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Metalloproteinase/Presenilin1 processing of ephrinB regulates EphB-induced Src phosphorylation and signaling

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Bidirectional signaling triggered by interacting ephrinB receptors (EphB) and ephrinB ligands is crucial for development and function of the vascular and nervous systems. A signaling cascade triggered by this interaction involves activation of Src kinase and phosphorylation of ephrinB. The mechanism, however, by which EphB activates Src in the ephrinB-expressing cells is unknown. Here we show that EphB stimulates a metalloproteinase cleavage of ephrinB2, producing a carboxy-terminal fragment that is further processed by PS1/γ-secretase to produce intracellular peptide ephrinB2/CTF2. This peptide binds Src and inhibits its association with inhibitory kinase Csk, allowing autophosphorylation of Src at residue tyr418. EphrinB2/CTF2-activated Src phosphorylates ephrinB2 and inhibits its processing by γ-secretase. These data show that the PS1/γ-secretase system controls Src activation and ephrinB phosphorylation by regulating production of Src activator ephrinB2/CTF2. Accordingly, γ-secretase inhibitors prevented the EphB-induced sprouting of endothelial cells and the recruitment of Grb4 to ephrinB. PS1 FAD and γ-secretase dominant-negative mutants inhibited the EphB-induced cleavage of ephrinB2 and Src autophosphorylation, raising the possibility that FAD mutants interfere with the functions of Src and ephrinB2 in the CNS.

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Introduction

Presenilin1 (PS1) is a polytopic transmembrane protein expressed in many tissues including the brain, where it is enriched in neurons and synaptic contacts (Sherrington *et al*, 1995; Elder *et al*, 1996; Georgakopoulos *et al*, 1999). PS1 is cleaved *in vivo* to yield N-terminal (PS1/NTF) and C-terminal (PS1/CTF) fragments that associate to form a functional heterodimer (Thinakaran *et al*, 1996). PS1 promotes the γ-secretase processing of APP and production of Aβ peptides, the structural unit of amyloid depositions of AD. Recent reports, however, show that, in addition to the classic γ-secretase cleavages of APP that take place at approximately the middle of its transmembrane sequence, the PS1/γ-secretase system promotes the ε-cleavage of several type I transmembrane proteins, including APP, Notch1 receptor, cadherins and CD44. This cleavage occurs close to the membrane/cytoplasm interface and is also sensitive to γ-secretase inhibitors. The resulting soluble peptides contain the cytoplasmic sequence of the cleaved proteins and have been shown to function as transcription factors and surface-to-nucleus communication signals (for reviews, see Kopan and Ilagan, 2004; Marambaud and Robakis, 2005).

The ephrinB family of proteins, including ephrins B1, B2 and B3, are homologous type I transmembrane polypeptides that act as ligands of the ephrinB receptor (EphB) tyrosine kinases (Tuzi and Gullick, 1994). Binding of ephrinB to EphB at cell–cell contacts triggers bidirectional intracellular signaling in both the EphB- and the ephrinB ligand-expressing cells (Boyd and Lackmann, 2001). Thus, bidirectional signaling has emerged as an important mechanism by which the ephrinB–EphB system regulates the output signal in cell–cell interaction and communication (Boyd and Lackmann, 2001; Schmucker and Zipursky, 2001; Adams, 2002). The EphB–ephrinB signaling regulates development and function of many tissues and organs, including the vascular and nervous systems (Palmer and Klein, 2003). For example, inactivation of the ephrinB2 gene results in severe vascular defects and midgestational embryonic lethality. Similar defects are caused by the removal of the cytoplasmic domain of ephrinB2, suggesting that reverse signaling through the cytoplasmic domain of ephrinB is necessary for normal vascular development (for a review, see Adams, 2002). In the developing CNS, EphB–ephrinB binding regulates cell migration, axon guidance and synaptogenesis, while in the adult brain it regulates memory-related functions, including synaptic plasticity and long-term potentiation (Contractor *et al*, 2002; Grunwald *et al*, 2004). Reverse signaling through the cytoplasmic domain of ephrinB2 is required for the pathfinding of axons that form the posterior tract of the anterior commissure (Cowan *et al*, 2004).

An early event following the EphB–ephrinB binding is the activation of Src kinase pp60src, which in turn phosphorylates the cytoplasmic domain of ephrinB at tyrosine residues. Tyrosine phosphorylation of ephrinB creates binding sites for SH2 domain adaptor proteins triggering the recruitment to ephrinB of cytosolic factors, including Grb4, that control actin dynamics and cell migration (Cowan and Henkemeyer, 2001).

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Furthermore, Src activity mediates the EphB-induced sprouting of endothelial cells, but the mechanisms by which the EphB–ephrinB interaction activates Src in the ligand-expressing cells remains unknown (Palmer *et al*, 2002). Recently, Src kinases have emerged as important factors for the induction of synaptic plasticity and for the regulation of NMDA receptor-dependent synaptic potentiation (Salter and Kalia, 2004). Src activation is regulated by c-terminal Src kinase (Csk) that binds Src and keeps it in an inactive conformation by inhibiting its autophosphorylation at residue tyr418 (Thomas and Brugge, 1997). Removal of the inhibitory Csk from the complex activates Src by allowing its autophosphorylation at tyr418 (Nada *et al*, 1993). Here we show that EphB stimulate a metalloproteinase (MP)/γ-secretase processing of ephrinB ligands to produce intracellular peptide ephrinB/CTF2, which contains the cytoplasmic sequence of ephrinB. Peptide ephrinB/CTF2 activates Src kinase by inhibiting its association with Csk kinase. Activated Src phosphorylates ephrinB and inhibits its processing by the PS1/γ-secretase system. Our data show that PS1 regulates the EphB-induced Src activation and signaling by promoting production of peptide ephrinB/CTF2.

Results

EphrinB ligands are cleaved by MP and PS1/γ-secretase

To investigate the role of PS1 in ephrinB processing, brain extracts from PS1 knockout mouse embryos (PS1−/−) (Marambaud *et al*, 2003; Baki *et al*, 2004) were probed with antibody C18 against the cytoplasmic sequence of ephrinB. Compared to PS1+/+ embryos, brains of PS1−/− embryos contained significantly higher amounts of a ~14 kDa carboxy-terminal fragment (Figure 1A, middle panel), although the levels of full-length ephrinB were similar in all brains (Figure 1A, upper panel). Due to the high homology and similar sizes of the cytoplasmic domains of ephrinBs, C18 antibody cannot distinguish between the three members of the ephrinB family of proteins (reviewed in Flanagan and Vanderhaeghen, 1998) and the detected 14 kDa fragment may derive from one or all members of the family. The immunoreactivity and apparent molecular mass of this fragment suggest that it contains the entire intracellular sequence of ephrinB. Accumulation of the 14 kDa fragment in PS1−/− brains suggests that it is metabolized by the PS1/γ-secretase system and may be derived from an MP cleavage of ephrinB analogously to other type I transmembrane proteins, including E- and N-cadherins (Marambaud *et al*, 2002, 2003). Indeed, MP inhibitor GM6001 inhibits accumulation of the 14 kDa fragment (termed ephrinB/CTF1) in PS1−/− mouse fibroblasts (Figure 1B, lanes 1 and 2). In contrast, γ-secretase inhibitors promote accumulation of ephrinB/CTF1 in WT cells (Figure 1B, lanes 3 and 4). Together, these data indicate that ephrinB/CTF1 is derived from ephrinB proteins by an MP activity and is subsequently metabolized by the PS1/γ-secretase system.

