

Phosphoinositide 3-kinase-dependent phosphorylation of the dual adaptor for phosphotyrosine and 3-phosphoinositides by the Src family of tyrosine kinase

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We recently identified a novel adaptor protein, termed dual adaptor for phosphotyrosine and 3-phosphoinositides (DAPP1), that possesses a Src homology (SH2) domain and a pleckstrin homology (PH) domain. DAPP1 exhibits a high-affinity interaction with PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 , which bind to the PH domain. In the present study we show that when DAPP1 is expressed in HEK-293 cells, the agonists insulin, insulin-like growth factor-1 and epidermal growth factor induce the phosphorylation of DAPP1 at Tyr¹³⁹. Treatment of cells with phosphoinositide 3-kinase (PI 3-kinase) inhibitors or expression of a dominant-negative PI 3-kinase prevent phosphorylation of DAPP1 at Tyr¹³⁹, and a PH-domain mutant of DAPP1, which does not interact with PtdIns(3,4,5) P_3 or PtdIns(3,4) P_2 , is not phosphorylated at Tyr¹³⁹ following agonist stimulation of cells. Overexpression of a constitutively active form of PI 3-kinase induced the phosphorylation of DAPP1 in unstimulated cells.

We demonstrated that Tyr¹³⁹ of DAPP1 is likely to be phosphorylated *in vivo* by a Src-family tyrosine kinase, since the specific Src-family inhibitor, PP2, but not an inactive variant of this drug, PP3, prevented the agonist-induced tyrosine phosphorylation of DAPP1. Src, Lyn and Lck tyrosine kinases phosphorylate DAPP1 at Tyr¹³⁹ *in vitro* at similar rates in the presence or absence of PtdIns(3,4,5) P_3 , and overexpression of these kinases in HEK-293 cells induces the phosphorylation of Tyr¹³⁹. These findings indicate that, following activation of PI 3-kinases, PtdIns(3,4,5) P_3 or PtdIns(3,4) P_2 bind to DAPP1, recruiting it to the plasma membrane where it becomes phosphorylated at Tyr¹³⁹ by a Src-family tyrosine kinase.

Key words: PH domain, PP2 inhibitor, protein phosphorylation, SH2 domain, Src tyrosine kinases.

INTRODUCTION

Stimulation of cells with insulin and growth factors activates members of the phosphoinositide 3-kinase (PI 3-kinase) family, which phosphorylate PtdIns(4,5) P_2 at the D-3 position of the inositol ring to generate the lipid second messenger, PtdIns(3,4,5) P_3 [1]. PtdIns(3,4,5) P_3 and its immediate breakdown product, PtdIns(3,4) P_2 , also thought to be a second messenger, interact with proteins that possess a certain type of pleckstrin homology (PH) domain [2], leading to the recruitment of these molecules to the plasma membrane and the activation of signal transduction pathways that regulate cell growth, proliferation, survival, differentiation and cytoskeletal changes [3].

Proteins possessing PH domains that interact with PtdIns(3,4,5) P_3 include the serine/threonine protein kinases, protein kinase B (PKB) [4], 3-phosphoinositide-dependent protein kinase-1 (PDK1) [5], Bruton's tyrosine kinase (BTK) [6], the Rho/Rac GTP exchange factor Vav [7], the adaptor protein Gab1 [8] and the ADP-ribosylation factor GTP exchange factor GRP1 [9]. Interaction of PtdIns(3,4,5) P_3 with PKB, BTK, Gab1 and Vav induces their translocation to the cell membrane, which is necessary for their phosphorylation and activation by specific upstream protein kinases. PKB is phosphorylated and activated by PDK1 [10], whereas BTK [6] and Vav [7] are phosphorylated and activated by members of the Src family of tyrosine kinases, and Gab1 is phosphorylated by receptor tyrosine kinases [8].

We [11] and subsequently others [12,13] have identified a widely expressed protein termed dual adaptor for phosphotyrosine and 3-phosphoinositides (DAPP1), which contains both a PH domain, that interacts specifically with PtdIns(3,4,5) P_3 /PtdIns(3,4) P_2 , as well as a phosphotyrosine-binding Src homology (SH2) domain. In the present paper, we demonstrate that the activation of PI 3-kinase in cells induces the phosphorylation of DAPP1 at Tyr¹³⁹, and provide evidence that a Src family of tyrosine kinase mediates the phosphorylation of DAPP1 at this site.

