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LOCALIZATION OF THE MYOTONIC DYSTROPHY KINASE IN HUMAN AND RODENT MUSCLE AND CENTRAL NERVOUS TISSUE

A thesis submitted to the School of Graduate Studies at the University of Ottawa in partial fulfillment of the requirements for the degree of Master of Science, Department of Microbiology and Immunology, Faculty of Medicine.

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ABSTRACT

Myotonic dystrophy (DM) is the most common form of inherited neuromuscular disease in adults and is characterized by progressive muscle wasting and myotonia. The mutation responsible for DM has been identified as the amplification of a polymorphic $(CTG)_n$ repeat in the 3' untranslated region of a gene encoding a serine/threonine kinase (DMK). We have produced a polyclonal rabbit antibody preparation against a fusion protein encoding C-terminal amino acids 471-629 of the human DMK gene. This antibody specifically detects products of both full length and truncated human DMK genes expressed in bacteria and in insect cells. On immunoblots, we observed protein species of ~74 and 82 kDa in human and rodent cardiac muscle and skeletal muscle, as well as rodent ependyma and choroid plexus. By immunofluorescence, DMK was found to localize postsynaptically at the neuromuscular junction of skeletal muscle, at intercalated discs of cardiac tissue and at the apical membrane of the ependyma and within the choroid plexus. We have also detected 2-3 species (~45-50 kDa) in brain tissue. Neuroanatomical evidence suggests synaptic localization for DMK in rodent cerebellum, hippocampus, midbrain and medulla. These results indicate that DMK may have a role in intercellular communication. Finally, we have demonstrated that DMK is present in adult and congenital DM tissues and that its distribution is no different than that observed in normal controls.

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LIST OF ABBREVIATIONS

AChE	acetylcholinesterase
AChR	acetylcholine receptor
AMP	adenosine monophosphate
ATP	adenosine triphosphate
bp	base pair
°C	degrees Celsius
C:G	cytosine-guanine base pairing
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CNS	central nervous system
CSF	cerebral spinal fluid
DM	myotonic dystrophy/dystrophia myotonica
DMK	DM kinase
DNA	deoxyribonucleic acid
DRPLA	dentatorubral pallidoluysian atrophy
GST	glutathione-S-transferase
HD	Huntington's disease
kb	kilobase
kDa	kilodalton
MJD	Machado-Joseph disease
mM	millimolar

mRNA	messenger RNA
NMJ	neuromuscular junction
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PK	protein kinase
SBMA	spinal and bulbar muscular atrophy
SCA1	spinal cerebellar ataxia
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
TBE	Tris-borate, EDTA buffer
Tris	tris(hydroxymethyl)aminomethane
µg	microgram
µl	microlitre
µM	micromolar
UTR	untranslated region

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CHAPTER I INTRODUCTION TO MYOTONIC DYSTROPHY

Myotonic dystrophy (DM) is an autosomal dominant neuromuscular disease. Through an international effort, the gene for DM was cloned in 1992 and the mutation subsequently identified (Aslandis et al., 1992; Mahadevan et al., 1992; Brook et al., 1992; Harley et al., 1992; Buxton et al., 1992; Fu et al., 1992). The clinical features of DM are extremely well described, however, very little is known about the underlying pathophysiology. Various approaches are being utilized in an attempt to decipher the effect(s) of the mutation on the normal physiological role of the DM gene product. Since its discovery, the DM gene has been characterized by several groups. The results of investigations of the molecular aspects of this disease, specifically RNA expression levels, have been contradictory (Carango et al., 1993; Fu et al., 1993; Hofman-Radvanyi et al., 1993; Sabourin et al, 1993). In addition, efforts made to characterize the protein species employing numerous peptide antiserum have also generated different results in terms of protein size, expression levels and subcellular localization (Koga et al.1993, Brewster et al, 1993; van der Ven et al., 1993; Étongue-Mayer et al., 1994). The development of an antibody which detects the authentic gene product would clarify some of these discrepancies. Determination of the cellular localization of the DM protein would allow working hypotheses to be posed regarding its normal function and its role in the pathophysiology of this disease.

Clinical characteristics of DM

The classic adult onset form of DM is characterized by myotonia, progressive muscle wasting and cardiac conduction defects (Harper, 1989). Other clinical symptoms such as diabetes, cataracts and intellectual impairment are also evident in this multisystemic disease. Typically, the onset for the adult form of DM is between 20-25 years of age. Variability in penetrance results in a broad range of disease manifestations from severe to mild. In fact, some individuals that carry the DM gene may be clinically asymptomatic. Congenital DM differs significantly from the adult onset form. The congenital form of DM is transmitted maternally and is generally more severe than the adult form. Congenital DM is associated with hypotonia, respiratory distress and mental retardation and is frequently fatal soon after birth. In some cases infants do survive, improve somewhat during their childhood but then present as the adult-onset form of the disease during adolescence.

Identification and characterization of the DM mutation

The DM mutation was identified through a positional cloning effort which defined the centromeric and telomeric borders of the DM locus on chromosome 19q13.3 (Smeets et al., 1991; Shutler et al., 1992). An unstable $(CTG)_n$ trinucleotide repeat in the 3' UTR of a gene encoding a serine/threonine kinase (DMK) was identified in 98% of individuals with DM (Fu et al., 1992; Brook et al., 1992; Mahadevan et al., 1992; Aslandis et al., 1992; Harley et al., 1992; Buxton et al., 1992). In normal individuals, this $(CTG)_n$

trinucleotide repeat sequence is highly polymorphic and can range from 5 to 35 repeat copies. Individuals carrying the DM protomutation, 35-80 $(CTG)_n$ repeats, can be asymptomatic or mildly affected. Relatively stable intergenerational behavior of the protomutation has been observed (Barceló et al., 1993). However, the threshold for repeat instability can also lie within the range of 35-80 $(CTG)_n$ copies and in some cases amplifications of up to 20 fold have been observed over one generation (Barceló et al., 1993). Amplifications as large as and greater than 2000 $(CTG)_n$ repeats have been observed in both adult and congenital forms of DM (Mahadevan et al., 1992; Tsilfidis et al., 1992; Barceló et al., 1993). Increases in the size of the $(CTG)_n$ repeat in DM families has been correlated with genetic anticipation, which is observed as an increasing degree of disease severity with decreasing age of onset (Hunter et al., 1992; Tsilfidis et al., 1992). Further, decreases in repeat size, documented in 6-7% of DM cases, have been correlated with decreased disease severity (O'Hoy et al.,1993; Harley et al., 1992; Hunter et al., 1992; Brook et al., 1992).

The amplification of the $(CTG)_n$ repeat appears to be the sole cause of DM, although there are rare cases (2%) of individuals with the clinical characteristics of DM that do not have the amplification nor any other mutation in the gene (Mahadevan et al., 1992). In the absence of spontaneous mutations, the relatively stable global incidence of DM (about 1 in 8500) suggests that there is a reservoir of individuals whose offspring are at risk of subsequent mutation. In order to investigate this possibility DM kindreds with asymptomatic or mildly affected individuals carrying the DM protomutation were examined for increased mutability due to their elevated $(CTG)_n$ copy number (Barceló et

al., 1993). Although stability of repeat transmission was observed through four generations, eventual transitions from the protomutation to full mutation would be predicted, accounting for the prevalence of DM globally. Interestingly, no transitions from the normal range into the protomutation range have been identified as of yet (Barceló et al, 1993). Nor is the mechanism underlying the trinucleotide repeat amplification in DM understood.

The DM mutation was found to be in complete linkage disequilibrium with a two allele insertion/deletion *Alu* element polymorphism located 5 kb telomeric to the $(CTG)_n$ repeat (Mahadevan et al., 1993a). In conjunction with this *Alu* element polymorphism, extensive haplotype analyses on DM individuals involving several polymorphisms and microsatellite markers strongly support a single common origin for the DM mutation (Mahadevan et al., 1992; Harley et al., 1992; Rubinzstein et al., 1994; Whiting et al.1995, in press). Three models for the origin of DM have been proposed: i) an ancestral mutation that occurred once, ii) a particular haplotype on which the $(CTG)_n$ repeat became inherently unstable, predisposing it to mutation or, iii) an ancient haplotype on which the (CTG)n repeat has evolved to its current size distribution (Imbert et al., 1993; Neville et al., 1994). A high resolution haplotype analyses of the DM locus clearly demonstrated that both models i) and ii) were possible (Neville et al., 1994).

The DM gene

The DM gene encodes 15 exons that span approximately 14 kb of genomic DNA on chromosome 19q13.3. Precise sequences for intron/exon boundaries, as determined

from genomic and cDNA clones isolated from brain, heart and muscle cDNA libraries, in both mouse and human have been previously described (Jansen et al., 1992; Mahadevan et al., 1993b). The full length mRNA for the human DM gene is 3.4 kb and encodes for a 629 amino acid protein with an N-terminal domain (exons 2-8) that is highly homologous to the family of cAMP-dependent serine/threonine kinases. There is also considerable conservation within exons 2 through 8 encoding the catalytic domain involved in substrate binding and catalysis. The central region, including exons 9 to 12, has significant homology to the α-helical coiled coil domains of myofibrillar and filamentous proteins. Exons 13 and 14 have no homology to any known protein or protein domain. These exons are alternatively spliced out in several cDNA clones that have been isolated from a human heart library (Mahadevan et al., 1993b). This splicing event results in the loss of the putative transmembrane domain (exon 15) of DMK, generating a protein with a predicted molecular weight of 59 kDa, as compared to the 69 kDa full length product. The DM mutation or $(CTG)_n$ lies beyond the translation stop codon within the 3' UTR of the gene.

DMK

Previous studies on DMK have indicated that it is a novel serine/threonine protein kinase, both structurally and functionally (Dunne et al.,1994; Étongue-Mayer et al.,1994). The catalytic domain in the DMK was found to have the closest homology to protein kinase A (PKA) and PKC. However, it was demonstrated that DMK activity was resistant to specific inhibitors of these PK's, as well as other classes of serine/threonine

kinases (Dunne et al., 1994). In addition, it has also been shown to autophosphorylate and transphosphorylate histone H1 (Tamai et al., manuscript in preparation; Étongue-Mayer et al., 1994). These preliminary analyses indicate that DMK may have restricted interactions with specific substrates involved in the regulation of one or more cellular pathways by phosphorylation. As DM is a multisystemic disease, it is likely that DMK has a specific regulatory role in a signal transduction pathway common to all the systems affected. However, until the substrate(s) for DMK have been determined and functional biochemical assays are performed its function remains speculative.

