGF. We confirmed that recombinant NGF-induced tuberin phosphorylation was also partially sensitive to PTX treatment, similar to that obtained with purified NGF (data not shown). To confirm that the inhibitory effect of PTX was caused by its ADP-ribosyltransferase activity, we pretreated the PC-12 cells with the B oligomer of PTX, which is devoid of enzymatic activity. In contrast to the PTX holoenzyme, the B oligomer was not able to attenuate the NGF-induced tuberin phosphorylation in PC-12 cells (Fig. 4B). Therefore, we hypothesized that PTX-sensitive $G_{i/o}$ proteins are involved, at least in part, in NGF-induced tuberin phosphorylation. To evaluate this hypothesis, PC-12 cells were transfected with expression vectors encoding RGSZ1, GAIP, or RGS3 and then selected by G418 treatment. All three RGS proteins were expressed to similar levels as determined by Western blot analysis (data not shown). Both RGSZ1 and GAIP function as GTPase-activating proteins for different G_i family members; they facilitate the conversion of Gα-GTP to Gα-GDP and thereby negatively regulate heterotrimeric G protein activation (Hollinger and Hepler, 2002). Overexpression of these RGS proteins should suppress $G_{i/o}$-dependent pathways. Our results demonstrated that overexpression of RGSZ1 and GAIP led to small but insignificant decrease in basal tuberin phosphorylation levels but markedly reduced the NGF-stimulated tuberin phosphorylation (Fig. 4C). However, the NGF-induced tuberin degradation was apparently unaffected by the overexpression of RGSZ1 or GAIP. In contrast, RGS3, an RGS protein that inhibits $G\alpha_q$-mediated signaling (Xie et al., 2000), did not inhibit the NGF-induced tuberin phosphorylation (Fig. 4C). These results further support a role of $G_{i/o}$ proteins in NGF-induced tuberin regulation. Inhibition of $G\alpha_{i/o}$ proteins by RGSZ1 and GAIP favors accumulation of $G\alpha_{i/o}$-GDP, an inactive form of $G\alpha_{i/o}$ that may readily form a complex with G$\beta\gamma$ protein and limit its functions. Therefore, NGF may use activated $G\alpha_{i/o}$, released G$\beta\gamma$, or both to regulate tuberin. To examine the role of Gα subunits in tuberin phosphorylation, we transfected PC-12 cells with cDNAs encoding various wild-type or constitutively active Gα subunits of the G_i subfamily (G_{i1}, G_{i2}, G_{i3}, G_{oA}, and G_{oB}). However, none of them could significantly induce tuberin phosphorylation (data not shown). To reveal the participation of G$\beta\gamma$ subunits in NGF-regulated tuberin phosphorylation, transducin (a G$\beta\gamma$ scavenger) was overexpressed in PC-12 cells. Transducin reduced NGF-stimulated tuberin phosphorylation by approximately 40% (Fig. 4D). The involvement of G$\beta\gamma$ in tuberin phosphorylation and degradation was then investigated by expressing $G\beta_1$ and $G\gamma_2$ in the PC-12 cells. Overexpression of $G\beta_1$ or $G\gamma_2$ alone did not significantly induce

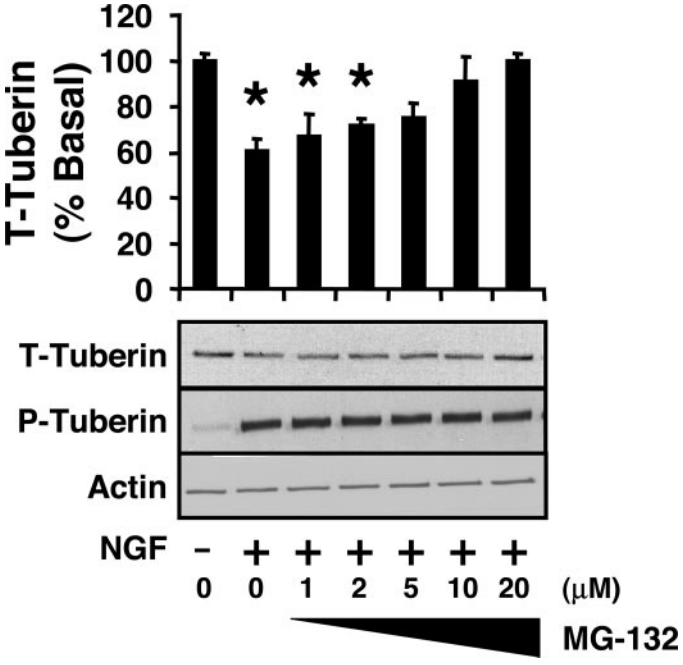

Fig. 2. Proteosome inhibitor MG132 attenuated the NGF-induced tuberin degradation in a dose-dependent manner. PC-12 cells were pretreated with various concentrations of MG132 for 30 min and then stimulated with 5 ng/ml NGF for 10 min. Tuberin phosphorylation (P-tuberin) and total protein levels (T-tuberin) were determined by Western blot analysis. The immunoblots shown represent one of three sets; two other sets yielded similar results. The bands detected in X-ray films were quantified, and the value of untreated basal control was set at 100%. Values shown represent the mean ± S.E. from three separate sets of immunoblots. * NGF significantly induced the degradation of tuberin compared with the corresponding untreated control (Dunnett's t test, $P < 0.05$).

tuberin phosphorylation. However, coexpression of $G\beta_1\gamma_2$ resulted in an induction of tuberin phosphorylation and degradation even in the absence of NGF stimulation (Fig. 4E). These findings suggest that NGF may use $G_{i/o}$ proteins to stimulate tuberin phosphorylation and degradation in a $G\beta\gamma$-dependent manner.