Since in fibroblasts we were unable to detect the product predicted from the γ-secretase cleavage of ephrinB/CTF1, we used HEK293 cells overexpressing exogenous mouse ephrinB2 cDNA. Treatment of these cells with phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which stimulates the MP cleavage (Vecchi and Carpenter, 1997), in combination with proteasomal inhibitor lactacystin, which

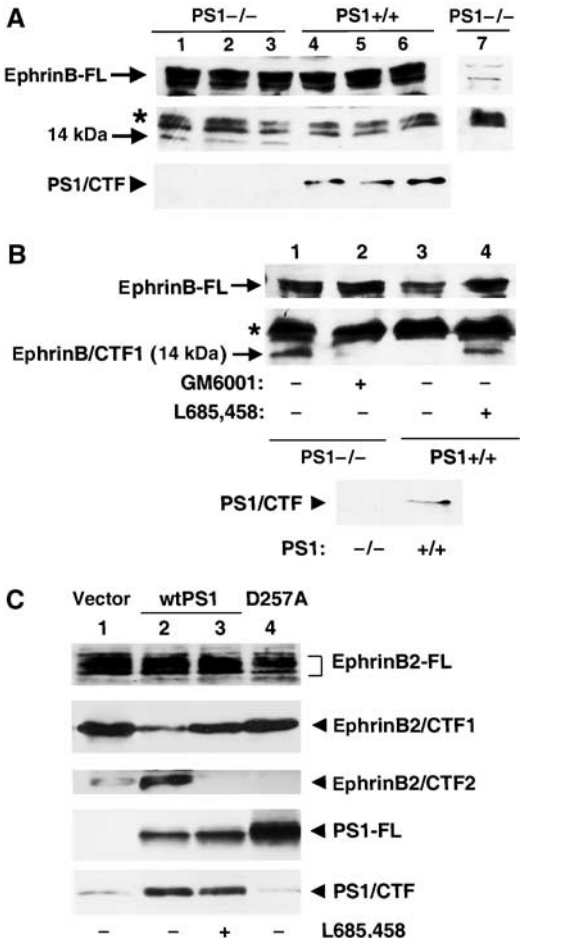

Figure 1 (**A**) Processing of ephrinB proteins in PS1 knockout mice. Brain tissue from three PS1 knockout (PS1−/−, lanes 1–3) or wild-type (PS1+/+, lanes 4–6) mouse embryos (E14.5) were solubilized in SDS solubilization buffer in the presence of protease inhibitors and protein extracts were probed on WBs either with anti-ephrinB C18 antibodies (lanes 1–6) or with C18 antibodies pretreated with the corresponding antigenic peptide (lane 7). Lower panel: Extracts were also probed with monoclonal antibody 33B10 against PS1/CTF (Georgakopoulos *et al*, 1999). Upper and lower arrows identify full-length ephrinB (ephrinB-FL) or a ~14 kDa ephrinB carboxyl-terminal fragment, respectively. *Nonspecific signal. (**B**) MP fragment ephrinB/CTF1 is detected in fibroblast cultures. PS1−/− mouse fibroblasts (lanes 1 and 2) were incubated either in the absence (lane 1) or in the presence (lane 2) of 2.5 μM MP inhibitor GM6001 for 30 min. PS1+/+ fibroblasts (lanes 3 and 4) were incubated either in the absence (lane 3) or in the presence (lane 4) of 0.5 μM L-685,458. Extracts from these cultures were probed with antibody C18 (upper two panels). The upper arrow indicates full-length ephrinB and the lower arrow indicates ephrinB/CTF1. Lower panel: Extracts from PS1−/− or PS1+/+ fibroblasts were probed with antibody 33B10. *Nonspecific signal. (**C**) PS1/γ-secretase fragment ephrinB2/CTF2 is detected in HEK293 cells overexpressing ephrinB2. HEK293 cells stably transfected with vector (lane 1), with wtPS1 (lanes 2 and 3) or with γ-secretase dominant-negative PS1 mutant D257A (lane 4) were transiently transfected with mouse ephrinB2 cDNA (Gale *et al*, 1996). All cultures were incubated with 100 ng/ml TPA for 2 h and then 10 μM of lactacystin was added to all cultures for 10 h. One culture was also treated with 0.5 μM of L-685,458 (lane 3). Extracts were probed with antibody C18 (upper three panels) or 33B10 (two lower panels). Mutant D257A has been described (Wolfe *et al*, 1999; Marambaud *et al*, 2002). Transfected full-length ephrinB2 is detected as several closely migrating bands, probably the result of post-translational modifications (Kalo *et al*, 2001).

prevents degradation of peptides derived by the γ-secretase cleavage (Kim *et al*, 2002), resulted in the detection of a ~12 kDa carboxy-terminal fragment, termed ephrinB2/CTF2, in the cell extract (Figure 1C, lane 1). Production of this fragment is stimulated by PS1, but is inhibited by both γ-secretase inhibitor L-685,458 and γ-secretase dominant-negative mutant PS1D257A (Wolfe *et al*, 1999) (Figure 1C). Co-immunoprecipitation (co-IP) experiments showed that ephrinB forms complexes with PS1 (Figure 1 in Supplementary data). Together, our data show that PS1 forms complexes with ephrinB ligands and promotes their metabolism through a process similar to that described for other PS1/γ-secretase substrates (Marambaud *et al*, 2002).

EphB stimulates MP/γ-secretase processing of ephrinB2 ligands

Since PS1 regulates processing of ephrinB ligands, we asked whether this processing might be induced by the EphB/ephrinB interaction. To examine this question, we treated ephrinB2-overexpressing PS1+/+ and PS1−/− fibroblasts with a clustered EphB2–Fc fusion construct which contains the EphB2 receptor ectodomain fused to the Fc portion of human IgG. This construct binds to and activates the ephrinB ligands, mimicking the receptor effects (Gale *et al*, 1996). Figure 2 shows that treatment of fibroblasts with EphB2-Fc for 10 min results in an increase of both the MP fragment ephrinB2/CTF1 and the γ-secretase fragment ephrinB2/CTF2 (lanes 1 and 2). Longer treatment resulted in a further accumulation of these fragments (Figure 2A in Supplementary data). Furthermore, production of the γ-secretase fragment is inhibited by γ-secretase inhibitors (lanes 2 and 4) and in the absence of PS1 (lanes 2 and 6). EphB2 stimulates the MP cleavage of ephrinB2 even in the presence of γ-secretase inhibitors (lanes 3 and 4) or in the absence of PS1 (lanes 5 and 6), suggesting that the MP cleavage is independent of the PS1/γ-secretase processing. Similar results were obtained using nontransduced fibroblasts. However, due to the low levels of endogenous ephrinB, we could only detect the MP fragment ephrinB/CTF1 in this system. The cellular levels of this fragment increased in response to EphB2-Fc treatment (Figure 2B in Supplementary data). Together, our data show that the EphB stimulates the MP cleavage of ephrinB2 and that the resulting peptide ephrinB2/CTF1 is further processed by the PS1/γ-secretase system to produce peptide ephrinB2/CTF2.

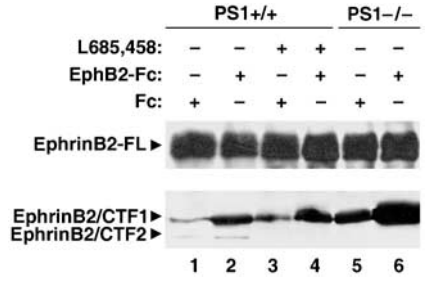

Figure 2 EphB2–Fc stimulates the MP/PS1/γ-secretase processing of ephrinB2. PS1+/+ fibroblasts transduced with ephrinB2 in pMX vector were treated with 2 μg/ml of preclustered EphB2–Fc for 10 min in the absence (lane 2) or presence (lane 4) of 0.5 μM γ-secretase inhibitor or with 2 μg/ml of Fc control in the absence (lane 1) or presence (lane 3) of γ-secretase inhibitor. PS1−/− fibroblasts were also treated with Fc (lane 5) or EphB2–Fc (lane 6) for the same amount of time. Preclustering of EphB2–Fc fusion protein was performed as described (Palmer *et al*, 2002). All samples were incubated in the presence of 10 μM lactacystin. Total extracts were probed on WBs with antibody C18. Detected proteins and fragments are indicated in the left of the figure. EphrinB2-FL: full-length ephrinB2.

