Use of double-stranded RNA-mediated interference to determine the substrates of protein tyrosine kinases and phosphatases

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Despite the wealth of information generated by genome-sequencing projects, the identification of *in vivo* substrates of specific protein kinases and phosphatases is hampered by the large number of candidate enzymes, overlapping enzyme specificity and sequence similarity. In the present study, we demonstrate the power of RNA interference (RNAi) to dissect signal transduction cascades involving specific kinases and phosphatases. RNAi is used to identify the cellular tyrosine kinases upstream of the phosphorylation of Down-Syndrome cell-adhesion molecule (Dscam), a novel cell-surface molecule of the immunoglobulin–fibronectin super family, which has been shown to be important for axonal path-finding in *Drosophila*. Tyrosine

phosphorylation of Dscam recruits the Src homology 2 domain of the adaptor protein Dock to the receptor. Dock, the orthologue of mammalian Nck, is also essential for correct axonal path-finding in *Drosophila*. We further determined that Dock is tyrosine-phosphorylated *in vivo* and identified DPTP61F as the protein tyrosine phosphatase responsible for maintaining Dock in its non-phosphorylated state. The present study illustrates the versatility of RNAi in the identification of the physiological substrates for protein kinases and phosphatases.

Key words: axonal path-finding, Dscam, signal transduction, Src-like tyrosine kinase.

INTRODUCTION

Protein phosphorylation is an essential covalent post-translational modification fundamental to the control of many cellular processes [1–3]. The importance of protein phosphorylation in cell physiology is reflected by the large number of protein kinases and protein phosphatases identified in sequencing projects of eukaryotic genomes. Analysis of the recently completed *Drosophila* genome predicts 251 protein kinases and 86 protein phosphatases [4]. In sharp contrast, our knowledge of cellular substrates and of the signal-transduction pathways in which these kinases and phosphatases participate is very limited.

Genetic analyses in *Drosophila* and in mammalian systems demonstrate that protein tyrosine phosphatase (PTP) and tyrosine kinase (TK) families are instrumental in controlling neuronal outgrowth and guidance. Within the growth cone, tyrosine-phosphorylated proteins are present at the tips of filopodia and the level of tyrosine phosphorylation is modulated by factors that influence axon outgrowth [5]. In addition, inhibitors that block TKs or phosphatases have dramatic effects on axon extension *in vitro* and alter axon path-finding behaviour *in vivo* [6,7]. In *Drosophila* embryos, four receptor PTPs, namely DLAR, DPTP69D, DPTP99A and DPTP10D, are selectively localized to CNS axons in late-stage embryos and are required for the directed guidance of specific axons [8,9]. One intracellular tyrosine phosphatase DPTP61F, associates with Dock, an adaptor protein previously shown to be involved in axonal guidance, but it has not been genetically linked to path-finding [10]. Less is known about the role of TKs, but Derailed, a receptor TK and Abl, an intracellular TK, have been shown to be important for the correct development of multiple axon pathways [11,12].

In the present study, we used RNA interference (RNAi) of gene expression to identify the protein TKs upstream of the *in vivo* phosphorylation of Dscam, a transmembrane receptor related to the human protein, Down-Syndrome cell-adhesion molecule (Dscam) [13]. Dscam is expressed on axons of the embryonic central nervous system and Dscam null mutations are early-larval-lethal. Furthermore, *Dscam* mutants display defects in the axonal guidance of the Bolwig's organ, which are similar to those seen in *dock* mutants [14]. This, in conjunction with the physical interaction between Dscam and Dock, suggests that Dscam acts as an axonal guidance receptor upstream of Dock. In an effort to understand the intracellular signal-transduction events initiated by Dscam phosphorylation, we began to investigate which TKs were responsible for its modification. Phosphorylation of Dscam is likely to mediate the assembly of an intracellular signalling complex organized by Dock, which is responsible for the intracellular conversion of axonal guidance cues. Using RNAi to knockout functionally *Drosophila* TKs, we have demonstrated that Src42A and Src64 are the kinases primarily upstream of *in vivo* Dscam phosphorylation.

In the course of our investigation on Dscam tyrosine phosphorylation, we determined that Dock also becomes tyrosine-phosphorylated *in vivo*. We have used RNAi to show that the PTP, namely DPTP61F, is essential for regulating Dock phosphorylation, making Dock the first physiological substrate identified for DPTP61F.