EGF Induced Tuberin Phosphorylation and Degradation via PI3K/Akt, but Not $G_{i/o}$ Proteins. Apart from the Trk receptors for NGF, EGF receptors are endogenously expressed in PC-12 cells and participate in the promotion of cell viability. It has recently been demonstrated that NGF and EGF can enhance cell viability in PC-12 cells via similar signaling pathways (Kawamata et al., 2003). Therefore, the effect of EGF on tuberin regulation was also examined in this study. Serum-starved PC-12 cells were treated with EGF at different times and concentrations. As illustrated in Fig. 5, A and B, EGF significantly induced tuberin phosphorylation and degradation in a time- and dose-dependent manner. PC-12 cells were treated with 3 ng/ml EGF for time periods of 1 to 15 min. Tuberin phosphorylation was increased in the PC-12 cells after 1 min of EGF treatment, and the phosphorylation

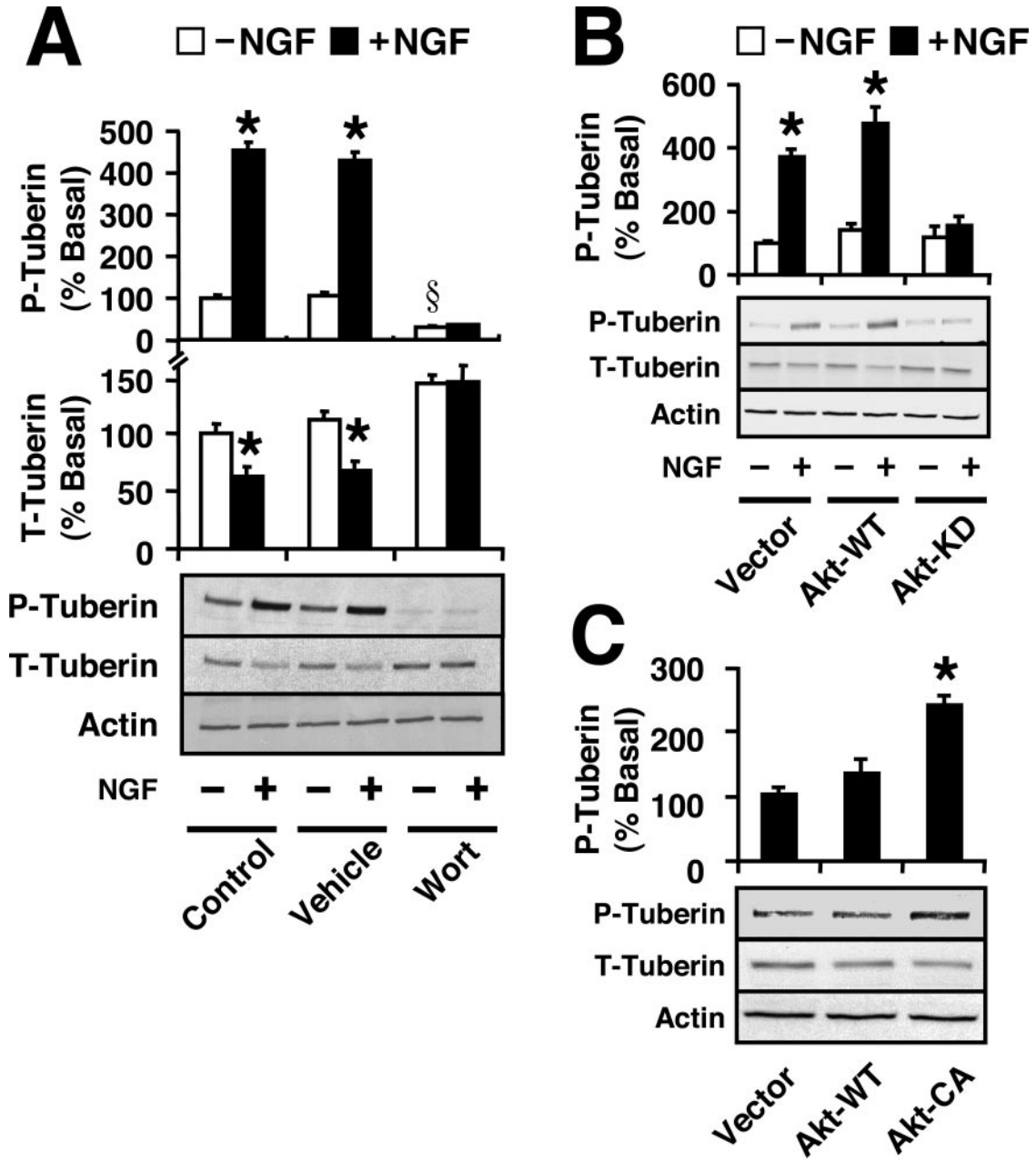

Fig. 3. NGF-regulated tuberin phosphorylation and degradation is PI3K/Akt-dependent. Parental PC-12 cells were pretreated with or without 100 nM wortmannin (Wort) for 30 min (A); PC-12 cells transfected with pcDNA3 vector, Akt-WT, Akt-KD, or Akt-CA were treated with or without 5 ng/ml NGF for 10 min (B and C). The phosphorylation level (P-tuberin) and the total protein level (T-tuberin) of tuberin were determined by Western blot analysis. The immunoblots shown represent one of three sets; two other sets yielded similar results. The bands detected in X-ray films were quantified, and the value of untreated basal control was set at 100%. Values shown represent the mean ± S.E. from three separate sets of immunoblots. *, NGF treatment or overexpression of Akt-CA significantly stimulated tuberin phosphorylation or degradation compared with the corresponding untreated or vector-transfected control (Dunnett t test, $P < 0.05$). § Wortmannin significantly inhibited basal tuberin phosphorylation compared with the corresponding control (Dunnett's t test, $P < 0.05$).

was sustained up to 15 min. However, unlike the NGF-stimulated tuberin regulation, EGF-induced tuberin phosphorylation and degradation were unaffected by PTX, indicating that EGF receptors cannot use $G_{i/o}$ proteins to regulate tuberin activity. To further confirm this hypothesis, we overexpressed RGSZ1 and GAIP in the PC-12 cells and checked for the tuberin phosphorylation again. As depicted in Fig. 5C, although both RGSZ1 and GAIP slightly decreased the basal tuberin phosphorylation level, neither RGSZ1 nor GAIP affected the EGF-mediated tuberin phosphorylation at 3 min. Furthermore, RGSZ1 and GAIP did not affect the EGF-induced tuberin degradation at 15 min (data not shown). Wortmannin completely blocked the EGF-mediated tuberin phosphorylation, indicating that PI3K/Akt pathway is important for both NGF- and EGF-induced tuberin regulation (Fig. 5D).

