DYRK1A is a dual-specificity protein kinase that is thought to be involved in brain development. We identified a single phosphorylated amino acid residue in the DYRK substrate histone H3 (threonine 45) by mass spectrometry, phosphoamino acid analysis, and protein sequencing. Exchange of threonine 45 for alanine abolished phosphorylation of histone H3 by DYRK1A and by the related kinases DYRK1B, DYRK2, and DYRK3 but not by CLK3. In order to define the consensus sequence for the substrate specificity of DYRK1A, a library of 300 peptides was designed in variation of the H3 phosphorylation site. Evaluation of the phosphate incorporation into these peptides identified DYRK1A as a proline-directed kinase with a phosphorylation consensus sequence (RPX(S/T/P)) similar to that of ERK2 (PX(S/T/P)). A peptide designed after the optimal substrate sequence (DYRKtide) was efficiently phosphorylated by DYRK1A (K_m = 35 μM) but not by ERK2. Both ERK2 and DYRK1A phosphorylated myelin basic protein, whereas only ERK2, but not DYRK1A, phosphorylated the mitogen-activated protein kinase substrate ELK-1. This marked difference in substrate specificity between DYRK1A and ERK2 can be explained by the requirement for an arginine at the P-3 site of DYRK substrates and its presumed interaction with aspartate 247 conserved in all DYRKs.

DYRK1A is a nuclear protein kinase that is ubiquitously expressed in rat tissues (1, 2). We have recently characterized a closely related isoform, DYRK1B, that is predominantly expressed in testis and muscle (3). The homolog of DYRK1A and DYRK1B in Drosophila, the protein kinase MNB, is encoded by the minibrain gene whose mutation results in specific defects in the development of the central nervous system (4). The human gene for DYRK1A is located in the “Down syndrome critical region” of chromosome 21, and the similarity of DYRK1A and MNB suggests that the triplication of the DYRKIA gene may play a role in mental retardation of patients with Down syndrome (5–9).

DYRK1A, DYRK1B, and MNB belong to a subfamily of protein kinases with structurally related catalytic domains and similar enzymatic properties (2, 10). At least 7 different DYRK-related kinases exist in mammals, of which DYRK1A and DYRK1B are targeted to the nucleus, whereas DYRK2 and DYRK3 are located in the cytoplasm (2, 9). Members of the DYRK family have also been found in lower eukaryotes, such as Yak1p in Saccharomyces cerevisiae (11), Pom1p in Schizosaccharomyces pombe (12), and YAKA in Dictostylium discoidium (13). Although mutations in YAK1, pom1, and yakA have diverse phenotypic consequences, it appears reasonable to generalize that DYRK-related kinases are involved in the regulation of growth and development.

The enzymatic activity of DYRK1A has been shown to depend on the presence of tyrosine residues in the activation loop, a result that suggests an activation mechanism similar to that of the MAP2 kinases (1). However, the participation of DYRK1A or other DYRK-related kinases in a particular signal transduction pathway has not been elucidated so far. The selective recognition of the correct substrates by protein kinases is an important biochemical mechanism that underlies the specificity of cellular responses to various stimuli. It is therefore important to define the structural determinants of substrate recognition by DYRK1A in order to understand its function in the regulation of cellular processes.

In this study, we have identified the phosphorylation site for DYRK1A in the in vitro substrate, histone H3. On the basis of this result, we designed a peptide library on cellulose paper (SPOT membrane) (14–17) to determine the consensus sequence for substrate recognition by DYRK1A. Sequence specificity of DYRK1A was similar to, but not identical with, that of the MAP kinase ERK2. This definition of the consensus phosphorylation sequence of DYRK1A should facilitate studies on physiological substrates of the kinase.

