dDYRK2: a novel dual-specificity tyrosine-phosphorylation-regulated kinase in *Drosophila*

Pamela A. LOCHHEAD*, Gary SIBBET*, Ross KINSTRIE*, Tava CLEGHON*, Margie RYLATT*, Deborah K. MORRISON† and Vaughn CLEGHON*¹

*The Beatson Institute for Cancer Research, Cancer Research UK, Garscube Estate, Switchback Road, Glasgow G61 1BD, U.K., and †Regulation of Cell Growth Laboratory, Center for Cancer Research, NCI-Frederick, Frederick, MD 21702, U.S.A.

Dual-specificity tyrosine-phosphorylation-regulated kinases (DYRKs) are an emerging family of protein kinases that have been identified in all eukaryotic organisms examined to date. DYRK family members are involved in regulating key developmental and cellular processes such as neurogenesis, cell proliferation, cytokinesis and cellular differentiation. Two distinct subgroups exist, nuclear and cytosolic. In *Drosophila*, the founding family member minibrain, whose human orthologue maps to the Down syndrome critical region, belongs to the nuclear subclass and affects post-embryonic neurogenesis. In the present paper, we report the isolation of dDYRK2, a cytosolic DYRK and the putative product of the smell-impaired *smi35A* gene. This is the second such kinase described in *Drosophila*, but the first to be characterized at the molecular and biochemical level. dDYRK2

is an 81 kDa dual-specificity kinase that autophosphorylates on tyrosine and serine/threonine residues, but appears to phosphorylate exogenous substrates only on serine/threonine residues. It contains a YXY motif in the activation loop of the kinase domain in the same location as the TXY motif in mitogen-activated protein kinases. dDYRK2 is tyrosine-phosphorylated *in vivo*, and mutational analysis reveals that the activation loop tyrosines are phosphorylated and are essential for kinase activity. Finally, dDYRK2 is active at all stages of fly development, with elevated levels observed during embryogenesis and pupation.

Key words: development, *Drosophila*, dual-specificity kinase, dual-specificity tyrosine-phosphorylation-regulated kinase (DYRK), minibrain.

INTRODUCTION

DYRK (dual-specificity tyrosine-phosphorylation-regulated kinase)/MNB (minibrain)/YAK kinases represent an emerging family of dual-specificity kinases that autophosphorylate on tyrosine, serine and threonine, but appear to phosphorylate exogenous substrates only on serine or threonine residues. DYRK family members have been identified in all eukaryotes examined to date. Although definitive functions have yet to be determined, work in *Saccharomyces cerevisiae*, *Drosophila* and mice implicate this family of kinases in such fundamental processes as the regulation of cell proliferation, cytokinesis, developmental responses (such as the transition from a growth to differentiation phase) and, in higher eukaryotes, proper brain development. Distantly related to MAPK (mitogen-activated protein kinase) and Cdk (cyclin-dependent protein kinase) [1–4], the DYRK family is characterized by the presence of several distinct amino-acid sequences in the kinase domain, including an SSC motif following subdomain VII, conserved sequences HCDLKPEN and YXYIQR-FYR(S/A)PE in subdomains VI and VIII respectively, and a YXY motif in the kinase-domain-activation loop between subdomains VII and VIII, and by a DYRK homology (DH) box immediately preceding the kinase domain [5]. All DYRK proteins appear to have extended N- and/or C-terminal regions that display little homology to other proteins except to closely related family members.

Functional studies have been conducted on yeast, *Drosophila* and mammalian DYRK family members. In the yeast *S.*

cerevisiae, the sole DYRK family member, Yak1p, functions as an antagonist to the Ras/cAMP-dependent protein kinase (PKA) pathway to negatively regulate cell growth [6–8]. The *Dictyostelium* counterpart, YakA, is essential for both a starvation-induced growth arrest and initiation of a developmental response, and has been suggested to function as a specific cell-cycle regulator to facilitate exit from the cell cycle and to mediate developmental events [9]. In the yeast *Schizosaccharomyces pombe*, DYRK family member Pom1 was identified as a gene that may position the growth and cytokinesis machineries by interaction with both the actin and microtubule cytoskeletons [10]. Experiments that modify the kinase activity of Pom1 indicate a negative role for Pom1p in microtubule growth at cell ends [11]. In *Drosophila*, mutation of the founding DYRK family member, minibrain (*mnb*), affects post-embryonic neurogenesis, resulting in specific reductions in the size of the optic lobes and central brain hemispheres [12]. The human orthologue of the minibrain gene, *Dyrk1A*, maps to the Down syndrome critical region and is overexpressed in Down syndrome brains, implicating a similar role for this kinase in mammalian neurogenesis [13]. Mice with a single copy of the *Dyrk1A* gene display region-specific reductions in the brain, indicating a conserved mode of action that determines normal growth and brain size in both mice and flies. The *Dyrk1A* mutant mice also exhibit significant reductions in body size, suggesting that, at least in mammals, this kinase may have a more general role in development than in just the brain [14].

To date, no *in vivo* substrates of DYRK family members have been identified definitively. However, *in vitro* kinase assays

Abbreviations used: CDS, coding sequence; DH, DYRK homology; DYRK, dual-specificity tyrosine-phosphorylation-regulated kinase; ECL®, enhanced chemiluminescence; EST, expressed sequence tag; FKHR, forkhead in rhabdomyosarcoma; GSK3, glycogen synthase kinase 3; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MIRK, minibrain-related kinase; MNB, minibrain; ORF, open-reading frame; PEST, Pro-Glu-Ser-Thr; Sf9 cells, *Spodoptera frugiperda* 9 cells; UTR, untranslated region; WT, wild-type.