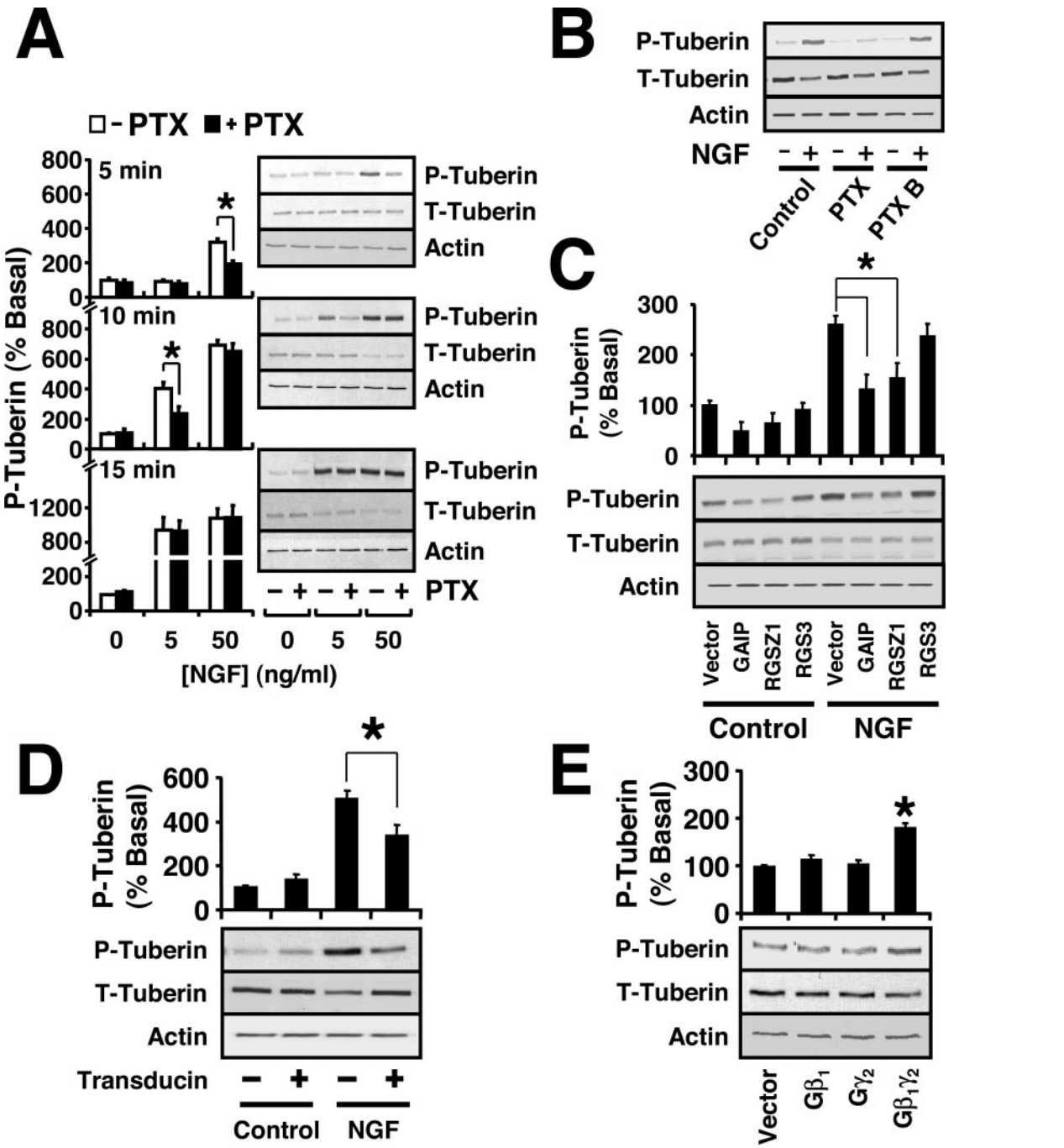

Fig. 4. Involvement of $G_{i/o}$ proteins in NGF-induced tuberin phosphorylation. PC-12 cells were pretreated with or without PTX (A; 100 ng/ml; 24 h), B oligomer of PTX (B; 100 ng/ml; 24 h), or transfected with pcDNA3 vector, GAIP, RGSZ1, and RGS3 (C), transducin (D), $G\beta_1$ and $G\gamma_2$ (E) before stimulation with 5 ng/ml NGF for 10 min or as indicated. Tuberin phosphorylation (P-tuberin) and total protein levels (T-tuberin) were determined by Western blot analysis. The immunoblots shown represent one of three sets; two other sets yielded similar results. The bands detected in X-ray films were quantified, and the value of untreated basal control was set at 100%. Values shown represent the mean ± S.E. from three separate sets of immunoblots. ∗ Tuberin phosphorylation was significantly different from the corresponding untreated or vector-transfected control (Dunnett's t test, $P < 0.05$).

