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SCIENCE DIRECT

Molecular Brain Research 138 (2005) 215 – 227

MOLECULAR BRAIN RESEARCH

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Research Report

Cyclin-dependent kinase 5 activators p35 and p39 facilitate formation of functional synapses

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Accepted 18 April 2005
Available online 23 May 2005

Abstract

Cyclin-dependent kinase 5 (Cdk5) has emerged as a key coordinator of cell signaling in neurite outgrowth. Cdk5 needs to associate with one of the regulatory proteins p35 or p39 to be an active enzyme. To investigate if Cdk5 plays a role in the establishment of functional synapses, we have characterized the expression of Cdk5, p35, and p39 in the neuroblastoma–glioma cell line NG108-15, and recorded postsynaptic activity in myotubes in response to presynaptic overexpression of Cdk5, p35, and p39. Endogenous Cdk5 and p35 protein levels increased with cellular differentiation and preferentially distributed to soluble pools, whereas the level of p39 protein remained low and primarily was present in membrane and cytoskeletal fractions. Transient transfection of a dominant-negative mutant of Cdk5 in NG108-15 cells and subsequent culturing on differentiating muscle cells resulted in a significant reduction in synaptic activity, as measured by postsynaptic miniature endplate potentials (mEPPs). Overexpression of either Cdk5/p35 or Cdk5/p39 resulted in a substantial increase in synaptic structures that displayed postsynaptic activities, as well as mEPP frequency. These findings demonstrate that Cdk5, p35, and p39 are endogenously expressed in NG108-15 cells, exhibit distinct subcellular localizations, and that both Cdk5/p35 and Cdk5/p39 are central in formation of functional synapses.

Theme: Excitable membranes and synaptic transmission
Topic: Presynaptic mechanisms

Keywords: Hybrid cell; Cellular differentiation; Miniature endplate potential; Synaptogenesis; Myotube; Acetylcholine

1. Introduction

Cdk5 is one of the few members in the serine/threonine protein kinase family of cyclin-dependent kinases that only has been implicated in mechanisms unrelated to cell cycle control [20,28,34,51,52]. For activity, Cdk5 is dependent on association with one of its regulatory proteins, p35 or p39. Cdk5 is ubiquitously expressed in mammalian tissues, but expression of the activators is enriched in brain. Cdk5 plays a major role in neurite outgrowth during neuronal differentiation [8]. Several mouse models have elegantly revealed the importance of Cdk5 in neuronal positioning and cytoskeletal dynamics during neurite outgrowth. Gene-targeted removal

Abbreviations: Cdk5, cyclin-dependent kinase 5; mEPPs, miniature endplate potentials; MAP, microtubule-associated protein; NFs, neurofilaments; dBcAMP, dibutyryladenosine cyclic monophosphate; EGFP, enhanced green fluorescent protein; GAP-43, growth-associated protein-43; SNAP-25, synaptosomal-associated protein of 25 kDa; RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; ECL, enhanced chemiluminescence; CCD, charge coupled device

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doi:10.1016/j.molbrainres.2005.04.014

of Cdk5 in mice leads to widespread disruption of neuronal layering and neonatal death [41]. Disruption of the p35 gene by homologous recombination results in aberrant layering of the neocortex [4]. Gene targeting of the p39 gene does not give a phenotype; however, the double p35/p39 knock-out results in a mouse mutant resembling that of $Cdk5^{-/-}$ mice [24], indicating that p35 and p39 are the only activators of Cdk5. p35 and p39 have been shown to have both distinct and compensatory functions during brain development [8,51].