**EXPERIMENTAL PROCEDURES**

**Protein Kinases and Substrates**—The expression plasmids for GST-DYRK1A, GST-DYRK2, GST-DYRK3, and GST-CLK3 have been described earlier (1, 2, 18). An NcoI site at base pair 1624 of the rat cDNA was used to create a C-terminal deletion (amino acids 500–763, numbering refers to Swiss-Prot accession number Q63470) in GST-DYRK1A-ΔC. GST-DYRK1A-ΔC was used in all phosphorylation reactions except for the experiment shown in Fig. 1A. To construct an expression plasmid for GST-DYRK1B, the open reading frame of the longest splice variant (3) was amplified by polymerase chain reaction from a human testis cDNA library (CLONTECH, catalog number HL1161x) with appropriate primers and inserted into the pGEX-2TK vector (Amersham Pharmacia Biotech). GST fusion proteins were partially purified from Escherichia coli by affinity adsorption to glutathione-Sepharose according to the manufacturer’s instructions (Amersham Pharmacia Biotech) (19). Recombinant, active ERK2 prepared

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1 Y. Weber and W. Becker, unpublished data.
2 The abbreviations used are: MAP, mitogen-activated protein; GST, glutathione S-transferase; MALDI, matrix-assisted laser desorption ionization; MBP, myelin basic protein; rH3, recombinant histone H3; RP-HPLC, reverse phase-high performance liquid chromatography; TOF, time-of-flight; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
from E. coli containing constitutively active MAP kinase/extracellular signal-regulated kinase kinase was obtained from New England Biolabs.

Protein substrates were purchased from the following suppliers: histone type II-S and MBF, Sigma; histone H3, H2B, and H4, Roche Molecular Biochemicals; GST-ELK, Peptide Institute, Inc. The combinator histone H3 (rH3) was prepared from bacterial inclusion bodies as described elsewhere (20). Point mutants of rH3 were generated by overlap extension polymerase chain reaction (21). The DYRK recombinant histone H3 (rH3) was prepared from bacterial inclusion bodies as described elsewhere (22–23), were purchased from Sigma.

**ASSAY CONDITIONS**—For the determination of kinetic parameters, assays were performed with 5 or 6 different substrate concentrations that were incubated for 8 min (4 min with DYRKtide) at 37 °C with 0.1–200 pmol/ml GST-DYRKIA-ΔC in phosphorylation buffer containing 25 mM Hepes, pH 6.8, 5 mM MgCl2, 5 mM MnCl2, 0.5 mM dithiothreitol, and 200 μM [γ-32P]ATP (100–150 μCi/ml). Incorporation of [γ-32P]ATP into histone was determined in triplicate by dotting aliquots of the reaction mixture onto Whatman P-81 ion exchange paper, washing in 5% phosphoric acid (10 times, 5 min each), and subsequent liquid scintillation counting. The position for linearity of the reaction, another aliquot was taken after 4 min (2 min for DYRKtide). The amount of [γ-32P]TP bound to P-S81 paper in assays without substrate was subtracted as background. Apparent 