¹ To whom correspondence should be addressed (e-mail v.cleghon@beatson.gla.ac.uk).

demonstrate that DYRK1A and MNB are able to phosphorylate the Gli1 transcription factor [15]. DYRK1A and DYRK2 phosphorylate the protein-synthesis-initiation factor eIF2B ϵ (eukaryotic initiation factor 2B ϵ), the microtubule-associated protein, tau [16] and STAT3 (signal transducer and activator of transcription 3) [17]. DYRK1A also phosphorylates the forkhead transcription factor in rhabdomyosarcoma, FKHR [18]; and Yak1p phosphorylates Pop2 (part of a multicomponent complex that regulates the transcription of many genes; [19]). *In vitro* studies also indicate that DYRK family kinases are proline-directed and display a strong preference for the presence of an arginine residue at the -2 or -3 position N-terminal to the phosphorylated residue [4,20]. DYRK kinases contain a YXY motif found in the same location as the TXY motif in MAPKs [2], and autophosphorylation of one or both of these residues appears to be essential for kinase activity [11,21,22].

We have used an anti-phosphotyrosine antibody-based screen to isolate *Drosophila* tyrosine and dual-specificity kinases. In the present paper, we describe the isolation and initial characterization of a novel *Drosophila* DYRK family member that we have termed dDYRK2, as it is the second such fly kinase described. dDYRK2 is the putative protein product of the *smi35A* gene which was identified based on the recessive olfaction defective behaviour of four P-element insertion fly lines [23]. We demonstrate that dDYRK2 is a dual-specificity kinase that autophosphorylates on tyrosine and serine/threonine residues, but phosphorylates exogenous substrates only on serine/threonine residues. We also characterize the role of tyrosine residues within the activation loop of the kinase domain. Finally, we demonstrate that dDYRK2 is expressed and tyrosine phosphorylated *in vivo*, and kinase activity can be detected at all stages of the *Drosophila* life cycle.

MATERIALS AND METHODS

Isolation of dDyrk2 cDNA

A *Drosophila melanogaster* Canton-S 2–14 h embryo expression library (Stratagene, cat #937602) was screened (3×10^5 total plaques) with the anti-phosphotyrosine antibody 4G10 (UBI). Bacteria were grown and infected with phage according to the manufacturer's protocol. To induce *Drosophila* protein expression, nitrocellulose filters were soaked in 10 mM IPTG (isopropyl β -D-thiogalactoside), dried to dampness and overlaid to plates. Plates were incubated overnight at room temperature (25 °C). Filters were then washed with TBS-T [50 mM Tris, pH 8.0, 137 mM NaCl and 0.05 % (v/v) Tween 20] at room temperature for 15 min followed by a second wash at 4 °C for 6 h. Filters were blocked with 2 % (w/v) BSA, washed with TBS-T at room temperature for 5 min, and probed with anti-phosphotyrosine antibody as described below in the Immunoblotting section. Immunoreactive plaques were visualized using ECL[®] (enhanced chemiluminescence; Amersham Biosciences). Positive plaques were isolated and subjected to two rounds of plaque purification. BlueScript vectors were then excised from phagemids using SOLR *Escherichia coli* bacteria according to the manufacturer's recommendations. Bluescript plasmid DNA was prepared, and the presence and size of inserts were determined by digestion of DNA with *EcoRI* and *XhoI*. Inserts were sequenced from both ends using T3 and T7 promoter primers. To isolate full-length *dDyrk2* cDNA, we followed the recommendations in the Stratagene Uni-ZAP XR library instruction manual. The GH *Drosophila* library was screened using a 1350 bp *PstI* fragment isolated from clone 13b.2. The probe was prepared using the Prime-It[®] II random primer labeling kit (Stratagene).

Baculovirus constructs

For the generation of dDYRK2-expressing baculovirus, an *EcoRI* site was cloned into the *dDyrk2* cDNA immediately upstream of the initiating ATG. The *EcoRI* fragment encoding the dDYRK2 protein was then cloned into the pVL1393 baculoviral vector (BD Biosciences) for expression in Sf9 (*Spodoptera frugiperda*) cells. dDYRK2 constructs encoding amino-acid point mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions.

Antibody preparation

The anti-dDYRK2 (α -dDYRK2) antibody is a rabbit polyclonal antibody generated against a purified synthetic peptide corresponding to amino-acid residues 709–722 of dDYRK2. The immunized rabbit serum was used at 0.2 μ l per immunoprecipitate and at a 1:15 000 dilution for immunoblotting.

Preparation of *Drosophila* lysates

Canton-S 0–22 h *Drosophila* embryos, larvae, pupae and adult flies (male and female) were collected and transferred into ice-cold lysis buffer [20 mM Tris/HCl, pH 8, 50 mM NaF, 137 mM NaCl, 1 mM sodium vanadate, 1 % (v/v) Nonidet P40, 5 mM sodium pyrophosphate and 10 % (v/v) glycerol]. The embryos were lysed using an Omni homogenizer (CamLab, Cambridge, U.K.), followed by homogenization with a Dounce homogenizer and the samples were then centrifuged at 16 000 g for 5 min to pellet the debris. The supernatant was removed for analysis and the protein concentration was determined using the method described by Bradford [24].

Immunoprecipitation of dDYRK2

Sf9 cell extract (0.15 mg) or lysed *Drosophila* embryos (6 mg) was incubated for 1 h on a rotating platform with Protein A–Sepharose conjugated to the α -dDYRK2 antibody. 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) Nonidet P40, 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].

