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EXTRACELLULAR PROTEOLYSIS OF REELIN BY TISSUE PLASMINOGEN ACTIVATOR FOLLOWING SYNAPTIC POTENTIATION

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Abstract—The secreted glycoprotein reelin plays an indispensable role in neuronal migration during development and in regulating adult synaptic functions. The upstream mechanisms responsible for initiating and regulating the duration and magnitude of reelin signaling are largely unknown. Here we report that reelin is cleaved between EGF-like repeats 6–7 (R6–7) by tissue plasminogen activator (tPA) under cell-free conditions. No changes were detected in the level of reelin and its fragments in the brains of tPA knockouts, implying that other unknown proteases are responsible for generating reelin fragments found constitutively in the adult brain. Induction of NMDAR-independent long-term potentiation with the potassium channel blocker tetraethylammonium chloride (TEA-Cl) led to a specific upregulation of reelin processing at R6–7 in wild-type mice. In contrast, no changes in reelin expression and processing were observed in tPA knockouts following TEA-Cl treatment. These results demonstrate that synaptic potentiation results in tPA-dependent reelin processing and suggest that extracellular proteolysis of reelin may regulate reelin signaling in the adult brain. © **2014 Published by Elsevier Ltd. on behalf of IBRO.**

Key words: Reelin, Tissue plasminogen activator (tPA), Hippocampus, Tetraethylammonium chloride, Long-term potentiation.

INTRODUCTION

The inside-out layering of the mammalian cortex requires the extracellular glycoprotein reelin, which is secreted by Cajal–Retzius cells (D'Arcangelo et al., 1995; Hirotsune et al., 1995). Loss of reelin or downstream signaling components (ApoER2/VLDLR, Disabled-1, etc.) leads to a roughly inverted cortex, perturbed hippocampal lamination, and cerebellar hypoplasia (Trommsdorff et al., 1999). In addition to playing a pivotal role in neuronal migration, reelin signaling is necessary for dendritic morphogenesis (Niu et al., 2004), synapse development (Groc et al., 2007; Qiu and Weeber, 2007; Niu et al., 2008; Rogers et al., 2011; Trotter et al., 2011), and synaptic plasticity (Weeber et al., 2002; Pujadas et al., 2010; Rogers et al., 2011; Trotter et al., 2013). Changes in reelin expression (Impagnatiello et al., 1998; Botella-Lopez et al., 2006; Chin et al., 2007; Herring et al., 2012), processing (Botella-Lopez et al., 2010; Duveau et al., 2011; Tinnes et al., 2011) and glycosylation (Botella-Lopez et al., 2006) have been associated with the pathoetiology of a range of neuropsychiatric and neurodegenerative diseases, underscoring the importance of understanding mechanisms that regulate reelin in the adult brain.

Reelin is processed at two main sites (Fig. 1A), between EGF-like repeats 2–3 (R2–3) and 6–7 (R6–7) (Jossin et al., 2004). Full-length reelin and the 5 potential fragments generated by reelin cleavage can be observed in both the developing and adult brain (Jossin et al., 2007; Krstic et al., 2012). The N-R2 region of reelin (Fig. 1A) is important for protein homopolymerization and signaling (Utsunomiya-Tate et al., 2000), but is not essential for lipoprotein receptor binding (Jossin et al., 2004). In fact, disruption of reelin aggregation with the CR-50 antibody, which binds the CR-50 region between N-R2 (Fig. 1A), perturbs neuronal migration *in vivo* (Nakajima et al., 1997). The N-R2 region has also been reported to bind $alpha_3beta_1$-integrins (Dulabon et al., 2000). Recently, it has been shown that reelin can be cleaved within the repeat 3 (R3) and that this produced N-terminal fragment is transported or diffuses to further regions than the larger fragments or full-length reelin (Koie et al., 2014). These results support a role of the N-terminal fragment in the range and duration of reelin signaling (Koie et al., 2014). The preponderance of known reelin functions require the R3–6 region (Fig. 1A), which is responsible for binding apoE receptor 2 (ApoER2), very-low-density-lipoprotein receptor (VLDLR) (D'Arcangelo et al., 1999), and amyloid precursor protein (APP) (Hoe et al., 2009). Consistent with these findings, application of reelin fragments

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Abbreviations: ADAMTS-4/5, a disintegrin and metalloproteinase with thrombospondin motifs 4 and 5; ApoER2, apoE receptor 2; APP, amyloid precursor protein; BDNF, brain-derived neurotrophic factor; DIFP, diisopropyl fluorophosphate; LTP, long-term potentiation; PAI-1, plasminogen activator inhibitor; PIC, protease inhibitor cocktail; SCs, Schaffer collaterals; tPA, tissue plasminogen activator; TEA-Cl, tetraethylammonium chloride; VLDLR, very-low-density-lipoprotein receptor.

