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Activation-Loop Autophosphorylation Is Mediated by a Novel Transitional Intermediate Form of DYRKs

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Summary

Autophosphorylation of a critical residue in the activation loop of several protein kinases is an essential maturation event required for full enzyme activity. However, the molecular mechanism by which this happens is unknown. We addressed this question for two dual-specificity tyrosine-phosphorylation-regulated protein kinases (DYRKs), as they autophosphorylate their activation loop on an essential tyrosine but phosphorylate their substrates on serine and threonine. Here we demonstrate that autophosphorylation of the critical activation-loop tyrosine is intramolecular and mediated by the nascent kinase passing through a transitory intermediate form. This DYRK intermediate differs in residue and substrate specificity, as well as sensitivity to small-molecule inhibitors, compared with its mature counterpart. The intermediate's characteristics are lost upon completion of translation, making the critical tyrosine autophosphorylation a "one-off" inceptive event. This mechanism is likely to be shared with other kinases.

Introduction

Protein kinases have developed a multitude of processes to regulate their activity. The best-understood mechanism is phosphorylation of the kinase activation loop (also called activation segment or T loop), reviewed in Johnson et al. (1996) and Nolen et al. (2004). This can be carried out by an upstream kinase as part of a signaling cascade, or a kinase can autophosphorylate its own activation loop by either an inter- or intramolecular mechanism. One family of kinases that activate themselves by autophosphorylation are the dual-specificity tyrosine-phosphorylation-regulated protein kinases (DYRKs).

DYRKs are an evolutionary conserved family of protein kinases that belong to the CMGC family of kinases and are distantly related to MAPKs (mitogen-activated protein kinases) and CDKs (cyclin-dependent protein kinases) (Becker and Joost, 1999; Himpel et al., 2000; Miyata and Nishida, 1999; Widmann et al., 1999). DYRK family members share a conserved kinase domain and adjacent N-terminal DH box (DYRK homology) but differ in their N- and C-terminal extensions. Two DYRK subclasses exist. Class 1 (nuclear) DYRKs are only present in multicellular organisms and contain an N-terminal nuclear localization signal and a C-terminal PEST or GAS region (regions typical in proteins targeted for degradation). Class 2 (cytosolic) DYRKs do not contain any other previously defined regions but are present in all eukaryotic organisms examined to date (Lochhead et al., 2003).

Functional studies have been conducted on yeast, *C. elegans*, *Drosophila*, and mammalian DYRK family members. In the yeast *S. pombe*, the class 2 DYRK family member Pom1 was identified as a gene that may be required for proper positioning of the growth and cytokinesis machineries by interaction with both the actin and microtubule cytoskeletons (Bahler and Pringle, 1998). Experiments modifying the kinase activity of Pom1 indicate a negative role for Pom1p in microtubule growth at cell ends (Bahler and Nurse, 2001). Genetic studies in *C. elegans* show that the only class 2 DYRK homolog, mbk-2, is essential for the completion of cytokinesis and the patterning of the first embryonic axis during the egg-to-embryo transition (Pang et al., 2004; Pellettieri et al., 2003; Quintin et al., 2003). In *Drosophila*, mutation of the founding DYRK family member, minibrain (*mnb*), a class 1 DYRK, affects postembryonic neurogenesis, resulting in specific reductions in the size of the optic lobes and central brain hemispheres (Tejedor et al., 1995). The human ortholog of the minibrain gene, *DYRK1A*, maps to the Down's syndrome critical region and is overexpressed in Down's syndrome brains, suggesting a similar role for this kinase in mammalian neurogenesis (Guimera et al., 1999). Mice with a single copy of the *DYRK1A* gene display region-specific reductions in the brain (Fotaki et al., 2002), indicating a conserved mode of action that determines normal growth and brain size in both mice and flies.

All DYRKs autophosphorylate a critical tyrosine residue in the activation loop and phosphorylate their substrates on serine and threonine residues (Bahler and Nurse, 2001; Himpel et al., 2001; Kentrup et al., 1996; Lee et al., 2000; Lochhead et al., 2003). Biochemical analysis of DYRK1A shows that Y321 in the activation loop is phosphorylated (Himpel et al., 2001). This is the second tyrosine in the sequence YXY, a motif that is shared with all DYRK family members. There is strong evidence that this phosphorylation is an autophosphorylation event, as mutation of the Mg^{2+}ATP-orientating lysine in the catalytic site of both class 1 and 2 DYRKs abolishes tyrosine phosphorylation (Bahler and Nurse, 2001; Himpel et al., 2001; Kentrup et al., 1996; Lee et al., 2000; Lochhead et al., 2003). Bacteria do not en-

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code tyrosine kinases (Kornbluth et al., 1988), and expression of DYRKs in this system produces a phosphorylated and active enzyme (Himpel et al., 2001; Kentrup et al., 1996; Lochhead et al., 2003). However, the mechanism of autophosphorylation is not known. In fact, the molecular mechanism of autophosphorylation of any protein kinase is poorly understood. We chose to address this question for two *Drosophila* DYRKs, MNB (minibrain) and dDYRK2, representatives of class 1 and 2 DYRKs, respectively. Here we demonstrate that dDYRK2 and MNB form transitional intermediates that phosphorylate Y358 or Y326 in the activation loop of the respective kinase domain by an intramolecular mechanism. Once dDYRK2 or MNB is fully translated and released from the ribosome, the transitional tyrosine-kinase activity is lost, and the molecules then function only as serine/threonine kinases. This is a novel mechanism by which protein kinases mature and explains how DYRKs autophosphorylate a tyrosine residue but phosphorylate their substrates on serine/threonine residues.

Results

Identification of Activation-Loop Phosphorylation Sites in dDYRK2 and MNB

Previously we demonstrated by mutational analysis that tyrosine residues in the activation loop of dDYRK2 (i.e., Y356 and Y358) were essential for tyrosine autophosphorylation and for kinase activity (Lochhead et al., 2003). Comparable tyrosines exist in MNB (i.e., Y324 and Y326), but phosphorylation of these sites has not been addressed. To identify phosphorylated residues in dDYRK2 and MNB, recombinant proteins were expressed in Sf9 cells and visualized by Coomassie staining (Figure 1A). Bands corresponding to dDYRK2 and MNB were excised from the gel and digested with trypsin. PHOS-select beads were then used to enrich the phosphopeptides, and the resulting samples were analyzed by MALDI and electrospray mass spectrometry. The most abundant phosphopeptides present in the MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) spectra were of mass 1123.5443 m/z and 1159.5552 m/z for dDYRK2 and MNB, respectively (data not shown). These phosphopeptide masses identify tryptic peptides derived from the activation loop of dDYRK2 (IYTYIQR, amino acids 355 to 362) and MNB (IYHYIQSR, amino acids 323 to 330). Fragmentation of these peptides revealed that Y358 of dDYRK2 and Y326 of MNB are phosphorylated residues (Figures 1B and 1C). This was also confirmed by inspecting the spectra obtained by electrospray LC-MS/MS (liquid chromatography-mass spectrometry/mass spectrometry) (data not shown). As no other phosphotyrosine was detected in dDYRK2 or MNB, immunoblotting with anti-phosphotyrosine antibodies will detect only phosphorylated Y358 in dDYRK2 and phosphorylated Y326 in MNB.

