Phosphorylation Reactions of Recombinant Human Myotonic Dystrophy Protein Kinase and Their Inhibition†

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ABSTRACT: The predicted protein kinase activity of the cloned gene product of the human myotonic dystrophy locus has been experimentally verified. Affinity-purified recombinant DM protein kinase became phosphorylated itself and transphosphorylated histone H1. These activities were not present in the bacterial host cells and were exhibited by DMPK and DMPKH, recombinant proteins which contain the protein kinase domain but exhibit distinct sizes, 43 and 66 kDa, respectively. DMPKH was further purified by velocity sedimentation on sucrose gradients; both activities migrated with the recombinant protein at 41 S, consistent with discrete multimeric particles. Phosphoamino acid analysis showed that threonine (predominantly) and serine were phosphorylated in both DMPKH and histone H1. Although PKA and PKC are the known types of protein kinase with closest sequence homology to the DM protein kinase domain, purified DMPKH was inhibited by 4 mM but not 0.04–0.4 mM H7 and H8, which inhibit PKA and PKC with K_i's of 0.4–15 μM. Specific inhibitors of other classes of multifunctional serine/threonine protein kinases such as casein kinases I (CKI-7) and II (heparin) and calcium/calmodulin-dependent protein kinase II (KN-62) did not inhibit DMPKH. DMPKH did not phosphorylate membrane-associated phosphoproteins such as acetylcholine receptor or spectrin which are known to be substrates for PKA, PKC, and CKI and -II, respectively. These experimental results suggest that the active center of the recombinant human myotonic dystrophy protein kinase may have properties distinct from the well-studied classes of serine/threonine protein kinases, in contrast to predictions based upon primary structure alone.

Myotonic dystrophy (DM)[1] is a multisystem disorder which can produce skeletal muscle weakness and myotonia, cardiac arrhythmias, characteristic cataracts of the eye lens, testicular atrophy, and insulin-resistant diabetes (Harper, 1989). The occurrence of this highly pleiotropic and variable complex phenotype is closely associated with an unstable expansion of a CTG tandem repeat in the 3′ untranslated region of a predicted DM protein kinase (Fu et al., 1992; Brook et al., 1992). The putative gene product of the DM locus is predicted to consist of a serine/threonine protein kinase catalytic domain near the amino terminal, a central α-helical coiled-coil domain, and a carboxyl-terminal transmembrane domain. To date, none of these functional and structural predictions have been verified experimentally.

The genomic sequence of the DM protein kinase gene and its intron–exon structure have recently been published (Mahadevan et al., 1993; Fu et al., 1993). The two groups differ on the location of the translation start site and the predicted amino acid sequence encoded by the first exon, but there is consensus on the sequences encoding the putative catalytic, coiled-coil, and transmembrane tail domains. The predicted patterns of alternative splicing and the isoforms so produced are also different in these reports; however, both studies predict that the RNA sequences which encode the putative transmembrane tail can be alternatively spliced, suggesting that under certain conditions or in certain locations a functional DM protein kinase may be expressed without a membrane-anchoring domain. Experiments detecting the naturally occurring DM protein kinase in skeletal muscle by reaction with specific antipeptide antibodies or the corresponding mRNA indicate that the endogenous levels are very low (Fu et al., 1993; Carango et al., 1993). The availability of biologically active recombinant DM protein kinase, therefore, appears necessary for many biochemical studies.

We report the expression in *Escherichia coli* and initial characterization of two enzymatically active forms of DM protein kinase. The protein products, DMPKH and DMPK, of two recombinant constructs which contain either the putative protein kinase and the α-helical coiled-coil domains or the kinase domain alone, respectively, both transphosphorylated the general protein substrate histone H1 and were phosphorylated themselves. In both the histone and kinase, threonine and, to an apparently lesser extent, serine but not tyrosine were phosphorylated. These studies and the complex domain structure predicted by the cDNA sequence suggest that DM kinase may represent a structurally and functionally distinct type of serine/threonine protein kinase.

EXPERIMENTAL PROCEDURES

Synthesis of DM Protein Kinase Constructs. Four oligonucleotide primers were synthesized complementary to specific DM protein kinase cDNA sequences (Fu et al., 1992). The sequences of the oligonucleotides (5′ to 3′) are: primer 1, CAGGTGTGCACATATGATCGTGGTGAGGCTT; primer 2, AACGTTGGATCCGTGTGGCTCAAGCAG; primer 3, AACGTTGGATCCGACAGCTGTGGCTCC; primer 4, AACGTTCCAACATATGTCAGTCTTCCAACGG.

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[1] Abbreviations: DM, myotonic dystrophy; DMPK, recombinant myotonic dystrophy protein kinase catalytic domain; DMPKH, DMPK and α-helical coiled-coil domain; DMPKHT, DMPKH and hydrophobic carboxyl-tail domain; PKA, cyclic AMP-dependent protein kinase; PKG, cyclic GMP-dependent protein kinase; PKC, protein kinase C; CKI/II, casein kinases I and II; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; PMSF, phenylmethanesulfonyl fluoride.