Effects of the DM mutation

The mechanism by which the DM mutation manifests its pathology has proven difficult to determine. The correlation between disease severity and $(CTG)_n$ copy number implies that there may be a quantitative effect of the $(CTG)_n$ amplification on gene expression. This phenomenon has been reported in seven other human diseases involving trinucleotide repeats. Unstable trinucleotide repeat amplifications, referred to as dynamic mutations (Richards et al., 1992), have been identified in both the coding and non-coding regions of these genes. Amplification of $(CAG)_n$ repeats within the coding region of the Huntington's disease gene (HD), Kennedy's disease gene and type 1 spinal and cerebellar ataxia (SCA1) results in the translation of polyglutamic tracts in the protein product. Alteration of these proteins, as such, is presumed to be responsible for reduced ligand binding in Kennedy's disease and a toxic gain of function in HD and SCA1 (The Huntington's Collaborative Research Group, 1993; Orr et al., 1993; Mhatre et al., 1993).

Alternatively, amplification of a $(CGG)_n$ repeat in the 5' untranslated region (UTR) of the fragile X syndrome gene (FMR-1) has a loss of function effect (Pieretti et al., 1991). Transcriptional repression of the FMR-1 mRNA, due to hypermethylation of a 5' CpG island, results in the loss of an RNA-binding protein in the brain (Knight et al., 1993; Siomi et al., 1993). In DM the $(CTG)_n$ repeat lies within the 3' UTR of the gene and represents a novel mechanism amongst dynamic mutations that is inherited as a dominant phenotype.

In order to understand the molecular mechanism underlying the dominant phenotype numerous research groups have attempted to examine DMK mRNA expression levels. Several lines of evidence regarding mRNA expression in DM individuals have been documented. Interestingly, conclusions drawn from analysis of DMK mRNA and protein from congenital DM tissues were contradictory to those from adult DM tissues. Reports have suggested that mRNA expression is consistently decreased in adult DM skeletal and cardiac tissues (Fu et al., 1993; Hofman-Radvanyi et al., 1993; Carango et al., 1993). These reports also demonstrated that in congenital DM tissues the mutant allele was not expressed. In addition, Hofmann-Radvanyi et al., detected a 20-30% decrease in the expression of the normal allele in adult DM tissues. The latter may indicate that both alleles are repressed by the $(CTG)_n$ amplification. In direct contrast to the above, it was shown that in congenital DM tissues the mutant DMK allele was expressed at levels equal to or greater than the controls and was attributed to a possible elevation in steady state levels of the DMK transcript (Sabourin et al., 1993). In addition, a immunofluorescence study using anti-DMK antibody reported that there were no differences between affected

and normal tissues (van der Ven et al., 1993). These contradictory conclusions may be reconciled only by the fact that inappropriate methodology may have been used, such as the problematic RT-PCR reaction performed through large repeat regions (Fu et al., 1993), which could have resulted in technical artifact. Also, differences in the integrity and specific degradation processes between normal and DM tissues may generate some sampling errors which account for the discrepancies in mRNA and protein levels (Roses, 1994). As DM is a multisystemic disease with variable age of onset, it may be that the disease gene is altered in its expression in a number of tissues and at different developmental stages. The effect that the mutation has on the normal gene function becomes more difficult to understand when attempts are made to reconcile the clinical differences between congenital and adult-onset DM by over- and under-expression models for DM.

In an effort to test these mutually exclusive hypotheses regarding DMK expression, transgenic and "knockout" mice models have been developed. In a comprehensive effort to mimic the DMK over-expression theory mouse lines have been established containing multiple copies of the human DM gene (Narang et al., unpublished data). Although there is no obvious phenotype in these mice, physiological and morphological analyses are currently underway. In addition, the development of mice lines with minigene constructs containing either 5, 60 or 100 $(CTG)_n$ repeats in the 3'UTR is also in progress. In order to identify a lethal or null mutation which would support the DMK under-expression theory, the "knockout" mouse was established (B. Wieringa, University of Nijmegen, unpublished data). The "knockout" mice do not express the

mouse DMK gene and do not show any obvious phenotype. These results would appear to favor the over-expression mechanism proposed by Sabourin et al.,1993. Alternatively, it is possible that the role of DMK may have some functional redundancy or its role is different in mouse. Therefore, the mouse "knockout" model may not be the same as a functional "null" mutation in the human.

Characterization and localization of DMK

The extensive analysis of tissue distribution is a necessary step toward understanding, or at least delineating a physiological role for DMK. Efforts to characterize the DMK protein species employing antisera generated from peptide immunogens were initiated by four different research groups. In human skeletal and cardiac tissues, the prominent protein species observed was between 52-55 kDa, although a 42 kDa species was also reported in brain tissue (van der Ven et al., 1993; Brewster et al., 1993; Fu et al., 1993). Another group identified a 62 kDa species in skeletal and cardiac muscle and a 53 kDa species, in addition to the larger species, in brain (Koga et al., 1994). Dunne et al. detected full length and truncated forms of DMK at 66 and 43 kDa respectively. It is noteworthy that the these species, with the exception of the 66 kDa protein, are smaller than the predicted protein size of ~69 and 59 kDa, as described above. However, the possibility that alternative splicing and/or post-translational modifications occur that reduce the protein size can not be ruled out.

These peptide-derived antisera were also used to detect DMK in tissues from DM individuals. Reduced expression of the 53- 55 kDa protein was observed in DM skeletal

muscle and cardiac tissue as compared to that in normal controls (Fu et al., 1993; Koga et al., 1994). However, van der Ven et al. report no differences in the expression of a 53 kDa protein species using both immunoblot and immunofluorescence analysis in control and DM skeletal muscle and cardiac tissues. The previous studies employing these antisera to localize DMK have also reported a variety of observations. Low levels of DMK have been found in the sarcoplasm of type I muscle fibers in normal controls (Brewster et al., 1993; van der Ven et al., 1993), as well as in specific sites of neuromuscular and myotendinous junctions in mature skeletal muscle and to intercalated discs in the myocardium (van der Ven et al., 1993). Localization at the cerebellar Purkinje cells has also been reported (van der Ven et al., 1993). As in the RNA expression level studies, the previously described reports differ in protein characterization and localization. This may also be attributable to tissue specific degradation processes and the lack of true matched controls.

The authenticity of the protein species described above can be challenged as an anti-DMK antibody generated by our group consistently detects DMK protein species larger than that previously reported. The species we detect are very similar to the size of the DMK protein expressed in bacteria and recombinant baculovirus. In addition, the tissue distribution of the DMK is consistent with previous reports on the RNA expression pattern in various tissues (Sabourin et al, 1993; Brook et al., 1992; Jansen et al., 1992). Furthermore, the 54 kDa species described in earlier reports, is still present in the "knockout mouse" tissue where the species we detect are absent (B. Wieringa, University of Nijmegen, unpublished data). Cumulatively, the inconsistencies in protein size and

expression levels raise questions regarding the specificity of these previously characterized antisera. These earlier reports should, therefore, be interpreted with caution due to the possibility that the antisera are detecting a cross-reactive species. In the present study, we demonstrated the specificity of our antibody for DMK and have pursued tissue distribution analyses using immunofluorescence in order to clarify the previous data and provide additional insight into the normal role of DMK.

In summary, DM is an inherited neuromuscular disease caused by amplification the of a $(CTG)_n$ repeat in the 3' UTR of the gene. The mutation can be transmitted by affected individuals of either sex to their offspring and its effects are multisystemic. The molecular mechanism underlying repeat amplification and the ultimate effect it has on gene function in this disease has not been defined. Although the gene product has been identified as a serine/threonine kinase (DMK), its function is not known. The objective of my research and the focus of this thesis involves the localization of the DMK protein species in human and rodent skeletal, cardiac and CNS tissues. In order to address this matter, an immunofluorescence study was conducted using our novel polyclonal anti-DMK antibody. The localization of DMK in affected and normal human tissues was also examined by immunofluorescence in order to identify any potential differences in distribution. This study is an initial step towards understanding the biology of DMK and provides insight into the role of this protein in the pathophysiology of the disease.

CHAPTER II CHARACTERIZATION OF THE MYOTONIC DYSTROPHY KINASE (DMK) PROTEIN IN HUMAN AND RODENT MUSCLE AND CENTRAL NERVOUS TISSUE. Elisabeth J. Whiting[1*], James D. Waring[2*], Katsuyuki Tamai[3], Martin J. Somerville[2], Maxwell Hincke[4], William A. Staines[4], Joh-E Ikeda[3], Robert G. Korneluk[1,2]. [1]Department of Microbiology and Immunology, University of Ottawa, Canada; [2]Molecular Genetics, Children's Hospital of Eastern Ontario, Ottawa, Canada; [3] GenoSPHERE Project, Tohkai University School of Medicine, Bohseidai, Isehara City, Kanagawa 259-11, Japan[4]Department of Anatomy and Neurobiology, University of Ottawa, Canada.*Co-authors
Hum. Molec. Genet. 4, 1063-72 (1995).

Background

With a global incidence of 1 in 8500 individuals adults, (DM) is the most common form of inherited neuromuscular disease. Classic DM is defined as an autosomal dominant multisystemic disorder in which a characteristic myotonia and progressive muscle wasting is accompanied by specific abnormalities in a number of other systems (Harper, 1989). In adult-onset DM, minor intellectual impairment, cataracts, and testicular and ovarian atrophy are commonly observed. Hypotonia, respiratory distress and mental retardation are present in severe congenital DM cases.

The molecular basis of DM has been identified as the amplification of an unstable trinucleotide $(CTG)_n$ repeat in the 3' untranslated region (UTR) of a transcript encoding a serine/threonine kinase (DMK) (Brook et al., 1992; Mahadevan et al., 1992; Fu et al., 1992; Dunne et al., 1994). Amplifications of similar repeats have been identified in other human diseases such as fragile X syndrome (FRAXA/FRAXE)(Kremer et al., 1991; Verkerk et al., 1991; Fu et al., 1991), Huntington's disease (The Huntington's collaborative Research Group), dentatorubral pallidoluysian atrophy (Koide et al., 1994) and most recently Machado-Joseph disease (MJD) (Kawaguchi et al., 1994). The DM trinucleotide repeat is highly polymorphic in normal individuals, ranging from 5 to 35 CTG

repeats (Barceló et al., 1993), while 2000 or more CTG repeats have been observed in congenital DM cases (Mahadevan et al., 1992). Increased repeat size has been correlated with increasing disease severity and an earlier age of onset, and is the basis for genetic anticipation observed in successive generations (Tsilfidis et al.,1992).

