Identification of novel PCTAIRE-1/CDK16 substrates using a chemical genetic screen

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ABSTRACT
PCTAIRE-1 (also known as cyclin-dependent protein kinase (CDK) 16), is a Ser/Thr kinase that has been implicated in many cellular processes, including cell cycle, spermatogenesis, neurite outgrowth, and vesicle trafficking. Most recently, it has been proposed as a novel X-linked intellectual disability (XLID) gene, where loss-of-function mutations have been identified in human patients. The precise molecular mechanisms that regulate PCTAIRE-1 remained largely obscure, and only a few cellular targets/substrates have been proposed with no clear functional significance. We and others recently showed that cyclin Y binds and activates PCTAIRE-1 via phosphorylation and 14–3-3 binding. In order to understand the physiological role that PCTAIRE-1 plays in brain, we have performed a chemical genetic screen in vitro using an engineered PCTAIRE-1/cyclin Y complex and mouse brain extracts. Our screen has identified potential PCTAIRE-1 substrates (AP2-Associated Kinase 1 (AAK1), dynamin 1, and synaptojanin 1) in brain that have been shown to regulate crucial steps of receptor endocytosis, and are involved in control of neuronal synaptic transmission. Furthermore, mass spectrometry and protein sequence analyses have identified potential PCTAIRE-1 regulated phosphorylation sites on AAK1 and we validated their PCTAIRE-1 dependence in cellular study and/or brain tissue lysates. Our results shed light onto the missing link between PCTAIRE-1 regulation and proposed physiological functions, and provide a basis upon which to further study PCTAIRE-1 function in vivo and its potential role in neuronal/brain disorders.

1. Introduction

PCTAIRE-1 (also known as CDK16), is an atypical Ser/Thr kinase of the CMGC family whose physiological function remains elusive. It shares high sequence homology with CDKs (50% sequence identity of kinase domain with CDK2) [1], but contains extended N- and C-termini compared to canonical CDKs, a characteristic of both the PCTAIRE (PCTAIRE-1, -2 [CDK17] and -3 [CDK18]) and the closely related PFTAIRE (PFTAIRE-1 [CDK14] and -2 [CDK15]) families. Although initially considered orphan kinases, we and others have shown robust PCTAIRE-1 interaction and activation by cyclin Y [3–5], a novel cyclin with essential functions in Drosophila development [6,7]. Moreover, we have identified two essential phosphorylation sites on cyclin Y that are essential for PCTAIRE-1 activation, and have shown that they are responsible for 14-3-3 adapter protein binding, which is required for cyclin Y-mediated PCTAIRE-1 activation [8,9].

Despite ubiquitous expression, PCTAIRE-1 activity was shown to be highest in the brain and testis [2,4,10,11]. In line with this, PCTAIRE-1 has been implicated in control of neurite outgrowth and neurotransmission [3,12], and has been shown to interact with components of the vesicle trafficking machinery such as COPII [13] and N-ethylmaleimide-Sensitive Fusion protein (NSF) [14]. In addition, genetic inactivation of PCTAIRE-1 in mice resulted in male infertility, suggesting an important function of this kinase in spermatogenesis [2]. Most recently, PCTAIRE-1 has been implicated in X-linked intellectual disability (XLID), as PCTAIRE-1 loss-of-function human variants have

Abbreviations: AAK1, AP2-Associated Kinase 1; A.T.C.C., American Type Culture Collection; CDK16, cyclin-dependent kinase 16; DTT, dithiothreitol; FBS, fetal bovine serum; GST, glutathione-sepharose transferase; HA, hemagglutinin; IPTG, Isopropyl β-D-thiogalactopyranoside; LB, Lysogeny Broth; LDL, low-density lipoprotein; PBS, phosphate-buffered saline; PMSF, phenylmethylsulphonyl fluoride; PNBM, p-nitrobenzyl mesylate

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been identified in XLID patients [15], which we have shown lose their ability to bind, and be activated by, cyclin Y14-3-3 complex [9]. Despite intensive research, the precise molecular mechanisms by which PCTAIRE-1 is regulated in physiological and/or pathological settings remained largely obscure, and only a few cellular targets have been proposed with no clear functional role/significance.

