Identification of the autophosphorylation sites and characterization of their effects in the protein kinase DYRK1A

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Protein kinases of the DYRK ('dual-specificity tyrosine-regulated kinase') family are characterized by a conserved Tyr-Xaa-Tyr motif (Tyr-319–Tyr-321) in a position exactly corresponding to the activation motif of the mitogen-activated protein kinase (MAP kinase) family (Thr-Xaa-Tyr). In a molecular model of the catalytic domain of DYRK1A, the orientation of phosphorylated Tyr-321 is strikingly similar to that of Tyr-185 in the known structure of the activated MAP kinase, extracellular-signal-regulated kinase 2. Consistent with our model, substitution of Tyr-321 but not of Tyr-319 by phenylalanine markedly reduced the enzymic activity of recombinant DYRK1A expressed in either *Escherichia coli* or mammalian cells. Direct identification of phosphorylated residues by tandem MS confirmed that Tyr-321, but not Tyr-319, was phosphorylated. When expressed in

COS-7 cells, DYRK1A was found to be fully phosphorylated on Tyr-321. A catalytically inactive mutant of DYRK1A contained no detectable phosphotyrosine, indicating that Tyr-321 is autophosphorylated by DYRK1A. MS identified Tyr-111 and Ser-97 as additional autophosphorylation sites in the non-catalytic N-terminal domain of bacterially expressed DYRK1A. Enzymic activity was not affected in the DYRK1A-Y111F mutant. The present experimental data and the molecular model indicate that the activity of DYRK1A is dependent on the autophosphorylation of a conserved tyrosine residue in the activation loop.

Key words: activation loop, molecular model, signal transduction, tyrosine phosphorylation.

INTRODUCTION

DYRK1A is a nuclear protein kinase distantly related to the mitogen-activated protein kinase (MAP kinase) family [1]. Human DYRK1A has been proposed as a candidate gene for mental retardation in Down syndrome because of its localization on chromosome 21 in the Down syndrome 'critical region', and because of its homology with the *minibrain* gene from *Drosophila* [2–4]. Recently, the transcription factors STAT3 (signal transduction and activators of transcription 3) and FKHR (forkhead in rhabdomyosarcoma) were identified as substrates of DYRK1A [5,6]. In lower eukaryotes, DYRK-related kinases were found to play essential roles in the control of growth and development [7–9].

DYRK1A is the prototype of a subfamily of protein kinases that are characterized by conserved sequence motifs in the catalytic domain [10–12]. These include a Tyr-Xaa-Tyr motif in a surface loop, referred to as an 'activation loop' or 'phosphorylation lip', that is located between the conserved subdomains VII and VIII of the catalytic domain. The position of the Tyr-Xaa-Tyr motif corresponds exactly to the Thr-Xaa-Tyr activation motif of the MAP kinases, suggesting that both families of kinases may be regulated by a similar mechanism. In support of this hypothesis, we have shown that the presence of these tyrosines is essential for full enzymic activity of DYRK1A [1]. More recently, the role of the Tyr-Xaa-Tyr motif has been demonstrated in functional assays of two different kinases of the DYRK ('dual-specificity tyrosine-regulated kinase') family: mutation of both tyrosines abolished the ability of DYRK1B to mediate the survival of colon carcinoma cell lines under serum-

free conditions [13], and the respective mutant of Pom1p was incapable of complementing the *pom1* mutation of *Schizosaccharomyces pombe*, which results in asymmetry during cell growth and division [14].

In contrast with members of the MAP kinase family, kinases of the DYRK family contain non-catalytic N-terminal and C-terminal segments of variable lengths that may be involved in the regulation of their activity [10,11]. These regions show no detectable sequence similarity to other known proteins. So far, no function has been attributed to these non-catalytic regions, except for the identification of a conserved nuclear localization signal in the N-terminal domains of DYRK1A, DYRK1B and the minibrain kinase from *Drosophila* [4,10,12,15].

To elucidate further the activation mechanism of DYRK1A, we have focused on the role of tyrosine phosphorylation. The autophosphorylation sites were identified by MS, and point mutants were employed to study their effects on the kinase activity of DYRK1A. A structural model of the kinase domain of DYRK1A was generated to support our hypothesis that DYRK1A is activated by a similar molecular mechanism to extracellular-signal-regulated kinase (ERK) 2.

