Vol. 199, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1994 Pages 89-92

THE MYOTONIN-PROTEIN KINASE PHOSPHORYLATES TYROSINE RESIDUES IN NORMAL HUMAN SKELETAL MUSCLE

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Received December 14, 1993

As a first approach to study the cellular events involved in myotonic dystrophy, we have produced a polyclonal antibody against a peptide sequence of the predicted gene product. This antibody specifically recognizes a 54 kDa protein in human skeletal muscle. This protein phosphorylates a co-polymer Glu/Tyr but not Myelin Basic Protein. This indicates that the myotonin-protein kinase has a tyrosine kinase activity in human skeletal muscle. This is the first demonstration of the kinase activity of the myotonin-protein kinase.

Myotonic dystrophy (DM) is an autosomal dominant inherited human genetic disease characterized by muscle weakness, myotonia, cardiac conduction defects, cataracts, and male baldness [1]. The molecular basis of DM is known to include a mutational expansion of a repetitive tri-nucleotide sequence (CTG)n located in the 3' untranslated-region of mRNA from a gene encoding a putative serine-threonine protein kinase product [2,3,4]. Recent studies have shown that the transcripts are expressed in various tissues, including the heart, the skeletal muscle, the liver and the brain, both in human and mouse [5,6]. Furthermore, several mRNA spliced forms have been identified in different human and mouse tissues [5,7]. To date, little is known about the myotonin-protein kinase (M-PK). In particular, the kinase activity of M-PK has not been so far demonstrated. In this study we have identified a 54 kDa protein product of the DM gene using specific antibodies and shown that this protein has a tyrosine kinase activity in human skeletal muscle.

METHODS

Production of M-PK antibodies. A computer analysis of the amino acid sequence of the human M-PK cDNA was used for prediction of the secondary structures, hydrophilicity, surface probability and antigenicity. A peptide (PSPRATDPPSHASRQ-Y) corresponding to the deduced amino acid residues 493-507 of the carboxyterminal region of human M-PK [2] was synthesized (Neo System, Strasbourg, France). This peptide had no homology with any known protein amino

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0006-291X/94 $5.00

acid sequence (Swiss prot 20, Genework, Intelligenetic Inc.). The peptide was coupled to keyhole limpet hemocyanin (KLH; Sigma Chemical Co., St Louis, Mo) through a tyrosine residue that was added at the amino terminal end of the peptide. Male New Zealand rabbits were immunized s.c. with 400 µg of KLH-peptide in complete Freund's adjuvant on day 0, 15 and 45. Booster injections were given every 4 weeks in incomplete adjuvant. Sera were collected 15-days after the third injection and after each boost. The titers of sera were tested by enzyme-linked immunoabsorbent assay (ELISA). The serum that gives the highest titer was designated p500. Immunoreactive antisera were purified by affinity chromatography on 1-ml columns of peptide linked to CNBr-activated Sepharose 4B according to manufacturer's instruction (Pharmacia, Picataway, NJ).

Production of M-PK fusion protein. To express the M-PK protein, the *Bsg*1/*Eco* R1 fragment of a human genomic clone (HMDM 722, a generous gift of Dr R.H. Korneluk, Ottawa) representing the coding sequence starting from threonine 91 and lacking conserved domains I, II, III , was filled-in for blunt ligation in the *Stu* 1 site of the expression vector pMal-c (New England Biolabs). The expression of the fusion protein was done according to the manufacturer's instructions.

Immunoblotting. For the fusion protein, 0.5 ml of induced and non-induced cultures were centrifuged for 5 sec. in a microfuge. The bacteria pellet was resuspended in 0.5 ml of Laemmli buffer [8], boiled for 5 minutes and submitted to SDS-PAGE in reducing conditions. Then, the proteins were transferred to a nitrocellulose membrane (BA S 85, Schleicher and Schull) and allowed to react with affinity-purified antibody (1/5.000) for 1 hour at room temperature. The reaction was visualized by using an enhanced chemiluminescence kit (ECL detection kit, Amersham). For human muscle, frozen skeletal muscle biopsies were homogenized in 10 volumes of gel loading buffer (10 mM Tris, pH 8.0 containing 1 mM EDTA, 40 mM dithiothreitol, 3.3% sodium dodecyl sulfate (SDS), 10% glycerol, 17 µg / ml phenyl methyl sulfonyl fluoride (PMSF), 5 µg / ml leupeptin, 5 µg / ml antipain and 5 µg / ml pepstatin) by using a Kontes homogenizer (pestle SZ 20). The protein concentration was determined according to the procedure described by Minamide and Bamburg [9]. 50 µg of protein was subjected to 12.5% polyacrylamide gel electrophoresis in the presence of 1% SDS according to the method of Laemmli [8]. After transferred onto a nitrocellulose membrane, the blots were incubated with the affinity purified antibody and revealed as above. Controls were performed using affinity-purified antibodies preadsorbed with unconjugated peptide (0.1mg/ml) overnight at 4°C.