MATERIALS AND METHODS

Materials

Insulin was from Novo-Nordisk (Copenhagen, Denmark), insulin-like growth factor-1 (IGF1) and epidermal growth factor (EGF) were from Life Technologies, PP2 and PP3 were from Calbiochem, Protease inhibitor tablets were from Roche, and wortmannin, LY 294002 and anti-FLAG M2 antibody were from Sigma. Antibodies against phosphotyrosine (PY99), Lyn and Lck were from Santa Cruz; partially purified Src and Lyn and the anti-Src antibody were from Upstate Biotechnology; anti-CD2 antibody was from Plasminogen. Lck was expressed in Sf9 cells infected with baculovirus vectors by Dr Andrew

Abbreviations used: BTK, Bruton tyrosine kinase; DAPP1, dual adaptor for phosphotyrosine and 3-phosphoinositides; EGF, epidermal growth factor; GST, glutathione S-transferase; IGF1, insulin-like growth factor-1; PKB, protein kinase B; PDK1, 3-phosphoinositide-dependent protein kinase-1; PI 3-kinase, phosphoinositide 3-kinase; PH, pleckstrin homology; SH2, Src homology; Y139F, Tyr¹³⁹ → Phe substitution.

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General methods

Molecular-biology techniques were performed using standard protocols. Site-directed mutagenesis was carried out using the QuikChange kit (Stratagene), following instructions provided by the manufacturer. DNA constructs used for transfection were purified from *Escherichia coli* using the Qiagen plasmid Mega kit according to the manufacturer's protocol, and their sequences were verified using an automated DNA sequencer.

Plasmids

Constructs encoding wild-type and mutant DAPP1 with an N-terminal glutathione S-transferase (GST) tag followed by a FLAG epitope tag were subcloned into either pEBG2T, for expression in HEK-293 cells (as described previously [11]), or pGEX-4T-2, for expression in *E. coli*. The constructs expressing the wild-type and kinase-dead PI 3-kinase catalytic subunit fused to the transmembrane domain of rat CD2 (termed p110* and p110-KD), and the dominant-negative (Δ p85 α) PI 3-kinase, have been described previously [14]. Constructs expressing Lyn, Itk and Bmx (in the pSR α vector), JAK2 (in the pSVK3 vector), Btk (in the pRK5 vector) and FAK (in the pCMV5 vector) were gifts from Hiroyuki Mano (Jichi Medical School, Tochigi, Japan). The construct expressing Lck (in the pCDNA vector) was kindly provided by Dr Bart Sefton (The Salk Institute, La Jolla, CA, U.S.A.). The construct expressing c-Src (in the pUSEamp vector) was purchased from UBI.

Transfection, cell stimulation and immunoblotting

HEK-293 cells were cultured on 10-cm-diameter dishes in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal-bovine serum, and transfections were carried out using a modified calcium phosphate method [15]. In all experiments, 5 μ g of DNA of the indicated construct was used per dish, except for experiments using the GST-DAPP1[W250L] mutant, in which 10 μ g of DNA was used in order to obtain expression equal to that of the wild-type GST-DAPP1. At 24 h post-transfection the cells were serum-starved for 16 h and incubated in the presence or absence of inhibitors prior to stimulation, as described in the Figure legends. The cells were lysed in 1 ml of ice-cold lysis buffer [which consisted of 50 mM Tris/HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1% (w/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 μ M microcystin-LR, 0.1% (v/v) 2-mercaptoethanol and one tablet of protease inhibitor cocktail per 50 ml of buffer]. The lysates were cleared by centrifugation at 13000 *g* for 10 min at 2 °C, and aliquots of supernatant (50 μ g of protein) were incubated for 1 h at 4 °C with 10 μ l of GSH-Sepharose. The beads were washed twice in lysis buffer containing 0.5 M NaCl, followed by two further washes in Buffer B [50 mM Tris/HCl (pH 7.5)/ 0.1 mM EGTA/ 0.1% (v/v) 2-mercaptoethanol]. The beads were resuspended in 1 vol. of sample buffer containing 100 mM Tris/HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol and 200 mM dithiothreitol, and the released proteins were subjected to SDS/PAGE. The gels were analysed by immunoblotting with the indicated antibodies. Briefly, membranes were blocked in 50 mM Tris/HCl (pH 7.5)/0.15 M NaCl/0.5% (v/v) Tween 20 (TBST) containing either 3 mg/ml BSA (for anti-phosphotyrosine blots) or 10% (w/v) skimmed milk (for all other blots). The nitrocellulose

membranes were immunoblotted in the same buffer with anti-phosphotyrosine PY99 antibody (at a dilution of 1:2000), anti-FLAG monoclonal antibody (1:10000) and anti-Src, Lck, Lyn, CD2 antibody (1:1000). Detection was performed using horseradish-peroxidase-conjugated secondary antibodies and ECL* (Amersham).