Treatment of PC-12 Cells with UK-14,304 Stimulated Tuberin Transient Phosphorylation. $G_{i/o}$ proteins are expressed in neuronal cells and can be activated by endogenous G_i-coupled receptors. Based on our findings that $G_{i/o}$ proteins are apparently involved in NGF-regulated tuberin activity, we predicted that G_i-coupled receptors can regulate tuberin phosphorylation in PC-12 cells. The α_2-adrenoceptor, which is endogenously expressed in PC-12 cells, couples to several G_i proteins (Chabre et al., 1994). Stimulation of PC-12 cells with 10 μM UK-14,304 (an α_2-adrenoceptor

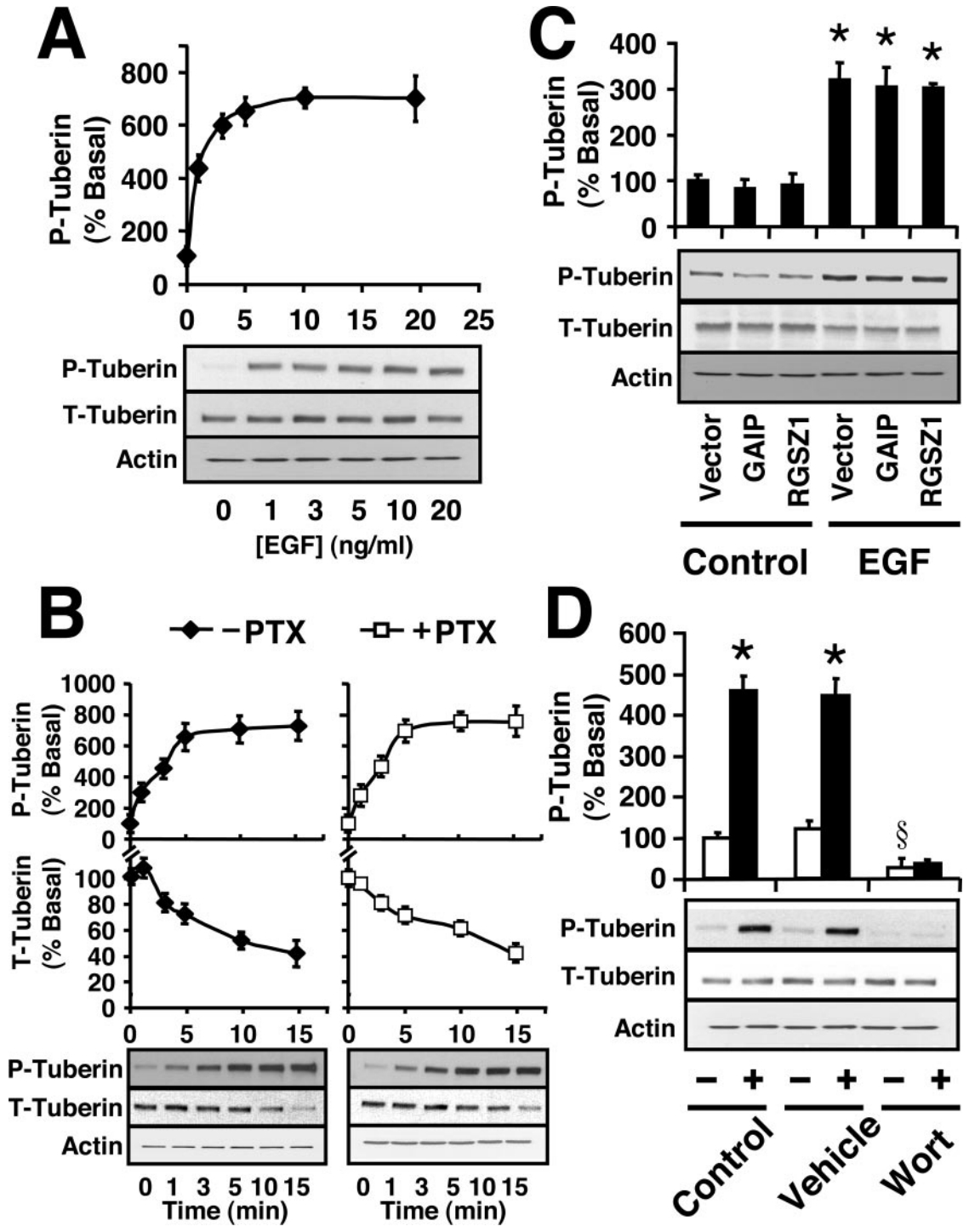

Fig. 5. EGF induced tuberin phosphorylation and degradation via PI3K/Akt, but not $G_{i/o}$ proteins. PC-12 cells were treated with different concentrations of EGF for 3 min (A) or 3 ng/ml EGF for various times with or without PTX pretreatment (100 ng/ml; 24 h) (B). PC-12 cells were transfected with pcDNA3 vector, GAIP, or RGSZ1 (C) or pretreated with or without 100 nM wortmannin (Wort) for 30 min (D) before treatment with or without 3 ng/ml EGF for 3 min. Tuberin phosphorylation (P-tuberin) and total protein levels (T-tuberin) were determined by Western blot analysis. The immunoblots shown represent one of three sets; two other sets yielded similar results. The bands detected in X-ray films were quantified, and the value of untreated basal control was set at 100%. Values shown represent the mean ± S.E. from three separate sets of immunoblots. *, EGF significantly stimulated the tuberin phosphorylation compared with the corresponding untreated basal (Dunnett's t test, $P < 0.05$). §, Wortmannin significantly inhibited basal tuberin phosphorylation compared with the corresponding control (Dunnett's t test, $P < 0.05$).

agonist) increased the levels of tuberin phosphorylation significantly (Fig. 6A). Using 10 μM UK-14,304, tuberin phosphorylation occurred 5 min after agonist stimulation and peaked at 10 min (Fig. 6B). However, as illustrated in Fig. 6B, UK-14,304 was only able to stimulate tuberin phosphorylation transiently, and the total protein level of tuberin was not affected by UK-14,304 treatment. To confirm the involvement of $G_{i/o}$ proteins and PI3K in UK-14,304-mediated tuberin phosphorylation, we treated PC-12 cells with PTX and wortmannin for 24 h and 30 min, respectively, before UK-14,304 stimulation. Both PTX and wortmannin effectively blocked UK-14,304-induced phosphorylation of tuberin (Fig. 6C). These data indicated that activated G_i-coupled receptors are able to transiently regulate tuberin phosphorylation level via $G_{i/o}$ and PI3K signaling.