Lambda phosphatase treatment

Immunoprecipitated dDYRK2 was incubated in a total volume of 50 μ l containing 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.1 mM MnCl₂, 0.1 mM EGTA, 2 mM dithiothreitol, 0.01 % (v/v) Brij 35 and 400 units of 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 of ice-cold buffer B. Control reactions were performed as above, but in the absence of phosphatase.

Immunoblotting

Immunoprecipitated dDYRK2 was subjected to SDS/PAGE; 4–12 % NuPAGE for low resolution (see Figures 3A and 6A) or on a large (15 cm $\times$ 15 cm) 7.5 % (w/v) polyacrylamide gel for high resolution (see Figures 3B and 3C), transferred on to nitrocellulose, blocked with 3 % (w/v) BSA in TBS-T and immunoblotted with either 1:15 000 dilution of anti-phosphotyrosine or

1:5000 anti-dDYRK2 antibody. Horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit antibody was used to detect anti-phosphotyrosine or anti-dDYRK2 antibody respectively, and visualized using the ECL[®] system. Endogenous dDYRK2 isolated from *Drosophila* tissue could only be visualized when analysed on the low-resolution gels (NuPAGE). This is most likely due to a more efficient transfer of proteins on to nitrocellulose from 1 mm thick NuPAGE gels than from 1.5 mm thick normal gels.

MBP (myelin basic protein) and histone kinase reactions

Immunoprecipitated dDYRK2 was incubated with either MBP, histone IV, histone 2B or histone II-S in a total volume of 50 μ l containing 2.5 μ M [γ -³²P]ATP, 10 mM MgCl₂, 30 mM Hepes, pH 7.4, and 2 mM dithiothreitol for 30 min at room temperature. The reaction was stopped by adding 15 μ l of NuPAGE sample buffer (Invitrogen) and run on a 4–12 % NuPAGE gel (Invitrogen). ³²P-incorporation was visualized by autoradiography.

Phosphoamino acid analysis

³²P-labelled MBP was prepared and subjected to SDS/PAGE as described above. The protein was transferred on to PVDF Hybond-P membrane (Amersham Biosciences) and was visualized by autoradiography. The radioactive band was excised from the membrane and the protein was hydrolysed in 6 M HCl (BDH) at 110 °C for 1 h. The resulting supernatant was dried in a SpeedVac and the pellet was resuspended in 5–10 μ l of distilled water. Hydrolysate (1–2 μ l) was mixed with 1 μ l of phospho-amino 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 ethanoic acid, 0.017 % (v/v) formic acid, 0.003 % (v/v) pyridine and 0.33 mM EDTA for 1 h at 300 V. To visualize the amino-acid standards, the plate was sprayed with ninhydrin (BDH) and ³²P-labelled amino acids were visualized by autoradiography.

Peptide kinase assay

Immunoprecipitated dDYRK2 was assayed at 30 °C for 10 min in a total volume of 50 μ l containing 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 0.1 % (v/v) 2-mercaptoethanol, 10 mM MgCl₂, 0.1 mM [γ -³²P]ATP (2×10^6 c.p.m./nmol) and 50 μ M Woodtide (UBI, Lake Placid, NY, U.S.A.) by the method described by Woods et al. [18].

RESULTS

Cloning of dDYRK2

As *E. coli* do not encode protein tyrosine kinases [25], we designed an anti-phosphotyrosine antibody screen to isolate *Drosophila* cDNAs encoding enzymically active tyrosine kinases. This strategy was used to screen 3×10^5 plaques from a Canton-S 2–14 h embryo library. Eleven positive plaques were isolated and sequenced: five of the clones corresponded to Src64B (CG7524), four to src42A (CG7873), one to Myt1 (CG10569) and one, the subject of the present paper, to a novel gene, which we have designated *dDyrk2*. At the same time that this sequence was submitted to the NCBI database, this same gene was implicated in smell and is therefore also known as *smi35A* [23,26]. However,

recent work on *smi35A* suggests that these genes may be different (R. Anholt, personal communication). Therefore, until this issue is resolved, we will use *dDyrk2* to denote this gene.

Our initial *dDyrk2* clone (13b.2) represented an incomplete cDNA since neither an initiating methionine codon nor a polyadenylation signal sequence was present. To isolate a full-length *dDyrk2* cDNA, a 1357 bp *Pst*I fragment was isolated from clone 13b.2 and was used to probe a cDNA library (GH library). Seven positive clones were isolated, all contained a complete ORF (open-reading frame) and the entire sequence of the longest clone was determined.

As shown in Figure 1, the *dDyrk2* cDNA was 4652 bp long comprising 823 bp of 5'-UTR (untranslated region), 2166 bp CDS (coding sequence) encoding 722 amino acids and 1663 bp of 3'-UTR, which included three polyadenylation signals. The first methionine codon was in the sequence context ACACAATG (underlined) with residues in positions – 2, – 3 and – 4 matching the Kozak consensus sequence [27]. A comparison of the cDNA sequence with the *Drosophila* genome demonstrated that the *dDyrk2* gene spans nearly 50 kb of DNA comprising 11 exons (Figure 2A) all with canonical donor/acceptor splice sites.

Examination of the Berkeley *Drosophila* Genome Project database (<http://www.fruitfly.org/>) revealed the existence of 48 separate *dDyrk2* EST (expressed sequence tag) entries. The majority, 42 entries, begin near the start of our cDNA with 31 of these entries extending the 5' boundary of exon 1 by 25 bases. In addition, we observed several examples of alternative splicing; namely, 3'-extension of exon 1, and 3'-truncation or complete absence of exon 2. As the ORF begins in exon 5, these differences do not affect the protein sequence. EST entries were derived from seven different libraries, which were embryonic (29 entries), adult head (18 entries) or ovarian (1 entry). All available sequence data support the existence of a single *dDyrk2* transcriptional start site with transcripts containing the same first exon and a second exon that may be absent or truncated, followed by nine additional exons.

