

Synaptic protein UNC-13 interacts with an F-box protein that may target it for degradation by proteasomes*

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Received: 26 May, 2005; revised: 18 November, 2005; accepted: 19 November, 2005
available on-line: 23 February, 2006

UNC-13 protein participates in regulating neurotransmitter release. In *Drosophila melanogaster*, proteasomal degradation controls UNC-13 levels at synapses. Function of the amino-terminal region of a 207 kDa form of *Caenorhabditis elegans* UNC-13 is unknown. Yeast two-hybrid and secondary yeast assays identified an F-box protein that interacts with this amino-terminal region. As F-box proteins bind proteins targeted for proteasomal degradation, this protein may participate in degrading a subset of UNC-13 proteins, suggesting that different forms of UNC-13 are regulated differently. Yeast assays also identified an exonuclease, a predicted splicing factor, and a protein with coiled-coil domains, indicating that UNC-13 may affect RNA function.

Keywords: synaptic neurotransmission, yeast two-hybrid, UNC-13, F-box

Release of neurotransmitters into synapses requires synaptic vesicle docking, priming and fusion with the neuronal plasma membrane. Proteins on the synaptic vesicles and proteins associated with the plasma membrane form a soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) complex that holds synaptic vesicles adjacent to the plasma membrane. Following maturation into a fusion-competent state, fusion of vesicles with the plasma membrane is triggered by the presence of calcium. UNC-13 proteins interact with several proteins involved in regulating neurotransmitter release including Doc2, β -spectrin, RIM-1, UNC-18, and syntaxin (Betz *et al.*, 1997; 2001; Orita *et al.*, 1997; Sakaguchi *et al.*, 1998; Sassa *et al.*, 1999). UNC-13 promotes a change in syntaxin from a closed to open conformation so that it can associate with synaptobrevin and SNAP-25 in the SNARE complex (Richmond *et al.*, 2001). UNC-13 function is regulated by signal transduction pathways. In *Drosophila melanogaster*, *G α S* and *G α q* pathways regulate abundance of *Drosophila* UNC-13 (DUNC-13) at synapses through proteasomal degradation (Arava-

mudan & Broadie, 2003). In *Caenorhabditis elegans*, *G α o* and *G α q* pathways regulate localization of a 167 kDa form of UNC-13 to synapses (Lackner *et al.*, 1999; Miller *et al.*, 1999; Nurrish *et al.*, 1999).

In *C. elegans*, expression of two different *unc-13* transcripts has been verified with Northern transfers. The more abundant transcript is 5.4 kb and codes for a 207 kDa protein and the less abundant transcript is 4.4 kb and codes for a 167 kDa protein. The 5' region of the two transcripts varies. There are 13 exons at the 5' end of the 5.4 kb transcript that are absent in the 4.4 kb transcript and one exon at the 5' end of the 4.4 kb transcript that is absent in the 5.4 kb transcript. The 3' region of both transcripts has 16 identical exons (Kohn *et al.*, 2000). The carboxyl-terminal region of the protein that is coded for by both transcripts interacts with syntaxin (Betz *et al.*, 1997). Functions of the dissimilar amino-terminal regions of the two UNC-13 forms have not been characterized. Antibodies recognizing the amino-terminal region of the 207 kDa protein label synapses in the nervous system and nuclei in the gut and gonad (Kohn *et al.*, 2000). Green fluorescent protein

*Preliminary report was presented at the 15th Biennial *C. elegans* Conference, University of California, 25–29 June 2005, Los Angeles, California, USA.

Abbreviations: 3AT, 3-aminotriazole; DUNC-13, *Drosophila melanogaster* UNC-13; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor; SD, synthetic dextrose.

fusion analysis of 167 kDa UNC-13 indicates that it is found along axons and that *Gao* and *Gaq* signal transduction pathways regulate localization of the protein to synapses (Lackner *et al.*, 1999; Miller *et al.*, 1999; Nurrish *et al.*, 1999).