The importance of Y358 in dDYRK2 was investigated further by mutational analysis. As shown in Figure 1D, alteration of Y358 but not Y356 to phenylalanine abolished recognition of this protein by anti-phosphotyrosine antibodies. Furthermore, phosphorylation of Y358 is required for enzyme activity, as the Y358F mutant has an activity level equivalent to the kinase-dead K227M mutant (Figure 1D). These results demonstrate that phosphorylation of Y358 is an essential maturation event required for full dDYRK2 activity.

Evidence that dDYRK2 and MNB Autophosphorylate by an Intramolecular Mechanism

Several lines of evidence indicate that tyrosine phosphorylation of the DYRKs occurs via autophosphorylation (Bahler and Nurse, 2001; Himpel et al., 2001; Kentrup et al., 1996; Lee et al., 2000; Lochhead et al., 2003). To determine if this is mediated by an inter- or intramolecular mechanism, we carried out a series of coexpression experiments in Sf9 cells using mutant forms of dDYRK2 or MNB. If phosphorylation occurs in *trans* (i.e., intermolecular), then kinase-inactive versions of the molecule would be phosphorylated when expressed in the presence of active wt enzyme. In contrast, if phosphorylation occurs via a *cis*- or intramolecular mechanism, then any mutation that compromised the catalytic activity of the molecule would also disrupt tyrosine autophosphorylation. For these studies, we used kinase-inactive versions of DYRK in which the Mg^{2+}ATP-orientating lysine (K227 in dDYRK2 and K193 in MNB) located in the small lobe was altered. As shown in Figure 2A, neither dDYRK2 K227M nor MNB K193M was tyrosine phosphorylated when expressed alone or when coexpressed with the wt enzyme in Sf9 cells. We extended these studies by determining if wt dDYRK2 or MNB could phosphorylate a peptide of their respective activation loop in vitro. As shown in Figure 2B, both kinases efficiently phosphorylated the synthetic exogenous substrate peptide Woodtide, but neither dDYRK2 nor MNB phosphorylated their activation-loop peptide. Furthermore, several different mutational forms of dDYRK2 that are unable to autophosphorylate Y358 in *cis* are also not phosphorylated by mature wt dDYRK2 in *trans* (Table 1). These results demonstrate that under no circumstance is intermolecular phosphorylation observed and are consistent with DYRK autophosphorylation being an intramolecular mechanism.

Mature dDYRK2 and MNB Cannot Autophosphorylate on Tyrosine Residues

In our initial attempts to identify the tyrosine residues phosphorylated in dDYRK2 and MNB, we conducted a series of in vitro kinase assays where either dDYRK2 or MNB was incubated in the presence of [γ-^{32}P]ATP. Surprisingly, phosphoamino acid analysis of the resulting products revealed that in vitro dDYRK2 and MNB autophosphorylate only on serine and threonine (Figure 3A). These results could be explained if the activation-loop tyrosines of dDYRK2 and MNB were stoichiometrically phosphorylated in vivo such that no further phosphate could be incorporated. To address this model, we first treated the kinases with lambda phosphatase. Both dDYRK2 and MNB were found to be resistant to dephosphorylation by phosphatase, with only a 50% reduction for both kinases seen after 15 min and no further reduction up to 60 min (Figure 3B). The reduction of tyrosine phosphorylation correlates with a 50% reduction in activity (Figure 3C), further demonstrating that the activation-loop phosphorylation is required for

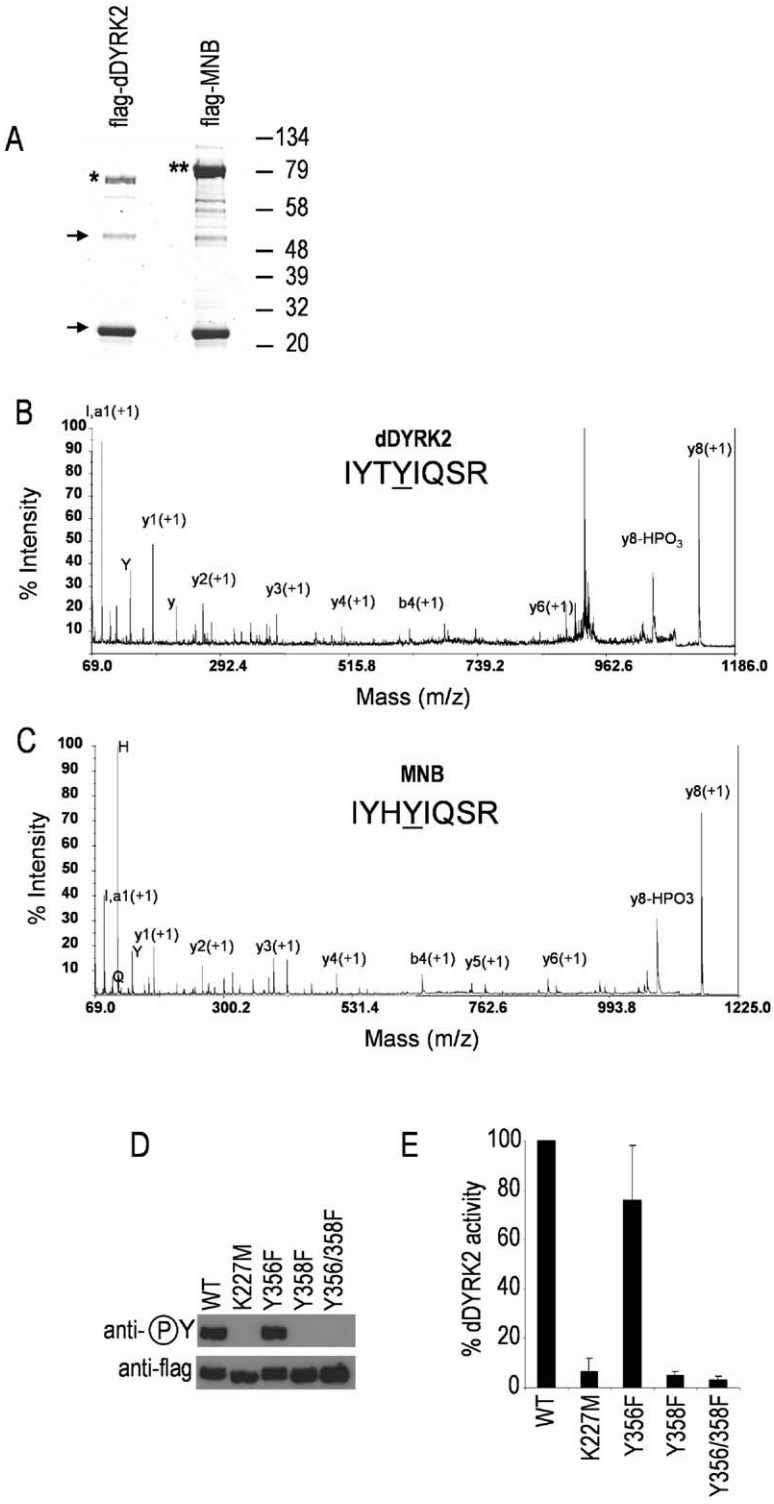

Figure 1. Identification of Activation-Loop Phosphorylated Residues in dDYRK2 and MNB

(A) Sf9 cell lysates containing FLAG-dDYRK2 or FLAG-MNB were incubated with anti-FLAG agarose, and the resulting immunocomplexes were subjected to SDS-PAGE and stained with Coomassie. *, FLAG-dDYRK2 protein; **, FLAG-MNB protein; arrows, IgG.