The conceptual translation of the dDYRK2 CDS (Figure 2B) indicates that the 722-amino-acid protein contains an N-terminal region of 197 amino acids, a central kinase domain of 297 amino acids and a C-terminal region of 228 amino acids. As shown in Figure 1, the dDYRK2 protein contains all the elements that distinguish DYRK family members including a YXY motif in the so-called 'kinase-domain-activation loop' and a DH box immediately preceding the kinase domain. As observed in other family members, the extended N- and C-terminal regions of dDYRK2 display little similarity with other proteins other than to closely related DYRK family members. dDYRK2 contained no nuclear localization sequences as are found in MNB/DYRK1-related family members (Figure 2C). A BLAST search identified the proteins most closely related to dDYRK2 (E-value < 10^{-100}) as a putative protein from *Anopheles gambiae* (EAA12191; E-value = 10^{-156}), human DYRK4 (XP.034551; E-value = 10^{-131}), a putative protein from *Oikopleura dioica* (AAL56407; E-value = 10^{-122}), human DYRK2 (NP.006473; E-value = 10^{-120}), a putative protein from *Caenorhabditis elegans* (NP.502494; E-value = 10^{-120}), a putative protein from *Neurospora crassa* (CAD21489; E-value = 10^{-116}), human DYRK3 (CAA73266; E-value = 10^{-110}), a second putative protein from *Anopheles gambiae* (EAA12365; E-value = 10^{-110}), dDYRK3 a putative protein from *Drosophila* (AAM48430; E-value = 10^{-109}), *S. pombe* Pom1p (NP.592974; E-value = 10^{-106}), a putative protein from *Trypanosoma cruzi* (AAL86608; E-value = 10^{-103}), and a second putative protein from *S. pombe* (CAA91166; E-value = 10^{-101}).

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The protein-kinase domain is shaded in grey, a DH box is shaded in black, and unusual amino acids within the kinase domain that define the DYRK family are boxed in white. Three putative polyadenylation signals are indicated with grey boxes. The nucleotide sequence has been submitted to the GenBank® nucleotide database with accession number AF168467.

To analyse dDYRK2, an antibody (α -dDYRK2) was raised against a peptide corresponding to residues 709–722 of the predicted pro-

tein (Figure 2B). This antibody was found to recognize proteins in lysates from *E. coli* expressing dDYRK2 (results not shown), *Drosophila* embryos (Figure 3A), and Sf9 cells infected with recombinant baculovirus expressing the *dDyrk2* cDNA (Figure 3B).

Figure 2 Schematic representation of the genomic region, mRNA and translated product of *dDyrk2*

(A) A comparison of the *dDyrk2* cDNA with a 50 kb region of genomic DNA (not drawn to scale) reveals that the transcript is made up of 11 exons. Non-coding regions are indicated as white boxes; regions encoding the ORF that begins in exon 5 are indicated as solid boxes. Individual exons, their sizes in bp, and the size of introns in kb are indicated. (B) and (C) Representation of the dDYRK2 and DYRK1A proteins. The locations of the DH box, protein-kinase domain, activation-loop tyrosine residues, histidine repeat, nuclear localization sequence (NLS) and PEST region are shown for (B) dDYRK2 and (C) DYRK1A.

The specificity of α -dDYRK2 was confirmed by competition studies in which the antibody was neutralized by co-incubation with the peptide against which the antibody was raised (Figures 3A and 3B).

All DYRK family members contain a YXY motif in the activation loop of the kinase domain and phosphorylation of either one or both of the tyrosine residues is required for enzymic activity in DYRK1A [21,22]. dDYRK2 contains this motif (YTY) at residues 356–358 (Figures 1 and 2B). Immunoblot analysis of dDYRK2 isolated from *Drosophila* embryos revealed that the endogenous protein is tyrosine-phosphorylated (Figure 3A, lower panel). To determine if it is the activation-loop tyrosines that are phosphorylated in dDYRK2, point mutants of the protein were generated [Y356D/Y358D (Tyr³⁵⁶ → Asp/Tyr³⁵⁸ → Asp) and Y356F/Y358F] and were expressed in Sf9 cells. As a control, we also expressed a putative kinase-inactive form of the protein, K227M, containing a mutation in the highly conserved lysine residue shown to associate with ATP in the catalytic domain and known to be critical for kinase function.

The α -dDYRK2 antibody recognized all forms of dDYRK2 expressed in Sf9 cells (Figure 3B, upper panel). Interestingly, the WT (wild-type), Y356D/Y358D and Y356F/Y358F forms all run as a doublet of approx. 80–90 kDa, whereas the K227M form runs as a single band which corresponds to the faster-migrating band (Figure 3B, upper panel). A similar doublet was observed when endogenous dDYRK2 was analysed with anti-phosphotyrosine (Figure 3A, lower panel). However, due to the size of protein gels needed to visualize endogenous dDYRK2 (see the Materials and methods section), this sensitivity in resolution was lost when using the α -dDYRK2 antibody. The size of the faster-migrating species of the endogenous and recombinant protein is consistent with the predicted 81 kDa molecular mass of conceptually translated dDYRK2, whereas the size of the larger 90 kDa protein is consistent with phosphorylation of dDYRK2, as has been observed for other family members. Assessment of tyrosine phosphorylation of the various Sf9-expressed proteins using a phosphotyrosine-specific antibody demonstrated that only WT-dDYRK2 was significantly phosphorylated on