The PS1/γ-secretase system regulates the EphB-induced phosphorylation of Src and ephrinB2

Binding of EphB to ephrinB ligands activates Src kinase in the ephrinB-expressing cells. The mechanism of this event, however, remains elusive (Palmer *et al*, 2002). We observed that treatment of fibroblasts with EphB2-Fc stimulates Src phosphorylation at residue tyrosine 418 (tyr418), an autophosphorylation event known to activate Src (Thomas and Brugge, 1997). To examine whether PS1 regulates the EphB-induced Src autophosphorylation, PS1+/+ and PS1−/− cells were treated with EphB2-Fc and phosphorylation of Src residue tyr418 was detected as described (Takasu *et al*, 2002). Figure 3A shows that EphB2–Fc stimulates phosphorylation of Src tyr418 in PS1+/+ but not in PS1−/− cells. In addition, γ-secretase inhibitor L-685,458 prevented the EphB2–Fc-induced phosphorylation of Src. These data indicate that the PS1/γ-secretase system mediates the EphB-induced activation of Src. Since the ephrinB/EphB system plays pivotal roles in the development and function of the CNS (Palmer and Klein, 2003), we asked whether the PS1/γ-secretase system acts similarly in neurons. Figure 3B shows that EphB2–Fc increased phosphorylation of Src tyr418 in PS1+/+ but not in PS1−/− mouse brain primary neurons. Furthermore, treatment of primary neurons with a γ-secretase inhibitor inhibited the EphB-induced (Figure 3B, lanes 1–3), but not the Reelin-induced (Figure 3 in Supplementary data), phosphorylation of Src, indicating that γ-secretase specifically mediates the EphB-induced phosphorylation of Src. Together, these data indicate that PS1 regulates the EphB-induced activation of Src in both neuronal and non-neuronal cells.

Binding of EphB to ephrinB ligands activates Src kinase that in turn phosphorylates the cytoplasmic domain of ephrinB (Palmer *et al*, 2002). That PS1 mediates the EphB-induced phosphorylation and activation of Src suggests that PS1 may also mediate phosphorylation of ephrinB. Indeed, Figure 3C (lanes 1–4) shows that, in PS1+/+ cells, inhibition of γ-secretase prevents the EphB-induced tyrosine phosphorylation of ephrinB2. Accordingly, PS1−/− cells contain low levels of phosphorylated ephrinB2 and EphB treatment fails to stimulate phosphorylation of ephrinB2 (lanes 5 and 6).

γ-Secretase activity regulates the EphB-induced recruitment of factor Grb4 to ephrinB and sprouting of endothelial cells

Recent evidence shows that EphB-induced Src phosphorylation of ephrinB tyrosines promotes sprouting activity of endothelial cells *in vitro*. In addition, tyrosine phosphorylation of ephrinB triggers the recruitment of cytosolic factor Grb4 to cytoplasmic ephrinB (Cowan and Henkemeyer, 2001). Since γ-secretase activity mediates the EphB-induced Src activation and ephrinB phosphorylation, we examined whether inhibition of γ-secretase activity prevents downstream effects, including Grb4 recruitment to ephrinB and

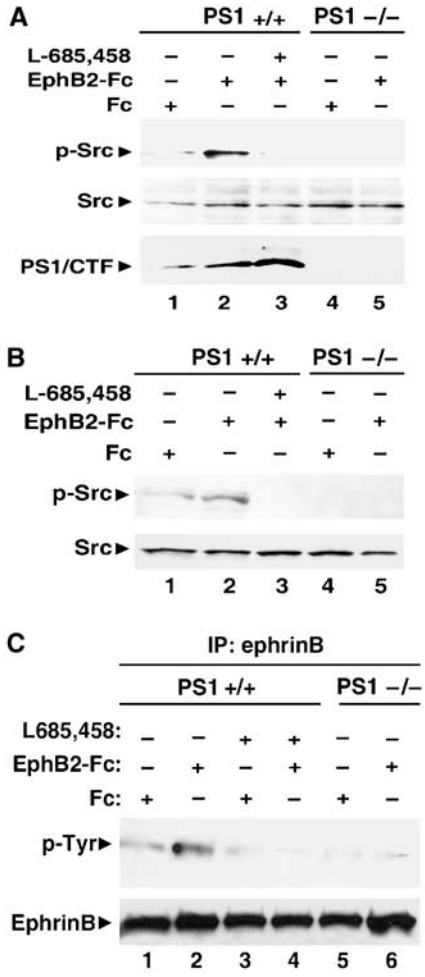

Figure 3 The PS1/γ-secretase system mediates the EphB2-induced phosphorylation of pp60Src in fibroblasts and primary neurons. (**A**) PS1 + / + or PS1 − / − fibroblasts were plated in medium containing 0.5% (v/v) FBS and treated for 6 h with 0.5 mM sodium suramin to dissociate any endogenous ephrinB from EphB (Stein *et al*, 1996). Cells were then washed and incubated for 90 min in Opti-MEM in the absence (lanes 1, 2, 4 and 5) or presence of 0.5 μM L-685,458 (lane 3) and then exposed for 5 min to 2 μg/ml of either preclustered EphB2–Fc or to Fc control protein in the presence or absence of L-685,458 as indicated in the figure. Cell lysates were analyzed on WBs using anti-pY^{418} antibody for detecting phosphorylated tyr418 of pp60Src (p-Src), or GD11 for detecting total pp60Src protein (Src). (**B**) Cortical neurons from PS1 + / + or PS1 − / − mice were cultured in NeuroBasal medium. After 8 days *in vitro* (DIV), a sample of PS1 + / + neurons was treated with 0.5 μM of L-685,458 overnight. Neurons were then exposed for 5 min to either 2 μg/ml of preclustered EphB2-Fc or to Fc control protein. Cell lysates were analyzed on WBs using either anti-pY^{418} (p-Src) or GD11 for total Src (Src). (**C**) PS1/γ-secretase mediates the EphB2-induced phosphorylation of ephrinB2. PS1 + / + (lanes 1–4) or PS1 − / − (lanes 5 and 6) fibroblasts were transduced by mouse ephrinB2 in pMX vector and then incubated in DMEM medium with 0.5% (v/v) FBS (see Figure 3A). Cells were then washed and transferred in Opti-MEM medium for 90 min in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 0.5 μM of L-685,458. At the end of the incubation, cells were treated with either 2 μg/ml EphB2-Fc (lanes 2, 4 and 6) or Fc (lanes 1, 3 and 5) for 5 min in the presence or absence of L-685,458, as indicated in the figure. Cells were treated with Triton X-100 extraction buffer and 1 mg of total protein extract was immunoprecipitated (IPed) with antibody C18. Obtained IPs were probed on WBs for phosphorylated ephrinB using anti-phosphotyrosine antibody pY99 (p-Tyr; upper panel), or for total ephrinB using anti-ephrinB antibody (lower panel).

