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ORIGINAL ARTICLE

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The Prader–Willi syndrome protein necdin interacts with the E1A-like inhibitor of differentiation EID-1 and promotes myoblast differentiation

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Abstract Proliferation and differentiation of muscle precursors are controlled by the activation of muscle-specific genes and inactivation of inhibitors of differentiation. Necdin is a multi-functional protein that is up-regulated during neural and myogenic differentiation. Necdin facilitates cell cycle exit and differentiation during development, but the role of necdin in embryonic myogenesis has not been described. In a cytoplasmic two-hybrid screen, we identified a novel interaction between necdin and the E1A-like inhibitor of differentiation (EID-1). EID-1 inhibits transcriptional activation of genes required for myogenic differentiation, and is degraded in myoblasts upon cell cycle exit. In a transactivation assay, necdin had no direct effect on myoD-responsive promoters in the presence of MyoD, but necdin did relieve the EID-1-dependent inhibition of these same promoters. *In vivo*, a normal number of MyoD-expressing myoblasts was present in primary embryonic limb bud cultures from mouse embryos with congenital necdin deficiency. In contrast, the number of myosin heavy chain-expressing myotubes in differentiating limb bud cultures cultured for 5 days was reduced compared with cultures from wild-type littermate controls. In the presence of necdin, steady-state levels of EID-1 were increased and the half-life of EID-1 was extended, and EID-1 was re-localized from the nucleus to the cytoplasm when necdin was co-expressed in transfected cells. Collectively, these data are consistent with a model whereby necdin promotes myoblast differentiation at least in part by relieving the inhibitory effect of EID-1.

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Introduction

NDN, encoding necdin, is one of four protein-coding genes genetically inactivated in individuals with Prader–Willi syndrome (PWS). PWS is marked by global developmental delay, hypoventilation, childhood-onset hyperphagia, severe obesity, and hypogonadism (Gunay-Aygun et al., 2001). Hypotonia of central origin is present prenatally, and reduced lean muscle mass with abnormal proportions of type 2 muscle fiber subtypes have been described in infants with PWS (Argov et al., 1984; Miike et al., 1988; Chitayat et al., 1989; Sone, 1994). Necdin belongs to the melanoma-associated antigen (MAGE) family of proteins characterized by the presence of a MAGE homology domain (MHD) (Barker and Salehi, 2002; Sasaki et al., 2005), and is most highly expressed in the nervous system, muscle and skin. The expression of necdin in neural precursor cells promotes and accelerates their differentiation (Hayashi et al., 1995; Uetsuki et al., 1996), and the loss of necdin in PWS could contribute to the neurological component of the phenotype. Necdin associates with centrosomal proteins during axonal outgrowth in sympathetic neurons (Lee et al., 2005), and facilitates TrkA signaling to promote survival of nerve growth factor-dependent nociceptive neurons (Salehi et al., 2000; Kuwako et al., 2005). In muscle satellite cells, necdin promotes differentiation after injury and protects myoblasts from apoptosis (Deponti et al., 2007). On some strain backgrounds, most mice carrying a genetic inactivation of gene encoding necdin (*Ndn*-null) die of a neonatal central respiratory defect (Gerard et al., 1999) and surviving *Ndn*-null mice have decreased sensitivity to pain (Kuwako et al., 2005).

While a number of proteins interact with necdin, the translation of these interactions into a molecular model

explaining the physiological defects observed in PWS is still forthcoming. Necdin interacts with and modulates the activity of two homeobox transcription factors, antagonizing the repressive action of Msx2 on the differentiation of mesenchymal progenitor cells, and potentiating gene activation by Dlx proteins during GABAergic neuronal differentiation (Masuda et al., 2000; Sasaki et al., 2002; Brunelli et al., 2004; Kuwajima et al., 2004; Brunelli and Cossu, 2005). Necdin interacts with the cell cycle regulatory transcription factors E2F1 and E2F4 (Taniura et al., 1998; Kurita et al., 2006), both of which are targets of retinoblastoma tumor suppressor protein (pRb) (Nevins, 1992). Together, these studies suggest that necdin may be important in the differentiation of multiple cell types through its interactions with DNA-binding transcription factors (Yoshikawa, 2000).

