CHAPTER 5

EphA4 protein kinase activity in hippocampal brain tissue of patients with Alzheimer’s disease

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in preparation
ABSTRACT
The dysfunction of synapses and the subsequent loss of neurons are major pathological hallmarks of Alzheimer's disease (AD). These events occur early and correlate well with cognitive decline observed in patients with mild cognitive impairment. Loss of synaptic functions involves aberrant signalling of the Ephrin receptor tyrosine kinase A4 (EphA4) which is highly expressed in the adult hippocampus.

We recently reported an altered distribution of EphA4 with neuritic amyloid beta plaques and tau tangles early in AD. Whether this re-localisation affects the EphA4 kinase activity remains elusive. To test this hypothesis, we investigated the EphA4 kinase activity in human hippocampal brain tissue lysates derived from AD and non-demented control cases. Since the currently available technologies to analyse the activity of one specific protein kinase in brain tissue lysates are limited, we developed a novel kinase immunodepletion assay (KID) and employed it in combination with kinase activity profiling.

We found that the KID method can be used to deplete EphA4 from brain tissue lysates and to determine the change in kinase activity after depletion of EphA4. The kinase activity of EphA4 was unchanged in brain tissue of AD patients compared to non-demented controls. These findings reveal that the re-localisation of EphA4 happens independently of its kinase activity.
INTRODUCTION
Alzheimer's disease (AD) is a neurodegenerative disorder that affects an increasing percentage of our ageing population. The pathological hallmarks of the disease are intracellular depositions of hyper-phosphorylated tau protein (pTau) leading to the formation of neurofibrillary tangles (NFTs) and the accumulation of extracellular amyloid beta (Aβ) deposits [1]. Diverse lines of evidence suggest that self-aggregated Aβ peptides lead to progressive synaptic dysfunction and subsequent neuronal death [2–4]. The loss of synapses is an early event in AD pathogenesis and correlates with the cognitive deficits observed in patients at early stages of AD [5–7].

Recent studies point towards a prominent role for the Erythropoietin-producing hepatocellular (Eph) protein kinase receptor family during aberrant synaptic functions associated with AD [8–10]. Eph receptors comprise the largest family of protein tyrosine kinases (PTK) and their membrane-bound ligands are called ephrins (Eph receptor-interacting proteins) [11]. Eph/ephrin signalling is highly complex and essential for a broad range of processes during neuronal development, synapse formation and maintenance and synaptic plasticity [12–17]. EphA4 forms heterotetramers, consisting of two EphA4 molecules and two ephrin molecules upon ligand binding. These tetramers keep two cells in close contact and are a requirement for downstream signalling by EphA4 [18,19]. Bidirectional modulation of synaptic functions by Eph/ephrin signalling has been observed [20]. Interestingly, the signalling between two Eph receptors requires a catalytically active kinase domain of the recipient Eph receptor (forward signalling) whereas the signalling of ephrins into the cell (reversed signalling) can take place independently from Eph receptor activity [21].

The Eph receptor family member EphA4 is highly expressed in the CA1-4 regions and the dentate gyrus of the adult hippocampus where it regulates spine morphology with the help of its ephrin A3 ligand [20,22–24]. It has been suggested that changes in hippocampal EphA4 signalling might precede the onset of memory decline in AD [14]. In a transgenic mouse model of AD overexpressing the human amyloid beta precursor protein (APP), reduced expression of EphA4 was linked to cognitive impairment [15]. Interestingly, synaptic damage induced by Aβ oligomers coincides with increased levels of EphA4 in synaptoneurosomes and cultured neurons [13]. That EphA4 mediates the Aβ-induced impairment of synaptic plasticity was demonstrated by depletion of EphA4 or blockage of the EphA4 activity through targeting its ligand-binding domain, resulting in reversed synaptic deficits in AD mouse models [25].

We recently reported that while the protein levels of EphA4 in the human hippocampus remained unchanged with progression of AD, the distribution of EphA4 in the hippocampi of AD patients compared to non-demented controls was altered [26]. In AD,
EphA4 immunoreactivity co-localized with neuritic amyloid plaques and tau tangles staining. Whether this re-localization is associated with altered EphA4 kinase activity is unknown. Yet, we hypothesized that the altered distribution goes hand in hand with aberrant EphA4 kinase activity. We therefore decided to determine the kinase activity of EphA4 in protein lysates prepared from brain tissue lysates of AD and non-demented controls. However, we quickly discovered that no method is available to directly measure the activity of an individual protein kinase in a complex mixture of proteins. We hence developed a protein kinase immunodepletion (KID) method that we used in combination with protein kinase profiling.