Finally, supporting our *in vivo* results, we show *in vitro* that Src42A can directly and efficiently phosphorylate Dscam's intracellular domain and that DPTP61F can directly dephosphorylate Dock. The approach outlined here will be widely used in sorting out the participation of kinases and phosphatases in other signal-transduction pathways.

Abbreviations used: Dscam, Down-Syndrome cell-adhesion molecule; dsRNA, double-stranded RNA; GST, glutathione S-transferase; MBP, myelin basic protein; PTP, protein-tyrosine phosphatase; RNAi, RNA interference; SH2, Src homology 2; TK, tyrosine kinase.

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EXPERIMENTAL

Cell culture and RNAi

Drosophila S2 cells were propagated as described previously [15]. Double-stranded RNA (dsRNA) treatment was as described previously [15a], except that 2×10^6 cells were seeded per well of six-well culture dishes.

Expression and purification of recombinant proteins

Full-length Dock and the intracellular domain of Dscam (residues 1641–2016) were expressed as N-terminal His₆-tagged fusion proteins using the PT7-LOH expression vector in *Escherichia coli* Novablue DE3 (Novagen Inc, Madison, WI, U.S.A.). The fusion proteins were expressed and purified using a single Ni-agarose purification step. The kinase domain of Src42A (amino acids 228–517) was cloned into pGEX6P1 (Pharmacia, Piscataway, NJ, U.S.A.) and expressed in *E. coli* BLR (Novagen Inc). The glutathione S-transferase (GST) fusion protein was purified as described previously [16]. All fusion proteins were 80–90% pure as determined by Coomassie Blue staining. Recombinant GST–DPTP61F was a gift from Dr Gregory Taylor (Department of Biological Chemistry, University of Michigan, Ann Arbor, MI, U.S.A.).

RNAi

dsRNA fragments of approx. 700 bp were prepared as described previously [15a]. All primers used in the PCR contained a 5'-T7 RNA polymerase-binding site (GAATTAATACGACTCACTATAGGAGA), followed by sequences specific for targeted genes. Primers used to generate specific dsRNA were as follows: Dack (GenBank® accession no. AF181642, sense primer 2197–2213, anti-sense 2807–2824), Src42A (accession no. D42125, sense primer 977–997, anti-sense 1710–1733), Src64 (accession no. M11917, sense primer 688–710, anti-sense 1393–1415), Shark (accession no. U37773, sense primer 990–1014, anti-sense 1713–1732), Tec29/Src28C (accession no. M16599, sense primer 437–457, anti-sense 1200–1221), Abl (accession no. M19692, sense primer 2318–2338, anti-sense 3497–3513), DPTP61F (accession no. L11253, sense primer 1127–1146, anti-sense 1734–1751), DPTP10D (accession no. M80465, sense primer 1109–1130, anti-sense 1824–1845), DPTP69D (accession no. M27699, sense primer 189–211, anti-sense 874–896), corkscrew (accession no. U19909, sense primer 16179–16201, anti-sense 7117–17134), DLAR (accession no. M27700, sense primer 1770–1792, anti-sense 2485–2509).

Antibody affinity purification

Polyclonal antibodies were raised in rabbits against N-terminal His₆-tagged Dock and Dscam fusion proteins (Cocalico, Reamstown, PA, U.S.A.). Antibodies were purified as described previously [17]. Polyclonal antibodies against Src42A and Src64 were generated using peptides corresponding to the C-terminus of coupled KHL (Research Genetics, Huntsville, AL, U.S.A.). 4G10 anti-phosphotyrosine antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.).

Western-blot analysis, immunoprecipitation and Src42A kinase assay

For Western-blot analysis, S2 cells were harvested, pelleted and lysed as described previously [17], using RIPA buffer [50 mM Tris (pH 8.0)/150 mM NaCl/1% Nonidet P40/0.5% deoxycholate/0.1% SDS/1 mM NaVO₄/10 mM NaF/0.4 mM EDTA/

10% glycerol] containing complete protease inhibitor cocktail tablets (Roche, Indianapolis, IN, U.S.A.). Cell lysates (50 µl) were added to 10 µl of 6× Laemmli loading buffer and analysed by SDS/PAGE (7.5% or 10% acrylamide). S2 cells were treated with pervanadate, which was prepared as described previously [10]. Immunoprecipitation and Western-blot analyses were performed as described previously [17]. Antibodies were diluted at the following concentrations: 4G10 (1:2000), anti-Dock (1:1000), anti-Dscam (1:1000). Goat anti-rabbit or anti-mouse IgG-conjugated horseradish peroxidase at 1:3000 (Bio-Rad Laboratories, Hercules, CA, U.S.A.) was used as secondary antibody. ECL® (enhanced chemiluminescence) Western-blotting detection system (Amersham International, Arlington, IL, U.S.A.) was used to detect the proteins.

