Phosphorylation of IκB-β Is Necessary for Neuronal Survival*

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Cerebellar granule neurons undergo apoptosis when switched from culture medium containing depolarizing levels of potassium (high potassium or HK) to nondepolarizing medium (low potassium or LK). We showed that in healthy neurons maintained in HK medium, IκB-β is phosphorylated at a novel site, Tyr-161. LK-induced neuronal apoptosis is accompanied by a decrease in the extent of IκB-β phosphorylation at this residue. Tyr-161 shares similarity to the consensus sequence for phosphorylation by the nonreceptor tyrosine kinases Abl and Arg. Arg phosphorylates Tyr-161 differentially in vitro, and LK treatment does cause a down-regulation of Arg activity. Moreover, treatment of neurons with two structurally distinct and highly selective Abl inhibitors, PD173955 and Gleevec, blocks HK-induced phosphorylation of IκB-β at Tyr-161 and induces neuronal apoptosis. Overexpression of wild-type IκB-β blocks LK-induced apoptosis, but this effect is abolished when Arg is pharmacologically inhibited. On the other hand, forced overexpression of IκB-β in which Tyr-161 is mutated inhibits survival in HK demonstrating the importance of this residue to neuronal survival. Phosphorylation of IκB-β enhances its association with p65/RelA causing an increase in NF-κB DNA binding activity. Our results identified IκB-β phosphorylation as a key event in neuronal survival and provided a mechanism by which this is mediated.

Apoptosis plays a critical role in the normal development of the nervous system by eliminating large numbers of superfluous neurons and ensuring proper neural connections. Aberrant apoptosis often occurs during adulthood leading to an unwanted loss of neurons such as that seen in neuropathological conditions, including Alzheimer, Parkinson, or Huntington disease and following ischemic stroke (reviewed in Ref. 1). Understanding the molecular mechanisms regulating apoptosis will therefore improve our understanding of neurodevelopment and will lead to the development of useful therapeutic strategies against neurodegenerative conditions.

One family of molecules that plays a pivotal role in the maintenance of neuronal survival in a variety of in vivo and in vitro experimental paradigms is NF-κB, a widely expressed transcription factor. Inhibition of NF-κB activity causes neuronal death in a variety of tissue culture paradigms of neurodegeneration (1–3). Reduced NF-κB activity has also been implicated in neurodegenerative diseases such as Alzheimer disease, Parkinson disease, and amyotrophic lateral sclerosis (reviewed in Ref. 4). In mammalian cells, there are five NF-κB proteins, p50, p52, p65 (RelA), RelB, and c-Rel, characterized by the presence of a conserved 300-amino acid Rel homology domain that is located toward the N terminus of the protein (reviewed in Refs. 5–8). Functional NF-κB is composed of homodimers and heterodimers of these proteins, typically p65/p50, which are held in the cytoplasm by association with members of the IκB protein family. Generally, but not always, activation of NF-κB is mediated by the phosphorylation of IκB proteins on two N-terminal serine or threonine residues by the IκB kinase (IKK)2 complex, which contains the catalytic subunits IκKα and IκKβ and the regulatory IκK/ NEMO protein. Phosphorylation by the IKK complex targets IκB for degradation via the ubiquitin–proteasome pathway. The released NF-κB thus translocates to the nucleus, where it binds to κB DNA motifs within the promoter regions of a variety of genes (6–8). In addition to nuclear translocation, more recent evidence indicates that maximal transcription activity of NF-κB requires protein-protein interaction and site-specific post-translational modifications, including phosphorylation and acetylation (6–8). Of the seven IκB family members, the best studied is IκB-α, which is phosphorylated by IKK at Ser-32 and Ser-36. Besides being a target of IKK, IκB-α can be phosphorylated at its C-terminal PEST domain by casein kinase-2 and DNA-PK (9–11). In contrast to IKK-mediated phosphorylation, however, IκB-α phosphorylation by these kinases does not cause its degradation (9–11). Another major member of the IκB family is IκB-β, which is phosphorylated by IKK at Ser-19 and Ser-23 (12). In comparison to IκB-α, relatively little is known about the functional significance of IκB-β. Although frequently assumed to be functionally interchangeable with IκB-α, more recent evidence indicates that IκB-β plays distinct roles within the cell (13, 14). Although association with IκB-β can inhibit NF-κB, in some situations, IκB-β can lead to increased NF-κB activity, although the precise mechanisms involved remain to be fully addressed (15, 16).