Possible effects of the CTG amplification on DMK expression include 1) an alteration in the steady-state levels of DMK mRNA, 2) interference with the normal splicing pattern for DMK RNA, 3) interruption of some normal regulatory role of DMK transcripts, or 4) a change in the efficiency of translation. Previous studies have suggested that the amplification of the CTG trinucleotide repeat is associated with a reduction in the steady-state mRNA levels of DMK in affected individuals based upon RT-PCR (Fu et al., 1993; Carango et al., 1993; Koga et al., 1994). In addition, two of these previous reports (Fu et al., 1993; Koga et al., 1994) showed that there was reduced levels of a 54 kDa protein species in the tissues of DM individuals compared with normal individuals. In contrast, we have previously demonstrated that the mutant DM allele is expressed regardless of repeat size, and that RNA levels from mutant alleles are as great as or greater than those from normal alleles (Sabourin et al., 1993). In support of the latter observations, we have localized DMK in human tissues and no differences were observed in the distribution or intensity of staining between tissues from normal and affected individuals.

Neither the normal function of DMK, or its role in pathophysiology of DM are understood. To further our understanding of this protein, we have generated a polyclonal anti-DMK IgG preparation (anti-DMK) directed against a glutathione-S-transferase

(GST)-DMK fusion protein. We have used this antibody to detect DMK isoforms in rodent and human tissues by immunoblot analysis and detected prominent protein species in heart, brain and skeletal muscle that were larger than the previously reported 52-54 kDa species (Fu et al., 1993; Carango et al., 1993; Koga et al., 1994; van der Ven et al., 1993; Étongue-Mayer et al., 1994). These species (~74 and 82 kDa) were more consistent with the size of DMK expressed in both bacteria and in insect cells from a novel full length cDNA. A survey of DMK distribution in clinically relevant tissues using histochemistry and immunofluorescence was performed. In addition, we have compared the distribution of DMK between normal and affected tissues using immunofluorescence.

Materials and Methods

Polyclonal Antisera Production and Recombinant DMK Expression.

A human fetal brain cDNA library (Stratagene) was probed with a B*am*HI genomic fragment which overlaps the 5' end of a previously isolated DM cDNA (HMDM2123) and the 3' end of the adjacent (centromeric) N9 gene (Mahadevan et al., 1993b). A cDNA was isolated which contains all 15 exons predicted from genomic sequence, as well as 326 nt. of 5' and 735 nt. of 3' non-coding sequence (NCS), and subcloned into pBluescript (pBSDMK) (Stratagene). For production of antiserum, a S*ac*I/H*ind*III fragment encoding the C-terminal 160 amino acids (aa.) (beginning in exon 11) was subcloned in the GST-fusion vector pGEX-KG (Guan et al., 1991). Bacterial lysates were prepared by sonication and IPTG-induced bacterial fusion protein was purified as described (Guan et al., 1991). Rabbits were immunized with ~100 μg of fusion protein in complete Freund's

adjuvent, and boosted with protein in incomplete Freund's adjuvent. Serum was precleared with IPTG-induced bacterial lysate containing GST protein (pGEX-KG vector) alone. The immunoglobulin fraction was isolated by ammonium sulfate precipitation and dialyzed against PBS. Anti-DMK immunoglobulin was further purified using immobilized GST-DMK fusion protein.

A product was PCR-amplified from a DMK cDNA with exons 13 and 14 spliced out (Mahadevan et al., 1993; Jansen et al., 1992) (HMDM2123) which corresponds to nt. 49 of the coding sequence (removing 17 aa.) to nt. 2680. The product was subcloned as an *Eco*RI/*Sal*I product in vector pAK-3 (pAKDMKΔ13-15), which contains a T7 promoter element preceding a multiple cloning site. A *Xho*I/*Hin*dIII 3' fragment from pAKDMKΔ13-15 was replaced with one from pBSDMK to create a non-spliced version (pAKDMK). After transfection in E.coli, strain BL21(DE3), which contains an integrated IPTG-inducible T7 polymerase, protein expression was IPTG-induced. Lysates were prepared by freeze-thaw of bacteria in 50 mM Tris-Cl, 4 mM β-ME, 1 mM PMSF and 1 mg/ml lysozyme. Lysates were adjusted to 10 mM $MgCl_2$, 10 μg/ml DNase I, incubated for 10 min on ice, adjusted to 0.1 % NP40, and sonicated to release inclusion bodies. Inclusion bodies were recovered by low speed centrifugation.

For expression in baculovirus, a 3' *Xba*I/*Hin*dIII fragment from pBSDMK was replaced with one from HMDM2123 (pBSDMKΔ13-15) to create a spliced version and both were subcloned into the baculovirus shuttle vector pVL1392/1393 (InVitrogen). Recombinant viruses were isolated using a MaxBac kit (InVitrogen) as suggested, and

viruses purified by standard technique (Summers et al., 1987). For expression, insect cells were infected with recombinant virus at an m.o.i. of >5 for two days.

Immunoblotting

For tissue extracts, human and rat tissues were homogenized in lysis buffer (150 mM NaCl, 50 mM Tris-Cl, pH 8.0) including 2 mM PMSF, 6 μg/ml aprotinin and 1 μg/ml leupeptin, adjusted to 1% NP40 and extracted 15 min on ice. Control whole tissue extractions were also performed by homogenizing tissues in lysis buffer which was supplemented with 5% SDS and 5% β-mercaptoethanol, and boiling for 5 min. DNA was sheared in these preparations to reduce viscosity by passing repeatedly through a 21 gauge needle. Protein concentration was determined using a Micro BCA protein assay kit. 20 μg of total tissue protein was loaded per lane. Tissue extracts were normally resolved using 10% polyacrylamide (29:1 acrylamide: bisacrylamide ratio) on a Biorad mini-Protean II apparatus. Molecular weights were calculated using a Protean II apparatus; log Mr vs. Rf plots were established using BioRad SDS-PAGE low range standards. Tissue extracts, bacterial inclusion bodies, or whole insect cells infected with recombinant virus were dissociated in sample buffer (50 mM Tris [pH 6.8], 5% SDS, 5% β-mercaptoethanol, 10% glycerol). Protein was resolved by discontinous SDS-PAGE and electrophoretically transferred to nitrocellulose using BioRad Trans-Blot cells. Standard localization and transfer efficiency were monitored using Ponceau S staining. Blots of bacterially-expressed products (Fig. 1B) were developed using a BioRad alkaline phosphatase color development system; all other blots were developed using an ECL kit (Amersham) as

recommended. Anti-DMK antibody was used at final concentrations 0.1-0.4 μg/ml. For tissue blots, anti-DMK was initially preincubated with a 10-fold excess of human liver extract as standard protocol to avoid non-specific background staining. This step proved unnecessary with anti-DMK preparations from later bleeds: the pattern observed was no different with anti-DMK that was not preincubated.

Immunofluorescence and Histochemical procedures

Human tissues were obtained at autopsy following informed consent. Tissues were collected 6-12 hours post-mortem, flash frozen with powdered dry ice and stored at -80° C. Rat tissues were collected immediately after sacrifice. Rats (300 to 350 gm) were anesthetized with pentobarbital and perfused transcardially with 60 ml of normal saline buffered with 10 mM sodium phosphate, pH 7.2 (PBS). Tissues were removed, flash-frozen and stored at -80°C.

Isolation of choroid plexus from the brain was carried out by both blunt and sharp dissection. For blunt dissection the brain was immediately cut along the coronal plane into blocks 3 to 4 mm thick and choroid plexus was drawn out from within the lateral and fourth ventricles using fine forceps. For sharp dissection, brain blocks were frozen onto microtome chucks and cut in a cryostat (Microm). The ependyma and periependyma were dissected from frozen sections.

All denervated rat muscles were a kind gift from Dr. B. Jasmin (University of Ottawa, Canada). Denervation of rat skeletal muscle was performed by severing the

sciatic nerve. Rats were sacrificed 10 days later and gastrocnemius muscles were removed, flash frozen and stored at -80°C.

Cryostat sections 16 μm in thickness were cut and thaw mounted on slides for immunostaining. Slide-mounted sections were preincubated with PBS followed by incubation with rabbit anti-DMK, rabbit anti-acetylcholinesterase (J. Massoulié, Laboratoire de Neurobiologie, CNRS UA 295, École normale superieure, Paris, France), sheep anti-Chol-1 (V.P. Whittaker, Neurochemistry Group, Max Planck Institute, Gottingen, Germany), or rat anti-tubulin (Serotec Yol1/34) in PBS containing 0.3% Triton X-100. Following incubation with primary antisera sections were rinsed with PBS and incubated with secondary antisera, FITC-labelled donkey anti-rabbit immunoglobulin (Amersham), CY3-labelled donkey anti-rabbit immunoglobulin (Sigma), biotinylated donkey anti-sheep immunoglobulin (Amersham), biotinylated goat anti-rat (Amersham), or streptavidin-Texas Red (Amersham). Sections were assayed for acetylcholinesterase activity to localize neuromuscular junctions (Karnovsky, 1964) by incubation with 2.5 mM acetylthiocholine iodide, 0.5 mM potassium ferrocyanide, 3 mM copper sulphate and 10 mM sodium citrate in 65 mM sodium malate buffer (pH 6.5) for 15 min at 10^{0} C. Sections were examined using a Zeiss fluorescence microscope with Normarski optics. Both single and double filters were used to visualize FITC and rhodamine fluorophores. For more detailed observations a Leica confocal laser microscope with a krypton/argon laser that allowed scanning for both FITC and rhodamine fluorophores within the same tissue sections was used.