(B and C) MALDI-MS/MS spectra of the tryptic phosphopeptide IYTYIQR from dDYRK2 (B) and IYHYIQR from MNB (C). The presence of the y6 ion in both spectra indicated that Y358 in dDYRK2 and Y326 in MNB were the phosphorylated residues (underlined), and the neutral loss of 243 Da (phosphotyrosine) between y5 and y4 provided further evidence for Y326 in MNB being the phosphorylated residue (C).

(D) Sf9 cell lysates containing either FLAG-wt, K227M, Y356F, Y358F, or Y356/358F were incubated with anti-FLAG agarose, and the immunocomplexes were subjected to SDS-PAGE and immunoblotted with anti-phosphotyrosine (upper panel) or anti-FLAG antibodies (lower panel).

(E) Immunocomplexes were prepared as above, and the enzyme activity was measured using Woodtide as substrate as described in Experimental Procedures. Results are presented as the means ± SD (n = 4).

activity. However, the resistance to dephosphorylation indicates that the phosphorylated activation-loop tyrosine is potentially buried in the mature kinase structure. We next tested the ability of the dephosphorylated DYRK molecules to rephosphorylate by incubating the phosphatase-treated samples with [γ-^{32}P]ATP in an in vitro kinase assay. As shown in Figure 3A, lambda-phosphatase-treated dDYRK2 and MNB autophosphorylate only on serine and threonine residues. The sample tested in this reaction contained both tyrosine- and non-tyrosine-phosphorylated DYRK molecules. These results therefore demonstrate that in vitro fully

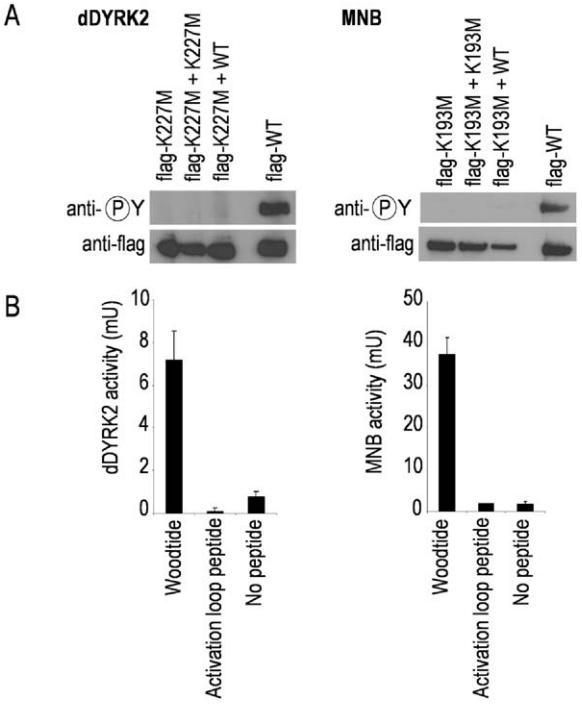

Figure 2. dDYRK2 and MNB Do Not *trans*-Phosphorylate

(A) FLAG-tagged versions of dDYRK2 (left panel) were expressed alone or in combination with nontagged K227M or wt dDYRK2 as indicated in Sf9 cells. Or FLAG-tagged versions of MNB (right panel) were expressed alone or in combination with nontagged K193M or wt MNB as indicated in Sf9 cells. The lysates were incubated with anti-FLAG agarose and the immunocomplexes subjected to SDS-PAGE and immunoblotted with either anti-phosphotyrosine (upper panel) or anti-FLAG (lower panel) antibodies.

(B) Immunocomplexes of either FLAG-wt dDYRK2 or FLAG-wt MNB were incubated in either the presence or absence of 50 μM Woodtide or 50 μM of their respective activation-loop peptides, and activity was determined as described in Experimental Procedures. Results are presented as the means ± SD (n = 4).

mature forms of dDYRK2 and MNB cannot autophosphorylate tyrosine residues by either an intra- or intermolecular mechanism, even when some of the phosphate has been removed.

dDYRK2 is Phosphorylated before Leaving the Ribosome

The above results and additional observations (see Discussion) led us to speculate that the tyrosine autophosphorylation event may be fundamentally different from the subsequent serine/threonine phosphorylation of substrates. Part of this disparity can be accounted for by fundamental differences in intra- versus intermolecular phosphorylation events. However, this explanation does not account for the apparent "one-off" characteristic of tyrosine autophosphorylation. To address the ephemeral nature of this process, we employed an in vitro translation system (rabbit reticulocyte lysate). As shown in Figure 4A, the kinetics of dDYRK2 expression (anti-FLAG immunoblot) parallels the levels of tyrosine phosphorylation (anti-phosphotyrosine immunoblot). dDYRK2 expression and tyrosine phosphorylation are both first detected after 20 min. As the levels of dDYRK2 expression increase, so to do the levels of tyrosine phosphorylation. Similar results were obtained with MNB (Figure 4B). The absence of any lag time between synthesis and autophosphorylation demonstrates that the autophosphorylation event is extremely rapid, consistent with it being an intramolecular event.

One model that would account for the observed experimental results is if tyrosine autophosphorylation occurred during an essential maturation process coupled to or completed immediately after the elongation step of protein translation. To address this, we purified ribosomes from rabbit reticulocyte lysate during translation and analyzed these samples to determine if dDYRK2 polypeptide chains were still attached to the ribosome. The samples were immunoblotted for both the nascent dDYRK2 chains and their phosphotyrosine status. Immunoblotting for the ribosomal protein L11 demonstrates that centrifugation through a sucrose cushion enriched for ribosomes compared with the supernatant (Figure 4C, lower panel); furthermore, wt and K227M dDYRK2 were copurified with the ribosomes (Figure 4C, middle panel). Wild-type but not K227M dDYRK2 is tyrosine phosphorylated to a level equivalent to that of fully translated and released wt dDYRK2 detected in the FLAG-immunoprecipitated sample of the supernatant (Figure 4C, upper panel). This indicates that dDYRK2 autophosphorylation on Y358 occurs while still bound to ribosomes. These results indicate that au-

Table 1. Summary of Autophosphorylation Results for dDYRK2 Mutants

Form of dDYRK2	Description of Form	Phosphorylation of Y358 When Expressed	Phosphorylation of Y358 In *trans* by Mature dDYRK2	Figure Number
K227M	Point mutation in small lobe	–	–	Figures 1, 2, 4
D324A	Point mutation in large lobe	–	N/T	Figure 4
ΔN and C noncatalytic termini[a]	Inactive kinase domain	–	–	Data not shown
Lambda-phosphatase treated	Removal of Y358 phosphorylation on mature kinase	N/A	–	Figure 3
Expressed with purvalanol A	Expression of full-length nonphosphorylated kinase	–	–	Figure 5
Activation-loop peptide	Consensus amino acid sequence lacking tertiary structure	N/A	–	Figure 2
ΔSD IX-C-term	Incomplete kinase domain	–	N/T	Figure 4

+, phosphorylation; –, no phosphorylation; N/A, not applicable; N/T, not tested.

[a] Removal of the noncatalytic N terminus abrogates Y358 autophosphorylation (data not shown).