Skeletal muscle formation requires the activation of a myogenic program by the sequential activity of bHLH transcription factors and associated chromatin remodeling proteins, and is dependent on pRb. The E1A-like inhibitor of differentiation-1 (EID-1, also known as CRI-1) was originally identified in two separate screens as a pRb-interacting protein (MacLellan et al., 2000; Miyake et al., 2000) and is implicated in transcriptional regulation during myogenesis. EID-1 inhibits MyoD-dependent transactivation by suppression of the acetyltransferase activity of p300, a transcriptional co-activator required for myogenic differentiation. EID-1 is degraded upon cell-cycle exit by ubiquitination and proteasomal degradation in an Rb-dependent manner (MacLellan et al., 2000; Miyake et al., 2000), a process that is antagonized by the activity of the RET finger protein RFP (Krutzfeldt et al., 2005). EID-1 is ubiquitously expressed in human tissue with greatest expression in cardiac and skeletal muscle, and brain (MacLellan et al., 2000; Miyake et al., 2000), and has similar expression in mouse tissues (Bavner et al., 2002).

In this study, we identified EID-1 as a necdin-interacting protein in a yeast two-hybrid screen. We demonstrated that necdin antagonizes the repressive effect of EID-1 at two muscle-specific promoters. Consistent with a model whereby necdin normally de-represses EID-1 during muscle differentiation, we found reduced numbers of myosin heavy chain (MHC) expressing cells in limb bud muscle cultures from *Ndn*-null mice. Our results suggest that necdin contributes to the normal myogenic differentiation program through interactions with the EID-1 inhibitor of differentiation.

Methods

Yeast two-hybrid screen

To identify necdin-interacting proteins, full-length necdin was cloned into the pSOS vector of the cytoplasmic yeast two-hybrid CytoTrap system to form the bait vector pSOS-Ndn (Stratagene, La Jolla, CA). The target library was constructed from fetal brain cDNA in the pMyr vector and purchased from Stratagene. An interaction screen of approximately one million target clones was carried out according to the manufacturer's instructions with appropriate controls.

Plasmids

Full-length necdin (325 amino acids) was cloned into pcDNA3.1 HisMaxC mammalian expression vector (Invitrogen, Carlsbad, CA) with an N-terminal Xpress epitope tag to form pcDNA3.1HisMaxXpressNdn (referred to as Xpress-Ndn) (Lee et al., 2005). Full-length EID-1 cDNA was cloned into a pCI-HA vector with an N-terminal in-frame HA tag (Promega Corp., Madison, WI) to generate HA-EID-1. The expression plasmids EMSV-MyoD, 4RTK-Luc, and β-MHC-Luc have previously been described (MacLellan et al., 2000; Ji et al., 2003). pRLTK was also from Promega Corp.

Cell culture

Ndn-null mice have been previously described and carry a replacement of the necdin gene with a LacZ reported cassette, resulting in complete loss of necdin function (Gerard et al., 1999). Mouse embryonic fibroblasts (MEFs) were obtained from *Ndn*-null and wild-type littermate embryos at embryonic day 16.5 (E16.5) that were minced, trypsinized at 37°C for 5 min, and then plated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). Primary embryonic limb bud cultures were prepared from the forelimbs of E10.5 necdin-null and wild-type littermates as described by Weston et al. (2000). *Ndn*-null embryos were identified by Lac-Z staining of spare tissue (Gerard et al., 1999). All animal study procedures were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Health Sciences Animal Policy and Welfare Committee for the University of Alberta.

FB14 human fibroblasts are normal for chromosome 15 and were obtained from the NIGMS Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research, Camden, NJ. FB16 human fibroblasts are from an individual with PWS carrying a deletion on chromosome 15q11-q13. Because of genomic imprinting that silences the maternal allele of the NDN gene on chromosome 15, FB16 fibroblasts are functionally necdin-null. This cell line was obtained from the Brain and Tissue Banks for Developmental Disorders, University of Miami, Florida. HEK293, GT1-7, U2OS, MEFs, FB14, and FB16 cell lines were cultured in DMEM supplemented with 10% FBS (Invitrogen). C2C12 cells were maintained in DMEM with 15% FBS and induced to differentiate using DMEM with 2% sheep serum. Primary limb bud cultures were maintained in 40% DMEM/60% F12 with 10% FBS (Invitrogen).

Antibodies

Mouse monoclonal anti-Xpress, anti-HA, and anti-γ-tubulin antibodies were from Sigma Co. (St. Louis, MO). Rabbit polyclonal anti-HA, anti-ID2, and anti-TFIID antibodies (SC-204, SC-489, N12) were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-MyoD was from Dako Corporation (Carpinteria, CA). The polyclonal rabbit anti-human EID-1 and polyclonal rabbit anti-necdin antibodies were from Upstate. The MF20 antibody (detecting MHC) developed by Dr. D.A. Fischman was obtained from the Developmental Studies Hybridoma Bank. The GN735/736 polyclonal anti-EID-1 antibody has been described previously (MacLellan et al., 2000).

Immunoprecipitation and immunoblot analysis

GT1-7 hypothalamic neuronal cells or C2C12 cells used for co-immunoprecipitation experiments were transfected using FuGene