MATERIALS AND METHODS

Expression of DYRK1A fusion proteins

The expression plasmids for GST-DYRK1A, GST-DYRK1A-ΔC, HA-DYRK1A and GFP-DYRK1A have been described earlier [1,10,16]. GST-DYRK1A-ΔN was constructed from an N-terminally truncated cDNA clone which had been isolated

Abbreviations used: ERK, extracellular-signal-regulated kinase; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, haemagglutinin; MBP, myelin basic protein; MAP kinase, mitogen-activated protein kinase; MS², collision-induced fragmentation; GSK, glycogen synthase kinase.

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from a rat brain library [1]. Glutathione S-transferase (GST) fusion proteins were expressed in *Escherichia coli* at 37 °C and were partially purified by affinity adsorption to glutathione-Sepharose (Amersham Pharmacia Biotech) in the presence of PMSF as described before [1]. Following SDS/PAGE and transfer to PVDF membranes, bands with kinase activity were identified by *in situ* autophosphorylation as described previously [1]. The concentration of active kinase in these bands was then estimated by comparison with known amounts of BSA present in adjacent lanes of Coomassie Brilliant Blue-stained SDS/PAGE gels.

To isolate haemagglutinin (HA) epitope-tagged and green fluorescent protein (GFP) fusion proteins from transiently transfected COS-7 cells, cells were lysed in buffer containing 50 mM Hepes, 150 mM NaCl, 2 mM EDTA, 25 mM NaF, 2 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 1 µg/ml pepstatin and 1 % Nonidet P-40. Fusion proteins were immunoprecipitated with either an HA-specific antibody (12CA5; Roche Diagnostics) or GFP-specific antiserum (Molecular Probes).

Kinase assays

Kinase activities of GST-DYRK1A constructs were determined with myelin basic protein (MBP, final concentration, 100 µg/ml; Sigma), histone H3 (10 µg/ml; Roche Diagnostics) or the peptide substrate DYRKtide (200 µM), as described in [16]. MBP and DYRKtide were incubated with the kinase for 4 min in the presence of 200 µM ATP; histone H3 was incubated with 100 µM ATP for 9 min. Phosphorylation of DYRKtide was assayed with the phosphocellulose method as described in [16]. Protein substrates (MBP, histone H3) were subjected to SDS/PAGE, and incorporation of ³²P was determined by liquid-scintillation counting of cut bands. Each data point was determined in triplicate, and the reaction was considered linear when incorporation of ³²P after 8 min (18 min for histone assays) was > 150 % of that determined at 4 min (9 min). For assays of HA-DYRK1A constructs, immunocomplexes were incubated for 20 min in phosphorylation buffer containing 200 µM DYRKtide in the presence of 10 µM [γ -³²P]ATP. The data were evaluated for statistical significance with the two-tailed Student's *t* test for independent samples, and differences were considered significant at *P* < 0.05.

Purification of DYRK1A from rat brain and GT1-7 cells

Five flasks (80 cm²) of confluent growing neuronal GT1-7 cells, a neuronal cell line derived from mouse hypothalamus [17,18], or 0.5 g of rat brain were lysed in 10 ml of guanidinium chloride/20 mM imidazole, pH 8.0, by sonication or with a Polytron homogenizer, respectively. DYRK1A was partially purified from the cleared lysates by immobilized metal affinity chromatography on Ni-charged nitrilotriacetic acid-agarose (Qiagen) according to the manufacturer's instructions. After a final wash with 0.1 M sodium phosphate/8 M urea, pH 5.9, bound proteins were eluted by boiling in Laemmli's sample buffer supplemented with 50 mM EDTA.

Assay of tyrosine phosphorylation

Tyrosine phosphorylation of GST-DYRK1A constructs was assayed by Western-blot analysis with anti-phosphotyrosine antibodies (PY20 from Transduction Laboratories; 4G10 from Upstate Biotechnology; PY99 from Santa Cruz Biotechnology). For quantitative evaluation, immunoreactive bands were detected with ¹²⁵I-labelled Protein A, and the bound radioactivity

was quantified with the help of phosphor storage screens (Cyclone, Packard). The data were normalized for GST immunoreactivity as determined from aliquots of the protein samples.