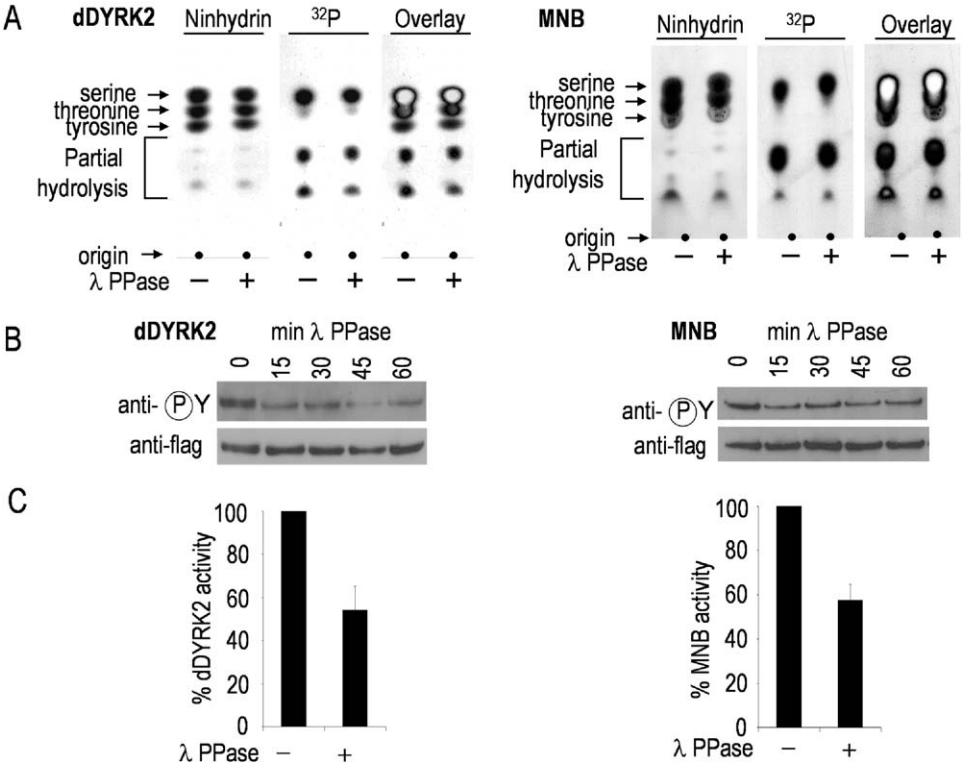

Figure 3. Mature dDYRK2 and MNB Do Not Autophosphorylate on Tyrosine

(A) Immunocomplexes of FLAG-wt dDYRK2 (left panel) or FLAG-wt MNB (right panel) were incubated in the presence or absence of lambda phosphatase for 30 min and then allowed to autophosphorylate in the presence of [γ-^{32}P]ATP. The proteins were then subjected to SDS-PAGE and transferred to PVDF membranes. ^{32}P-labeled FLAG-dDYRK2 and FLAG-MNB were excised from the filter, hydrolyzed, and normalized for counts, and the amino acids were separated by TLC. Ninhydrin was used to visualize amino acids, and autoradiography was used to visualize phosphorylated amino acids. An overlay of these images shows which amino acids are phosphorylated.

(B) Sf9 cell lysates containing either FLAG-dDYRK2 (left panel) or FLAG-MNB (right-panel) were incubated with anti-FLAG agarose, and the immunocomplexes were treated with lambda phosphatase for the times indicated, subjected to SDS-PAGE, and immunoblotted with anti-phosphotyrosine (upper panel) or anti-FLAG antibodies (lower panel).

(C) Immunocomplexes were prepared as above and treated with lambda phosphatase for 30 min, and the enzyme activity was measured using Woodtide as substrate as described in Experimental Procedures. Results are presented as the means ± SD (n = 4).

tophosphorylation occurs at dDYRK2's inception and suggest the existence of a maturational intermediary capable of tyrosine autophosphorylation.

dDYRK2 Requires a Fully Translated and Functional Kinase Domain for Autophosphorylation

To address the catalytic properties of the intermediate form of the dDYRK2 kinase domain, we generated various C-terminal-deletion mutants. As proteins are translated from N to C terminus, we inserted stop codons in between the various kinase subdomains, starting after subdomain VIII, where Y358 is situated. A schematic diagram of the truncated molecules is outlined in Figure 4D. The FLAG-tagged truncated molecules were expressed in the rabbit reticulocyte system, immunoprecipitated, and immunoblotted for their expression and their tyrosine phosphorylation status. As shown in Figure 4E, the expected sizes of molecules were detected (lower panel). dDYRK2 autophosphorylation requires full expression of all the canonical kinase subdomains (Δ C-term), as mutants lacking any of these abolish autophosphorylation (ΔSD IX-C-term, ΔSD X-C-term, and ΔSD XI-C-term).

It is clear that the maturation intermediary requires the conserved Mg^{2+}ATP-orientating lysine for autophosphorylation (see above); however, it is unknown if this form of the kinase domain requires other conserved catalytic residues. To determine if dDYRK2 autophosphorylation requires D324, the aspartic acid in the catalytic loop of the large lobe, we mutated this residue to alanine and expressed it in the rabbit reticulocyte lysate. D324 is indeed essential for autophosphorylation of dDYRK2, as no phosphotyrosine is detected in this mutant (Figure 4F). These results demonstrate that the molecular mechanism of phosphotransfer for the maturation intermediate to autophosphorylate is at least in part similar to the mechanism employed by the mature form of dDYRK2 to phosphorylate exogenous substrates.

Drug-Sensitivity Studies Show Fundamental Differences in DYRK Tyrosine Autophosphorylation and Serine/Threonine Substrate Phosphorylation

We next used the in vitro translation system to ask if any known small-molecule inhibitors of DYRKs would be able to block the autophosphorylation event and