We have been studying the molecular mechanisms underlying neuronal survival using primary cultures of cerebellar granule neurons. These neurons undergo apoptosis when shifted from medium containing serum and depolarizing concentrations of potassium (high K+ medium, HK) to medium containing low potassium (LK) (17). NF-κB is required for the survival of granule neurons by HK (2). Most interestingly, however, neither the levels nor intracellular distribution of the five NF-κB proteins nor those of IκB-α and IκB-β are altered in neurons primed to undergo apoptosis by LK treatment (2). We have reported previously that one factor involved in the down-regulation of NF-κB activity by LK treatment is a lowering interaction between p65 and cAMP-response element-binding protein-binding protein (CBP), an alteration that is associated with hyperphosphorylation (3).

In this study, we show that cerebellar granule neuron survival by membrane-depolarizing stimuli such as HK involves phosphorylation of IκB-β. HK-induced phosphorylation of IκB-β occurs at a novel site, Tyr-161. We also report that phosphorylated IκB-β associates with...

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2 The abbreviations used are: IKK, IκB kinase; HK, high potassium; LK, low potassium; DAPI, 4,6-diamidino-2-phenylindole; DTTH, dihydrothiothixone; PMSF, phenylmethylsulfonyl fluoride; EMSA, electromobility shift assay; DMEM, Dulbecco’s modified Eagle’s medium; GFP, green fluorescent protein; EGFP, enhanced GFP; GST, glutathione S-transferase; EMMA, electromobility shift mobility assay; oligos, oligonucleotides; PB, phosphate buffer.
NF-κB and stimulates its DNA binding activity. In neurons primed to die by LK treatment, there is a reduction in IκB-β phosphorylation and binding activity of NF-κB. We present evidence that HK-induced phosphorylation of IκB-β is likely mediated by nonreceptor tyrosine kinases, Abl/Arg, molecules shown previously to regulate neuronal morphogenesis and axon guidance in the developing nervous system (18–22). Overexpression of wild-type IκB-β prevents LK-induced apoptosis in neurons, whereas addition of PD173955, a highly selective Abl/Arg inhibitor, abolishes the neuroprotection effect by IκB-β. Moreover, when IκB-β (Y161F) mutant is overexpressed in neurons, neuronal survival in HK is reduced. Our results also show that mutation on Tyr-161 of IκB-β decreases the interaction between IκB-β and p65, which is similar to the effect of LK treatment and HK treatment with the presence of Abl inhibitor. Thus, our results identify IκB-β phosphorylation as a key event in neuronal survival and provide a mechanism by which this is mediated.

EXPERIMENTAL PROCEDURES

Materials—Unless specified otherwise, all chemicals were purchased from Sigma. PD173955 was a kind gift from Dr. Bayard Clarkson (Memorial Sloan-Kettering Cancer Center, New York). CGP57148B (Gleevec) was obtained from Novartis Pharma AG (Switzerland). Both chemicals were dissolved in MeSO. Antibodies for p65 (sc-797), IκB-β (sc-945), IκB-α (sc-371), GST (sc-138), c-Abl (sc-23), Arg (sc-2708), and c-Jun (sc-1694) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Specific peptides for IκB-β (sc-945P) and p35 (sc-820P) were from Santa Cruz Biotechnology as well. Anti-IκB-β, c-Abl, and Arg antibodies were also used in immunoprecipitation experiments. Anti-IκB-β and p65 antibodies were used in supershift experiments as well.

Cell Culture and Treatments—Granule neuron cultures were obtained from dissociated cerebella of 7–8-day-old rats as described previously (17). Cells were plated in Basal Eagle’s Medium with Earle’s salts (BME) supplemented with 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine (Invitrogen), and 100 μg/ml gentamycin on dishes coated with poly-L-lysine in 24-well dishes at a density 1.0 × 10⁶ cells/well, 1.2 × 10⁵ cells/60-mm dish, or 3.0 × 10⁴ cells/100-mm dish. Cytosine arabinofuranoside (10 μM) was added to the culture medium 18–22 h after plating to prevent replication of non-neuronal cells. Unless indicated otherwise, cultures were maintained for 6–7 days prior to experimental treatments. For treatment, the cells were rinsed twice and then maintained in LK medium (serum-free BME medium, 5 mM KCl) or HK medium (serum-free BME medium, supplemented with 20 mM KCl). Unless indicated otherwise in the figure legends, treatment of cultures with pharmacological inhibitors was initiated 15 min prior to rinsing and was maintained through the subsequent incubation in LK or HK medium. The control cultures were treated with MeSO.