MS

Coomassie Brilliant Blue-stained gel bands containing GFP-DYRK1A, GST-DYRK1A-ΔC and GST-DYRK1A-ΔC-K188R (unphosphorylated control) were excised, cut into smaller pieces and washed for 15 min in water on a vortex-mixer. The gel pieces were then subjected to the following steps, each for 30 min in 250 µl of 200 mM ammonium bicarbonate/50 % MeCN (per 1 cm × 2 mm gel band) at room temperature, on a tube rotator. Solutions were discarded after steps 1, 3 and 4: step 1, wash; step 2, reduce with 5 mM tris(2-carboxyethyl)phosphine; step 3, then add 20 µl of 0.25 M iodoacetamide; step 4, wash; step 5, dry under vacuum. Gel pieces were re-swollen at 0 °C in 50 µl of 100 mM ammonium bicarbonate containing 10 µg/ml modified trypsin (Promega). After 45 min, excess solution above the gel was removed; digestion was at 37 °C for 15 h. Peptides in the supernatant were bound to a Millipore C₁₈ ZipTip according to the manufacturer's instructions and washed with 1 % formic acid. Peptides were eluted to a Protana nanospray needle using 2–3 µl of 0.2 % formic acid/70 % methanol and examined on a Finnigan LCQ ion-trap mass spectrometer fitted with a Protana nanospray interface. In addition to analysing all significant peaks present, searches were made in zoomscan for signals which may have arisen from any peptide containing a potential tyrosine-phosphorylation site. For each signal corresponding to a phosphorylated mass, the unphosphorylated sample was examined to ensure absence of the signal. Each signal was subjected to collision-induced fragmentation (MS²) to confirm its identity and establish the site of phosphorylation. Database searches were done over the worldwide web using Mascot (<http://www.matrixscience.com>).

Molecular modelling

A three-dimensional model of the kinase domain of DYRK1A was built by using the X-ray structure of the phosphorylated MAP kinase ERK2 (PDB accession code 2erk) as a template. A sequence alignment of DYRK1A and ERK2 was generated and optimized by taking into account the solvent accessibilities for all amino acids of ERK2 and corresponding amino acids of DYRK1A. A secondary structure prediction for DYRK1A confirmed that the predicted structural elements were correctly aligned with the corresponding elements of the ERK2 structure. According to this alignment, amino acid residues were exchanged in the template. Insertions and deletions in DYRK1A were modelled using a database-search approach included in the software package WHATIF XX (<http://www.cmbi.kun.nl/whatif>). Finally, these model structures were energy-minimized using the steepest descent. The structural representations were generated with the RIBBON program XX (<http://www.cmbi.kun.nl/whatif>). All programs were run on a Silicon Graphics Indigo² work station.

RESULTS

Tyr-321 in the activation loop is required for full activity of DYRK1A

In order to assess the role of the tyrosines in the activation loop for enzymic activity of DYRK1A, point mutants of DYRK1A (Y319F, Y321F and Y319F/Y321F) were expressed in *E. coli* as recombinant fusion proteins with GST. For comparison, GST-

Figure 1 Kinase activity of GST-DYRK1A mutants expressed in *E. coli*

The specific kinase activities of GST-DYRK1A (WT) and the indicated mutants were calculated from incorporation of ^{32}P into DYRKtide or MBP, as described in the Materials and methods section. The data represent means $\pm$ S.D. from three experiments with two different preparations of GST-DYRK1A fusion proteins. * $P < 0.05$, mutants versus wild-type DYRK1A.

DYRK1A-K188R was included as a mutant with markedly reduced kinase activity [1]. As described previously [1], the GST-DYRK1A fusion proteins were purified from *E. coli* as C-terminally truncated proteins with an apparent molecular mass of 90 kDa (see below and Figure 4B); the calculated mass of full-length GST-DYRK1A was 114 kDa. The specific activity of the constructs was determined in kinase assays with MBP and the peptide substrate, DYRKtide. As we have previously shown for the phosphorylation of histone [1], substitution of both tyrosines by phenylalanine resulted in a reduction of kinase activity by 60–85% (Figure 1). A similar decrease was caused by the mutation of Tyr-321 alone, whereas exchange of Tyr-319 failed to reduce the kinase activity of DYRK1A. Indeed, surprisingly, DYRK1A-Y319F exhibited enhanced kinase activity towards the peptide substrate, but not in MBP assays. Except for this result, qualitatively similar results were obtained with both substrates (Figure 1), although the kinase activity of DYRK1A-Y321F and DYRK1A-Y319F/Y321F was more strongly reduced in the MBP assay (15% of wild-type activity) than with DYRKtide (39%). This difference may indicate that the replacement of Tyr-321 by phenylalanine affects the access of the substrate protein to the active site rather than the catalytic reaction itself.

To determine the degree of autophosphorylation during bacterial expression, phosphotyrosine was detected immunochemically with a specific antibody (Figure 2). The lack of immunoreactivity of the kinase-negative construct, DYRK1A-K188R, provides evidence for the specificity of the antibody. Notably, significant amounts of phosphotyrosine were detected in DYRK1A-Y319F/Y321F. This result indicates that DYRK1A autophosphorylates not only on the tyrosines in the activation loop. Indeed, results shown below provide evidence that the monoclonal antibody used here (PY20) predominantly recognized a phosphorylated tyrosine residue in the N-terminal domain of DYRK1A. Accordingly, the reduced amount of immunologically detectable phosphotyrosine in DYRK1A-Y321F and DYRK1A-Y319/321F correlated well with the reduced kinase activity of these mutants (Figure 1).

