Differing substrate specificities of members of the DYRK family of arginine-directed protein kinases

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Abstract The mammalian DYRK (dual specificity tyrosine phosphorylated and regulated kinase) family of protein kinases comprises a number of related, but poorly understood enzymes. DYRK1A is nuclear while DYRKs 2 and 3 are cytoplasmic. We recently showed that DYRK2 phosphorylates the translation initiation factor eIF2B at Ser539 in its ε-subunit and thereby 'primes' its phosphorylation by glycogen synthase kinase-3. Here we have used peptides based on the sequence around Ser539 to help define the specificity of DYRK2/3 in comparison with DYRK1A. These kinases require an arginine N-terminal to the target residue for efficient substrate phosphorylation. This cannot be replaced even by lysine. A peptide with arginine at −2 is phosphorylated much less well by all three kinases than one with arginine at −3. Replacement of the +1 proline by alanine almost completely eliminates substrate phosphorylation, but valine here does allow phosphorylation especially by DYRK2. This study reveals both similarities and differences in the specificities of these arginine-dependent protein kinases.

Key words: Protein kinase; DYRK; Proline; Minbrain; Initiation factor; eIF

1. Introduction

Members of the dual specificity tyrosine phosphorylated and regulated kinase (DYRK) group of kinases [1] have recently been shown to phosphorylate translation initiation factor eIF2B and the microtubule-associated protein τ in vitro [2]. Of particular potential significance is the fact that they do so at residues whose phosphorylation is known to be important in priming the phosphorylation of adjacent serine residues (at −3) by glycogen synthase kinase-3 (GSK3) [2]. This finding thus identified members of the DYRK family as candidates for the role of 'priming kinase' for the phosphorylation of these substrates by GSK3, an enzyme known to play manifold roles in cellular physiology, including responses to hormones, such as insulin, and growth factors.

In the case of eIF2B, the sites phosphorylated by DYRKs and GSK3 lie in the catalytic domain of the largest (ε) subunit of this guanine nucleotide-exchange factor [2–4]. GSK3-mediated phosphorylation inhibits the activity of mammalian or insect eIF2B [3,5]. Since hormones such as insulin, which activate eIF2B [6–8], inhibit GSK3 [9,10], this observation provides a potential signalling mechanism for the stimulation of eIF2B by insulin, which is involved in switching on overall protein synthesis.

The DYRKs comprise a poorly understood group of protein kinases [1]. The gene for DYRK1A lies within the so-called Down syndrome critical region on chromosome 21 [11–15]. The *Drosophila* homologue Minibrain (MNB) appears to play an important role in the development of the nervous system [16], suggesting that triplication of the DYRK1A may be involved in the mental retardation associated with Down syndrome. Closely related enzymes in lower eukaryotes (Yak1 in budding yeast [17]; YakA in *Dictyostelium discoideum* [18]) play key roles in nutrient signalling. The regulation of eIF2B activity is also modulated by nutrients [19,20]. Mammalian DYRKs comprise a family of related protein kinases, encoded by at least seven different genes in mammals [21,22]. Our earlier studies showed that eIF2Bε was phosphorylated by DYRK1A and DYRK2 [2]. eIF2B is a cytoplasmic protein while DYRK1A is nuclear [21], making it unlikely that it is responsible for the phosphorylation of the priming site in eIF2B in vivo. In contrast, DYRK2 is cytoplasmic [21]. Given that, like DYRK2 but unlike DYRK1A, DYRK3 has no nuclear localisation signal [21], this isoform is also likely to be cytoplasmic. It is thus clearly possible that DYRK2 and DYRK3 also phosphorylate other cytoplasmic proteins, and that this may important in nutrient signalling, to prime GSK3-mediated phosphorylation of these proteins or for other reasons. The substrate specificities of the cytoplasmic DYRK isoforms (2 and 3) have not previously been examined. The present study therefore provides the first detailed information on the basis of their substrate specificities. It reveals important features of their sequence requirements for efficient phosphorylation of substrates, and subtle differences between their individual specificities.