Plasmid Construction and Mutagenesis—pGEX-KG-IκB-β constructs containing full-length wild-type and the S19A/S23A mutation of IκB-β were the generous gifts from Dr. Richard B. Gaynor (Eli Lilly Co.). The truncation mutants were generated by PCR and cloned into pGEX-KG vector. Site-specific mutations in IκB-β were generated with the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA). The full-length IκB-β containing wild-type or mutations on S19A/S23A and Y161F, respectively, were subcloned into pEGFP-N1 (Clontech) vector at the N terminus of enhanced green fluorescence protein (EGFP). All plasmid constructs were confirmed by sequencing.
**IκB-β Phosphorylation in Neuronal Survival**

**FIGURE 2. Differential phosphorylation of GST-IκB-β proteins by cellular extracts from HK- or LK-treated neurons.** Bacterially expressed GST-IκB-β (wt), (S19A/S23A, shown as dm), C-terminally truncated (1–204dm), and N-terminally truncated (195–359) proteins were bound to glutathione-agarose beads. These beads were incubated with cellular extracts prepared from cerebella granular agarose beads. These beads were incubated with (γ-32P)ATP, A, an autoradiographic image (top panel) for phosphorylation of full-length GST-IκB-β. Coomassie Blue staining of GST-IκB-β proteins that were used as substrates are shown in the 2nd panel. Coomassie Blue staining image for supernatants after GST pull-down assay are shown in the 3rd panel. Levels of phosphorylated GST-IκB-β (top panel) were quantified by using ImageQuant and normalized with levels of both IκB-β protein (2nd panel) and input proteins (3rd panel). The value for phosphorylation of wild-type (wt) GST-IκB-β at HK was set to 1, and others were valued based on the relative levels to the P-GST-IκB-β at HK. The H/L bars refer to the ratio of P-GST-IκB-β at HK versus LK (bottom panel). B, representative autoradiographic image for truncated forms of GST-IκB-β (top panel). Western blot with GST antibody (2nd panel), and Western blot with α-Tubulin antibody (3rd panel). Levels for P-GST-IκB-β were quantified and normalized with GST-IκB-β and α-tubulin levels. The values for phosphorylation of both GST-IκB-β proteins at HK were set at 100, and others were valued based on the relative levels to the P-GST-IκB-β at HK. Results from three separate experiments were analyzed and shown as mean ± S.E. (4th panel). C, schematic structure of GST-IκB-β fusion proteins is as follows: wild-type IκB-β (1st lane), IκB-β with mutations of serine residues 19 and 23 to alanine (2nd lane), referred as IκB-β(dm), IκB-β(dm) with residues 205–359 deleted (3rd lane), 1–204dm, and IκB-β with residues 1–194 deleted (4th lane). The positions of the ankyrin (Ank) repeats, the PEST domain, and serine and/or tyrosine residues that were mutated are shown.

The end of incubation, 1/20 volume of 10% Nonidet P-40 was added. Cells were vortexed for 30 s and then subjected to centrifugation for 30 s at 14,000 rpm. Supernatants were collected as cytosolic proteins.

Nuclei from neurons were resuspended in buffer containing 20 mM HEPES, pH 7.9, 50 mM KCl, 420 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, protease inhibitor mixture and extracted on ice for 30 min, followed by centrifugation at 14,000 rpm for 5 min at 4 °C. The supernatants were collected as cellular extracts. Protein concentrations of the cellular proteins were determined by the Bradford assay using Bio-Rad software (Amersham Biosciences).

**Western Blots**—50 μg of cellular extracts or protein complex pulled down by GST fusion protein or antibodies were resolved on SDS-polyacrylamide gels. The proteins were then transferred to nitrocellulose membranes. Membranes were blocked and incubated with the various antibodies mentioned previously. After washes, membranes were incubated with horseradish peroxidase-conjugated secondary antibody. Following the incubation, membranes were washed extensively and developed with ECL luminal reagent (Amersham Biosciences). The image was captured on x-ray film. Data were quantified using ImageQuant software (Amersham Biosciences).

**Immunoprecipitation**—100–200 μg of cellular lysates from treated neurons was incubated with control and specific antibodies (1 to 2 μg) on ice for 30 min. Protein A/G-agarose beads were added to the mixture, and further incubation was carried out at 4 °C overnight. After incubation, beads were pelleted by centrifugation followed by three washes with buffer containing 10 mM HEPES, pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, and protease inhibitor mixture. The beads were then used in variety of experiments. Supernatants from the immunoprecipitation were collected and resolved on SDS-polyacrylamide gels for Western blotting of α-tubulin antibody.

