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PABPN1 overexpression leads to upregulation of genes encoding nuclear proteins that are sequestered in oculopharyngeal muscular dystrophy nuclear inclusions

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Oculopharyngeal muscular dystrophy (OPMD) is an adult-onset disease caused by expanded $(GCN)_{12–17}$ stretches encoding the N-terminal polyalanine domain of the poly(A) binding protein nuclear 1 (PABPN1). OPMD is characterized by intranuclear inclusions (INIs) in skeletal muscle fibers, which contain PABPN1, molecular chaperones, ubiquitin, proteasome subunits, and poly(A)-mRNA. We describe an adenoviral model of PABPN1 expression that produces INIs in most cells. Microarray analysis revealed that PABPN1 overexpression reproducibly changed the expression of 202 genes. Sixty percent of upregulated genes encode nuclear proteins, including many RNA and DNA binding proteins. Immunofluorescence microscopy revealed that all tested nuclear proteins encoded by eight upregulated genes colocalize with PABPN1 within the INIs: CUGBP1, SFRS3, FKBP1A, HMG2, HNRPA1, PRC1, S100P, and HSP70. In addition, CUGBP1, SFRS3, and FKBP1A were also found in OPMD muscle INIs. This study demonstrates that a large number of nuclear proteins are sequestered in OPMD INIs, which may compromise cellular function.

Keywords: Muscular dystrophy; OPMD; PABPN1; Polyalanine; Intranuclear inclusion

Introduction

Oculopharyngeal muscular dystrophy (OPMD, MIM 164300) is an autosomal dominant disease of late onset characterized by progressive eyelid drooping (ptosis), swallowing difficulties (dysphagia) and proximal limb weakness. OPMD has a worldwide distribution and is particularly frequent among French Canadians due to a founder effect (Brais, 2003). The OPMD locus was mapped to chromosome 14q11.1 by linkage analysis (Brais et al., 1995) and the mutation responsible for the disease was found to be within the polyadenylate-binding protein nuclear 1 (*PABPN1*) gene (Brais et al., 1998). In dominant OPMD, a $(GCN)_{10}$ repeat coding for a polyalanine domain at the N-terminal part of the protein is expanded to a $(GCN)_{12–17}$ repeat (Brais, 2003). The pathological hallmark of the disease is the presence of intranuclear inclusions (INIs) in skeletal muscle fibers (Tomé and Fardeau, 1980). These inclusions contain tubular filaments that are generally arranged in palisades or tangles. They were shown to contain PABPN1, along with molecular chaperones (HSP40, HSP70) and components of the proteasome-ubiquitin pathway (ubiquitin and 20S proteasomal subunit) (Bao et al., 2004; Calado et al., 2000b). In situ hybridization with a poly(T) probe further revealed that INIs contained large amounts of poly(A)-RNA (Calado et al., 2000b).

PABPN1 plays a major role in the polyadenylation of all mRNAs. PABPN1, in conjunction with CPSF, stimulates poly(A) polymerase (PAP) to promote the elongation of the poly(A) tail (Kerwitz et al., 2003; Kuhn and Wahle, 2004). In addition, PABPN1 has been shown to bind nascent mRNA transcripts, to act

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as a regulator of the poly(A) tail length (Bienroth et al., 1993; Wahle, 1991, 1995) and to shuttle between the nucleus and the cytoplasm bound to the poly(A) tail of mRNAs (Calado et al., 2000a). PABPN1 has in its C-terminal region an RNA binding motif (RNP-type) (Kuhn et al., 2003), a nuclear localization signal (NLS) (Calado and Carmo-Fonseca, 2000) and two oligomerization domains (Fan et al., 2001). A few PABPN1 interacting proteins have been identified: HNRPA/B, HNRPA1 (Fan et al., 2003), HNRPC (Calapez et al., 2002), HSP40 (DNAJ), and BRG1 (Kim et al., 2001). PABPN1 when overexpressed by transfection, has been shown to interact with SKIP and to stimulate muscle-specific gene expression by upregulating both myogenin and MyoD levels and stimulating E-box mediated transcription (Kim et al., 2001). Of all these proteins, only HNRPA1 and HSP40 have been studied in OPMD muscle, and shown to be localized in the INIs (Bao et al., 2004; Fan et al., 2003).

While much is known about PABPN1's C-terminal region, the role of the N-terminal polyalanine domain remains elusive. OPMD is a member of a growing number of polyalanine diseases (Brais, 2003). To date, eight other diseases genes have been found to code for expanded polyalanine domains in their proteins: HOXD13 (Muragaki et al., 1996), RUNX2 (Mundlos et al., 1997), ZIC2 (Brown et al., 1998), HOXA13 (Goodman et al., 2000), FOXL2 (Crisponi et al., 2001), PHOX2B (CCHS) (Amiel et al., 2003; Sasaki et al., 2003), ARX (Bienvenu et al., 2002), and SOX3 (Laumonnier et al., 2002). All are nuclear proteins and, except for PABPN1, they are all transcription factors (Brown and Brown, 2004; Lavoie et al., 2003). However, among polyalanine diseases, INIs have only been described in OPMD. PABPN1 is known to be aggregate prone (Kuhn et al., 2003; Scheuermann et al., 2003). The C-terminal domain of PABPN1 has been shown to be essential for its nuclear accumulation (Fan et al., 2001; Kuhn and Wahle, 2004; Kuhn et al., 2003). The biophysical properties of polyalanine were suggested to play an important role in the accumulation of PABPN1 in OPMD muscle nuclei (Brais, 2003; Kuhn and Wahle, 2004). Different lines of evidence suggest that polyalanine oligomers form resistant macromolecules in vivo and in vitro (Scheuermann et al., 2003). Polyalanine oligomers containing more than 8 alanines in a row form fibrils spontaneously (Blondelle et al., 1997). These oligomers form stable β-sheet structure in vitro that are very resistant to a wide range of proteases, denaturants, pH, and temperatures (Forood et al., 1995).