Measurement of kinase activity. Human adult skeletal muscles were homogenized in 10 volume of gel loading buffer as above. The homogenate was centrifugated at 12.000 RPM for 2 min. and the supernatant was collected. The proteins were determined as described above. The supernatants were incubated with 50 µl of affinity-purified antibody overnight at 4°C. The antigen / antibody complex was immunoprecipitated using 50 µl protein A sepharose according to the instructions of the manufacturer (Sigma, St-Louis). After washing twice in solution A (1% Triton X-100, 50 mM Hepes pH 7.4), twice in solution B (0.1% Triton X-100, 0.1% SDS, 50 mM Hepes pH 7.4), the M-PK was eluted with 0.1M glycine pH 3.0 and centrifugated. After neutralization with 1M Tris pH 8.0 , the supernatant was used for kinase assay (10). Aliquots were mixed with 50 mM Hepes buffer (pH 7.4) containing 40 mM $MgCl_2$, and 200 µg of Myelin Basic Protein (MBP) or 230 µg of a co-polymer Glu / tyr (4:1) (PGT) (Sigma, St Louis, Mo). The reaction was initiated by adding [γ-^{32}P]-ATP (final concentration 25 µM, 32 µCi/nmol). After 30 minutes, the reaction was stopped by spotting 50 µl aliquots on chromatography paper squares (Whatman 3 MM, 2 x 2 cm) which were then placed in a 10% trichloracetic acid solution containing 10 mM sodium pyrophosphate for 60 min at 4 °C (5 ml / paper square). The squares were washed twice for 30 min at room temperature, rinsed with 100% ethanol and air dried. Radioactivity was determined by liquid scintillation counting. Incubation in the absence of substrate was used as control. Control values were subtracted from the total counts.

RESULTS AND DISCUSSION

The affinity purified antibody specifically recognized the human M-PK proteins expressed as a fusion protein in Echerischia Coli. A 97 KDa band protein which is the expected size of the fusion protein (43K for the maltose binding protein and 54 KDa for M-PK) (Fig. 1B), was detected on

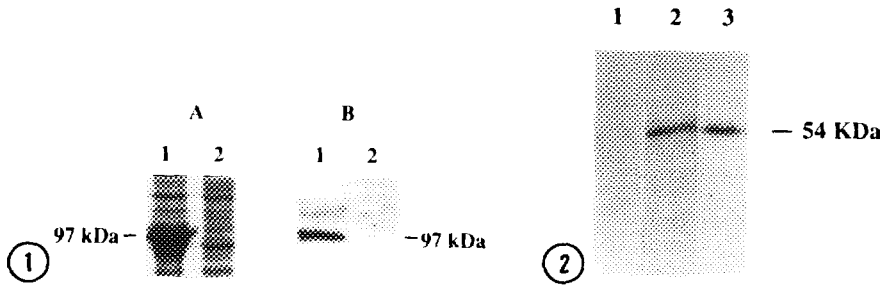

Figure 1. Immunoblotting of bacterially expressed M-PK.
The fusion protein was resolved by SDS-PAGE, transferred to nitrocellulose, and probed as described in materials and methods. Panel A: Coomassie blue staining. 20 µl of sample was applied per lane: 1, induced culture; 2, non-induced culture. Panel B: immunoblots; 2 µl of samples was applied per lane: 1, induced culture; 2, non induced culture.