Src42A kinase and DPTP61F phosphatase assays

S2 extracts used for Src42A kinase reactions were made using RIPA buffer without deoxycholate and SDS (RIPA kinase). Immunoprecipitation was done as follows: lysates were cleared by centrifugation at 14000 g, 4 °C for 10 min, incubated at 4 °C for 1 h with 50 µl of Protein A-agarose beads (BRL Life Technologies), pre-bound with Src42A antisera (10 µl of antisera/200 µl of 50% bead slurry). Beads were washed three times with 1 ml of RIPA kinase, twice with 1 ml of RIPA kinase containing 1 M NaCl and twice with kinase buffer [50 mM Tris (pH 7.2)/62.5 mM MgCl₂/12.5 mM MnCl₂/1 mM EGTA/0.1 mM NaVO₄/1 mM dithiothreitol]. Immunoprecipitated Src42A beads (10 µl) were mixed with 10 µg of myelin basic protein (MBP), acid-denatured enolase or Dscam, and incubated for 30 min at 25 °C in the presence of 60 µM of ATP spiked with 5 µCi of [γ-³²P]ATP (6000 Ci/mmol). Reactions were terminated by the addition of 13.3 µl of 6× Laemmli loading buffer, boiled for 2 min and analysed by electrophoresis and autoradiography. To test whether DPTP61F can directly dephosphorylate Dock, purified GST–DPTP61F fusion protein was incubated with purified His₆-tagged Dock phosphorylated by preincubation with immunoprecipitated Src42A or GST–Src42A as described above. Kinase reactions were terminated by the addition of EDTA to a final concentration of 15 µM. Aliquots of phosphorylated Dock (approx. 10 µg) were incubated for 1 or 10 min with 2.5 µg of affinity-purified GST–DPTP61F. Reactions were terminated by the addition of 6× Laemmli loading buffer, and the phosphorylation of Dock was monitored by electrophoresis and autoradiography.

RESULTS AND DISCUSSION

The *Drosophila* guidance receptor Dscam is synthesized in S2 cells as a 270 kDa protein, which is readily visible on Western-blot analyses using antibodies directed against the C-terminus of the protein (Figure 1). Furthermore, Dscam is tyrosine-phosphorylated in these cells, and dsRNAs directed against Dscam effectively abolish expression of this protein, confirming the identity of the tyrosine-phosphorylated protein (Figure 1). To determine the cellular kinases responsible for this phosphorylation, we ablated the expression of several TKs using RNAi.

We began by testing a number of cytoplasmic TKs, including Src42A, Src64, Abl, Tec29, Dack and Shark. Src42A, Src64, Abl and Tec 29 were chosen for analysis because the Src family kinases have been shown to be involved in the development of the mammalian nervous system [18]. Dack was considered a likely candidate, since it is found in a complex with Dock and

Figure 1 Dscam produced in S2 cells is tyrosine-phosphorylated

Protein extracts from wild-type S2 cells (—) and S2 cells treated with dsRNA directed against Dscam (+) were separated by SDS/PAGE and Western-blot analysis was performed using Dscam and pTyr antibodies. The higher-mobility tyrosine-phosphorylated band serves as an internal control for the specificity of the Dscam knockout.

