Calmodulin-Activated Phosphorylation of Dystrophin†

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ABSTRACT: Purified dystrophin glycoprotein complex (DGC) contains an endogenous protein kinase activity which phosphorylates dystrophin. Mg^{2+} (or Mn^{2+}) and ATP are required for this phosphorylation. Ca^{2+}–calmodulin increases the rate of phosphorylation of dystrophin 12-fold relative to the EGTA control, while other protein kinase activators, cAMP and cGMP, have no effect. Phosphorylation of other proteins in the DGC preparation was observed, with a 59-kDa protein also being phosphorylated in a calmodulin-dependent manner. These phosphorylations were all on serine residues. The DGC protein kinase activity also phosphorylates syntide-2, a peptide substrate for CaM kinase II, and antibodies raised against CaM kinase II cross-react with DGC blotted onto nitrocellulose. Further, purified, baculovirus-expressed CaM kinase II phosphorylates dystrophin and also phosphorylates at least one of the peptides of dystrophin which is phosphorylated by the DGC protein kinase activity, as shown by tryptic peptide maps. CaM kinase II also phosphorylates other proteins present in the DGC preparation that are phosphorylated by the endogenous protein kinase. Finally, dystrophin sequence 2618–3074, produced by recombinant techniques, is phosphorylated by both the DGC protein kinase and purified CaM kinase II. Since dystrophin and two other DGC components have also been shown to bind calmodulin, two important components of signal transduction—calmodulin binding and protein phosphorylation—operate in the DGC.

Duchenne muscular dystrophy is marked by a defective gene on the X chromosome that results in the deficiency or absence of its product, dystrophin, in patients afflicted with this degenerative myopathy (Hoffman et al., 1987). Dystrophin is a sarcolemmal cytoskeletal protein associated with a complex of proteins and glycoproteins, the dystrophin glycoprotein complex (DGC)[1] (Ervasti et al., 1991).

Protein phosphorylation and calmodulin (CaM) binding to cellular proteins are two important forms of signal transduction. That signal transduction may be relevant to the etiology of muscular dystrophy was suggested by the Ca^{2+}-dependent CaM binding to three proteins in the DGC (Madhavan et al., 1992). Ca^{2+} and CaM levels are often also altered in dystrophic tissue (Turner et al., 1988; Hudecki et al., 1986). Furthermore, Ca^{2+}–CaM is thought to serve an important role in regulating cellular cytoskeletal functions (Kakiuchi, 1985). CaM activates various enzymes, many of which are protein kinases, and several CaM binding proteins are substrates for protein kinases (Cheung, 1980; Klee & Vanaman, 1982). Dystrophin homologues such as spectrin and fodrin bind CaM and are also phosphorylated (Reinhart et al., 1986; Sobue et al., 1982). Thus protein phosphorylation and CaM binding are often interrelated. While this manuscript was in preparation, Milner et al. (1993) showed that dystrophin is phosphorylated *in vivo* and Luise et al. (1993) showed that dystrophin is phosphorylated by protein kinases endogenous to the sarcolemma. In purified sarcolemma, Luise et al. found a cAMP- and cGMP-dependent protein kinase activity as well as a Ca^{2+}–CaM-dependent one. Here, using purified DGC, we report the phosphorylation of dystrophin in a Ca^{2+}–CaM-dependent manner by a protein kinase activity endogenous to the DGC preparation. Thus, the Ca^{2+}–CaM-dependent protein kinase Luise et al. report is retained in the DGC preparation. Other proteins in the DGC preparation are also phosphorylated in the absence or presence of Ca^{2+}–CaM. Further, dystrophin and these proteins are phosphorylated by exogenously added CaM kinase II. We also show that CaM kinase II phosphorylates a recombinant fragment of dystrophin (sequence 2618–3074), as does the protein kinase endogenous to the DGC preparation. The focus of this paper is on the phosphorylation of dystrophin.

MATERIALS AND METHODS

Digitonin from Sigma was purified as described (Sharp et al., 1987). [γ-^{32}P]ATP (5000 Ci/mmol) was from Amersham Corp. The prestained molecular weight markers mixture was from Bio-Rad. Succinylated wheat germ agglutinin agarose was from Vector Labs. NCL-DYS2, a monoclonal antibody against the COOH-terminus end of dystrophin, was from Novo Castra Labs (U.K.). Antibodies against CaM kinase II were the kind of gift of Dr. Marita King. CaM was purified (Gopalakrishna & Anderson, 1982) from bovine brain. All other reagents and chemicals were of the highest purity available.

Protein concentrations of the microsomes and the soluble proteins were determined according to the methods of Lowry et al. (1951) and Bradford (1976), respectively, using BSA as the protein standard. The concentration of BSA and glycogen phosphorylase was adjusted using their absorptivity values (Fasman, 1989).