**Mass Spectrometry**—To achieve maximal incorporation of phosphate into H3, conditions for phosphorylation were modified as follows. Seven nmol of rH3 were incubated at a concentration of 10 μM in phosphorylation buffer containing 500 μM ATP with 180 pmol GST-DYRK1 (for 105 min at 30 °C. Fresh aliquots of the kinase (90 pmol) were taken at 15, 30, 45, and 60 min for liquid scintillation counting. To allow tracing of the phosphorylated histone in further steps, an equivalent, but 5-fold down-scaled reaction, was carried out in the presence of [γ-32P]ATP (500 μCi/ml). After removing unincorporated [γ-32P]P by precipitation with trichloroacetic acid, products of both reactions were combined and lyophilized. A sample of 100 μg of lyophilized phosphorylated rH3 was dissolved in 3 μl guanidinium HCl, pH 4.5, containing 10% (v/v) acetonitrile and 9.4% (v/v) trifluoroacetic acid and fractionated by RP-HPLC (Sephasil C-18 SC 2.1/10 column connected to a SMART system, Amersham Pharmacia Biotech) in a linear gradient of acetonitrile (0.42% (v/v) per min) in 0.1% (v/v) trifluoroacetic acid at a flow of 100 μl/min. Phosphorylated rH3 was detected by Cerenkov counting, and its identification was confirmed by SDS-PAGE and autoradiography of the dried gel. For mass spectrometry, about 5–10 μg of lyophilized, phosphorylated, or unphosphorylated rH3 were dissolved in 1 μl of 33% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid. After addition of 1 μl of matrix solution (saturated solution of sinapic acid in 50/30 (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid), 0.5 μl of this solution was subjected to mass spectrometry (MALDI-TOF mass spectrometry—Biflex III TOF, Bruker-Franzen Analytik). Spectra were produced by accumulating data from 200 laser shots (nitrogen laser, 337 nm, 300 μJ, 10 Hz). To calculate specific enzyme activities, the amount of intact, radiolabeled phosphoamino acids was measured by liquid scintillation counting, and its identification was confirmed by SDS-PAGE and autoradiography, gels were destained only until standards could just be resolved by film autoradiography (exposure times of 1–3 d) and with the PhosphorImager system (Molecular Dynamics).

**RESULTS**

In Vitro Phosphorylation of Histones by Recombinant DYRK1A—We have previously shown that calf thymus histones can be used as in vitro substrates for DYRK1A (1, 2). The phosphorylation of histones was determined by using histone H2A, data not shown), histone H3 was the best substrate for DYRK1A (Fig. 1). Fig. 1 shows also that a truncated construct of DYRKIA lacking the non-catalytic C-terminal domain (GST-
DYRK1A-ΔC) is an active protein kinase with the same substrate specificity as undeleted DYRK1A. GST-DYRK1A-ΔC was used for all further experiments of this work since this construct appeared to have a higher specific activity than GST-DYRK1A when purified from recombinant E. coli.

To avoid potential problems due to post-translational modifications or sequence microheterogeneity of histone H3 isoforms, we decided to use recombinant histone H3 (rH3) for the following experiments. Construction of the expression plasmid and the method to prepare bacterially expressed rH3 are presented elsewhere.

Mass Spectrometry and Phosphoamino Acid Analysis—In order to determine the number of phosphorylation sites in rH3, we analyzed unphosphorylated and phosphorylated rH3 by MALDI-TOF mass spectrometry (Fig. 2, A and B). Unphosphorylated rH3 was detected at a mass of 15,930 Da, close to the calculated mass of 15,925 Da (assuming cleavage of the N-terminal methionine). After phosphorylation by DYRK1A, a new peak appeared with a separation plus 81 Da, corresponding with the addition of a single phosphate moiety to rH3 (80 Da). By phosphoamino acid analysis, only phosphothreonine was detected in DYRK1A-phosphorylated rH3 (Fig. 2C). Taken together, these data indicate that a single threonine residue in rH3 is phosphorylated by DYRK1A under our conditions.

Determination of the DYRK1A Phosphorylation Site in rH3 by Protein Sequencing—Endoproteinase Glu-C (protease V8) was used to generate peptides of phosphorylated rH3 suitable for sequencing. Even after prolonged digestion, two radioactively labeled bands were detected after electrophoretic separation in Tricine/SDS-polyacrylamide gels (Fig. 3A). The sizes of these bands (4.4 and 4.9 kDa) did not match any of the expected cleavage products, indicating partial or nonspecific cleavage of the protein. Elution of these bands from the gel did not yield enough material to identify the phosphorylated amino acid by protein sequencing. The proteolytic fragments were therefore fractionated by RP-HPLC (Fig. 3B). A single peak contained most of the $^{32}$P radioactivity, and this material was further analyzed by automated Edman degradation. The N-terminal sequence, together with the size of the fragment, suggested that the residues Thr-32, Thr-45, and Thr-58 were the potential phosphorylation sites in this fragment. It should be noted that the cleavage site giving rise to this peptide (C-terminal of Lys-25) did not correspond with the known specificity of endoproteinase Glu-C (C-terminal of Glu or Asp). The irregular cleavage was reproduced with different batches of the proteinase and rH3. This surprising finding cannot easily be explained by an inadvertent modification of the recombinant H3, since we confirmed the amino acid sequence around the cleavage site by N-terminal sequencing of the protein.