Dscam [19], and Shark was chosen to represent all the cytoplasmic TKs known before the completion of the genome-sequencing project. Among the protein TKs examined, only dsRNA specific for Src42A dramatically decreased Dscam tyrosine phosphorylation. Surprisingly, the dsRNA specific for the other *Drosophila* Src isoform Src64, had no detectable effect on Dscam phosphorylation (Figure 2A, upper panel). In contrast, synergy was observed when Src64 dsRNA was used in combination with Src42A dsRNA, demonstrating a limited functional redundancy between the two Src isoforms. Consistent with our results, Src kinase functional redundancy has been reported in mice knockout studies (reviewed in [20,21]) and recently observed in *Drosophila* embryonic development [22]. Western-blot analyses using Src-specific antibodies verified the efficacy of the dsRNA treatment for ablating the expression of Src42A and Src64 (results not shown). dsRNAs directed against Dack did not significantly reduce the level of Dscam tyrosine phosphorylation. This is somewhat surprising, since Dack was isolated as a binding partner of Dock's Src homology 2 (SH2) domain and would therefore be in the ideal position to phosphorylate Dscam. The elimination of the Abl, Shark and Tec29 TKs also does not effect the level of Dscam tyrosine phosphorylation. The different levels of Dscam tyrosine phosphorylation cannot be attributed to a discrepancy in protein loading, as equal amounts of Dscam are present in each lane (Figure 2A, lower panel). The low level of tyrosine phosphorylation detected on Dscam in the absence of both the Src isoforms may be explained by a low residual activity of these kinases. In fact, as we have described previously, RNAi typically reduces protein levels by approx. 90–95%. Alternatively, other kinase(s) may be responsible for the residual tyrosine phosphorylation. Our results clearly demonstrate that Src42A and Src64 are the major kinases upstream of Dscam phosphorylation in an intact cellular context.

To demonstrate that Src42A can directly phosphorylate Dscam, we tested the ability of recombinant and immunoprecipitated Src42A to phosphorylate the intracellular domain of Dscam *in vitro*. Both the catalytic domain of Src42A produced in bacteria as a GST fusion protein and immunoprecipitated Src42A were capable of phosphorylating Dscam, as well as common artificial substrates of Src, including MBP and enolase (Figure 2B). In the GST–Src42A-treated samples, autophosphorylated GST–Src42A is visible as a phosphorylated protein with a mobility slightly lower than that of Dscam.

In the course of our study we observed that Dock immunoprecipitated from pervanadate-treated S2 cells was tyrosine-phosphorylated (Figure 3). Consistent with our results, tyrosine phosphorylation of mammalian Nck has also been observed [23]. To date, little is known about the ability of intracellular TKs

Figure 2 Src42A and Src64 are the major TKs for Dscam

(A) Profile of tyrosine-phosphorylated proteins in various S2 cell knockouts. Protein extracts from untreated S2 cells (—) or S2 cells treated with the indicated dsRNAs directed against cytoplasmic TKs were separated by SDS/PAGE and Western-blot analysis was performed using pTyr antibodies. An arrow denotes the relative mobility of Dscam. In the lower panel, the Western-blot analysis was stripped and re-probed with Dscam antibodies to confirm equal loading. (B) *In vitro* kinase assay using immunoprecipitated Src42A and bacterially expressed GST–Src42A. Src42A was immunoprecipitated from S2 cells using either pre-immune serum (control) or the affinity-purified antibody specific for Src42A generated as described in the Experimental section. GST–Src42A (kinase domain) was produced and isolated from bacteria as described in the Experimental section. Approx. 10 µg of recombinant Dscam, MBP or enolase was used as a substrate for Src42A. Phosphorylated proteins were separated by SDS/PAGE, stained with Coomassie Blue to verify equal loading (results not shown) and visualized by autoradiography.

Figure 3 Dock is tyrosine-phosphorylated in S2 cells

S2 cells were either left untreated or vanadate-treated in the presence of dsRNA for Dock. Cell extracts were separated by SDS/PAGE and Western-blot analysis was performed using antibodies recognizing Dock or pTyr. An arrow indicates the protein band corresponding to Dock.

to phosphorylate Dock. Using dsRNA directed against Src42A, we can demonstrate an approx. 50% reduction in Dock tyrosine phosphorylation (results not shown). Therefore we conclude that although Src42A is upstream of Dock phosphorylation in S2 cells, Dock can also be phosphorylated by other TKs. Since little

Figure 4 DPTP61F is responsible for maintaining Dock in an unphosphorylated state