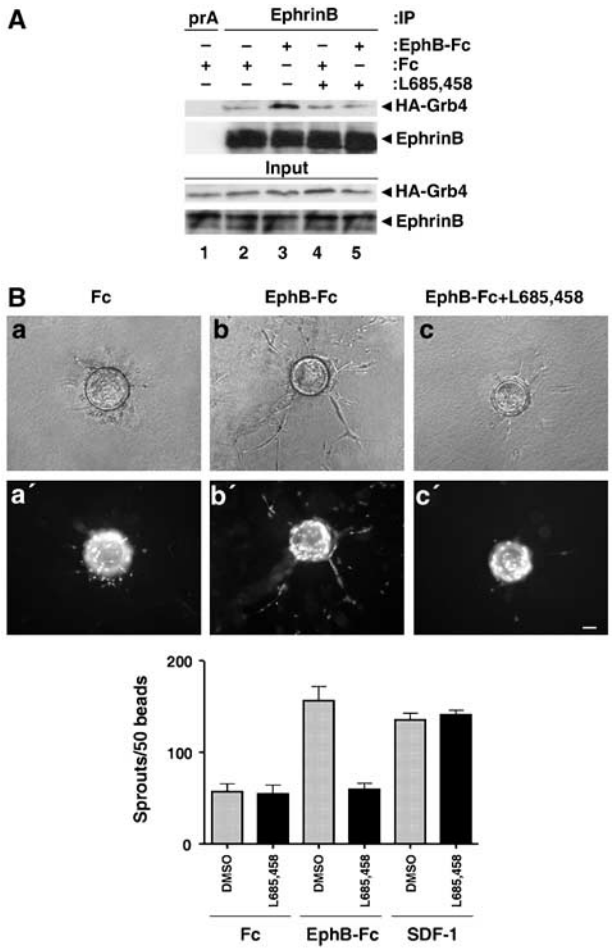

Figure 4 (**A**) EphB-induced recruitment of Grb4 to ephrinB is mediated by γ-secretase. PS1 + / + fibroblasts transduced with ephrinB2 in pMX vector were transiently transfected with HA-tagged Grb4 and treated with 2 μg/ml of EphB2–Fc for 10 min in the absence (lane 3) or presence (lane 5) of 0.5 μM γ-secretase inhibitor or with 2 μg/ml of Fc control in the absence (lanes 1 and 2) or presence (lane 4) of γ-secretase inhibitor. Cell extracts were IPed with antibody C18 (lanes 2–5) or protein A (lane 1, prA) and IPs were analyzed on WB with anti-HA antibody (first panel) or C18 (second panel). Total levels of HA-Grb4 and ephrinB are shown in the two lower panels (Input). (**B**) EphB-induced endothelial cell sprouting is mediated by γ-secretase. BAMEC cells were grown on MC beads and incubated in three-dimensional fibrin gels with inducers as described (Palmer *et al*, 2002) in the presence or absence of 0.5 μM L-685,458. Upper panel: Phase-contrast photomicrographs of sprouts induced by EphB4–Fc in the absence (b) or presence (c) of L685,458. Hoechst 333258 nuclear staining shows the number of endothelial cells that make up individual sprouts (a′–c′). Scale bar: 100 μM. Lower panel: Quantitative analysis of sprout formation (see Materials and methods) expressed as the number of capillary sprouts with lengths exceeding the diameter of the MC bead for every 50 MC counted. Bars represent the mean ± s.e. of three independent experiments.

endothelial cell sprouting activity. Figure 4A shows that γ-secretase inhibitor L-685,458 inhibits the EphB-induced recruitment of HA-tagged Grb4 (Chen *et al*, 2000) to ephrinB. Furthermore, Figure 4B shows that this inhibitor prevents the EphB-induced, but not the SDF-1-induced, sprouting of endothelial cells. Together, these data show that, by regulating the EphB-induced Src activation, the PS1/γ-secretase system also controls signaling events downstream of Src.

Peptide ephrinB2/CTF2 binds Src kinase and promotes its autophosphorylation even in the absence of PS1

That PS1/γ-secretase mediates the EphB-induced Src autophosphorylation suggested that the product of the γ-secretase processing of ephrinB2 may play a role in this process. To examine whether peptide ephrinB2/CTF2 interacts with Src, a cDNA encoding this peptide fused to three c-myc tags was constructed for detection purposes (ephrinB2/CTF2-myc3; GeneScript Co., NJ). Figure 5A shows that this construct co-IPs with Src kinase (upper panels) and the reverse is true, Src co-IPs with construct ephrinB2/CTF2-myc3 (lower panels). In addition, this construct stimulates phosphorylation of Src tyr418 in both PS1+/+ and PS1−/− cells (Figure 5B). In contrast, N-Cad/CTF2, the product of the γ-secretase cleavage of N-cadherin (Marambaud *et al*, 2003), shows no effects on Src phosphorylation (Figure 5B, lanes 3 and 6). Overexpression of ephrinB2 was not sufficient to increase Src phosphorylation in the absence of EphB-induced processing of ephrinB (Figure 4 in Supplementary data). Together, these data indicate that the product of the γ-secretase cleavage of ephrinB2 promotes Src phosphorylation.

To further examine the correlation between the levels of cellular ephrinB2/CTF2 and Src phosphorylation, we treated PS1+/+ and PS1−/− cells overexpressing ephrinB2/CTF2-myc3 with lactacystin, which inhibits degradation of ephrinB2/CTF2 (Figure 1C). Cells treated with lactacystin displayed increased levels of both ephrinB2/CTF2-myc3 and Src phosphorylation at tyr418, although the levels of Src protein remained unchanged (Figure 5C, lanes 2–3 and 5–6). This effect was specific to Src, since phosphorylation of MAPK was not affected (Figure 5C, lower panels). Withdrawal of lactacystin from the medium resulted in the degradation of ephrinB2/CTF2-myc3 and in the dephosphorylation of Src tyr418 (data not shown). These data indicate a correlation between peptide ephrinB2/CTF2 and Src phosphorylation, providing further support for the conclusion that this peptide promotes Src activation. That ephrinB2/CTF2 promotes Src phosphorylation in PS1−/− cells (Figure 5B) indicates that this peptide acts downstream of PS1.

Peptide ephrinB2/CTF2 stimulates phosphorylation of ephrinB2 and inhibits its γ-secretase processing in an Src-dependent manner

Combined with reports that Src phosphorylates ephrinB (Palmer *et al*, 2002), our findings that peptide ephrinB2/CTF2 promotes phosphorylation of Src tyr418, an Src-activating event (Thomas and Brugge, 1997), predict that this peptide may stimulate tyrosine phosphorylation of ephrinB. We used C6 glioma cells that express detectable levels of endogenous ephrinB to test this prediction. Figure 6A shows that exogenous ephrinB2/CTF2 stimulates tyrosine phosphorylation of ephrinB without affecting the total levels of the protein (lanes 1–3). Furthermore, the ephrinB2/CTF2-induced tyrosine phosphorylation of ephrinB is mediated by Src kinase because pharmacological inhibitors of Src activity or a dominant-negative Src mutant (DN-Src; Igishi and Gutkind, 1998) inhibited the phosphorylation of ephrinB (Figure 6A). To test whether ephrinB2/CTF2 regulates the γ-secretase cleavage of ephrinB2, we transfected ephrinB2-expressing fibroblasts with ephrinB2/CTF2-myc3 to distinguish it from endogenous ephrinB2/CTF2. Exogenous