Results

A GST-DMK fusion protein was used to elicit a polyclonal antibody preparation (anti-DMK) against a C-terminal segment of DMK. To provide a source of DMK protein with which the antisera could be characterized, a full-length cDNA was isolated (see Materials and Methods). In addition, a cDNA was generated which corresponds to a splice-isoform cDNA which has exons 13 and 14 removed (Mahadevan et al., 1993b; Jansen et al; 1992). This deletion results in termination of translation after two amino acids of exon 15 (DMKΔ13-15). Both proteins (Fig.1A) were expressed in bacteria under the control of IPTG-inducible T7 polymerase, and in insect cells using recombinant baculoviruses. Anti-DMK is clearly reactive to both DMK and DMKΔ13-15 produced in bacteria. The species identified were specifically induced by IPTG (data not shown) and were detected in whole cell lysates or in inclusion bodies (Fig. 1B). No reactivity against vector expressing GST alone was observed (data not shown). Two major species were detected for DMK (~82 and 69 kDa) while only one was observed for DMKΔ13-15 (~72 kDa). This suggests that the presence of exons 13-15 reveal a (more N-terminal) processing site. The difference between the full length DMK species and DMKΔ13-15 is consistent with the coding region removed, but they are larger than predicted (69 and 59 kDa, respectively). Both cDNA's were also used to create recombinant baculoviruses (AcDMK and AcDMKΔ13-15, respectively). In infected insect cells, anti-DMK recognizes a single species of ~85 kDa for AcDMK, while a closely spaced doublet (~82 and 81 kDa) is detected for AcDMKΔ13-15 (Fig. 1C). The basis for this difference is not

Figure 2-1 Immunoblot analysis of human DMK and DMKΔ13-15 expressed in bacteria and in insect cells using recombinant baculovirus. (A) Full-length DMK and a truncated version constructed using the 3' end of a splice isoform cDNA are illustrated. Exon boundaries are indicated by vertical lines. The PKA-homologous domain is designated by dark shading. The domain homologous to myosin heavy chain which has a predicted coiled-coil helical domain is indicated by light shading. (B) DMK and DMKΔ13-15 were expressed in bacterial strain BL21(DE3) from vector pAK-3 (under T7 polymerase control) by IPTG induction of integrated host T7 polymerase. For detection of DMK, isolated inclusion bodies were blotted to nitrocellulose after electrophoresis and incubated with anti-DMK. Alkaline-phosphatase-coupled goat anti-rabbit IgG second antibody was used. Two major species were detected for DMK (lane 2, ~82 and 69 kDa) and only one species (~72 kDa) was detected for DMKΔ13-15 (lane 1). (C) Recombinant baculoviruses were isolated and used to infect SF9 insect cells. Infected cells were harvested and analyzed two days post-infection. Horseradish peroxidase-coupled goat anti-rabbit IgG second antibody was used (and for all other immunoblots below) in conjunction with enhanced chemiluminescence detection. No products were detected in control insect cells infected with wild type baculovirus AcMNPV (lane 1). We detected one species (~85 kDa) for AcDMK (lane 2) and detected a closely spaced doublet for AcDMKΔ13-15 (lane 3). In comparison, human heart extract exhibits a minor species similar in size to DMKΔ13-15 (lane 4).

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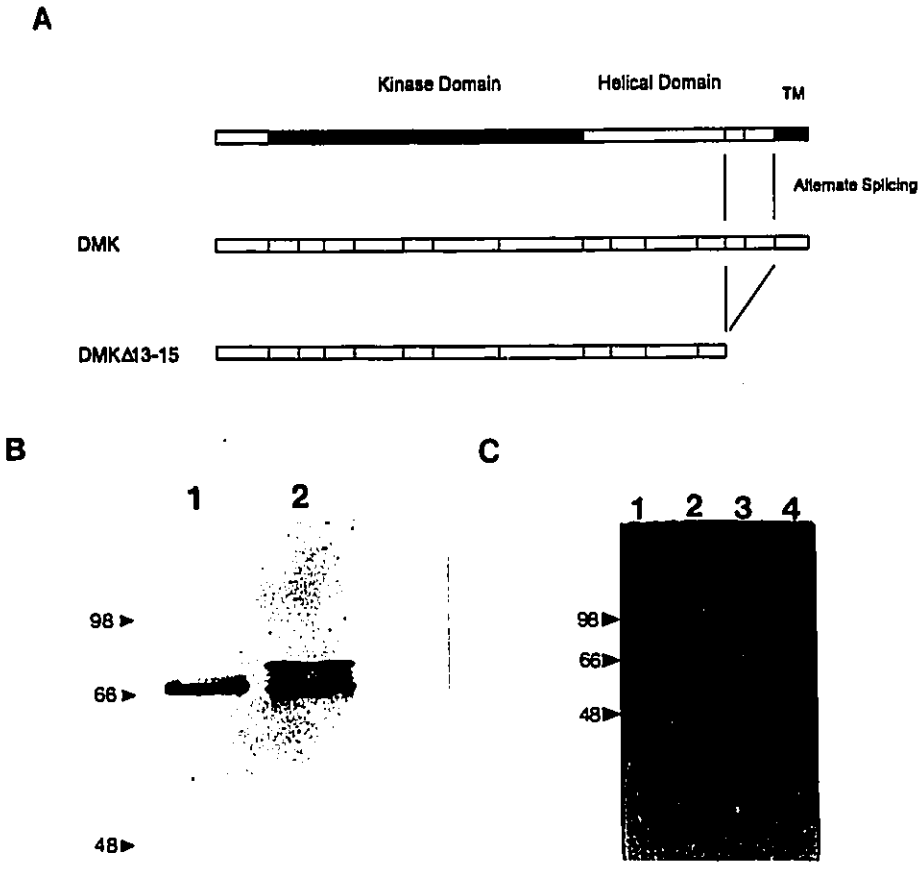
A
Kinase Domain
Helical Domain
TM
Alternate Splicing
DMK
DMKΔ13-15
B
1
2
98
66
48
C
1
2
3
4
98
66
48

known, but indicates further post-translational modification of DMKΔ13-15. No species were detected for the wild-type virus-infected cells.

In order to examine tissue for the presence of DMK, human cardiac muscle, diaphragm, forebrain, pectoral muscle, and rat quadricep extracts were immunoblotted with anti-DMK (Fig. 2A). Two prominent species (~82 and 74 kDa), were detected for cardiac and skeletal muscle, while minor amounts were observed in diaphragm and forebrain. No species were detected in human liver and pancreas (data not shown). Human forebrain also exhibited 2-3 species at ~45-50 kDa (Fig. 2A). Closer examination revealed that the 82 kDa human skeletal muscle species was slightly larger than the corresponding rat species (Fig. 2B). In human samples, the upper band was prevalent in skeletal muscle, while the lower band appeared to be predominant in cardiac muscle, although the exact distribution varied over different experiments. In contrast, the pattern was identical for rat skeletal and cardiac muscle (Fig. 2B).

Anti-DMK was then used to localize DMK by immunofluorescence on cryostat sections from rat tissues. In adult skeletal muscle, DMK immunoreactivity was observed only at neuromuscular junctions (Fig. 3A), as previously described (van der Ven et al., 1993). This was confirmed by staining adjacent sections with anti-DMK (Fig. 4A) and performing a histochemical reaction that detects acetylcholinesterase concentrated at the basal lamina of the NMJ (Fig.4B). To investigate the localization of DMK at NMJ's, antibodies to presynaptic and postsynaptic markers were used to compare immnofluorescence in control and denervated rat skeletal muscle (examined 10 days after denervation). Acetylcholinesterase (AChE), a postsynaptic marker, maintains the same

Figure 2-2 Immunoblot analysis of human and rodent tissues. (A) Non-ionic detergent extracts were prepared from human cardiac (lane 1), diaphragm (lane 2), forebrain (lane 3), pectoralis (lane 4) and rat quadriceps (lanes 4 and 5). Two prominent species (74 and 82 kDa) were detected in muscle samples; only minor amounts of these species were observed in diaphragm (lane 2) and forebrain (lane 3). A group of 2-3 species (between 45 and 50 kDa) was also observed in forebrain (lane 3). (B) For comparison, human pectoralis (lane 1), rat quadricep (lane 2), human cardiac (lane 3), and rat cardiac (lane 4) tissue extracts were analysed together. The major human skeletal muscle species was slightly larger than the rat species (lanes 1 and 2, respectively). In human samples the upper band was more prevalent in skeletal muscle (lane 1) and the lower band prominent in the cardiac tissue (lane 3). In rat samples the upper band was predominent in both skeletal (lane 2) and cardiac tissue (lane 4).

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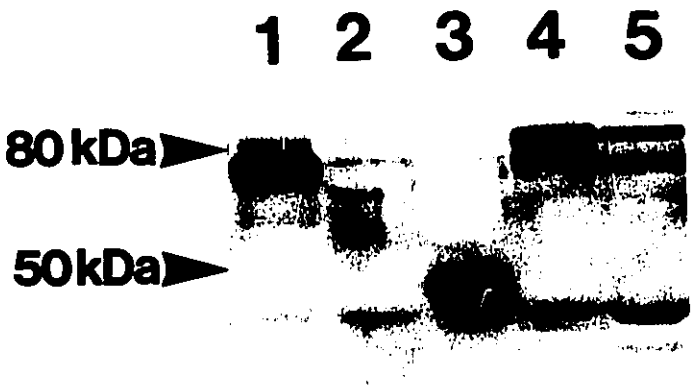
A



B

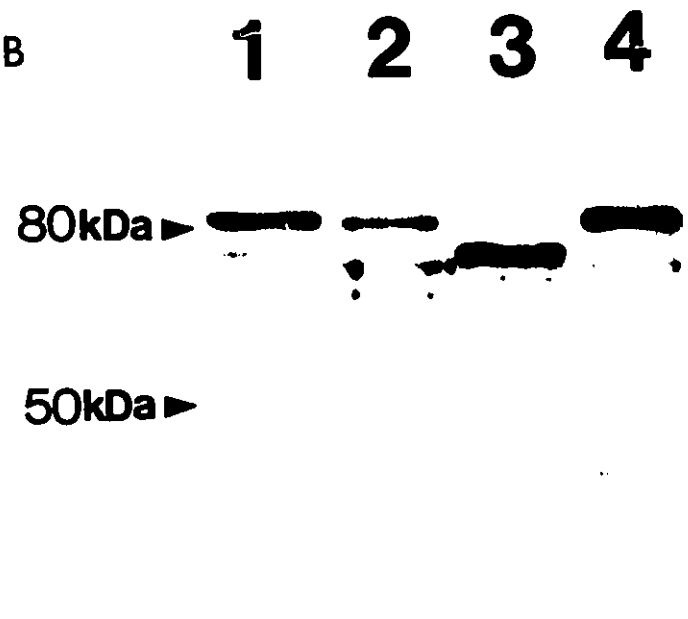
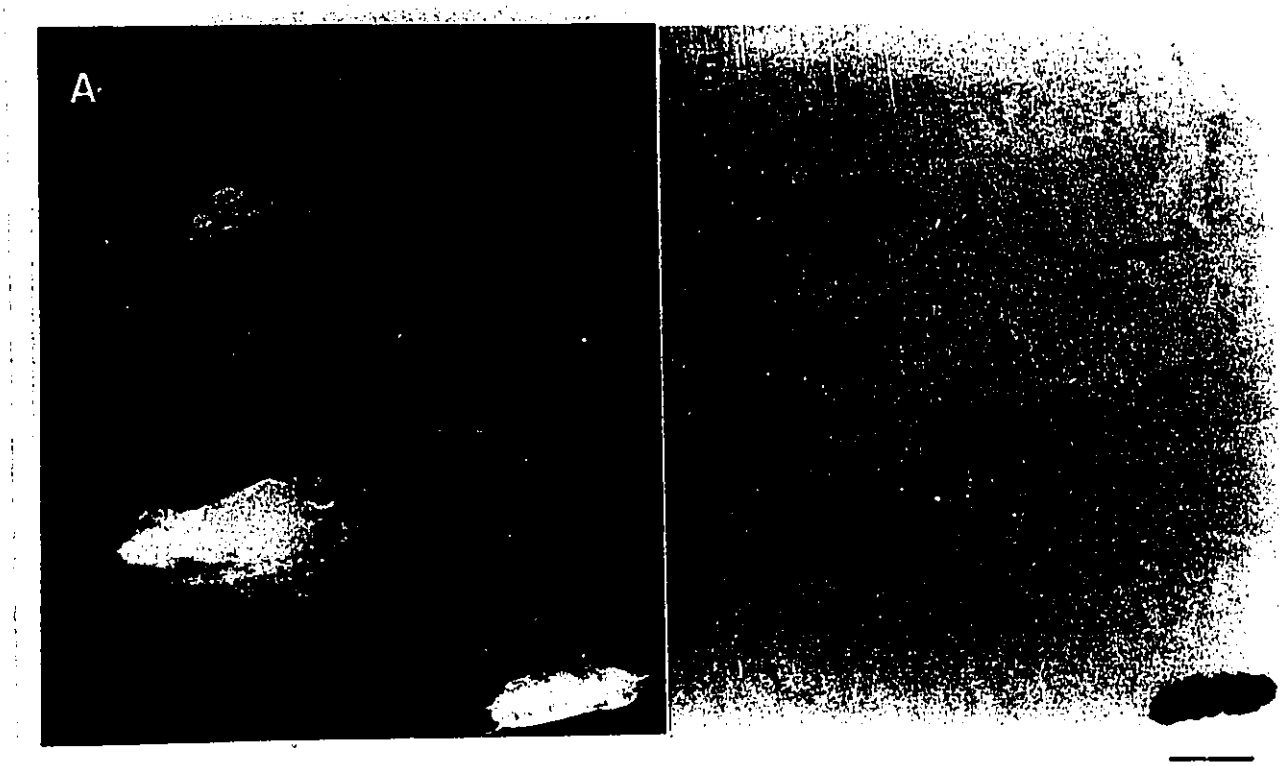