Expression of GST-DAPP1 and GST-DAPP1[Y139F] (Tyr¹³⁹ → Phe substitution) in *E. coli*

The pGEX-4T-2 constructs encoding wild-type DAPP1 and DAPP1[Y139F] were transformed into BL21 *E. coli* cells and a 0.5 l culture was grown at 37 °C in Luria broth containing 100 μ g/ml ampicillin until the D_{600} was 0.6. Isopropyl- β -D-thiogalactoside (250 μ M) was added and the cells were cultured for a further 16 h at 26 °C. The cells were lysed and the GST-DAPP1 fusion proteins purified by affinity chromatography on GSH-Sepharose beads, as described previously for ERK2 [16].

In vitro phosphorylation of DAPP1 by Src, Lyn and Lck

A solution (20 μ l) containing bacterially expressed GST-DAPP1 or GST-DAPP1[Y139F] (1 μ g), purified Src (1 unit), Lck (1 unit) and Lyn (1 unit), 5 mM manganese acetate, 5 mM magnesium acetate, 100 μ M [γ -³²P]ATP, 50 mM Tris/HCl (pH 7.5) and 0.1% (v/v) 2-mercaptoethanol was incubated for 30 min at 30 °C. The reactions were stopped by the addition of SDS and the samples were subjected to SDS/PAGE (7.5% gel), and phosphorylation of DAPP1 was revealed by autoradiography. Src, Lyn and Lck were assayed using the peptide substrate KVEKIGEGTYGVVYK [17], and 1 unit of activity was determined as that which phosphorylates 1 nmol of peptide substrate in 1 min.

Mapping the residue phosphorylated in DAPP1 by Lck

A solution (50 μ l) containing GST-DAPP1 (5 μ g), 5 μ g of Sf9 cell lysate expressing Lck (or Sf9 cell lysate not expressing Lck, as a control), 5 mM manganese acetate, 5 mM magnesium acetate, 100 μ M [γ -³²P]ATP, 50 mM Tris/HCl (pH 7.7) and 0.1% (v/v) 2-mercaptoethanol was incubated for 30 min at 30 °C. The reactions were stopped by the addition of SDS and 2-mercaptoethanol to final concentrations of 1% (w/v) and 1% (v/v) respectively, and heated for 5 min at 95 °C. After cooling to ambient temperature, 4-vinylpyridine was added to a concentration of 2.5% (v/v), and the sample was kept on a shaking platform for 1 h at 30 °C to alkylate cysteine residues. The sample was then electrophoresed by SDS/PAGE (7.5% gel), and the ³²P-labelled GST-DAPP1 was eluted from the gel and digested with trypsin and chromatographed on a reverse-phase C₁₈ column as described previously for PKB [15]. DAPP1 was not significantly phosphorylated when incubated in the control Sf9 cell lysate not expressing Lck.

RESULTS

DAPP1 is phosphorylated at Tyr¹³⁹ in HEK-293 cells

We sought to test whether DAPP1 was phosphorylated on tyrosine residues following stimulation of cells with extracellular agonists. Full-length human DAPP1 was expressed in human HEK-293 cells as a GST-fusion protein, the cells were serum-starved for 16 h, and then stimulated with either insulin, IGF1 or EGF. The cells were lysed, GST-DAPP1 was purified by affinity chromatography on GSH-Sepharose, and then GST-DAPP1