2. Materials and methods

2.1. Synthetic peptides

Synthetic peptides were prepared and purified by Dr Graham Bloomberg (University of Bristol, Bristol, UK).

2.2. Protein kinases

Vectors encoding DYRK2 and DYRK3 as glutathione-*S*-transferase (GST)-fusion proteins were kindly provided by Dr Walter Becker (Aachen, Germany). Proteins were expressed in *Escherichia coli* [21] and purified over glutathione agarose. Bound proteins were eluted from the resin with 20 mM glutathione. The purified enzymes were dialysed against a buffer comprising 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 15 mM β-mercaptoethanol and 30% (v/v) glycerol to re-

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Table 1
Sequences around residues known to be phosphorylated by members of the DYRK group of protein kinases

Protein	Residue	Local sequence	DYRKs shown to act on it	References
FKHR	Ser329	ISGRL**S**PIM	DYRK1A	[31]
eIF2Bε	Ser539	DSRAG**S**PQL	DYRK1A, 2	[2]
Tau	Thr212	PGRSR**T**PLP	DYRK1A	[2]
Histone H3	Thr45	RYRPG**T**VAL	DYRK1A	[23]
(optimal peptide, DYRKtide)	–	FRPA**S**PLR	DYRK1A	[23]

Sequences around residues known to be phosphorylated by members of the DYRK family are shown. The residue phosphorylated by the DYRK enzyme is shown bold and the N-terminal arginyl residue is underlined.

move residual glutathione, and were stored at −80°C. DYRK1A, expressed as a GST-fusion protein in *E. coli*, was kindly provided by Dr Chris Armstrong (Division of Signal Transduction Therapy, University of Dundee, Dundee, UK).

2.3. Protein kinase assays

Protein kinase assays were performed in reaction mixtures (50 μl) at 30°C. Peptides were dissolved in 20 mM HEPES–KOH, pH 7.5, and stored at −20°C. Reactions contained substrate peptide at the stated concentration, [γ-^{32}P]ATP (30 cpm/nmol, final concentration 0.1 mM), $MgCl_2$ (1 mM) and DYRK2 or DYRK3, diluted in the above dialysis buffer supplemented with 0.1 mg/ml bovine serum albumin as a carrier protein. At the appropriate times, samples were removed, spotted onto 1.5×1.5 cm squares of phosphocellulose P81 peptide (Whatman) which were then washed four times with 1% (v/v) orthophosphoric acid. Radioactivity was determined by the Čerenkov method. The amount of kinase used was selected to give reaction kinetics within the linear range based on pilot experiments performed with each batch of kinase.

3. Results

To date, sites of phosphorylation have been identified for members of the DYRK group in four proteins (Table 1). In all cases, an arginyl residue is located two or three positions N-terminal to the site phosphorylated by the DYRK. Studies using a panel of peptide substrates suggested that an arginine at −3 was very important for substrate phosphorylation by DYRK1A [23], and replacement of the arginine by alanine in 'Woodtide' (a synthetic peptide based on the site in FKHR) was reported to reduce drastically phosphorylation by DYRK1A or DYRK3. However, no systematic study of the specificity requirements of DYRK2, and no information at all for DYRK3, has previously been presented.

To address this, we generated a panel of peptides based on the sequence around Ser539 in eIF2Bε which was designed to explore the role of the arginyl residue N-terminal to the target serine and the importance of the proline at +1 (Table 2). In each of these peptides, the residue corresponding to Ser535 (the site for phosphorylation by GSK3) was changed to Ala, to give peptides containing only one phosphorylatable residue, and two arginine residues were added at the extreme N-terminus to ensure the peptide would be bound to phosphocellulose paper, for ease of assay (this is a widely used feature of synthetic peptides used for protein kinase assays).