Figure 2-3 Immunofluorescence analysis of rat tissues. Cryostat sections of quadriceps (A), cardiac muscle (C), and cerebral ventricle (E) were incubated with anti-DMK, or with anti-DMK preabsorbed with 1 μg/ml GST-DMK immunogen and 1μg/ml glutathione-agarose (B, D, and E, respectively). Staining at NMJ's in rodent quadricep (A), intercalated discs in myocardium (C) and at the apical membrane of the ependyma and choroid plexus (E) was reduced to the level of background staining after preabsorbtion (B, D, and F). Calibration bar = 17μm.

Figure 2-4 Identification of NMJ's. Acetylcholinesterase histochemistry was performed on cryostat sections of rat quadricep (A) and its adjacent section was incubated with anti-DMK (B). DMK co-localized at the sites of NMJ's. Calibration bar = 17 μm.


A

localization before (Fig. 5A) and after denervation (Fig. 5B). The presynaptic marker, Chol-1, an antigen identified in the plasma membrane of the cholinergic electromotor nerve terminals, shows a similar distribution to that of AChE in control muscle (Fig. 5E), but completely disappears in denervated muscle (Fig. 5F), as expected. The distribution and intensity of DMK in control muscle (Fig. 5C) remains the same in the denervated rat muscle (Fig. 5D), demonstrating that DMK is localized to the postsynaptic side of the NMJ. In rodent cardiac muscle, staining of intercalated discs with anti-DMK was observed (Fig. 3C). DMK immunoreactivity in rodent brain was heterogeneous. The most prominent staining was observed in specialized epithelial cells of the cerebroventricular system (Fig. 3E). More specifically, DMK immunoreactivity was observed in the ependyma and choroid plexus. In the ependyma DMK localized at the apical membrane (Fig. 6A), as demonstrated by the staining of ependymal cilia in adjacent sections using an antibody against tubulin (Fig. 6B). To demonstrate that our antisera was not recognizing cross-reactive proteins, we preabsorbed anti-DMK with the DMK-GST immunogen prior to tissue analysis. Immunofluorescence was reduced to background levels in these sections (Fig. 3B, D, F).

Further analysis of rodent brain by immunofluorescence revealed that DMK localized at discrete synaptic sites in a large number of brain regions. In the hippocampus, DMK immunoreactivity was observed in alignment with the apical and basal processes of interneurons (Fig. 7A). The substantia nigra had dense fields of punctate staining, similar to afferent neurotransmitter staining observed in this region (Christensson-Nylander, et al.,1986) (Fig. 7B.)

Figure 2-5 Synaptic localization of DMK in rat muscle. Rat quadricep sections from normal (A, C, E) and denervated (B, D, F) muscle were stained with AChE (A and B), anti-DMK (C and D), or anti-Chol-1 antisera (E and F). AChE (post-synaptic) staining remains in denervated muscle while Chol-1 (presynaptic) disappears, as expected. The distribution and intensity of DMK straining in denervated muscle (D) demonstrates that it localizes post-synaptically at the NMJ. Calibration bar = 17 μm.

Figure 2-6 Immunolocalization of DMK in the ependyma and choroid plexus of rat brain. Sections of rat cerebral ventricle were stained with anti-DMK (A) or with anti-tubulin (B). DMK in the ependyma (ep) (small arrows in A) is localized at the apical membrane near the base of the cilia which are identified by immunostaining of tubulin (small arrows in B). Localization of DMK in the choroid plexus (ch) (large arrows in A) is less concentrated than in the ependyma. Calibration bar=17 μm.

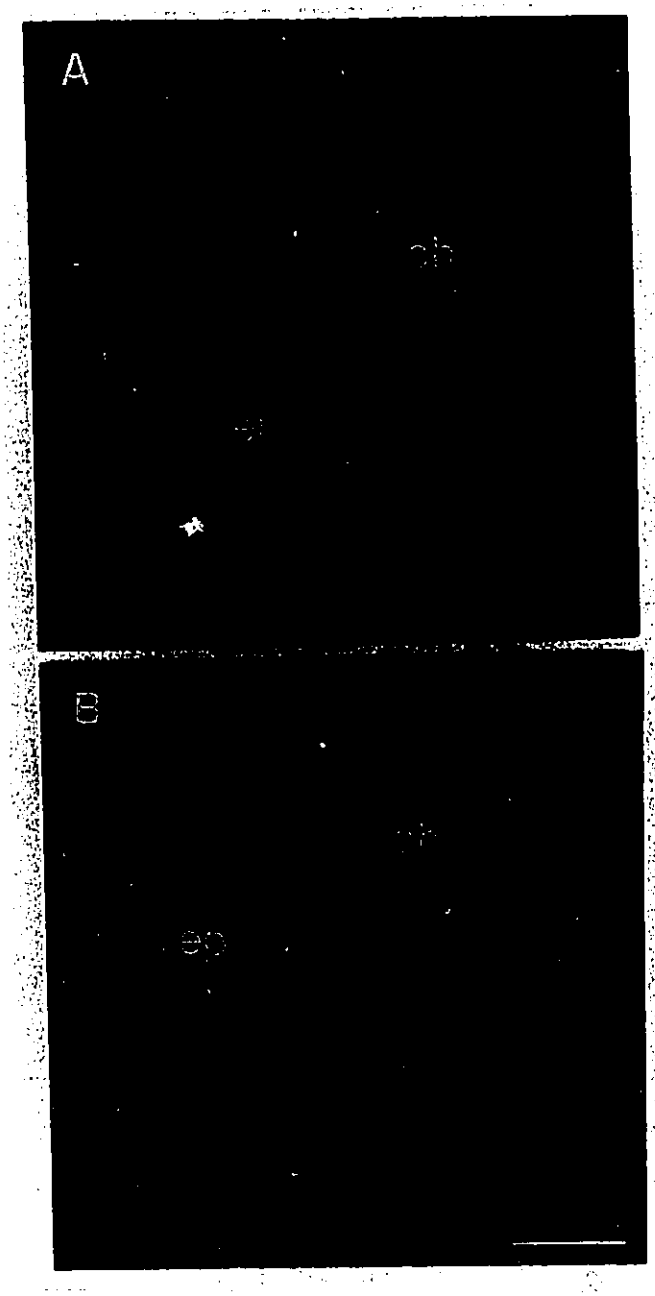
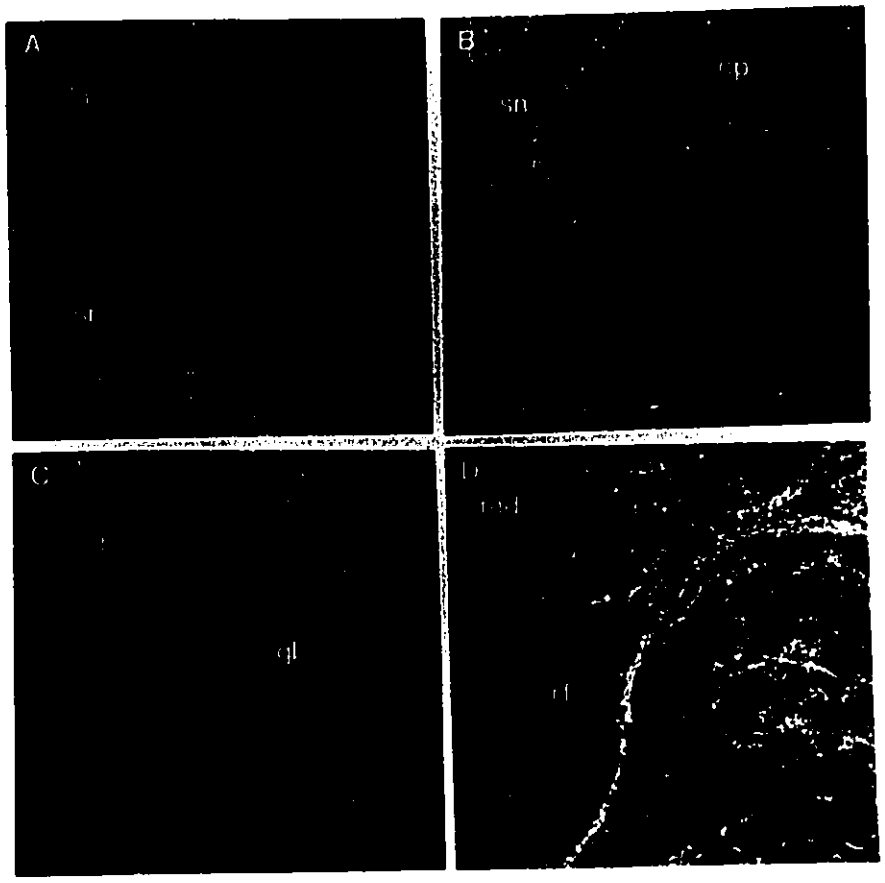

A
B

Figure 2-7 Localization of DMK at synaptic sites in rat brain. Sections of rat hippocampus (A and B), cerebellum (C) and medulla (D) were stained with anti-DMK. hp, hippocampus; so, stratum oriens; sr, stratum radiatum; cp, cerebral peduncle; sn, substantia nigra; cb, cerecellum; gl, granular layer; md, medulla; rf, reticular formation. Calibration bar = 17 μm


A
B
sn
C
gl
D

Also, scattered cells in the cerebral peduncle, likely to be astrocytes, were stained (Fig. 7B). Prominent staining of DMK within the cerebellum localized to the synaptic glomeruli (Fig. 7C). Intense DMK-immunoreactivity was observed in individual cells and dendritic processes within the medulla, particularly the gigantocellular population (Fig. 7D). Immunoblots of brain samples were unique in the number of species identified (see Fig. 2A). We therefore attempted to localize different DMK species from microdissected rat brain. Frontal cortex (and other samples examined) exhibited only the 45-50 kDa. species (Fig. 8A). However, the ependyma and choroid plexus of dissected ventricles (lateral, third and fourth) exhibited larger species; comparison with rat and human skeletal muscle tissue confirmed the similarity of these DMK isoforms (~74 and 82 kDa, Fig. 8B). This suggests that within the brain, isoforms in common with those found in skeletal and cardiac muscle may be restricted to the ependyma and choroid plexus.