Several groups have produced cell models for OPMD by overexpressing mutant PABPN1 using transfection methods in HeLa and Cos7 cells (Abu-Baker et al., 2003; Bao et al., 2002, 2004; Fan et al., 2001; Kim et al., 2001; Ravikumar et al., 2002; Shanmugam et al., 2000). All have shown inclusion formation and some have demonstrated an association between aggregate formation and cell death (Abu-Baker et al., 2003; Bao et al., 2002; Fan et al., 2001). More recently, the first transgenic mouse model of OPMD was obtained by expressing mutant (13 Ala) human *PABPN1* under the control of a strong ubiquitous (CAG) promoter (Hino et al., 2004). In these mice, muscle disease is progressive and associated with INIs formation. Despite the known function of PABPN1 and the easy in vitro production of INIs, the pathophysiology of OPMD is still poorly understood. It is possible that the misfolding of mutant PABPN1 is an important factor as judged by the presence of the molecular chaperones HSP40 and HSP70 in the INIs. This is supported by the protective effect of either the overexpression of these chaperones (Abu-Baker et al., 2003; Bao et al., 2002, 2004), as well as treatment with Congo red and doxycycline, both known to interfere with β-sheet formation (Bao et al., 2004). Many INI-dependent and -independent pathological mechanisms have been proposed to be involved in OPMD (Brais, 2003; Fan and Rouleau, 2003; Kuhn and Wahle, 2004) including autophagy (Ravikumar et al., 2002) and mRNA trapping by the INIs (Calado and Carmo-Fonseca, 2000). However, as in the field of polyglutamine disease (Michalik and Broeckhoven, 2003), the exact role of the INIs in the pathophysiology of OPMD is still debated (Brais, 2003; Kuhn and Wahle, 2004).

The knowledge that the INIs contain poly(A)-mRNAs, that PABPN1 is an important mRNA processing factor, and that its overexpression influenced the expression of certain transcription factors led us to hypothesize that the formation of INIs may influence the expression level of genes that could play a role in OPMD. In order to identify genes and possibly pathways that are relevant to inclusion formation and OPMD pathogenesis, we used an adenoviral *PABPN1* expression model, which reproducibly led to INIs formation within 48 h in the majority of cells in culture. We used Affymetrix human HG-U133A gene chips to detect changes in the level of gene expression induced by PABPN1 expression. In this paper, we describe the significant effect that PABPN1 expression accompanied by INIs formation has on the expression profile of infected cells. Furthermore, by studying proteins coded by upregulated genes, we were able to identify new proteins that colocalize with PABPN1 in OPMD nuclear inclusions.

Materials and methods

Cell lines and cell culture

Human A549tTA cells were cultured as previously described (Massie et al., 1998a,b). Proliferating A549tTA cells were maintained in DMEM (Wisent, St. Bruno, QC) supplemented with 5% of TSA FBS (Tet-free system approved fetal calf serum; Clontech, Palo Alto, CA) and 30 μg/ml phleomycin (Sigma, St-Louis, MI) until one passage before experiments. BMAdE1 78–42, BMAdE1 220–8 (Massie, U.S. patent 5,891,6690) and 293A cells were maintained in DMEM containing 10% FBS (Wisent) and 239ACymR cells were maintained in DMEM containing 5% FBS supplemented with Geneticin G418 at 500 μg/ml (Massie et al., 1998b).

Plasmids and viruses

The *PABPN1* bovine cDNA (provided by E. Whale) cloned in the expression vector pGM10-pabp2 was modified by PCR-directed mutagenesis (Nemeth et al., 1995). The following two primers were used to add three alanine codons to the normal *PABPN1* bovine cDNA: 5′-CAG GAT CCC ATA TGG CAG CAG CAG CGG CGG CGG CGG CGG CGG CAG CAG CA-3′ and 5′-CGA GCC AGG CCC AGG GCC CGG AGC TCC CG-3′. The wt *PABPN1*-10Ala and mutated *PABPN1*-13Ala PCR products were cut with *Nde*I and *Sac*I and cloned back into the pGM10-pabp2 plasmid downstream of a sequence encoding a 6XHis-tag. The His-tagged cDNAs were digested by *Nco*I and *Bam*HI and cloned in the *Pme*I site of the adenoviral shuttle vector pAd5TR5-K7-GFP (Massie et al., 1998a,b). To generate recombinant viruses by homologous recombination, the *Fse*I digested shuttle vector was co-transfected with *Cla*I digested viral DNA in 293A cells. The recombinant clones were plaque purified in BMAdE1 78–42 cells.