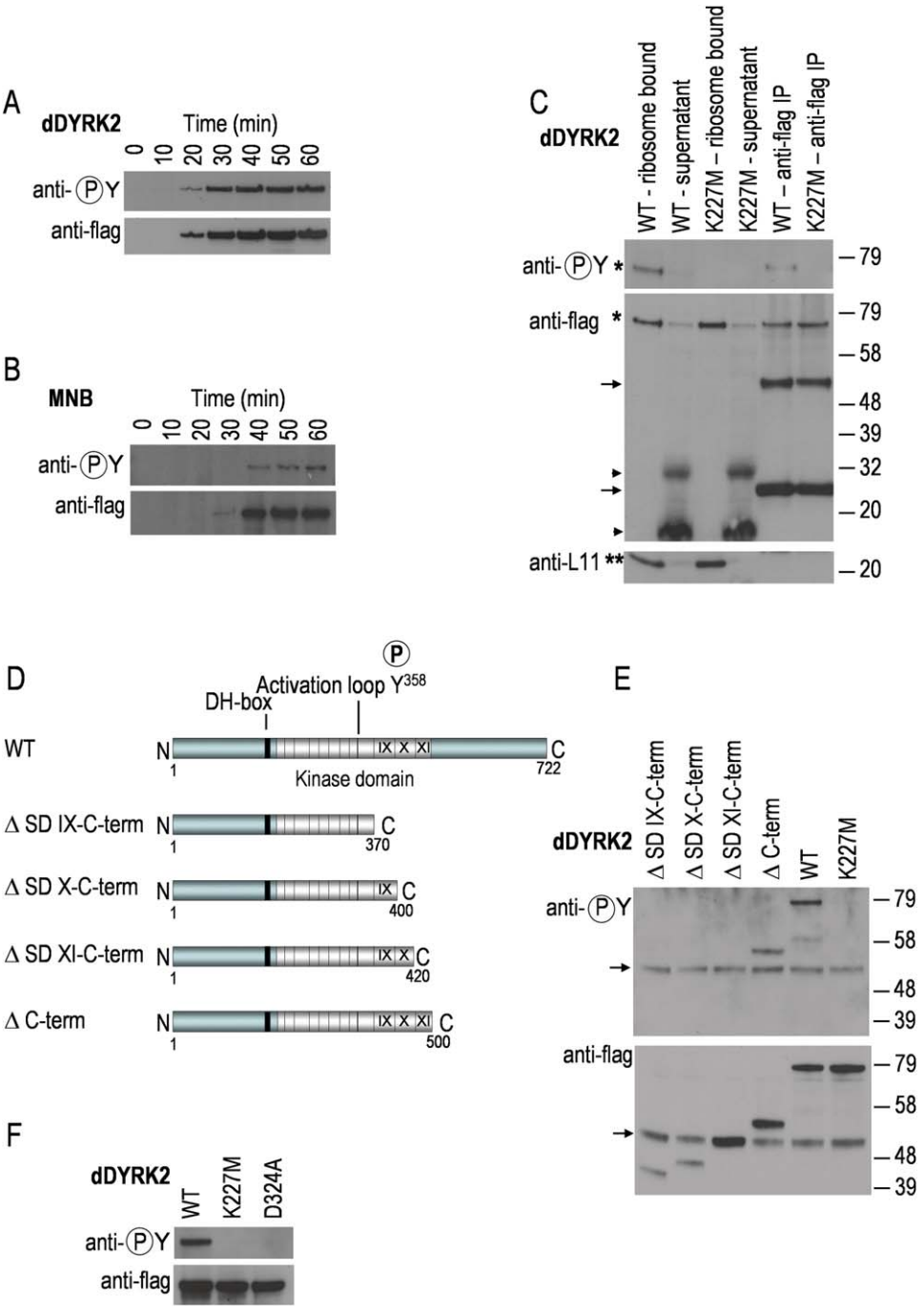

Figure 4. dDYRK2 Autophosphorylation Is Coupled to Translation

(A) FLAG-wt dDYRK2 was transcribed and translated in rabbit reticulocyte lysates for the times indicated. Samples were then incubated with anti-FLAG agarose, and immunocomplexes were subjected to SDS-PAGE and immunoblotted with either anti-phosphotyrosine (upper panel) or anti-FLAG (lower panel) antibodies.

(B) FLAG-wt MNB was transcribed and translated in rabbit reticulocyte lysates for the times indicated and analyzed as in (A).

(C) FLAG-wt dDYRK2 was expressed in rabbit reticulocyte lysate for 25 min, and then translation stopped. Ribosomes were purified through a 0.5 M sucrose cushion and resuspended in sample buffer. The supernatant from the sucrose cushion was incubated with anti-FLAG agarose. The ribosomal pellet, 5 μl of neat supernatant, and the supernatant immunocomplexes were subjected to SDS-PAGE and immunoblotted with either anti-phosphotyrosine (upper panel), anti-FLAG (middle panel), or anti-L11 (lower panel) antibodies. *, FLAG-dDYRK2; **, L11; arrows, IgG; arrowheads, nonspecific bands.

(D) Schematic representation of kinase-subdomain (SD) deletion mutants of dDYRK2. Kinase domain is in gray with the subdomain boundaries marked, and the domains analyzed are marked with roman numerals. The amino acid numbers of the mutants are indicated.

(E) The FLAG-tagged kinase-subdomain-deletion mutants of dDYRK2 outlined in (D) were expressed in rabbit reticulocyte lysate and prepared as in (A).

(F) FLAG-tagged wt, K227M, and D324A were expressed in rabbit reticulocyte lysate and prepared as in (A).

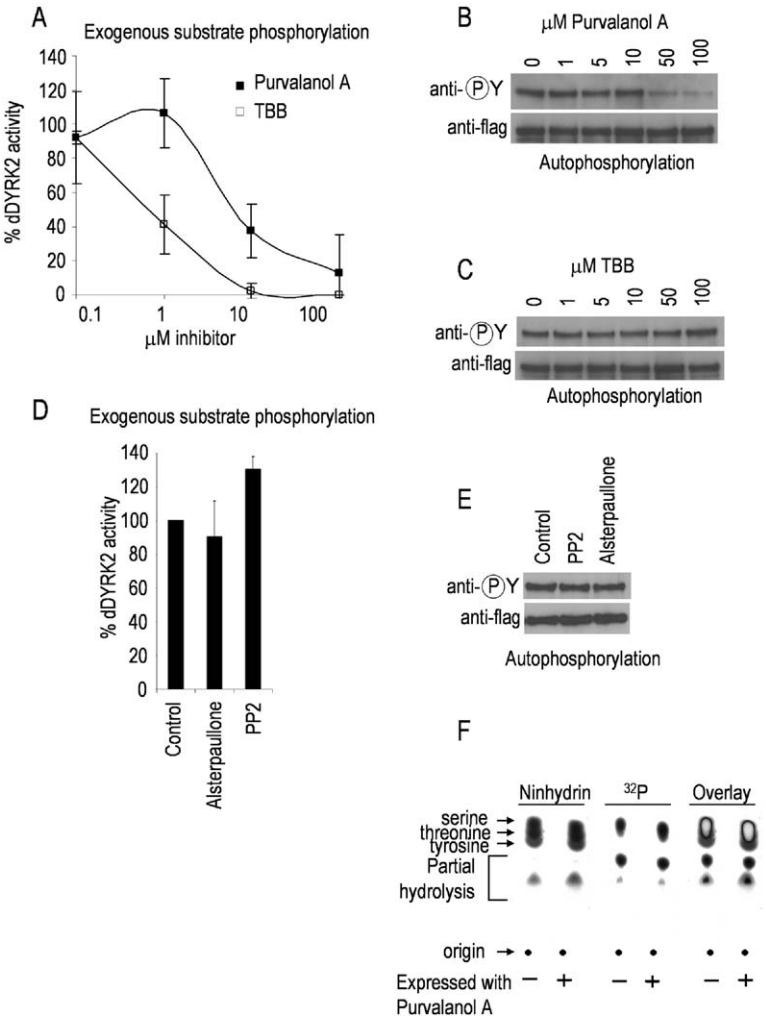

Figure 5. The Effect of Purvalanol A and TBB on dDYRK2 Exogenous Substrate Phosphorylation and Nascent Kinase Autophosphorylation

(A) FLAG-wt dDYRK2 was preincubated in the presence or absence purvalanol A or TBB at the concentrations indicated for 30 min on ice. Kinase activity was measured against Woodtide as described in Experimental Procedures. Results are presented as the means ± SEM (n = 3).

(B) FLAG-wt dDYRK2 was expressed in rabbit reticulocyte lysate in the presence of purvalanol A at the concentrations indicated and then incubated with anti-FLAG agarose. The immunocomplexes were subjected to SDS-PAGE and immunoblotted with either anti-phosphotyrosine (upper panel) or anti-FLAG (lower panel) antibodies.

