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Classification of tyrosine kinases from *Dictyostelium discoideum* with two distinct, complete or incomplete catalytic domains

Kristin Adler, Günther Gerisch, Ulrike von Hugo, Andrei Lupas, Anton Schweiger*

Max-Planck-Institut für Biochemie, D-82152 Martinsried, Germany

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Abstract **Two new kinases of *Dictyostelium discoideum* were identified by screening of a λgt11 expression library with a phosphotyrosine specific antibody. Amino-acid sequences derived from cDNA and genomic clones indicate that DPYK3 is a protein of 150 kDa and DPYK4, a protein of 75 kDa. The C-terminal fragments of each protein were produced in *Escherichia coli* and shown to be autocatalytically phosphorylated at tyrosine residues. A common feature of these kinases is the presence of two different sequence stretches in tandem that are related to kinase catalytic domains. The sequence relationships of DPYK3 and 4 to other protein kinases, and the positions of their catalytic domain sequences within the phylogenetic tree of protein kinases were analysed. Domains I of both kinases and domain II of DPYK3 constitute, together with the catalytic domains of two previously described tyrosine kinases of *D. discoideum*, a branch of their own, separate from the tyrosine kinase domains in sensu strictu. Domain II in DPYK4 is found on a different branch close to serine/threonine kinases.**

Key words: *Dictyostelium*; Dendrogram; Protein tyrosine kinase

1. Introduction

During development of the eukaryotic microorganism *Dictyostelium discoideum*, cell interactions are mediated by a series of chemical signals [1]. These include cAMP and folic acid that act as chemoattractants, and as ligands in signal transduction pathways controlling gene activities in development. Genistein, an inhibitor of protein tyrosine kinases, suppresses the chemotactic response to folate [2]. Two glycoproteins are involved in the sensing of cell density at different stages of development, prestarvation factor (PST) and conditioned medium factor (CMF) [3]. Different types of protein kinases are known to be involved in one or several pathways linked to these extracellular signals. Protein kinase A is essential for early development up to the aggregation stage, and plays a role in the differentiation of spores and stalk cells [4]. ERK1 and ERK2, two serine/threonine kinases that are phosphorylated at tyrosine residues, control growth and development [5,6]. In particular, ERK2 is implicated in the cAMP-receptor mediated activation of adenylyl cyclase [7]. Tyrosine phosphorylation is also part of the stress responses in *D. discoideum* cells. The most prominent substrate phosphorylated under conditions of oxygen depletion, heat shock, or heavy metal treatment is actin [8]. A single tyrosine (Tyr-53) close to the area of subunit interaction in actin filament formation is phosphorylated under these conditions [9].

Little is known about the structure and role of tyrosine kinases in *D. discoideum*. The primary structures of two non-receptor kinases, DPYK1 (=SplA) and DPYK2, have been published by Tan and Spudich [10]. The elimination of one of them (SplA) by gene disruption indicates that this kinase is required for viable spore production [11]. In this paper we describe two new tyrosine kinases of *D. discoideum*, DPYK3 and DPYK4, and the positions of their catalytic consensus domains in a dendrogram of protein kinases. A characteristic of both kinases is the presence of two of these domains, a peculiarity that will be discussed in terms of its putative phylogenetic origin.

2. Material and methods

2.1. Library screening and DNA sequencing

A λgt11-cDNA expression library of *D. discoideum* AX3, purchased from Clontech, was screened with iodinated anti-phosphotyrosine mAb 5E2 [12]. The cDNA inserts of phages from 11 plaques recognized by mAb 5E2 were amplified by PCR with λgt11 specific primers, subcloned into pUC19 and sequenced. Based on cDNA and genomic DNA, the DPYK3 and DPYK4 sequences were completed using sequence specific PCR, inverted PCR, and enrichment mediated PCR approaches [13,14]. The continuity of the obtained sequence fragments was confirmed by the sequencing of two overlapping PCR products of genomic DNA of *D. discoideum* AX2 covering the entire DPYK3 coding region including one intron and by the sequencing of one continuous PCR product of genomic DNA comprising the entire DPYK4 coding region plus two introns.

2.2. Expression in E. coli

A β-Gal/DPYK3 fusion protein containing amino acids 918–1338 of DPYK3 encoded by the original λgt11-cDNA clone was expressed in *E. coli* Y1089. Cells were grown in LB medium with 200 μg/ml ampicillin at 30°C. At an optical density of 0.5 at 600 nm, the temperature was increased to 42°C for 20 min. Cells were induced with 10 mM IPTG and incubated for 1 h at 37°C.

A PCR product encoding amino acids 296–647 of DPYK4 was cloned behind a His tag into the plasmid pQE32 (Qiagen). The correct sequence of the construct was confirmed and the DNA expressed in *E. coli* M15/pREP (Qiagen) cultivated in LB medium with 2% glucose, 200 μg/ml of ampicillin and 25 μg/ml of kanamycin at 37°C to an optical density of 0.8 at 600 nm. Samples were taken from bacteria harvested after 2 h of induction with 2 mM IPTG, or from non-induced bacteria.