To determine the degradation cycle in which the labeled phosphate was released, the HPLC eluates of automated Edman degradation were collected for liquid scintillation counting (Fig. 3C). A very similar profile as in Fig. 3C was obtained by evaluation of the collected S2 washes (see “Experimental Procedures”). As noted by others, $^{32}$P-containing products of the Edman reaction were inefficiently recovered from the reaction chamber, leading to extensive tailing of radioactivity peaks into the following cycles (Fig. 3C). However, the significant increase in eluted radioactivity identified Thr-45 as the phosphorylated residue, whereas there was no increase after cleavage of the other relevant threonine residue, Thr-32.

Phosphorylation of Mutated Versions of rH3 by DYRK1A—To confirm this result by an independent method, we generated mutants of each threonine in the $^{32}$P-containing proteolytic fragment (T32A, T45A, and T58A) and tested their phosphorylation by GST-DYRK1A-ΔC (Fig. 4). Incorporation of $^{32}$P into rH3 was abolished when Thr-45 was exchanged for alanine, whereas substitution of other threonine residues did not affect phosphorylation of rH3 by DYRK1A. This result shows that

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3 P. Panzer, H. Kentrup, and W. Becker, unpublished data.
4 Numbering of amino acids in histone H3 (Swiss-Prot accession number P06351) refers to the processed protein lacking the initiator methionine.
subjected to autoradiography (polyacrylamide gels. Gels were stained by Coomassie Blue (aliquots were taken and analyzed by electrophoresis on Tricine/SDS-

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endoproteinase Glu-C. 600

digested with 12 nmol of phosphorylated rH3 was digested with endoproteinase Glu-C, and reaction products were fractionated on a C-18 re-

versed phase column with a linear gradient of acetonitrile as described

under “Experimental Procedures.” The corresponding radioactivity in

each fraction was quantitated by Cerenkov counting (right panel). B, separation of peptides by RP-HPLC. 12 nmol of phosphorylated rH3 was digested with endopro-

teinase Glu-C, and reaction products were fractionated on a C-18 re-

versed phase column with a linear gradient of acetonitrile as described

under “Experimental Procedures.” The corresponding radioactivity in

each fraction was quantitated by Cerenkov counting (open bars). C, Edman degradation of the phosphopeptide. The 32P-containing peptide was subjected to automated Edman degradation. For each cleavage cycle, the released radioactivity was determined in column eluates of RP-HPLC. Numbered residues of rH3 corresponding with the respective cleavage cycles are indicated below the bars. Only cycles around the relevant threonine residues are shown.

Thr-45 is the only residue in rH3 efficiently phosphorylated by DYRK1A.

Phosphorylation of rH3 by DYRK-related Kinases—We have previously shown that DYRK-related kinases exhibit similar, but not identical, substrate specificity toward different histones (2). In order to assess whether these kinases recognize the same target site in rH3, we compared the phosphorylation of wild type rH3 and rH3-T45A by bacterial GST fusion proteins of DYRK1B, DYRK2, and DYRK3 (Fig. 5). With all kinases of the DYRK family, incorporation of 32P into H3 was greatly reduced or completely abolished in the mutant protein, clearly indicating that Thr-45 is the main phosphorylation site in histone H3 for DYRK-related kinases. For comparison, GST-CLK3 was included as a more distantly related protein kinase (18). Large amounts of the kinase were required to achieve detectable phosphorylation of rH3, and no difference was observed between rH3 and rH3-T45A (Fig. 5).