The impaired neuronal positioning found in the knock-out mouse models of Cdk5 and p35 could partly be caused by deregulated axon guidance. During the last few years, a number of evidences have pointed towards a role for Cdk5 in transducing cell-surface-derived signals into several intracellular pathways, resulting in cytoskeletal rearrangements. For instance, Cdk5 phosphorylation of β-catenin disrupts N-cadherin-mediated cell adhesion, thereby promoting neuronal migration [25]. Cdk5 has also been implicated in integrin and netrin 1 signaling [7,26,29]. In the presynaptic terminal, a growing list of actin-modulating proteins are being identified as Cdk5 substrates. Cdk5 is involved in growth cone collapse during axon guidance and positioning, in several ways linked to rac-signaling. In semaphorin 3A-induced growth cone collapse, Cdk5 phosphorylates collapsin response mediator protein-2 (CRMP-2) [3,13]. Phosphorylation of ras guanine nucleotide releasing factor 2 (RasGRF2) downregulates rac, affecting the distribution of microtubule-associated protein (MAP) 1b [22], and Cdk5 inhibits pak1. The latter two proteins are involved in cross-talk between different types of cytoskeleton [7,46]. Furthermore, trio, a rho GDP/GTP exchange factor (GEF), phosphorylated by Cdk5, was shown to induce actin reorganization and influence secretory granule movement [62]. During neurite outgrowth, Cdk5 might influence cytoskeletal stability, for instance, through phosphorylation of neurofilaments (NFs) and MAPs [50]. Aberrant Cdk5 phosphorylation of cytoskeletal substrates has been demonstrated in several neuronal pathologies, reviewed in [50]. Cdk5 activity has also been linked to synaptic proteins like munc18-1 and synapsin I [9,32,49]. Cdk5 operates as a negative regulator of dopaminergic and glutamatergic transmission in the striatum, but promotes hormone secretion from endocrine cells [6,30,31,62], suggesting several functional roles of Cdk5 in regulated exocytosis.

Upon dibutyryl cAMP-induced differentiation, NG108-15 cells acquire a neuron-like phenotype, hence offering a tool to investigate process outgrowth and synaptic formation [56]. They express, for example, N-type Ca^{2+} channels, and can be stimulated to release acetylcholine [17,21]. NG108-15 cells do not form functional autaptic synapses, but when co-cultured with differentiating muscle cells, functional D-tubocurarine-sensitive cholinergic synapses are formed [18,23,36]. By measuring miniature endplate potentials (mEPPs) in postsynaptic myotubes demonstrating cell–cell contacts with emerging processes from NG108-15 cells, it is possible to analyze the frequency of spontaneous transmitter release and the rate of formation of synaptic-like contacts.

In the present study, we first characterized the mRNA and protein expression of Cdk5 and the regulatory subunits p35 and p39 in differentiating NG108-15 cells and examined the subcellular distribution of the different proteins. Secondly, we investigated if a dominant-negative mutant of Cdk5 influenced the formation of synaptic structures in the neuroblastoma-myotube co-culture model by measuring postsynaptic activities. Thirdly, by transient overexpression of Cdk5 in combination with the different Cdk5 activators, p35 and p39, we analyzed if there were any alterations in the number of synaptic-like structures formed in this in vitro model of the neuromuscular junction and if the frequency of mEPPs was altered.

2. Materials and methods

2.1. Expression vectors

The templates for p35 and p39 were inserted into the bicistronic pIRES2-EGFP expression vector (Clontech) as described previously [30]. Both the p35 and p39 expression constructs were analyzed by DNA sequencing. Cdk5 expression constructs encoding wild-type (wt) and a dominant-negative (dn) form of Cdk5 were kindly provided by Dr. S. van den Heuvel [61]. In the dominant-negative Cdk5 construct, Asp (D) at codon 144 is mutated to Asn (N), a site involved in the phosphotransfer reaction. Although Cdk5 is still capable of binding p35 and p39, this amino acid substitution results in a catalytically inactive kinase [37,59].

2.2. Antibodies

Primary antibodies used were a rabbit polyclonal anti-Cdk5 antibody, C8 (Santa Cruz Biotechnology), a rabbit polyclonal anti-p35 antibody, C19 (Santa Cruz Biotechnology), which also recognizes the truncated p35-variant p25, and two rabbit polyclonal anti-p39 antibodies; one was a generous gift from Dr. J. Wang and the other one was raised against a synthetic peptide corresponding to the last 14 amino acids of the p39 protein (Innovagen). For Western blotting, except for blocking peptide experiments, the anti-p39 antibody from Dr. Wang was used, and for immunocytochemistry, the newly generated anti-p39 antibody was used. As cell organelle markers, a mouse monoclonal anti-syntaxin 1 antibody, HPC-1 (Sigma), a mouse monoclonal anti-synaptotagmin I antibody (Wako Chemicals) and a mouse monoclonal anti-synaptophysin antibody, SVP-38 (Sigma), were used. Mouse monoclonals for growth-associated protein-43 (GAP-43, Boehringer Ingelheim) and synaptosomal-associated protein of 25 kDa (SNAP-25), SMI81 (Sternberger Monoclonals), were used as markers for growth cones and neuronal cells. Secondary antibodies used for Western blotting were horseradish peroxidase-conjugated anti-rabbit and anti-mouse immunoglobulins (Dako Corporation and Rockland), and for immunocytochemistry, Alexa Fluor 488 and 633 anti-mouse