Purification of DGC. Salt-extracted rabbit skeletal muscle microsomes were prepared (Ervasti et al., 1991) and stored frozen at −85 °C prior to use. Microsomes (0.8–1.0 g total protein) were digitonin solubilized and purified by succinylated wheat germ agglutinin agarose affinity chromatography using the described method (Ervasti et al., 1991) except that a 20-

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[1] Abbreviations used: DGC, dystrophin glycoprotein complex; CaM, calmodulin; PVDF, polyvinylidene difluoride; CaM kinase II, baculovirus-expressed, calmodulin-dependent, multifunctional protein kinase II-α315 mutant; proteins are denoted by "p" and glycoproteins by "gp" followed with a number giving the apparent M_r on SDS–gel electrophoresis in kilodaltons, e.g., p59; TLC, thin-layer chromatography; BSA, bovine serum albumin.

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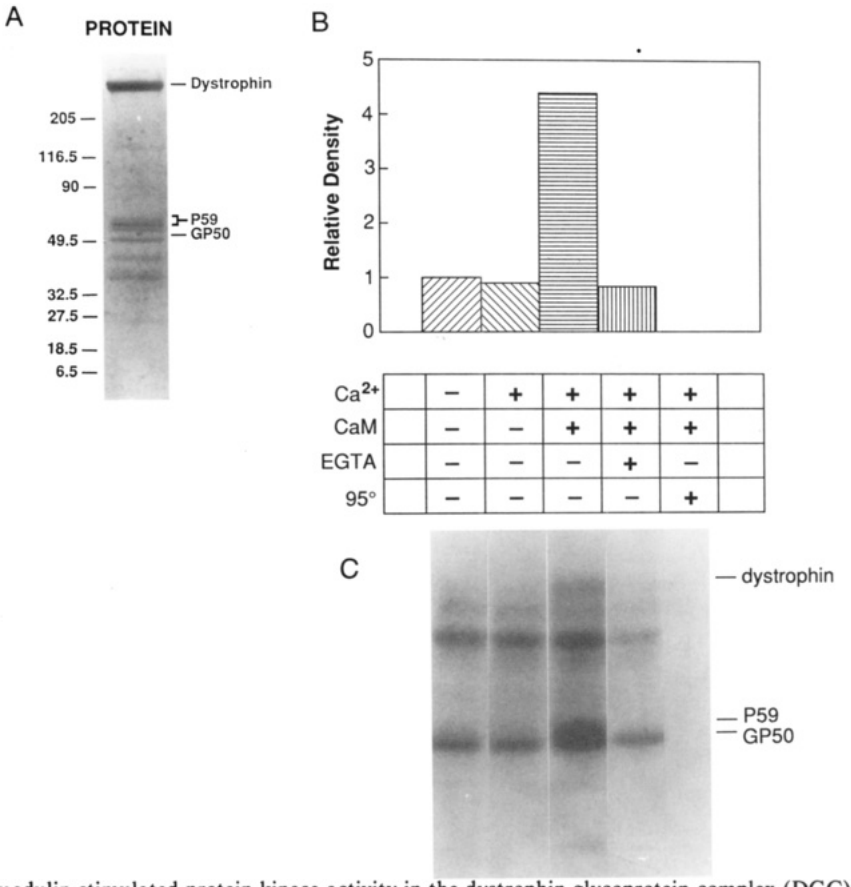

FIGURE 1: Ca^{2+}- and calmodulin-stimulated protein kinase activity in the dystrophin glycoprotein complex (DGC) preparation from rabbit skeletal muscle microsomes. (A) Coomassie Blue-stained SDS–polyacrylamide 5–15% gradient gel of the DGC. Dystrophin is seen as the major component in the preparation. The positions of p59 and gp50 are also shown. The M_r values (in kilodaltons) of the molecular weight standards are shown on the left. (B) Densities of the phosphorylated dystrophin band as determined by densitometry of the autoradiogram in panel C. DGC, 5 μL, was phosphorylated by incubation for 30 min in 20 μL either without additives (25 mM HEPES, pH 7.4, 10 mM $MgCl_2$, and 10 μM [γ-^{32}P]ATP (44 400 cpm/pmol), control, column 1) or in the presence of 10 μM Ca^{2+} (column 2), 10 μM Ca^{2+}–1 μM CaM (column 3), or 10 μM Ca^{2+}–1 μM CaM–500 μM EDTA (column 4). These conditions were used throughout unless otherwise stated. An aliquot of the DGC was also heated at 95 °C for 1 min prior to phosphorylation (column 5). (C) Autoradiogram of phosphorylated DGC. The effect of Ca^{2+} and CaM on the phosphorylation of the DGC components is shown here.

mL column was used. The *N*-acetylglucosamine-eluted material was further purified using a 3-mL Whatman DE-52 column essentially as described by Ervasti et al. (1991). The 175 mM NaCl eluate from the DE-52 column was concentrated to 0.6 mL using Amicon Centriprep 100 concentrators, and this concentrated material is referred to as DGC.