Figure 3 Specificity of α -dDYRK2 antibody and expression of recombinant protein

(A) *Drosophila* embryo (0–22 h) lysates were incubated with the α -dDYRK2 antibody either in the presence or absence of peptide 709–722, as indicated. Immunoprecipitates were then subjected to SDS/PAGE and were immunoblotted using the α -dDYRK2 (upper panel) or α -pY (anti-phosphotyrosine; lower panel) antibodies. Results are representative of three replicates. (B) Sf9 cell lysates containing WT-, Y356D/Y358D-, Y356F/Y358F- or K227M-dDYRK2 were incubated with α -dDYRK2 antibody in the presence or absence of peptide 709–722, as indicated. Immunocomplexes were then subjected to SDS/PAGE and were immunoblotted using α -dDYRK2 (upper panel) or α -pY (lower panel) antibodies. Results are representative of three replicates. (C) Sf9 cell lysates containing either WT- or K227M-dDYRK2 were immunoprecipitated and incubated either in the presence or absence of λ phosphatase (λ PPase), as indicated. Immunocomplexes were then subjected to SDS/PAGE and were immunoblotted with α -dDYRK2 (upper panel) or α -pY (lower panel) antibodies. Results are representative of three replicates.

tyrosine (Figure 3B, lower panel). These results suggest that dDYRK2 is phosphorylated on at least one of the tyrosine residues in the activation loop and that it is not tyrosine phosphorylation itself that is responsible for the slower-migrating form of the protein.

There are many post-translational modifications, including phosphorylation, that can account for the same protein migrating at different rates during SDS/PAGE. To determine if dDYRK2 runs as a doublet due to phosphorylation, we incubated dDYRK2 with various phosphatases and assessed the effects by SDS/PAGE. Treatment of dDYRK2 with λ phosphatase, which removes phosphate from tyrosine and serine/threonine residues, converted the protein into a single species which migrated at approx. 80 kDa (Figure 3C) similar to that observed for the kinase-dead K227M form of the protein, indicating that the observed SDS/PAGE mobility shift is due to phosphorylation. In contrast, treatment with serine/threonine- or tyrosine-specific phosphatases did not have the same effect (results not shown), suggesting that dDYRK2 may be phosphorylated on serine/threonine and tyrosine residues.

dDYRK2 has phospho-serine/threonine activity against exogenous proteins

Histones and MBP are non-specific substrate proteins that are commonly used to measure tyrosine- and serine/threonine-kinase activity, and to ascertain which residues are phosphorylated. We therefore incubated immunoprecipitates of WT- or K227M-dDYRK2 with histones IV, 2B or II-S, or MBP in the presence of [γ - 32 P]ATP. The reaction products were then subjected to SDS/PAGE and the phosphorylated proteins were visualized by autoradiography. WT-dDYRK2 was found to phosphorylate histones IV, 2B and II-S, and MBP with differing efficiency (Figures 4A and 4B). We quantified the phosphorylation of MBP by WT- and K227M-dDYRK2 and found that the WT protein was 30-fold more effective than K227M-dDYRK2 (Figure 4B).

To determine which amino acids dDYRK2 phosphorylates, MBP was incubated with WT-dDYRK2 in the presence of [γ - 32 P]ATP. MBP was then hydrolysed and subjected to TLC (Figure 4C). WT-dDYRK2 phosphorylated serine and threonine, but not tyrosine residues in MBP (Figure 4B). Similar results were obtained using histones as *in vitro* substrates (results not shown). These results indicate that dDYRK2 has phospho-serine/threonine-kinase activity against exogenous proteins.

Development of a peptide-based assay to measure dDYRK2 activity

To obtain a more specific, rapid and quantitative *in vitro* assay to measure dDYRK2 activity, we tested the ability of recombinant dDYRK2 to phosphorylate the short, commercially available synthetic peptide Woodtide. Woodtide (KKISGRSLSPIMTEQ) corresponds to the amino-acid sequence surrounding Ser³²⁹ of the putative DYRK1A substrate, FKHR [18]. Using this method, kinase activity was detected with WT-, but not K227M-dDYRK2 (Figure 5A), consistent with the kinase activity measured using MBP as substrate (Figure 4A).

To characterize kinase activity further, we tested the dependency of dDYRK2-mediated phosphorylation for magnesium and manganese ions. dDYRK2 was found to phosphorylate Woodtide 10-fold more efficiently in the presence of magnesium compared with dDYRK2 in the presence of manganese (Figure 5B). Interestingly, when these ions were present at equal concentrations, manganese had an inhibitory effect on dDYRK2 activity (Figure 5B, closed bars). To determine if this observation was unique to dDYRK2, we performed identical experiments on recombinant DYRK1A. In the presence of magnesium, 5-fold more activity was measured from DYRK1A compared with DYRK1A in the presence of manganese; furthermore, manganese appeared to inhibit DYRK1A in the presence of magnesium (Figure 5B, open bars). The inhibitory effect of manganese ions on DYRK1A was not as marked as that on dDYRK2; however, this result does suggest a common effect between DYRK family members.

