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STIMULUS-EVOKED ERK-DEPENDENT PHOSPHORYLATION OF ACTIVITY-REGULATED CYTOSKELETON-ASSOCIATED PROTEIN (ARC) REGULATES ITS NEURONAL SUBCELLULAR LOCALIZATION

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Abstract—Activity-regulated cytoskeletal-associated protein (Arc) is implicated as a master regulator of long-term synaptic plasticity and memory formation in mammalian brain. Arc acts at synapses and within the nucleus, but the mechanisms controlling Arc localization and function are little known. As Arc transcription and translation are regulated by extracellularsignal-regulated kinase (ERK) signaling, we asked whether Arc protein itself is phosphorylated by ERK. GST-fused Arc of rat origin was able to pull down endogenous ERK2 from rat hippocampal lysates. Using a peptide array, we show that ERK binds a non-canonical docking (D) motif in the C-terminal domain of Arc, and this interaction is abolished by phosphorylation of Tyr309. Activated ERK2 phosphorylated bacterially expressed Arc *in vitro* at all five predicted sites, as confirmed by phospho-specific protein staining and LC–MS/MS analysis. In neuroblastoma cells expressing epitope tagged-Arc, we demonstrate ERK-dependent phosphorylation of Arc in response to activation of muscarinic cholinergic receptors with carbachol. Using phosphosite-specific antibodies, this stimulus-evoked phosphorylation was shown to occur on Ser206 located within the central hinge region of Arc. In cultured hippocampal neurons expressing phosphomutant Arc under control of the activity-dependent promoter, we show that Ser206 phosphorylation regulates the nuclear:cytosolic localization of Arc. Thus, the neuronal activity-induced phosphomimic exhibits enhanced cytosolic localization relative to phosphodeficient and wild-type Arc. Furthermore, enhanced Ser206 phosphorylation of endogenous Arc was detected in the dentate gyrus cytoskeletal fraction after induction of long-term potentiation (LTP) in live rats. Taken together, this work demonstrates stimulus-evoked ERK-dependent phosphorylation and regulation of Arc protein.

Key words: activity-regulated cytoskeleton-associated protein (Arc), extracellularsignal-regulated kinase (ERK), hippocampus, long-term potentiation, mitogen-activated protein kinase (MAPK), phosphorylation.

INTRODUCTION

Mounting evidence supports a role for Arc as a vertebrate-specific gene specialized for mediating stable forms of activity-dependent synaptic plasticity (Bramham et al., 2010; Korb and Finkbeiner, 2011; Shepherd and Bear, 2011). Synthesis of Arc protein is required for long-term potentiation (LTP), long-term depression (LTD), and homeostatic plasticity, as well as postnatal development of the visual cortex and long-term memory formation (Guzowski et al., 2000; Plath et al., 2006; Rial Verde et al., 2006; Shepherd et al., 2006; Messaoudi et al., 2007; Waung et al., 2008; Béïque et al., 2010; McCurry et al., 2010).

Arc interacts with different protein partners in different cellular compartments to mediate diverse effects on neuronal function and plasticity. In dendritic spines, Arc regulates endocytic trafficking of AMPA-type glutamate receptors and actin cytoskeletal dynamics (Chowdhury et al., 2006; Shepherd et al., 2006; Messaoudi et al., 2007; Peebles et al., 2010; Okuno et al., 2012; DaSilva et al., 2016; Nair et al., 2017). In the nucleus, Arc forms complexes with proteins involved in the regulation of transcription and chromatin state (Korb et al., 2013; Wee et al., 2014; Oey et al., 2015). Recent structural analysis shows that Arc has two structured regions flanking a central, mostly disordered hinge region (Myrum et al., 2015; Zhang et al., 2015) Furthermore, a loose tertiary structure within and between domains indicates that Arc is flexible and capable of undergoing major conformational changes (Myrum et al., 2015).

The picture emerging is one of Arc as a multifunctional activity-induced hub protein for mediating and organizing long-term synaptic plasticity (Nikolaienko et al., 2017). To understand how plasticity is controlled in the brain, information is needed on the molecular regulation of Arc protein. Remarkably, the role of protein phosphorylation,

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Abbreviations: Arc, activity-regulated cytoskeleton-associated protein; EDTA, ethylenediaminetetraacetic acid; ERK, extracellularsignal-regulated kinase; HCD, Higher-Energy Collision; HFS, high-frequency stimulation; LTP, long-term potentiation; PSD95, postsynaptic density protein-95; SARE, synaptic activity-response element.

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the major mechanism for regulation of protein activity in cells (Ubersax et al., 2007), is largely unexplored for Arc.

Previous work showed an important role for extracellularsignal-regulated kinase (ERK) in regulating the synthesis of Arc. Indeed, Arc transcription (Waltereit et al., 2001; Ying et al., 2002; Kawashima et al., 2009; Panja et al., 2009; Pintchovski et al., 2009), postsynaptic mRNA localization (Huang et al., 2007), and translation (Waung et al., 2008; Panja et al., 2009, 2014; Soulé et al., 2012) are all ERK-dependent processes. Using a combination of *in vitro* and *in vivo* assays, we demonstrate stimulus-evoked, ERK-dependent phosphorylation of Arc. Using affinity-purified Arc from neuroblastoma cells, we specifically observe ERK catalyzed phosphorylation on Ser206 located within Arc's disordered hinge region. In cultured hippocampal neurons expressing phosphomutant Arc in response to excitatory stimulation, we show that Ser206 phosphorylation promotes the early cytosolic retention of Arc. This same modification is detected on endogenous Arc following LTP induction in dentate gyrus of live rats. The results identify Arc as an ERK substrate, and further implicate ERK as a coordinate regulator of Arc transcription, translation and protein function.