To further understand function of UNC-13 we investigated the role of the amino-terminal region of the 207 kDa form of UNC-13 expressed in *C. elegans*. We identified interacting proteins using a yeast two-hybrid screen. The strength of the protein-protein interactions was examined using three secondary yeast screens. Four proteins that have strong protein-protein interactions with the amino-terminal region of 207 kDa UNC-13 were identified. One is an F-box protein that may regulate levels of 207 kDa UNC-13 at synapses *via* proteasomal degradation. The other three are ERI-1, which regulates RNA interference, a protein predicted to be a splicing factor, and a protein with two coiled-coil domains. Interaction with these three proteins suggests that in addition to playing a role in regulating neurotransmitter release, UNC-13 may be involved in regulating the processing and function of RNA.

MATERIALS AND METHODS

Plasmids for yeast two-hybrid screen. cDNA corresponding to *unc-13* exons 1-13 was made using Super ScriptTM II Reverse Transcriptase (InvitrogenTM Life Technologies, Carlsbad, California, USA) according to manufacturer's directions. Reverse primer oRK107 5'CTT AAT TCC GAG ATC AGC ACA 3' was used to generate the cDNA and forward primer oRK105 5'CACC ATG GAT GAC GTT GGA GAT 3' was used in the subsequent PCR reaction. *unc-13* cDNA was transferred into a plasmid using the pENTRTM Directional TOPO Cloning Kit (InvitrogenTM Life Technologies, Carlsbad, California, USA) according to manufacturer's directions. Following sequence verification, the *unc-13* cDNA was transferred into the pDEST32 vector coding for the Gal4 DNA binding domain using the ProQuestTM Two-Hybrid System with Gateway[®] Technology (InvitrogenTM Life Technologies, Carlsbad, California, USA) according to manufacturer's directions. A positive clone of *unc-13* cDNA corresponding to exons 1-13 in the pDEST32 vector (pDB-UNC13N) was purified using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, California, USA), according to manufacturer's instructions, and verified by sequencing at the Children's Hospital of Philadelphia (Philadelphia, PA, USA).

Yeast two-hybrid screen. The screen was carried out using bait and prey vectors, yeast strain, and controls included in the ProQuestTM Two-Hybrid System with Gateway[®] Technology (Invitro-

genTM Life Technologies, Carlsbad, California, USA). Empty bait vector pDBLeu and UNC-13 bait vector pDB-UNC13N were transformed into yeast strain MaV203 and plated on minimal media lacking leucine (synthetic dextrose (SD)-Leu). Empty activation vector pEXP-AD502 was transformed into yeast strain MaV203 with pDBLeu or pDB-UNC13N and plated on minimal media lacking leucine and tryptophan (SD-Leu-Trp). Controls A through D correspond to different levels of protein-protein interactions with control A having no interaction, control B having a weak interaction, control C having a moderately strong interaction and control D having a strong interaction. 3-Aminotriazole (3AT) concentration needed to titrate basal levels of *HIS3* expression was determined by plating MaV302 cells with pDBLeu and pEXP-AD502, MaV302 cells with pDB-UNC13N and pEXP-AD502, and controls A-D on SD-Leu-Trp-His plates containing concentrations of 3AT at 10 mM, 25 mM, 50 mM, 75 mM, and 100 mM. All plates lacking histidine in the screen had 50 mM 3AT added. MaV203 cells with pDB-UNC13N were transformed with cDNA library (Li *et al.*, 2004) and were plated on SD-Leu-Trp and SD-Leu-Trp-His+50 mM 3AT plates. Thirteen *C. elegans* genomes were screened.