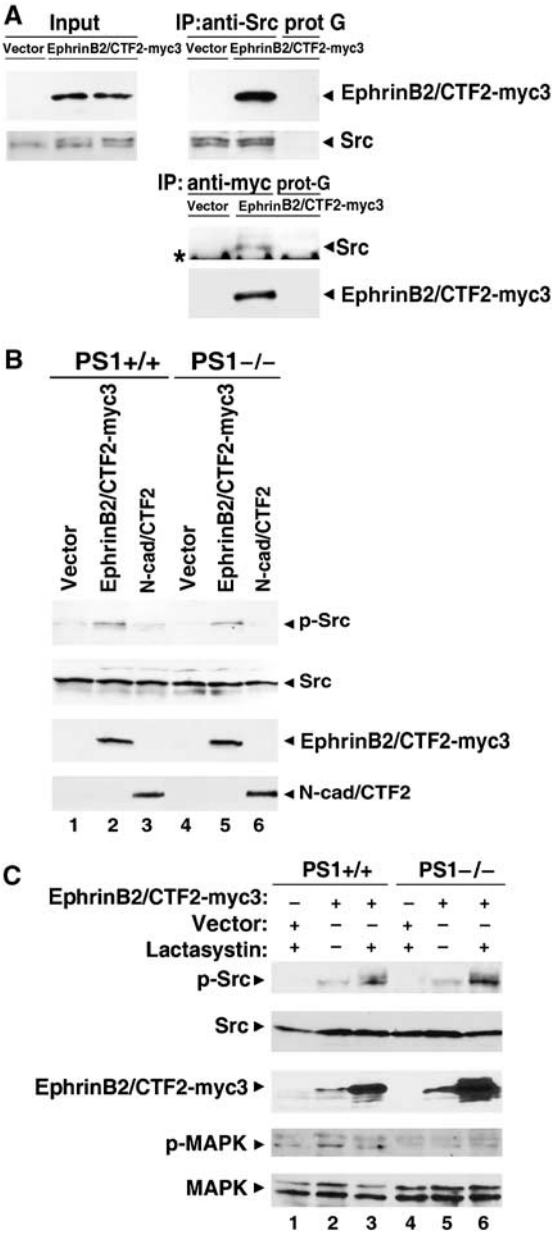

Figure 5 (**A**) EhrinB2/CTF2 complexes with Src. HEK293 cells were transfected with either vector cDNA (vector) or vector encoding ephrinB2/CTF2 fused to three c-myc epitopes (ephrinB2/CTF2-myc3) as indicated in the figure. In all, 1 mg of extract in Triton X-100 buffer was IPed with either anti-Src GD11 or protein G (prot G) as indicated and IPs were probed on WBs for ephrinB2/CTF2-myc3 and Src protein (two upper right panels). A similar aliquot was IPed with anti-myc antibody 9E10 and IPs probed for Src and ephrinB2/CTF2-myc3 (two lower right panels). Input is shown in the left panel. *IgG. (**B**) EphrinB2/CTF2 promotes Src phosphorylation. PS1+/+ or PS1−/− fibroblasts were transfected with vector (lanes 1 and 4), ephrinB2/CTF2-myc3 (lanes 2 and 5) or N-cad/CTF2 (lanes 3 and 6) as indicated in the figure. Cell lysates were analyzed on WBs with either anti-pY^{418} (upper panel) or GD11 (second panel). The two lower panels indicate levels of expression of exogenous peptides. Arrowheads indicate phosphorylated Src (p-Src), total Src (Src), ephrinB2/TCF2-myc3 and N-Cad/CTF2. (**C**) Decrease of cellular ephrinB2/CTF2 parallels decrease in Src phosphorylation. PS1+/+ (lanes 1–3) or PS1−/− (lanes 4–6) fibroblasts transduced by pMX vector (lanes 1 and 4) or pMX-ephrinB2/CTF2-myc3 (lanes 2, 3, 5 and 6) were treated without (lanes 2 and 5) or with 10 μM lactacystin (lanes 1, 3, 4 and 6) for 10 h. Cell extracts were probed on WBs for p-Src, total Src, ephrinB2/CTF2-myc3, p-MAPK and MAPK as indicated by the arrowheads.

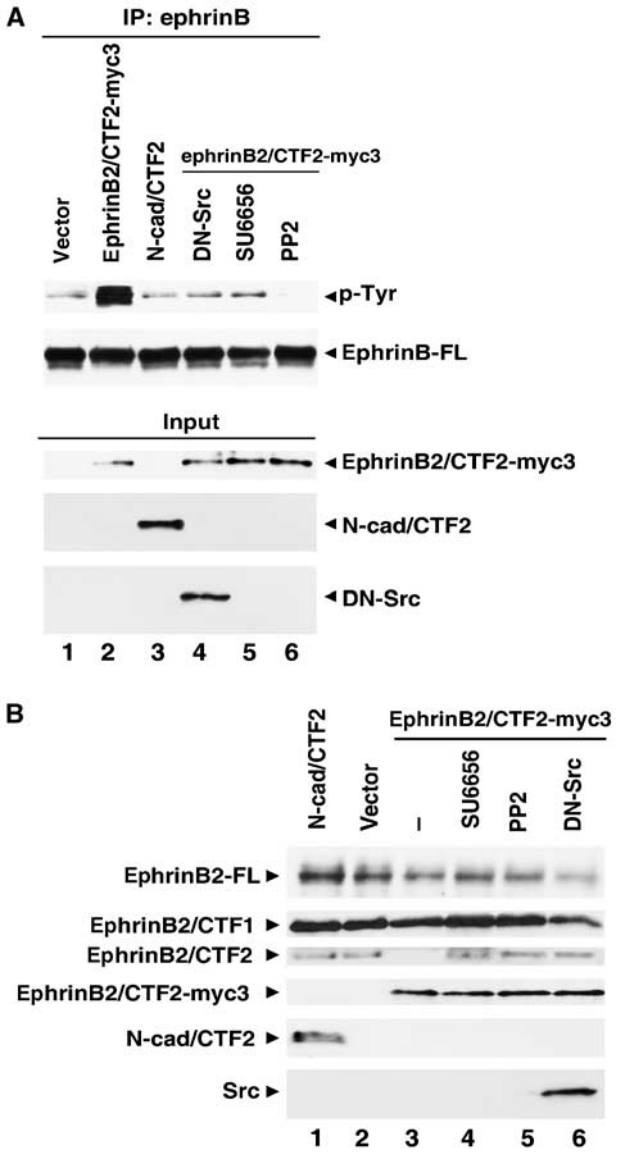

Figure 6 (**A**) EphrinB2/CTF2 promotes ephrinB phosphorylation in an Src-dependent manner. C6 rat glioma cells were transfected as follows: vector (lane 1), ephrinB2/CTF2-myc3 alone (lanes 2, 5, and 6), N-cad/CTF2 (lane 3) or ephrinB2/CTF2-myc3, and dominant-negative (DN) Src (lane 4) as indicated. At 24 h following transfection, cells in samples 5 and 6 were treated with 1 μM SU6656 or 10 μM PP2, respectively, for 1 h. Extracts in Triton X-100 extraction buffer were IPed with antibody C18 and obtained IPs were probed with anti-phosphotyrosine pY99 (p-Tyr, upper panel) or C-18 (ephrinB-FL, second panel). The three lower panels indicate levels of transfected proteins (input). (**B**) EphrinB2/CTF2 inhibits γ-secretase processing of ephrinB2 in an Src-dependent manner. PS1 + / + cells were transduced with pMX-ephrinB2 and then transiently transfected with N-cad/CTF2 (lane 1), vector alone (lane 2), ephrinB2/CTF2-myc3 (lanes 3–5), or cotransfected with ephrinB2/CTF2-myc3 and dominant-negative Src (DN-Src, lane 6). At 24 h following transfection, all cultures were made 10 μM in lactacystin and then incubated for an additional 10 h before harvesting. Cultures 4 and 5 were treated with 1 μM SU6656 (lane 4) or 10 μM PP2 (lane 5) 1 h prior to harvesting. Cell lysates were probed for endogenous ephrinB2/CTF2 using C18 (panel 3). Expression of exogenous protein is indicated in panels 4–6. Arrowheads indicate ephrinB2 (ephrinB2-FL), ephrinB2/CTF1, endogenous ephrinB2/CTF2, exogenous ephrinB2/CTF2 (ephrinB2/CTF2-myc3), N-cadherin/CTF2 (N-cad/CTF2) and Src.

ephrinB2/CTF2-myc3, but not N-Cad/CTF2, inhibited accumulation of the endogenous ephrinB2/CTF2, suggesting a feedback inhibitory mechanism that controls the PS1/γ-secretase processing of ephrinB (Figure 6B, lanes 1–3). Furthermore, Figure 6B shows that the inhibitory effect of ephirnB2/CTF2 on ephrinB2 processing is mediated by Src activity because Src inhibitors (lanes 4 and 5) or a DN-Src (lane 6) prevent the ephrinB2/CTF2 effect on the γ-secretase processing of ephrinB2. Together, these data indicate that ephrinB2/CTF2 promotes phosphorylation of ephrinB and inhibits its processing by activating Src.

The PS1/γ-secretase system regulates Src–Csk association

Csk kinase binds Src kinase, decreasing both phosphorylation of Src residue tyr418 and Src activity (Lee *et al*, 2003). Since the PS1/γ-secretase system stimulates phosphorylation of Src tyr418, we asked whether this system affects the Src–Csk association. Immunoprecipitates from PS1 + / + and PS1−/− mouse brain extracts showed an inceased Csk–Src association in the absence of PS1 (Figure 7A), indicating that PS1 may downregulate the Src–Csk complex. Indeed, Figure 7B (lanes 1 and 3) shows that PS1 + / + fibroblast cells contain lower levels of the Src–Csk complex than PS1−/− cells, and that exogenous PS1 downregulates the association of Src with Csk in PS1−/− cells (lanes 3 and 4). Furthermore, inhibition of γ-secretase increases the Src–Csk association (lanes 1 and 2). These data show that the PS1/γ-secretase system regulates the association of Src with its inhibitor Csk. Transfection of PS1−/− fibroblasts with ephrinB2/CTF2-myc3 suppressed the Src–Csk association (Figure 7C, lanes 2 and 3), while N-Cad/CTF2 was inactive, indicating that peptide ephrinB2/CTF2 specifically downregulates the Src–Csk complex even in the absence of PS1. Similar results were obtained using untagged ephrinB2/CTF2 (data not shown). The role of ephrinB2/CTF2 in the EphB-induced Src activation is further supported by the data of Figure 7D, showing that EphB treatment of fibroblasts, a condition that stimulates production of ephrinB2/CTF2 (see Figure 2), decreases the levels of the endogenous Src–Csk complex and this decrease depends on γ-secretase activity. Together, our data show that the PS1/γ-secretase system inhibits the association of Src with Csk via the production of peptide ephrinB2/CTF2.