EXPERIMENTAL PROCEDURES

Antibodies and antibody production

Primary antibodies were mouse anti-Arc (Santa Cruz Biotechnology, Dallas, TX, USA, C7, sc-17839; Antibody Registry: AB_626696; WB dilution 1:3000), rabbit anti-Arc (Synaptic Systems, Göttingen, Germany, #156003; Antibody Registry: AB_887694; WB dilution 1:3000), rabbit anti-ERK2 (Santa Cruz Biotechnology, Dallas, TX, USA, C14, sc-154; Antibody Registry: AB_2141292; WB dilution 1:2000), rabbit anti-phospho-ERK (Cell Signaling Technology, Danvers, MA, USA, 9101S; Antibody Registry: AB_331646; WB dilution 1:3000), rabbit anti-total-ERK (Cell Signaling Technology, Danvers, MA, USA, 4695S; Antibody Registry: AB_390779; WB dilution 1:3000), mouse anti-HA (Covance, Princeton, NJ, USA, HA.11 clone 16B12; Antibody Registry: AB_10063630; WB dilution 1:5000), goat anti-GST (GE Healthcare Life Sciences, Little Chalfont, UK, # 27-4577-01; Antibody Registry: AB_771432; WB dilution 1:500), mouse anti-GAPDH (Thermo Fisher Scientific, Waltham, MA, USA, MA5-15738; Antibody Registry: AB_10977387; WB dilution 1:3000), rabbit anti-NMDAR2B (Thermo Fisher Scientific, Waltham, MA, USA, OPA1-04022; Antibody Registry: AB_325652; WB dilution 1:3000), rabbit anti-Histone H4 Acetylated (Bio-Rad Laboratories, Hercules, CA, USA, AHP418; Antibody Registry: AB_2116715; WB dilution 1:3000), mouse anti-PSD 95 (Thermo Fisher Scientific, Waltham, MA, USA, MA1-045; Antibody Registry: AB_325399; WB dilution 1:3000; ICC dilution 1:1000), chicken anti-MAP2 (EnCor Biotechnology, Gainesville, FL, USA, CPCA-MAP2; Antibody Registry: AB_2138173; ICC dilution 1:5000). HRP-conjugated anti-mouse, anti-rabbit and anti-goat secondary antibodies were from Merck, Billerica, MA, USA (WB dilutions 1:15,000). Fluorescent dyes for immunostaining of neurons were Alexa Fluor chicken 405 and mouse 568 (Thermo Fisher Scientific, Waltham, MA, USA, ICC dilutions 1:500). Anti-pS170/pT175 and anti-pS206 polyclonal antibodies were generated by immunizing rabbits with KLH-conjugated phosphopeptides (GYDYTVS*PYAIT*P and C + GEDGQPS*PGVDT respectively), and then cross-affinity purified using corresponding phosphorylated and non-phosphorylated peptides (immunization and purification – Eurogentec, Seraing, Belgium; WB dilution 1:5000).

Constructs

The rat ERK2 coding sequence was excised from pcDNA3-HA-ERK2 WT (a gift from John Blenis; Addgene plasmid # 8974) and cloned into the pGEX-4T-3 vector (GE Healthcare Life Sciences, Little Chalfont, UK). A cDNA fragment encoding full-length Arc was produced by PCR using rat brain cDNA and the High Fidelity PCR enzyme mix (Thermo Fisher Scientific, Waltham, MA, USA). The PCR product (NC_005106.4:1 15910325.0.115909138) was cloned into the pGEX-4T-3 vector and into the lentiviral expression vector CD511B-1 with a StrepII-HA tag at its C-terminus. The plasmid pfMSCV C-Strep-HA IRES GFP-Gw containing the StrepII-HA tag sequence was a gift from Giulio Superti-Furga. To create fluorescently tagged Arc constructs (*SARE*::mArc-sfGFP wt and mutants), the mouse Arc coding sequence (NC_000081.6:74672372.0.74671185) was cloned into the lentiviral expression vector CD511B-1 lacking GFP reporter gene, under the control of the *SARE-ArcMin* promoter (NC_000081.6:74679364.0.746 79261 + NC_000081.6:74672793.0.74672571) with an sfGFP fluorescent protein fused to its C-terminus flanked by the Arc 5′UTR (NC_000081.6:74672570.0.74 672373) and 3′UTR (NC_000081.6:74671184.0.746691 22). We used plasmid pGL4.11 SARE-ArcMin containing the *SARE-ArcMin* promoter sequence as previously described (Kawashima et al., 2009). The plasmid pcDNA5-FRT-TO-mCherry-sfGFP containing sfGFP sequence was a gift from Michael Knop. The plasmid pCS6-Arc-VenusTS2HA2 containing the mouse Arc CDS together with 5′UTR and 3′UTR sequences was a gift from Michael Lin. pMD2.G and psPAX2 plasmids used for virus packaging were provided from Didier Trono (Addgene plasmids # 12259 and # 12260 respectively). Mutagenesis was performed using QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions. All constructs were verified by sequencing.

Cell culture, infection and lysate preparation

HEK293FT and SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Sigma–Aldrich, St. Louis, MO, USA), L-glutamine and penicillin/streptomycin at 37 °C in a humidified incubator with 5% CO_2. For virus packaging sub-confluent HEK293FT cell cultures were co-transfected with CD511B-1 *CMV*::rArc-StrepII-HA, psPAX2 and pMD2.G (10:9:1 ratio) using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. 48 and 72 h