Secondary yeast screens. Secondary screens were carried out according to ProQuestTM Two-Hybrid System with Gateway[®] Technology (InvitrogenTM Life Technologies, Carlsbad, California, USA) protocols. Colonies that grew on the SD-Leu-Trp-His+50 mM 3AT plates following transformation with a cDNA library and controls A-D were streaked on SD-Leu-Trp and SD-Leu-Trp-His+50 mM 3AT plates. Yeast on the SD-Leu-Trp plate were replica plated onto a SD-Leu-Trp, a YPD plate with a nitrocellulose filter on top, SD-Leu-Trp-His+50 mM 3AT plate, SD-Leu-Trp-Ura, and SD-Leu-Trp-His+50 mM 3AT + cycloheximide. The SD-Leu-Trp and YPD + nitrocellulose filter plates were incubated immediately and remaining plates were replica cleaned and then incubated. The nitrocellulose filter was assayed for β -galactosidase expression according to manufacturer's instructions. Four plasmids from yeast expressing all three reporter genes and sensitivity to cycloheximide were purified using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, Ca, USA), according to manufacturer's instructions, and verified by sequencing at the Children's Hospital of Philadelphia (Philadelphia, PA, USA).

RESULTS AND DISCUSSION

In a yeast two-hybrid screen for proteins interacting with the amino-terminal region of *C. elegans* 207 kDa UNC-13, seven candidates were

identified. Candidates were tested with four secondary yeast screens to examine the strength of the protein–protein interactions. Four candidates grew in the absence of histidine and in the absence of uracil following replica plating and replica cleaning (Fig. 1). These candidates therefore express both the *HIS3* and *URA3* reporter genes as a result of protein–protein interactions with the amino-terminal region of 207 kDa UNC-13. All four candidates grew in a similar manner as control D, indicating a strong protein–protein interaction. None of the candidates grew in the presence of cycloheximide following replica plating and replica cleaning (Fig. 1). Growth on cycloheximide would have indicated that the yeast cells lost the pDB-UNC13N bait plasmid that expresses the dominant gene *CYH2^S* and sensitizes cells to cycloheximide. In a β -galactosidase assay, the same four candidates that grew in the absence of histidine and uracil turned dark blue and

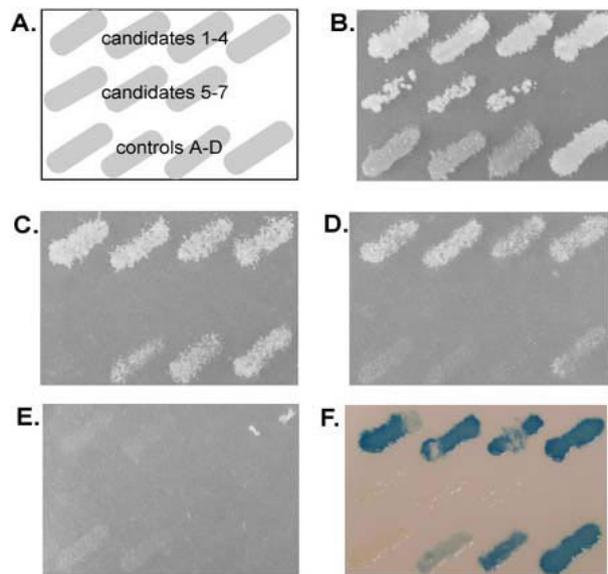


Figure 1. Secondary yeast screens analyzing strength of protein–protein interactions.

(A) Identities of yeast patches are described. The top row in each panel includes candidates one through four. The second row includes candidates five through seven. The bottom row includes controls A through D. (B) All candidates for protein–protein interactions and all controls are shown growing on plate lacking leucine and tryptophan. (C) Candidates one through four and controls B, C, and D are shown growing on a plate lacking leucine, tryptophan, and histidine, with 50 mM 3AT. Candidates five through seven and control A show no growth. (D) Candidates one through four and control D are shown growing on plates lacking leucine, tryptophan, and uracil. Candidates five through seven show no growth and controls A, B, and C show little growth. (E) In the presence of cycloheximide candidates three through seven and controls C and D show no growth. Slight growth is seen for candidates one and two and controls A and B. (F) β -Galactosidase expression assay shows candidates one through four as dark blue. Candidates five through seven did not turn blue. Control A is not blue and controls B, C, and D have increasing intensity of blue color.

were the same intensity as control D, indicating a strong protein–protein interaction. The remaining three candidates were the same color as control A, indicating no protein–protein interaction (Fig. 1). The four cDNAs coding for proteins that interact with the amino-terminal region of 207 kDa UNC-13 were sequenced and identified as 1) *C. elegans* gene coding for a protein with F-box and FTH domains (GenBank accession number NM_074480); 2) *C. elegans* gene *eri-1* (GenBank accession number NM_171245), coding for a protein with exonuclease and SAP domains that is involved in regulating RNA interference; 3) *C. elegans* gene coding for an RNA recognition motif and PWI domain that is predicted to be a splicing factor (GenBank accession number NM_073733); and 4) *C. elegans* gene coding for a protein with two coiled-coil domains (GenBank accession number NM_67630).

