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Preferential targeting of p39-activated Cdk5 to Rac1-induced lamellipodia

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ABSTRACT

Cdk5 is a member of the cyclin-dependent kinase (Cdk) family that plays a role in various neuronal activities including brain development, synaptic regulation, and neurodegeneration. Cdk5 requires the neuronal specific activators, p35 and p39 for subcellular compartmentalization. However, it is not known how active Cdk5 is recruited to F-actin cytoskeleton, which is a Cdk5 target. Here we found p35 and p39 localized to F-actin rich regions of the plasma membrane and investigated the underlying targeting mechanism in vitro by expressing them with Rho family GTPases in Neuro2A cells. Both p35 and p39 accumulated at the cell peripheral lamellipodia and perinuclear regions, where active Rac1 is localized. Interestingly, p35 and p39 displayed different localization patterns as p35 was found more at the perinuclear region and p39 was found more in peripheral lamellipodia. We then confirmed this distinct localization in primary hippocampal neurons. We also determined that the localization of p39 to lamellipodia requires myristoylation and Lys clusters within the N-terminal p10 region. Additionally, we found that p39–Cdk5, but not p35–Cdk5 suppressed lamellipodia formation by reducing Rac1 activity. These results suggest that p39–Cdk5 has a dominant role in Rac1-dependent lamellipodial activity.

Introduction

Cdk5 is a member of the cyclin-dependent kinase (Cdk) family that unlike the cell cycle-related Cdks, is involved in migration, neurite outgrowth, synaptic activity, and cell death in postmitotic neurons (Barnett and Bibb, 2011; Cheung et al., 2006; Hisanaga and Endo, 2010; Su and Tsai, 2011). Activation of Cdk5 is mediated by the regulatory p35 and p39 subunits, which are expressed predominantly in neurons (Hisanaga and Endo, 2010). In support of this, Cdk5$^{-/-}$ mice display perinatal lethality and show heavy disturbances of neuronal organization in various brain regions (Gilmore et al., 1998; Ohshima et al., 1996). However, in examining the role of the p35/p39 regulatory subunits it was found that p35-deficient mice are viable and fertile, but show lamination defects in the cerebral cortex (Chae et al., 1997), whereas p39 deficiency does not induce any apparent phenotype (Ko et al., 2001). Thus, while the in vivo functions of p39 are currently unknown, since p35$^{-/-}$/p39$^{-/-}$ mice display phenotypes identical to those of Cdk5$^{-/-}$ mice (Ko et al., 2001), it is likely that p39–Cdk5 has its own functions that are mostly taken over for by p35–Cdk5 in p39$^{-/-}$ mice.

Structurally, p39 is an isoform of p35 with 57% amino acid sequence homology (Tang et al., 1995; Zheng et al., 1998). Although p39 can activate Cdk5 (Tang et al., 1995), only a few biochemical and cell biological studies have examined p39–Cdk5 (Asada et al., 2008, 2012; Humbert et al., 2000; Patzke and Tsai, 2002; Saito et al., 2013; Tang et al., 1995; Yamada et al., 2007), due to the labile properties of the p39–Cdk5 complex and the lack of specific antibodies against p39. While many membrane bound proteins are known substrates for Cdk5 (Borquez et al., 2013), the subcellular localization of active Cdk5, which is closely related to function, is determined by p35 or p39 (Cheung et al., 2006; Hisanaga and Endo, 2010; Su and Tsai, 2011). Both p35–Cdk5 and p39–Cdk5 are mainly localized to plasma membranes and endomembranes at the perinuclear region (Asada et al., 2008, 2012; Paglini et al., 1998; Patrick et al., 1999), with membrane association mediated by myristoylation of p35 and p39 (Asada et al., 2008; Patrick et al., 1999). On the other hand, Cdk5 can also act on cytoskeletal structures including neurofilaments, microtubules, and F-actin (Kesavapany et al., 2003; Xie et al., 2006; Ye et al., 2012). Among them, F-actin functions mainly on the cytoplasmic side of plasma membranes. While there are a few reports describing the association of p35 or p39 with actin cytoskeletal proteins (He et al., 2011; Humbert et al., 2000; Ledee et al.,

Abbreviations: ca, constitutive active; Cdk, cyclin-dependent kinase; F-actin, filamentous actin; kn, kinase negative.