In peptide 1, the sequence around Ser539 of rat eIF2Bε is retained, apart from the modifications noted above. In peptide 2, the proline in the +1 position relative to Ser539 is altered to alanine. Peptides 3 and 4 contain arginines at the −2 and −1 positions rather than at −3 as in eIF2Bε. In peptide 5, the arginine residue at −3 is replaced by another basic residue, lysine.

DYRK2 readily phosphorylated the parent peptide, peptide 1 (Fig. 1A,C), consistent with its ability to phosphorylate the protein on whose sequence it is based, eIF2Bε [2]. Each of the above modifications to the peptide sequence drastically reduced the ability of DYRK2 to phosphorylate the peptide. The only variant that was significantly phosphorylated was that containing an arginine at the −2 position (peptide 3, Fig. 1A), but even this peptide was poorly phosphorylated relative to the parent peptide. Peptide 2 was not phosphorylated significantly by DYRK2, showing the importance of a proline at the +1 position (Fig. 1C), and consistent with the presence of such a residue in this position adjacent to the two known phosphorylation sites for DYRK2, in eIF2Bε and Tau [2] (Table 1). Shifting the arginine to position −1 (peptide 4) also abolished phosphorylation. Even replacing the arginine at −3 by a similarly charged (basic) residue (lysine, peptide 5) rendered the peptide an extremely poor substrate for DYRK2. This is rather unusual in view of the specificities of a number of other protein kinases which use basic residues as a recognition motif but can accept either lysine or arginine [24].

In the case of DYRK3, the overall pattern was similar to that observed for DYRK2 (Fig. 1B,D). DYRK3 readily phosphorylated the parent peptide (peptide 1), consistent with its ability to phosphorylate eIF2Bε (data not shown), the protein from which this peptide is 'derived'. Again, the only modification that is tolerated is 'moving' the arginine to the −2

Table 2
Peptides used in this study

Peptide number	Sequence	Comments
1	RREELDARAG**S**PQL	Corresponds to sequence around Ser539 in eIF2Bε, but Ser535 is changed to Ala
2	RREELDARAG**S**AQL	As 1; Pro at +1 changed to Ala
3	RREELDAARG**S**PQL	As 1; Arg now at −2
4	RREELDAAAR**S**PQL	As 1; Arg now at −1
5	RREELDAKAG**S**PQL	As 1; Arg at −3 changed to Lys
6	RREELDARAG**S**VQL	As 1; Pro at +1 replaced by Val
Woodtide [2]	KKISGRL**S**PIMTEQ	

Sequences are shown using the single letter code. The residue phosphorylated by DYRK family members is shown bold.