The distribution of DMK in normal and DM tissues was compared. In adult cardiac tissues, no obvious differences were observed in its localization at intercalated discs between the affected individual (Fig 9A) and the normal control (Fig. 9C). In order to determine the relative intensity of DMK we compared the normal background staining, due to lipofusion, in the affected individual (Fig.9C) and in the matched control (Fig. 9D). No differences in intensity were observed. Examination of fetal tissues revealed that DMK was present at this early stage in development in both the normal and congenital samples, at the sites of NMJ's in fetal skeletal muscle from a congenital DM infant (Fig. 10A and B) and in control neonatal skeletal muscle (Fig. 10C and D).

Figure 2-8 Immunoblot analysis of microdissected rat brain. (A) Extracts from frontal cortex (lane 1), and from the ependyma and choroid plexus of the two lateral, third and fourth ventricles (lanes 2-5, respectively) were analyzed. (B) For comparison, ependyma (lane 1) and choroid plexus (lane 2) extracts were analyzed together with human pectoralis (lane 3) and rat quadriceps (lane 4). The ependyma and choroid plexus contain species at ~74 and 82 kDa similar in size to those found in skeletal muscle.

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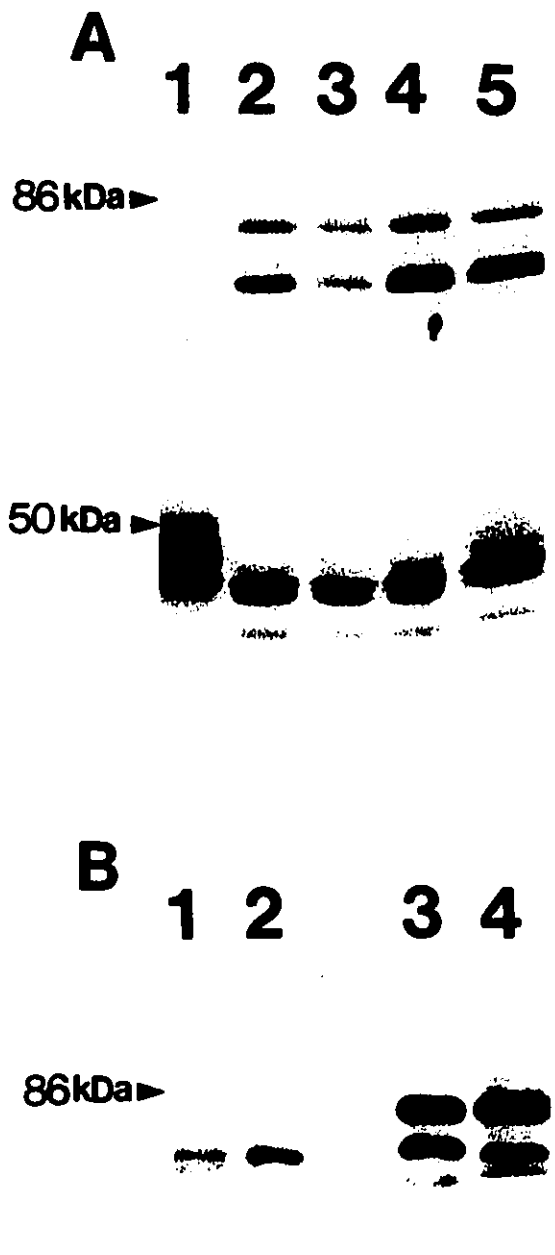

A
1 2 3 4 5
86kDa
50kDa
B
1 2 3 4
86kDa

Figure 2-9 Distribution of DMK in normal and affected cardiac tissue. Cryostat sections of adult cardiac tissue from an affected individual, 50 yrs, (A,B) and a normal individual, 70 yrs, (C, D) were incubated with anti-DMK (A,C). DMK localized at the intercalated discs in both the normal and affected tissues. Comparison of normal background staining (lipofusion) in the same sections (B,C) reveals intensity of DMK staining is relatively equal. Calibration bar = 17μm

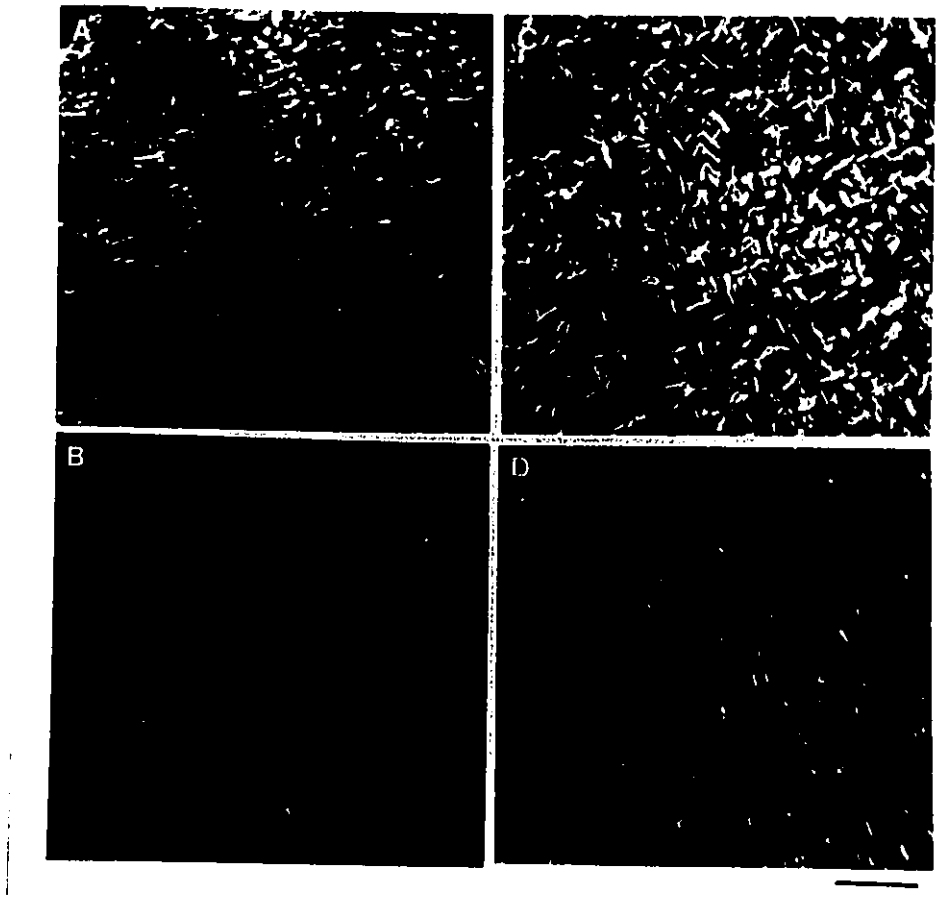
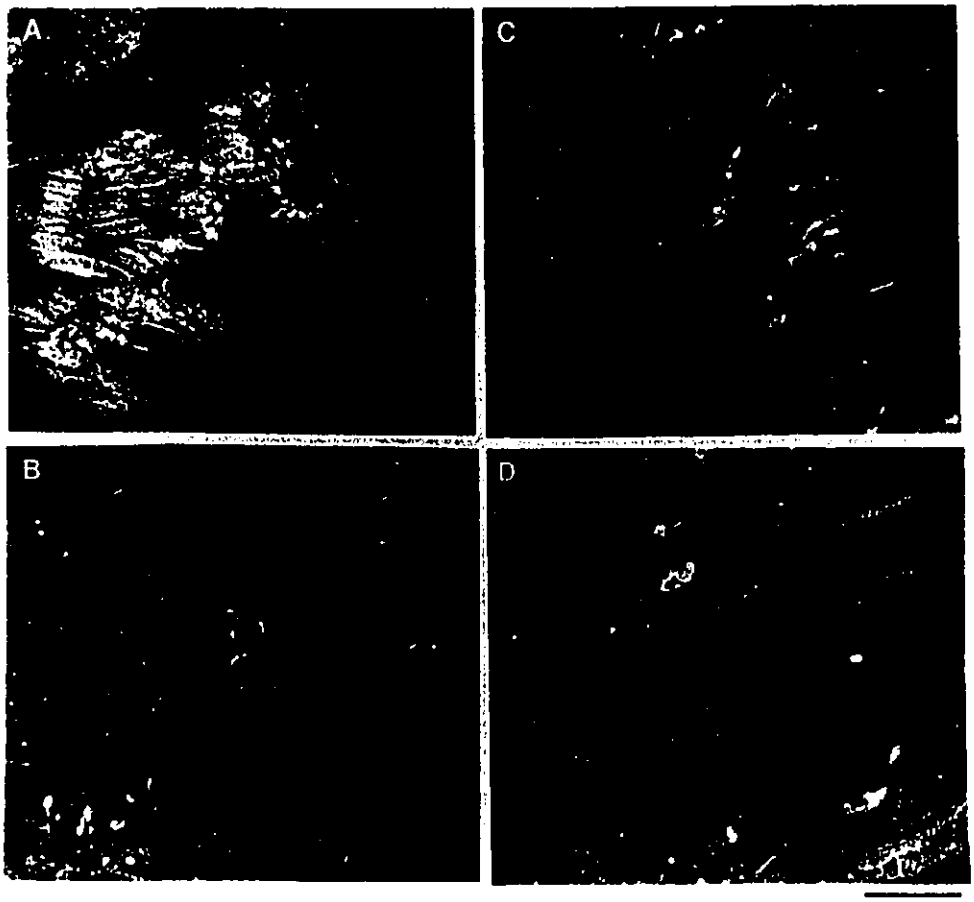