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Primer 1 was used with primer 2 or 3 or 4 to generate three constructs by PCR amplification: DMPK, DMPKH, and DMPKHT (Figure 1). The PCR products were subcloned into the vector pET15b (Novagen). The fidelity of the PCR products was verified by sequencing the corresponding subcloned plasmid inserts by the dideoxy chain termination method (Sanger et al., 1977) using Sequenase 2.0 (U.S. Biochemical). The calculated molecular weights including 2672 of vector-encoded flanking sequences are the following: DMPK, 44 866; DMPKH, 56 043; and DMPKHT, 67 550.

Expression of Recombinant PCR Products. Recombinant proteins were expressed in *E. coli* strain BL21([DE3]pLysS) (Novagen). Cells were harvested 3 h after induction by IPTG and lysed in a French pressure cell at 8000 psi in 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM PMSF, 8 mM diisopropyl fluorophosphate, 50 mM NaCl, and 1 μg/mL each benzoyl-L-arginine ethyl ester (Sigma), N^{α}-*p*-tosyl-L-arginine methyl ester (Sigma), soybean trypsin inhibitor, leupeptin, chymostatin, and pepstatin (Boehringer Mannheim). The lysates were clarified by centrifugation at 39000*g* for 20 min and then filtered through 0.45 μm nylon membranes. Samples from the procedure were analyzed by SDS–PAGE.

Purification of DMPK and DMPKH. Recombinant proteins were purified by the following affinity-based method. All steps were performed at 4 °C. Lysates were equilibrated in 20 mM Tris-HCl, pH 8.0, 1 mM PMSF, 8 mM diisopropyl fluorophosphate, 4 mM imidazole, and 500 mM NaCl. The samples were then applied to a prepared His-Bind column (Novagen) and washed, and the recombinant proteins were eluted according to the manufacturer's protocol. The flow rate was 30 mL/h. Fractions were analyzed by SDS–PAGE, and those fractions containing recombinant protein were pooled and dialyzed against 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM PMSF, and 50 mM NaCl. The dialysate was applied in some preparations to a Sephadex G-150 column and developed with dialyzing buffer without effect upon activity. The purified DMPK or DMPKH fractions were analyzed by SDS–PAGE.

Gel Electrophoresis, Radioautography, and Densitometry. 7.5% SDS–PAGE was performed as described previously by this laboratory (Epstein et al., 1988). Samples were dissolved or diluted into gel loading buffer (60 mM Tris-HCl, pH 6.8, 1 mM bromophenol blue, 10% glycerol, 1% SDS, and 1% β-mercaptoethanol), heated at 90 °C for 5 min, cooled to room temperature, and then placed directly on SDS–PAGE. For radioautography, gels were dried under vacuum and exposed to Kodak XAR-5 film with an intensifier screen at −80 °C for various times as required. Densitometry of dried SDS–PAGE gels and of radioautograms was performed in the reflectance and absorbance modes, respectively, on the Model 620 video densitometer (BioRad, Richmond, CA) according to the manufacturer's instructions as previously modified by this laboratory (Deitiker & Epstein, 1993).

Sedimentation Velocity. Linear 5–20% (v/v) gradients in sucrose with final volumes of 10 mL were constructed. The sucrose solutions also contained 100 μM ATP, 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM dithiothrietol, and 1 μg/mL each of pepstatin, chymostatin, leupeptin, soybean trypsin inhibitor, benzoyl-L-arginine ethyl ester, and tosyl-L-arginine methyl ester; 800 μg of DMPKH in 1.0 mL of 20 mM Tris-HCl, pH 8.0, and 1 mM PMSF was layered on top of the gradient. The sample–gradient combinations were spun in an SW 41.1 swinging-bucket rotor (Beckman) at 28 000 rpm for 5 h. One-milliliter fractions were collected by puncture from the bottom of each tube; 100 μL of each fraction was

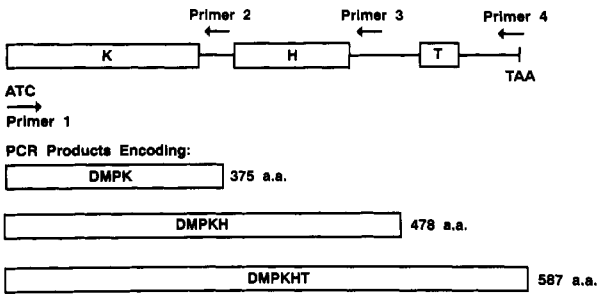

FIGURE 1: Recombinant myotonic dystrophy protein kinase constructs. Primers 1–4 were used pairwise to amplify a DM protein kinase cDNA by PCR.

incubated under standard kinase assay conditions and analyzed by SDS–PAGE and radioautography.

