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N-myristoylation regulates the axonal distribution of the Fragile X-related protein FXR2P

Emily E. Stackpole [a], Michael R. Akins [a,b], Justin R. Fallon [a,*]

[a] Department of Neuroscience, Brown University, Providence, RI 02912, USA
[b] Department of Biology, Drexel University, Philadelphia, PA 19104, USA

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ABSTRACT

Fragile X syndrome, the leading cause of inherited intellectual disability and autism, is caused by loss of function of Fragile X mental retardation protein (FMRP). FMRP is an RNA binding protein that regulates local protein synthesis in the somatodendritic compartment. However, emerging evidence also indicates important roles for FMRP in axonal and presynaptic functions. In particular, FMRP and its homologue FXR2P localize axonally and presynaptically to discrete endogenous structures in the brain termed Fragile X granules (FXGs). FXR2P is a component of all FXGs and is necessary for the axonal and presynaptic localization of FMRP to these structures. We therefore sought to identify and characterize structural features of FXR2P that regulate its axonal localization. Sequence analysis reveals that FXR2P harbors a consensus N-terminal myristoylation sequence (*MGXXXS*) that is absent in FMRP. Using click chemistry with wild type and an unmyristoylatable G2A mutant we demonstrate that FXR2P is N-myristoylated on glycine 2, establishing it as a lipid-modified RNA binding protein. To investigate the role of FXR2P N-myristoylation in neurons we generated fluorescently tagged wild type and unmyristoylatable FXR2P (WT and G2A, respectively) and expressed them in primary cortical cultures. Both $FXR2P^{WT}$ and $FXR2P^{G2A}$ are expressed at equivalent overall levels and are capable of forming FMRP-containing axonal granules. However, $FXR2P^{WT}$ granules are largely restricted to proximal axonal segments while granules formed with unmyristoylatable $FXR2P^{G2A}$ are localized throughout the axonal arbor, including in growth cones. These studies indicate that N-terminal myristoylation of the RNA binding protein FXR2P regulates its localization within the axonal arbor. Moreover, since FMRP localization within axonal domains requires its association with FXR2P, these findings suggest that FXR2P lipid modification is a control point for the axonal and presynaptic distribution of FMRP.

1. Introduction

Fragile X syndrome (FXS) is the leading cause of inherited intellectual disability and autism (Cohen et al., 2005; Hernandez et al., 2009; Pickett and London, 2005). Individuals with FXS typically present with variable degrees of intellectual impairment, hyperactivity, anxiety and seizures (Garber et al., 2008). In addition, essentially all FXS patients demonstrate autistic features such as language impairments, social anxiety, and inappropriate emotional responses (Hernandez et al., 2009). FXS is almost always caused by the transcriptional silencing of the Fragile X mental retardation gene *FMR1*, resulting in the loss of its protein product, the RNA binding protein FMRP (Siomi et al., 1993).

Investigations of FMRP function have largely focused on its role in the somatodendritic compartment, where it regulates protein synthesis and influences synaptic plasticity (Bagni and Greenough, 2005; Bassell and Warren, 2008; Bear et al., 2004). However, FMRP is also localized to axonal and presynaptic sites in both cultured neurons and the intact brain (Akins et al., 2012; Antar et al., 2006; Christie et al., 2009; Feng et al., 1997; Price et al., 2006; Till et al., 2010). Several lines of evidence indicate that FMRP has important roles in these axonal and presynaptic domains. Enduring synaptic plasticity in *Aplysia* neurons requires both postsynaptic and presynaptic FMRP (Till et al., 2010). In *Drosophila*, FMRP controls axonal arborization as well as presynaptic function and structure in an activity-dependent manner, possibly in part through local regulation of *dscam* translation (Cvetkovska et al., 2013; Kim et al., 2013; Tessier and Broadie, 2008; Zhang et al., 2001). In mammals, FMRP in the presynaptic neuron is required for synapse formation, synaptic activity and presynaptic short-term plasticity (Deng et al., 2011, 2013, Ferron et al., 2014; Hanson and Madison, 2007). Finally, axonal and presynaptic FMRP is poised to function as a translational regulator since FMRP binds mRNAs that encode approximately one-third of the presynaptic proteome (Darnell et al., 2011).