PS1 FAD mutations inhibit production of ephrinB2/CTF2 and phosphorylation of pp60src

Recent evidence suggests that PS1 FAD mutations inhibit the PS1-dependent ε-cleavage of N-cadherin, decreasing production of peptide N-Cad/CTF2 that functions as a transcriptional repressor (Marambaud *et al*, 2003). Additional reports show that specific FAD mutations inhibit the ε-cleavage of APP and Notch1 receptor (Chen *et al*, 2002). Figure 8 (A and B) shows that several PS1 FAD mutations strongly inhibit production of ephrinB2/CTF2, suggesting that these FAD mutations may interfere with the ability of PS1 to mediate the EphB-induced Src autophosphorylation. Indeed, Figure 8C shows that although exogenous WT PS1 restores the ability of PS1−/− cells to respond to EphB2–Fc treatment by increasing phosphorylation of Src kinase at tyr418, several PS1 FAD mutants, including ΔE9, A260V, E280G and G384A, as well as dominant-negative PS1 mutant D257A, are unable to do so. These data show that PS1 FAD mutations may affect

the ability of PS1 to mediate the EphB-induced activation of Src kinase. Two additional PS1 FAD mutations, including I213T and M146L, tested in our system showed no significant inhibition of the production of ephrinB2/CTF2. In agreement with our data that ephrinB2/CTF2 mediates the EphB-induced Src phosphorylation, these mutations had no effect on the EphB-induced phosphorylation of Src either (data not shown).

Discussion

Binding of EphB receptor tyrosine kinases (RTKs) to transmembrane ephrinB ligands at the surface of adjacent cells initiates a cascade of signaling events in both the receptor- and the ligand-expressing cells. Thus, ephrinB ligands have unique signaling properties because they induce both forward signaling by stimulating the EphB and reverse signaling through their cytoplasmic domain into their cells. Recent work indicates that ephrinB signaling plays crucial roles in the development of both the vasculature, where it is required in endothelial and endocardial cells for angiogenesis (Adams *et al*, 2001; Gerety and Anderson, 2002), and the nervous system, where ephrinBs play crucial roles in axon guidance and synaptic plasticity (Santiago and Erickson, 2002; Cowan *et al*, 2004; Grunwald *et al*, 2004). In the adult brain, binding of presynaptic ephrinB to postsynaptic EphB induces rapid dendritic spine morphogenesis (Penzes *et al*, 2003). Recent evidence, however, indicates that in the CA1 area of the hippocampus the EphB/ephrinB system is used inverted with postsynaptic ephrinB functioning as a receptor to promote synaptic plasticity. These data indicate that ephrinB-mediated reverse signaling functions in both pre- and postsynaptic cells (Grunwald *et al*, 2004). A well-established consequence of the EphB/ephrinB binding is the ephrinB-dependent activation of Src kinase, which then phosphorylates tyrosine residues of ephrinB, leading to recruitment of cellular factors to cytoplasmic ephrinB and to rearrangement of the actin cytoskeleton (Cowan and Henkemeyer, 2001; Palmer *et al*, 2002). In addition, the activities of both cytoplasmic ephrinB and Src are required for the ephrinB-mediated angiogenic response of endothelial cells (Adams *et al*, 2001; Palmer *et al*, 2002). The mechanism, however, by which the signal generated by the EphB/ephrinB interaction travels to Src kinases in the ephrinB-expressing cell is unclear. Here we show that EphB2–Fc stimulates the phosphorylation of Src kinase at the critical residue tyr418, an autophosphorylation event that activates Src (Thomas and Brugge, 1997). The mechanism of this event involves an EphB-induced MP cleavage of ephrinB2 producing a carboxy-terminal ephrinB2 fragment that is further cleaved by the PS1/γ-secretase system to produce peptide ephrinB2/

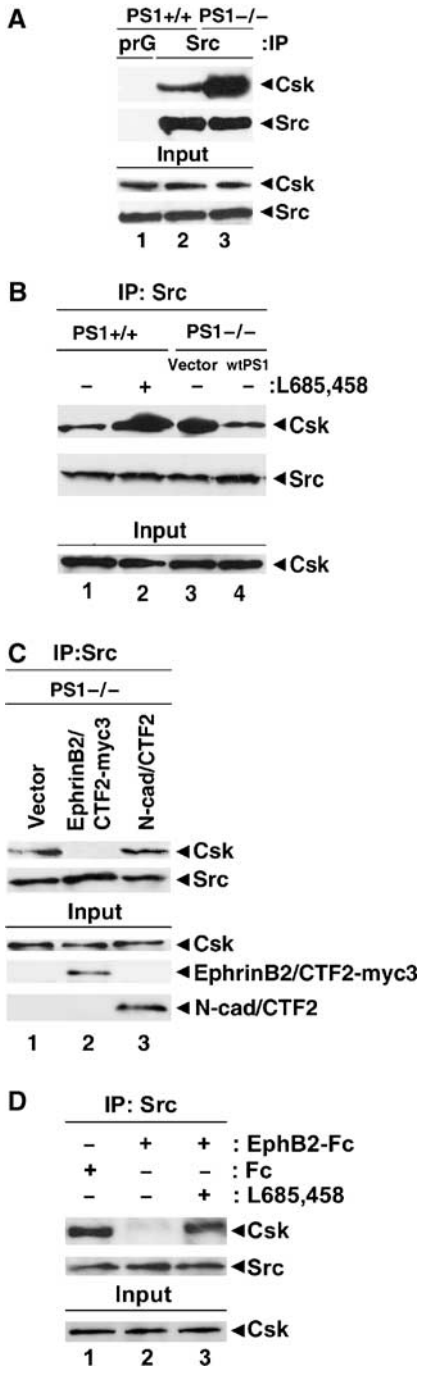

Figure 7 PS1/γ-secretase downregulates the Src–Csk complex. (**A**) Brain extracts from PS1+/+ and PS1−/− embryos were IPed with antibody GD11 (lanes 2 and 3) and obtained IPs were probed on WB with anti-Csk antibody (upper panel) or anti-Src polyclonal antibody (second panel). Extract from PS1+/+ brains was also IPed with proteinG (lane 1, prG) and the IP was treated as above. In the lower two panels, the total levels of Csk and Src are shown (input). (**B**) PS1+/+ fibroblasts were incubated in the presence or absence of 0.5 μM L-685,458 (lanes 1 and 2) and extracts were IPed with anti-Src GD11 and analyzed on WB with polyclonal anti-Csk (upper panel) or anti-Src antibodies (middle panel). Extract from PS1−/− fibroblast cultures transduced by either pMX vector (lane 3) or pMX-WTPS1 (lane 4, wtPS1) were IPed with GD11 and resultant IPs were analyzed as above. In the lower panel, the total Csk levels are shown (input). (**C**) PS1−/− fibroblasts were transfected with vector (lane 1), ephrinB2/CTF2-myc3 (lane 2) or N-cad/CTF2 (lane 3). Cell extracts were IPed with GD11 and IPs were probed with anti-Csk or anti-Src antibodies as in (B) (upper two panels). Input panels indicate the cellular levels of total Csk, ephrinB2/CTF2-myc3 and N-cad/CTF2. (**D**) PS1+/+ fibroblasts were incubated in the absence (lanes 1 and 2) or presence (lane 3) of 0.5 μM γ-secretase inhibitor L-685,458. Cells were then treated with either 2 μg/ml Fc (lane 1) or EphB2-Fc (lanes 2 and 3) and extracts were IPed with GD11. IPs were probed on WB with anti-Csk or anti-Src antibodies (upper and middle panel). Input shows the levels of the total.