Assay of Phosphorylation Activity and Inhibition. Dialyzed samples were assayed for protein kinase activity in kinase assay solution: 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 3 μg of histone H1 (Boehringer-Mannheim), 100 μM ATP, and 10 μCi of [γ-^{32}P]ATP. Assays were initiated by the addition of ATP after preincubation of the kinase samples. Unless noted otherwise, 15 μg of lysate or 3.75 μg of purified protein was routinely incubated in 50 μL of kinase assay buffer for 1 h at 30 °C. Using a Betascope 603 β-scanner, phosphorylations of histone H1 were linear with time over an hour under these conditions. In some instances, proteins from assay incubations were precipitated in 10% trichloroacetic acid at 4 °C, and the pellets were washed once with 70% ethanol at 4 °C. The pellet was dissolved in gel loading buffer and analyzed directly by SDS–PAGE. Alternatively, assay samples were directly prepared for SDS–PAGE. For inhibition studies, specific inhibitors at concentrations indicated were added to the kinase assay solution along with the kinase samples and preincubated for 15 min before addition of ATP.

Amino Acid Analysis of Incubated Histone H1 and DMPKH. Histone H1 and DMPKH were incubated in kinase assay buffer as above, separated by SDS–PAGE, and transferred to nitrocellulose, and the nitrocellulose strips containing each protein were flushed with argon, sealed, and incubated in 6 N HCl for 1 h at 110 °C. Hydrolysates were separated on K2 250 μm thin-layer cellulose plates (Whatman) by electrophoresis in pyridine/acetic acid/water (1:10:90), pH 3.5, toward the anode at 500 V for 4.5 h, and then by electrophoresis in formic acid/acetic acid/water (1:4:45), pH 2.1, in the orthogonal direction. One nanomole each of nonradioactive phosphoserine, phosphothreonine, and phosphotyrosine (Sigma) was added to each sample before thin-layer electrophoresis as standards for identification of the radioactive phosphoamino acids by ninhydrin staining.

RESULTS

Expression of Soluble Recombinant DM Protein Kinase. Since DM protein kinase is predicted to contain several structurally and functionally distinct domains, as shown in Figure 1, the expression of three different forms of the protein was attempted by synthesizing the different constructs in Figure 1 by PCR with the appropriate oligonucleotide primers. Only the DMPKHT product would contain the putative membrane-spanning tail; DMPKH and DMPKHT would both contain the α-helical coiled-coil region, and all three potential products would contain the DMPK catalytic domain. DMPK and DMPKH were expressed in BL21([DE3]-pLysS) as proteins with apparent molecular weights of 43 000 and 66 000, respectively, based upon SDS–PAGE (Figures 2 and 3). The molecular weight agrees closely with the calculated molecular

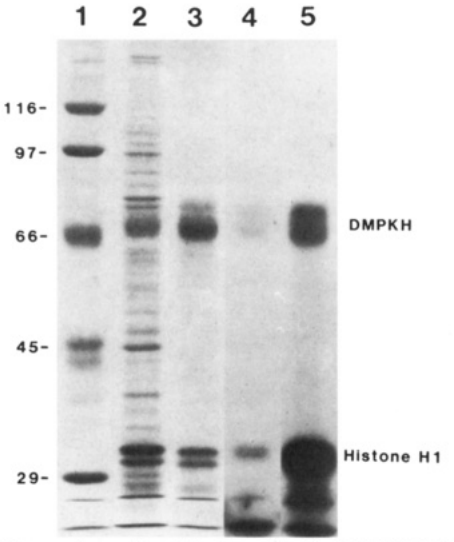

FIGURE 2: Expression and purification of active DMPKH. DMPKH with polyHis leader was purified by binding to a Ni affinity column and release upon competition with imidazole. Both samples were incubated for detection of protein kinase activity and analyzed by 7.5% SDS–PAGE and radioautography. Lanes 1–3, Coomassie blue staining; lanes 4, 5, radioautograph of lanes 2, 3. Lane 1, molecular weights ($\times 10^{-3}$) of marker proteins; lanes 2 and 4, 15 μg of total lysate protein expressing DMPKH; lanes 3 and 5, 375 μg of purified DMPKH each incubated with 3 μg of histone H1, 10 μCi of [γ-^{32}P]-ATP, and 100 μM ATP as indicated in the text.

weight of 44 866 for DMPK; however, DMPKH migrates more slowly than expected from its calculated molecular weight of 56 043. Although it has been suggested that the polyHis leader produces this abnormally slow migration on SDS–PAGE (Fu et al., 1993), the different behavior of the two constructs suggests that the slow mobility is specific to the addtional structure of DMPKH. The DMPKHT construct was not recovered except in reverse orientation (20/20 colonies), which suggests that this product may have been lethal in the *E. coli* host due to leaky expression.