**Analysis of Phosphorylation on Endogenous IκB-β**—100-mm dishes of 7–8-day-old neurons were washed twice with warm, phosphate-free DMEM (Invitrogen) and incubated in phosphate-free DMEM containing 20 mM KCl overnight. The cultures were then incubated for 6 h in medium containing [32P]orthophosphate (MP Biomedicals, Irvine, CA) with the indicated treatment. After being lysed in ice-cold RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% SDS, 1 mM Na3VO4, 50 mM NaF, 30 mM β-glycerophosphate, 1 mM EDTA, protease inhibitors mixture), the lysates were subjected to immunoprecipitation as described, and the proteins were separated on SDS-polyacrylamide gel. After electrophoretic transfer to nitrocellulose membrane, labeled proteins were visualized by autoradiography. Results were obtained by scanning on Storm860 (Amersham Biosciences). Data were quantified using ImageQuant software (Amersham Biosciences).

**GST Pull-down and In Vitro Kinase Assay**—GST-IκB-β proteins were bound to glutathione-agarose beads. The beads were incubated with whole cell lysates prepared from HK- and LK-treated neuronal
cultures. The whole cell lysates were generated from cultures plated in 100-mm dishes (30 × 10⁶ cells/dish) and lysed in a volume of 250 μl. The kinase assay was performed in the kinase reaction buffer containing 20 mM HEPES, pH 7.9, 100 mM KCl, 5% glycerol, 0.2 mM EDTA, 4 μM ATP, 10 μCi of [γ-³²P]ATP, 5 mM NaF, 1 mM Na₃VO₄, 40 μM MgCl₂, protease inhibitor mixture at 30 °C for 30 min. The beads were pelleted by brief centrifugation. After addition of 4× SDS sample buffer to the pellet, the samples were heated at 95 °C for 5 min. The proteins were resolved on 10% SDS-polyacrylamide gels followed by autoradiography. Results were obtained by scanning with Storm860. Data were quantified using ImageQuant.

**RESULTS**

**Phosphorylation of IκB-β but Not IκB-α Is Reduced during Neuronal Apoptosis**—Cerebellar granule neurons undergo apoptosis when switched from HK medium to LK medium. Although cell death begins at about 16 h, previous studies have shown that commitment to death occurs within 6 h after the switch to LK medium (23–26). As shown in Fig. 1A, IκB-β is phosphorylated in HK medium, and LK treatment leads to a reduction in the level of IκB-β phosphorylation. The reduction in IκB-β phosphorylation is detectable as early as 4 h after LK treatment, suggesting that it is causally involved in the induction of neuronal apoptosis (Fig. 1A). In contrast to IκB-β, the phosphorylation level of IκB-α is similar in HK or LK medium (Fig. 1B). The overall pattern of protein phosphorylation (Fig. 1C) and protein levels of both IκB-α and IκB-β are not altered by the treatment with HK and LK conditions (Fig. 1D), indicating that the reduced phosphorylation of IκB-β is specific.

**HK-induced IκB-β Phosphorylation Occurs at Site(s) Independent of IKK**—It is known that IKK phosphorylates IκB-β at Ser-19 and Ser-23. We examined whether HK-induced IκB-β phosphorylation was medi-
Mapping Apoptosis-regulated Phosphorylation Site—To map the site within IκB-β that is differentially regulated during LK-induced apoptosis, several deletion constructs were generated and used in *in vitro* kinase assays with extracts from HK- and LK-treated neurons (Fig. 2C). As shown in Fig. 2B, GST-IκB-β fragments spanning residues 1–204S19A/S23A displayed differential phosphorylation similar to that seen with the full-length GST-IκB-β protein. In contrast, fragment spanning residues 195–359 were not differentially phosphorylated. Another fragment spanning residues 1–168S19A/S23A also displayed elevated phosphorylation levels in HK compared with LK (data not shown). These results localize the HK-induced phosphorylation site to the region spanning residues 1–168 of IκB-β.

To determine whether the apoptosis-regulated phosphorylation site was a Tyr residue or a Ser/Thr residue, we used genistein, a broad spectrum pharmacological inhibitor of tyrosine kinases. Addition of genistein to the *in vitro* kinase assay abolished the differential phosphorylation of GST-IκB-β (Fig. 3A), suggesting that the HK-induced phosphorylation occurred at a Tyr residue. In comparison, other inhibitors, such as the phosphatidylinositol 3-kinase inhibitor wortmannin, the Akt inhibitor ML-9, and the casein kinase-II inhibitor 5,6-dichlorobenzimidazole riboside, did not affect the differential phosphorylation pattern (Fig. 3A). Western blot analysis of GST-IκB-β in the *in vitro* kinase assay with a phosphotyrosine antibody also showed increased immunoreactivity in HK compared with LK, providing additional evidence that the HK-induced phosphorylation of IκB-β occurred at Tyr residue(s) (Fig. 3B). The higher level of phosphotyrosine immunoreactivity was abolished with the addition of genistein (Fig. 3B). To confirm that tyrosine phosphorylation on IκB-β protein also occurred in *vivo*, immunoprecipitation was performed with IκB-β antibody following by Western

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FIGURE 5. PD173955 inhibits Abl/Arg-mediated phosphorylation on Tyr-161 of GST-1x8-B. 