The tyrosine mutants of DYRK1A were also expressed in COS-7 cells as fusion proteins with the HA epitope tag at the N-

Figure 2 Tyrosine autophosphorylation of GST-DYRK1A mutants

Bacterial fusion proteins of wild-type DYRK1A (WT) and the indicated mutants were subjected to Western-blot analysis with phosphotyrosine-specific (PY20) or GST-specific antibodies. The primary antibody was detected with ^{125}I -labelled Protein A. The left-hand panels show autoradiographs of representative Western blots. For quantitative comparison of the different constructs, band intensities were determined with the help of a PhosphorImager, and data were normalized for GST immunoreactivity (right-hand panel). The data represent means $\pm$ S.D. from three different preparations of GST-DYRK1A fusion proteins. * $P < 0.05$, mutants versus wild-type DYRK1A.

Figure 3 Kinase activity of DYRK1A point mutants expressed in COS-7 cells

COS-7 cells were transiently transfected with wild-type HA-DYRK1A, the indicated mutants or blank vector (Control). Fusion proteins were isolated by immunoprecipitation, and immunocomplexes were subjected to a kinase assay with DYRKtide as substrate. Incorporation of ^{32}P into DYRKtide was determined in triplicate by dotting aliquots of the reaction on to phosphocellulose paper. To control for equal expression of the fusion proteins, a Western blot of total cellular lysates was developed with an HA-specific antibody (lower panel).

terminus. DYRKtide kinase activity of the tagged proteins was determined by immunocomplex kinase assay (Figure 3). The effects of the individual mutations on kinase activity qualitatively paralleled those found in the bacterial fusion proteins. Quantitatively, mutants of Tyr-321 exhibited a stronger reduction of

Figure 4 Effects of the deletion of the N- and C-terminal domains on the kinase activity of DYRK1A

(A) Schematic illustration of the deletion constructs of GST-DYRK1A. The C-terminal truncation of the bacterial fusion proteins is indicated by broken lines. (B) Detection of phosphotyrosine. Western blots containing aliquots of the deletion constructs as purified from recombinant *E. coli* were probed with antibodies against phosphotyrosine (α PY, PY20) or GST (α GST). The arrowhead points to a truncated fusion protein comprising GST plus the N-terminal domain of DYRK1A. Molecular-mass markers (in kDa) are indicated on the right. (C) Kinase activity towards DYRKtide. The data represent means $\pm$ S.D. from three experiments with different preparations of the constructs. * $P < 0.05$, mutants versus wild-type DYRK1A.

activity after mammalian expression than as bacterial fusion proteins.

Tyrosine phosphorylation and activity of C- and N-terminal DYRK1A deletion constructs

Deletion mutants of GST-DYRK1A were constructed to study the role of regions outside the catalytic domain (Figure 4A). As

Figure 5 Tyrosine autophosphorylation of DYRK1A point mutants

(A) Anti-phosphotyrosine Western blot. GST-DYRK1A-ΔNΔC, GST-DYRK1A-ΔC (WT) and the indicated mutants of GST-DYRK1A-ΔC were immunoblotted with two different phosphotyrosine-specific antibodies (PY20, 4G10). Aliquots of the protein samples were stained with Coomassie Brilliant Blue (CB) after SDS/PAGE. Essentially identical results were obtained with three different preparations of the proteins. Molecular-mass markers (in kDa) are indicated on the right. (B) Kinase assay. Kinase activity was assayed with histone H3 and is given as means $\pm$ S.D. from three experiments with different preparations of the kinases. * $P < 0.05$, GST-DYRK1A-ΔNΔC versus GST-DYRK1A-ΔC.

noted above, bacterially expressed GST-DYRK1A was purified as a C-terminally truncated protein of about 90 kDa (Figure 4B). Similarly, GST-DYRK1A-ΔN was obtained as a truncated