Conditions for growth, induction, and solubility of DMPKH and DMPK were optimized. Both growth and induction of the recombinant cells at 25 °C generated severalfold more soluble DMPHK and DMPK than either or both phases at 37 °C.

Purification of DMPKH and DMPK. In order to prepare enzymatically active recombinant DM kinase forms for biochemical studies and for production of antibodies specifically reactive with native enzyme in tissues, DMPKH and DMPK were purified without the use of denaturing conditions. Greater than 50% of the bacterially expressed DMPKH and DMPK were soluble in nondenaturing buffers and were extensively purified. The insert sequences were ligated into the vector pET15b which encodes a fusion leader peptide containing polyhistidine residues (Novagen). Soluble DMPKH and DMPK were purified by sequential chromatography on nickel(II)-affinity and, optionally, Sephadex G150 gel filtration columns.

The DMPKH so purified was 87% of the total chromatographed protein as determined by densitometry of SDS–PAGE of the pooled peak Sephadex fractions. Figure 2, lanes 2 and 3, compares the protein compositions of the lysate and purified protein from the DMPKH-expressing strain.

DMPKH and DMPK Exhibit Protein Kinase Activity. To determine whether DMPKH or DMPK showed protein kinase activity, the incorporation of [^{32}P]phosphate from [γ-^{32}P]-ATP into added histone was studied. Figure 2, lanes 4 and 5, shows that the phosphorylation of histone increased

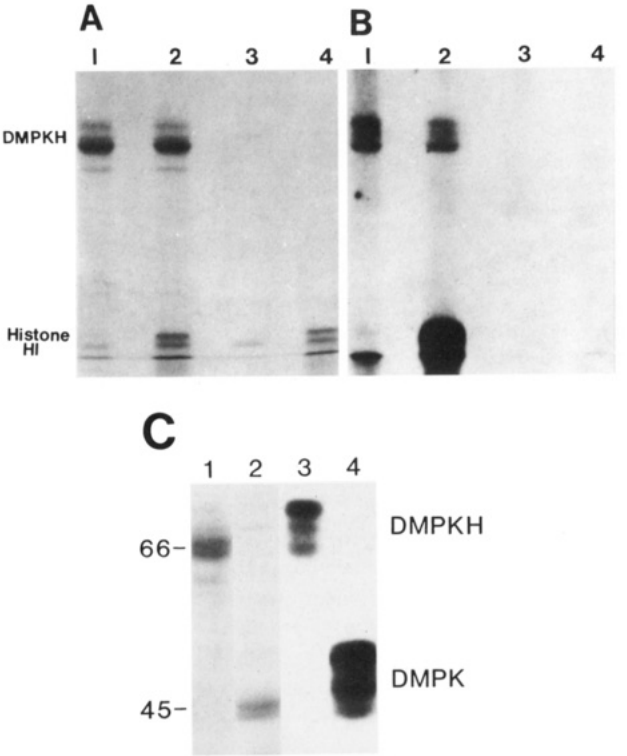

FIGURE 3: Affinity binding of recombinant and host cell lysates. Lysates from the *E. coli* host strain transformed with DMPKH (A, B, C), DMPK (C), or control (A, B) were run over nickel affinity columns. Equal amounts of the lysates and equal volumes of the resulting affinity column eluants from each strain were compared. The affinity-bound proteins were incubated with the standard kinase assay mixture for 1 h and then separated by SDS–PAGE. In (A) Coomassie blue staining and in (B) radioautography of (1) DMPKH, (2) DMPKH and histone H1, (3) control, and (4) control and histone H1. In (C), Coomassie blue staining of (1) DMPKH and (2) DMPK and radioautography of (3) DMPKH and (4) DMPK.

considerably with the purification of DMPKH from the lysate. Phosphorylations of histone H1 were linear with time over an hour. Interestingly, DMPKH was itself phosphorylated, and the modified protein appeared in several closely spaced bands in the 66 kDa region of the SDS–PAGE radioautogram. The histone H1 preparation, itself, did not show any incorporation; however, DMPKH and DMPK showed significant self-phosphorylation in the absence of histone.

Figure 3A,B shows that the incorporation activities were specific to protein purified from the recombinant DMPKH cells and not to species purifiable from the host bacteria. No comparable radioactive species in the DMPKH or DMPK regions of SDS–PAGE were seen in control eluant. The histone incorporated in an hour about 10-fold of the [^{32}P]-phosphate as DMPKH at a molar ratio of histone to DMPKH of 2:1. The levels of DMPKH and DMPK were at these high levels to permit detection of their self-phosphorylation; histone phosphorylation was still proportionally dependent upon DMPKH concentration (see Figure 6C).