Determination of the Consensus Phosphorylation Sequence for DYRK1A—In order to ascertain that the amino acid residues flanking Thr-45 indeed determine substrate recognition by DYRK1A, a peptide containing 15 amino acids surrounding Thr-45 of histone H3 (H338–52) was synthesized and tested as a substrate for DYRK1A. To exclude the possibility that Tyr-41 might contribute to phosphate incorporation, a second peptide was synthesized that contained a phenylalanine at this position (H338–52Y41F). Both peptides were phosphorylated by DYRK1A to a similar degree, whereas selected peptide substrates of other kinases (Kemptide, angiotensin) were not recognized (Fig. 6). For the further definition of the consensus phosphorylation sequence, we took advantage of the SPOT assay which offers the possibility to compare simultaneously a large number of different peptides as substrates for protein kinases (16, 17, 29). Based on the sequence of H338–52, a series of 300 peptides was designed, each harboring a single amino acid exchange. The peptides were synthesized as individual spots on a cellulose paper and phosphorylated by GST-DYRK1A-ΔC. Relative incor-

poration of radioactivity into each peptide was evaluated by autoradiography with the help of a storage phosphor screen (Fig. 7). The most striking effects of amino acid substitutions were observed at the phosphorylation site (P), at the position C-terminal to the phosphorylation site (P +1), and at two N-terminal positions (P −2 and P −3). First, exchange of the valine for proline at the P +1 position resulted in a 10-fold increase of peptide phosphorylation. DYRK1A also prefers a proline at the P −2 site. Second, an arginine residue is absolutely required at the P −3 site. Third, serine is preferred versus threonine as the phosphate acceptor, and no tyrosine phosphorylation was detected in this experiment. Taken together, these results indicate that RXS/TYP represents the optimal substrate sequence for DYRK1A. This consensus sequence is similar to that of ERK1/2 (PXST/V/P) (30, 31) and indicates that DYRK1A is a proline-directed kinase. By evalu-

ating the 18 peptides lacking a phosphorylatable residue at this site, the background of noncovalently bound radioactivity was calculated to be 17.0 ± 6.0 relative units (mean ± S.D.). A measure for scattering between identical peptides was ob-

tained by comparing phosphate incorporation into the reference peptide, H338–52 that was present in 15 spots on the paper (open bars in Fig. 7B). These spots contained 67.9 ± 14.1
Phosphorylation of rH3 and rH3-T45A by DYRK-related kinases. GST fusion proteins of the indicated kinases were incubated with wild type rH3 (WT) or rH3-T45A in the presence of [γ-32P]AT. Reaction products were separated by SDS-PAGE, and incorporation of 32P into histone was visualized with the help of a storage phosphor autoradiography system (A). Reaction conditions (amounts of kinase and concentrations of ATP) and detection thresholds were adjusted to achieve comparable band intensities. The Coomassie stain (B) shows equal loading of the histones.

**Fig. 5.** Phosphorylation of rH3 and rH3-T45A by DYRK-related kinases. GST fusion proteins of the indicated kinases were incubated with wild type rH3 (WT) or rH3-T45A in the presence of [32P]AT. Reaction products were separated by SDS-PAGE, and incorporation of 32P into histone was visualized with the help of a storage phosphor autoradiography system (A). Reaction conditions (amounts of kinase and concentrations of ATP) and detection thresholds were adjusted to achieve comparable band intensities. The Coomassie stain (B) shows equal loading of the histones.

**Fig. 6.** Phosphorylation of peptides by GST-DYRK1A-ΔC. GST-DYRK1A (26 pmol/ml) was incubated with the indicated peptides (H338–52, H338–52Y41F, Kemptide, angiotensin II, each at 100 μM) or without substrate (control) in the presence of 500 μM [32P]AT. Incorporation of phosphate into peptides was determined by binding to phosphocellulose paper. The plot shows incorporation of phosphate into the substrates over time.