Figure 6 Identification of DYRK1A autophosphorylation sites by MS

Phosphorylated peptides were identified in tryptic digests of DYRK1A-ΔC by ion-trap MS. Phosphorylation sites in these peptides were determined by MS². (A) MS² analysis of the [M+2H]²⁺ ion of phosphorylated IQYIQSR. Peptide monoisotopic masses were 1149.5 Da (calculated) and 1149.4 Da (observed). The fragmentation data clearly identify Tyr-4 as the site of phosphorylation. (B) MS² analysis of phosphorylated HINEVYYAK. Peptide monoisotopic masses were 1215.5 Da (calculated) and 1215.4 Da (observed). The b₆/y₃ ions are diagnostic of Tyr-6 being phosphorylated. (C) Collision-induced fragmentation of y₅ ion (MS³ analysis). [M+H]⁺ monoisotopic masses were 723.3 Da (calculated) and 723.2 Da (observed). The fragmentation of the y₅ ion from the sample shown in (B) gives ions a₂, b₂ and b₃, clearly indicating that Tyr-6 is phosphorylated in HINEVYYAK. (D) MS² analysis of phosphorylated KLSVDLIK. Peptide monoisotopic masses were 994.5 Da (calculated) and 994.4 Da (observed).

protein of 66 kDa (calculated mass 97 kDa). The C-terminally deleted fusion proteins migrated close to their expected molecular masses (86 and 69 kDa). In Western blots, very little phosphotyrosine was detected in the N-terminally deleted constructs (GST-DYRK1A-ΔN and GST-DYRK1A-ΔNΔC; Figure 4B, left-hand panel), suggesting that the majority of the immunoreactive phosphotyrosine was located in the N-terminus of DYRK1A.

Kinase assays with DYRKtide clearly showed that the catalytic domain, as represented in DYRK1A-ΔNΔC (amino acids 150–499), was sufficient for enzymic activity (Figure 4C). However, deletion of the N-terminal region (amino acids 1–149) suppressed the kinase activity (Figure 4C, compare DYRK1A-ΔN with wild-type, and DYRK1A-ΔNΔC with DYRK1A-ΔC). The C-terminally deleted constructs showed an increase of activity; however, this difference was not statistically significant (DYRK1A-ΔC versus wild-type, $P = 0.12$; DYRK1A-ΔNΔC versus DYRK1A-ΔN, $P = 0.07$).

Autophosphorylation of Tyr-111 accounts for immunoreactivity with phosphotyrosine-specific antibodies

In order to identify the presumed autophosphorylation site in the N-terminal region of DYRK1A, point mutants were generated for each of the seven tyrosines in the N-terminal domain. GST-DYRK1A-ΔC fusion proteins were partially purified from recombinant *E. coli* and subjected to Western-blot analysis with two different phosphotyrosine-specific antibodies (Figure 5A). GST-DYRK1A-ΔNΔC was included as a control that lacks all N-terminal tyrosines. Histone kinase assays showed that none of the point mutations affected the enzymic activity of DYRK1A (Figure 5B). Two constructs had a strongly reduced content of immunoreactive phosphotyrosine: no phosphotyrosine could be detected in GST-DYRK1A-ΔC-Y111F, and immunoreactivity was reduced by $64.3 \pm 9.6\%$ in GST-DYRK1A-ΔC-Y104F (mean $\pm$ S.D. from three experiments with PY20). These results strongly suggest that phosphorylated Tyr-111 represents the epitope detected by both antibodies. The reduced immunoreactivity of GST-DYRK1A-ΔC-Y104F suggests that the presence of Tyr-104 is necessary for efficient phosphorylation of Tyr-111. Increased immunoreactivity with one of the antibodies (PY20) was observed in GST-DYRK1A-ΔC-Y112F, probably due to improved binding of this specific monoclonal antibody to the neighbouring phospho-Tyr-111. Both antibodies detected a truncated fragment of about 40–45 kDa, corresponding to protein containing GST and the N-terminal domain of DYRK1A, which was labelled much more strongly in DYRK1A-ΔC-Y112F than in the other constructs.

Identification of autophosphorylation sites by MS

To identify directly the autophosphorylation sites in DYRK1A, tryptic digests of bacterially expressed DYRK1A-ΔC were analysed by MS, as described in the Materials and methods section. Peptides and sites of phosphorylation were identified from the peptide mass and the fragmentation pattern following collision-induced dissociation. Of all peaks examined, three peptides were found to be phosphorylated; HINEVYYAK (corresponding to residues 106–114 of DYRK1A), IQYIQSR

The major fragmentation product represents loss of H_2PO_4 from the doubly charged peptide. Fragmentation peaks from β -eliminated peptide are shown as a 'y' series. The expected fragment ions (a, b, y and y⁺⁺ series according to the Biemann nomenclature [38]) from the given sequences are given. The fragments observed experimentally in the mass spectra are underlined; ● denotes water loss; *, ammonia loss; ++, doubly charged ion.