Figure 4 Phosphorylation of histones and MBP by dDYRK2 *in vitro*

(A) Histones IV, 2B and II-S were incubated with WT- or K227M-dDYRK2 immunocomplexes in the presence of [γ -³²P]ATP, as indicated. The protein was subjected to SDS/PAGE and was visualized by autoradiography. Results are representative of duplicate experiments. (B) WT-, Y356D/Y358D-, Y356F/Y358F- or K227M-dDYRK2 immunocomplexes were incubated with MBP in the presence of [γ -³²P]ATP, as indicated. The protein was subjected to SDS/PAGE and was visualized by autoradiography or was quantified by phosphorimager analysis. Results are means $\pm$ S.D. (upper panel) and a representative image (lower panel) of duplicate experiments. (C) WT-dDYRK2 immunocomplexes were incubated with MBP in the presence of [γ -³²P]ATP. The protein was subjected to SDS/PAGE and was transferred on to PVDF membranes. ³²P-labelled MBP was excised from the filter, hydrolysed 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. Results are representative of three replicates.

As dDYRK2 was isolated from a *Drosophila* embryo library and the protein can be visualized by immunoblotting (Figure 3A), we hypothesized that dDYRK2 activity could be detected from *Drosophila* embryos. Embryos were collected, lysed and the α -dDYRK2 antibody was used to immunoprecipitate dDYRK2. As a control, the peptide used to raise α -dDYRK2 was included

in a separate reaction in order to neutralize immunoprecipitation of dDYRK2 activity. As illustrated in Figure 5(C), dDYRK2 isolated from *Drosophila* embryos was able to phosphorylate the Woodtide peptide. Therefore an assay used to measure DYRK1A [18] can also be used to measure dDYRK2 activity present in lysates from Sf9 cells and *Drosophila* embryos.

dDYRK2 requires tyrosine phosphorylation for activity

To test the role of Tyr³⁵⁶ and Tyr³⁵⁸ in the activation loop, we measured the activity of WT-, Y356F/Y358F-, Y356D/Y358D- and K227M-dDYRK2 using MBP (Figure 4B) and Woodtide (Figure 5A) as exogenous substrates. Each of the dDYRK2 proteins was incubated with MBP in the presence of [γ -³²P]ATP. The reactions were then subjected to SDS/PAGE, visualized by autoradiography, and ³²P-incorporation into MBP was quantified by phosphorimager analysis. As illustrated in Figure 4(B), substitution of phenylalanine (Y356F/Y358F) or aspartic acid (Y356D/Y358D) for the activation-loop tyrosine residues significantly reduced the phosphorylation of MBP by dDYRK2 with only 3–4 % of the WT activity remaining. Similar results were obtained using Woodtide as a substrate where substitution of the two tyrosine residues decreased dDYRK2 activity to 10 % of the WT protein. Although mutation of the activation-loop tyrosine residues decreased activity, both mutant proteins (Y356F/Y358F and Y356D/Y358D) appeared to retain some residual activity as substrate phosphorylation was elevated over that of the putative kinase-dead K227M-dDYRK2 protein (Figures 4B and 5A).

To assess further the role of tyrosine phosphorylation in the regulation of dDYRK2 activity, we immunoprecipitated the protein from *Drosophila* embryos and incubated the immunocomplex with λ phosphatase. As illustrated in Figure 6(A), λ -phosphatase treatment decreased tyrosine phosphorylation of the dDYRK2 by approx. 50 %. The activity of dDYRK2 was tested at the same time in duplicate samples using the Woodtide peptide *in vitro* kinase assay. Consistent with the idea that tyrosine phosphorylation is required for kinase activity, λ -phosphatase treatment decreased both baculovirus-expressed (results not shown) or *Drosophila*-immunoprecipitated dDYRK2 activity by 50 % (Figure 6B). These results demonstrate that tyrosine phosphorylation is required for full activity of dDYRK2.

dDYRK2 is expressed and is active throughout all stages of *Drosophila* development

Next, we examined various *Drosophila* life stages for the presence of dDYRK2 activity. Protein lysates were prepared from embryos, crawling third instar larvae, pupae, and adult males and females. dDYRK2 was immunoprecipitated from lysates containing equal amounts of total protein and the samples were subjected to immunoblot analysis, but detecting dDYRK2 protein by immunoblotting proved difficult. We were able to detect protein in both the embryo (Figures 3A and 6A) and pupa stages (results not shown); however, a high background obscured our analysis of the other life stages. In these experiments, dDYRK2 levels were consistently 2–4-fold higher in the pupa than in the embryo. As an alternative approach, we used the Woodtide peptide assay to measure dDYRK2 activity at the various stages of the *Drosophila* life cycle. As illustrated in Figure 7, dDYRK2 activity was detected in all stages of development, and this activity could be abolished by prior neutralization of the antibody with peptide 709–722 (results not shown). Interestingly, the highest level of

Figure 5 Development of a rapid and quantitative assay to measure dDYRK2 activity *in vitro*

(A) Woodtide was incubated with WT-, Y356D/Y358D-, Y356F/Y358F- or K227M-dDYRK2 immunocomplexes in the presence of [γ - 32 P]ATP, as indicated. The assay was performed as described in the Materials and methods section. Results are means $\pm$ S.D. ($n = 3$). (B) WT-dDYRK2 from Sf9 cell lysates (closed bars) or DYRK1A (open bars) kinase activity was measured as in (A) in the presence of 10 mM MgCl₂ (Mg), 10 mM MnCl₂ (Mn) or 5 mM MgCl₂ and 5 mM MnCl₂ (Mg + Mn), as indicated. Results are means $\pm$ S.D. ($n = 3$). (C) *Drosophila* embryo (0–22 h) lysates were incubated with the α -dDYRK2 antibody either in the presence or absence of the peptide 709–722, as indicated. dDYRK2 activity was measured as in (A). Results are means $\pm$ S.D. ($n = 3$).