**Phosphorylation of peptides by GST-DYRK1A-ΔC.** GST-DYRK1A (26 pmol/ml) was incubated with the indicated peptides (H338–52, H338–52Y41F, Kemptide, angiotensin II, each at 100 μM) or without substrate (control) in the presence of 500 μM [32P]AT. Incorporation of phosphate into peptides was determined by binding to phosphocellulose paper. The plot shows incorporation of phosphate into the substrates over time.

**Comparison of Different Substrates for DYRK1A by Kinetic Analysis—**An optimized peptide substrate for DYRK1A based on the data in Fig. 7 was synthesized (DYRKtide; RRRFRPSPLRGPK) in order to compare the kinetic parameters of different substrates. Table I shows kinetic parameters of the phosphorylation of DYRKtide and peptide H338–52 by DYRK1A. Consistent with the results of the SPOT assay, DYRKtide was phosphorylated with a higher Vmax than H338–52, and the apparent Km was also lower for DYRKtide than for H338–52. Similar kinetic constants were obtained with different preparations of the kinase (Table I). The Vmax/Km ratio was calculated as an overall measure of the ability of the peptides and proteins to function as a substrate of DYRK1A. This value was 26-fold higher for DYRKtide than for H338–52. For comparison, kinetic parameters were also determined for the protein substrates, rH3 and MBP. We considered MBP a potential substrate for DYRK1A because it is efficiently phosphorylated by proline-directed kinases such as ERK1 and ERK2 (32, 33). The Vmax/Km ratio was about 25-fold higher for MBP than for rH3, indicating that MBP is an excellent substrate for DYRK1A.

**DYRK1A and ERK2 Have Different Substrate Specificities—**The optimal phosphorylation sequence for DYRK1A as derived from the SPOT assay (Fig. 7, FRPSPPLRG) resembles the consensus sequence for the MAP kinase, ERK1, as determined in a peptide library screen (Ref. 31, TGPLSPGPWF). We have therefore compared the ability of DYRK1A and ERK2 to phosphorylate DYRKtide and protein substrates in vitro. Only at the highest concentration of DYRKtide tested (2 mM), minute phosphorylation by ERK2 was detectable, thus precluding the determination of kinetic parameters. However, the Km value for the phosphorylation of DYRKtide by ERK2 can be extrapolated to be at least 100-fold higher (>3 mM) than that for its phosphorylation by DYRK1A (30–35 μM, see Table I).

The transcription factor ELK-1 is a well characterized substrate of ERK2 (34). Phosphorylation of the GST-ELK-1 construct by ERK2 was so efficient that the phosphorylated protein could be detected on the Coomassie-stained gel by its slower migration (Fig. 8A). In contrast, DYRK1A failed to phosphorylate ELK-1, despite the presence of 9 potential phosphorylation sites with a proline at the P +1 position (Fig. 8C). Furthermore, ERK2 failed to phosphorylate rH3. DYRK1A and ERK2 also exhibited different site selectivity toward MBP. Phosphoamino acid analysis showed that DYRK1A phosphorylated MBP on serine and threonine residues (Fig. 8B). Consistent with previous reports, ERK2 phosphorylated MBP only on threonine (i.e. Thr-97 (33, 35)).

**DISCUSSION**

In the present paper we have defined the consensus sequence for substrate phosphorylation by the protein kinase DYRK1A. Three positions in the vicinity of the phosphorylated serine or threonine residue were found to be particularly important for substrate recognition, the optimal phosphorylation site sequence for DYRK1A being Arg-Pro-X-Ser/Thr-Pro (RPX(S/T)P, where X is a variable amino acid, and the phosphorylated amino acid is underlined).