(C) FLAG-wt dDYRK2 was expressed in rabbit reticulocyte lysate in the presence of TBB at the concentrations indicated and then prepared as in (B).

(D) FLAG-wt dDYRK2 was preincubated in the presence or absence of 100 μM alsterpaullone or PP2 for 30 min on ice. Kinase activity was measured against Woodtide as described in Experimental Procedures. Results are presented as the means ± SEM (n = 3).

(E) FLAG-wt dDYRK2 was expressed in rabbit reticulocyte lysate in the presence or absence of 100 μM alsterpaullone or PP2 and then prepared as in (B).

(F) FLAG-wt dDYRK2 was expressed in rabbit reticulocyte lysate in the presence or absence of 100 μM purvalanol A and immunoprecipitated with anti-FLAG agarose. The immunoprecipitates were allowed to autophosphorylate in the presence of [γ-^{32}P]ATP. The proteins were then subjected to SDS-PAGE and transferred to PVDF membranes. ^{32}P-labeled FLAG-dDYRK2 and FLAG-MNB were excised from the filter, hydrolyzed, and normalized for counts, and the amino acids were separated by TLC. Ninhydrin was used to visualize amino acids, and autoradiography was used to visualize phosphorylated amino acids. An overlay of these images shows which amino acids are phosphorylated.

produce non-tyrosine-phosphorylated protein. Purvalanol A and TBB are known inhibitors of DYRK1A (Bain et al., 2003; Sarno et al., 2003). These small-molecule inhibitors also inhibit dDYRK2 activity toward the exogenous substrate Woodtide in vitro, TBB being more potent than purvalanol A (Figure 5A). However, only purvalanol A but not TBB inhibits dDYRK2 autophosphorylation in the rabbit reticulocyte lysate (Figures 5B and 5C). Purvalanol A can also inhibit CDKs; therefore, to control for these kinases being involved in dDYRK2 autophosphorylation, we used alsterpaullone, a known CDK but not DYRK inhibitor (Bain et al., 2003). Alsterpaullone had no effect on either dDYRK2 activity toward Woodtide or its autophosphorylation. Furthermore, PP2, a tyrosine-kinase inhibitor (Hanke et al., 1996), also had no effect (Figures 5D and 5E). This provides further evidence that dDYRK2 autophosphorylates in the rabbit reticulocyte lysate as well as indicating that the mechanism of dDYRK2 autophosphorylation is different from the mechanism that it uses to phosphorylate exogenous substrates.

Our model makes a strong prediction: drugs that inhibit autophosphorylation may act irreversibly. As the purvalanol A-treated non-tyrosine-phosphorylated DYRK molecules represent intermediates in maturation, we asked if they represent functional intermediates and if the effects of purvalanol A could be reversed, allowing DYRK to complete autophosphorylation in vitro. Figure 5F demonstrates that full-length DYRK molecules that have never been phosphorylated as a consequence of treatment with purvalanol A are unable to autophosphorylate on tyrosine. This result supports our model that autophosphorylation of the DYRK activation-loop tyrosine is accomplished by the action of a transitional intermediate and is a one-off event.

Discussion

We initiated these studies to answer two questions concerning the regulation of DYRK catalytic activity. First, how does a serine/threonine kinase autophosphorylate on tyrosine residues? And second, why are DYRK proteins unable to phosphorylate these same tyrosine residues in vitro?

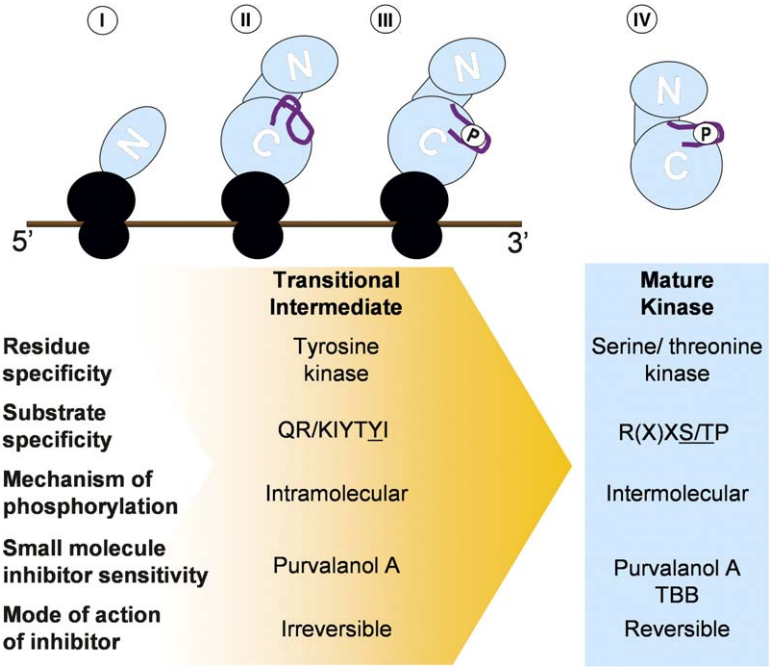

Figure 6. Model of the Mechanism of DYRK Autophosphorylation

As DYRK mRNA is translated, the N-terminal polypeptide begins to form secondary and tertiary structure (I). During folding of the polypeptide, an intermediate is formed, and the activation loop is the first substrate seen by the nascent kinase domain (II). The intermediate possesses tyrosine-kinase activity that phosphorylates the critical activation-loop tyrosine (III). The fully translated and activation-loop phosphorylated DYRK is now in its mature conformation. The tyrosine-kinase activity is lost, and the kinase functions as a serine/threonine kinase (IV). The residue and substrate specificities and sensitivities to small-molecule inhibitors are outlined below.

Here we suggest that a canonical kinase domain can adopt a functional intermediate during maturation of the enzyme that is capable of intramolecular phosphotransfer. As proteins are translated, the N terminus becomes available for folding before the C terminus, leading to the formation of folded translational intermediates typically consisting of ~50–300 amino acids. Certain intermediates have been shown to exhibit transient properties that differ from the full-length protein. Once translation is complete, proteins then assume their mature conformation (Fedorov and Baldwin, 1997). We postulate that the existence of an intramolecular transitional intermediate provides the simplest explanation for the observed behavior of DYRK proteins (Figure 6), based on the following evidence. First, full-length dephosphorylated (phosphatase-treated) or nonphosphorylated (purvalanol A-expressed) DYRKs cannot rephosphorylate the activation-loop tyrosine, but newly synthesized kinase can, in parallel with translation. Second, purification of ribosome bound nascent tyrosine-phosphorylated DYRK polypeptides demonstrates that DYRK tyrosine autophosphorylation occurs prior to disengagement from the ribosome. Third, the functional DYRK intermediate formed during translation has different residue and substrate specificities, as well as different sensitivities to small-molecule inhibitors, compared to its mature counterpart. For example, the dDYRK2 transitional intermediate phosphorylates a tyrosine residue in the sequence KIYTYI (where the underlined residue is phosphorylated), compared to the mature kinase that phosphorylates a serine or threonine residue in the preferred substrate sequence, R(X)XS/TP (Campbell and Proud, 2002). We believe that it is the intramolecular nature of the reaction that accounts for differences in residue and substrate specificities, for, as the protein matures, the first substrate encountered is the molecule's own activation-loop region, and phosphorylation of the second tyrosine in the YXY motif is inevitable. While many studies have concentrated on specificity determinants of intermolecular phosphorylation events, we know of none that addresses intramolecular constraints. Our work suggests that intramolecular determinants will differ not only with respect to surrounding consensus sequences but even with respect to the amino acid phosphorylated. The difference in sensitivity to two structurally distinct chemical inhibitors (TBB and purvalanol A) suggests that there are differences in the folded structure of the transitional intermediate compared with the mature kinase. TBB (Szyszka et al., 1995) and purvalanol A (Gray et al., 1998) are structurally distinct ATP-competitive inhibitors, with purvalanol A being the significantly larger of the two. Therefore, it is conceivable that the intermediate forms a different kinase structure that is only sensitive to the larger of the two molecules, or that nonconventional inhibitor:protein interactions are formed that are not conserved in the mature kinase. These results, taken together, indicate that the transitional intermediate kinase domain has a functionally different structure compared with its mature counterpart.

