DYRKs are a new family of dual-specificity tyrosine-regulated kinases with emerging roles in cell growth and development. Recently, we discovered that DYRK3 is expressed primarily in erythroid progenitor cells and modulates late erythropoiesis. We now describe 1) roles for the DYRK3 YTY signature motif in kinase activation, 2) the coupling of DYRK3 to cAMP response element (CRE)-binding protein (CREB), and 3) effects of DYRK3 on hematopoietic progenitor cell survival. Regarding the DYRK3 kinase domain, inactivation of Tyr333 (but not Tyr331) within subdomain loop VII–VIII was critical for activation. Tyr331 plus Tyr333 acidification (Tyr mutated to Glu) was constitutively activating, but kinase activity was not affected substantially by unique N- or C-terminal domains. In transfected 293 and HeLa cells, DYRK3 was discovered to efficiently stimulate CRE-luciferase expression, to activate a CREB-Gal4 fusion protein, and to promote CREB phosphorylation at Ser133. Interestingly, this CREB/CRE response was also supported (50% of wild-type activity) by a kinase-inactive DYRK3 mutant as well as a DYRK3 C-terminal region and was blocked by protein kinase A inhibitors, suggesting functional interactions between protein kinase A and DYRK3. Finally, DYRK3 expression in cytokine-dependent hematopoietic FDCW2 cells was observed to inhibit programmed cell death. Thus, primary new insight into DYRK3 kinase signaling routes, subdomain activities, and possible biofunctions is provided.

Mammalian DYRKs1 (1, 2) and HIPKs (3) are recently described subfamilies of MAPK-related protein kinases that target Ser/Thr sites, yet also appear to be activated by tyrosine (auto)phosphorylation at a conserved YXX motif (or loop) between consensus kinase subdomains VII and VIII (hence, the nomenclature dual-specificity tyrosine-regulated kinases, DYRKs) (1, 4). At least seven DYRK isoforms have been described (5) that appear to derive from four unique genes (dyrk1–4).2 Within this family, DYRK1 has been best studied to date, is expressed at high levels in brain (6), maps to the critical region of the Down’s syndrome locus (7), and precipitates learning and memory defects when expressed in transgenic mice (8). Also, mutation of a dyrk1 gene homolog in Drosophila (MNB, for mini-brain kinase) disrupts neuroblast formation in the outer proliferation center and limits optic and central lobe development (9). By comparison, HIPKs contain a DYRK-type kinase domain, but, as a separable subfamily, differ in possessing N-terminal domains that interact with NK homeoproteins (3). HIPK2 has been best studied and recently has been discovered to play an important role in p53 regulation during radiation-induced apoptosis (10).

Other DYRKs have not been well studied. Recently, our laboratory (11) and Lord et al. (12) discovered that DYRK3 is selectively expressed at high levels in hematopoietic cells of erythroid lineage. Using an antisense oligonucleotide approach, it has also been demonstrated, in primary murine and human hematopoietic progenitor cells, that inhibition of DYRK3 expression significantly and specifically affects the production of colony-forming units-erythroid (the penultimate precursor of erythroblasts). Based on apparently arginine- and proline-directed substrate sequences (13) and on the in vitro ability of DYRK1 to phosphorylate eukaryotic synthesis initiation factor-2Be and tau microtubule-associated protein at priming sites (14), DYRK1 has been suggested to act as a glycogen synthase kinase-priming kinase. Via in vitro kinase assays, DYRK1 has also been shown to be capable of phosphorylating forkhead transcription factor FKHR (forkhead in rh-adrenosarcoma) (15), CREB (16), and STAT3 (signal transducer and activator of transcription-3) (17). However, factors that regulate DYRK3 and the nature of DYRK targets in general remain otherwise unclear.