Immuno precipitation (IP) was performed using anti-Arg or c-Abl antibody (Ab) with cellular extracts prepared from neurons treated for 6 h under HK, LK, or HK with PD173955 (2 μM) conditions. In vitro kinase assay was conducted on immunoprecipitated complex with GST-Ix8-B (1–168dm) or GST-Ix8-B (1–168dmY161F) as substrate. A, results relating to immunoprecipitation with Arg antibody are shown as follows: autoradiograph for phosphorylation of GST-1x8-B (top panel), Western blot with GST antibody (middle panel), and Western blot with α-tubulin antibody on the samples of supernatants from immunoprecipitation (bottom panel). B, quantified result for A is shown. Levels of P-GST-1x8-B were normalized with levels of input GST-1x8-B and α-tubulin. Values of P-GST-1x8-B at HK are set to 100. Others are valued based on the relative level to the P-GST-1x8-B at HK. The results are expressed as the mean ± S.D. of three separate experiments. C, results relating to immunoprecipitation with c-Abl antibody are shown as follows: autoradiograph for phosphorylation of GST-1x8-B (top panel), Western blot with GST antibody (middle panel), Western blot with α-tubulin antibody on the samples of supernatants from immunoprecipitation (bottom panel). D, quantified result for C is shown. Procedure for data processing is the same as mentioned above in B. E, the membrane used in A is reprobed with the Tyr(P) antibody (top) and Arg antibody (bottom). F, the membrane used in C is reprobed with Tyr(P) antibody (top) and c-Abl antibody (bottom).

Blotting with Tyr(P) antibody. Results in Fig. 3C reveal that a higher level of Tyr(P) immunoreactivity was detected with the HK-treated neuronal lysate, whereas Western blotting results with the 1x8-B antibody show similarity to total 1x8-A immunoreactivity. These in vivo and in vitro results suggest that HK-induced phosphorylation on 1x8-B happens on tyrosine residue(s).

Analysis of the amino acid sequence of 1x8-B in the region between residues 1 and 168 reveals four tyrosine residues, Tyr-52, Tyr-85, Tyr-114, and Tyr-161, that are conserved among rat, mouse, and human 1x8-B proteins. Among them, Tyr-85, Tyr-114, and Tyr-161 are identified by NetPhos2.0 (www.cbs.dtu.dk/services/NetPhos/) as potential phosphorylation sites (Fig. 4A). We investigated whether any of these residues represented the apoptosis-regulated phosphorylation site by using GST-1x8-B in which each of these three sites were mutated in in vitro kinase assays (Fig. 4D). As shown in Fig. 4, B and C, only mutation of Tyr-161 reduced the extent of phosphorylation seen with HK extracts.

Apopotosis-regulated Phosphorylation of 1x8-B Is Mediated by Arg in Vitro—Tyr-161 resides within the consensus sequence for phosphorylation by the nonreceptor tyrosine kinase Abl (analyzed by ScanSite server at scansite.mit.edu). Two Abl kinases are expressed in mammalian cells, c-Abl and Arg. As a step toward examining whether these kinases were responsible for HK-induced 1x8-B phosphorylation, we immunoprecipitated c-Abl or Arg from neuronal cultures treated with HK or LK medium. The ability of the immunoprecipitated kinase to phosphorylate GST-1x8-B was analyzed using a construct containing residues 1–168S19A/S23A of 1x8-A and another construct in which Tyr-161 was mutated to Phe. As shown in Fig. 5, A and B, Arg phosphorylated 1x8-B to a greater extent in HK. The elevated phosphorylation in HK was abolished when Tyr-161 was mutated. PD173955 is a highly selective inhibitor of c-Abl and Arg (27, 28). As shown in Fig. 5A, treatment of neuronal cultures with PD173955 blocks the ability of Arg to phosphorylate GST-1x8-B in HK medium. As seen with the GST-1x8-B construct, treatment of neuronal cultures with PD173955 also blocked HK-mediated phosphorylation of endogenous 1x8-B (Fig. 6, E and F). However, phosphorylation of the GST-1x8-B proteins by c-Abl immunoprecipitants did not exhibit the differential pattern (Fig. 5, C and D). These results suggest that Arg is likely the kinase that mediates the differential phosphorylation of Tyr-161 in 1x8-B. It has been reported that activity of c-Abl and Arg is tightly regulated by phosphorylation on tyrosine residues in these two kinases. Higher kinase activity of c-Abl/Arg correlates to higher tyrosine phosphorylation in these two proteins. We performed Western blot experiments to examine the tyrosine phosphorylation status of immunoprecipitated c-Abl and Arg in HK, LK, and HK with PD173955-treated neurons. HK treatment induces the tyrosine phosphorylation on Arg (Fig. 5E) and only slightly higher levels of tyrosine phosphorylation on c-Abl (Fig. 5F). PD173955 treatment abolishes the tyrosine phosphorylation on both Arg and c-Abl (Fig. 5, E and F), indicating that this drug does affect its targets in vivo. Moreover, the levels of immunoprecipitated Arg and c-Abl remain similar, regardless of the treatment, when the same blots were probed with Arg and c-Abl antibody, respectively. These data suggest that the regulation of Arg and...
c-Abl activity under the conditions we used occurs at the activity level, and not because of a change in the protein level.