Figure 7 Tyrosine phosphorylation of DYRK1A *in vivo*

(A) Endogenous DYRK1A. DYRK1A was partially purified from GT1-7 cells or rat brain by adsorption to Ni-chelating agarose. COS-7 cells transfected with the blank vector (pSVL) or pSVL-HA-DYRK1A were used as controls. Phosphotyrosine was detected by Western-blot analysis with the help of a specific antibody (PY99) and enhanced chemiluminescence (α PY). Thereafter, the blot was stripped and incubated with a DYRK1A-specific antibody (α DYRK1A). Arrows point to DYRK1A bands. (B) Point mutants of GFP-DYRK1A. Wild-type GFP-DYRK1A and the indicated point mutants were immunoprecipitated from transiently transfected COS-7 cells with the help of a GFP-specific antiserum. Western blots were developed with the indicated monoclonal antibodies (α PY, PY99, α GFP).

(318–325) and KLSVDLIK (95–102). The peptide containing Tyr-104 (TYK) was not seen in labelled or unlabelled form and may have been too small to be retained by the reverse-phase micro-column during the clean-up procedure. For phosphorylated HINEVYYAK and IYQYIQR, signals of similar intensity corresponding to the unmodified form were also present, suggesting that the phosphorylation was not complete. As the phosphorylated and unphosphorylated forms will not have had the same ionization characteristics, their relative levels could not accurately be determined from this experiment. These observations were confirmed by matrix-assisted laser-desorption ionization MS analysis of the digest (results not shown).

With each phosphorylated peptide, the site of modification was determined by MS² analysis. For peptide IYQYIQR, the MS² analysis was conclusive (Figure 6A), identifying the structure as IYQpYIQR. For peptide HINEVYYAK the phosphorylation site was unclear from the MS² analysis, as the two tyrosine residues are adjacent, which means that only the b6 and y3 ions

are indicative of the phosphorylation site; these were in poor yield although the data favoured the first tyrosine (Figure 6B). However, further investigation using collision-induced fragmentation of selected ions from the MS² stage of analysis (particularly y5) gave conclusive evidence that the site of label was HINEVpYYAK (Figure 6C). Thus, the MS analysis provided conclusive evidence that Tyr-321, but not Tyr-319, and Tyr-111, but not Tyr-112, were phosphorylated in bacterially expressed DYRK1A. A third site, Ser-97, was found to be phosphorylated, giving rise to the peptide KLpSVDLIK (Figure 6D), the result of an incomplete tryptic cleavage. No fully cleaved or unphosphorylated version of this peptide was found, suggesting that the modification was complete.

For each phosphorylation site discovered above, only the unphosphorylated peptides were identified in the kinase-negative mutant DYRK1A- Δ C-K188R. Peptide LSVDLIK lacked the N-terminal Lys in this digest, suggesting that in autophosphorylated DYRK1A the trypsin cleavage at Lys-95 is affected by the close proximity of phospho-Ser-97.

Tyrosine phosphorylation of DYRK1A *in vivo*

Taking advantage of the histidine repeat in the C-terminal region of DYRK1A [1], we employed immobilized-metal affinity chromatography to partially purify DYRK1A from rat brain and GT1-7 cells [17,18]. COS-7 cells transfected with HA-DYRK1A or empty vector were used as controls. On Western blots (Figure 7A) the anti-DYRK1A antibody recognized a pair of bands of about 90 kDa and a minor band of 82 kDa, the same pattern of bands as obtained by Okui et al. with a different antibody [19]. The upper bands were also detected by the phosphotyrosine-specific antibody, indicating that DYRK1A is phosphorylated on tyrosine residues *in vivo*.

Next, we studied tyrosine phosphorylation of GFP-DYRK1A and its point mutants after immunoprecipitation from transfected COS-7 cells (Figure 7B). Phosphotyrosine was clearly detected in wild-type DYRK1A and DYRK1A-Y111F, but not in DYRK1A-K188R and DYRK1A-Y321F, indicating that the kinase activity of DYRK1A is required for its tyrosine phosphorylation. In contrast with bacterially expressed GST-DYRK1A-Y111F, immunochemically detectable phosphotyrosine was not reduced in GFP-DYRK1A-Y111F. GFP-DYRK1A-Y111F was also indistinguishable from the wild-type construct in its subcellular localization, as determined by fluorescence microscopy, and in its catalytic activity towards DYRKtide (results not shown).

To identify *in vivo* tyrosine-phosphorylation sites by MS, GFP-DYRK1A was isolated from transiently transfected COS-7 cells by immunoprecipitation with a GFP-specific antibody. Analysis of tryptic digests showed that peptide IYQYIYSR was quantitatively phosphorylated on Tyr-321, whereas peptides containing Ser-97 and Tyr-111 were present only in their unphosphorylated forms.