The Expression of Tuberin Attenuated NGF-Promoted Survival Protection of PC-12 Cells. Numerous reports have demonstrated that tuberin acts as an important upstream inhibitory protein of mTOR (Inoki et al., 2002; Tee et al., 2002). Inhibition of mTOR strongly affected the viability of PC-12 cells (Kawamata et al., 2003). Based on our findings that NGF rapidly induced tuberin degradation via PI3K/Akt in serum-deprived PC-12 cells, tuberin may act as an important inhibitory factor of the prosurvival machinery. In agreement with this idea, Akt has been reported to play a critical role in NGF-promoted PC-12 cell survival (Zheng et al., 2002). Therefore, overexpression of nonphosphorylable tuberin in PC-12 cells may affect the ability of NGF to promote cell survival. To test this hypothesis, Xpress-tagged wild-type tuberin (tuberin-WT) and nonphosphorylable tuberin mutant (tuberin-NP) were transfected into PC-12 cells, and NGF-dependent survival of these cells was determined by the MTT assay. Overexpression of tuberin-WT and tuberin-NP in the transfectants was confirmed by immunodetection using anti-Xpress or anti-tuberin antibodies (Fig. 7A). Treating the cells with NGF induced degradation of endogenous as well as Xpress-tagged tuberin-WT, but not tuberin-NP (Fig. 7A); the intactness of tuberin-NP was demonstrated by the anti-Xpress antibody. This result is consistent with the finding of Dan et al. (2002), who reported that nonphosphorylatable tuberin inhibited the Akt-mediated degradation of tuberin. Serum deprivation induced cell death in approximately 50% of the population in all three transfectants, whereas 100 ng/ml NGF significantly promoted the survival of serum-deprived empty vector control cells and the tuberin-WT transfectants (Fig. 7B). In contrast, the protective effect of NGF on serum-deprived cells was lost in the