Abl Inhibitors Induce Apoptosis in HK Condition and Reduce IκB-β Phosphorylation in Vivo—The above results indicated that elevated IκB-β phosphorylation in HK happens at a site(s) not sensitive to IKK. We also showed that Tyr-161 of IκB-β is related to the induced phosphorylation and that Arg is the candidate kinase for this phosphorylation. We then conducted experiments to address the biological relevance of the above observations with regulating apoptosis in neurons. We treated neuronal cultures with PD173955, a highly selective inhibitor of Abl and Arg (27). As shown in Fig. 6A, treatment with PD173955 blocked HK-mediated survival in a dose-dependent manner. This result was confirmed using a second and structurally distinct inhibitor of Abl/Arg, Gleevec (29, 30), as shown in Fig. 6B. Arguing against a nonspecific toxic effect of these inhibitors is the finding that treatment with PD173955 caused nuclear condensation and the characteristically nonrandom fragmentation of DNA (Fig. 6, C and D). To confirm that the reduction in neuronal survival observed after pharmacological inhibition of Arg/Abl involved a reduction in IκB-β phosphorylation, we performed 32P metabolic labeling experiments. IκB-β was immunoprecipitated from lysates of neuronal cultures that were labeled with [32P]orthophosphate and treated with HK or HK with PD173955 for 6 h. Lysates prepared from these neurons were immunoprecipitated with control IgG (Con. IgG) or IκB-β antibody. Immunoprecipitants (IP) were resolved on SDS-polyacrylamide gel followed by autoradiograph to Phosphorlmager screen. Image for phospho-IκB-β is shown (top). Same membrane was reprobed with IκB-β antibody (bottom), whereas the total tyrosine phosphorylation profile for the HK or HK/PD-treated neurons is shown in F. IB, immunoblot.

FIGURE 6. Treatment with specific c-Abl inhibitors causes apoptosis and reduced IκB-β phosphorylation in HK-treated neurons. Granular neurons were treated with various concentrations of c-Abl inhibitor PD173955 or Gleevec in HK. Viability of cells during the treatment was quantified by counting DAPI-stained cells. Survival rate for HK-treated neurons is set to 100. Values for other treatments were calculated based upon the relative level to the value of HK-treated neurons. Results from three separate experiments are analyzed and shown as mean ± S.E. *, p < 0.05; **, p < 0.005 on unpaired Student’s t test when results from chemical treatment are compared with that of the HK treatment. A, viability of neurons with PD173955 treatment. B, viability of neurons with Gleevec treatment. C, DAPI staining images of neurons treated with PD173955. D, DNA fragmentation analysis was performed. The image of an agarose gel with DNA samples prepared from HK, LK, or HK with PD173955 and LK with PD173955-treated neurons. E, neurons were metabolically labeled with [32P]orthophosphate and treated with HK or HK with PD173955 for 6 h. Lysates prepared from these neurons were immunoprecipitated with control IgG (Con. IgG) or IκB-β antibody. Immunoprecipitants (IP) were resolved on SDS-polyacrylamide gel followed by autoradiograph to Phosphorlmager screen. Image for phospho-IκB-β is shown (top). Same membrane was reprobed with IκB-β antibody (bottom), whereas the total tyrosine phosphorylation profile for the HK or HK/PD-treated neurons is shown in F. IB, immunoblot.
Phosphorylated IkB-β Associates with p65 and Regulates NF-κB Binding Activity—In non-neuronal cell lines, IkB-β has been found to associate with p65 under some conditions causing an increase in NF-κB DNA binding activity (16). NF-κB activity in cerebellar granule neurons is higher in HK (2). Because the phosphorylation of IkB-β is also higher in HK, we examined whether IkB-β associated with p65 in neurons and, if so, what effect the phosphorylation of IkB-β had on its interaction with p65. Full-length GST-IkB-β-bound glutathione-agarose beads were incubated with cellular extracts prepared from neurons treated with HK or LK medium. Following the pull-down assay, p65 that associated with the exogenously added GST-IkB-β was then studied by Western blot with a p65 antibody. As shown in Fig. 7A, association between GST-IkB-β and p65 was detectable under both HK and LK conditions. As observed with IkB-β phosphorylation, the extent of interaction was reduced in LK consistent with the possibility that association between p65 and IkB-β was regulated by the phosphorylation of IkB-β. To investigate this further, endogenous IkB-β was immunoprecipitated from 32P-labeled HK- or LK-treated neuronal cultures, and the phosphorylation of IkB-β was greatly reduced when PD173955 was added to the in vitro kinase reaction. Not unexpectedly and consistent with what was observed in intact neurons, phosphorylation of GST-IkB-β was also reduced in LK-treated cultures. The amount of tyrosine phosphorylation correlated well with the amount of p65 that associated with GST-IkB-β. Thus, association between GST-IkB-β and p65 was clearly detectable when HK lysates were used but was barely detectable in the presence of PD173955, LK-treated lysates, or GST-IkB-βY161F. This mutant IkB-β displayed significantly lower tyrosine phosphorylation than wild-type IkB-β, and no interaction between p65 and IkB-β Y161F was observed.