Figure 3C compares the phosphorylation activities of DMPKH and DMPK. The triad of closely spaced radioactive species moves from about 66 kDa with DMPKH to about 43 kDa with DMPK, corresponding to the regions containing their protein bands, further suggesting that autophosphorylation and not a contaminating bacterial kinase is responsible for these bands. The number and intensity of these bands vary from experiment to experiment. This may represent different states of phosphorylation of DMPKH or DMPK which has been observed in several other phosphoproteins

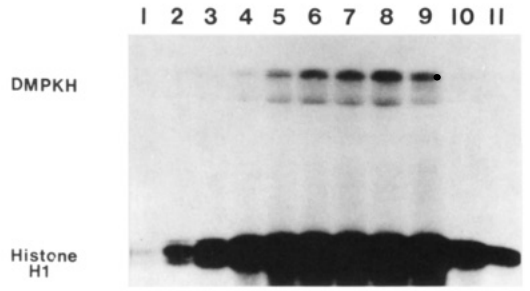

FIGURE 4: Cosedimentation of DMPKH and kinase activities. Affinity-purified DMPKH was placed on a 5–20% sucrose gradient and sedimented at 28 000 rpm for 5 h in an SW 41.1 rotor. Individual fractions were collected bottom (1) to top (11); histone H1 was added, incubated under standard kinase conditions with high specific activity ATP, and then separated by SDS–PAGE. The profile of Coomassie blue staining was very similar to the radioautography of DMPKH.

(Gould & Hunter, 1988; Whalen & Stewart, 1993). The greater intensity of the more slowly migrating species may reflect either more phosphorylation sites per molecule or that these species represent a more active enzyme or both. Histone H1 was similarly phosphorylated in the presence of either recombinant protein.

These observations indicate that the bacterially expressed recombinant proteins were responsible for the incorporation of [^{32}P]phosphate into histone H1, consistent with transphosphorylation by the protein kinase activity of either DMPK or DMPKH. The incorporation of [^{32}P]phosphate by the recombinant proteins themselves may have been the result of self-phosphorylation either by autophosphorylation or by transphosphorylation by other recombinant molecules. In preliminary experiments, we have not been able to renature SDS–treated DMPKH by methods used for other kinases (Hager & Burgess, 1980; Shabanowitz et al., 1992) in attempts to further test this question.

DMPKH and Phosphorylation Activity Cosediment as a Multimer. As a means of further characterizing and purifying DMPKH, we sedimented the affinity-purified preparation on sucrose gradients. Figure 4 shows that phosphorylatable DMPKH cosediments with the activity responsible for transferring [^{32}P]phosphate to histone H1, confirming the likely protein kinase activity of DMPKH. Coomassie blue staining of the DMPKH showed that the protein content also cosedimented with these activities (data not shown).

The peak of DMPKH sedimented at 41 S. This apparent sedimentation constant is consistent with spherical particles of average molecular weight 1.2×10^6 (Deiteker & Epstein, 1993). With a calculated molecular weight of 56 043 for DMPKH including the fusion sequences, this particle would be a multimer with an average of approximately 21 DMPKH subunits. Electron microscopy of negatively stained samples from the peak fractions showed discrete particles whose dimensions, about 30–50 nm, were consistent with the sedimentation results (data not shown). No amorphous or very large aggregates were seen. The presence of 150 mM NaCl did not affect the mobility of DMPKH on Superose 6 FPLC (data not shown).

Serines and Threonines Become Phosphorylated in both Histone H1 and DMPKH. To verify covalent phosphoryl transfer and characterize its specificity in the phosphorylation experiments, [^{32}P]phosphate-labeled DMPKH and histone H1 were hydrolyzed in constant-boiling HCl, and the products were separated by two-dimensional electrophoresis on thin-layer cellulose. The radioautograms of Figure 5A,B show that both proteins after incubation together in kinase assay

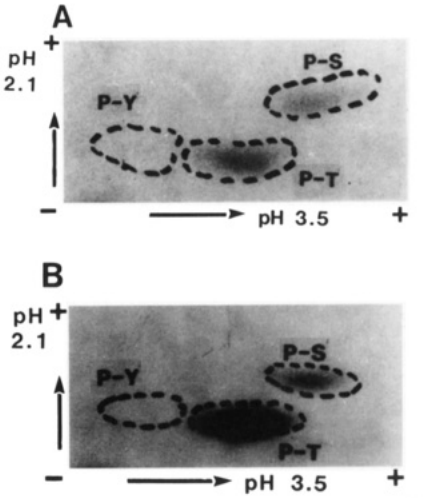

FIGURE 5: Phosphoamino acid analysis of DMPKH and histone H1 after incubation. DMPKH (A) and histone H1 (B) were incubated in standard kinase buffer, separated by SDS–PAGE, eluted, and then subjected to two-dimensional electrophoresis and radioautography. Dotted lines represent the migration of phosphoserine (P-S), phosphothreonine (P-T), and phosphotyrosine (P-Y).

solution contained radioactive phosphothreonine and, to a lesser extent, phosphoserine. No radioactive phosphotyrosine was detected at these or longer exposures.