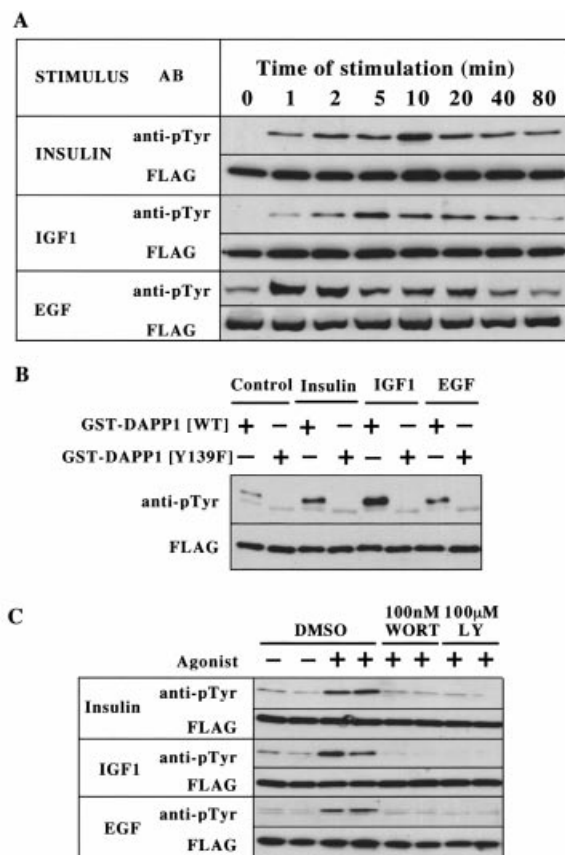


Figure 1 DAPP1 is phosphorylated at Tyr¹³⁹ in stimulated cells

(A) HEK-293 cells were transiently transfected with DNA constructs expressing wild-type GST-DAPP1. At 24 h post-transfection the cells were serum-starved for 16 h and stimulated with insulin (100 nM), IGF1 (100 ng/ml) or EGF (100 ng/ml) for the indicated times. The cells were lysed, and GST-DAPP1 was purified from aliquots of the supernatant by affinity chromatography on glutathione-Sepharose. The purified protein was separated by SDS/PAGE (7.5% gel) and immunoblotted using an anti-phosphotyrosine (anti-pTyr) or a FLAG antibody to detect GST-DAPP1 (GST-DAPP1 is also FLAG-epitope tagged [11]). (B) As in (A), except that the HEK-293 cells were transiently transfected with DNA constructs expressing wild-type (WT) GST-DAPP1 or the GST-DAPP1 [Y139F] mutant. The cells were stimulated with insulin (10 min) and IGF1 (10 min) or EGF (5 min). (C) As in (A), except that the cells were incubated for 10 min prior to stimulation with 100 nM wortmannin (WORT) or 100 µM LY294002 (LY) or DMSO. The cells were stimulated either for 10 min with insulin or IGF1 or for 5 min with EGF. These results are representative of at least three independent experiments in (A) and (C) and two experiments in (B).

was immunoblotted with an anti-phosphotyrosine antibody (Figure 1A). These agonists induced a rapid phosphorylation of DAPP1 within 1–2 min, which was sustained for 80 min after stimulation with insulin, but was more transient with IGF1 and EGF (Figure 1A).

Tyr¹³⁹ of DAPP1 is located between the SH2 and PH domains of DAPP1 and is the only tyrosine residue in the DAPP1 protein not located in the SH2 or PH domains. To our knowledge no tyrosine residue in a SH2 or PH domain has previously been shown to be phosphorylated. We therefore speculated that Tyr¹³⁹ was the site of phosphorylation on DAPP1. To investigate this we mutated Tyr¹³⁹ → Phe (Y139F) and expressed this mutant in HEK-293 cells as a GST-fusion protein. Stimulation of cells with insulin, IGF1 or EGF failed to induce any detectable phosphorylation of the GST-DAPP1[Y139F] mutant (Figure 1B), indicating that Tyr¹³⁹ was indeed the site of phosphorylation.

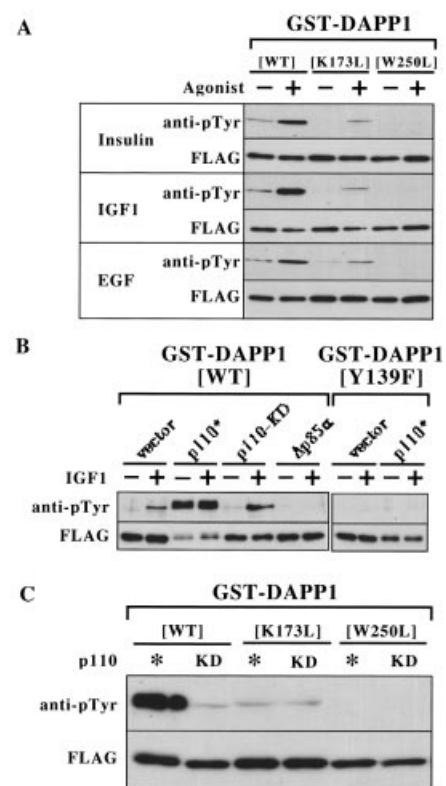


Figure 2 Phosphorylation of DAPP1 is dependent on the PH domain and requires PI 3-kinase