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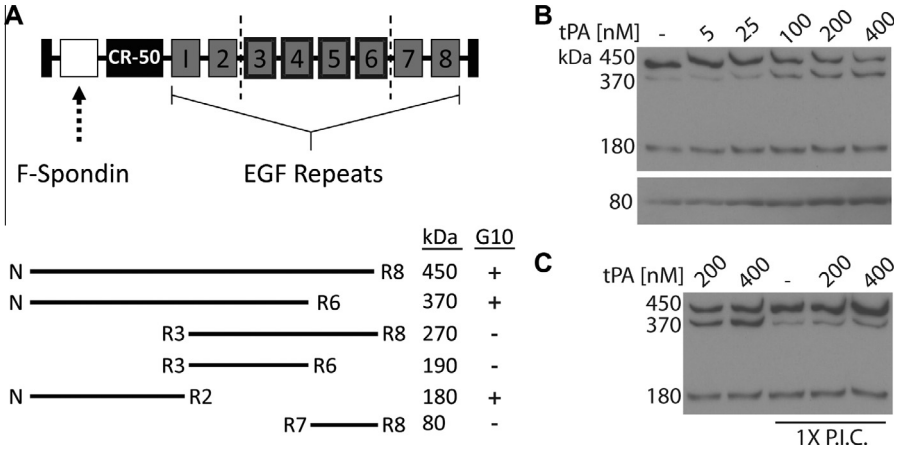

Fig. 1. Modulation of Reelin processing by tPA. (A) Reelin is cleaved between epidermal growth factor (EGF) repeats 2–3 (R2–3) and 6–7 (R6–7), resulting in 5 potential fragments (370, 270, 190, 180, and 80 kDa). The anti-reelin G10 primary antibody detects full-length reelin (450 kDa), and the 370- and 180-kDa fragments. Ab14 was used to detect the 80-kDa fragment. (B) Recombinant reelin (50 nM) was incubated with 0–400 nM tPA at 37 °C for 15 min. tPA concentration-dependently increased processing of recombinant reelin between R6 and 7. Both the 370 and 80-kDa reelin fragments were increased, while the level of full-length reelin was decreased by tPA treatment. The 180-kDa fragment was not altered. (C) Inclusion of the broad-spectrum Halt protease inhibitor cocktail (1X P.I.C.) with tPA (200 nM and 400 nM) inhibited reelin processing. Experiments were performed in triplicate.

containing R5–6 to *reeler* cortical explants is sufficient to induce Disabled-1 phosphorylation and normalize cortical lamination (Jossin et al., 2004). The highly-charged C-terminal region (R7-C) may be involved in reelin folding, secretion (D'Arcangelo et al., 1997; de Bergeyck et al., 1997), and signaling efficacy (Nakano et al., 2007), but is not known to bind to receptors.

While significant progress has been made in delineating downstream mechanisms of reelin signaling, upstream mechanisms operative in the developing and adult nervous system remain elusive. Similar to other extracellular signaling molecules, a critical locus of reelin signaling regulation is at the level of transcription (Erbel-Sieler et al., 2004; Wang et al., 2004; Chen et al., 2007; Miller and Sweatt, 2007; Cubelos et al., 2008), although receptor availability (Duit et al., 2010; Hong et al., 2010; Balmaceda et al., 2014) and secretion (Duveau et al., 2011) may also play a role. However, these mechanisms only serve to adjust the level of reelin signaling, as they are not sufficient to initiate the reelin signal by themselves. In support of this view, preventing extracellular proteolysis of reelin by inhibiting metalloproteinases blocks signaling in the developing cortex and disrupts corticogenesis (Lambert de Rouvroit et al., 1999; Jossin et al., 2007) and also impairs reelin processing in an epilepsy model (Tinnes et al., 2013). These findings imply that reelin is tethered to the extracellular matrix following secretion, where it remains inactive until liberated by proteolysis to initiate downstream signaling.

Recent *in vitro* studies have identified a disintegrin and metalloproteinase with thrombospondin motifs 4 and 5 (ADAMTS-4/5) and tissue plasminogen activator (tPA) as candidate enzymes capable of generating major reelin fragments (Krstic et al., 2012). Although little is known about the function of ADAMTS-4 and -5 in the brain, tPA represents a promising reelin protease candidate due to its high expression in the hippocampus and important role in learning and memory (Qian et al., 1993; Barnes and Thomas, 2008). Moreover, activity-dependent secretion and activation of tPA has been found to be critical for synapse formation and hippocampal synaptic plasticity (Pang et al., 2004; Nagappan et al., 2009), processes which also require reelin signaling (Weeber et al., 2002; Pujadas et al., 2010; Rogers et al., 2011). The extent to which tPA cleaves reelin *in vivo* and its potential significance have not been previously addressed. In this study we present evidence that tPA is a critical regulator of reelin processing following synaptic potentiation.

EXPERIMENTAL PROCEDURES

Animals

Both C57BL/6 J (wild-type) and tPA knockout mice (B6.129S2_Plat^{tm1mlg}/j) were obtained from the Jackson Laboratory. For experiments, males at approximately 4 months of age were used. Animals were group housed in a standard 12-h light/dark cycle and fed ad libitum standard mouse chow. All animal care protocols were followed in accordance with the Institutional Animal Care and Use Committee of the University of South Florida.

Chemicals and reagents

Plasminogen (P7397), activated plasmin (P1867), Diisopropyl fluorophosphate (DIFP; D0879), tetraethylammonium chloride (TEA-Cl; 86616) were obtained from Sigma. Recombinant tissue plasminogen activator (tPA; #176) was obtained from American Diagnostica Inc. Plasminogen activator inhibitor-1 (PAI-1, 528205) and aprotinin (616371) were purchased from Calbiochem. HyClone Dulbecco's Phosphate-Buffered Saline (DPBS) without magnesium and calcium and Halt Protease Inhibitor Cocktail (78425) were obtained from Thermo Scientific. Mammalian protein extraction reagent (M-PER) was obtained from Thermo Pierce. Reelin G10 antibody (MAB5364) was obtained from Millipore and