We found no significant changes in protein kinase activity of EphA4 in human AD brains compared to non-demented controls. Our findings show that the altered distribution of EphA4 in the hippocampus does not correlate with an altered kinase activity and raises the interesting possibility that EphA4 signalling during synaptic plasticity might function independently of its activity.

**MATERIAL & METHODS**

**Case selection**

Human brain tissue was obtained from the Netherlands Brain Bank (NBB, Amsterdam, The Netherlands). Prior to death, all donors gave written informed consent according to the Declaration of Helsinki for the use of their brain tissue and medical records for research purposes. The study was approved by the ethics committee of the NBB. Dementia status at death was determined on the basis of clinical information available during the last year of life. Neuropathological diagnosis was performed as previously described [27]. Cases with and without clinical neurological disease diagnosis were processed identically. Patients with co-morbidities like Parkinson’s disease, frontotemporal dementia (FTLD) or dementia with Lewy-bodies were excluded from the study.

In total, 10 patients with confirmed AD and 10 non-demented controls were included. Age, gender, clinical diagnosis, pH of the cerebrospinal fluid (CSF) and Braak score for neurofibrillary tangles (NFTs) and amyloid beta of all cases used in this study are listed in Table 1. *Post mortem* delay (PMD) of all cases was between 2½ and 9 hours with a median of less than 6 hours.
Preparation of brain tissue lysates

Twenty 10 µm thick frozen hippocampal tissue slices were cut and lysed as previously described [26,27]. The supernatants were pooled, snap frozen in 100 µl aliquots and stored at -80 °C. The protein concentration was determined using the Bradford Lowry Assay (Bio-Rad Protein Assay) with BSA as the standard. Protein lysates are susceptible to freeze-thawing, which can result in up to 30 % loss of kinase activity [28,29]. All 100 µl protein lysates were thawed, aliquoted into smaller portions and frozen one more time before using them for protein kinase immunodepletion. The workflow is represented in Figure 1.

Protein kinase immunodepletion (KID)

Modification of protein A/G

Protein A/G (0.024 µM, Pierce Biotechnology, Rockford, USA) is a recombinant fusion protein containing immunoglobulin G (IgG) binding domains of both protein A and protein G and four Fc binding domains [30]. The surface of a porous support material was used to immobilize chemically activated protein A/G molecules [30]. First protein A/G was modified by attaching an acetyl protected thiol group as follows: an aliquot of 1.2 mg of protein A/G was dissolved in 390 µl of phosphate-buffered saline (PBS, pH 7.4) and incubated with ten-fold molar excess of N-succinimidyl S-acetylthioacetae (SATA,

### Table 1 - Patient characteristics.

<table>
<thead>
<tr>
<th>Case number (previous study)</th>
<th>Braak stage (tau)</th>
<th>Braak stage (amyloid)</th>
<th>protein concentration [mg/ml]</th>
<th>clinical diagnosis</th>
<th>Gender</th>
<th>Age [years]</th>
<th>PMD [hours]</th>
<th>Brain weight [g]</th>
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CON = non-demented control; AD = Alzheimer’s disease; PMD = Post mortem delay; NA = not available; column 2 = case numbers for previously published studies ([26,27]).
Pierce Biotechnology) in 3.5 µl dimethylsulfoxide. The SATA solution was prepared by dissolving 1 mg of SATA in 65 µl of DMSO. Of this solution, 3.5 µl was added to the 390 µl ProtA/G solution. (DMSO, Merck, Darmstadt, Germany). The reaction mixture was incubated for 45 min at room temperature and dialyzed against PBS pH 5.0, for 2 hours and again overnight at room temperature using a Slide-a-Lyzer® Cassette (Pierce Biotechnology). Dialysed modified protein A/G samples were stored at 4 °C [30].