Figure 6 Treatment of embryonic dDYRK2 with λ phosphatase decreases tyrosine phosphorylation and activity

(A) dDYRK2 was immunoprecipitated from 0–22 h *Drosophila* embryo lysates and were incubated either in the presence or absence of λ phosphatase, as indicated. The protein was subjected to SDS/PAGE and was immunoblotted with α -dDYRK2 (upper panel) or α -pY (anti-phosphotyrosine; lower panel) antibodies. Results are representative of three experiments. (B) dDYRK2 was prepared as in (A) and the kinase activity was determined as detailed in Figure 5. Results are means $\pm$ S.D. ($n = 3$).

dDYRK2 activity was detected in the pupa, with almost 2.5-fold more activity than that seen in the embryo. This increase in activity is similar to an increase in dDYRK2-protein level observed in the pupa. Therefore the relative specific activity of dDYRK2 appears to be similar in both life stages. Larvae contained the least kinase activity with only 10% of that seen in the embryo, whereas adults possessed 50% of the activity detected in embryos. No

Figure 7 dDYRK2 expression and activity during *Drosophila* development

Drosophila embryo, larva, pupa, and adult male and female lysates were incubated with the α -dDYRK2 antibody. Kinase activity was determined as detailed in Figure 5. Results are means $\pm$ S.D. ($n = 3$).

difference in activity was observed between adult males and females suggesting there is no sex-specific role for this kinase.

DISCUSSION

In the present paper, we describe the cloning and characterization of dDYRK2, a novel member of the DYRK family of protein kinases. The DYRKs are an emerging family of kinases that are grouped together by unique sequence characteristics in their kinase domains; however, outside of the kinase domain there is little similarity, apart from a short sequence in the N-terminus termed the DH box (see the Introduction). These differences in amino-acid sequences in the N- and C-terminal regions suggest that there are discrete roles for different members of the DYRK family. Based on intracellular localization, there are two sub-groups of DYRK kinases: nuclear and cytosolic. The founding

DYRK family member MNB, its mammalian orthologue DYRK1A (Figure 2C) and the related DYRK1B all contain a potential bi-partite nuclear localization signal within the N-terminal region that is not present in mammalian DYRK2, 3 or 4, *S. pombe* Pom1p, or dDYRK2 (Figure 2B). DYRK1A is indeed a nuclear protein [5], whereas DYRK2 [5], Pom1p [10] and dDYRK2 (P. A. Lochhead and V. Cleghon, unpublished work) localize to the cytoplasm. Therefore, the identification of dDYRK2 in *Drosophila* demonstrates that both subgroups of DYRKs are represented in *Drosophila*; furthermore, our characterization of this protein is the first to be reported for a cytosolic DYRK family member.

One of the main focuses to date in the study of all DYRK family members has been to define the role of the YXY motif in the kinase-domain-activation loop. This motif is located in the same position as the TXY motif in the activation loop of MAPKs [2]. Phosphorylation of both residues in the TXY motif is required for the activation of MAPK [28–31]. By analogy, DYRK activity has been proposed to be regulated in a similar manner. Our mutational analysis of dDYRK2 (Figures 4B and 5A), as well as similar studies performed on DYRK1A [21,22], DYRK1B/MIRK (minibrain-related kinase) [32] and Pom1p [11], have shown that the activation-loop tyrosine residues are required for full activity. Tyrosine phosphorylation is also required for full activity of dDYRK2 isolated from *Drosophila* embryos, as treatment of immunoprecipitated dDYRK2 with λ phosphatase, which decreases its tyrosine phosphorylation, also decreases its kinase activity (Figure 6). Similar observations have been made with treatment of Yak1p with the tyrosine phosphatase PTP-1B, which decreases YAK1p activity [33]. These results indicate that phosphorylation of at least one of the tyrosines in the dDYRK2 YXY motif is required for enzyme activity both *in vitro* and *in vivo*, and potentially provides a mechanism by which dDYRK2 activity could be regulated.

A more detailed study of overexpressed DYRK1A [22] and a more distantly related DYRK, Yak1p [33], indicates that only the second tyrosine in the YXY motif (Tyr³²¹ for DYRK1A and Tyr³⁵⁰ for Yak1p) is phosphorylated. This pattern of phosphorylation is more reminiscent of GSK3 β (glycogen synthase kinase 3 β), a kinase distantly related to the DYRK family. Tyr²¹⁶ in the activation loop of GSK3 β aligns with Tyr³²¹ in DYRK1A. Phosphorylation of Tyr²¹⁶ is required for GSK3 β activity [34]. However, the activation-loop sequence of GSK3 β does not contain the YXY motif found in DYRKs. Further investigation into the phosphorylation of the DYRK activation-loop tyrosines is required to determine if one or both of the tyrosines are phosphorylated in dDYRK2 and the other DYRK family members *in vivo*.

Substitution of an acid amino acid for a phosphorylated residue can mimic the effects of phosphorylation in some kinases. However, substitution of aspartic acid residues for Tyr³⁵⁶ and Tyr³⁵⁸ in dDYRK2 decreased kinase activity to levels seen with substitution of phenylalanine and thus did not mimic the effects of phosphorylation (Figures 4B and 5A). Identical results have been obtained when Tyr⁸⁵⁷ and Tyr⁸⁵⁹ in the YXY motif of Pom1p were mutated to aspartic acid [11], suggesting a common effect between DYRK family members. This mutational analysis also demonstrates a similarity with MAPK since when the TXY motif in the activation loop is mutated to acidic amino acids, there is also a reduction in kinase activity [35].