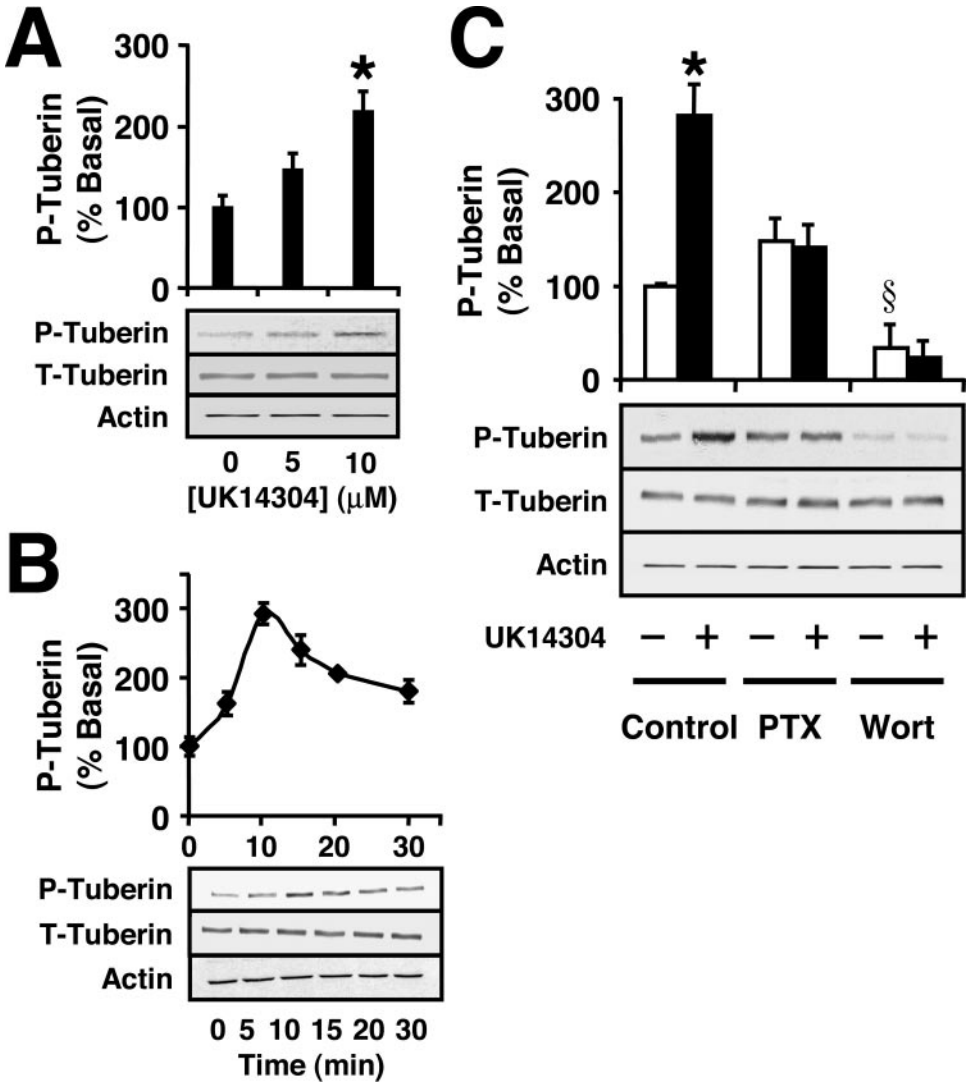

Fig. 6. UK-14,304 induced tuberin phosphorylation via PI3K/Akt and $G_{i/o}$ proteins. PC-12 cells were treated with different concentrations of UK-14,304 for 15 min (A) or 10 μM UK-14,304 for various times (B). PC-12 cells were pretreated with or without 100 ng/ml PTX and 100 nM wortmannin (Wort) for 24 h and 30 min, respectively, before UK-14,304 treatment (10 μM; 15 min) (C). Tuberin phosphorylation (P-tuberin) and total protein levels (T-tuberin) were determined by Western blot analysis. The immunoblots shown represent one of three sets; two other sets yielded similar results. The bands detected in X-ray films were quantified, and the value of untreated basal control was set at 100%. Values shown represent the mean ± S.E. from three separate sets of immunoblots. *, UK-14,304 significantly stimulated the tuberin phosphorylation compared with the corresponding untreated basal (Dunnett's t test, $P < 0.05$). §, Wortmannin significantly inhibited basal tuberin phosphorylation compared with the corresponding control (Dunnett's t test, $P < 0.05$).

tuberin-NP transfectants. These data suggested that controlling the total level of tuberin as well as the phosphorylation is important for NGF-mediated cell survival and further revealed the connection between degradation of tuberin and neuronal survival.

NGF Induced the Phosphorylation and Degradation of Tuberin in Primary Cortical Neurons. To confirm the regulatory role of NGF on tuberin phosphorylation and degradation in neurons, we prepared primary cortical neurons from E18 mice. As demonstrated in Fig. 8A, 50 ng/ml NGF (5 min) induced the phosphorylation of tuberin in primary cortical neurons. Both PTX and wortmannin effectively attenuated the NGF-induced phosphorylation of tuberin, indicating the involvement of $G_{i/o}$ proteins and PI3K/Akt. To detect changes in total protein level of tuberin, we stimulated the primary cortical neurons for 15 min. Our results demonstrated that tuberin phosphorylation was sustained up to 15 min, whereas the total tuberin level was lowered (Fig. 8B). In contrast, NGF had no effect on the protein level of actin (Fig. 8B). These data confirmed the in vivo role of NGF in tuberin regulation.

Discussion

The role of tuberin in the regulation of translational control is well established. The serum-deprived PC-12 cell has emerged as a major model in the study of neuronal survival, and the induction of this process by NGF has been extensively documented. However, the importance of tuberin in NGF-regulated neuronal survival has not been appreciated. In the current study, we showed for the first time that exposure of serum-deprived PC-12 cells and primary cortical neurons to NGF increases the phosphorylation of tuberin, thus leading to decreased tuberin protein level via a proteosome-mediated mechanism. Likewise, EGF can induce tuberin phosphorylation and degradation in PC-12 cells. Moreover, we found that the decrease in tuberin level contributes to the ability of NGF to promote survival in serum-deprived PC-12 cells.

Several lines of evidence have highlighted the importance of tuberin degradation in the ability of NGF to induce survival of serum-deprived neuronal cells. First, NGF stimulated tuberin phosphorylation and induced tuberin degradation dramatically in serum-deprived PC-12 cells and primary cortical neurons. Second, the ability of NGF to promote survival in serum-deprived PC-12 cells was inhibited by overexpression of nonphosphorylatable tuberin. The finding that phosphorylation of tuberin participated in the regulation of cell viability process in PC-12 cells is consistent with the observations of Inoki et al. (2003), who

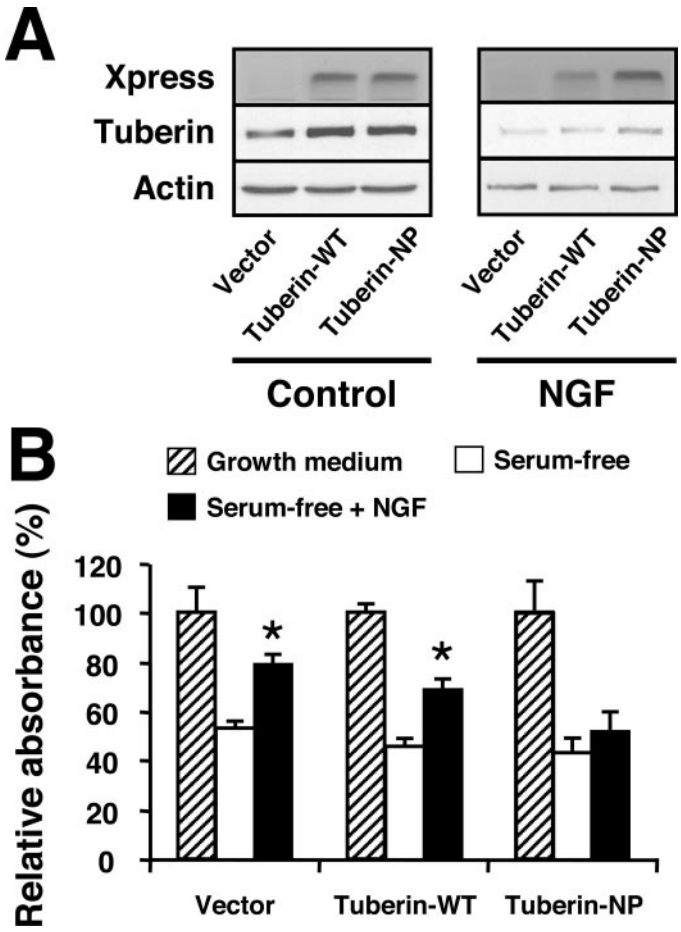

Fig. 7. Overexpression of tuberin attenuated NGF-promoted survival protection of PC-12 cells. PC-12 cells were transfected with pcDNA3 vector, tuberin-WT, or tuberin-NP as described under *Materials and Methods*. Transfectants were treated with or without 100 ng/ml NGF for 48 h. The protein levels of endogenous tuberin or exogenous tuberin-WT and tuberin-NP were determined by immunodetection using anti-tuberin or anti-Xpress antibodies as indicated (A). Different transfectants treated with 100 ng/ml NGF under serum-free conditions were grown for 3 days. The survival of these cells was determined by using the MTT assay. The absorbances detected were quantified, and the value for cells cultured in normal growth medium was arbitrarily set at 100% (B). Values shown represent the mean ± S.E. from three separate sets of experiments. *, NGF significantly promoted survival of cells compared with the untreated control (Dunnett's t test, $P < 0.05$).