In order to elucidate the biological function of PCTAIRE-1 and explore proposed links with neuronal regulation/function, we have undertaken a chemical genetic approach (also known as ‘Shokat’ method) [16] to identify novel and physiologically relevant PCTAIRE-1 substrates. Here we have identified AP2-associated protein kinase 1 (AAK1), dynamin 1 and synaptojanin 1 as putative substrates of PCTAIRE-1, all of which are proposed as key components for receptor internalisation and synaptic transmission [17,18]. Through protein sequence and mass spectrometry analyses, we have identified potential PCTAIRE-1 regulated phosphorylation sites on AAK1 and have validated them in a cellular study and/or brain tissues lysates derived from wild-type (WT) or PCTAIRE-1/CDK16-null mice.

2. Materials and methods

2.1. Materials

PCTAIRE-tide peptide substrate for kinase assays was synthesised by GL Biochem. (γ-32P)ATP was from PerkinElmer. Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Jackson ImmunoResearch. Glutathione-Sepharose 4B and Protein-G-Sepharose were from GE Life Sciences, FLAG M2- and HA-agarose were from Sigma. P81 paper was from Whatman. All cell culture reagents were from ThermoFisher.

2.2. Antibodies

Anti-14-3-3-3 (K-19) antibodies were from Santa Cruz Biotechnology. AAK1 (A302-145A) antibody was from Bethyl Laboratories. Dynamin 1 (3G4B6) (3G4B6) antibody was from Cell Signaling Technology. Synaptotagmin 1 (24677-1-AP) antibody was from Proteintech. Thiophosphate ester antibodies for immunoblotting (ab92570) and immunoprecipitation (ab133473) were from Abcam. Anti-haemagglutinin (HA) antibody was from Covance Research Products. The cloning of CDK16, transcript variant 1 (NCBI Acc. NM_003406.3) have been described earlier [9]. The coding region of AAK1 (isoform2) corresponding to NCBI acc. BC104842.1 (Uniprot acc. Q2MB12-2) was amplified from Image clone 8143845. The amplified PCR products were cloned into different bacterial and mammalian expression vectors. Site-directed mutagenesis was performed using the QuikChange method (Agilent). The sequences of all the constructs were verified in house, utilizing the BigDye Terminator v3.1 kit and 3500xL Genetic Analyser (ThermoFisher).

2.3. Plasmids

All plasmid constructs were generated using standard molecular biology techniques. The cloning of CDK16, transcript variant 1 (NCBI Acc. NM_006201.5) and 14-3-3-3 (Acc. NM_003406.3) have been described earlier [9]. The coding region of AAK1 (isoform2) corresponding to NCBI acc. BC104842.1 (Uniprot acc. Q2MB12-2) was amplified from Image clone 8143845. The amplified PCR products were cloned into different bacterial and mammalian expression vectors. Site-directed mutagenesis was performed using the QuikChange method (Agilent). The sequences of all the constructs were verified in house, utilizing the BigDye Terminator v3.1 kit and 3500xL Genetic Analyser (ThermoFisher).

2.4. Cell culture

COS1 cells (A.T.C.C.) were maintained in high-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) FBS under 5% CO2. Cells were transfected with DNA using poly-ethylenimine and harvested 24 h post-transfection. Cells were washed with ice-cold PBS and scraped into lysis buffer (50 mM Tris/HCl, pH7.5, 1 mM EDTA, 1 mM EGTA, 0.27 M sucrose, 1% (w/v) Triton X-100, 50 mM NaF, 5 mM Na2P2O7, 1 mM Na3VO4, 1 mM DTT, 1 mM benzamide and 0.5 mM PMSF). Lysates were clarified at 17,000 g for 10 min at 4 °C and stored at −80 °C. Protein concentration was determined using Bradford reagent and BSA standard.

2.5. Immunoprecipitation

Tissue lysates were incubated with 2–5 μg of AAK1 antibody, 10 μl of dynamin 1, 2.5 μl synaptotagmin 1 antibody, or 2 μg of thiophosphate ester antibody, and 5 μl of Protein-G-Sepharose for 1 h at 4 °C. FLAG- and HA-tagged proteins were isolated from cells using 5 μl of FLAG M2- or HA-agarose respectively. Immune-complexes were pelleted at 500 g for 1 min and washed twice with 0.1 ml of lysis buffer plus 0.5 M NaCl, twice with 0.1 ml of buffer A (50 mM Tris/HCl, pH 8, 0.1 mM EGTA and 1 mM DTT) and either eluted with Laemmli sample buffer for analysis by immunoblotting, or assayed directly for kinase activity as previously described [9], or separated by SDS-PAGE, trypsin digested and analysed by mass spectrometry.