(A) HEK-293 cells were transiently transfected with DNA constructs expressing wild type (WT) GST-DAPP1 or mutants of GST-DAPP1 that do not interact with 3-phosphoinositides (GST-DAPP1[K173L] or GST-DAPP1[W250L]). At 24 h post-transfection the cells were serum-starved for 16 h prior to stimulation with 100 nM insulin (10 min), 100 ng/ml IGF1 (10 min) or 100 ng/ml EGF (5 min). The cells were lysed, and GST-DAPP1 was purified and immunoblotted using an anti-phosphotyrosine antibody (anti-pTyr) or a FLAG antibody to detect GST-DAPP1. (B) As in (A), except that HEK-293 cells were transiently co-transfected with DNA constructs expressing membrane-targeted wild-type PI 3-kinase (p110*), membrane-targeted kinase dead PI 3-kinase (p110-KD), a dominant-negative PI 3-kinase (Δp85α) or empty pCMV5 vector (vector). At 24 h post-transfection the cells were serum-starved for 16 h and then left unstimulated (–) or stimulated with IGF1 for 10 min (+). The expression of PI 3-kinase constructs was verified by immunoblotting with anti-CD2 antibody. p110* and p110-KD were expressed at similar levels, and Δp85α was expressed at a 5-fold higher level (results not shown). (C) As in (A), except that the cells were co-transfected with the indicated forms of PI 3-kinase. The cells were lysed without stimulation. These results are representative of two separate experiments.

Phosphorylation of DAPP1 is downstream of PI 3-kinase

In order to establish whether the activation of PI 3-kinase was required for phosphorylation of DAPP1 at Tyr¹³⁹, we added the structurally unrelated PI 3-kinase inhibitors wortmannin (100 nM) or LY 294002 (100 µM) to the tissue-culture medium at concentrations that inhibit the insulin-induced activation of PKB in these cells [18] prior to stimulation of the cells. These inhibitors prevented tyrosine phosphorylation of DAPP1 induced by insulin, IGF1 or EGF (Figure 1C).

Two mutants of GST-DAPP1, which do not interact with 3-phosphoinositides {GST-DAPP1[K173L] (Lys¹⁷³ → Leu) and GST-DAPP1[W250L] (Trp²⁵⁰ → Leu)} [11], were not significantly phosphorylated on tyrosine following stimulation of cells with insulin, IGF1 or EGF (Figure 2A). Overexpression of DAPP1

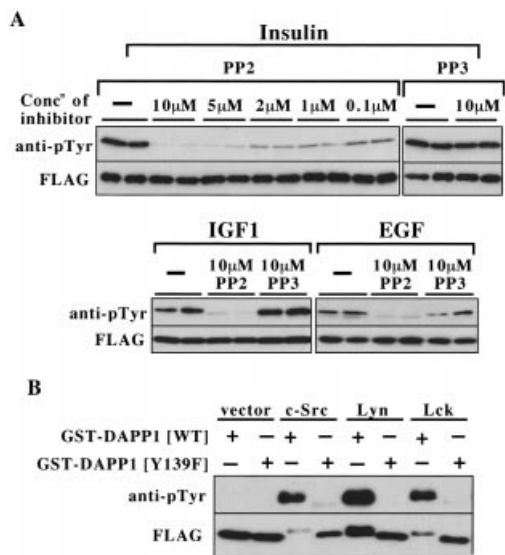


Figure 3 Phosphorylation of DAPP1 at Tyr¹³⁹ by Src-family kinases *in vivo*

(A) HEK-293 cells were transiently transfected with DNA constructs expressing wild-type GST-DAPP1. At 24 h post-transfection, the cells were serum-starved for 16 h and incubated with DMSO (—) or the indicated concentrations of PP2 or PP3 for 20 min before stimulation with 100 nM insulin (10 min), 100 ng/ml IGF1 (10 min) or 100 ng/ml EGF (5 min). The cells were lysed and GST-DAPP1 was purified and immunoblotted using an anti-phosphotyrosine antibody (anti-pTyr) or a FLAG antibody to detect GST-DAPP1. (B) As in (A), except that HEK-293 cells were co-transfected with DNA constructs expressing wild-type (WT) GST-DAPP1 or GST-DAPP1[Y139F], together with constructs expressing Src, Lyn, Lck or empty pCMV5 vector (vector). At 24 h post-transfection, the cells were serum-starved for 16 h and lysed without stimulation. Expression of Src, Lyn and Lck was verified by immunoblotting of cell lysates with appropriate antibodies and these kinases were expressed at similar levels in cells expressing either GST-DAPP1 [WT] or GST-DAPP1 [Y139F] (results not shown). These results are representative of two separate experiments.

with a membrane-targetted, constitutively activated form of PI 3-kinase (termed p110* [14]) induced a high level of tyrosine phosphorylation of wild-type GST-DAPP1, but not of GST-DAPP1(Y139F), in unstimulated cells, which was not increased further by stimulation with IGF1 (Figure 2B). In contrast, a membrane-targetted, catalytically inactive mutant of PI 3-kinase (termed p110-KD) failed to induce tyrosine phosphorylation of DAPP1 and the overexpression of a dominant-negative mutant of PI 3-kinase (termed Δp85α) inhibited the IGF1-induced tyrosine phosphorylation of DAPP1 (Figure 2B). Moreover, overexpression of the constitutively activated form of PI 3-kinase did not induce tyrosine phosphorylation of the mutants of GST-DAPP1, which no longer interact with 3-phosphoinositides (GST-DAPP1[K173L] and GST-DAPP1[W250L]) (Figure 2C).