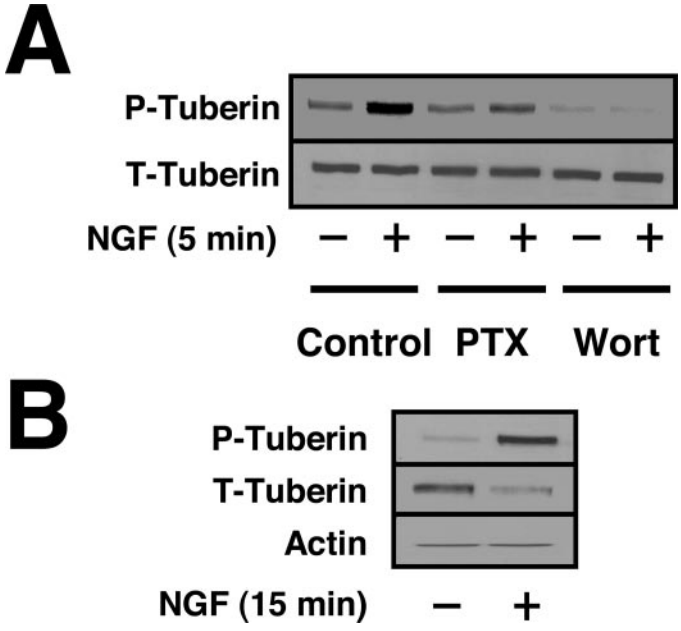

Fig. 8. NGF induced tuberin phosphorylation and degradation via PI3K/Akt and $G_{i/o}$ proteins in primary cortical neurons. Primary cortical neurons were pretreated with or without PTX (100 ng/ml; 24 h) or wortmannin (Wort; 100 nM; 30 min) before stimulation with NGF (50 ng/ml) for 5 min. Tuberin phosphorylation (P-tuberin) and total protein levels (T-tuberin) were determined by Western blot analysis (A). Primary cortical neurons were treated with 50 ng/ml NGF for 15 min. P-tuberin, T-tuberin, and actin levels were determined by Western blot analysis (B). The immunoblots shown represent one of three sets; two other sets yielded similar results.

reported that stimulation of tuberin phosphorylation by AMP-activated protein kinase protected cells from energy deprivation. An increasing body of evidence supports the notion that tuberin acts as a GTPase-activating protein for the small G protein Ras homolog enriched in brain to suppress mTOR and p70 S6 kinase (for review, see Li et al., 2004). mTOR and p70 S6 kinase are known to be involved in insulin-mediated neuronal cell survival (Wu et al., 2004). mTOR transmits positive signals to p70 S6 kinase and participates in the inactivation of the elF4E inhibitor 4E-BP1, resulting in activation of translation. In TSC2 null murine neuroepithelial progenitor cells, p70 S6 kinase was highly activated as assessed by phosphorylation at both Thr^{389} and Thr^{421}, and 4E-BP1 was present exclusively in the inactivated state (Onda et al., 2002). These observations demonstrated that tuberin acts as an important inhibitory factor to suppress the activity of mTOR and its downstream signaling. It is therefore plausible that mTOR signaling can be activated to allow cell survival by removal of tuberin. Consistent with this hypothesis, TSC2 null murine neuroepithelial progenitor cells displayed persistent growth even when growth factors were withdrawn (Onda et al., 2002). Additional evidence showing that inhibition of mTOR abolishes NGF-promoted neuronal survival has been obtained by treating the PC-12 cells with rapamycin, a specific inhibitor for mTOR, which strongly blocked the effect of NGF on PC-12 cell viability (Kawamata et al., 2003). This indicates that mTOR is involved in the NGF-induced prosurvival process in PC-12 cells. It is reasonable to deduce that NGF may induce mTOR activity to promote survival of neuronal cells by the degradation of tuberin.

Binding of NGF to TrkA activates the PI3K signaling pathway, leading to activation of Akt in sympathetic neurons (Crowder and Freeman, 1998). Treatment with the PI3K inhibitor wortmannin and overexpression of Akt-KD completely blocked the NGF-induced tuberin phosphorylation and degradation. These findings suggest that PI3K/Akt signaling is required for NGF-induced tuberin regulation in neuronal cells. The activation of Akt has previously been implicated in the regulation of tuberin activity in non-neuronal cell lines that overexpress the constitutively active Akt mutant (Dan et al., 2002). Engagement of TrkA by NGF also leads to activation of Erk1/2. Liu et al. (2003) reported that activation of Erk1/2 is required for the NGF-promoted prosurvival effect. However, in our study, MEK/Erk activation was not required for the NGF-induced tuberin regulation because U0126, an MEK1/2 inhibitor, did not prevent the NGF-induced tuberin phosphorylation and degradation. Both MEK/Erk and PI3K/Akt pathways have been implicated in the NGF-promoted neuronal survival. However, most of the findings from other groups are similar to ours and support the notion that MEK/Erk is dispensable for NGF-promoted prosurvival effects (Yao and Cooper, 1995).