Previous work from our laboratory has shown that in the intact brain FMRP and its homologues FXR1P and FXR2P localize to endogenous axonal and presynaptic granules termed FXGs (Fragile X granules; Akins et al., 2012; Christie et al., 2009). These granules are expressed within a restricted subset of neurons throughout the mammalian brain including corticocortical and thalamacortical fibers, olfactory sensory neuron

* Corresponding author at: Department of Neuroscience, Brown University, Box G-LN, Providence, RI 02912, USA.
E-mail address: Justin_Fallon@brown.edu (J.R. Fallon).

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axons, hippocampal CA3 associational axons and cerebellar parallel fibers (Akins et al., 2012). FXR2P is a component of all FXGs, while FMRP and FXR1P are only detected in a circuit-selective subset (Christie et al., 2009). Moreover, FXR2P, but not FMRP, is required for FXG expression (Christie et al., 2009). Taken together, these studies indicate that FXR2P is a key regulator of both FXG expression and the axonal and presynaptic localization of FMRP.

Here we sought to characterize the mechanisms that regulate the axonal distribution of FXR2P. Database searches revealed that FXR2P is the only Fragile X protein family member that contains a consensus N-terminal myristoylation motif (*MGXXXS*). N-myristoylation is the covalent attachment of myristate, a 14-carbon saturated fatty acid, to the N-terminal glycine (G2). This irreversible fatty-acid modification occurs co-translationally following the removal of initiator methionine residues (Towler et al., 1987; Wilcox et al., 1987). N-myristoylation is observed in a wide range of proteins including src family kinases, protein kinase A, NAP-22, MARCKS and rapsyn (Aderem et al., 1988; Carr et al., 1982; James and Olson, 1989; Musil et al., 1988; Resh, 1994; Takasaki et al., 1999). The primary function of myristoylation is to regulate protein targeting by promoting interactions with the plasma membrane, lipid microdomains or hydrophobic pockets of other proteins (Resh, 2004; Sorek et al., 2009; Tanaguchi, 1999).

Here we demonstrate that FXR2P is N-terminally myristoylated by virtue of its second glycine. We utilized a cultured neuron system to assess the role of N-myristoylation for FXR2P localization in axons. While both wild type and unmyristoylatable FXR2P form axonal granules containing FMRP, these forms show strikingly different localization within the axonal arbor. Granules containing wild type FXR2P are largely restricted to the proximal domain of the axonal arbor, while granules formed from mutant FXR2P invade the entire axon arbor including the growth cone. Taken together, these results show that N-terminal myristoylation regulates the localization of the RNA binding protein FXR2P within the axonal arbor.

2. Results

2.1. FXR2P contains a consensus N-myristoylation motif

Since FXR2P is the only Fragile X family member required for FXG expression, we performed database analyses to search for features unique to this protein. As shown in Fig. 1A, we observed that the N-termini of all mammalian FXR2P sequences queried contain a consensus N-terminal myristoylation sequence (*MGXXXS*). This sequence is not