Figure 2. Detection of M-PK by Western blot in human skeletal muscle.
Total muscle extracts were resolved by SDS-PAGE gels as described in materials and methods. Panel 1: immunoblots of total tissue extracts incubated with preabsorbed affinity-purified antibodies. Panel 2: Incubation with affinity-purified antibodies (1/500). Panel 3: The immunoprecipitated M-PK was eluted, resolved by SDS-PAGE, transferred to nitrocellulose and probed with affinity-purified antibodies.

immunoblots. No immunoreactivity was observed in uninduced cultures (Fig. 1B). No protein band was observed with preimmune or preabsorbed sera (not shown).

A 54 KDa protein band was detected on immunoblots of human skeletal muscle probed with affinity purified antibodies. (Fig. 2). The immunoreactivity was specific, since no protein band was detectable with preabsorbed sera. In further experiments, we examined the ability of the affinity-purified antisera to immunoprecipitate the endogenous M-PK. This antibody also immunoprecipitated a protein band of a similar molecular weight as observed on immunoblots (Fig.2). The size of the M-PK protein recognized by this antibody is in good agreement with the size of M-PK protein reported by others [7,11]. Although several putative spliced forms of mRNAs of M-PK have been reported in human muscles [5,7], only one protein band was recognized by this antibody. Similar results have been recently reported [7,11]. This suggests either that these spliced forms do not occur *in vivo* or that the spliced proteins have a similar molecular weight and cannot be discriminated. This latter possibility is supported by recent data showing that the putative alternative splicing deleted nucleotide sequences that encode less than 60 amino acid residues [5,7].

In order to determine the kinase activity of M-PK, we examined the capacity of the immunoprecipitated protein to phosphorylate specific substrates, e.i. MBP and PGT. As shown in Fig. 3, the M-PK phosphorylated PGT but not MBP. Since MBP is a usefull substrate for two serine / threonine kinases such as protein kinase C and protein kinase A (12), this indicates that M-PK has a tyrosine protein kinase activity in human muscle. This is in direct contrast with the conserved DIKPD-sequence motif which is a strong indicator of the enzyme's serine / threonine specificity. The tyrosine specificity may correspond either to an intrinsic property of M-PK (for review see 13) or to a distinct activity of spliced isoforms. This antibody was raised against a peptide sequence located in a putative splicing site of human M-PK, it is therefore possible that only selected isoforms have a tyrosine kinase activity. However, this possibility is unlikely since

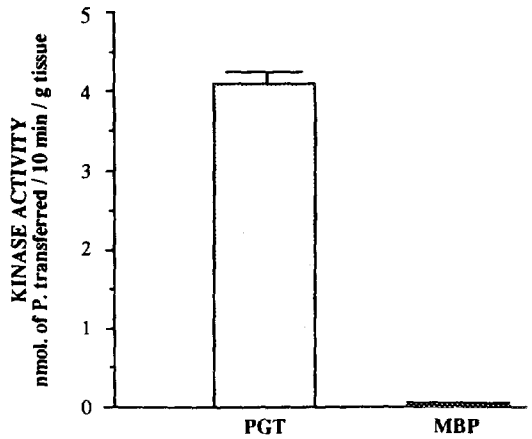

Figure 3. Substrates phosphorylation by human M-PK.
M-PK was immunoprecipitated and eluted as described in materials and methods. An aliquot of the eluate was incubated for 30 minutes in the presence of PGT or MBP and [γ-^{32}P]-ATP. Results are expressed as nmol of Phosphorus transferred / 10 minutes / g of tissue and represent the mean ± S.D. of two independent determinations. Each determination was done in triplicate.

this antibody also immunoprecipitated a 54 kDa protein with a similar tyrosine kinase activity in mouse muscle (not shown) where no splicing site in this region has been identified. Alternatively, we can not rule out the possibility of a tyrosine kinase that has been co-immunoprecipitated in the muscle. However, this does not explain why a serine / threonine kinase activity was not detected in human muscle.

Overall, our results strongly suggest that M-PK is a tyrosine protein kinase in human skeletal muscle opens a broad range of physiological questions about its role in the pathophysiology of DM.

ACKNOWLEDGMENTS

We are grateful to Dr.R.H. Korneluck for providing the M-PK cDNA. This work was supported by the Association Française des Myopathies. P.M. is supported by fellowships from FCAR.

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