Figure 1 - Experimental workflow. Brain tissue was obtained from frozen hippocampal sections (N=20) and lysed. Brain tissue lysates were incubation with either Rb (mock), EphA4 or Src antibody (A). Lysates were applied to FAEC arrays with covalently coupled protein A/G. Each PamChip® contains 4 arrays, allowing for duplicates of each condition to be measured on the same chip (B). After depletion of EphA4 (or Src), antibody-sample-complexes were manually aspirated from the depletion arrays and transferred to PTK arrays for the determination of the remaining kinase activity (C). Fluorescence was captured with a CCD camera, followed by image and data analysis with BioNavigator® software (PamGene) (D).
Activation and coupling of protein A/G to the PamChip® surface

Each immunodepletion experiment started with the deacetylation of the thiol moiety of SATA protein A/G as follows: for each array 1.6 µl (4.9 µg) modified protein A/G was incubated with 0.25 µl hydroxylamine solution containing 0.5 M hydroxylamine and 0.25 mM ethylenediaminetetraacetic acid [EDTA], pH 7.4, for 2 hrs at room temperature. The protein solution was diluted by adding 15 µl of 0.2 M sodium acetate buffer (pH 5.0) and applied to the surface of a fully activated PamChip® array without peptides (fully activated empty chip, FAEC) and the arrays were immediately placed in a PamStation 12. The protein A/G solution was pumped through the membrane (8 cycles of 1 min) to allow covalent binding, and the arrays were washed two times with ThiolPEG (2-[2-(2-mercapto-ethoxy)-ethoxy]-ethanol, MercaChem B.V., Nijmegen, The Netherlands). The arrays were blocked with 30 µl Thiol-PEG by pumping the solution up and down through the array (30 cycles of 30 sec).

After this step, the arrays were washed three times with PBS containing 0.01 % Tween 20, followed by a solution of 30 µl PBS containing 1 % BSA for 30 cycles of 30 seconds each, aspirated (10 sec) and washed again with PBS containing 0.01 % Tween 20.

Immunodepletion

While the protein A/G was coupled to the arrays, 1 µl anti-Eph receptor A4 rabbit polyclonal antibody (#5396, Abcam, Cambridge, UK) was added to the hippocampal lysate. The lysate volume was completed to 9 µl with M-PER lysis buffer. As a positive control anti-Src rabbit monoclonal antibody (36D10) (#2109, Cell Signalling Technologies, Danvers MA, USA) was used and anti-phospho-Retinoblastoma (Rb; Ser807/811) rabbit monoclonal antibody (D20B12) (#8516, Cell Signalling Technologies) as a negative control (mock depletion). After 2 hrs incubation at 4 °C, 30 µl of buffer containing 4.0 µl 10x PK buffer (1x PK buffer contains 50 mM Tris/HCl, 10 mM MgCl$_2$, 1 mM EDTA, 2 mM DTT, 0.01 % Brij, pH 7.5), 4.0 µl 10x PTK additive (PamGene, International BV, 's-Hertogenbosch, the Netherlands), 0.4 µl 1M DTT was added to the antibody-lysate mixture and the solution was transferred onto the array containing covalently bound protein A/G. The mixture was pumped up and down (30 cycles, 30 min) in order to bind the antibody-antigen complex to protein A/G. After the depletion of the antibody-protein complex on this array, 35 µl of the incubation mixture was manually aspirated from the array. This kinase depleted sample was used immediately for protein kinase activity profiling.

Protein kinase activity profiling

Protein kinase activity profiles were generated using the PamChip® protein tyrosine (PTK) peptide microarray system from PamGene International B.V. ('s-Hertogenbosch, The Netherlands) as described previously [27,28,31]. The kinase-depleted sample (35
µl) was added to 5 µl assay mixture containing 0.5 µl 10x PK buffer, 0.5 µl 10x PTK additive (PamGene), 0.05 µl 1M DTT, 0.05 µl 1 % BSA and 0.6 µl PY20-FITC antibody (0513B, AdB Serotec, Kidlington, UK). The enzymatic reaction was started by adding 0.4 µl 4 mM ATP (Sigma Aldrich, St. Louis, USA) and the sample was quickly transferred to the PTK PamChip® and pumped up and down (1 cycle per min) for 60 cycles to allow phosphorylation of the peptides on the chip. PY20-FITC antibody was used to detect phosphorylation. Pictures were taken with a CCD camera every 5 cycles at exposure times of 20, 50 and 100 ms. For each sample, immunodepletion followed by kinase profiling was performed in three independent experiments. For the following samples the experiment was performed 6 times: TIS300, 301, 303, 304, 312 and 314 (EphA4 depletion); TIS301, 303, 307, 315, 318 and 319 (Src depletion).