A
C
B
D

Figure 2-10 Immunofluorescence analysis of normal and congenital fetal skeletal muscle. Cryostat sections of DM fetal skeletal muscle (A,B) and neonatal skeletal muscle (C,D) were incubated with anti-DMK. DMK localized at the NMJ in both the normal and affected tissue. Calibration bar = 17μm


A
C
B
D

Discussion

We report the characterization and localization of authentic DMK protein species using a novel polyclonal immunoglobin preparation directed against DMK. This anti-DMK Ig clearly reacted to DMK produced in two foreign expression systems. We have also shown that preadsorption with our immunogen reduces the immunofluorescence of tissue sections to background levels. The antibody detects protein species larger than those previously reported, as described above. The anti-peptide sera employed in these previous studies, not only reacted specifically with bacterially produced antigen, but one also shares a very similar immunofluorescence profile in human and rodent tissues as reported here (van der Ven et al., 1993). In order to assess the localization and expression levels of DMK between normal and myotonic individuals accurately the correct identity of DMK itself must first be resolved. We report evidence, in addition to several observations made during the preparation of this manuscript, that clearly confirm the authenticity of the 74 and 82 kDa species identified by our anti-DMK antibody.

First, DMK expressed in mammalian system (COS) cells is detected by our anti-DMK as two isoforms identical in size to the two species seen in human tissues (our unpublished observations; B. Perryman, University of Colorado, personal communication). If the tissue species detected were not DMK, they would be coincidentally sized the same as DMK produced in COS cells. Further, DMK produced in insect cells is only slightly larger than the tissue species. This difference may be due to differential processing between mammalian and insect cells.

The second line of evidence is derived from recently established trangenic mice lines which overexpress the human DMK gene. Our anti-DMK detects species in wild-type mouse muscle by immunoblot which comigrate with the human species detected. Notably, no species are detected in wild type mouse brain (Narang et al., manuscript in preparation). However, brain from transgenic mice express two novel species which comigrate with human and wild-type mouse muscle species. While it is not conclusive that the human muscle species, wild-type mouse muscle species and human species produced *de novo* in mouse brain are identical, their identity in size makes this the most likely conclusion. The absence of detectable species in wild-type mouse brain is presumably due to much lower levels of DMK expression; in accordance we have found it difficult to observe immunofluorescence in mouse brain. The ease of DMK detection in transgenic mice brain compared to the relative difficulty of detecting the human brain species, illustrated in this report, we attribute to the higher avidity of the anti-DMK preparation currently employed.

Finally, "knock-out" mice which do not express the mouse DMK gene have been generated (B. Wieringa, University of Nijmegen, personal communication). The species identified by our anti-DMK in control mouse muscle in this study are completely absent in the "knock-out" mouse muscle (B. Wieringa, University of Nijmegen, personal communication; P. Strong, Hammersmith Hospital, London, personal communicaton). Unless the ablation of the mouse DMK gene in the "knock-out" down regulates the expression of a cross reactive protein species it would seem apparent that the species detected in wild-type mouse are authentic DMK isoforms. We believe that the above

observations, cummulatively confirm the specificity of our antibody preparation for DMK in tissues as detected by immunoblot.

The larger-than-predicted size for the detected tissue species (and DMK produced in expression systems) may be due to post-translational modification(s). Alternatively, there may be some structural feature of DMK that is resistant to denaturation and retards its mobility on SDS-PAGE. In support of the latter, DMK produced by *in vitro* translation also migrates slower than predicted (Waring et al., manuscript in preparation). The difference between the 74 and 82 kDa tissue forms may be accounted for by the deletion of exons 13 and 14 from a splice-isoform cDNA. The 74 kDa species is predominant in human heart, the organ from which this truncated cDNA was first isolated. A similar size difference was observed between the two DMK isoforms expressed in bacteria. However, there was no considerable size difference observed between DMK and DMKΔ13-15 expressed in insect cells. It is also noteworthy that both forms of DMK expressed in insect cells are phosphorylated (Waring et al., manuscript in preparation), as is DMK expressed in bacteria (Dunne et al., 1994). The degree of phosphorylation between the two proteins expressed in insect cells may, therefore, account for this difference. In addition, we detected species in rat tissues which are slightly different from the human species in both size and isoform pattern. Also, our antibody detects hamster species which are unique in their size and isoform patterns (B. Perryman, University of Colorado, personal communication). This suggests that DMK expression may be conserved in vertebrates but differ between species in mRNA or protein-processing patterns. Further characterization of tissue species will determine if there are in fact

products of alternatively spliced transcripts and identify, if any, tissue-specific differences in transcript processing.

We have colocalized DMK with acetylcholinesterase in the basal lamina of the NMJ in skeletal muscle and have confirmed that it is concentrated at the postynaptic side of the junction. We have also localized DMK to the intercalated discs in cardiac muscle. Its presence at these sites suggests that DMK may have a role in signal transduction and intercellular communication. Also, discrete localization of DMK within different brain regions and to specialized regions of individual neurons suggests a similar action of this kinase in central nervous system function. However, the relationship between its localization and its function in the cardiac conduction abnormalities, myotonia and intellectual impairment observed in DM individuals remains to be resolved. Closer examination of its tissue distribution, localization and identification of its substrates will be required to determine its role at these sites.

We used our antibody, convinced of its specificity for DMK, in order to compare the distribution of DMK in normal and affected tissues. Clinical manifestations of DM such as, the progressive weakening and deterioration of skeletal muscle in adult DM and the lack of muscle development in congenital DM suggest that the alterations in DMK expression levels, isoform patterns or localization would be apparent in a comparison of normal and DM tissues. In previous studies, DMK protein levels have been compared in normal and affected tissues and results have shown reduced levels of the protein in both adult and congenital DM cases (Koga et al., 1994; Fu et al., 1993). However, our results, as well as those reported previously by van der Ven et al., indicate that there are no

differences in the localization or prevalence of DMK in adult and congenital DM tissues compared to that which is observed in control samples. Further, no significant differences between the protein levels of DMK in normal and affected tissues, from both adult and fetal samples, were observed in immunoblot analysis from our group (Sabourin et al., unpublished data). These preliminary experiments suggest that the DMK protein may not be involved in the pathophysiology of DM. Further investigation into the effects of the mutation will be required to elucidate the pathological mechanism in DM.

In summary, we believe that our anti-DMK preparation detects authentic DMK species in tissues that are clinically relevant in myotonic dystrophy. Using this antibody we have localized DMK to specific cellular sites in skeletal, cardiac and CNS tissue which suggest it may have a role in intercellular communication. Further, we have demonstrated that DMK is present in affected tissues and by comparison to normal tissue is not any more or less prevalent, nor altered in its distribution. Additional biochemical studies on DMK are required to determine its function, substrate(s), and more importantly, its involvement in the pathophysiology of this multisystemic disease.

CHAPTER III GENERAL DISCUSSION

The characterization and localization of the DM protein using a novel antibody has been presented. These experiments have provided insight into the function of DMK and its possible role in the pathophysiology of DM. We suggest that DMK may be involved in intercellular communication given the specific localization of DMK at specialized cellular sites in skeletal and cardiac muscle, as well as in brain. Although the function of DMK has not yet been determined, its localization to the postsynaptic side of the NMJ in skeletal muscle, to intercalated discs in cardiac muscle and discrete synaptic sites in the brain appears to be consistent with the DM phenotype. The presence of DMK at these sites suggests that aberrant phosphorylation by DMK may be responsible for clinical characteristics such as myotonia, cardiac conduction abnormalities and mental retardation commonly observed in DM individuals.

Myotonia is characterized by muscle stiffness caused by increased excitability and delayed relaxation of muscles after voluntary contraction. Clinical and electrophysiological data indicate that myotonia results from structural or functional defects in the muscle cell membrane (sarcolemma). Contraction of skeletal muscle occurs only in response to activity in the motor nerves which innervate the muscle fiber. Myogenic contraction is initiated by excitatory neural activity at the specialized junction, between the terminal motor nerve and muscle fiber, referred to as the neuromuscular junction (NMJ). Upon stimulation the neurotransmitter, acetylcholine, is released from synaptic vesicles at the nerve (presynaptic) terminal and subsequently taken up by postsynaptic receptor sites along the sarcolemma. The uptake of acetylcholine depolarizes

the sarcolemma causing release of calcium which triggers muscle contraction. A disruption somewhere along this excitation-contraction coupling pathway and/or the failure of the muscle membrane to rapidly return to its resting potential could result in myotonia. DMK is found at the postsynaptic side of the neuromuscular junction (NMJ). The relationship between the localization of DMK and its function at this site is difficult to predict due to the concentration of numerous proteins at the NMJ. Nonetheless, events involved in muscle contraction such as, the degradation of acetylcholine (ACh) and activation and inactivation of ion channels involved in membrane depolarization could very well be regulated via phosphorylation by DMK.

In other inherited muscular disorders, myotonia has been attributed to alterations in the sodium or chloride channels of skeletal muscle. The adr (arrested development of righting response) mouse model for human myotonias, has clearly demonstrated that primary defects in the Clc-1 chloride channel alters the stability of muscle membrane resting potential (Gronemeier et al., 1994, Jockusch et al., 1994). Similarly, in myotonia congenita (Tomsen's disease) a defect in the sodium channel that results in their re-opening is believed to be the major cause of muscle membrane hyperexcitability (Iaizzo et al., 1991, Lerche et al., 1993). Also, membrane hyperexcitability in hyperkalemic periodic paralysis (hyperPP) and potassium-aggravated myotonia (PAM), both of which are inherited as dominant traits, is thought to result from a persistent inward sodium current due to an alteration in the sodium channel (Hoffman et al., 1995). Interestingly, in hypokalemic periodic paralysis (hypoPP) the failure of muscle membrane excitation has been linked to mutations in a voltage-sensitve calcium channel in skeletal muscle, the $\alpha 1$

subunit of the dihydropyridine (DHP) receptor (Hoffman et al., 1995). This receptor works in conjunction with the ryanodine receptor, a calcium release channel in the sarcoplasmic reticulum, mediating changes in membrane potential into intracellular calcium release. The ion channels are defective in the diseases described above, yet there have been no such defects identified in DM. However, their activation and/or inactivation could potentially be regulated by DMK.