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2005; Qi et al., 2004), it is largely unknown how membrane-bound, active Cdk5 affects the actin cytoskeleton.

Organization of F-actin is regulated by Rho small GTPases (Arimura and Kaibuchi, 2005; Ridley, 2012; Visvikis et al., 2010). Among the Rho family proteins, Cdk5 appears most closely involved with Rac1 (Lai et al., 2012; Nikolic, 2002), which is essential for dendritic remodeling, neurite outgrowth, spine formation, and neuronal migration (Lai et al., 2009; Watabe-Uchida et al., 2006), all of which involve Cdk5. However, the relationship between Cdk5 and Rac1 is not clearly understood at the molecular level. While early studies indicated that p35–Cdk5 regulates Pak1 Ser/Thr kinase downstream of Rac1 (Nikolic et al., 1998), several subsequent studies have implicated Cdk5 in the regulation of Rac1 guanine nucleotide exchange factors (GEFs) or GTPase activating proteins (GAPs), both of which are upstream of Rac1 (Causeret et al., 2007; Kesavapany et al., 2004; Lai et al., 2012; Xin et al., 2004). Furthermore, Cdk5 regulates several actin-binding lamellipodia proteins such as WAVE1/2 and Neurabin1 by phosphorylation (Causeret et al., 2007; Kim et al., 2006; Miyamoto et al., 2008). Thus, the interaction between Cdk5 kinase and Rac1 activity is quite complicated and as a result, not yet fully determined.

We have observed that the Cdk5 activators p39 and p35 localize in the F-actin-rich submembrane regions of COS-7 cells (Asada et al., 2008). While p39 and p35 have been shown to associate with actin (He et al., 2011; Humbert et al., 2000) there has been no further analysis on the targeting mechanism. Here we investigated the association of p39, as well as p35, with Rac1-induced lamellipodia in Neuro2A cells and found that myristoylation and lysine clusters in the N-terminal p10 region are required for targeting both p39 and p35 to Rac1-induced submembranous lamellipodia. We also found a stronger accumulation of p39, which has an effect on cell shape, suggesting that p39–Cdk5 has a dominant role in Rac1-dependent lamellipodial activity in neurons.

Results

Differential localization of p39 and p35 Cdk5 activators in F-actin-rich submembrane regions

While both p39 and p35 Cdk5 activators have been found in the submembranous F-actin rich regions of COS-7 cells (Asada et al., 2008; Humbert et al., 2000), here we used Neuro2A neuroblastoma cells to study the localization of p39 and p35 to F-actin structures in more detail so as to determine their subcellular localization in cells similar to neurons. First, we examined the localization of p39 and p35 in Neuro2A cells by transfection with expression vectors encoding p39-myc or p35-myc, followed by visualization with anti-myc antibody. Both p39 and p35 were found in both the perinuclear region (Fig. 1A, arrow) and cell periphery (Fig. 1A, arrowheads), but the precise distribution was different between them, with p35 localized more to the perinuclear region and p39 found more in the cell periphery. p39 was found in lamellipodia-like structures (Fig. 1A, arrowheads in upper left panel) showing partial colocalization with F-actin (Fig. 1A, double arrowheads in the upper right panel). Also, as shown in Fig. 1B, we observed various colocalization patterns of p39 or p35 with F-actin. For example, p39 colocalized with F-actin at the frontal region of the lamellipodia-like protrusions (left panels) and p35 showed scattered colocalization with F-actin (right panels).

p39 preferentially localizes to Rac1-induced lamellipodia

Cortical F-actin structures are regulated by Rho-family small GTPases (Amano et al., 2010; Ridley, 2006; Visvikis et al., 2010). Here we examined the localization of p39 or p35 with Rho family protein-induced F-actin structures by exogenous expression of the constitutively active Rho family proteins RhoA V14, Rac1 V12, and Cdc42 V12 in Neuro2A cells, which induced the formation of F-actin structures typical for each of them, namely stress fibers, lamellipodia, and filopodia (Supplemental Fig. S1). When these proteins were coexpressed with p39 or p35, both p39 and p35 showed localization to Rac1 V12-induced lamellipodia and -perinuclear accumulation (Fig. 1C). Neither p39 nor p35 localized with RhoA V14-dependent stress fibers (Supplemental Fig. S2), while p39 displayed localization to Cdc42 V12-induced filopodia along the surface of cells (Supplemental Fig. S2). Because both p39 and p35 exhibited localization with Rac1 V12 at the lamellipodia and perinuclear regions, as shown in control Neuro2A cells (Fig. 1A), our study focused on the interaction of p39 or p35 with Rac1.