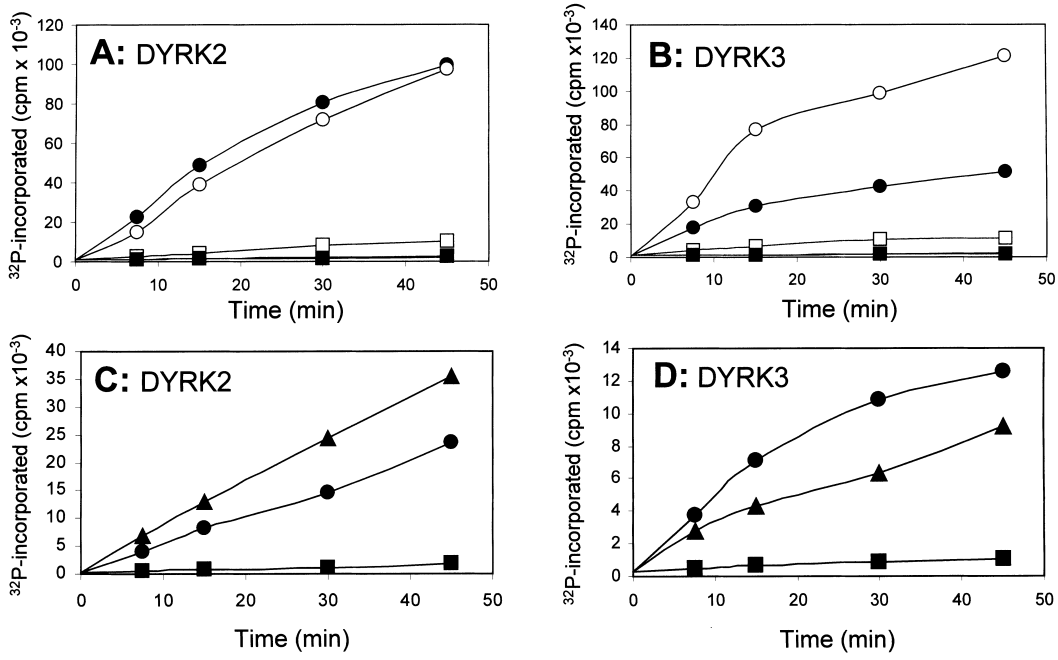

Fig. 1. Phosphorylation of peptides based on the sequence around Ser539 in eIF2Bε by DYRK2 and DYRK3. Assays were performed as described in Section 2 using DYRK2 (panels A,C) or DYRK 3 (panels B,D), samples being removed from the incubations at the times indicated. All peptides were present at 50 μM. Panel A displays results obtained using peptides in which the basic residues N-terminal to the serine were substituted or 'moved' relative to the serine: peptide 3 (□), peptide 4 (■), and peptide 5 (■) (lines for peptides 4 and 5 are coincident). Also shown is the phosphorylation of Woodtide (○). Panel B shows data for peptides in which the residue in the +1 position relative to the serine were substituted: peptide 2 (■) and peptide 6 (▲). Peptide 1 (●) served as a control for experiments in both panels. For details of peptides see Table 2. Similar findings were obtained in nine separate experiments (three for Woodtide) using three different batches of DYRK3 and two different batches of DYRK2. The activities of DYRK2 (panel C) and DYRK3 (panel D) were also measured using Woodtide as substrate.

position. The only obvious difference between the patterns of activity observed for DYRK2 and DYRK3 is that the activity of DYRK3 against peptide 3 was not as severely reduced compared to peptide 1, as is the case for DYRK2 (cf. Fig. 1A,B). Thus, when normalised for their activities against peptide 1, DYRK3 showed substantially higher activity against the peptide with arginine at −2 than DYRK2 did. This issue is discussed further below.

From these data we can conclude that a proline at the +1 position is very important for the phosphorylation of substrates by DYRK2 and that an arginine residue at the −1 position, or even conservative replacement of the arginine at −3 by lysine, almost completely abolishes phosphorylation by DYRK2 or DYRK3.

In histone H3, which is a substrate for DYRK1A [23], the +1 position is occupied by a valyl residue. We therefore synthesised an additional peptide ('6' in Table 2) containing valine at this position. When tested at 50 μM, this peptide was phosphorylated quite well by DYRK2 or DYRK3 (Fig. 1C,D).

To learn more about the effects of modifications on the activity of DYRK2 and DYRK3 against different peptides, we determined their kinetic parameters for the phosphorylation of the two best substrates, peptides 1 and 3, by performing assays at a range of peptide concentrations. Such an analysis was not possible for the other peptides (2, 4 and 5) as the activities of DYRK2 and DYRK3 against them were so low. Both kinases showed a relatively lower K_m for the peptide with arginine at −3 as compared to the corresponding peptide where the arginine is at −2. For DYRK2, this change raised the apparent K_m more than 10-fold, from less that 50 μM to more than 0.5 mM. For DYRK3, the effect on the K_m was about four-fold, the K_m with peptide 1 being about 0.1 mM while for peptide 3 the value was 0.4 mM (Table 3). Each kinase also displayed a decreased V_m with peptide 3 as compared to peptide 1. Thus, for this series of peptides, an argi-