To examine more directly the significance of IkB-β phosphorylation to its association with p65, we performed GST pull-down and in vitro kinase assay with nonradioactive ATP with lysates from HK- or LK-treated neuronal cultures. As shown in Fig. 7C, GST-IkB-β pulled down from HK-treated cultures was tyrosine-phosphorylated. The extent of tyrosine phosphorylation was greatly reduced when PD173955 was added to the in vitro kinase reaction. Not unexpectedly and consistent with what was observed in intact neurons, phosphorylation of GST-IkB-β was also reduced in LK-treated cultures. The amount of tyrosine phosphorylation correlated well with the amount of p65 that associated with GST-IkB-β. Thus, association between GST-IkB-β and p65 was clearly detectable when HK lysates were used but was barely detectable in the presence of PD173955, LK-treated lysates, or GST-IkB-βY161F. This mutant IkB-β displayed significantly lower tyrosine phosphorylation than wild-type IkB-β, and no interaction between p65 and IkB-β Y161F was observed.

To examine more directly the significance of IkB-β phosphorylation to its association with p65, we performed GST pull-down and in vitro kinase assay with nonradioactive ATP with lysates from HK- or LK-treated neuronal cultures. As shown in Fig. 7C, GST-IkB-β pulled down from HK-treated cultures was tyrosine-phosphorylated. The extent of tyrosine phosphorylation was greatly reduced when PD173955 was added to the in vitro kinase reaction. Not unexpectedly and consistent with what was observed in intact neurons, phosphorylation of GST-IkB-β was also reduced in LK-treated cultures. The amount of tyrosine phosphorylation correlated well with the amount of p65 that associated with GST-IkB-β. Thus, association between GST-IkB-β and p65 was clearly detectable when HK lysates were used but was barely detectable in the presence of PD173955, LK-treated lysates, or GST-IkB-βY161F. This mutant IkB-β displayed significantly lower tyrosine phosphorylation than wild-type IkB-β, and no interaction between p65 and IkB-β Y161F was observed.

To examine whether Abl/Arg-mediated phosphorylation of IkB-β affected the activity of NF-κB during neuronal apoptosis, we performed EMSA. As shown in Fig. 8A and as reported previously (2), the DNA binding activity of NF-κB activity is reduced in neurons primed to die by HK treatment. NF-κB binding activity is also reduced in HK with the presence of PD173955 (Fig. 8B). In contrast, neither LK nor PD173955 had a substantial effect on the DNA binding activity of Sp1 or TFIID. The DNA binding activity of NF-κB (Fig. 8C) was also reduced when the nuclear extracts were co-incubated with IkB-β or p65 antibody (Fig. 8D), suggesting the presence of these proteins in the complex. Taken together, these results suggest that IkB-β and NF-κB are associated in a DNA-binding complex that is stimulated by Arg-mediated phosphorylation of IkB-β.

Overexpression of Wild-type or Y161F Mutant of IkB-β Affects Neuronal Survival—To address further the in vivo function of IkB-β and the relationship of Tyr-161 phosphorylation to neuronal survival, we transiently expressed wild-type IkB-β or a mutant form of the protein in which Tyr-161 was mutated (IkB-βY161F) in cultured neurons. As shown in Fig. 9, forced expression of wild-type IkB-β protects neurons from HK-induced cell death. Consistent with the requirement for Arg-mediated phosphorylation, inclusion of PD173955 blocked protection by wild-type IkB-β. Further underscoring the importance of Arg-mediated IkB-β phosphorylation to neuronal survival is the finding that overexpression of IkB-βY161F decreases neuronal survival in both HK and LK conditions.