The phosphorylation activity associated with DMPKH as indicated by these experiments was that of a serine/threonine protein kinase, consistent with the predictions based on sequence homology (Fu et al., 1992; Brook et al., 1992).

DMPKH Specificity Differs from Multifunctional Protein Kinase Activities. As a means of comparing the protein kinase activity of DMPKH to those of well-characterized serine/threonine protein kinases such as PKA, PKG, and PKC which also share significance sequence homology with DM protein kinase, the effects of the competitive inhibitors H7 and H8 upon DMPKH were studied. H7 and H8 differentially inhibit PKA, PKG, and PKC with K_is between 0.4 and 15 μM (Hidaka et al., 1984). Figure 6A shows that 4 mM H7 or H8 inhibits the phosphorylation of both histone H1 and DMPKH whereas the tyrosine inhibitor genistein had no detectable effect. These results are consistent with the demonstrated serine/threonine specificity of the protein kinase activity associated with DMPKH. The effects of H8 were further studied under conditions of increased sensitivity and specificity. The radioautogram of Figure 6B shows that neither 40 nor 400 μM H8 inhibits either DMPKH or histone H1 phosphorylation, even though the ATP concentration was lowered from 100 to 20 μM in order to increase the sensitivity of the kinase to the inhibitors. Control experiments showed that 40 μM H7 or H8 nearly completely inhibited the phosphorylation of histone H1 by commercial PKC (Boehringer Mannheim), as expected from the published K_i (Hidaka et all, 1984). These results suggest that the kinase activity associated with DMPKH may be qualitatively different from the activities of PKA, PKG, and PKC.

Specific inhibitors of other serine/threonine protein kinases were assayed for their effects on DMPKH–histone H1 phosphorylation. No inhibition was obtained with inhibitors CKI-7 of CKI (Chijiwa et al., 1989), heparin of CKII (Hathaway et al., 1980), or KN-62 of CaMKII (Tokumitsu et al., 1990) at concentrations up to 90-fold the published IC_{50}s. The inhibition of the DMPKH-associated activity did not appear to be similar to any of the major classes of multifunctional serine/threonine protein kinases although it is clearly a serine/threonine kinase.

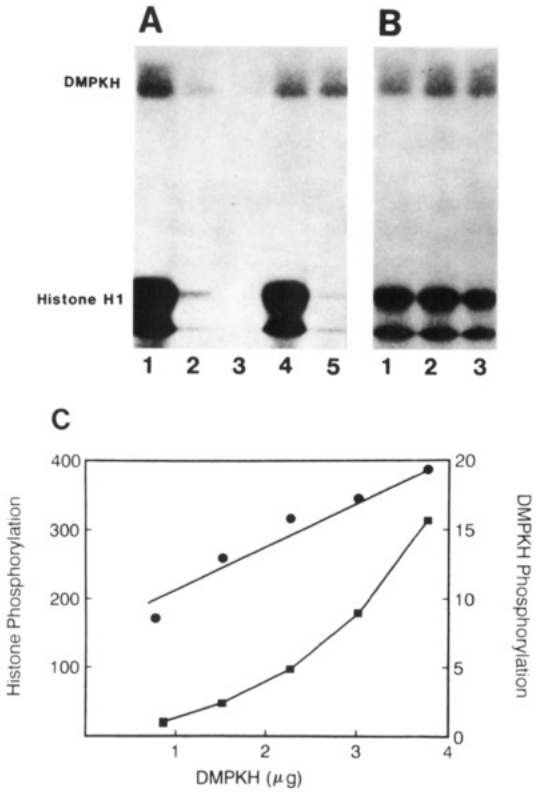

FIGURE 6: Inhibition of DMPKH by high concentrations of H7 and H8. In (A) and (B), all assays were performed with 3.75 μg of affinity-purified DMPKH with or without 3 μg of histone H1 and concentrations of inhibitors as indicated. In (C), the concentration of DMPKH was varied to indicate the linear dependence of transphosphorylation upon it. (A) Lanes 1–5, all incubations with a total of 100 μM ATP: (1) no inhibitor; (2) 4 mM H7; (3) 4 mM H8; (4) 148 μM genistein; (5) no histone or inhibitor. (B) Lanes 1–3, all incubations with 20 μM ATP: (1) 40 μM H8; (2) 400 μM H8; and (3) no inhibitor. (C) Histone H1 and DMPKH phosphorylation as a function of DMPKH concentration. Phosphorylation was measured by densitometry of the absorbance on radioautograms. Circles represent histone H1 phosphorylation; squares represent DMPKH phosphorylation.