Autophosphorylation by a transitional intermediate is a previously unrecognized aspect of protein-kinase structure and function that is unlikely to be unique to the DYRK family of protein kinases. For instance, autophosphorylation of the serine/threonine kinase GSK3β is intramolecular (Cole et al., 2004) and on a tyrosine residue (Y216) whose surrounding sequence differs from its substrate phosphorylation sequence (Hughes et al., 1993). In addition, no phosphate is incorporated into tyrosine when GSK3 isolated from mammalian cells is incubated with Mg^{2+}ATP (Hughes et al., 1993) Although there is some evidence for rephosphorylation of

Y216 in bacterially expressed GSK3β, it is far less efficient than autophosphorylation of newly synthesized kinase and highlights GSK3 as a kinase that, like DYRKs, may autophosphorylate by passing through a maturational intermediate.

Another candidate is ERK7, a member of the MAPK family of protein kinases (Abe et al., 1999). ERK7 is constitutively active and autophosphorylates by an intramolecular mechanism on its TEY motif in the activation loop (which is shared with all MAPK family members) (Abe et al., 2001). The consensus phosphorylation sequence for ERK7 substrates is not known; however, other ERKs are serine/threonine kinases, suggesting that the tyrosine autophosphorylation mechanism could be the same as described here for the DYRKs.

The potential of kinases to autophosphorylate by a transitional intermediate is not limited to phosphorylation of tyrosine residues. Kinases that are constitutively phosphorylated on serine or threonine residues in the activation loop include the cAMP-activated protein kinase (PKA). PKA is a holoenzyme composed of two catalytic and two regulatory subunits. Catalytic subunits isolated from animal cells are fully phosphorylated on the activation-loop residue T197, after which the subunits are assembled to give the mature holoenzyme (Smith et al., 1999). PKA can autophosphorylate on T197 when expressed in bacterial cells, while expression of PKA in the presence of H-89 (a small-molecule inhibitor of PKA) produces catalytic subunits that are not phosphorylated on T197 and are unable to rephosphorylate (Steinberg et al., 1993), reminiscent of results observed with purvalanol A-expressed dDYRK2. Although controversy exists as to the kinase responsible for phosphorylation of T197 (Cauthron et al., 1998; Moore et al., 2002), it is likely that autophosphorylation may be the major route of T197 phosphorylation (Cheng et al., 1998; Williams et al., 2000). We propose that this phosphorylation may use a maturational intermediate form of PKA, in a mechanism similar to that of DYRKs. The molecules listed here are by no means exclusive, and examples of other protein kinases that activate using this mechanism remain to be identified.

Kinases that are autophosphorylated by a maturation intermediate are likely to have developed additional mechanisms to regulate their activity. For example, they may only be expressed when and where they are required, and the synthesis and degradation of the kinase may be extremely important. This seems be the case for DYRK1A/MNB, as its expression in the developing nervous system is only at specific times (Hammerle et al., 2002; Hammerle et al., 2003a). Furthermore, DYRK1B expression alters during the cell cycle (Lee et al., 2000). Other possibilities include inhibitory phosphorylations on other regions of the kinase, such as the S9 phosphorylation site in GSK3β, which inhibits the kinase's activity in a reversible manner (Cross et al., 1995). Or, the kinase's activity could be regulated by other proteins, such as the catalytic subunits of PKA interacting with its regulatory subunits (Smith et al., 1999).

Protein kinases are attractive targets for therapy of many diseases. DYRK1A is overexpressed in Down's syndrome, and this is thought to be responsible for many of the developmental defects associated with the disorder, reviewed in Hammerle et al. (2003b). Thus, inhibiting DYRK1A kinase activity may be a good target for this disease. Also, overexpression of DYRK1A (de Wit et al., 2002), DYRK1B (Lee et al., 2000), and DYRK2 (Miller et al., 2003) has been observed in various types of cancer, although it is not known what role these DYRKs play in the etiology of the disease. It will be of interest to determine if some of the cellular effects demonstrated by purvalanol A, such as cell cycle arrest and cell death (Villerbu et al., 2002), may be through inhibition of DYRKs.

The identification of the one-off nature of protein-kinase maturation intermediates highlights that DYRKs and other kinases that activate in this way can be targeted at two levels, the transitory intermediate and the mature active enzyme. Purvalanol A is a known inhibitor of the mature form of DYRKs, but we have shown that it can also prevent the activation of the kinase by blocking autophosphorylation. Similar results were seen for MNB in the rabbit reticulocyte lysate system (results not shown), implying that this sensitivity is shared by all DYRK family members. Transitory intermediates also provide opportunities for intervention that are not presented by the mature enzyme. Some inhibitors may be specific to the transitional event and may not be revealed by screens on mature molecules, while a possible transitional requirement for additional proteins, such as molecular chaperones, may offer novel targets for therapy.

In conclusion, recognition of intramolecular autophosphorylation mediated by a transitional intermediate form of the DYRK protein kinases demonstrates that at least a subset of these highly studied proteins have additional activation mechanisms and adopt alternative transient functional structures from the same polypeptide chain as their mature counterpart. A major challenge in the future will be to visualize this transitional intermediate kinase form.

Experimental Procedures

Materials

The pBluescript containing the MNB cDNA was provided by Francisco Tejedor (Instituto de Neurociencias, Unidad de Neurobiologia del Desarrollo, CSIC y Universidad Miguel Hernandez, Campus de San Juan, Alicante, Spain). Purvalanol A was obtained from Sigma. Alsterpaullone and PP2 were obtained from Calbiochem. TBB was a gift from Lorenzo Pinna (Venetian Institute for Molecular Medicine, University of Padova, Italy). The peptides of the dDYRK2 and MNB activation loops were synthesized by Protein and Peptide Chemistry, Cancer Research UK, London Research Institute, Lincoln's Inn Field, London). The anti-L11 antibody was provided by Karen Vousden (The Beatson Institute for Cancer Research, Glasgow, United Kingdom).

Baculovirus Constructs

The generation of the FLAG-wt, K227M, and Y356/358F dDYRK2 has been described previously (Lochhead et al., 2003). The other dDYRK2 constructs encoding amino acid point mutations and stop codons (for deletions) were generated with the primers outlined in Table S1 (see the Supplemental Data available with this article online) using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's recommendations.