It should be noted that these blots (Figures 7A and 7B) were developed with a different anti-phosphotyrosine antibody (PY99) from those presented above (Figures 2–5), since PY20 did not detectably react with the mammalian fusion proteins. Apparently, PY20 selectively recognizes phosphorylated Tyr-111 in bacterially expressed GST-DYRK1A, but does not detect phosphorylated Tyr-321.

Structural model of DYRK1A

A three-dimensional model of the catalytic domain of DYRK1A was built based on the crystallographic co-ordinates of

Figure 8 Structural model of DYRK1A

(A) Ribbon diagrams of phosphorylated ERK2 (left) and DYRK1A (right). The model comprises amino acids 138–526 of DYRK1A. Structural motifs outside the catalytic core domain (as defined by the structure of protein kinase A [23]) are designated according to the nomenclature established for the MAP kinase family (β_{1L0} , β_{2L0} , α_{L16}). The phosphorylation lip is highlighted in blue. The segment of 21 amino acids with no counterpart in ERK2 was omitted from the model of DYRK1A, as indicated by the arrow. (B) Phosphorylation lip and neighbouring structures in ERK2 and DYRK1A. (C) Cluster of negatively charged amino acids in the region N-terminal of the β_1 sheet of DYRK1A (DYRK homology box).

phosphorylated ERK2 [20]. The general fold of the catalytic domain is probably conserved in DYRK1A, and most of the DYRK1A residues could be easily accommodated within the catalytic domain, as defined by phosphorylated ERK2 (Figure 8A). Deletions and insertions compared with ERK2 were minor,

except for a single large insertion of 21 amino acids between the α_G and α_H helices (arrow in Figure 8A); this insertion could not be modelled. Given that Tyr-321 is phosphorylated in active DYRK1A, the conformation of the activation loop can be well adjusted to the known structure of phosphorylated ERK2 (Figure 8B). In striking analogy to ERK2, the phosphate of Tyr-321 (Tyr-185 in ERK2) can interact with Arg-325 (Arg-189 in ERK2) and Arg-328 (Arg-192 in ERK2). In ERK2 and SAPK (stress-activated protein kinase)/p38 γ , phosphorylation of Tyr-185 is a prerequisite for the correct conformation of the P + 1 specificity pocket, and the phosphorylated tyrosine is positioned to bind the substrate proline directly [20,21]. Consistent with our recent identification of DYRK1A as a proline-directed kinase [16], the structure of the P + 1 pocket is well conserved in the model of DYRK1A. In contrast, none of the arginines (Arg-68, Arg-146 and Arg-170) that ligate with phospho-Thr-183 in ERK2 is present in DYRK1A, and the model provides no clues concerning the role of Tyr-319 in the activation loop of DYRK1A.

The structural conservation of ERK2 and DYRK1A appears to exceed the limits of the catalytic kinase domain as defined by sequence alignments (subdomains I–XI [22]) and structural analyses (L1 loop– α_I helix [23]). This core domain comprises amino acids 159–479 of DYRK1A. In addition, amino acids 138–158 of DYRK1A fit well the β_{1L0} and β_{2L0} strands of ERK2, and residues 480–526 superimpose the L16 loop and the α_{L16} helix of ERK2 with minimal backbone deviation (Figure 8A). In DYRK1A, the sequence corresponding with the β_{1L0} and β_{2L0} strands harbours a cluster of negatively charged amino acids (Figure 8C) that is absent in MAP kinases. Sequence alignments have already shown that this structural motif is conserved within the DYRK family, the cdc2-like kinase (CLK) family and in the PRP4 kinases (DYRK homology box) [11]. Notably, this region is incompletely represented in the N-terminally deleted mutants of DYRK1A, GST-DYRK1A- ΔN and GST-DYRK1A- $\Delta N\Delta C$ (lack of residues 138–148), which showed reduced kinase activity (Figures 4C and 5B).

DISCUSSION

The present paper provides strong evidence that phosphorylation of Tyr-321 is required for full activity of DYRK1A. Tyr-321 is phosphorylated in recombinant DYRK1A, and the exchange of Tyr-321 for phenylalanine suppressed kinase activity of DYRK1A. Tyr-321 is located within the activation loop close to the active site of the catalytic domain and resembles the activating phosphorylation site of ERK2, Tyr-185. Given that Tyr-321 is phosphorylated, DYRK1A can adopt an active conformation strikingly similar to the known structures of phosphorylated ERK2 and p38 γ [18,19]. In contrast with the MAP kinases, phosphorylation of Tyr-321 appears to be catalysed by DYRK1A itself.