Phosphorylation of DAPP1 is prevented by inhibitors of the Src family of tyrosine kinases

Tyr¹³⁹ of DAPP1 lies in a sequence motif Ile¹³⁸-Tyr-Glu¹⁴⁰, which is predicted to correspond to the optimal consensus motif for phosphorylation by Src-family tyrosine kinases [19]. We therefore tested the effect of PP2, a specific inhibitor for this class of tyrosine kinase, and PP3, a structurally related compound that does not inhibit Src-family tyrosine kinases [20,21]. PP2 inhibited the Src family kinase members tested with an IC₅₀ of ≈ 5 nM *in vitro* and does not significantly inhibit any other class of tyrosine kinase tested [20,21] or 25 serine/threonine protein kinases tested

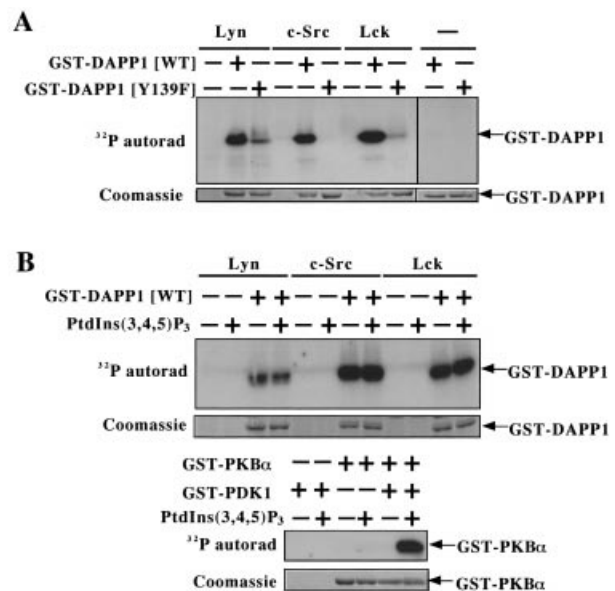


Figure 4 Phosphorylation of DAPP1 at Tyr¹³⁹ by Src-family kinases *in vitro*

(A) The indicated Src-family tyrosine kinases were incubated with purified wild-type (WT) GST-DAPP1 or GST-DAPP1[Y139F] in the presence of [γ -³²P]ATP as described in the Materials and methods section. The proteins were separated by PAGE (7.5% gel), the gel was stained with Coomassie Blue and autoradiographed to detect phosphorylated DAPP1. (B) As in (A), except that the reactions were performed in the presence of lipid vesicles containing 100 μ M phosphatidylcholine and 100 μ M phosphatidylserine with (+) or without (—) 10 μ M *sn*-1-stearoyl-2-arachidonoyl-*D*-PtdIns(3,4,5)P₃ (prepared as described in [28]). In a parallel reaction it was verified that GST-PDK1 only phosphorylated GST-PKBα in the presence of lipid vesicles containing PtdIns(3,4,5)P₃. (C) DAPP1, which had been phosphorylated with Lck using [γ -³²P]ATP to 0.8 mol of ³²P/mol of protein (see the Materials and methods section), was digested with trypsin and chromatographed on a Vydac 218TP54 C₁₈ column (Separations Group, Hesperia, CA, U.S.A.), equilibrated with 0.1% (v/v) aqueous trifluoroacetic acid. The column was developed with a linear acetonitrile gradient (diagonal line) at a flow rate of 0.8 ml/min and fractions of 0.4 ml were collected. Of the radioactivity applied to the column 85% was recovered from the major ³²P-peptide eluting at 18.8% acetonitrile. (D) A portion of the major ³²P-peptide (5 pmol) was analysed on an Applied Biosystems 492A sequencer, and the phenylthiohydantoin amino acids identified after each cycle of Edman degradation are shown, using the single-letter code. A portion of this peptide (2000 c.p.m.) was also coupled covalently to a Sequelon arylamine membrane and analysed on an Applied Biosystems 470A sequencer; the ³²P radioactivity released was measured after each cycle of Edman degradation.