Table 3
Kinetic parameters for phosphorylation of peptides with arginine at −2 or −3 by DYRK2 and DYRK3

Substrate	Kinase					
	DYRK1A		DYRK2		DYRK3	
	K_m (μM)	Relative V_{max}	K_m (μM)	Relative V_{max}	K_m (μM)	Relative V_{max}
Peptide 1 (Arg at −3)	>1000	n.d.	45 ± 2.3	100	97 ± 3.6	100
Peptide 3 (Arg at −2)	>1000	n.d.	526 ± 15	51 ± 2.7	402 ± 18	66 ± 3.0
Peptide 6 (Val at +1)	n.d.	n.d.	>500	n.d.	>1000	n.d.

Kinetic parameters were determined by assaying the activity of DYRK2 and DYRK3 against the indicated peptide substrate across a range of substrate concentrations. Data are based on at least three parallel determinations. Relative V_{max} values are normalised to the activity observed against peptide 1, arbitrarily set at 100. N.d. = not determined, due to low activity of the corresponding kinase for the peptide or problems with the solubility of the peptide at the high concentrations required for the experiment.

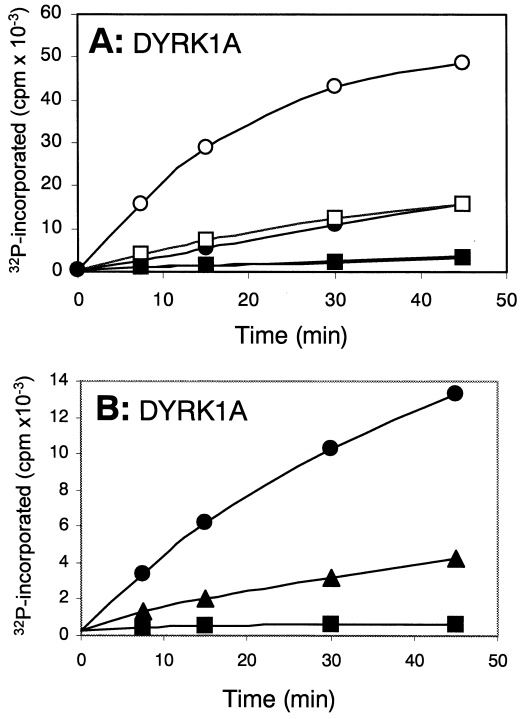

Fig. 2. Phosphorylation of peptides based on the sequence around Ser539 in eIF2Bε by DYRK1A. Assays were performed as described in Section 2 using DYRK1A, samples being removed from the incubations at the times indicated. All peptides were present at 50 μM. Panel A displays results obtained using peptides in which the basic residues N-terminal to the serine were substituted or 'moved' relative to the serine; symbols used are as for Fig. 1A,B. Panel B shows data for peptides in which the residue at the +1 position relative to the serine was substituted; symbols as Fig. 1C,D. Peptide 1 served as a control for experiments in both panels. For details of peptides see Table 2. The activities of DYRK1A was also measured using Woodtide as substrate (○), but here DYRK1A was used at one twentieth the amount used in the other reactions.

nine in position −3 gives a substrate for which DYRK2 and DYRK3 display a lower K_m and higher V_{max} than when this residue is located in position −2, thus explaining the differences in rates of phosphorylation seen in Fig. 1. Similar studies using peptide 6, with valine at +1, yielded non-linear data, perhaps due to problems with the solubility of this more hydrophobic peptide at the relatively high concentrations required for attempts to study the K_ms of DYRK2 and DYRK3 for this peptide. We estimate that the K_m values of DYRK2 and DYRK3 for this peptide are in excess of 500 and 1000 μM, respectively, although we were unable to determine these values with accuracy. Given these high K_m values, the fact that DYRK2 and DYRK3 phosphorylated these peptides rapidly when tested at 50 μM suggests that the V_{max} for DYRK2 and DYRK3 against these peptides is also high.