2.6. Immunoblotting

Cell/tissue lysates were denatured in Laemmli buffer, separated by Tris/glycine SDS-PAGE and transferred onto a PVDF membrane. Membranes were blocked for 1 h at room temperature in 20 mM Tris/HCl (pH7.6), 137 mM NaCl, 0.1% (v/v) Tween-20 (Tris-buffered saline with Tween-20; TBST) containing 5% (w/v) skimmed milk. Membranes were incubated in primary antibody prepared in TBST containing 5% (w/v) BSA overnight at 4 °C. Signal detection was performed using HRP-conjugated secondary antibodies and enhanced chemiluminescent reagent.

2.7. Preparation of GST-tagged cyclin Y in E. coli

GST-tagged human cyclin Y in pGEX-6p was expressed in E. coli BL21-CodonPlus (DE3)-RIL cells and isolated by column purification using glutathione-Sepharose 4B. Briefly, transformed E. coli cells were cultured in lysogeny broth (LB) medium until an OD600 of 0.6 was attained and induced with 0.2 mM IPTG for 16 h at 18 °C. Cells were pelleted by centrifugation at 5000 g for 30 min, lysed in 50 mM Tris/HCl, pH 7.5, 0.25 M NaCl, 0.1 mM EDTA, 1 mM benzamide and 1 mM DTT by sonication and clarified by centrifugation at 30,000 g for 30 min. Supernatants were incubated with Glutathione-Sepharose 4B for 1 h at 4 °C, washed extensively with lysis buffer and eluted with 20 mM reduced glutathione in 50 mM Tris/HCl, pH 8. Preparations were gel filtered over Sephadex G-25 to remove excess glutathione and the GST-tag was retained. Protein concentration was estimated by densitometry of Coomassie Blue (Life Technologies)-stained gels using BSA standards. Protein preparations were snap-frozen in liquid nitrogen and stored at −80 °C in 50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 0.1 mM EDTA and 10% (v/v) glycerol.

2.8. Preparation of tissue lysates

Animal studies were approved by the local Ethical Committee of the Canton of Vaud (license number 2770) or Medical University of Innsbruck (license number: BMWF-66.011/0017-II/3b/2014). WT C57BL/6N mice were obtained from Charles River Laboratories. Two-month old male CDK16 WT and knockout mice (C57BL/6 background)
FLAG-PCTAIRE-1/HA-cyclin Y immune complexes (30 μU activity) isolated from cell lysates were assayed for phosphotransferase activity in a final assay volume of 20 μl containing 50 mM HEPES, pH 7.5, 0.1 mM EGTA, 10 mM magnesium acetate, 1 mM ATP S (or N6-phenylethyl-ATP S), 1 mM DTT and 0.2 μg E. coli-purified recombinant GST-cyclin Y. Reactions were incubated for 1 h at 30 °C with constant mixing. Reactions were then alkylated in 2.5 mM p-nitrobenzyl mesylate (PNBM) for an additional hour at room temperature. The reaction was terminated by adding Laemmli sample buffer and analysed by immunoblotting using anti-thiophosphate ester antibody Ab 51-8.

2.10. Substrate screen in mouse brain using analogue-specific (AS)-PCTAIRE-1

Brain lysates (50–500 μg) were incubated with 1 mM N6-phenylethyl-ATP S and 1% (w/w) COS1-purified AS (or non-AS)-PCTAIRE-1 complexed with either FLAG- or HA-WT cyclin Y (or S12A and S363A mutants) in 50 mM HEPES, pH 7.5, 150 mM NaCl, and 10 mM magnesium acetate for 10 min at room temperature with constant mixing. Reactions were terminated using 20 mM disodium EDTA and 0.1% (w/v) SDS, and the samples alkylated in 2.5 mM PNB M for 1 h at room temperature. Samples were then either eluted with Laemmli sample buffer and immunoblotted or gel-filtered over Sephadex G-25 column (to remove excess PNB M). Samples were then immunoprecipitated using anti-thiophosphate ester antibody at 4 °C, and immunoprecipitates either immunoblotted using anti-thiophosphate ester antibody or the proteins separated by SDS-PAGE, digested with trypsin and analysed by mass spectrometry. Alternatively, gel-filtered lysates were immunoprecipitated using AAK1, dynamin 1 or synaptojanin 1 antibodies as described above and immunoblotted using anti-thiophosphate ester antibody.