For the generation of MNB-expressing baculovirus, either a NotI site and FLAG tag were inserted into *mnb* cDNA immediately upstream of the initiating ATG with the primers outlined in Table S1 (described as above) and the NotI EcoRI fragment encoding the

FLAG-MNB protein or the EcoRI fragment encoding MNB protein was cloned into the pVL1393 baculoviral vector (BD Biosciences) for expression in Sf9 cells. The other MNB constructs encoding amino acid point mutations were generated with the primers outlined in Table S1.

Plasmid Constructs

The EcoRI insert encoding the FLAG-dDYRK2 protein was subcloned into pcDNA3 for expression using T7 RNA polymerase in the Rabbit Reticulocyte Lysate TNT system (Promega). The FLAG-MNB-expressing plasmid was generated using the primers and conditions outlined above. T3 RNA polymerase was used to express the protein in the Rabbit Reticulocyte Lysate TNT system (Promega).

In Vitro Transcription and Translation

The FLAG-dDYRK2 and FLAG-MNB were expressed in the Rabbit Reticulocyte Lysate TNT system (Promega) using either T7 or T3 RNA polymerase, respectively, as outlined by the manufacturer, for 45 min unless the time is indicated. For small-molecule inhibitor experiments, purvalanol A, TBB, alsterpaullone, or PP2 was added prior to the initiation of transcription.

Purification of Ribosome Bound Nascent Chains

The in vitro transcription and translation were carried out as above for 25 min (first full-length chains begin to appear). The reaction was stopped by adding 50 μl of ice-cold 2× buffer C (100 mM Tris-HCl [pH 7.5], 2 mM EDTA, 2 mM EGTA, 100 mM NaF, 10 mM NaPPi, 2 mM $NaOVa_4$, and 2× Complete minitab protease inhibitor). Fifty microliters of the stopped reaction was placed on top of 150 μl 0.5 M sucrose cushion in buffer C and spun at 100,000 rpm for 6 min at 4°C in a Beckman TLA-100.3 rotor. The pellet was washed twice in buffer B and resuspended in sample buffer.

Immunoprecipitation of dDYRK2 or MNB

Sf9 cell extract (0.15 mg) or rabbit reticulocyte lysate was incubated for 1 hr on a rotating platform with anti-FLAG agarose. The immunocomplex was pelleted and washed twice with 1 ml of ice-cold buffer A (20 mM Tris-HCl [pH 8], 50 mM NaF, 500 mM NaCl, 1 mM sodium vanadate, 1% [v/v] NP-40, 5 mM sodium pyrophosphate, 10% [v/v] glycerol, and 0.1% [v/v] 2-mercaptoethanol) and twice with 1 ml of ice-cold buffer B (50 mM Tris-HCl [pH 7.5], 0.1 mM EGTA, and 0.1% [v/v] 2-mercaptoethanol).

Identification of Phosphorylated Residues

Immunoprecipitated dDYRK2 or MNB was subjected to SDS-PAGE (4%–12% NuPAGE) and stained using Simply Blue Safe Stain (Invitrogen). The excised proteins were alkylated with 4-vinylpyradine and digested with 5 μg/ml trypsin in 20 mM ammonium bicarbonate/0.1% (w/v) N-octyl glucoside (Calbiochem). The peptides were extracted from the gel with addition of 60 μl acetonitrile, and the supernatant was dried in a speedvac and then resuspended in 80 μl buffer D (30% acetonitrile/0.25 M acetic acid). The sample was then incubated for 45 min with the addition of 20 μl buffer D containing 3 μl PHOS-select beads. The beads were collected onto a μC 18 Zip-Tip, washed three times with 25 μl buffer D, eluted with 2 × 25 μl 0.4 M NH_4OH, dried, and reconstituted in 10 μl 50% acetonitrile/0.1% TFA. 0.5 μl of the sample was analyzed on a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, California) in MS and MS/MS modes (1 KeV plus air in CID). Further analysis was performed by LC-MS/MS on a Q-TOF2 mass spectrometer (Micromass, Manchester, United Kingdom) as described previously (Way et al., 2002). Sites of phosphorylation were assigned by manual inspection of resultant MS/MS spectra as well as by database searching using the Mascot (Matrixscience) search engine run on an in-house server.

Lambda-Phosphatase Treatment

Immunoprecipitated dDYRK2 or MNB was incubated in a total volume of 50 μl containing 50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 0.1 mM $MnCl_2$, 0.1 mM EGTA, 2 mM dithiolthreitol, 0.01% BRIJ 35, and 400 U lambda phosphatase for 30 min at 30°C. The reaction was terminated by washing twice with 1 ml of ice-cold buffer A and twice with 1 ml ice-cold buffer B. Control reactions were performed as above but in the absence of phosphatase.

Immunoblotting

Immunoprecipitated dDYRK2 or MNB or ribosome-purified dDYRK2 was subjected to SDS-PAGE (4%–12% NuPAGE), transferred to nitrocellulose, blocked with 3% BSA TBS-T, and immunoblotted with either 1:15,000 dilution of anti-phosphotyrosine, 1:5,000 dilution of anti-FLAG antibody, or 1:1,000 dilution of anti-L11 antibody. HRP-conjugated anti-mouse was used to detect anti-phosphotyrosine or anti-FLAG antibody, and HRP-conjugated anti-rabbit was used to detect anti-L11, then visualized using the enhanced chemiluminescence system (Amersham).

Phosphoamino Acid Analysis

Immunoprecipitated FLAG-dDYRK2 or FLAG-MNB was incubated in buffer B with 100 μM [γ-^{32}P]ATP (2 × 106 cpm/nmol) for 30 min with agitation. The ^{32}P-labeled dDYRK2 or MNB was subjected to SDS-PAGE as described above. The protein was transferred to PVDF Hybond-P membrane (Amersham) and visualized by autoradiography. The radioactive band was excised from the membrane and the protein hydrolyzed in 6 N HCl (Aristar) at 110°C for 1 hr. The resulting supernatant was dried in a speedvac and the pellet resuspended in 5–10 μl distilled water. One to two microliters of hydrolysate was mixed with 1 μl of phosphoamino acid standards (1 mg/ml of phospho-S, phospho-T, and phospho-Y in a 1:1:1 ratio). The amino acids were separated on a plastic-backed TLC cellulose plate (Merck) using fresh buffer containing 1.5 M glacial acetic acid/0.017% (v/v) formic acid/0.003% (v/v) pyridine/0.33 mM EDTA for 1 hr at 300 V. To visualize the amino acid standards, the plate was sprayed with ninhydrin (BDH), and ^{32}P-labeled amino acids were visualized by autoradiography.

Peptide Kinase Assay

Immunoprecipitated FLAG-dDYRK2 or FLAG-MNB was assayed using 50 μM Woodtide (UBI) or 50 μM activation-loop peptide with two additional lysines attached to the N terminus to allow it to bind to P81 paper (KKSSCYVDRKIYTYIQSRFY for dDYRK2 or KKSSC QLGQRIYHYIQSRFY for MNB) as described previously (Lochhead et al., 2003). For experiments with small-molecule inhibitors, immunoprecipitated dDYRK2 or MNB was preincubated in the presence or absence of 10 μM purvalanol A, TBB, alsterpaullone, or PP2 for 30 min on ice, then assayed as outlined above.

Statistical Analysis

All statistical analysis was performed using Microsoft Excel 2002.

Supplemental Data

Supplemental Data include one table and are available with this article online at http://www.cell.com/cgi/content/full/121/6/925/DC1/.

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