2.3. Protein and phosphoamino acid analysis

For Western blot analysis of bacterial lysates, cell pellets of 1 ml culture were frozen in liquid nitrogen and resuspended in 100 μl SDS-sample buffer. Proteins from 300 μl culture were separated by SDS-PAGE, blotted and incubated with anti-His-tag mAb 232-470-5 [15] or anti-phosphotyrosine mAb 5E2.

For immunoprecipitation of the β-Gal/DPYK3 fusion protein, cells from 1 l induced culture of *E. coli* Y1089 were pelleted, frozen, and resuspended in 5 ml of buffer containing 50 mM Tris-HCl, pH 8.0, 1.5 mM EDTA, 150 mM NaCl, and 1.5 mg lysozyme. After 15 min on ice, 1 mM PMSF, 200 units/ml of aprotinin, 0.5 μg/ml of bestatin, and 1 μg/ml each of antipain, leupeptin, pepstatin A (Sigma), and 100 μM sodium orthovanadate were added, and the cells were disrupted by mild sonication. The extract was centrifuged at 10000 rpm for 15 min,

*Corresponding author. Fax: (49) (89) 8578-3885.

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and the supernatant (usually 100 μl) incubated for immunoprecipitation with 10 μg of mAb 5E2 or anti-β-Gal mAb (Promega, Heidelberg) as described [16]. Immunoprecipitates were labelled with [γ-^{32}P]ATP, separated by SDS-PAGE and the β-Gal/DPYK3 protein subjected to phosphoamino acid analysis according to [16].

2.4. Sequence analysis and dendrogram construction

Sequence similarity searches against GenBank were performed on the World Wide Web using the BLAST protocol at the National Center for Biotechnology Information NCBI [17] (http://www.ncbi.nlm.nih.gov). Searches for coiled-coil regions were made with the COILS program at the Swiss Institute for Cancer Research [18] (http://ulrec3.unil.ch/software/COILS_form.html). The sequences used to construct the dendrogram in Fig. 6 were retrieved from the SwissProt database (http://expasy.hcuge.ch/sprot/sprot-top.html) or from GenBank via the Entrez server at NCBI [19] (http://www3.ncbi.nlm.nih.gov/Entrez/). The sequences were edited down to the conserved protein kinase domain as defined by a comparison of domains of known structure (Fig. 2). For this comparison, coordinates 1IRK, 1CDK, and 1CSN were retrieved from the Protein Data Bank (http://www.pdb.bnl.gov/) and superimposed to yield a minimal rms deviation in backbone atoms. The resulting alignment agreed with the automated output generated by the Dali server [20] (http://www.embl-heidelberg.de/dali/dali.html). The alignment of the *Dictyostelium* kinase domains to the kinases of known structure was performed using MACAW [21]. Dendrograms were constructed using the Darwin server [22] (http://cbrg.inf.ethz.ch/), ClustalW [23], and the Fitch module of Phylip [24]. For ClustalW, the dendrogram was subjected to 1000 bootstrap cycles. In cases of discrepancy between the dendrograms, which were minor, the branching pattern that two of the three programs agreed on was used.

3. Results

3.1. Sequences of tyrosine kinases DPYK3 and DPYK4

During screening for tyrosine kinases from *D. discoideum*, phages were isolated from 11 plaques of a λgt 11 expression library of *D. discoideum* cDNA. These plaques were recognized by a phosphotyrosine-specific antibody, indicating that the cDNAs encoded sequences of tyrosine kinases that are capable of autophosphorylation and/or phosphorylation of tyrosine residues in bacterial host proteins. The sequences of the cDNA clones represented four different proteins. Two clones encoded sequences of DPYK1, and five clones sequences of DPYK2 [10]. One of our cDNA clones encoded a C-terminal fragment of a new kinase, DPYK3, and three of these clones encoded C-terminal fragments of a fourth kinase, DPYK4.

Based on the isolated cDNA fragments, the complete sequences of the coding regions of DPYK3 and 4 were obtained by cDNA and genomic sequencing (Fig. 1). Comparison of the C-terminal regions with other kinase sequences indicated that DPYK3, as well as DPYK4, contains two segments in tandem, each segment related to a catalytic domain sequence of other protein kinases (Fig. 2).

3.2. Autophosphorylation activity of DPYK3 and DPYK4 C-terminal fragments

In order to determine whether the C-terminal domains I in DPYK3 and DPYK4 have catalytic activity, cDNA fragments encoding portions of these kinases, as outlined in Fig. 3, were expressed in *E. coli* and autophosphorylation of the products was assayed. A β-Gal fusion of the DPYK3 sequence from Pro-918 to the C-terminal Ser-1338, which had a predicted molecular mass of 164 kDa, was precipitated from the bacterial extracts either with anti-phosphotyrosine or anti-β-galactosidase antibody and was recognized, after SDS-PAGE by