This motif resembles the consensus sequences for MAP kinases (PX(S/T)P) and the cyclin-dependent kinases ((S/T)P, X(S/P)) and the cyclin-dependent kinases correlates well with their structural relationship as illustrated by their classification in the protein kinase superfamily (Fig. 9A). Other structurally related kinases, GSK3 and members of the CLK family, are also known to phosphorylate serine or threonine residues followed by a proline (39, 40). Molecular modeling of CDK2-substrate (41) and ERK2-substrate complexes (42) suggested that a pocket formed by the amino acids preceding subdomain VIII of the catalytic domain (185–193 of ERK2; 162–170 of CDK2) determines P +1 specificity. Particularly, the conserved arginine side chain (Arg-192 in ERK2, Arg-169 in CDK2, and Arg-328 in DYRK1A) is thought to recognize a unique backbone conformation of the substrate that results from the presence of the proline (42, 43). This sequence motif is highly conserved in MAP kinases, cyclin-dependent kinases, and members of the DYRK family (Fig. 9B). It appears likely that related kinases with this sequence structure will also turn out to be proline-directed kinases, e.g. HIPK (homeodomain-interacting protein kinase (44)), also called ANPK, androgen receptor interacting nuclear protein kinase (45)), protein kinase PRP4 (46), and MAK (male germ cell-associated kinase (47)). However, dependence on a proline at the P +1 site is apparently not absolute in all of these kinases, since GSK3 (39) and DYRK1A (this paper) can also phosphorylate other target sites.

In addition to identifying DYRK1A as a proline-directed kinase, the present data show also striking differences in the selection of phosphorylation sites by DYRK1A and ERK2. Although DYRKtide contains the established consensus site for ERK2 (PX(S/T)P, 30–32), it was a very poor substrate of ERK2 so...
that kinetic parameters could not be determined. The results of the SPOT assay indicate that an arginine residue at the P-2 site of the substrate is important for substrate recognition by DYRK1A (Fig. 8). This position is not known to be relevant for substrate recognition by MAP kinases but may determine the ability or inability of DYRKtide to be phosphorylated by
DYRK1A and ERK2, respectively. This hypothesis is supported by molecular modeling of the DYRK1A-DYRKtide complex. Given that the peptide is oriented in the same way as in the binary peptide-kinase complexes of other kinases (cAMP-dependent kinase, phosphorylase kinase, (48–50)), the arginine in the P–2 position is likely to interact with Asp-247 of DYRK1A (in the sequence LYDLL). This residue is conserved in all members of the DYRK family (10), whereas the equivalent position in ERK2 is occupied by Lys-112 (in LYKLL), obviously precluding the analogous interaction with the P–2 arginine in the substrate. CLK kinases also have an acidic residue at this position, and it is interesting to note that the only known phosphorylation site for kinases of this family, Ser-164 of MBP (40), is preceded by an arginine in the P–2 position (RSGSP). The sequence surrounding this residue matches quite well the optimal phosphorylation site of DYRK1A, and it appears likely that Ser-164 is one of the phosphorylation sites for DYRK1A in MBP.

Results obtained with protein substrates are consistent with the conclusions drawn from the peptide assays. As predicted from the results of the SPOT assay, MBP is efficiently phosphorylated by DYRK1A. Furthermore, the different substrate specificities of DYRK1A and ERK2 were corroborated by the assay of a physiologically relevant substrate of ERK2, ELK-1. The inability of DYRK1A to phosphorylate ELK-1 may again be explained by the lack of an arginine at P–2 in all potential phosphorylation sites for proline-directed kinases (Fig. 8C).

DYRK1A appears to be less dependent on binding to sequences distant to the target residue as it phosphorylates the peptide, DYRKtide, at a $K_m$ value much lower than that of ERK2 for its optimized peptide substrate (35 $\mu M$ for DYRKtide versus 450 $\mu M$ for ERK2).