2.11. Trypsin digestion and MS analysis of mouse brain

Immunoprecipitated proteins were denatured in Laemmli buffer, alkylated using iodoacetamide and separated by SDS-PAGE on NuPAGE 4–12% bis-tris gels (Life Technologies). Gels were stained with colloidal Coomassie Blue. The HA-AAK1 band (50 μM) for 30 min, separated by SDS-PAGE on NuPAGE 4–12% bis-tris gels, and stained with colloidal Coomassie Blue. The HA-AAK1 band was excised, destained, in-gel reduced, then dried with acetonitrile followed by SpeedVac concentration. Samples were digested with 60 μl of 2 μg/ml trypsin (sequencing grade, Promega) in 50 mM triethyl-lammonium bicarbonate (TEAB), pH 8, overnight. Peptides were extracted with an equal volume of acetonitrile, dried and redissolved in 60 μl of 5% acetonitrile/10 mM TEAB. The peptide mixtures were separated using the same LC-MS and chromatography system as described above.

The ten most abundant precursors detected in the Orbitrap at 30000 resolution (2–4+ charge state) were selected for MS/MS in the LTQ Velos. To ensure the best fragmentation spectrum for any phosphopeptide was obtained, the precursors were fragmented using multistage activation (MSA) of the precursor mass (m/z) minus 49, 32 and 24.25 Da (this represents the loss of H3PO4 from a 2+, 3+ or 4+ precursor ion). Raw files were searched using Mascot 2.4.1 against the Swiss Prot database (human only, 148,212 entries), allowing for carbamidomethyl modification of cysteine, oxidation of methionine and phosphorylation of serine/threonine/tyrosine. Potential phosphorylation sites were manually inspected.

3. Results

3.1. Identification and optimisation of analogue-specific PCTAIRE-1 mutant for chemical genetic screen

Previous studies of the structure and function of protein kinases have identified a conserved amino acid (termed the ‘gatekeeper’ residue) in the ATP-binding pocket that controls nucleotide and small molecule binding [20–22]. Substitution of the (usually bulky) side chain at the gatekeeper residue with a small (typically Ala or Gly) one creates an empty space that can preferentially recognise ATP analogues with bulky groups at the N6 ring position [23–26]. This has been exploited in a chemical genetic approach to uniquely label and identify protein kinase substrates [16,25,27]. The mutant (termed ‘Analogue-Specific (AS)’) kinase preferentially utilises a bulky N6-ATP S analogue to selectively thio phosphorylate its substrates (Fig. 1A). An alkylation reaction then converts the modification to a thiophosphate ester, which can be affinity purified using a thiophosphate ester antibody for further identification (Fig. 1A) [16]. To identify the gatekeeper residue in PCTAIRE-1, we performed sequence and structure alignment with CDK2 (Fig. 1A and data not shown), for which this screen has been previously performed [28], and identified Phe240 as the PCTAIRE-1 gatekeeper residue.

It has been reported that mutating the gatekeeper residue often severely diminishes kinase activity, for which rescue mutations must be empirically determined [29]. We therefore tested a Phe240-to-Ala PCTAIRE-1 mutant for both kinase activity against PCTAIRE-1 and cyclin Y1/14-3-3 binding, and indeed we observed both were robustly decreased, confirming that this gatekeeper residue in PCTAIRE-1 is required for full protein kinase activity.