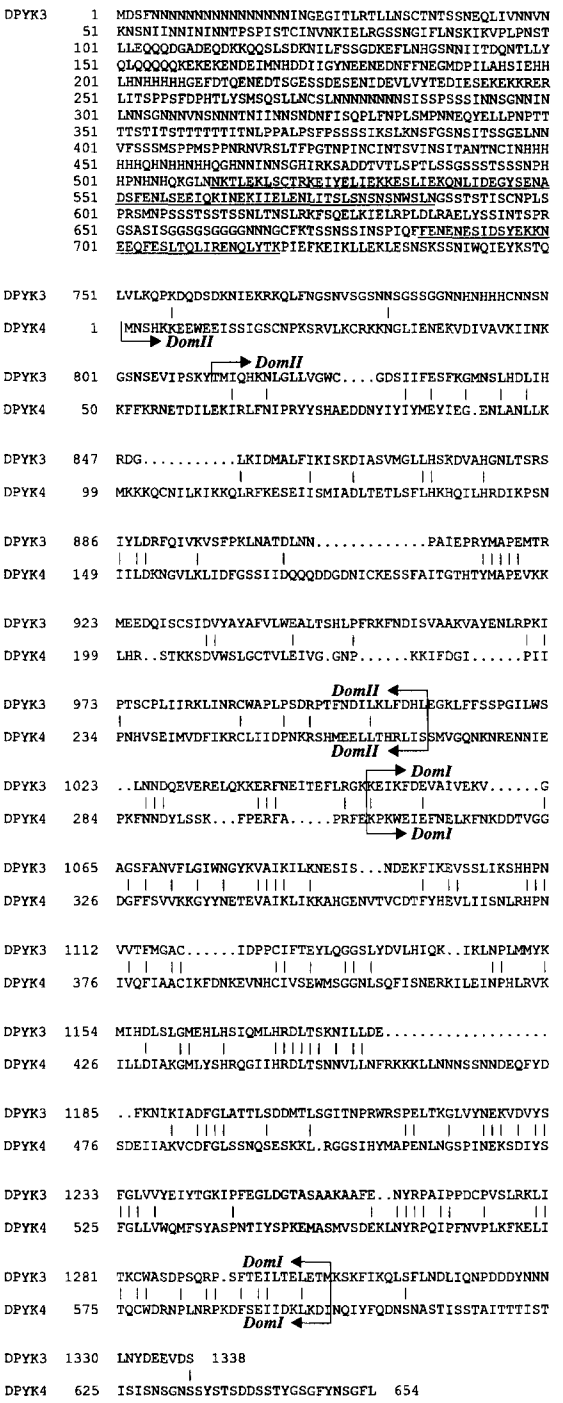

Fig. 1. Comparison of the predicted amino acid sequences of the protein tyrosine kinases DPYK3 and DPYK4 from *D. discoideum*. Predicted coiled-coil regions are underlined. The arrows indicate borders of catalytic domains (DomI and DomII) as specified in Fig. 2. The DNA sequences have been assigned GenBank accession numbers U64830 (DPYK3) and L76171 (DPYK4).

Coomassie blue staining of the precipitate (Fig. 4A, lane 1) or by labelling immunoblots with anti-phosphotyrosine antibody (Fig. 4A, lanes 2,3). As independent proof of tyrosine phosphorylation, proteins precipitated with anti-phosphotyrosine or anti-β-galactosidase antibody were incubated with [γ-^{32}P]ATP. Analysis of the proteins by SDS-PAGE revealed strong [^{32}P]phosphate incorporation at the position of the