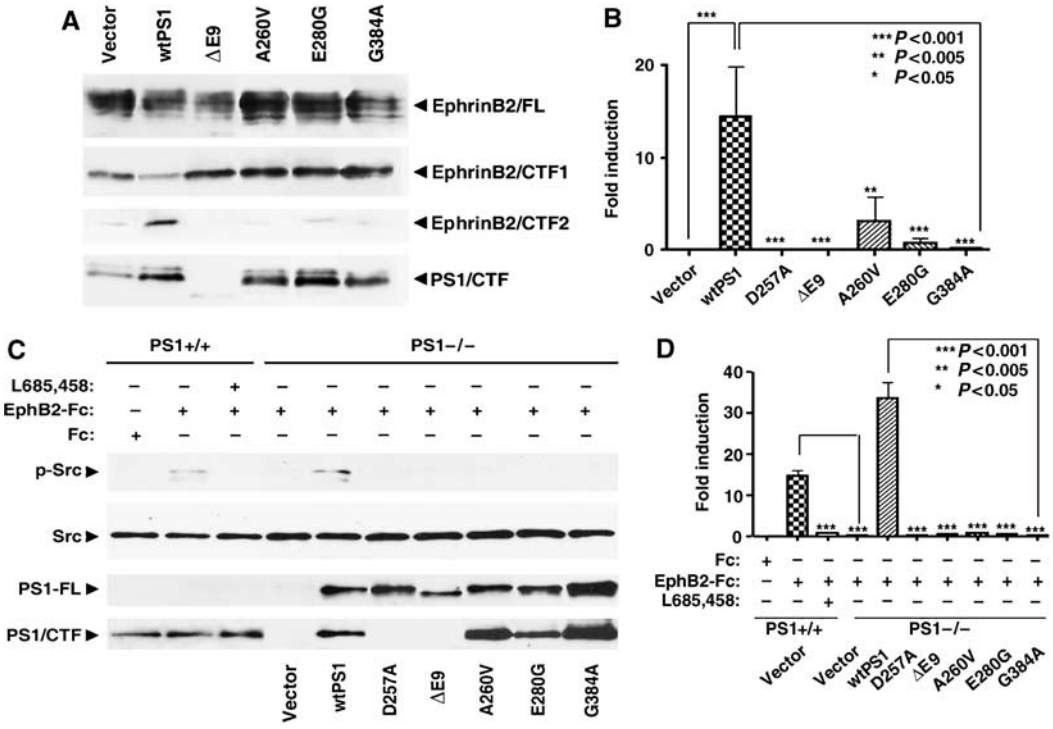

Figure 8 PS1/FAD mutations inhibit the γ-secretase cleavage of ephrinB2 and the EphB2-induced Src phosphorylation. (**A**) HEK293 cells stably transfected with vector, wtPS1 or PS1/FAD mutants ΔE9, A260 V, E280G and G384A were transiently transfected with ephrinB2 and then treated with TPA and lactacystin as described in Figure 2B. Extracts were analyzed on WB with anti-ephrinB antibody C18 or anti-PS1 antibody 33B10. Arrowheads indicate full-length ephrinB2 (ephrinB2-FL), ephrinB2/CTF1, ephrinB2/CTF2 and PS1/CTF. (**B**) Densitometric quantitation of ephrinB2/CTF2 production. EphrinB2/CTF2 levels were normalized to ephrinB2/CTF2 produced from vector-transfected cells. Bars represent the mean ± s.e. of three independent experiments. (**C**) PS1 + / + cells were treated for 5 min either with Fc or with EphB2-Fc in the presence or absence of 0.5 μM γ-secretase inhibitor L-685,458 as indicated. PS1 − / − cells transduced with pMX vector alone (vector), WT PS1 (wtPS1) or the PS1/FAD mutants indicated in the figure were treated with EphB2–Fc as above. Lysates were analyzed on WB for [pY^{418}] Src (p-Src), total Src, full-length PS1 (PS1-FL) and PS1/CTF. (**D**) Densitometric quantitation of [pY^{418}]Src phosphorylation induced by EphB2. [pY^{418}]Src levels were normalized to [pY^{418}]Src induced in vector-expressing PS1 + / + cells. Bars represent the mean ± s.e. of three independent experiments.

CTF2. This peptide binds to Src kinase, stimulating its autophosphorylation at tyr418.

Src activity is downregulated by Csk kinase, which forms complexes with Src, thus inhibiting its autophosphorylation and activation. Removal of Csk from the complex activates Src by allowing its autophosphorylation at residue Tyr418 (Nada *et al*, 1993). Our data show that ephrinB2/CTF2 inhibits the association of Csk with Src and that conditions that inhibit production of ephrinB/CTF2, including treatment with γ-secretase inhibitors or absence of PS1, increase the cellular levels of Src/Csk complexes. Overexpression of ephrinB2/CTF2 in cells lacking PS1 reduces the complex of Src with Csk and increases Src autophosphorylation. Together, these data show that ephrinB2/CTF2 activates Src downstream of PS1 by inhibiting its association with Csk. Accordingly, treatment of cell cultures with EphB2–Fc downregulates the Csk/Src complex in a γ-secretase-dependent manner. Depletion of ephrinB2/CTF2 from the cell coincides with Src dephosphorylation at residue tyr418, suggesting that peptide ephrinB2/CTF2 acts as a switch that controls Scr activity. Our data reveal a novel signaling cascade where EphB binding to ephrinB stimulates processing of ephrinB by MPs. The resultant MP fragment is further processed by the PS1/γ-secretase system to produce peptide ephrinB2/CTF2 that binds Src and promotes its dissociation from Csk kinase, thus allowing the autophosphorylation and activation of Src. Degradation of ephrinB2/CTF2 results in Src dephosphorylation and deactivation.

Since Src phosphorylates the cytoplasmic domain of ephrinB ligands (Palmer *et al*, 2002), our data suggested that the PS1/γ-secretase system might regulate the EphB-induced phosphorylation of ephrinB through production of peptide ephrinB/CTF2. Indeed, EphB2–Fc is unable to stimulate phosphorylation of either Src or ephrinB2 in the absence of PS1 or in the presence of γ-secretase inhibitors. Furthermore, peptide ephrinB2/CTF2 stimulates phosphorylation of ephrinB2 in an Src-dependent manner. Together, our data show that PS1 regulates the EphB-induced phosphorylation of ephrinB via the γ-secretase cleavage of ephrinB. The physiological importance of the PS1/γ-secretase activity in the ephrinB-mediated reverse signaling induced by EphB is shown by our data that this activity mediates critical cellular functions downstream of Src, including sprouting of endothelial cells and recruitment of cytosolic factor Grb4 to ephrinB.

Our data show that peptide ephrinB2/CTF2, the product of the MP/PS1/γ-secretase processing of ephrinB, inhibits processing of ephrinB in an Src-dependent manner. The cell possibly uses this 'feedback' inhibition mechanism to limit production of ephrinB2/CTF2, thus controlling the extent of signaling cascades evoked by EphB–ephrinB interactions. By limiting ephrinB cleavage, this mechanism may also allow the recruitment of factors to the cytoplasmic domain of this

protein, a process vital to cellular functions like regulation of actin dynamics and vascular remodeling (Huot, 2004). In addition to ephrinB2/CTF2, several other peptides with signal transduction activities, including N-Cad/CTF2 of N-cadherin (Marambaud *et al*, 2003), NICD of Notch1 (Schroeter *et al*, 1998) and AICD of APP (Cao and Sudhof, 2001), are produced by a process involving MP and γ-secretase processing (for a review, see Marambaud and Robakis, 2005). Similar to ephrinB2/CTF2, the cellular levels of these signaling peptides are very low, and it has been suggested that this is due to their rapid degradation. Our data on the inhibition of ephrinB2 processing by ephrinB2/CTF2, however, suggest the existence of possible feedback mechanisms, where the signaling peptides produced by the MP/γ-secretase cleavages may inhibit processing of their precursors, thus controlling the extent of signaling.