2.12. Mass spectrometry analysis of HA-AAK1

COS1 cells were transfected with HA-tagged human AAK1 with or without co-transfection with FLAG-cyclin Y and FLAG-PCTAIRE-1 WT or D304A (kinase-inactive) mutant, and lysates were prepared 24 h later as described above. HA-AAK1 was immunoprecipitated from 1 mg of lysate using HA-agarose, washed twice with 0.5 ml of lysis buffer plus 0.5 M NaCl, twice with 0.5 ml of buffer A and eluted with Laemmli sample buffer. Eluted HA-AAK1 was alkylated with iodoacetamide (50 mM) for 30 min, separated by SDS-PAGE on NuPAGE 4–12% bis-tris gels, and stained with colloidal Coomassie Blue. The HA-AAK1 band was excised, destained, in-gel reduced, then dried with acetonitrile followed by SpeedVac concentration. Samples were digested with 60 μl of 2 μg/ml trypsin (sequencing grade, Promega) in 50 mM triethyl-lammonium bicarbonate (TEAB), pH 8, overnight. Peptides were extracted with an equal volume of acetonitrile, dried and redissolved in 60 μl of 5% acetonitrile/10 mM TEAB. The peptide mixtures were separated using the same LC-MS and chromatography system as described above.

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Previous studies have identified Ser153 as an inhibitory PCTAIRE-1 site, and that Ser153Ala substitution resulted in increased enzymatic activity [2,12]. In addition, we have observed that introducing both N- (Δ105) and C-terminal (Δ20) truncations also increases PCTAIRE-1 activity of the resulting truncated PCTAIRE-1 spanning residues 106–476 [9]. We have therefore introduced both of these modifications to the F240A PCTAIRE-1 gatekeeper mutant, and observed that kinase activity and cyclin Y/14-3-3 binding was significantly rescued to ~50% that of full-length WT PCTAIRE-1 (Fig. 1B). We therefore decided to use this version of PCTAIRE-1 (106–476, S153A, F240A) to perform the chemical genetics screen, and will refer to it as AS-PCTAIRE-1. As a control, we decided to use the equivalent of AS-PCTAIRE-1, but lacking the F240A mutation (106–476, S153A), and will refer to it as non-AS-PCTAIRE-1. Both kinase versions were then co-expressed with FLAG-WT-cyclin Y and purified using FLAG-agarose to produce active kinases. To test whether the purified active AS-PCTAIRE-1 complex is able to selectively utilise N6-phenylethyl ATPγ S, we compared the ability of both AS- and non-AS-PCTAIRE-1 (each co-purified with cyclin Y) to thio phosphorylate E. coli-purified GST-cyclin Y substrate. Instead of ATP, either ATPγ S or N6-phenylethyl ATPγ S was used. Indeed, while both kinases equally utilised ATPγ S to thio phosphorylate GST-cyclin Y, only AS-PCTAIRE-1 was able to utilise the N6-phenylethyl ATPγ S (Fig. 1C), confirming the validity of the gate-keeper mutant.

### 3.2. PCTAIRE-1 thio phosphorylation pattern in cell/tissue lysates shows cyclin Y is an in vitro thio phosphorylation substrate

We next sought to determine the most suitable cell/tissue in which to explore PCTAIRE-1 substrates. Since PCTAIRE-1 expression and activity have consistently been shown to be highest in mouse brain and testis [2,4,11], we decided to test these tissues. We therefore incubated active AS-PCTAIRE-1 (low and high amounts), along with N6-phenylethyl ATPγ S, with either brain or testis lysates, and the resulting materials were immunoblotted to detect thio phosphorylated proteins. Unexpectedly, while we detected several distinct bands in brain lysates, no significant labelling (thio phosphorylation) was observed in testis, with the exception of a band corresponding to a protein at ~37kDa, which was also detected in brain (Fig. 2A). We repeated the labelling experiment using brain lysates from three different mice to confirm reproducibility along with a negative control (i.e. lysis buffer without tissue/cell lysate). We confirmed that the thio phosphorylation labelling pattern in all three individual brain lysates was nearly identical (Fig. 2B). Interestingly, we detected the same ~37kDa band in the control sample (Fig. 2B), indicating that it must originate from the active AS-PCTAIRE-1 preparation. Since PCTAIRE-1 phosphorylates cyclin Y on two sites (Ser12 and Ser336) [9], and cyclin Y’s predicted molecular mass is ~39 kDa, we suspected that the FLAG-WT-cyclin Y in the active AS-PCTAIRE-1 complex is being thio phosphorylated. We therefore repeated the labelling experiment using active AS-PCTAIRE-1 co-purified with either FLAG-WT-cyclin Y or phospho-deficient mutants (S12A, S336A or S12A/S336A), and observed that the band at ~37 kDa disappeared in the S12A-cyclin Y mutant (and S12A/S336A mutant) (Fig. 2C). This is consistent with our previous study that S12 is preferred phosphorylation site in cell-free assay [9].