The activity of DYRK3 seemed less sensitive to the position of the arginine residue, especially when one considers the K_m, at least using peptides based on the sequence of eIF2Bε. To address this a little further, we assayed the relative activities of DYRK2 and DYRK3 against another peptide in which the arginine is at the −2 position, i.e. Woodtide, a peptide whose sequence is based on that around the DYRK phosphorylation site in FKHR (Tables 1 and 2). At the fixed concentration chosen (50 μM), DYRK2 phosphorylated peptide 1 and Woodtide at similar rates, while DYRK3 displayed substantially higher activity against Woodtide (Fig. 1). Since the sequence of this peptide differs in many respects from those of the eIF2Bε-based peptides used in this study, one cannot draw any conclusions about specificity from this observation, other than the point that again DYRK3 shows relatively higher activity than DYRK2 against a peptide with the arginine in the −2 rather than in the −3 position. To assess whether phosphorylation by DYRK2 and DYRK3 occurred only at the expected serine, the more C-terminal of the two in this peptide, Woodtide, was labelled by either kinase, freed of ATP and then subjected to mass spectrometric (M/S) analysis. In each case, the singly phosphorylated derivative (m/z = 1667.9) but not the doubly phosphorylated species (m/z = 1747.8) was observed, showing that the peptide was only phosphorylated at one site by DYRK2 or DYRK3. M/S M/S analysis yielded a series of peptides in which C-terminal residues had been removed. The series clearly demonstrated that peptides from which the dehydroalanine generated by β-elimination of phosphoserine had been lost all still contained serine, indicating that only the more C-terminal serine was actually phosphorylated by DYRK2 or DYRK3 (data not shown).

Previous studies on the specificity of DYRK family kinases have focused only on DYRK1A [23]. We therefore compared the ability of DYRK2 and DYRK3 to phosphorylate the peptides under study here with the activity of DYRK1A against the same peptides. DYRK1A phosphorylated the eIF2Bε-based peptide (peptide 1) and peptide 3 (with Arg at −2) with similar efficiencies (Fig. 2A), but showed very little activity against peptides 4 and 5. Thus, an arginine at −2 or −3 appears to be crucial for efficient phosphorylation by DYRK1A. However, the activity against even peptides 1 and 3 was very much (about 100-fold) less than that against Woodtide, used as a control, when initial rates were compared. Other features of Woodtide, which differ in several respects from the eIF2Bε-based peptides, render it a much better substrate for DYRK1A: such a great difference in activity was not observed for DYRK2 and DYRK3 (Fig. 1). Consistent with this, the K_m values of DYRK1A for peptides 1 and 3 were too high (>1 mM) to determine with accuracy.

The activity of DYRK1A against peptide 2 (with Ala rather than Pro at +1) was even lower than that seen against peptides 4 and 5 (Fig. 2B). DYRK1A showed some activity against peptide 6 (Val at +1), in contrast to DYRK2 and DYRK3 which showed relatively better activity against this peptide when compared to their activities against peptide 1 (Fig. 1C,D).

4. Discussion

The data presented here are the first characterisation of the substrate specificities of DYRK2 and DYRK3. Our findings clearly show that these poorly understood enzymes show similar substrate specificities. This study reveals that, for efficient phosphorylation of the peptide substrates tested here, both enzymes require an arginine at −2 or −3, the latter position being preferred. The arginine cannot, perhaps surprisingly, be replaced by lysine. A comprehensive study of the substrate requirements of DYRK1A [23] revealed that this enzyme also shows a very strong requirement for an N-terminal ar-