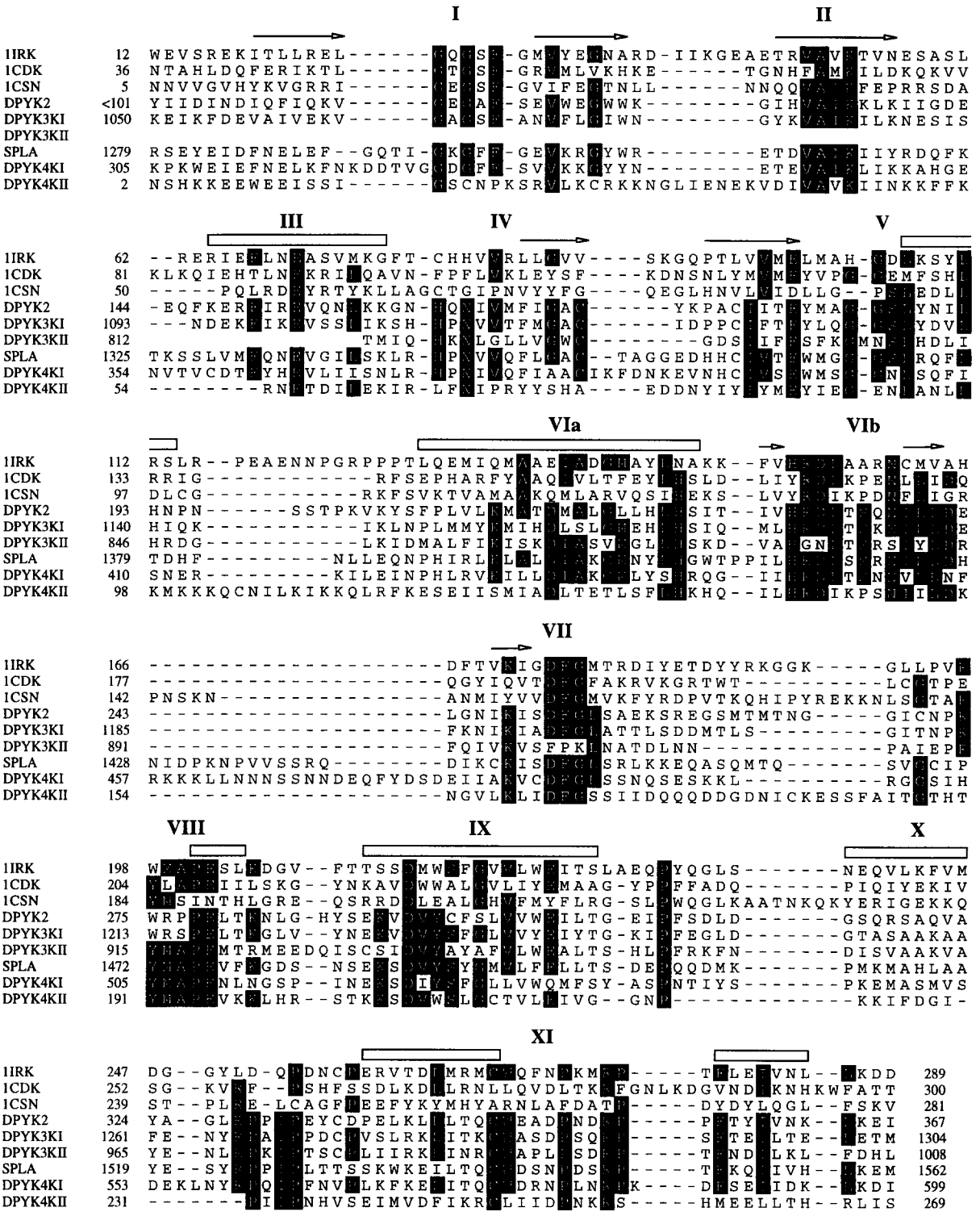

Fig. 2. Alignment of *Dictyostelium* protein kinase domains with three protein kinases of known structure: insulin receptor tyrosine kinase domain (1IRK); cAMP-dependent kinase catalytic subunit (1CDK); and casein kinase 1 (1CSN); KI and KII refer to kinase domains I and II of DPYK3 and DPYK4, respectively. The consensus secondary structure as defined by Dali [20] is shown above the sequences (arrows, β-strands; open boxes, α-helices). Roman numerals indicate 12 distinct motifs defined by Hanks and Hunter [25]. Residues conserved in a majority of the sequences in the alignment are shown in reverse type. The numbering of DPYK2 is preceded by an arrowhead, as this sequence has been published only as a C-terminal fragment [10].

fusion protein (Fig. 4A, lanes 4,5), and phosphoamino acid analysis of the labelled band showed that only tyrosine residues were phosphorylated under these conditions in vitro (Fig. 4B).

A 42 kDa fragment of DPYK4, comprising domain I of this kinase, was produced in *E. coli* as a His-tagged protein. Anti-His-tag antibody indicated significant production in induced bacteria of the undegraded fragment (Fig. 5). Anti-

phosphotyrosine antibody recognized proteins only in bacteria that produced this DPYK4 fragment. In addition to the strongly phosphorylated band of the DPYK4 fragment itself, several bands of higher M_r were labelled by the anti-phosphotyrosine antibody, suggesting that bacterial proteins served as substrates for the tyrosine kinase activity of this fragment (Fig. 5).

3.3. Analysis of the DPYK3 primary structure

The DPYK3 protein is deduced to contain 1338 residues, which corresponds to a calculated molecular mass of 150 kDa. The protein contains neither transmembrane segments nor SH2 domains. Following Tyr-811, DPYK3 contains two protein kinase domains (domain II, Thr-812 to Leu-1008; domain I, Lys-1050 to Met-1304), connected by a linker of 41 residues. Domain I is well-conserved and, as demonstrated in this article, is functional as a protein tyrosine kinase. Domain II lacks most of the smaller lobe of the protein kinase domain. In view of this truncation, it is questionable whether this domain could bind ATP. It also diverges significantly from the kinase sequence consensus and lacks several residues that are conserved in all known functional kinases [25]. The Asp in motif VIb, which probably accepts the proton from the attacking substrate hydroxyl group, is replaced by Asn-880, and Asn in this motif, which determines the conformation of the catalytic loop, is replaced by Ser-885. Also, Asp in motif VII, which chelates the Mg^{2+} ion essential for the transfer of the γ-phosphate group, is replaced by Phe-898 (Fig. 2). It thus appears highly unlikely that domain II acts as a catalytic domain. However, this domain may still be able to interact via its large lobe with regulatory proteins, and may thus modulate kinase-mediated signal transduction.