A previous study has demonstrated the involvement of PTX-sensitive $G_{i/o}$ proteins in NGF-regulated Erk1/2 activation (Rakhit et al., 2001). Furthermore, other studies have shown that TrkA could form complexes with some G protein signal regulators, such as the G protein-coupled receptor kinase 2 and GAIP/GAIP-interacting protein (C terminus) (Lou et al., 2001; Rakhit et al., 2001), thereby providing a putative link between TrkA and G protein signaling pathways. We have observed that treatment with PTX and overexpression of RGSZ1 and GAIP attenuated the ability of NGF to induce tuberin phosphorylation. These results suggest that NGF induces tuberin phosphorylation via $G_{i/o}$ proteins. Additional evidence showing that NGF uses G protein signaling pathways to regulate tuberin was obtained by using cells transfected with the $G\beta\gamma$ scavenger transducin. Overexpression of transducin in PC-12 cells significantly decreased NGF-induced tuberin phosphorylation and degradation. Based on these findings, the $G\beta\gamma$ complex released upon activation of $G_{i/o}$ proteins is apparently involved in NGF-induced tuberin regulation. Because free $G\beta\gamma$ has been shown to activate PI3Kγ by its recruitment to the membrane, it is reasonable to infer that released $G\beta\gamma$ stimulates tuberin phosphorylation via the PI3K/Akt pathway. The mechanism by which TrkA receptors induce $G\beta\gamma$ release remains to be determined. However, other studies have reported that growth factor receptors, such as insulin receptor tyrosine kinase, can interact with PTX-sensitive G proteins (Luttrell et al., 1990). Furthermore, insulin-like growth factor 1 has been shown to activate G_i to release $G\beta\gamma$ subunits (Hallak et al., 2000). These findings further reveal and strengthen the important connection between $G_{i/o}$ protein signaling and NGF-induced tuberin regulation. However, not all trophic factor receptors are able to use $G_{i/o}$ proteins to regulate tuberin. In the current study, we demonstrated that both PTX treatment and overexpression of GAIP and RGSZ1 did not affect the EGF-mediated tuberin phosphorylation and degradation. Different recruitment of scaffolding adaptor components to the activated TrkA and the EGF receptor complex may provide an explanation for the involvement of $G_{i/o}$ signaling in NGF-regulated function, but not in EGF. EGF rapidly induced marked dephosphorylation of C3G and rapid recruitment of the C3G/CrkL/Shp2 complex to EGF receptor via the adaptor Casitas B-lineage lymphoma (Cbl) (Galisteo et al., 1995; Wu et al., 2001). In contrast to EGF treatment, C3G is persistently tyrosine phosphorylated after NGF treatment. NGF induces the binding of C3G/CrkL/Shp2 to Grb2-associated binder 2 (Gab2) and TrkA (Wu et al., 2001). In other words, TrkA is able to recruit Gab2 to form a complex, whereas the EGF receptor binds with another adaptor protein Cbl. It is interesting that although both Cbl and Gab2 serve as PI3K adaptors, only Gab2 can be phosphorylated and regulated by the G_i-coupled *N*-formyl-methionyl-leucyl-phenylalanine receptor (Momose et al., 2003). Thus, it seems that Gab2 may serve as a bridge to link TrkA, $G_{i/o}$ proteins, and PI3K.

In addition to trophic factors, the G_i-coupled opioid receptors have been reported to activate the PI3K/Akt pathway, thus leading to cell survival (Polakiewicz et al., 1998; Dermitzaki et al., 2000; Iglesias et al., 2003). Furthermore, increasing evidence shows that GPCRs often cooperate with receptor tyrosine kinases in the regulation of numerous signal transduction pathways (Lowes et al., 2002). Here, we have also demonstrated that activation of the G_i-coupled α_2-adrenoceptor by UK,14–304 induced tuberin phosphorylation in a $G_{i/o}$- and PI3K-dependent manner. The finding that PI3K is downstream of G_i-coupled receptor is in line with other previous studies (Polakiewicz et al., 1998; Kam et al., 2004). However, we observed that the UK-14,304-induced tuberin phosphorylation profile is different from the NGF-stimulated one. UK-14,304 was only able to stimulate

tuberin phosphorylation transiently, but NGF could maintain tuberin in a phosphorylated state persistently. This finding is consistent with the results of the transient effect of PTX on NGF-induced tuberin phosphorylation. In agreement with our findings, Dermitzaki et al. (2000) observed that stimulated G_i-coupled κ- and δ_2-opioid receptors transiently prevented PC-12 cells from cell death after short periods of serum withdrawal. More recently, Iglesias et al. (2003) reported that the μ-opioid receptor promotes neuronal survival via a $G_{i/o}$-coupled, PI3K/Akt-dependent signaling cascade.

In conclusion, we have presented evidence that NGF and EGF induce the phosphorylation and degradation of the tuberin via PI3K/Akt to protect neuronal cells. $G_{i/o}$ protein signaling is involved in NGF-induced tuberin regulation, but not in EGF-dependent regulations. $G_{i/o}$ may be directly regulated by the Trk receptor, or it may indirectly act as a modulator of NGF-induced responses. In this scenario, tuberin functions as an inhibitory factor to affect the prosurvival machinery. Removal of tuberin may constitute a mechanism by which trophic factors and G_i-coupled receptors can promote neuronal cell survival. However, different studies have shown that other Akt-mediated actions, such as inhibition of Bad and phosphorylation of Forkhead family of transcription factors (Zheng et al., 2002), are involved in neurotrophin-induced survival. These effects of Akt may act independently or synergistically with degradation of tuberin, leading to cell survival. It remains to be determined whether degradation of tuberin can indeed cooperate with other prosurvival effects in neuronal cell lineages.

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