Colocalization of p39 and p35 with Rac1 V12 is shown in Fig. 1C. Interestingly, p39 was found primarily on the lamellipodial structures surrounding the entire cell that are also rich in Rac1 V12, whereas p35 localized to the perinuclear region so as to surround the nucleus. We designated the distribution of p39 the peripheral ring (Fig. 1C, dashed line in the upper panel) and the distribution of p35 the perinuclear ring (Fig. 1C, dashed line in the lower panel). This peripheral lamellipodia and perinuclear localization of active Rac1 has been previously shown in several cultured cells including PC12 cells (Navarro-Lerida et al., 2012; Nikolic et al., 1998; Xin et al., 2008). Fluorescent intensities along yellow lines in the merges are shown in the right side of Fig. 1C and clearly indicate a greater colocalization of p39 with Rac1 V12 than p35. However, closer examination revealed that even in the peripheral ring the p39 staining did not completely overlap with Rac1 V12 localization (Fig. 1D), suggesting that p39 and Rac1 V12 may associate indirectly.

Both p39 and p35 are Cdk5 activators, and can recruit active Cdk5 to particular subcellular regions (Asada et al., 2008, 2012). To examine whether p39 or p35 localized to Rac1-induced lamellipodia is capable of recruiting Cdk5, we expressed kinase negative (kn) Cdk5 together with p39 or p35 in the presence of Rac1 V12. Because Cdk5 activity is known to affect actin organization by phosphorylation of several actin-binding proteins, we used knCdk5 to determine the localization of p39–Cdk5 or p35–Cdk5 without the influence of its kinase activity on F-actin structures (Fu et al., 2007; Kim et al., 2006; Miyamoto et al., 2008; Nikolic et al., 1998; Rashid et al., 2001). In fact, as described later in Fig. 5, activation of Cdk5 by p39 changed the cells to a round shape, which was inappropriate for a subcellular localization study. When expressed alone, knCdk5 was distributed diffusely in the cytoplasm (Fig. 2, upper). However, when coexpressed with p39, knCdk5 was found to localize to the peripheral lamellipodia, although free knCdk5 was still detected in the center of cells (Fig. 2, middle). In contrast, p35 strongly recruited knCdk5 to perinuclear particles but showed less localization to the peripheral lamellipodia (Fig. 2, bottom). The localization of F-actin to the peripheral lamellipodial ring is shown in the upper left panel of Fig. 2. These results confirm that p39 and p35 determine the localization of Cdk5 with p35 doing so with a stronger capacity, which is consistent with our previous in vitro binding results (Saito et al., 2013; Yamada et al., 2007).

Localization of p39 and p35 to the Rac1-induced peripheral lamellipodial ring is dependent on the N-terminal p10 region of p39 and p35

Next, we examined how p39 or p35 localizes to Rac1-induced lamellipodia. Both p39 and p35 are isoforms composed of two domains, the N-terminal p10 ($p10^{p39}$ and $p10^{p35}$) and C-terminal Cdk5 activation domain (p29 and p25; Fig. 3A, C). We first examined which of these fragments localizes to Rac1-dependent structures. We found that while p29 was diffusely distributed in the central region of cells with a smaller localization to Rac1 V12-induced lamellipodia (Fig. 3B, E, p29), $p10^{p39}$ accumulated at both the peripheral ring and perinuclear Rac1 V12 (Fig. 3B, lower) similar to the full-length p39, although the percentage localized to peripheral ring was reduced (Fig. 3E). We also found that p25 in the central cytoplasm was excluded from the peripheral lamellipodia (Fig. 3D, E, p25) and while the strongest staining was found at the region close to Rac1 V12, there was no evidence of