Western blot analysis
After depletion of the hippocampal lysate, 10 µl of the recovered lysate, containing approximately 2.5 µg of protein, were analysed by Western blotting. Proteins were separated using a 10 % Mini-PROTEAN® TGX gel (Biorad, Berkeley, USA) and subsequently transferred onto PROTRAN Nitrocellulose Transfer Membrane (GE Healthcare, Buckinghamshire, UK) using standard procedures as described by the manufacturers. The membrane was blocked with PBS (Biorad) containing 5 % BSA and 0.1 % Tween 20 (PBS-BSA-Tween) for one hour at room temperature while shaking. EphA4 and Src, antibodies were diluted 1:1.000 in PBS-BSA-Tween and used for incubation of the membrane overnight at 4 °C while shaking. Anti-EphA4 mouse monoclonal antibody (#6101471, Becton-Dickinson, New Jersey, USA) and anti-Src rabbit monoclonal antibody (36D10 #2109; Cell Signalling Technologies) were used. Incubation with the secondary goat anti-mouse or goat anti-rabbit IgG conjugated to horseradish peroxidase (1:100.000 in PBS-BSA-Tween) was performed for 1 h at 4 °C while rotating. Membranes were washed 3x 10 mins with 10 ml PBS-BSA-Tween rotating at room temperature between each antibody incubation step. Antibodies were detected using SuperSignal® West Femto (Thermo Scientific) in combination with Carestream® Kodak® Biomax® MS film (Sigma-Aldrich) according to the manufacturer’s instructions. The film was developed in GBX developer (Kodak, New York, USA) and fixed in GBX fixer (Kodak). An anti-actin antibody (A2066-100UL -Sigma) was used to stain the actin loading control (1:100.000).

Kinase activity profiling data analysis
The fluorescence intensity of each peptide on a 144-peptide array was analysed using BioNavigator 6.2 software (PamGene International B.V., ‘s-Hertogenbosch, The Netherlands). In order to quantify the signal, to increase the dynamic range and filter out time differences, the slopes of the peptide-fluorescence minus background per time unit gave the kinase activity versus exposure time for all samples. Saturated signals
were excluded and a visual quality control was performed to exclude defective arrays from the analysis. The fluorescence intensity of each peptide was calculated by taking the mean value from the last three time points of incubation (last three images taken). Negative values were set to 1. The ratios between the mock depletion and the depletion with EphA4, respectively Src, were calculated for each replicate. Then the average ratio was expressed as percentage of the remaining activity.

RESULTS
In order to investigate whether the re-localisation of EphA4 in AD patients compared to non-demented controls [26] was associated with changes in protein kinase activity of EphA4, we developed a new method that allowed the removal of one specific protein kinase from brain lysates. The kinase immunodepletion (KID) assay was used to deplete EphA4 from hippocampal tissue lysates allowing a comparison between the kinase activity of the depleted lysate with the lysate after mock depletion (negative control) and providing information about EphA4 activity (Figure 1).

Protein Kinase Immunodepletion (KID) method
The KID method is based on FAEC technology, whereby the surface of the porous support material (PamChip®) was used to immobilise activated protein A/G [30]. For the immunodepletion of EphA4, an anti-EphA4 rabbit polyclonal was used. An antibody raised against Src was used as a positive control (Figure 1A and 1B). As a negative control we used the antibody against Retinoblastoma (Rb) that has no kinase activity. Antibodies were added to the brain lysate for 2 hours after which the lysates were applied to the FAEC chip containing protein A/G, to allow binding of the antibody-antigen complex. After 30 min the protein lysates were collected and subjected to Western blot analysis and/or kinase profiling. For optimization, a concentration series of each antibody was tested to determine the optimal concentration required for full depletion (data not shown).