Homologs of mammalian DYRKs interestingly also occur in Saccharomyces cerevisiae and Dictyostelium (Yak1p and YakA, respectively) (18, 19); and recently, each of these DYRK-like kinases has been linked to PKA signaling pathways (20, 21). In Dictyostelium, YakA is required for cAMP (and PKA)-directed differentiation to sporulating stalks due to nutrient withdrawal (21, 22), whereas in S. cerevisiae, Yak1p may directly affect PKA function by phosphorylating Bcy1p, the single PKA regulatory subunit of budding yeast (23). Based on these observations, the prospect that DYRK3 might also somehow engage a PKA (and possibly CREB-linked) pathway was investi...
First, DYRK3 activity is shown to depend upon intactness of Tyr\textsuperscript{333} within its predicted (auto)phosphorylation loop, and loop acidification is proved to be activating. This is unlike ERK2, for example, which possesses an equivalently positioned \textit{TXY} loop, but is not affected markedly by tyrosine acidification (24). Second, DYRK3 is shown to act via kinase domain- as well as unique C-terminal domain-dependent mechanisms to regulate CREB and CRE response pathways via PKA-dependent routes. Finally, DYRK3 expression in FDC hematopoietic progenitor cells is revealed to modulate apoptosis due to cytokine withdrawal. Overall, this work advances an understanding of DYRK3 activation, action mechanisms, and possible cellular functions.

**EXPERIMENTAL PROCEDURES**

**DYRK3 Constructs**—The full-length murine DYRK3 cDNA used in these studies was prepared by expressing a bacterial artificial chromosome-derived \textit{dyrk3} gene fragment in COS cells. cDNAs generated by this process were cloned into a \textit{H9261} ZAP vector, excised from a derived phagemid library, adapted at 5\textit{H11032} and 3\textit{H11032} termini with EcoRI and XhoI sites, and cloned into the mammalian expression vector pEFNeo-Myc6. The DYRK3 point mutants Y331A, Y333A, Y331E/Y333E, and K202R were prepared from pEFNeo-Myc-wtDYRK3 using the Stratagene PCR-based XL site-directed mutagenesis kit and the following primers: 5\textit{H11032}-CGA GTA TCA GAA GCT TGC CAC GTA TAT CCA GTC C-3\textit{H11032} plus 5\textit{H11032}-GGA CTG GAT ATA CGT GGC AAG CTT CTG ATA CTC G-3\textit{H11032} (Y331A construct), 5\textit{H11032}-GAA GCT TTA CAC GGC TAT CCA GTC CCG C-3\textit{H11032} plus 5\textit{H11032}-GCG GGA CTG GAT TTC CGT CTC AAG CTT CTG ATA C-3\textit{H11032} (Y333A construct), 5\textit{H11032}-CGA TCA GAA GCT TGA GAC GGA AAT CCA GTC C-3\textit{H11032} plus 5\textit{H11032}-GTT TCT CAT TGC GCA CCA TTC TCA GGG CCA CGTBACT GCC G-3\textit{H11032} (K202R construct).
Figure 4. Murine DYRK3-Y331E/Y333E phosphorylates MBP in a time course comparable with wtDYRK3. The activities of DYRK3-Y331E/Y333E and wtDYRK3 (together with the negative control construct DYRK3-K202R) were tested in a time course format using immunoprecipitates from transfected 293 cells plus MBP and [γ-32P]ATP as substrates. As shown in Fig. 3, the expression levels and immunoprecipitates for all forms of DYRK3 were essentially equivalent (data not shown). Analyses revealed similar activities for wtDYRK3 and DYRK3-Y331E/Y333E regarding rates and levels of [32P]-labeled MBP product formation.

Figure 5. DYRK3 and DYRK3-K202R activate the CREB/CRE response pathway. A, 293 cells were transfected with pCRE-Luc, pSEAP, and either pEFNeo-wtDYRK3 or empty pEFNeo vector. At 36 h post-transfection, cells were harvested, and luciferase activity was assayed. Shown are means ± S.D. of triplicate analyses from two independent experiments. B, DYRK3-Y331E/Y333E and DYRK3-K202R were also tested as described for A for activity in activating this CREB/CRE response pathway. Interestingly, significant activity was retained by kinase-inactive DYRK3-K202R.

Expression of DYRK3 Constructs—293 cells (American Type Culture Collection, Manassas, VA) were maintained in Opti-MEM I (Invitrogen) and 7% fetal bovine serum (FBS) plus PSF (100 units/ml penicillin, 100 μg/ml streptomycin, 0.5 μg/ml amphotericin B). DYRK3 constructs in pEGNeo vectors were transfected and expressed using 15 μg of plasmid DNA plus 30 μl of FuGENE 6 (Roche Molecular Biochemicals) per 100-mm dish of 293 cells at 50% confluency. Lysates were prepared at 48 h post-transfection. In assays of phospho-CREB, the 293 cell medium was changed to 0.5% FBS in Opti-MEM I 2 h prior to transfections.