**TABLE I**

| Substrate | $K_m$ | $V_{max}$ | $V_{max}/K_m$ |
|-----------|-------|-----------|--------------|
| H338–52   | 298 ± 23 | 10.10 ± 0.19 | 0.0622       |
| (2)       | 274 ± 30  | 8.88 ± 0.39   |              |
| DYRKtide  | 32.7 ± 9.1 | 14.1 ± 1.30  | 0.682        |
| (2)       | 35.9 ± 7.7 | 9.3 ± 0.69    |              |
| rH3       | 1.10 ± 0.09 | 0.55 ± 0.02   | 0.54         |
| (2)       | 1.07 ± 0.12 | 0.83 ± 0.02   |              |
| MBP       | 0.14 ± 0.01 | 2.26 ± 0.06   | 13.23        |
| (2)       | 0.12 ± 0.02 | 1.19 ± 0.11   |              |

*a* Mean values calculated from both data sets.

**FIG. 8.** DYRK1A and ERK2 exhibit different specificities toward protein substrates. The indicated substrates (MBP, GST-ELK-1307–428, rH3, and rH3-T45A) were phosphorylated by GST-DYRK1A-D or ERK2 in the presence of [32P]ATP. Amounts GST-DYRK1A-D and ERK2 were adjusted to give the same MBP kinase activity. Reaction products were separated by SDS-PAGE and detected by autoradiography of the dried gel (A, upper panel). The Coomassie stain of the gel is shown in the lower panel. Aliquots of the reaction products were subjected to phosphoamino acid analysis as described (B). Phosphorylation sites for ERK2 in ELK-1 are shown in C (32).

**FIG. 9.** Structural similarity of known and putative proline-directed kinases. A, relationship of the DYRK family, the MAP kinase family, and other kinases. The dendrogram was constructed with the CLUSTAL program (61) on the basis of a sequence alignment of the catalytic domains. Only representative members of the different kinase families were included in the analysis. B, sequence conservation of the P +1 specificity region. The conserved subdomain VII of the catalytic domain (nomenclature according to Ref. 62) is highlighted by shading, and the arrow points to the arginine residue (boxed) that presumably determines proline specificity (see text). Symbols used in the consensus: $\Phi$, hydrophobic aliphatic residue, $\theta$, aromatic residue.

**J. Groetzinger and W. Becker, unpublished data.**
Substrate Specificity of the Protein Kinase DYRK1A

μM for ERK tide (56).

Peptides on SPOT papers have previously been employed to study the substrate specificity of protein kinases (16, 17, 29). By using degenerate peptide libraries in an iterative strategy, it is possible to determine the optimal substrate sequence without prior knowledge on a phosphorylation site (16). In the present work, by starting with a reference peptide derived from a known phosphorylation site, we were able to evaluate and compare the effect of all possible amino acid substitutions in the vicinity of the phosphorylation site in a single experiment. In the recent years new approaches have been successfully used to determine substrate specificities of protein kinases by phosphorylation of degenerate peptide libraries (31, 41, 57, 58). Optimal residues determined by such assays were selected in the respective position in a large number of different peptides. The method of Wu et al. (59) allows the identification of individual peptides in a random library that are well phosphorylated but provides no information on the importance of specific residues. The systematic approach applied in the present study makes it unlikely that major determinants of substrate specificity escaped detection, including negative determinants (60). However, more complex effects of combined substitution of two or more residues are not readily detectable with this approach.

In addition to sequence specificity, the SPOT assay revealed the residue specificity of DYRK1A. Like many protein kinases (60), DYRK1A preferred serine over threonine in substrate peptides and did not detectably phosphorylate tyrosine (Fig. 7). This result is consistent with our recent finding that DYRK1A phosphorylated none of its tested substrates on tyrosine (2). Our previous detection of phosphotyrosine in DYRK-phosphorylated substrates escaped detection, including negative determinants (60). This makes it unlikely that major determinants of substrate specificity remain to be determined.

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