In a dendrogram of protein kinases (Fig. 6), both domains I and II of DPYK3 are found in a branch that is mainly defined by the four *Dictyostelium* kinases DPYK1 (SplA), DPYK2, DPYK3, and DPYK4. This branch is approximately equidistant to the branches containing the Raf and the TGFβ/activin-like receptor families. Collectively, these three branches

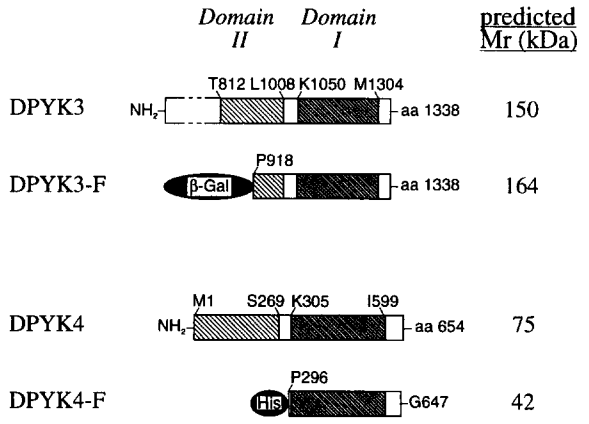

Fig. 3. Diagram of the DPYK3 and DPYK4 domains and their expression constructs. Designations of the proteins are given on the left, their predicted molecular masses on the right. Domains I and II refer to the putative protein kinase consensus domains. Amino acid residues forming the plausible boundaries of these domains or of the expressed fragments of the proteins are indicated. Fragments are expressed as fusion proteins with β-galactosidase (β-Gal) or with a histidine tag (His).

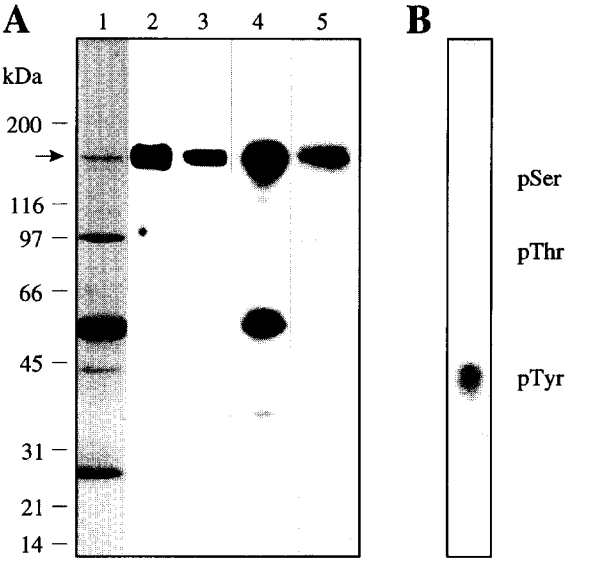

Fig. 4. Immunoprecipitation of a β-Gal-DPYK3 fusion protein to demonstrate tyrosine phosphorylation. Soluble fractions of induced bacteria producing a fusion of the DPYK3 C-terminal fragment as displayed in Fig. 3 were used for immunoprecipitation with anti-phosphotyrosine antibody (lanes 1,2,4) or anti-β-galactosidase antibody (lanes 3,5). Precipitated proteins were separated by SDS-PAGE. (A) Lane 1, proteins in a precipitate stained with Coomassie blue; lanes 2,3, immunoblots probed with iodinated anti-phosphotyrosine antibody; lanes 4,5, precipitated proteins labelled with [γ-^{32}P]ATP. (B) Phosphoamino acid analysis of the fusion protein shown in A, lane 5.

form a group of kinases that is most closely related to, but clearly distinct from, the protein tyrosine kinase (PTK) group.

The N-terminal region of DPYK3 up to Tyr-811 appears to be unrelated to any protein in current sequence databases. This region is characterized by several stretches of high sequence entropy. First, this region contains several repeats of single amino acid residues which are reminiscent of Asn and Gln stretches in other proteins from *D. discoideum* [26], for instance, in two histidine kinases from this organism [27,28]. The most prominent repeats in DPYK3 are stretches of up to 16 Asn residues. Second, the N-terminal region of DPYK3 contains a cluster of 17 hydroxyamino acids, including 13 threonine residues from Thr-349 to Thr-365. Third, two clusters of histidine residues are located in this region. The first of these clusters, from His-199 to His-208, comprises 8 histidine residues, the second, from His-448 to His-461, contains 11 of these residues.

The N-terminal region contains also a predicted coiled-coil segment of approx. 10 heptads between Asn-512 and Asn-587, as well as a region of lower, but probably still significant, coiled-coil scores between Phe-686 and Lys-719. The first predicted coiled-coil region contains a strong discontinuity between Leu-541 and Asp-543.