The activity of DMPKH with respect to several well-known membrane-associated protein substrates of multifunctional serine/threonine protein kinases that could be potential candidate substrates was assayed. The γ and δ subunits of *Torpedo* acetylcholine receptor contain phosphorylation sites for PKA, and the δ subunit contains a site for PKC (Huganir & Greengard, 1990). The incubation of purified *Torpedo* acetylcholine receptor with DMPKH produced no phosphorylation of the γ and δ subunits. The β-spectrin subunit of human erythrocyte spectrin contains phosphorylation sites for CKI and CKII (Simkowski & Tao, 1980). After inhibition of the endogenous spectrin-associated kinase activity by the specific CK inhibitors used above which do not affect DMPKH activity, added DMPKH did not phosphorylate spectrin. These results agreed with the inhibitor studies that the DMPKH activity differed from PKA, PKC, and CK. As a control experiment for both these inhibition studies and the protein kinase assays of DMPKH generally, the dependence of histone H1 and DMPKH phosphorylation upon DMPKH concentration was measured by the absorbance-based densitometry of gel radioautograms. Figure 6C shows that the dependence of histone phosphorylation was approximately linear in the region which corresponded to the concentration used throughout this report. In contrast, DMPKH phosphorylation showed a hyperbolic increase with DMPKH concentration. The molar ratio of histone H1 to DMPKH varied from 2:1 to 10:1 in this experiment. The molar ratios of inhibitors to DMPKH varied from 26:1 to 2600:1, which indicates that the DMPKH protein and not the inhibitors was the limiting reactant in the experiments of Figure 6A,B.

DISCUSSION

We have expressed the putative human DM protein kinase as two recombinant forms in *E. coli*. The DMPK and DMPKH proteins both contain the putative protein kinase catalytic domain and both lack the putative membrane-spanning tail of the complete predicted DM gene product (Fu et al., 1992; Brook et al., 1992). Analysis of the genomic sequences of the DM locus indicates that this tail may be absent in some gene products because of alternative splicing of the exon encoding it. DMPKH contains additionally the predicted α-helical coiled-coil domain whose function is presently unknown. The availability of these recombinant proteins is important for many biochemical and immunological studies because the amounts of DM protein kinase in skeletal muscle and other tissues appear to be very low (van der Ven et al., 1993; Carango et al., 1993).

The bacterially expressed DMPK and DMPKH showed protein kinase activity with respect to the general protein kinase substrate histone H1 and to themselves. These activities were detected only in recombinant bacterial lysates and their derivatives. Both phosphorylating activities cosedimented at 41 S with DMPKH, and the self-phosphorylating activity moved with DMPK at 43 kDa and with DMPKH at 66 kDa. The phosphorylation of histone H1 and itself by DMPKH yielded phosphothreonine as the major modified amino acid and phosphoserine as a lesser species. Both activities showed similar behavior to a spectrum of protein kinase inhibitors. They were inhibited only by millimolar concentrations of H7 and H8 as are all multifunctional serine/threonine protein kinases but not by lower concentrations of H7 or H8 as are PKA, PKG, and PKC. Neither activity was affected by high concentrations of inhibitors specific to serine/protein kinases such as CK I and II or CaMK II or of tyrosine kinase.

We consider that both protein kinase activities observed in the DMPK and DMPKH preparations were most likely produced by the recombinant proteins and not some contaminating bacterial activity. The latter species would have had to be induced in recombinant rather than control bacterial cells, and then to have copurified through affinity chromatography, gel filtration, and velocity sedimentation steps with recombinant protein and the two phosphorylation activities. Moreover, it would have had to have shown the unusual pattern of inhibition by specific reagents and to have exhibited a similar preponderance of threonine over serine phosphorylation. The nonlinear dependence of DMPKH phosphorylation upon DMPKH concentration, in contrast to the linear dependence of histone phosphorylation, is also consistent with this conclusion. We believe the cumulative weight of the evidence supports the conclusion that the activity is associated with the recombinant protein, although we cannot completely rule out the alternative. The work presented here serves as a necessary step for additional experiments leading to more definitive conclusions as regaining of activity from excised gel bands containing DMPK or DMPKH or loss of activity by site-directed mutagenesis of the predicted active centers of DMPK or DMPKH.

The DM protein kinase is a molecule with unusual structural and functional features. The predicted coiled-coil and membrane-spanning domains would be unique for any protein kinase (Knighton et al., 1991). The amino acid sequence of

the catalytic domain shows strong homology to regions of PKA, PKG, and PKC; however, neither inhibitors nor substrates for these multifunctional enzymes appeared to functionally interact under specific conditions with recombinant DM protein kinase. The DM enzyme, thus, appears to be a serine/threonine protein kinase with restricted specificity of interaction with substrates and inhibitors. Further experiments defining more quantiatively the kinetics of the recombinant enzyme are required. The multimeric nature of the recombinant DM kinase is also an unusual finding; the protein structures are clearly not amorphous, nonspecific aggregates; however, more structural analysis is required to define detailed features as has been done with brain CaMK II (Clari & Moret, 1985).