The F-box protein identified in this screen could play a role in regulating levels of UNC-13 available at synapses. DUNC-13 is degraded in *Drosophila melanogaster* by proteasomes responding to *Ga*S and *Ga*q pathways (Aravamudan & Broadie, 2003). F-box proteins are components of ubiquitin ligase complexes (Skowrya *et al.*, 1997). The F-box protein binds to a target protein that is then ubiquitinated. Following ubiquitination, the target protein is degraded by proteasomes (Hershko & Ciechanover, 1998). There are 326 predicted F-box proteins in *C. elegans*, most of which have unknown functions (Kipreos & Pagano, 2000). As the F-box protein identified in this study interacts with the amino-terminal end of the 207 kDa UNC-13 product, an amino-acid sequence absent from the 167 kDa product, it would potentially target only a subset of UNC-13 proteins for degradation. There are 20 lysine residues in 207 kDa UNC-13 that could be targets for ubiquitination (Pickart, 2001). Ninety eight additional lysine residues are found in the 1206 amino acids in co mMon between the 207 kDa and 167 kDa forms of UNC-13. A mechanism for degrading a subset of UNC-13 proteins would allow levels of the 207 kDa and 167 kDa protein products expressed from the *unc-13* gene to be differentially regulated, suggesting that the two protein products have different functions.

Three additional proteins that interact with the amino-terminal end of 207 kDa UNC-13 were identified in this screen and implicate *unc-13* in roles beyond regulating neurotransmitter release. The *eri-1* gene codes for an exonuclease that negatively regulates RNA interference. The protein is expressed in the cytoplasm of a subset of neurons and in the gonad. Few mRNAs in the *C. elegans* nervous system are degraded as a result of RNA interference and it has been suggested that ERI-1 may be involved in limiting cell-type specificity of RNA interference (Kennedy *et al.*, 2004). A protein with RNA recog-

dition and PWI domains that it is predicted to be a splicing factor and a protein with two coiled-coil domains were also identified as interacting with 207 kDa UNC-13. A previous yeast two-hybrid screen indicated that the protein with two coiled-coil domains also interacts with the product of the *hpl-2* gene in *C. elegans* (Li *et al.*, 2004). *hpl-2* is involved in formation of the germline, silencing of transgenes in the germline, and in vulval development (Couteau *et al.*, 2002). Interactions between UNC-13 and proteins that function in the nervous system, nuclei, and germline convey the significance of antibodies specific for the amino-terminal end of 207 kDa UNC-13 labeling synapses as well as nuclei in the gut and gonad (Kohn *et al.*, 2000). Direct protein-protein interactions between UNC-13 and ERI-1 may include UNC-13 in a mechanism regulating RNA interference in the nervous system. Interactions with a splicing factor indicate a possible role for 207 kDa UNC-13 in regulating processing of RNA in nuclei of the gut and gonad. Association with a coiled-coil domain protein that interacts with HPL-2 suggests that UNC-13 protein in gonad nuclei could participate in cell-type specific silencing of transgenes and formation of the germline.

Acknowledgements

We thank Philip Meneely, Nate Favini, and Lauren Ortiz for their help and support with the yeast two-hybrid screens. We also thank Philip Meneely for providing high quality *C. elegans* RNA for cDNA production. We thank Theresa Moser for her technical support in the laboratory and Rebecca Roberts for critical reading of the manuscript.

This material is based upon work supported by the National Science Foundation under Grant No. 0092733 to R.E.K.

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