### 3.3. Mass spectrometry analysis of thio phosphorylated brain lysates identifies in vitro PCTAIRE-1 substrates

We therefore co-expressed and purified AS- and non-AS-PCTAIRE-1...
complexes containing S12A-cyclin Y and incubated each with brain lysates along with N6-phenylethyl-ATPγ S, followed by alkylation with PNBM. After gravity gel filtration to remove excess PNBM, lysates were affinity-purified using anti-thiophosphate ester antibody, separated by SDS-PAGE (Fig. 3A, left panel), and in-gel digested with trypsin. The resulting peptides were analysed by LC-MS/MS. Samples immunoblotted prior to trypsin digestion showed distinct labelling of mouse brain-derived proteins at ~75, 100 and 150kDa (Fig. 3A, right panel). Using three replicates in brains from different mice, we identified several peptides enriched in the AS-PCTAIRE-1-incubated lysates that were also absent (or present in significantly lower amounts) in the non-AS-PCTAIRE-1-incubated control lysates. These included peptides mapping to dynamin 1, synaptojanin 1, and AAK1 (Fig. 3B), all of which are highly involved in vesicle trafficking and neuronal regulation/synaptic transmission [30], in line with PCTAIRE-1 proposed physiological roles [1,12,13,31,32].

3.4. PCTAIRE-1 thiophosphorylates AAK1, dynamin 1, and synaptojanin 1 in vitro

We sought to confirm whether these proteins were indeed thiophosphorylated in the brain samples where the in vitro labelling reactions were performed. We immunoprecipitated AAK1, dynamin 1 or synaptojanin 1 from the brain lysates following the labelling reaction and immunoblotted for detection of thiophosphorylation. All three proteins showed thiophosphorylation only in the AS-PCTAIRE-1-incubated samples (Fig. 4A–C). Immunoblotting of the samples from pre- and post-immunoprecipitation confirmed almost complete depletion of the thiophosphorylation signal at the predicted molecular weights of both dynamin 1 and synaptojanin 1 (Fig. 4A, right panel and Fig. 4B and C). Only a partial depletion and no apparent thiophosphorylation signal in the lysates was observed for AAK1 (Fig. 4A) possibly due to its lower abundance reflected by total spectrum/peptide count shown in Fig. 3B. Due to recent reports indicating AAK1’s involvement in vesicle formation and trafficking [33,34], we next sought to identify specific phosphorylation sites on AAK1. We affinity purified HA-tagged AAK1 expressed in COS1 cells either alone or with cyclin Y and WT (or kinase-inactive, D304A) PCTAIRE-1, and subjected it to mass spectrometry analysis. This identified an increased abundance of phosphopeptides containing Thr389 which increased when AAK1 was co-expressed with WT PCTAIRE-1/cyclin Y compared to other conditions (Supplementary Fig. 1). Taken together, in vitro analysis suggested that AAK1, dynamin...
1, and synaptojanin 1 are potential candidates for PCTAIRE-1 substrates in brain, with identification of a specific site (Thr389) phosphorylation on AAK1.

3.5. Identification and validation of PCTAIRE-1-mediated phosphorylation sites on AAK1 in cells and mouse brain tissues

Next, we sought to determine whether AAK1 is a PCTAIRE-1 substrate in a cellular context and also at the endogenous level in mouse brain tissue. Analysis of the protein sequence of human AAK1 identified several proline-directed Ser/Thr residues (S-P motifs), some of which resemble the preferred substrate sequence for PCTAIRE-1 (SPKAR) [4]. Based on this and results from the mass spectrometry analysis (Supplementary Fig. 1), we have generated phospho-specific antibodies against Thr389 (*TPRKR: * denotes a phosphorylated residue), Thr170 (*TPIIH), Ser624 (*SPKTQ), and Ser678 (*SPRTS). In order to validate