3.4. Analysis of the DPYK4 primary structure

The DPYK4 protein is deduced to contain 654 residues. With its calculated molecular mass of 75 kDa, DPYK4 has only half the size of DPYK3, due to the lack of a portion in its sequence corresponding to the N-terminal region of DPYK3 (Fig. 1). DPYK4 is made up of two kinase domains connected by a linker of 35 residues (domain II, Met-1 to Ser-269; domain I, Lys-305 to Ile-599). Only domain I of DPYK4

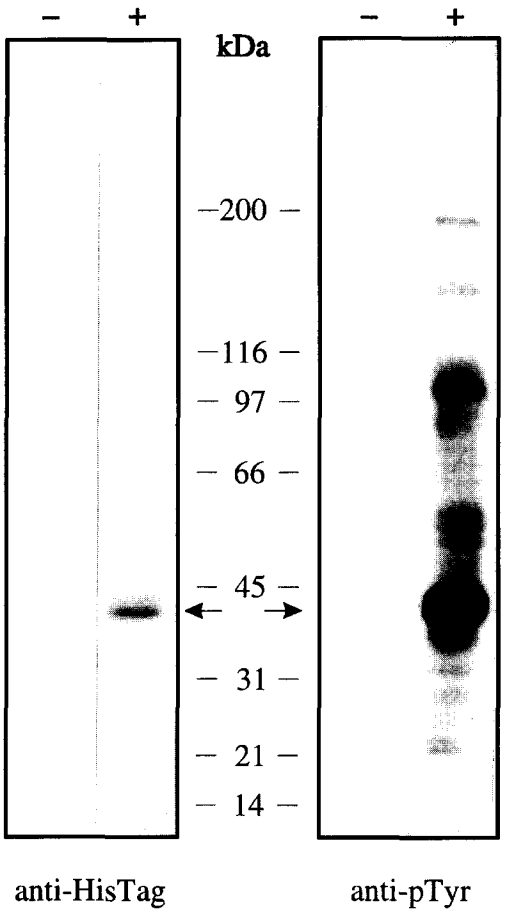

Fig. 5. Western blots of bacterial lysates showing that domain I of DPYK4 has protein tyrosine kinase activity. Bacteria expressing the His-tagged carboxy-terminal domain of DPYK4 were untreated (–) or induced with IPTG (+), and equivalent amounts of lysates were subjected to SDS-PAGE and blotting onto nitrocellulose. The blots were probed with anti-His tag or anti-phosphotyrosine antibody.

is a member of the *Dictyostelium* tyrosine kinase branch (Fig. 6). This domain is well conserved and shown in this article to be functional as a protein tyrosine kinase. Domain II is equally well-conserved. Since of the invariant residues only a glycine in motif I appears to be missing (Fig. 2), it is likely that domain II is functional as a protein kinase. This domain is part of a group of Ser/Thr protein kinases that contains members of the MAP kinase family and some dual-specificity kinases.

4. Discussion

4.1. Two kinase domains in DPYK3 and DPYK4

Both proteins described in this article contain two protein kinase domains. In this respect these proteins resemble the JAKs of mammalian cells [29], but because of the distant position of their catalytic domains in a dendrogram (PTK group), the *Dictyostelium* kinases are not considered to be homologs of these mammalian kinases. Whereas domain I in DPYK3 and 4 is highly conserved, domain II is more divergent and, in the case of DPYK3, also apparently truncated. We have demonstrated that in both proteins domains I are active as tyrosine kinases, but we have not determined the activity, if any, of domains II. On the basis of phylogenetic considerations, it appears likely that domain II in DPYK4 is derived from a Ser/Thr kinase. For domain II of DPYK3, the same considerations would indicate tyrosine kinase activity, but because of truncation and lack of sequence conservation this domain is most likely inactive. By analogy to JAKs, whose N-terminal domain seems to have only regulatory function [30], domain II of DPYK3 may still be able to bind proteins and thus have regulatory activity.

Domains I and II of DPYK3, as well as domain I of DPYK4 belong to a branch of protein tyrosine kinase domains that are defined by the *Dictyostelium* kinases (DPYK1–4). This branch is embedded in a group that consists mostly of Ser/Thr kinases, but nevertheless is more closely related to the tyrosine kinase superfamily than to the Ser/Thr kinase superfamily.

Domain II of DPYK4 is clearly distinct and belongs to a group of kinase domains that are related to catalytic domains of the Ser/Thr kinase superfamily. Because of the strong divergence between domains I and II of DPYK4, it appears likely that the protein evolved by fusion of a tyrosine kinase with a Ser/Thr kinase domain. In the case of DPYK3, the two domains are more closely related to each other. It is therefore unclear whether this protein evolved by fusion of two related kinases or by tandem gene duplication and subsequent divergence.

4.2. The coiled-coil region and histidine clusters in DPYK3

A number of non-receptor protein kinases, such as Nima or Fes/Fps, contain coiled-coil segments, occasionally of considerable length. Moreover, many receptor tyrosine kinases, which do not contain coiled coils, are fused to heterologous coiled-coil proteins during oncogenic activation [31]. For example, the Trk oncogene has been found to be activated by no less than three different fusions, to tropomyosin, to TPR, and to TFG, all three containing coiled-coil segments. The activation is thought to result from the conversion of the receptor kinase into a constitutive oligomer.

The detection of a second potential coiled-coil region in DPYK3, albeit with lower scores, raises the possibility of a further regulatory mechanism by analogy to the heat-shock transcription factors. It is thought that, prior to activation, these factors are monomers containing an intramolecular coiled coil [32]. During activation, the intramolecular interaction between the coiled-coil helices is disrupted, allowing them to form an intermolecular, trimeric coiled coil. Only the trimeric form of the transcription factor is able to activate transcription from heat-shock promoters. By analogy, it is conceivable that DPYK3 alternates between monomeric and oligomeric forms as part of a regulatory cycle.