The mechanism by which FAD mutations promote AD-like neurodegeneration is under intense investigation. Although attention has been focused on the influence of these mutations on amyloid and soluble Aβ species, it remains unclear that Aβ-based theories can explain the neuropathology and heterogeneity of AD (for reviews, see Terry, 1996; Neve and Robakis, 1998; Robinson and Bishop, 2002). Furthermore, the effects of PS1 FAD mutations on Aβ production correlate neither with the age of disease onset nor with the severity of neuropathology (Cruts and Van Broeckhoven, 1998; Neve and Robakis, 1998; Murayama *et al*, 1999), suggesting that additional FAD mutation-induced changes in PS1 activities may contribute to the pathogenesis of FAD. Our data show that PS1 FAD mutations may have a strong inhibitory effect on the production of ephrinB2/CTF2, and accumulating evidence indicates that PS1 FAD mutations can inhibit the PS1/γ-secretase-dependent ε-cleavage of other substrates, including N-cadherin, APP and Notch1 (Chen *et al*, 2002; Marambaud *et al*, 2003), thus inhibiting production of peptides with important signaling functions (Marambaud and Robakis, 2005). The consequences of these inhibitory effects on neurodegeneration and AD remain to be explored. Our data, however, raise the possibility that PS1 FAD mutations may interfere with the ability of PS1 to mediate the EphB-induced Src autophosphorylation at Tyr418. Src kinases are multipotent cellular factors involved in a multitude of cellular activities in addition to their effects on the phosphorylation of ephrinBs. In the brain, the Src family of kinases regulates important neuronal functions such as synaptic activity in the hippocampus and LTP during learning and memory formation (Lu *et al*, 1998; Zhao *et al*, 2000). By inhibiting production of ephrinB2/CTF2 and Src activation, PS1 FAD mutations may affect a number of Src-dependent signal transduction cascades triggered by the EphB–ephrinB interactions.

In summary, our data show that an MP/PS1/γ-secretase processing of ephrinB ligands mediates the EphB-induced activation of Src kinase and production of peptide ephrinB2/CTF2. This peptide activates Src by binding to it and inhibiting its association with inhibitory kinase Csk. Activated Src initiates a signaling cascade that involves phosphorylation of ephrinB, recruitment of cytosolic factors to ephrinB and stimulation of spouting activity in endothelial cells. PS1 FAD mutants unable to promote production of ephrinB2/CTF2 may inhibit Src-dependent signaling cascades initiated by the EphB/ephrinB interaction.

Materials and methods

Chemicals and antibodies

L-685,458, lactacystin, compound E and Src kinase inhibitors SU6656 and PP2 were from Calbiochem; GM6001 from Chemicon; TPA, fibrinogen, thrombin, aprotinin, microcarrier (MC) beads (Cytodex-3) and Hoechst 33258 from Sigma. EphB2-Fc, EphB4-Fc, Fc and stromal-derived factor 1 (SDF-1) were from R&D Systems. Rabbit anti-human Fc was from Jackson Immunoresearch. Anti-myc tag (9E10), anti-HA tag (F-7), anti-phosphotyrosine monoclonal (pY99), anti-ephrinB (C-18), anti-Src (N-16) and anti-Csk (C-20) polyclonal antibodies were from Santa Cruz Biotechnology. Polyclonal anti-p-MAPK and anti-MAPK antibodies were from Cell Signaling Technology. Antibody 33B10 against PS1/CTF has been described (Georgakopoulos *et al*, 1999). Anti-transferrin receptor and anti-Pan ephrinB monoclonal antibodies were from Zymed. Anti-*N*-cadherin monoclonal antibody was from BD Transduction Laboratories. Polyclonal antibody against phospho-Src tyr418 (pY^{418}) was from Biosource International and anti-Src monoclonal antibody (clone GD11) from Upstate Biotechnology.

Cell lines, transfections and transductions

HEK293 and mouse glial C6 cells were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS) as described (Baki *et al*, 2001). All transfections were performed with 1 μg of cDNA using the Lipofectamine Plus™ Reagent (Invitrogen) according to manufacturer's protocol. PS1+/+ and PS1−/− mouse fibroblasts and stably transfected HEK293 cell lines were grown in the presence of G418. To transduce cells using pseudotype retrovirus, phoenix-alpha packaging cells were cotransfected with pMX retroviral vectors (Onishi *et al*, 1996) and pVSV-G plasmid (Clontech) using Fugene 6 reagent (Roche). At 48 h after transfection, culture media supernatants were harvested and passed through 0.4 μm filters. PS1+/+ and PS1−/− mouse fibroblasts were incubated with filtered transducing supernatant in the presence of 10 μg/ml polybrene (Sigma) for 24 h. Transduction efficiency determined by GFP expression using FACS analysis was 80–90%. To insert WT or mutant PS1 cDNAs into pMX retroviral vector, we used *Eco*RI and *Not*I digestions. To insert ephrinB2/CTF2-myc3 into pMX vector we used *Eco*RI and *Xho*I digestion, and for full-length ephrinB2 *Xho*I and *Not*I digestion.

Receptor stimulation

Mouse fibroblasts were transferred to low FBS medium (0.5%) 16 h before the experiment and sodium suramine (0.5 mM) was added 6 h prior to experiment to eliminate engagement of ephrinB to endogenous EphB. Cells were then washed twice with PBS, incubated for 90 min in Opti-MEM (Invitrogen) and then stimulated with 2 μg/ml preclustered EphB2-Fc (R&D Systems) or 2 μg/ml control Fc for the indicated times. Primary neurons and endothelial cells were not treated with sodium suramine. Preclustered multimers of EphB2-Fc or Fc were generated by preincubation of EphB2–Fc or Fc with rabbit anti-human Fc IgG (Jackson Immunoresearch) for 1 h at 4°C at a ratio of 10:1 as described (Palmer *et al*, 2002).

Reelin stimulation

HEK293 cells stably transfected with Reelin were kindly provided by Dr E Förster (University of Freiburg, Germany). Reelin-containing and control conditioned medium was prepared as described (Bock and Herz, 2003) and concentrated 100-fold by using 100 kDa cutoff centrifugal filters (Millipore). Primary neurons were stimulated with approximately 5 nM Reelin or the same volume of control medium for 20 min at 37°C.

Mouse embryo preparation and primary neuronal cell cultures

PS1+/+ and PS1−/− mouse embryos were collected at E14.5 and brains were solubilized as described (Baki *et al*, 2001). In all, 100 μg of extract was analyzed on Western blots (WBs). Cortical neuronal cultures were prepared from E16 PS1+/+ and PS1−/− mouse brains as described (Marambaud *et al*, 2003). Neurons were maintained 8 days *in vitro* in NeuroBasal medium (Gibco) and then stimulated with 2 μg/ml EphB2-Fc or Fc.

Sprouting assays

Bovine adrenal microvessel endothelial cells (BAMEC; VEC Technologies) were grown in complete medium (MCDB131; VEC

Technologies) on Cytodex-3 MC beads. The *in vitro* sprouting angiogenesis and quantitation of sprouting were performed as described (Nehls and Drenckhahn, 1995; Palmer *et al*, 2002). Cells were stimulated with 4 μg Fc or EphB4-Fc, or with 100 ng/ml SDF-1 in the presence or absence of 0.5 μm L685,458. The number of capillary sprouts exceeding in length the diameter of the MC bead (~175 μm) was determined for every 50 MC beads counted. Cells were photographed on an Olympus IX70 microscope with Retiga Exi camera.

Immunoprecipitations (IPs) and immunoblotting

Cells or brains from PS1+/+ and PS1−/− embryos were extracted in TX-100 extraction buffer as described (Georgakopoulos *et al*, 1999) and extracts were precleared with protein A or protein G for 1 h and IPed for 16 h. IPs were precipitated with protein A or protein G for 1 h at 4°C, washed and analyzed on WBs.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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