Hippocampal brain tissue lysates of AD patients (n=10, Braak V and VI) and non-demented controls (n=10, Braak I and II) who died of unrelated non-neurological causes were used for EphA4 depletion. In order to compare our results with those from our previous study on the localisation of EphA4 in the human hippocampus, 15 out of 20 the cases were used again [26] and an additional 5 well characterized cases were selected from another previous study [27]. For the AD cases (Braak stage V and VI), mainly samples showing a relatively high kinase activity were selected to allow measurement of activity after depletion.
To confirm protein depletion, KID treated brain lysates were analysed using western blotting. Figure S1 shows the presence of bands for EphA4 (~108 kDa) and Src (~50 kDa) in the mock depleted hippocampal tissue lysates of TIS304. These bands were not detected after depletion. The presence of a single band of 108 kDa indicates that full length EphA4 is depleted and not just the kinase domain.

Protein kinase activity profiling

In the next step, the kinase activities of the depleted and mock-depleted lysates were compared. We found that as a result of the additional freeze-thaw step, protein kinase activity was lower in general. In addition, we found a substantial difference between samples with regards to the degree and amount of phosphorylated peptides, making a comparison of all (144) peptides between samples difficult (data not shown). However, all samples had decent phosphorylation of the peptide CD79A. Therefore, CD79A was used as the sole measure (read-out) for the remaining cellular PTK activity after depletion. Figure S2 shows an example of the kinetics of the phosphorylation of peptide CD79A. When the antibody against EphA4 was used for depletion, the phosphorylation was reduced. Use of the Src antibody resulted in a larger reduction (Figure 3). A depletion without a primary antibody gave the same results as depletion with the Rb antibody (data not shown). One control case (TIS318) showed high signal intensity in comparison to the other cases.

Protein kinase activities were calculated using the BioNavigator 6.2 software (PamGene International BV, 's-Hertogenbosch, The Netherlands) as described previously [27]. Visual quality control of the array was performed and mechanically damaged arrays were excluded. To integrate signals obtained at different exposure times, the slope of the signal versus exposure time was calculated and saturated signals were excluded. Subsequently, the ratio between the mock depletion and the EphA4 or Src depletion was calculated for each replicate. The average ratio was expressed for the representative peptide, CD79A, in percentage remaining activity. The remaining phosphorylation of peptide CD79A was 60-80 % in the case of EphA4 depletion (Figure 3A), and 5-20 % for Src depletion (Figure 3B). We found that there was no significant difference between AD and controls (Figure 3). In summary we show that depletion of a specific kinase can be achieved by using the KID method resulting in a significant reduction of kinase activity.
Figure 2 – Peptide (CD79A) phosphorylation after control (mock) depletion. Bar graph for all 20 samples after depletion with Rb control antibody (= negative control; left: non-demented controls; right: Alzheimer’s disease cases). These values have been used to calculate the ratios shown in Figure 3. Fluorescence is shown for the representative peptide CD79A.

Figure 3 – Percentage of activity remaining after EphA4 and Src depletion. Percentage tyrosine protein kinase activity remaining for each sample after depletion of EphA4 (A) or Src (B). For percentage calculation, the values with mock depletion (Figure 2) were used as a negative control. Left: non-demented controls; right: Alzheimer’s disease samples. Scale in [%] activity remaining after depletion.
DISCUSSION
Synaptic loss is considered an early event in AD pathogenesis and as such an interesting target for pharmacological intervention [2,32]. EphA4 signalling plays a crucial role during synaptic formation and plasticity in the human brain [33,34]. In the hippocampi of AD patients, EphA4 levels remained unchanged compared to controls. However, the distribution of EphA4 changed towards a co-localization with the hallmarks of AD (Aβ and tau) [26].

We hypothesized that the observed altered distribution of EphA4 in AD patients might be the result of an aberrant function of the kinase. Therefore, we investigated the protein kinase activity of EphA4 in human hippocampus derived from AD (n=10) and non-demented control cases (n=10). Current technologies to investigate the activity of one specific protein kinase in brain tissue lysate are limited. Protein kinase activity is often inferred from Western blot analysis showing that a particular site of a kinase is phosphorylated [35]. However, most protein kinases only become activated by a series of consecutive phosphorylation events. Their activity may also require the interactions with other proteins. To monitor the kinase activity of protein kinase B (PKB, also known as Akt) for example, Western blotting is generally used to detect phosphorylated threonine T308 and serine S473. However, Akt activity is not only affected by the phosphorylation of T308 by 3-phosphoinositide-dependent kinase 1 (PDK1) [36] and S473 by mammalian target of rapamycin C2 (mTORC2) [37]. In addition, the phosphorylation of the tyrosine residues Y315, Y326 by breast tumor kinase (Brk), also known as protein tyrosine kinase 6 (PTK6), and Y474 (by an unknown kinase) increase the kinase activity of Akt [38,39]. Src catalyzed phosphorylation of recombinant Akt with T308 and S473 already phosphorylated, increased the Akt activity about two-fold (R. Hilhorst, unpublished observation). Assessing the phosphorylation of T308 and S473 by blot is hence insufficient to determine the activity of Akt.