(S. Davis, H. Reddy and P. Cohen, personal communication). PP2 potentially inhibited the insulin-induced tyrosine phosphorylation of DAPP1 (Figure 3A), even at very low concentrations of this drug (0.1 μ M). In contrast, PP3, even at a concentration of 10 μ M, did not inhibit insulin-mediated tyrosine phosphorylation significantly (Figure 3A). PP2 also inhibited the tyrosine phosphorylation of DAPP1 induced by IGF1 and EGF (Figure 3A).

Expression of Src, Lck or Lyn in cells induces phosphorylation of DAPP1

HEK-293 cells were transfected with constructs expressing either the wild-type GST-DAPP1 or the GST-DAPP1[Y139F] mutant, together with constructs encoding the Src-family tyrosine kinases Src, Lyn or Lck. This resulted in the wild-type GST-DAPP1, but not the GST-DAPP1[Y139F] mutant, becoming tyrosine phosphorylated in unstimulated cells (Figure 3B). Phosphorylation was accompanied by a decrease in the electrophoretic mobility of DAPP1 and was not increased further by stimulating cells with insulin, IGF1 or EGF (results not shown). Expression of DAPP1 with other tyrosine kinases, including the focal adhesion kinase, Janus kinase-2 and the Tec tyrosine kinases (BTK, Itk and BMX), failed to induce phosphorylation of wild-type GST-DAPP1 (results not shown).

Src, Lyn and Lck phosphorylated wild-type GST-DAPP1 *in vitro*, but the GST-DAPP1[Y139F] mutant was either not phosphorylated or phosphorylated to a very low extent (Figure 4A). The phosphorylation of DAPP1 by Src, Lyn and Lck *in vitro* was not affected by the presence of lipid vesicles containing PtdIns(3,4,5) P_3 . However, in parallel experiments, PDK1 only phosphorylated PKB in the presence of lipid vesicles containing PtdIns(3,4,5) P_3 (Figure 4B), as reported previously [22,23]. The site on DAPP1 phosphorylated by Lck was also mapped by phosphorylating DAPP1 with Lck to 0.8 mol of P_i /mol of protein, digesting the ^{32}P -labelled DAPP1 with trypsin and subjecting the resulting peptides to chromatography on a C_{18} column at pH 1.9. Only one major ^{32}P -labelled peptide, eluted at 18.8% acetonitrile, was observed (Figure 4C). Its sequence commenced at residue 132 of DAPP1, and a single burst of radioactivity occurred after the eighth cycle of Edman degradation (Figure 4D). Its molecular mass (1515.66), determined by matrix-assisted laser-desorption ionization-time-of-flight ('MALDI-TOF') MS, was identical with that expected (1515.74) for the tryptic peptide comprising residues 132–143 and phosphorylated at a single position. These results demonstrate that DAPP1 is phosphorylated at Tyr¹³⁹ *in vitro* by Lck.

DISCUSSION

In the present work we demonstrate that DAPP1 becomes phosphorylated at a single tyrosine residue (Tyr¹³⁹) following stimulation of HEK-293 cells with agonists such as insulin, IGF1 and EGF. The phosphorylation of DAPP1 on Tyr¹³⁹ is likely to be downstream of PI 3-kinase as inhibitors of PI 3-kinase (Figure 1C) and expression of a dominant-negative PI 3-kinase mutant prevented agonist-induced DAPP1 tyrosine phosphorylation (Figure 2B). Furthermore, the expression of a constitutively active PI 3-kinase in cells induced phosphorylation of DAPP1 at Tyr¹³⁹ in unstimulated cells (Figure 2B). DAPP1 mutants that are unable to interact with PtdIns(3,4,5) P_3 /PtdIns(3,4) P_2 were not phosphorylated at Tyr¹³⁹ in either stimulated cells (Figure 2A) or cells overexpressing active PI 3-kinase (Figure 2C), suggesting that DAPP1 needs to interact with PtdIns(3,4,5) P_3 /PtdIns(3,4) P_2 in order for it to become phosphorylated at this site. Furthermore, the time course of DAPP1 phosphorylation (Figure 1A) closely paralleled the activation of PKB in these cells by insulin, IGF1 [18] and EGF (M. Shaw, personal communication). DAPP1 also possesses an SH2 domain at its N-terminus and, in order to investigate the role of this domain, we mutated a conserved Arg residue (Arg⁶¹ to a Glu) that, in principle, should prevent the SH2 domain of DAPP1 from binding to tyrosine-phosphorylated proteins. We observed that when the GST-DAPP1[R61E] mutant was expressed in unstimulated cells, it was phosphorylated on tyrosine at a low

level. However insulin, IGF1, EGF or overexpression of constitutively active PI 3-kinase did not induce any further tyrosine phosphorylation of the GST-DAPP1[R61E] mutant (results not shown). This indicates that the SH2 domain of DAPP1 is likely to be playing a role in enabling DAPP1 to become phosphorylated at Tyr¹³⁹ in response to agonists that induce PI 3-kinase activation. Further work is required to identify the proteins with which the SH2 domain of DAPP1 interacts, and the role that they play in enabling DAPP1 to become phosphorylated at Tyr¹³⁹.