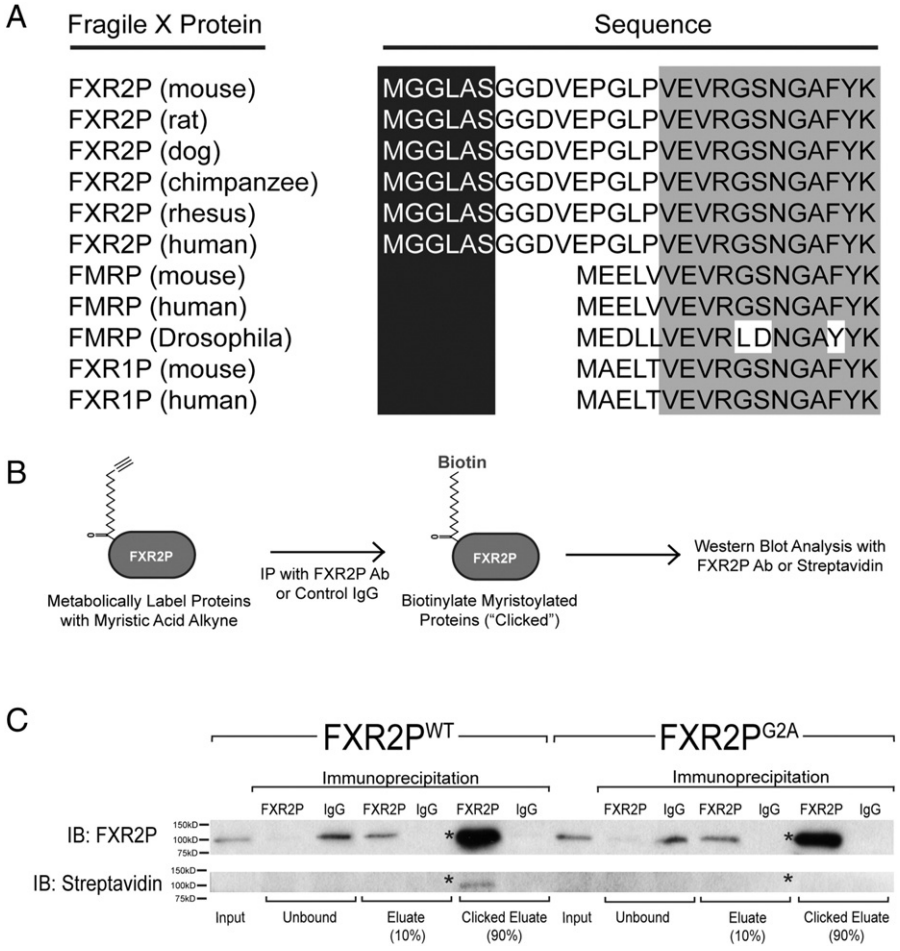

Fig. 1. FXR2P is N-terminally myristoylated. (A) The N-terminus of FXR2P from the six mammalian species analyzed contains a conserved N-terminal myristoylation consensus sequence (*MGXXXS*). This consensus sequence is not present in either FMRP or FXR1P in any species examined (for clarity, only mouse, human and *Drosophila* are shown). (B) Schematic of click chemistry-based approach used to detect FXR2P myristoylation (see Experimental methods section). (C) Western blot demonstrating N-terminal myristoylation of $FXR2P^{WT}$ but not $FXR2P^{G2A}$. COS-7 cells transfected with either $FXR2P^{WT}$ or $FXR2P^{G2A}$ were incubated with a biotinylatable analog of myristic acid. Lysates were collected ('input') and then immunoprecipitated with either FXR2P antibody or a control IgG. Ninety percent of immunoprecipitates were subjected to the click-iT reaction to biotinylate proteins that had incorporated the myristic analog. Analysis of the Western blots with an FXR2P antibody demonstrated the comparable immunoprecipitation of $FXR2P^{WT}$ and $FXR2P^{G2A}$ (*asterisk*; ~100 kD), while neither was detected when a control IgG was used for the immunoprecipitation. Anti-biotin Western blotting detected protein that incorporated the myristic analog. $FXR2P^{WT}$ is biotinylated when the click reaction is performed after immunoprecipitation with an FXR2P antibody (*asterisk* marks ~100 kD band corresponding to clicked FXR2P). $FXR2P^{G2A}$ is not biotinylated after immunoprecipitation with an FXR2P antibody and click reaction. Equivalent results were observed in four independent experiments.