Cell Lysates, Immunoprecipitations, and Western Blotting—293 cell lysates were prepared by collecting cells in phosphate-buffered saline (138 mM NaCl, 2.7 mM KCl, 1.2 mM KH2PO4, and 8.1 mM Na2HPO4, pH 7.4) plus 5 mM Na2EDTA; washing cells with phosphate-buffered saline; and incubating each plate equivalent for 5 min initially in 1.5 ml of 10 mM NaCl, 6 mM MgCl2, 0.2 mM NaVO4, 1 mM diethiothreitol, and 10 μM Tris, pH 7.4, containing 0.5 mM phenylmethylsulfonyl fluoride plus a protease inhibitor mixture (P-8340, Sigma). Cells were then collected (5 min at 2000 × g) and incubated for 15 min in this buffer supplemented with 0.3% Triton X-100. Supernatants were recovered (5 min at 5000 × g), and pelleted nuclei were extracted by gentle rocking for 30 min in 150 μl of 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM Na2EDTA, 20% glycerol, and 20 mM HEPES, pH 7.9. Triton X-100 and nuclear extracts were then combined. For direct Western blotting, aliquots were denatured in 1.6 mM SDS, 100 mM diethiothreitol, 0.3 mM bromphenol blue, 5% glycerol, and 60 mM Tris-HCl, pH 6.8. Anti-Myc antibody 9E10 (1:2000; Invitrogen) and anti-phospho-CREB (1:1000), anti-CREB (1:1000), and anti-phospho-PKAz1 (1:2000) antibodies (Upstate Biotechnology, Inc.) were used. In immunoprecipitations, lysates were precleared (30-min incubation) using 10 μl of protein G-agarose CL-4B (Sigma) prewashed with 25 mM NaCl and 25 mM Tris, pH 7.6. Samples were then incubated stepwise at 4 °C with 5 μg of anti-Myc antibody 9E10 for 90 min and with 30 μl of protein G-agarose for 30 min. Immune complexes were washed twice at 4 °C with 0.2% Nonidet P-40, 50 mM NaCl, and 50 mM Tris-HCl, pH 7.6, and twice at 23 °C with 5 mM MgCl2, 1 mM Na2EDTA, 25 mM β-glycerol phosphate, 0.5 mM dithiothreitol, 0.2 mM NaVO4, and 25 mM MOPS, pH 7.2. Protease and phosphatase inhibitors were included at all steps. Typically, one-third of the washed and aspirated immune complexes were denatured for Western blotting, and two-thirds were used in kinase assays. Electrophoresis and ECL Western blotting were performed as detailed previously (25).

In Vitro Kinase Assays—In kinase assays, washed immunoprecipitates were reacted at 30 °C with 40 μl of a solution containing 1.25 mM EGTA, 0.25 mM NaVO4, 0.25 mM dithiothreitol, 5 mM β-glycerol phosphate, 15 mM MgCl2, 125 μM ATP, 10 μCi of [γ-32P]ATP (3000 Ci/mmol), 20 μg of myelin basic protein (MBP), and 6.25 mM MOPS, pH 7.2, plus 5 μM protein kinase C inhibitor peptide (catalog no. 12-121, Upstate Biotechnology, Inc.), 0.5 μM PKA inhibitor peptide (catalog no. 12-151), and 5 μM compound R24571 (catalog no. 20-116). This solution was prepared using the following reagents from Upstate Biotechnology, Inc.: assay dilution buffer I (catalog no. 20-108), inhibitor mixture (catalog no. 20-116), kinase substrate mixture (catalog no. 20-115), and magne-
sium/unlabeled ATP mixture (catalog no. 20-113). At the indicated intervals, supernatants were recovered and denatured in SDS sample buffer. 32P-Labeled MBP products were assayed by SDS-PAGE and phosphorimaging (Storm Scanner, Amersham Biosciences).