Fig. 3. Mass spectrometry analysis of mouse brain lysates thiophosphorylated by AS-PCTAIRE-1 complex identified PCTAIRE-1 targets. (A) Mouse brain lysates (500 μg) were incubated with PhEt-ATPγ S and purified non-AS- or AS-PCTAIRE-1 complexed with HA-cyclin Y S12A. Reactions were terminated and the samples alkylated using PNBM. Samples were then desalted (to remove excess PNBM) followed by overnight immunoprecipitation using anti-thiophosphate ester antibody. Immunoprecipitates were either immunoblotted or separated by SDS-PAGE, stained with colloidal Coomassie Blue, and the gel pieces (indicated by boxes) excised and analysed by mass spectrometry. (B) Total peptide spectral counts of all hits were manually analysed and filtered according to relative abundance between WT and AS samples, and proteins with the highest ratio were selected for further analysis. AS, analogue-specific. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Identification/Confirmation of distinct band identities thiophosphorylated by AS-PCTAIRE-1 complex in mouse brain. (A) Brain lysates (50 μg) were incubated with PhEt-ATPγ S and purified non-AS- or AS-PCTAIRE-1 complexed with HA-cyclin Y S12A. Reactions were terminated and the samples alkylated using PNBM. Samples were then immunoprecipitated (IP) using AAK1 antibody and immunoblotted (IB) using anti-thiophosphate ester (Thio) antibody. (B) Brain lysates (50 μg) were treated as in (A) then immunoprecipitated using dynamin 1 (Dyn1) antibody and immunoblotted. (C) Brain lysates (50 μg) were treated as in (A) then immunoprecipitated using synaptojanin 1 (Synj1) antibody and immunoblotted. Results are representative of 2–3 independent experiments. WT, wild-type. AS, analogue-specific. nAS, non-AS. Pre/Post, pre- or post-immunoprecipitation lysates.
specificity of the antibodies and assess if these phosphorylation sites are regulated in a PCTAIRE-1 dependent manner, we ectopically expressed either HA-AAK1 WT or phospho-deficient mutants (T170A, T389A, S624A, S678A, T170A/T389A/S624A/S678A) together with FLAG-cyclin Y and WT or D304A PCTAIRE-1 in COS1 cells (Fig. 5A). Immunoblot analysis revealed that all the antibodies are specific to the individual site raised, and the levels of phosphorylation increased markedly (T170, T389, S678) or modestly (S624) in PCTAIRE-1 WT-expressed compared to D304A-expressed cells. We next wanted to confirm if these phosphorylation sites are regulated in a PCTAIRE-1 dependent manner in mouse brain. Immunoblot of whole mouse brain extract has shown that there is ~50% reduction in Ser624 phosphorylation in PCTAIRE-1/CDK16 knockout compared to WT (Fig. 5B). In contrast, there was no significant decrease in Ser678 phosphorylation in the PCTAIRE-1/CDK16 knockout lysates (Fig. 5B). We have also tested p-Thr170 and p-Thr389 antibodies, but we did not detect specific signal due possibly to lower sensitivity of the antibodies.

4. Discussion

We and others have previously established that PCTAIRE-1 activation requires binding of a cyclin Y/14-3-3 complex [2,8,9]. However, cellular regulation and the in vivo consequences of this activation (mediated by the PCTAIRE-1/cyclin Y/14-3-3 complex) remain elusive. While KAP0 [35] and p27 [36] have been proposed as PCTAIRE-1 substrates in the context of spindle orientation and cancer cell mitosis, respectively, only one PCTAIRE-1 substrate, NSF, relating to brain function has been proposed without robust supporting evidence [14]. We have therefore undertaken a chemical genetic screen to identify PCTAIRE-1 substrates in the brain and here report three previously unknown PCTAIRE-1 targets, namely AAK1, dynamin 1, and synaptojanin 1.