The regulation of kinase activity in DYRK family members remains largely unanswered. The phosphorylation of the DYRK YXY activation-loop motif is believed to result from constitutive autophosphorylation. Bacteria do not express tyrosine kinases [25], and bacterially expressed dDYRK2 is tyrosine-

phosphorylated (see the Results section), indicating that this is due to autophosphorylation. In addition, in Sf9 cells, WT-, but not kinase-inactive (K227M)-dDYRK2, is tyrosine-phosphorylated. These results are consistent with studies on DYRK1A where WT, but not kinase-dead DYRK1A, is phosphorylated in the activation loop [22], and bacterially expressed DYRK1A is tyrosine-phosphorylated [21,22]. However, there is also evidence that DYRK family members may be regulated by upstream activators. First, DYRK1A expressed in *E. coli* is not stoichiometrically phosphorylated at Tyr³²¹, whereas DYRK1A exogenously expressed in COS-7 cells is [22]. This implies that there is either an increase in autophosphorylation of Tyr³²¹ in DYRK1A expressed in COS-7 cells, or that COS-7 cells express a Tyr³²¹ kinase. Secondly, stimulation of hippocampal H19-H17 cells with FGF (fibroblast growth factor) increases tyrosine phosphorylation of DYRK1A [36]. This study did not determine if this increase in tyrosine phosphorylation is on Tyr³²¹ or on tyrosine residues outside the activation loop. Also, it is unclear if this increase in tyrosine phosphorylation is mediated by an increase in autophosphorylation or by an upstream kinase. Finally, DYRK1B/MIRK is phosphorylated and activated by the p38 MAPK activating enzyme MKK3 (MAPK kinase 3) *in vitro* [37], but, again, it is not known if this increase in phosphorylation occurs in the activation loop, or at another phosphorylation site within the protein.

dDYRK2 is phosphorylated on residues other than those in the activation loop. Mutation of Tyr³⁵⁶ and Tyr³⁵⁸ to phenylalanine or aspartic acid blocks tyrosine phosphorylation, but does not eliminate the band shift observed in WT-dDYRK2 (Figure 3B). λ phosphatase, which dephosphorylates tyrosine and serine/threonine residues, eliminates the band shift (Figure 3C). Taken together, these results imply that serine and threonine residues are phosphorylated in dDYRK2, but these sites remain to be identified. Other DYRK family members are phosphorylated on residues outside of the activation loop. For example, bacterially expressed DYRK1A is phosphorylated on Tyr¹¹¹ and Ser⁹⁷, residues in the N-terminus of DYRK1A [22]. No studies were carried out on the role of Ser⁹⁷, but mutation of Tyr¹¹¹ to phenylalanine does not diminish kinase activity *in vitro* [22]. It therefore remains to be seen if these phosphorylation events have a role in regulating kinase activity in cells.

Mammalian nuclear DYRKs, DYRK1A [21] and DYRK1B/MIRK [5,32] contain a PEST (Pro-Glu-Ser-Thr) sequence. These sequences are typical of proteins that are targeted for degradation. DYRK1B/MIRK is regulated by protein degradation, as stimulation of the 'classical' MAPK pathway in colon carcinoma cells decreases DYRK1B/MIRK protein levels [32]. The DYRK1B/MIRK degradation signal is thought to be a consequence of phosphorylation by MAPK at a yet unidentified site [32]. Neither dDYRK2, nor any of the other cytosolic DYRKs have a PEST domain or any regions that would suggest that they are regulated in a similar manner.

dDYRK2 is expressed and is active in the embryo, larva, pupa and adult fly (Figure 7), indicating that dDYRK2 may have a physiological role in fly development. It is interesting that there are two peaks of dDYRK2 activity (embryo and pupa). A global analysis of gene expression of the fly genome during development demonstrated that many genes that have a peak of expression in the embryo also have a peak of expression in the pupa, and genes that have a peak of expression in larva also have a peak of expression in the adult [38]. This does not rule out a role for dDYRK2 in the larva and adult, but does imply that the role in the *Drosophila* life stages may be different. The fact that dDYRK2 has peaks of activity during embryogenesis and pupation is interesting, as these are times of rapid cellular growth and differentiation, two

cellular processes which other DYRK family members have been proposed to have a role in regulating.

dDYRK2 is implicated as the product of the *smi35A* gene (R. Anholt, personal communication and [26]); however, this important connection is yet to be formally proven. *smi35A* was originally isolated in a P-element mutagenesis screen to identify genes involved in odour-guided-avoidance responses in adult flies. The fly line containing the P-element in the *smi35A* gene was referred to as k11509 [23]. Based on the location of this P-element, dDYRK2 was considered to be the protein product of *smi35A* (R. Anholt, personal communication). Our lab has been unable to reproduce a smell-impaired phenotype with the k11509 line, and recent work in the Anholt lab (R. Anholt, personal communication) indicates that this phenotype may be lost in these flies. In addition, our analysis of k11509 embryos reveals that dDYRK2 is expressed and tyrosine-phosphorylated in a similar fashion as observed in WT embryos (V. Cleghon, unpublished work). However, recent preliminary work in our lab with *dDyrk2* mutant flies is consistent with a smell impaired phenotype (V. Cleghon and P. A. Lochhead, unpublished work). While further analysis is clearly warranted to demonstrate that the *smi35A* phenotype is attributable to *dDyrk2*, it is worth noting that *mnb* (encodes a DYRK family member) mutant flies exhibit poor odour-discrimination learning [12].

Now that dDYRK2, a representative of the cytosolic subgroup of DYRKs, has been identified in *Drosophila*, it is our major goal to investigate the spatial and temporal expression pattern of this kinase and to understand what role dDYRK2 has during embryogenesis, pupation and its putative role in smell-recognition in adults. Thus, with the aid of genetic tools that *Drosophila* provide, we hope to gain an understanding of the role(s) of these kinases *in vivo*.

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