**CREB/CRE Reporter Experiments**—In experiments using 293 cells, 6 × 10^5 cells were plated (six-well plate format) and cultured overnight. Four hours prior to transfection, the medium was changed to 1% FBS in Opti-MEM I. Cells were then transfected (in triplicate) with pEFNeo vectors encoding wtDYRK3, DYRK3-Y331E/Y333E, DYRK3-K202R, or empty pEFNeo plus an EFV-encoding vector (pEYFP-C1) (upper panel). At 36 h post-transfection, transfected cells were isolated by fluorescence-activated cell sorting (lower panels), and luciferase activity levels were assayed. Shown are normalized mean luciferase activities ± S.D. Results are representative of two independent experiments.

**PKA Inhibitor Experiments**—(R)p-cAMP-S and H-89 (N-[2-(p-bromo-cinnamylamino)ethyl]-5-isoquinolinesulfonamide·2HCl, BIOMOL Research Labs Inc.) were prepared in methanol and 50% ethanol, respectively, as 100-fold concentrated stocks and applied to 293 cells at 2 h prior to transfections. In pilot experiments, the activities of these com-
Fig. 7. The unique C-terminal subdomain of DYRK3 can engage a CREB/CRE response pathway. To further investigate the bases for activities of DYRK3-K202R, cDNAs encoding the unique C- and N-terminal domains of DYRK3 were prepared (as diagrammed in the upper panel) and cloned into pEFNeo. 293 cells were transfected with these constructs (in parallel with wtDYRK3 and DYRK3-K202R) plus pCRE-Luc (and pSEAP). At 36 h post-transfection, lysates were prepared and assayed for luciferase activity (normalized means ± S.D. of triplicates)(center panel). For each form of DYRK3, expression levels were also assayed (anti-Myc epitope Western blot) (lower panel). K, kDa.

pounds in this system (and potential toxicities) were tested over broad-range concentrations in transfections using pCRE-Luc plus pFC-PKA (Stratagene) and trypan blue cell viability assays.

FDC Cells and Assays of Programmed Cell Death—FDC cells were maintained in Opti-MEM I containing 7% FBS plus 3.5% WeHI3 cell-conditioned medium (as a source of IL-3). For stable expression of wtDYRK3 and Bcl-xL, FDCW2 cells were washed once with ice-cold Opti-MEM I medium and resuspended at 1 × 10^6 cells/ml in Opti-MEM I. pEFNeo constructs encoding Myc-wtDYRK3 or Bcl-xL (60 µg) were transfected into FDCW2 cells (0.8 ml) using a Gene Zapper 450/2500 electroporation cuvette (Bio-Rad). Transfected cells were then selected by 100 µg/ml G418. Derived lines were expanded to 8 × 10^6 cells/ml in medium containing 1 mg/ml G418. Derived lines were also assayed (anti-Myc epitope Western blot) (Fig. 2, lower panel) and assayed for luciferase activity (normalized means ± S.D. of triplicates)(center panel). For each form of DYRK3, expression levels were also assayed (anti-Myc epitope Western blot) (lower panel). K, kDa.

RESULTS

Roles for the DYRK3 YTY Motif in Kinase Activation—Studies of DYRK3 are limited, and little is known regarding the nature of activation mechanisms and/or targets. Experiments first sought to assess ways in which Tyr331 and/or Tyr333 within the DYRK3 unique predicted YTY activation loop might affect kinase activity. As shown in Fig. 1 (lower panel), this YTY motif lies between kinase subdomains VII and VIII and is related to motifs within PRP4 and ERK1/2 kinases, but occurs in DYRK3 as a SSSFEYQKYTYQSRFYR sequence (bounded by kinase DFG and APE signature sequences). To test how DYRK3 kinase activity might be affected by this motif, cDNAs were prepared that encoded the Y331A, Y333A, or Y331E/Y333E mutation (Fig. 1, upper panel). As an additional control, Lys302 within the DYRK3 predicted ATP-binding site (kinase subdomain II) was mutated to generate kinase-inactive DYRK3 (K202R). Next, 293 cells were transfected with pEFNeo vectors encoding these DYRK3 forms, and DYRK3 kinase assays were performed using anti-Myc epitope immunoprecipitates plus [γ-32P]ATP and MBP. These experiments revealed that intactness of Tyr333 (but not Tyr331) is important for DYRK3 kinase activity (Fig. 2, upper panels). Replicate quantitative analyses showed that DYRK3-Y333A retained little to no activity, whereas -75% of the wild-type activity was retained by DYRK3-Y331A (Fig. 2, lower panel). cDNAs were also prepared and cloned into a pEFNeo vector that encoded N-terminal plus kinase domains (NK construct) or kinase plus C-terminal domains (KC construct). These forms were also expressed and assayed for in vitro kinase activities. Each form proved to possess activity approximating that of wtDYRK3 (data not shown), suggesting that these domains may not substantially affect kinase domain activity.