It is a unique feature of MAP kinases that they are activated by dual phosphorylation of a Thr-Xaa-Tyr motif in the activation loop. DYRK-related kinases are characterized by a corresponding Tyr-Xaa-Tyr motif which is conserved in DYRK-related kinases from mammals, invertebrates, yeasts and *Dictyostelium*. The proposed active conformation of DYRK1A differs from the structure of phosphorylated ERK2 by the lack of a counterpart of phospho-Thr-183 (Figure 7B). The experimental data also indicate that the corresponding Tyr-319 is not phosphorylated in recombinant DYRK1A, and that this tyrosine is not important for kinase activity *in vitro*. Recent data show that the yeast homologue of DYRK1A, Yak1p, is also specifically phosphorylated on the second tyrosine of the Tyr-Xaa-Tyr motif [24].

Although both tyrosines of the activation loop are conserved in the DYRK family, only the second tyrosine is present in other kinases more distantly related to the DYRK and MAP kinase families [25], e.g. homeodomain-interacting protein kinases (HIPKs), PRP4, male-germ-cell-associated kinase (MAK), MAK-related kinase (MRK) [26–31] and glycogen synthase kinase (GSK) $3\alpha/\beta$. Among these, only GSK3 and its yeast homologues have been shown to be regulated by tyrosine phosphorylation [32–35]. In mammalian GSK3, the insulin-induced dephosphorylation of the tyrosine in the activation loop is an inactivation mechanism independent of the better-known Akt-mediated phosphorylation of Ser-9 in the N-terminus of GSK3 [33].

The activating phosphorylation sites of MAP kinases are targeted by protein kinases that operate upstream in signal pathways. When expressed in *E. coli*, MAP kinases only inefficiently autophosphorylate the tyrosine in the activation loop (25–45% [36]). Similarly, bacterially expressed DYRK1A was only partially phosphorylated on Tyr-321, whereas phosphorylation was stoichiometric when DYRK1A was expressed in COS-7 cells. In a striking contrast with the MAP kinases, DYRK1A appears to autophosphorylate the tyrosine in the activation loop, since a kinase-negative mutant of DYRK1A contained no detectable phosphotyrosine. Autophosphorylation appears to argue against a regulatory role of tyrosine phosphorylation in DYRK kinases. However, the under-phosphorylation of DYRK1A in *E. coli* may indicate that an activator of autophosphorylation is required for full activation of DYRK1A. It might also be speculated that dephosphorylation of Tyr-321 in DYRK1A is regulated, similar to the insulin-dependent dephosphorylation of Tyr-216 in GSK3 β [32].

In addition to Tyr-321, Ser-97 and Tyr-111 are autophosphorylated in recombinant GST-DYRK1A. Although we cannot absolutely exclude the possibility that DYRK1A is phosphorylated by a bacterial kinase, this appears extremely unlikely in view of the results obtained with the point mutants. Immunochemically detected phosphorylation of Tyr-111 was not only abolished by the inactivating K188R mutation, but was also reduced in DYRK1A-Y321F, which exhibited reduced kinase activity. Furthermore, although hypothetical kinase genes have been identified in *E. coli*, there is presently no evidence that *E. coli* contains a serine-, threonine- or tyrosine-specific protein kinase that is capable of phosphorylating exogenous proteins [37].

The autophosphorylation of the N-terminal residues in GST-DYRK1A was efficient and specific, because phosphorylation of Ser-97 is stoichiometric, and because of the high selectivity for Tyr-111 among seven tyrosines in the N-terminal part of DYRK1A. Unexpectedly, Ser-97 and Tyr-111 were found to be unmodified when DYRK1A was expressed in COS-7 cells, and it is an open question as to why this phosphorylation does not take place in mammalian cells. The identification of both a serine and a tyrosine residue as the targets of autophosphorylation by DYRK1A confirms our previous observation that DYRK1A is a dual-specificity protein kinase [1]. The sequence around Ser-97 (RKLpSV) resembles the phosphorylation site of DYRK1A in histone H3 (RPGpTV) and contains the arginine residue in P-3 that is important for substrate recognition by DYRK1A [16]. In contrast, DYRK1A does not phosphorylate tyrosine in exogenous substrates, and the sequences around Tyr-111 and Tyr-321 do not match the consensus sequence for DYRK1A phosphorylation [16]. Thus, the specificity in the autophosphorylation of individual tyrosines of DYRK1A is clearly determined by a different molecular mechanism from that responsible for the specific recognition of exogenous substrates.

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