Similar to myotonia, electrocardiographic abnormalities are typically caused by structural or functional defects in the cardiac conduction system. Contraction of cardiac cells is similar to the myogenic contraction mechanism of skeletal muscle, but is involuntary, contracting rhythmically and automatically. Specialized gap junctions connect cardiac myocytes allowing the passage of ions and intercellular second messengers from cell to cell that mediate normal cardiac conduction. Sodium and calcium channels, have been localized to the intercalated discs (Kieval et al., 1992) and as in skeletal muscle, defects in these voltage-gated sodium channels have been implicated in cardiac arrhythmias, such as Long QT syndrome (Wang et al., 1995). Intercalated discs are responsible for cardiac cell to cell adhesion, the transmission of action potentials and force of contraction between cardiac cells. The presence of DMK at these junctions suggests that it may indirectly participate, via phosphorylation, in normal cardiac conduction and rhythm. Again, aberrant phosphorylation may be responsible for the cardiac conduction defects characteristic of DM. Connexins, a family of membrane spanning proteins, which assemble into gap junction channels, have also been immunolocalized to the intercalated discs (el Aoumari et al., 1990). Most cardiac gap

junctions contain connexins 40, 43 and 45, whose variable distribution may be responsible for electrophysiologic differences in intercellular activities and conduction (Davis et al., 1994). In addition, the phosphorylation of these connexins has been suggested to be involved in the assembly and activity of the cardiac cell junctions (Musil et al., 1990). The localization of DMK to the intercalated discs suggests it could be responsible for the phosphorylation of connexins or the regulation of the constituent ion channels. The presence of DMK at both the NMJ and the intercalated discs indicates that it is more likely to be involved in the conduction systems that maintain normal muscle contraction than in the actual contractile apparatus.

Mental retardation is not common in the adult onset DM, although minor intellectual impairment has been reported (Sinforinani et al., 1991; Franzese et al., 1991). In contrast, prominent mental retardation and developmental delay is observed in the majority of severe congenital DM cases (Harper, 1989). Synaptic transmission and plasticity in the CNS involves numerous biochemical events that are mediated by cellular factors and the generation of second messengers, as well as by protein phosphorylation. The possibility that DMK is involved in the regulation of ion transport is supported by its presence in the choroid plexus and ependyma, both of which are specialized epithelial cells involved in the production and secretion of cerebral spinal fluid (CSF). Interestingly, connexin43 is also present at the gap junctions in the ependyma. It may be that DMK has an association with this connexin that could be similar to its cardiac counterpart. Also, chloride channels which are expressed in both epithelial and non-epithelial cells, have several functions including stabilization of membrane potential, as in skeletal muscle, as

well as signal transduction and transepithelial transport (Thiemann et al., 1992). The presence of DMK at synaptic sites in various regions of the brain would seem to indicate that it has a role in signal transduction and synaptic transmission. Interestingly, voltage-gated sodium and calcium channels in the apical dendrites of hippocampal pyramidal neurons have recently been shown to be involved in the coordination of synaptic plasticity and integration (Spurston et al., 1995; Magee et al., 1995). Ultimately, the identification of the DMK substrate(s) and additional biochemical assays will be necessary in order to understand the normal function of DMK.

Early experimental and clinical data suggested that DM arises from structural and functional alterations in the muscle cell membrane (Roses and Appel, 1974; Harper, 1989). In fact, decreased protein phosphorylation of muscle membrane has been reported in DM muscle samples (Roses and Appel, 1974). Also, previous reports have shown increases of calcium uptake in sarcoplasmic vesicles of muscle tissue and erythrocytes suggesting that DM may result from a cellular abnormality of Ca^{2+} transport (Plishker et al., 1978 ; Hockings et al., 1993). The localization of DMK at the sites described above, is indicative of its role in cellular crosstalk, which may involve both phosphorylation and Ca^{2+}transport . Although, the function of DMK is yet to be established, its localization has provided some insight into the pathophysiology of the disease of which very little is known.

Particular clinical manifestations observed in congenital DM, such as the delay of muscle maturation, imply that DMK may have a role in development. Specifically, the localization of DMK at the NMJ suggests that it could be involved in synaptogenesis. The formation of this junction requires coordination between the developmental cues of the

muscle fiber and nerve terminals. Accordingly, studies on synaptogenesis of the NMJ have demonstrated the interdependence between the development of the muscle and the nerve through denervation experiments. For example, denervation of mature skeletal muscle has been shown to reactivate the repressed transcription of myogenin (Buonanno et al., 1993). In this respect, it is possible to hypothesize that DMK may regulate the cellular signals involved in the development of synaptic contacts or cell junctions. Furthermore, the extent to which the developmental events are disrupted may account for the varying degrees of severity observed in DM, as well as the differences between adult and congenital DM.

The hypotonia observed in congenital DM and the muscular dystrophy typical of adult-onset DM muscular dystrophy, suggests that DMK may be involved in myogenesis. Kinase domain homology between DMK and PKC permits the suggestion that DMK could potentially share similar inhibitory functions and/or have a role in cell signals mediated by muscle-specific genes. PKC is involved in the control of myogenesis, as well as depolarization-triggered cascades (Huang et al.,1992). PKC has also been shown to be a component of the pathway coupling membrane excitation and acetylcholine receptor (AChR) gene control, both of which are ultimately involved in the development of contractile properties of the postsynaptic muscle cell (Huang et al., 1992). Further, it has been demonstrated that activators of PKC can induce membrane instability, causing myotonia, by interfering with the normal chloride conductance (Brinkmeier et al., 1987). Interestingly, a previous report identified binding motifs in the muscular chloride channel gene (Clc-1) which suggest that its activity may be regulated by myogenic factors (Klocke

et al., 1994). The expression of the myogenic factors is also indirectly regulated by levels of intracellular cyclic AMP, as well as through second messengers and growth factors via phosphorylation (Li et al., 1992). DMK may be involved in the regulation of the myogenic factors and/or the maintenance of muscle cell differentiation via phosphorylation of its substrate(s).

Differences in the expression level of DMK may result from the $(CTG)_n$ amplification and could potentially cause aberrant phosphorylation of its substrate(s). In previous studies, reduced DMK protein levels have been reported in affected tissues by comparison to controls (Fu et al.,1993; Koga et al.,1994; van der Ven et al., 1993). As discussed previously in this Chapter, these results should be viewed with caution due to differences in tissue sampling, quality of the specimens used in these assays (Roses, 1994) and the specificity of the antibodies that were used. Moreover, our study has shown that the distribution and subcellular localization of DMK does not appear to be altered by the mutation. Furthermore, we have determined by immunoblot analysis that there were no apparent differences in the protein levels or isoform patterns between affected tissues as compared to normal controls (Sabourin et al., unpublished data). These experiments imply that either the protein may not be involved in the pathophysiology of DM or that minimal changes in DMK expression have considerable effects.

Alternatively, the $(CTG)_n$ repeat length mutation could interfere with the expression, stability and/or splicing of the DM mRNA. Both increased (Sabourin et al., 1993) and decreased (Fu et al., 1993; Carango et al., 1993; Hofman-Radvanyi et al., 1993) DMK mRNA levels in affected tissues and cells have been reported. As described

previously, problems in assay conditions and tissue sampling make it difficult to interpret these data (Roses, 1994) and determine which mechanism, if either, may be responsible for DM. Reconciliation of these opposing conclusions could be established upon determining whether the stability or the transcription rate of the mutant DMK allele is altered. In this regard, increased CAT activity has been observed in constructs of the DM 3' UTR containing the larger (CTG)n repeats (Caroline Ang and Monica Narang, unpublished data). It is also possible that the secondary structure of the mutant DMK mRNA may confer increased stability due to the excess in C:G base pairing which is inherently more stable thermodynamically. Also, aberrant binding of tissue specific RNA binding factors have been shown to prevent normal degradation and/or interfere with polyadenylation and translation (Jackson, 1993; Santoro et al., 1991). The DM mutation may have similar consequences on the stability of the DMK message. However, additional studies are required in order to be certain of the involvement of DMK mRNA in the pathology of DM.

The study of the DMK 3' UTR may be important to the understanding the pathology of DM. Recent studies on the function of 3' UTR mRNA's have demonstrated that they have a variety of roles, such as the cytoplasmic regulation of mRNA, mRNA localization, stability and translation, as well as the regulation of signals controlling growth and differentiation. As an example, alteration of a stem loop structure in the 3' terminus of a replication-dependent histone mRNA was found to disrupt the efficiency of its degradation and translation (Williams et al., 1994). Also, cellular factors involved in growth and differentiation may be regulated by their interaction with specific sequences

encoded by UTR's, as in the case of AU-rich elements in the 3'UTR of several oncogene transcripts and the iron-responsive element sequence in the 3'UTR of the transferrin receptor transcript (Gillis et al., 1991; Koeller et al., 1991). More importantly, 3' UTR's have been found to have a regulatory role in muscle development, such as the sarcomeric actin genes (Gunning et al., 1984). In addition, Rastinejad and Blau (1993), have demonstrated that the expression of the 3'UTR from differentiation-specific RNA's actually inhibits cell division and promotes differentiation. These studies suggest that 3' UTR's are involved in feedback loops which maintain the cell cycle and regulate factors or other RNA's that are involved in differentiation and growth. The amplification of the DM $(CTG)_n$ repeat could alter the normal function of its 3' UTR, interfering with any one of these associated functions. If the function of the DM gene 3' UTR is altered, the effects may vary depending on the size of the repeat amplification, accounting for the genetic anticipation observed in this disease and the clinical differences between adult and congenital DM. Further investigation of the DM mutation and the function of the 3' UTR will determine if this is, in fact, the mechanism responsible for the pathology of DM.

Conclusions

The objective of this thesis was to determine the cellular localization of DMK and provide insight into the normal biology of the protein. DM is a multisystemic disease that has variable age of onset and a variety of clinical characteristics ranging from mild to severe. A $(CTG)_n$ repeat amplification in the 3' UTR of the gene is believed to be responsible for this dominant phenotype, however the underlying molecular mechanism(s) are not yet understood. We have developed an antibody directed against DMK in order to characterize and localize the DM protein species. I have presented an immunofluorescence analysis, using this antibody, of the localization of DMK in rodent and human skeletal muscle, cardiac tissue and CNS. The localization of DMK at specific cellular sites suggests a possible function in signal transduction pathways. Based on the results from this study, models for the normal role of DMK can be tested and new hypotheses for the mechanism of its pathophysiology can be developed. Additional biochemical analyses of DMK and the identification of its substrate(s) will provide further insight into the biology of DMK and contribute to the understanding of the pathogenesis of myotonic muscular dystrophy.

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