The prominent clusters of histidine residues in the N-terminal domain of DPYK3 are reminiscent of hisactophilins, proteins of *Dictyostelium* cells that contain three histidine-rich loops on their surface [33]. Hisactophilins can shuttle between cytoplasm, plasma membrane and the nucleus in response to cytoplasmic pH-changes that modulate the charge of histidine residues [34].

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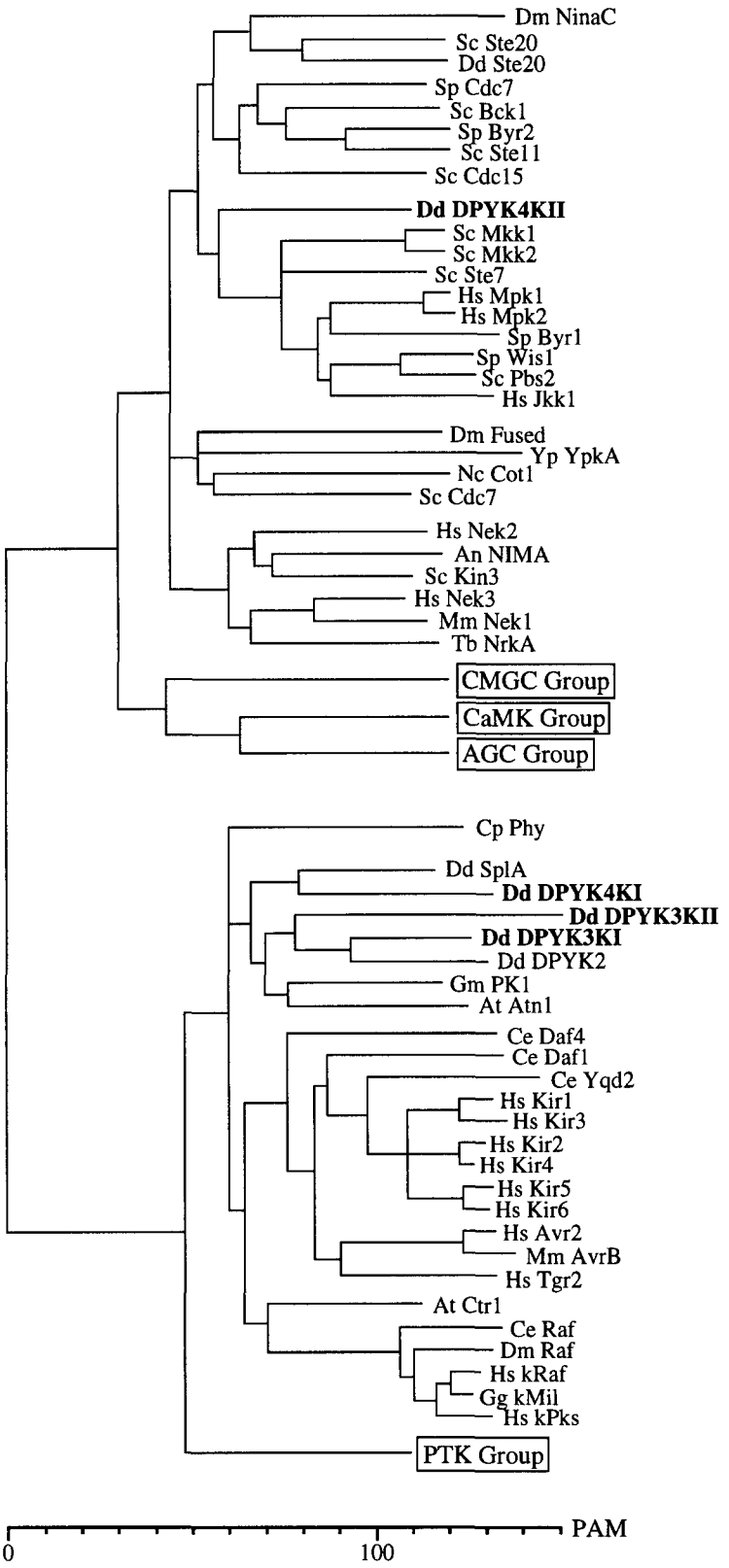

Fig. 6. Dendrogram of protein kinase domains, based on the dendrogram of Hanks and Hunter [25]. The branch length is given in PAM (percent accepted mutation) units. The branches containing the DPYK3 and DPYK4 kinase domains KI and KII were recalculated as described in Section 2 and are shown in detail. Their positions relative to the main kinase families, cyclin-dependent kinases (CMGC), calcium/calmodulin-dependent kinases (CaMK), cAMP-dependent kinases (AGC), and protein tyrosine kinases (PTK), are taken from Hanks and Hunter [25]. The protein name abbreviations are taken from the SwissProt and GenBank databases. The organisms are: An, *Aspergillus nidulans*; At, *Arabidopsis thaliana*; Ce, *Caenorhabditis elegans*; Cp, *Ceratodon purpureus*; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster*; Gg, *Gallus gallus*; Gm, *Glycine max*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Nc, *Neurospora crassa*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Tb, *Trypanosoma brucei*; Yp, *Yersinia pseudotuberculosis*.