Further biochemical and cytological studies based upon the availability of active recombinant enzyme will be necessary for understanding the role of DM protein kinase in multiple kinds of cells including muscle, eye lens, heart, and brain both in normal physiology and in inherited disruption by myotonic dystrophy. In muscle, alterations in its membrane excitability following neuronal stimulation and in its metabolic regulation by insulin (Harper, 1989) may suggest that the enzyme studied here plays a specific role in the regulatory cascades of protein kinases common to signal transduction pathways of both processes (Merickel et al., 1981; Pelech & Sanghera, 1992).

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REFERENCES

Brook, J. D., McCurrach, M. E., Harley, H. G., Buckler, A. J., Church, D., Aburatani, H., Hunter, K., Stanton, V. P., Thirion, J.-P., Hudson, T., Sohn, R., Zemelman, B., Snell, R. G., Rundle, S. A., Crow, S., Davies, J., Shelbourne, P., Buxton, J., Jones, C., Juvonen, V., Johnson, K., Harper, P. S., Shaw, D. J., & Housman, D. E. (1992) *Cell 68*, 799–808.

Carango, P., Noble, J. E., Marks, H. G., & Funanage, V. L. (1993) *Genomics 18*, 340–348.

Chijiwa, T., Hagiwara, M., & Hidaka, H. (1989) *J. Biol. Chem. 264*, 4924–4927.

Clari, G., & B. Moret, V. (1985) *Mol. Cell. Biochem. 68*, 181–187.

Coleman, T. R., Tung, Z., & Dunphy, W. G. (1993) *Cell 72*, 1919–1929.

Deitiker, P. R., & Epstein, H. F. (1993) *J. Cell Biol. 123*, 303–311.

Epstein, H. F., Berliner, G. C., Casey, D. L., & Ortiz, I. (1988) *J. Cell Biol. 106*, 1985–1995.

Fu, Y.-H., Pizzuti, A., Fenwick, R. G., King, J., Rajnarayan, S., Dunne, P. W., Dubel, J., Nasser, G. A., Ashizawa, T., deJong, P., Wieringa, B., Korneluk, R., Perryman, M. B., Epstein, H. F., & Caskey, C. T. (1992) *Science 255*, 1256–1258.

Fu, Y.-H., Friedman, D. L., Richards, S., Pearlman, J. A., Gibbs, R. A., Pizzuti, A., Ashizawa, T., Perryman, M. B., Scarlato, G., Fenwick, R. G., & Caskey, C. T. (1993) *Science 260*, 235–238.

Gould, K. L., & Hunter, T. (1988) *Mol. Cell Biol. 8*, 3345–3356.

Hager, D. A., & Burgess, R. R. (1980) *Anal. Biochem. 109*, 76–86.

Harper, P. S. (1989) *Myotonic Dystrophy*, 2nd ed., Saunders, London, Philadelphia, Toronto, Sydney, and Tokyo.

Hathaway, G. M., Lubben, T. H., & Traugh, J. A. (1980) *J. Biol. Chem. 255*, 8038–8041.

Heald, R., McLaughlin, M., & McKeon, F. (1993) *Cell 74*, 463–474.

Hidaka, H., Inagaki, M., Kawamoto, S., & Sasaki, Y. (1984) *Biochemistry 23*, 5036–5041.

Huganir, R., & Greengard, P. (1990) *Neuron 5*, 555–567.

Knighton, D. R., Zheng, J., ten Eyck, L. F., Ashford, V. A., Xuong, N.-H., Taylor, S. S., & Sowadski, J. M. (1991) *Science 253*, 407–414.

Mahadevan, M. S., Amemiya, C., Jansen, G., Sabourin, L., Baird, S., Neville, C. E., Wormskamp, N., Segers, B., Batzer, M., Lamerdin, J., deJong, P., Wieringa, B., & Korneluk, R. G. (1993) *Hum. Mol. Genet. 2*, 299–304.

Merickel, M., Gray, R., Chauvin, P., & Appel. S. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*; 648–652.

Pelech, S. L., & Sanghera, J. S. (1992) *Science 257*, 1355–1356.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

Shabanowitz, J., Hunt, D. F., & Sturgill, T. A. (1992) *Biochem. J. 285*, 701–705.

Simkowski, K. W., & Tao, M. (1980) *J. Biol. Chem. 266*, 6450–6461.

Suter, B., & Steward, R. (1991) *Cell 67*, 917–926.

Tokumitsu, H., Chijiwa, T., Hagiwara, M., Mizutani, A., Terasawa, M., & Hidaka, H. (1990) *J. Biol. Chem. 265*, 4315–4320.

van der Ven, P. F. M., Jansen, G., van Kuppevelt, T. H. M. S. M., Perryman, M. B., Lupa, M., Dunne, P. W., ter Laak, H. J., Jap, P. H. K., Epstein, H. F., & Wieringa, B. (1993) *Hum. Mol. Genet. 11*, 1889–1894.

Whalen, A. M., & Steward, R. (1993) *J. Cell Biol. 123*, 523–534.