Based on the above findings, efforts were also made to assay the predicted (auto)phosphorylation of Tyr333 (and possibly Tyr331). In experiments using several distinct anti-phosphotyrosine antibodies, however, these events were not clearly detected (as compared with JAK2 (Janus kinase-2) kinase as a parallel and positive control). In addition, DYRK3 expression levels in stably transfected 293 lines and in SI9 lines (baculovirus system) were low, and this complicated direct analyses of phosphorylated sites. We therefore chose to test the possible effects of YTY loop acidification on DYRK3 activity. Interestingly, acidification of this loop to Y331E/Y333E yielded a DYRK3 form that possessed -75% of the wild-type activity (Fig. 3). This DYRK3-Y331E/Y333E mutant also phosphorylated MBP in a time course comparable with wtDYRK3 (Fig. 4).
DYRK3 engages a CREB/CRE response pathway via kinase domain- and C-terminal domain-dependent mechanisms. In *S. cerevisiae* and *Dictyostelium*, the DYRK kinase homologs Yak1p and YakA have recently been revealed to function within PKA-integrated signaling routes (20, 21), and human DYRK1A has been suggested to phosphorylate CREB (16). These reports prompted tests of the ability of DYRK3 to possibly modulate a CREB/CRE signaling pathway. This was investigated initially by cotransfecting 293 cells with a pEFPNeo vector encoding wtDYRK3 (or empty pEFPNeo as a control) plus a pCRE-Luc transcriptional reporter. pCRE-Luc was reproducibly stimulated 4–5-fold by wtDYRK3, and DYRK3-Y331E/Y333E likewise proved to efficiently activate pCRE-Luc (Fig. 5A). Interestingly, yet somewhat unexpectedly, pCRE-Luc was also stimulated significantly by the kinase-inactive construct DYRK3-K202R (Fig. 5B). To examine these effects in an independent and CREB transactivation domain-specific system, the activities of DYRK3 constructs were also tested in CCL-2 HeLa cells. These cells stably express a CREB-Gal4 fusion protein that is activated upon phosphorylation of the CREB kinase-inducible activation domain and report activation via a single stably integrated Gal4-binding element-luciferase reporter cassette. To control for transfection efficiencies (and to analyze DYRK3 activities within transfected cell populations), CCL-2 HeLa cells were transfected with pEFPNeo-DYRK3 vectors plus an EYFP expression vector (pEYFP-C1). At 60 h post-transfection, EYFP-positive cells were retrieved by fluorescence-activated cell sorting, and luciferase activities due to DYRK3 expression were assayed (Fig. 6). These analyses revealed CREB kinase-inducible transcription domain activation by wtDYRK3 and DYRK-Y331E/Y333E and again demonstrated significant activation by kinase-inactive DYRK-K202R.

Next, to investigate possible bases for the above apparent CREB/CRE targeting of kinase-inactive DYRK3-K202R, cDNAs encoding the unique N- and C-terminal subdomains of DYRK3 were prepared, cloned into pEFPNeo vectors, and tested in 293 cells for possible activation of this response pathway. Luciferase assays revealed that the DYRK3 C-terminal domain (but not the N-terminal domain) possessed activity essentially equivalent to that of DYRK3-K202R (Fig. 7, upper panels). Western blot analyses of cell lysates also showed that each Myc epitope-tagged form was expressed at a comparable level (Fig. 7, lower panel). Together, these findings indicate that DYRK3 can affect this signaling pathway via not only kinase domain-, but also C-terminal subdomain-mediated routes.