References

[1] Parent, C.A. and Devreotes, P.N. (1996) Annu. Rev. Biochem. 65, 411–440.
[2] Browning, D.D., The, T. and O'Day, D.H. (1995) Cell. Signal. 7, 481–489.
[3] Clarke, M. and Gomer, R.H. (1995) Experientia 51, 1124–1134.
[4] Reymond, C.D., Schaap, P., Veron, M. and Williams, J.G. (1995) Experientia 51, 1166–1174.
[5] Segall, J.E., Kuspa, A., Shaulsky, G., Ecke, M., Maeda, M., Gaskins, C., Firtel, R.A. and Loomis, W.F. (1995) J. Cell Biol. 128, 405–413.
[6] Gaskins, C., Clark, A.M., Aubry, L., Segall, J.E. and Firtel, R.A. (1996) Genes Dev. 10, 118–128.
[7] Knetsch, M.L.W., Epskamp, S.J.P., Schenk, P.W., Wang, Y., Segall, J.E. and Snaar-Jagalska, B.E. (1996) EMBO J. 15, 3361–3368.
[8] Jungbluth, A., Von Arnim, V., Biegelmann, E., Humbel, B., Schweiger, A. and Gerisch, G. (1994) J. Cell Sci. 107, 117–125.
[9] Jungbluth, A., Eckerskorn, C., Gerisch, G., Lottspeich, F., Stocker, S. and Schweiger, A. (1995) FEBS Lett. 375, 87–90.
[10] Tan, J.L. and Spudich, J.A. (1990) Mol. Cell. Biol. 10, 3578–3583.
[11] Nuckolls, G.H. and Spudich, J.A. (1995) in: The Protein Kinase Facts Book I (Hardie, G. and Hanks, S. eds.) pp. 374–375, Academic Press, San Diego, CA.
[12] Fendly, B.M., Winget, M., Hudziak, R.M., Lipari, M.T., Napier, M.A. and Ullrich, A. (1990) Cancer Res. 50, 1550–1558.
[13] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
[14] Williamson, B.D. and Rutherford, C.L. (1994) BioTechniques 17, 670–672.
[15] Faix, J., Steinmetz, M., Boves, H., Kammerer, R.A., Lottspeich, F., Mintert, U., Murphy, J., Stock, A., Aebi, U. and Gerisch, G. (1996) Cell 86, in press.
[16] Schweiger, A., Mihalache, O., Muhr, A. and Adrian, I. (1990) FEBS Lett. 268, 199–202.
[17] Madden, T.L., Tatusov, R.L. and Zhang, J. (1996) Methods Enzymol. 266, 131–141.
[18] Lupas, A. (1996) Methods Enzymol. 266, 513–525.
[19] Schuler, G.D., Epstein, J.A., Ohkawa, H. and Kans, J.A. (1996) Methods Enzymol. 266, 141–162.
[20] Holm, L. and Sander, C. (1996) Methods Enzymol. 266, 653–662.
[21] Schuler, G.D., Altschul, S.F. and Lipman, D.J. (1991) Proteins 9, 180–190.
[22] Gonnet, G.H. and Benner, S.A. (1991) Comput. Biochem. Res. ETH 154, 1–18.
[23] Higgins, D.G., Thompson, J.D. and Gibson, T.J. (1996) Methods Enzymol. 266, 383–402.
[24] Felsenstein, J. (1996) Methods Enzymol. 266, 418–427.
[25] Hanks, S.K. and Hunter, T. (1995) FASEB J. 9, 576–596.
[26] Kimmel, A.R. and Firtel, R.A. (1982) in: The Development of *Dictyostelium discoideum* (Loomis, W.F. ed.) pp. 233–324, Academic Press, San Diego, CA.
[27] Schuster, S.C., Noegel, A.A., Oehme, F., Gerisch, G. and Simon, M.I. (1996) EMBO J. 15, 3880–3889.
[28] Wang, N., Shaulsky, G., Escalante, R. and Loomis, W.F. (1996) EMBO J. 15, 3890–3898.
[29] Harpur, A.G., Andres, A.-C., Ziemiecki, A., Aston, R.R. and Wilks, A.F. (1992) Oncogene 7, 1347–1353.
[30] Velazquez, L., Mogensen, K.E., Barbieri, G., Fellous, M., Uzé, G. and Pellegrini, S. (1995) J. Biol. Chem. 270, 3327–3334.
[31] Rodrigues, G.A. and Park, M. (1994) Curr. Opin. Gen. Dev. 4, 15–24.
[32] Rabindran, S.K., Haroun, R.I., Clos, J., Wisniewski, J. and Wu, C. (1993) Science 259, 230–234.
[33] Habazettl, J., Gondol, D., Wiltscheck, R., Otlewski, J., Schleicher, M. and Holak, T.A. (1992) Nature 359, 855–858.
[34] Hanakam, F., Albrecht, R., Eckerskorn, C., Matzner, M. and Gerisch, G. (1996) EMBO J. 15, 2935–2943.