ginyl residue, although in this case it appeared to be essential that this residue lay in the −3, rather than the −2, position. Using the present set of peptides, arginyl residues at −2 or −3 seemed to be roughly equally acceptable (Fig. 2A). For all three kinases, a basic residue at −1 would not allow phosphorylation. For DYRK1A, the earlier study showed that a proline at −2 was an important requirement, whereas this does not seem to be the case for DYRK2 or DYRK3, since neither the protein substrates so far identified for this enzyme nor the peptide substrates used here have a proline in this position. DYRK1A did phosphorylate Woodtide about two orders of magnitude more rapidly than the eIF2Bε-based peptides, although the many differences in sequence between these substrates make it hard to discern the reason(s) for this striking difference. In common with DYRK1A, this study shows that replacement of the proline at +1 by alanine almost completely eliminates phosphorylation by DYRK2 and DYRK3 (and also, in keeping with the earlier report, DYRK1A). Interestingly, replacement of the +1 proline by a hydrophobic residue, valine, yielded a peptide that was a good substrate for DYRK2, and (poorer) substrate for DYRK3 or DYRK1A. This suggests that DYRK2 in particular may be able to phosphorylate Ser–Val (or perhaps, although this was not tested here, Ser–Leu/Ile) sites in proteins; however, the K_m of DYRK2 for the valine-containing substrate was very high (mM), so these such sites may not be physiological substrates.

The work of Himpel et al. [23] previously showed that the requirement for an N-terminal arginine residue distinguished DYRK1A from ERK2, a member of another major group of protein-directed kinases. These authors suggested that, based on the three-dimensional structures of other kinases that phosphorylate target sites with adjacent N-terminal basic residues, such as cAMP-dependent protein kinase, Asp-247 might interact with the arginine at −3 in the substrate [23]. In view of our finding that DYRK2 and DYRK3 also show a requirement for an arginine in this position, it is therefore interesting to note that the position corresponding to Asp247 is also occupied by an acidic residue in DYRK2 (Glu229) and DYRK3 (Glu254). Other DYRK-related kinases such as DYRK1A [22] and DYRK4 [21], the *Dictyostelium* homologue YakA [18] and the *Drosophila* MNB protein [16] also possess a Glu at this position, raising the possibility that the requirement for an arginine N-terminal to the target residue in their substrates is a common feature of the specificity of members of the DYRK family of protein kinases. However, their substrate specificities are clearly not identical as exemplified by the observation [21] that DYRK2 and DYRK3 can phosphorylate histone H2B while DYRK1A cannot.

The peptides used here form the basis of simple assays for the activities of DYRK2 and DYRK3. The sequences of these peptides are based on the sequence around the GSK3 site in eIF2Bε. Sequences are now known for this protein from a number of species. The eIF2Bε proteins from mammals and from the fruitfly *Drosophila melanogaster* are phosphorylated by GSK3 [3,5,25] and contain a serine (or threonine, *Drosophila*) at the 'priming position' (+4 relative to the GSK3 site). In every case, this is followed by a proline and preceded at −3 by an arginine [3,5] (porcine sequence SRTGSP, accession number AW416134.1). This is consistent with the idea that members of the DYRK family such as the cytoplasmic forms DYRK2 and DYRK3 are responsible for phosphorylating the priming site in mammals, although further work will be required to prove this. On the other hand, the sequence adjacent to the GSK3 sites in glycogen synthase, another cytoplasmic GSK3 substrate that requires priming, does not contain a DYRK consensus sequence, suggesting that DYRKs may not be the priming kinase in this case. Skurat and Roach [26,27] have provided data that sites 3a/b in glycogen synthase may be phosphorylated independently of one another, while phosphorylation of the other two GSK3 sites in glycogen synthase, 3c/d, is thought to be primed by phosphorylation of site 5 by casein kinase 2 [28,29]. Similarly, the sequence around the probable priming phosphorylation site in ATP-citrate lyase [30] fails to conform to the DYRK2/3 consensus. It is therefore possible that other protein kinases, perhaps so far uncharacterised members of the DYRK family with different substrate specificities, phosphorylate the priming sites in these GSK3 substrates.

This characterisation of the substrate specificities of DYRK family kinases may assist the identification of further potential substrates for this little-studied group of enzymes.

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