**Roles for PKA in DYRK3 Activation of CREB/CRE Signaling**—As indicated above, evidence has recently been provided that the DYRK-related Yak1p kinase of *S. cerevisiae* may interact with PKA (especially via the PKA regulatory subunit Bcy1) (23). Mammalian PKAs are more complex and include at least four regulatory subunits and three catalytic subunits (and useful antibodies to specific PKA subunits also are limiting) (26). Therefore, to test possible roles for PKA in our discovered DYRK3/CREB/CRE response pathway, we employed the specific PKA inhibitors (R<sub>p</sub>)-cAMP-S and H-89 (27, 28). In pilot experiments, concentrations/doses of each that efficiently inhibited PKA (but did not compromise 293 cell viability) were determined in cells transfected transiently with a PKA catalytic subunit expression vector (pFC-PKA). Based on reported Ki values for intact cells (27–29), (R<sub>p</sub>)-cAMP-S was tested at 15–135 μM and H-89 at 6.25–25 μM, and concentrations of 100 μM (R<sub>p</sub>)-cAMP-S and 20 μM H-89 proved effective when administered at 2 h prior to transfection (data not shown). When...
administered at these doses to 293 cells transfected with pEF-Neo-wtDYRK3 (and pCRE-Luc), H-89 and (R)p-cAMP-S each efficiently blocked the ability of DYRK3 to activate a CREB/CRE response (Fig. 8). In addition, each inhibitor also efficiently blocked the activities of the DYRK3-Y331E/Y333E, DYRK3-K202R and DYRK3-CT constructs (Fig. 9). These outcomes (plus the above-detailed experiments) support a model for DYRK3 action whereby DYRK3 can interact via not only its kinase domain, but also its unique C-terminal region, with PKA in a way that activates CREB/CRE responses.

Experiments were next performed to more directly demonstrate DYRK3-dependent phosphorylation of CREB at a critical site (Ser133) within the CREB kinase-inducible transactivation domain. Here, 293 cells were transfected with pEFNeo-Myc-wtDYRK3 (or empty pEFNeo as a negative control), and possible effects on the phosphorylation of endogenous CREB were assayed using an anti-phospho-Ser133 CREB antibody (Fig. 10). Under the conditions specified, endogenous CREB phosphorylation proved to be clearly stimulated upon wtDYRK3 expression. In addition, this event (DYRK3-dependent CREB phosphorylation) also proved to be inhibited by the PKA inhibitor H-89 (Fig. 10, lower panels). Using the singularly available antibody to one phospho-PKA regulatory subunit, whether PKAreg/H9251II might be a target of DYRK3 was also tested, but with negative results. Whether this outcome relates to antibody sensitivity or possibly PKAreg isoform specificity is presently unresolved.

DYRK3 Inhibits Apoptosis Due to Cytokine Withdrawal in FDC Hematopoietic Progenitor Cells—In a final series of experiments, the possible effects of DYRK3 on progenitor cell growth, cell cycle, and/or survival were tested via the ectopic stable expression of wtDYRK3 in hematopoietic FDCW2 cells. FDCW2 cells depend strictly upon IL-3 as a growth and survival factor (30) and correspond closely to a myeloid progenitor cell population, but retain the capacity to activate erythroid gene expression in response to ectopically expressed GATA-1 (30). Also, FDCW2 cells express only low level endogenous DYRK3 (11). FDCW2 cells were electrotransfected with pEF-Neo-Myc-wtDYRK3 (or empty vector as an initial control), and stably transfected lines were selected in G418. In FDCW2-Myc-wtDYRK3 cells, expression of Myc-DYRK3 was confirmed (Fig. 11). Intact DYRK3 (70 kDa) as well as proteolyzed forms (30

**Table I**

| FDCW2 cell line                        | FDCW2 | FDCW2-pEFNeo-Myc-wtDYRK3 | FDCW2-pEFNeo-Bcl-xL |
|----------------------------------------|-------|--------------------------|--------------------|
| Parental FDCW2                         | 100   | 73 ± 6.8                 | 58.3 ± 4.9         |

* Percent maximal death (i.e. FDCW2 cells) as assayed at 18 h post-cytokine withdrawal.