(A) Cell extracts were prepared from untreated S2 cells or S2 cells treated with dsRNAs corresponding to the indicated tyrosine phosphatases. These extracts were separated by SDS/PAGE and Western-blot analysis was performed using antibodies directed against phosphotyrosine or Dock. An arrow indicates the mobility of Dock. (B) Phosphatase assay using recombinant DPTP61F and GST–Src42A-phosphorylated Dock. 32 P-labelled Dock was either untreated (—) or treated with GST–DPTP61F for 1 min or 10 min (1', 10') as described in the Experimental section. The reduction of phosphorylated Dock was monitored by SDS/PAGE followed by autoradiography.

or no tyrosine phosphorylation of Dock was detected in the absence of PTP inhibitors, we anticipated that Dock was constitutively dephosphorylated *in vivo*. As Dock was initially identified as a protein that interacts with the tyrosine phosphatase DPTP61F, we speculated that DPTP61F was responsible for dephosphorylating Dock *in vivo*. Blocking the DPTP61F expression by RNAi was sufficient to augment Dock tyrosine phosphorylation (Figure 4A). Other phosphatases involved in neuronal development, namely DPTP69, DPTP10D, DLAR and corkscrew, were examined for their possible role in Dock dephosphorylation [24]. The fact that dsRNAs specific for these four additional PTPs were ineffective in increasing Dock tyrosine phosphorylation highlights the selectivity of DPTP61F for Dock. We have shown that DPTP61F and Dock constitutively associate with each other in cultured cells and in embryos [10]. Our results provide compelling evidence that DPTP61F is the phosphatase responsible for maintaining the dephosphorylated state of Dock *in vivo*.

Finally, we demonstrate that DPTP61F can directly dephosphorylate Dock *in vitro*. To prepare tyrosine phosphorylated Dock, recombinant Dock purified from *E. coli* was phosphorylated *in vitro* using GST–Src42A. The kinase reaction was stopped by the addition of EDTA and the phosphorylated Dock was then incubated with GST–DPTP61F, which efficiently removed its tyrosine phosphorylation (Figure 4B). This distinguishes Dock as the first physiological substrate identified for the PTP, DPTP61F.

Recently, Nck has been reported to associate with the PTP–PEST [25]. Nck's second SH3 domain and the proline-rich

motif PXXPXXXS present in the C-terminus of PTP–PEST mediate this interaction. Similarly, we have shown that the second SH3 domain of Dock interacts with the DPTP61F peptide GSGGSDELPPPLPPRVQ, containing the same proline motif. We show that DPTP61F dephosphorylates Dock and, by extension, speculate that the ubiquitously expressed PTP–PEST is responsible for regulating the tyrosine phosphorylation level of Nck in mammals. There is evidence that the tyrosine phosphorylation of Dock and Nck is conserved during evolution and that PTPs are dedicated to regulate this phosphorylation, suggesting the possibility that tyrosine phosphorylation of these adaptor proteins plays a novel and critical regulatory role for this family of adaptor proteins.

Our observations identify a novel function for Src kinases in axonal path-finding in *Drosophila*. Loss of Fyn, a mammalian Src family member, has been associated with a variety of neuronal abnormalities, and neurons from Fyn-deficient mice are impaired in their ability to extend processes [26]. Supporting a model in which loss of Src42A phosphorylation would prevent the association of the Dock SH2 domain with Dscam, *src42A* null mutants have been shown to display a defect in R cell projection, strikingly similar to the phenotype of *Dock* null mutant (J. C. Clemens, unpublished work). Further experiments are required to define in more detail how this phenotype is linked to the Dscam signal-transduction pathway.

It is clear that using *Drosophila* cell culture to study signal-transduction pathways has several advantages compared with the recently reported mammalian RNAi experiments [27]. First, ablation of endogenous protein expression in *Drosophila* cells using RNAi is effective and reproducible. Expression of the targeted protein is typically decreased to less than 5% of its usual expression level, resulting in a functional knockout of the protein of interest. Since the *Drosophila* cells take up the dsRNA without transfecting, the irreproducibility inherent in transfections is avoided. Furthermore, transfection efficiency is not a concern as it is in mammalian RNAi experiments. Secondly, owing to the smaller size of the *Drosophila* genome, there are fewer proteins with functional redundancy. This is particularly true for the PTP and Src kinase families, in which compensatory interactions among family members may ameliorate the phenotypic consequences of single-gene knockouts. Thus initially dissecting the complex signal-transduction pathways involved in axonal guidance in *Drosophila* cells will complement and facilitate the study of these pathways in mammalian neuronal cells.

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