EphA4 becomes activated upon ligand (ephrin) binding, which is followed by phosphorylation of the tyrosine (Y) residues in the kinase domain (Y779) and the juxtamembrane segment (Y596 and Y602 [35,40]). Other potential phosphorylation sites of EphA4 are unknown, which makes it impossible to infer EphA4 kinase activity by determining the state of its phosphorylation sites. The KID method that we present here allows the determination of kinase activity of EphA4 in a brain lysate. The KID method is based on depletion of EphA4 from a hippocampal tissue lysate using a specific antibody. Kinase activity profiles of the depleted and non-depleted samples can then be compared. Thereby we were able to obtain information about the activity of EphA4 without the need of antibodies directed against specific phospho-sites. We show that the KID method has a wide dynamic range and gives highly reproducible results even when using as little as 10 µg of total brain protein lysate. EphA4 activity
did not change significantly in brain tissue lysates of AD compared to non-demented controls.

Eph4A signalling follows a multistep process of induced kinase activity as well as higher-order clustering [12]. The EphA4 kinase activity is strictly correlated with the degree of enzymatic activation through dimerization [41]. It was shown that the higher-order clustering does not lead to an increase in EphA4 kinase activity, but rather to the recruitment of a new set of cytoplasmic effectors. The altered distribution of EphA4 in AD hippocampus may reflect a decreased function of EphA4 [26]. However, changes in synaptic plasticity in AD might not be due to a loss of EphA4 protein kinase activity but may instead reflect aberrant signalling mechanisms of EphA4 and our results suggest, that EphA4 mediates synaptic plasticity in a kinase-independent manner.

EphA4-mediated ephrin reverse signalling is required for synaptic plasticity in the hippocampus. The deletion of EphA4 blocks CA3-CA1 long-term potentiation (LTP) in mice [42]. This observation indicates that forward-signalling-dependent spine morphogenesis that involves kinase activity of the Eph receptors [23,33] is not a major mechanism in the regulation of synaptic plasticity [21,43]. On the contrary, while EphA4 signalling leads to the induction of ephrin B mediated reverse signalling and cis-interaction (interaction between Eph receptors and proteins that are co-expressed on the same cell [44]) with synaptic proteins such as N-Methyl-D-aspartate receptors (NMDAR), its kinase activity remains the same [45,46].

The complexity of kinase-dependent and kinase-independent EphA4 signalling, raises challenges in understanding its signalling mechanisms during synaptic plasticity and pathological synaptic dysfunction in early stages of AD [47]. Whether EphA4 kinase activity directly contributes to the formation of neuritic plaques and tangles remains to be seen. By using a new KID method and showing that EphA4 kinase activity is unchanged in AD while EphA4 re-distribution has occurred, opens this possibility for further investigation.

ACKNOWLEDGEMENTS

We thank the Netherlands Brain Bank for providing human brain tissue for this study, Annemieke Rozemuller for neuropathological evaluation of the autopsy material, Tjado H. J. Morrema and Nils Lievense for technical assistance.
REFERENCES


36 Alessi DR, James SR, Downes CP, Holmes...


SUPPLEMENTARY FIGURES

Figure S1 – Western blot analysis (TIS304). Mock depleted and EphA4 and Src depleted tissue lysates of TIS304. Lysates were loaded in duplicates. Antibodies were detected using SuperSignal® West Femto in combination with Carestream® Kodak® Biomax® MS film. An anti-actin antibody was used as a loading control. Left: depleted, right: mock depleted (negative control). Actin was used as a loading control.

Figure S2 – Kinetics of protein kinase activity (TIS315). Kinetics for EphA4 (blue), Src family kinase (green), depleted samples, in comparison to Rb (pink) mock depletion for the peptide CD79A. Shown is the median fluorescence intensity of the CD79A peptide of 3 replicates of TIS315 in arbitrary units (a.u.). Each dot represents one cycle as indicated on the x-axis.