**Fig. 11.** DYRK3-dependent attenuation of apoptosis due to cytokine (IL-3) withdrawal. IL-3-dependent FDCW2 cells ectopically expressing Myc-wtDYRK3 were prepared by electroporation and selection in G418. The possible effects of DYRK3 on IL-3-dependent proliferation or survival after IL-3 withdrawal were then assayed in these FDCW2-Myc-wtDYRK3 ('FDC DYRK3') cells and parental FDCW2 (FDC) cells. In assays of proliferation ('[3H]dThd incorporation; upper left panel), no apparent effects of DYRK3 on proliferation were observed. In contrast, DYRK expression significantly (and reproducibly in independent experiments) attenuated cell death due to IL-3 withdrawal (upper right and lower left panels). Myc-DYRK3 in FDCW2-Myc-wtDYRK3 cells was detected as a primary 70-kDa species, but 40- and 30-kDa forms were also detected (lower right panel, square and oval). PI, propidium iodide.
and 40 kDa) were observed (here and in repeated independent experiments). The possible effects of ectopically expressed Myc-DYRK3 on either proliferation or apoptosis due to cytokine (IL-3) withdrawal were then assayed. In [3H]dThd incorporation assays (Fig. 11, upper left panel), no significant effects of DYRK3 on proliferation were detected. In contrast, ectopically expressed DYRK3 interestingly proved to significantly attenuate cell death due to cytokine withdrawal (Fig. 11, upper right and lower left panels). To better gauge the magnitude of this effect, FDCW2 cells were electrophoretically transfected with the pEFNeo-Bcl-xL construct, and lines expressing Bcl-xL were isolated and assayed in parallel for inhibition of apoptosis due to cytokine withdrawal. In parallel assays, the effects of DYRK3 on this process were gauged to be strong (i.e., ~2-fold greater than those exerted by Bcl-xL) (Table 1).

**DISCUSSION**

The main goals of this investigation were to analyze ways in which the YTY motif of DYRK3 kinase might contribute to kinase activation, to initially define possible effects of DYRK3 on progenitor cell growth and/or survival, and to attempt to discover intracellular signaling routes that DYRK3 might engage. As discussed above, the DYRK3 XYY loop together with several unique kinase domain sequence components structurally best define DYRK3 as a novel group of dual-specificity kinases. These additionally conserved components include an SSC motif in subdomain VII; lack of an arginine that is highly represented in subdomain VIB in other kinases; and unusual DYRK characteristic cysteine, NLY, and glycine residues in subdomains IV–V, V, and VIII–IX, respectively (1, 4). Regarding the DYRK3 YTY motif, our observation that intactness of Tyr333 (but not Tyr331) within the DYRK3 activation loop is important for catalysis is consistent with a primary role for Tyr333 in phosphorylation-induced activation. In support of this notion (and while this work was in progress), Himpel et al. (31) reported that in DYRK1 kinase, the (auto)phosphorylation of the second Tyr residue of a corresponding YTY loop (Fig. 1) correlates with kinase activation. In our study, catalytic roles for the YTY motif of DYRK3 were also assessed by acidification to mimic predicted phosphorylation. Mutation of Tyr331 and Tyr333 to glutamate proved to activate DYRK3 kinase function (or Ser218 and Ser222 of the ERK kinase MKK1 (MAPK kinase-1)) as a second example), for which acidification leads to constitutive catalysis (24), suggests a regulatory route through which DYRK3 and possibly other DYRKs might be involved. Both PKA (37, 38) and CREB (38, 39) are known to play important roles in cell growth, survival, and/or differentiation in several specific tissues and lineages (including erythroid progenitor cells) (40, 41). The specific ways by which DYRK3 modulates these regulators should likewise be interesting to discover.

Finally, DYRK3 has been shown to attenuate apoptosis due to cytokine withdrawal in IL-3-dependent hematopoietic FDCW2 cells (Fig. 11 and Table 1), and this occurred independently of any detectable effect on mitogenic potential. Roles for DYRKs in cell survival have not previously been well studied or described. However, the DYRK3-related kinase HIPK2 has recently been discovered to mediate p53 activity following radiation-induced damage (10, 42), and p53 is well known to affect cell survival pathways (43, 44). By comparison, the specific mechanisms by which DYRK3 modulates these regulators should likewise be interesting to discover.

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