Given that both brain and testis tissues have the most abundant PCTAIRE-1 expression and activity [2,4,37,38] we hypothesised that PCTAIRE-1 may influence the amount of receptor internalised in the testis lysates and/or additional optimisation is necessary to more sensitively/ specifically detect potentially very modest differences in thio phosphorylation labelling between samples. Furthermore, PCTAIRE-1 activation in testis might be most important during early development, leading to loss or decreased detection of PCTAIRE-1 phosphorylation events in adult testis lysates. It is clear that further studies are required to elucidate the precise role of PCTAIRE-1 in the testis, especially since cyclin Y-like 1 (CCNYL1), a novel cyclin with functions overlapping with cyclin Y [6,39,40], and which we have shown robustly activates PCTAIRE-1 [9], has been reported to cooperate with PCTAIRE-1 to regulate spermatogenesis [41], and male mice lacking either PCTAIRE-1/CDK16 or CCNYL1 (but not cyclin Y) were reported to be infertile [2,41].

Among the PCTAIRE-1 substrates identified through the in vitro screen, we have identified PCTAIRE-1 kinase activity dependent sites (T170, T389, S624, S678) on AAK1 at cellular level, and among them we confirmed that phosphorylation of S624 is at least partially dependent on PCTAIRE-1 at endogenous levels in brain. It would be of major interest to investigate whether AAK1 phosphorylation is altered in specific cell-types/regions in the brain under certain pathophysiological states (e.g. in XLID patients). In addition, it should be confirmed whether dynamin 1 and synaptojanin 1 are cellular and pathophysiology relevant PCTAIRE-1 substrates.

AAK1 is a kinase shown to function in receptor-mediated internalisation of the LDL (low-density lipoprotein) and transferrin receptors [42,43]. It is reportedly activated by binding to clathrin on coated membranes being internalised [44] and phosphorylates AP2 on the invaginating membrane, thereby increasing affinity for receptors to be internalised [42,43]. One way in which PCTAIRE-1 may influence this process would be to affect AAK1 phosphorylation of AP2, thereby influencing the amount of receptor internalised. AAK1 has also been reported as a substrate of NDR (nuclear Dbf2-related) 1 kinase with roles in dendrite aborisation [45], further associating AAK1 with important roles in the brain. Since dynamin 1 and synaptojanin 1 are closely linked to receptor internalisation via vesicle scission [46-49] and uncoating [50-53], respectively, it would be important to further investigate and determine how PCTAIRE-1 activation may influence these cellular processes.

In addition to the above-mentioned targets, we have also identified peptides mapping to proteins such as clathrin (heavy chain), dynamin 1 and synaptojanin 1.
neuroglycan C, collagen response mediator protein 2 (CRMP2), microtubule-associated protein 2 (MAP2), and syntaxin-binding protein 1 (STXBP1) (data not shown). Interestingly, neuroglycan C (also known as chondroitin sulfate proteoglycan 5 (CSPG5)), is a transmembrane proteoglycan that is involved in dendritic branching [54] and synaptic regulation [55]. Moreover, neuroglycan C has been identified as a downstream target of plant homeodomain finger 6 (PHF6) [56], mutations of which cause Börjeson-Forssman-Lehmann syndrome (BFLS), an XLID disorder [57,58]. It would therefore be interesting to further investigate whether neuroglycan C is indeed a physiological PCTAIRE-1 target, and how this might link to its proposed role in XLID.

In summary, our chemical genetics screen has identified potential new PCTAIRE-1 targets AAK1, dynamin 1, and synaptotagmin 1 in mouse brain that have been demonstrated to regulate crucial steps of receptor endocytosis, and are involved in control of neuronal synaptic transmission. We have identified potential PCTAIRE-1 regulated phosphorylation sites on AAK1 and have validated their PCTAIRE-1 dependence in a cellular context and/or in brain tissue lysates. Our results shed light onto the missing link between PCTAIRE-1 regulation and proposed physiological functions, and provide a basis upon which to further study PCTAIRE-1 function in vivo and its potential role in neuronal/brain disorders.

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Declaration of interest

S.N.S., M.D., C.C., and K.S. are employees of the Nestlé Research (Switzerland).

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Author contribution statement

K.S. and S.N.S. designed the experiments, analysed the results and drafted the manuscript. S.N.S. performed the experiments described in Figs. 1, 2, 4. Experiments described in Fig. 5 were jointly performed by S.N.S. and C.C. D.S. performed the mass spectrometry analyses described in Figs. 3 and 4D. M.D. performed all the molecular cloning and mutagenesis. S.S. and S.G. provided CDK16/PCTAIRE-1 knockout brain tissue. All authors reviewed, provided feedback, edited and approved the final manuscript.

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