The Src family of protein kinases contain eight members, some which are broadly expressed (e.g. Src) and others whose expression is restricted to certain tissues (such as Lck and Lyn, which are mainly expressed in haematopoietic cells) [24]. These kinases possess overlapping substrate specificities, are known to play key functions in regulating signal-transduction pathways and have also been shown to play a role in several human malignancies [25]. Src-family tyrosine kinases are activated by both receptor tyrosine kinases, as well as by receptors that are not protein kinases. Here we demonstrate that DAPP1 is likely to be a physiological substrate for the Src family of tyrosine kinase, as the Src-family inhibitor PP2 prevented agonist-induced tyrosine phosphorylation of DAPP1 in cells. HEK-293 cells express detectable levels of Src and Lyn (results not shown), which may be the kinases responsible for the phosphorylation of transfected GST-DAPP1 at Tyr¹³⁹ observed in our experiments. Src, Lyn or Lck phosphorylated DAPP1 on Tyr¹³⁹ *in vitro*.

Tyr¹³⁹ is conserved in human, mouse and sea-squirt (*Haliocynthia roretzi*) DAPP1 [National Center for Biotechnology Information (NCBI) accession number AV383515], which would be predicted if phosphorylation of this residue is of physiological importance. Furthermore, co-expression of DAPP1 with Src, Lyn or Lck induced a very high level of phosphorylation of DAPP1 at Tyr¹³⁹, even in unstimulated cells, which was not increased further by agonist stimulation of cells. As Src-family kinases activate the PI 3-kinase pathway in many cells [1], it is possible that the overexpression of Src, Lyn or Lck in HEK-293 cells induces the activation of PI 3-kinase, thereby promoting DAPP1 phosphorylation in unstimulated cells. As Src-family tyrosine kinases are located at the plasma membrane by virtue of myristoylation and palmitoylation of their N-termini [26], it is likely that the role of PtdIns(3,4,5) P_3 is to recruit DAPP1 to the cell membrane, where it can be phosphorylated with Src-family tyrosine kinases.

One of the general mechanisms by which PtdIns(3,4,5) P_3 /PtdIns(3,4) P_2 functions in cells is to recruit PH-domain-containing proteins to the plasma membrane, enabling them to become phosphorylated and hence activated (see the Introduction). This also appears to be the case for DAPP1 which, like BTK, Vav and Gab1, becomes phosphorylated at tyrosine residues following its interaction with PtdIns(3,4,5) P_3 /PtdIns(3,4) P_2 in cells. DAPP1, like BTK and Vav, is phosphorylated by Src-family kinases *in vitro* at a similar rate in the presence or absence of PtdIns(3,4,5) P_3 (Figure 4B), suggesting that the interaction of DAPP1 with 3-phosphoinositides does not induce a conformational change that converts it into a substrate for the Src family of tyrosine kinases. This is in contrast with PKB, which is converted into a substrate for PDK1 through its interaction with PtdIns(3,4,5) P_3 (Figure 4B) [27].

Consistent with the above model, Clark and colleagues [12] have recently demonstrated that transfected DAPP1 is recruited to the plasma membrane of cells through its PH domain following activation of PI 3-kinase. Those authors also showed that the endogenous DAPP1 in B cells is phosphorylated on a tyrosine residue(s) in response to activation of the B-cell receptor. They

also present some evidence that DAPP1 may interact with phospholipase $C\gamma 2$ through its SH2 domain, and, when DAPP1 is overexpressed in cells, it negatively inhibits transcriptional responses, regulated by nuclear factor, of activated T cells [12]. It will be important to establish whether DAPP1 in B cells is also phosphorylated at Tyr¹³⁹ by Src-family kinases following the activation of PI 3-kinase in response to activation of the B-cell receptor.

In summary, the results presented here indicate that DAPP1 is likely to be a direct cellular target for both PI 3-kinase and Src-family tyrosine kinases, and that the phosphorylation of DAPP1 at Tyr¹³⁹ may play an important role in triggering signal-transduction pathways that lie downstream of both PI 3-kinase and the Src family of tyrosine kinases. Physiological processes regulated by phosphorylation of DAPP1 should therefore be inhibited by either PI 3-kinase or Src inhibitors.

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