**DISCUSSION**

We report that IkB-β is phosphorylated in cerebellar granule neurons and that the level of phosphorylation is reduced in neurons primed to apoptosis by HK treatment. The higher phosphorylation of IkB-β in HK medium is not because of IKK. A mutant form of IkB-β with mutations in the two IKK-mediated phosphorylation sites and a C-terminally truncated form of IkB-β lacking the two phosphorylation sites still display differential phosphorylation in HK versus LK medium. We have mapped the apoptosis-regulated phosphorylation site to Tyr-161. A comparison of sequences from different mammalian species reveals that this is an evolutionarily conserved residue within IkB-β and hence one that may be important for the function of the protein. Indeed, over-
expression of a mutant form of IκB-β in which Tyr-161 is mutated to a nonphosphorylatable residue inhibits neuronal survival even in HK.

Tyr-161 bears the consensus sequence for phosphorylation by the Abl nonreceptor tyrosine kinases. The Abl family consists of c-Abl and its paralogue Arg. Both c-Abl and Arg are expressed in most neurons during development and in the adult brain with Arg being much more abundant than c-Abl in the adult brain (19). Although the functional significance of these Abl kinases in the brain are unclear, roles in neuronal migration, axonal guidance, and synaptic communication have been suggested (18, 20, 22, 31). In E10.5 precludes analysis of the roles of these proteins in brain development and maturation (19).

Consistent with their involvement in IκB-β phosphorylation, the activities of c-Abl and Arg are reduced when neurons are switched from HK to LK medium. Treatment of neurons with PD173955, a specific Abl inhibitor, reduced GST-IκB-β phosphorylation in HK-treated cultures to a level comparable with that observed in LK medium. Although both c-Abl and Arg interact with IκB-β, the differential pattern of endogenous IκB-β phosphorylation is recapitulated in vitro only by Arg implicating it in the phosphorylation of IκB-β. Underscoring the importance of Abl-mediated phosphorylation of IκB-β to neuronal survival is our finding that inhibition of this phosphorylation event using two distinct pharmacological inhibitors, PD173955 and Gleevec, leads to cell death.

How does the phosphorylation of IκB-β at Tyr-161 help neuronal survival? We find that IκB-β associates with p65, and association is enhanced by IκB-β. Mutation of Tyr-161, or the pharmacological inhibition of Abl kinases, reduces the association of IκB-β with NF-κB. The importance of NF-κB to neuronal survival has been well established in a number of neuronal paradigms (1, 2). In cerebellar granule neurons, the activity of NF-κB is reduced by LK treatment (2, 33). A similar reduction of NF-κB activity is observed when IκB-β phosphorylation is inhibited by PD173955 treatment. The crystal structure (Protein Data Bank codes 1K3Z, crystal form I, and 1OY3 crystal form II) of a IκB-β–p65 complex reported recently reveals that this complex can bind to DNA (34). We have confirmed that IκB-β bound to p65 can bind DNA.5 Studies in other laboratories have shown that IκB-β bound to p65 can enhance NF-κB DNA binding activity within the nucleus (15, 16). Taken together, these observations suggest that the phosphorylation of IκB-β increases NF-κB activity by stimulating DNA binding.

The best studied member of the IκB protein family is IκB-α. Although IκB-α and IκB-β have often been assumed to be interchange-

5 L. Liu and R. D'Mello, unpublished observations.
able, a portion of IκB-β that is important for its subcellular localization is missing in IκB-α (34). Tyr-161 resides within this region. EMSA results from our laboratory using recombinant IκB-α shows that it inhibits the DNA binding of p65. In cerebellar granule neurons and other neuronal cell types, overexpression of IκB-β inhibits NF-κB activity and induces apoptosis, whereas overexpression of either wild-type or a mutant form of IκB-β in which IKK-mediated phosphorylation sites are altered results in the survival of neuronal cells under apoptotic conditions. Thus, in the context of neuronal survival, IκB-α and IκB-β have different effects.

In contrast to other members of the IκB family of proteins, mice lacking IκB-β have yet to be generated, and hence the physiological function of IκB-β is poorly understood. Our results implicate a novel role for IκB-β in the regulation of neuronal survival. This action of IκB-β is regulated by its phosphorylation by Abl kinases and leads to the activation of NF-κB. By understanding in more detail the molecular events leading to the inactivation of c-Abl and Arg in dying neurons and the mechanism by which reduced IκB-β phosphorylation ultimately leads to neuronal death may shed insight into the mechanisms underlying neurodegenerative diseases.

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