Phosphorylation reactions were done at 30 °C using conditions described in the legend for Figure 1. Assay mixtures without DGC and ATP were preincubated (30 °C, 3 min); DGC was then added, followed by incubation for 1 min more. Reactions were initiated by adding [γ-^{32}P]ATP and terminated by mixing with SDS sample buffer (Laemmli, 1970) at indicated times and heating (95 °C, 1 min). Purified, baculovirus-expressed CaM kinase II (Takeuchi-Suzuki et al., 1992) was at concentrations described in the figure legends. Syntide-2 was used to 20 μM final concentration (Takeuchi-Suzuki et al., 1992).

Electrophoresis, Autoradiography, and Blotting. Electrophoresis of phosphorylated samples was according to the method of Laemmli (1970), using 0.75-mm-thick, 5–15% gradient acylamide gels. Gels were stained with Coomassie Blue R-250; staining and destaining was in 40% methanol, 10% acetic acid. The destained gels were exposed to X-OMAT film. The Millipore BioImage system was used for densitometry of autoradiograms.

For some experiments, gels were electroblotted onto 0.2-μm PVDF membranes for protein staining (Amido Black) and antibody detection of dystrophin. Transfer buffer was 10% methanol, 25 mM Tris, 192 mM glycine, pH 8.3, run for 80 min at 110 V. Destained blots (showing total protein) were placed in water for 1 h prior to drying and autoradiography. Other blots, after being blocked with 5% nonfat milk, were probed with NCL–DYS 2 (1:100 dilution) for dystrophin and 0.1 μg/mL biotinylated wheat germ agglutinin for glycoproteins in the DGC. In other experiments, equal amounts (2 μg) of CaM kinase II, DGC, BSA, and a total protein extract from rat brain were blotted directly onto nitrocellulose, blocked with 10 mg/mL BSA, and probed with the antibodies against CaM kinase II (1:1000 dilution). Goat anti-mouse or -rabbit IgG–horseradish peroxidase-conjugated secondary antibody was used for detecting dystrophin and CaM kinase II antibodies, respectively, and streptavidin-biotinylated alkaline phosphatase conjugates for glycoproteins, using methods previously described (Jarrett & Madhavan, 1991; Madhavan et al., 1992).

Phosphoamino acid analysis was performed on gel electrophoresis slices containing dystrophin and the 59- and 50-kDa phosphorylated proteins. After phosphorylation for 30 min using 50-μCi carrier-free [γ-^{32}P]ATP with Ca^{2+}–CaM, the DGC was fractionated by electrophoresis (Laemmli, 1970). After the gel was stained and destained, the Coomassie-stained protein bands were excised and minced. The proteins were eluted by incubating the gel pieces in 50 mM ammonium bicarbonate and hydrolyzed by heating (110 °C, 1 h) in 6 M

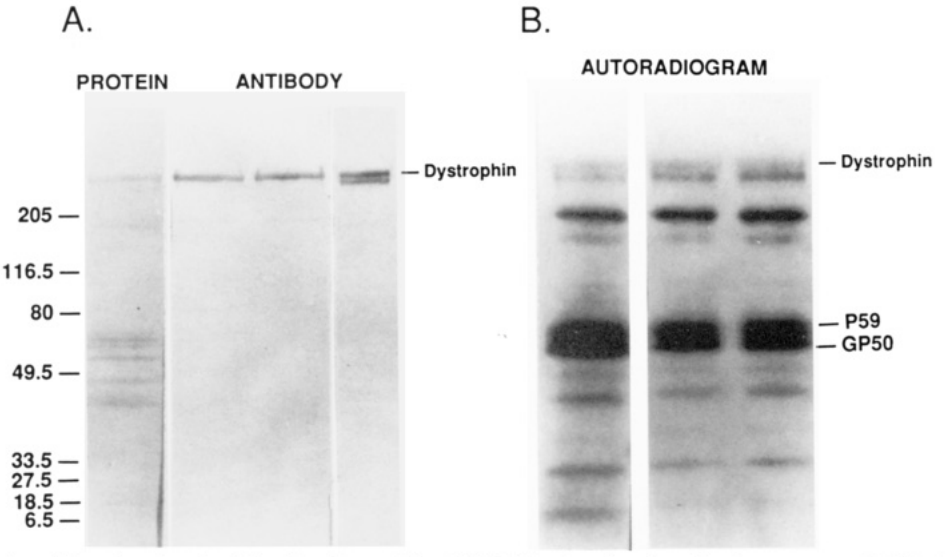

FIGURE 2: Identification of the phosphorylated band as dystrophin. (A) DGC, phosphorylated in the presence of Ca^{2+}–CaM for 60 min, was electrophoresed, blotted onto 0.2-μm PVDF membrane, and either stained for total protein using Amido Black stain or probed for dystrophin using a monoclonal antibody, NCL–DYS 2. The M_r values (in kilodaltons) of the molecular weight markers are shown on the left. (B) Autoradiogram of the gel blot showing that dystrophin is phosphorylated. The blots shown in panel A were attached to a support and autoradiographed after radioactive alignment markers were placed so that the phosphorylated bands could be aligned with the antibody staining.

HCl. Hydrolyzed samples, dried *in vacuo*, were dissolved in 10 μL of pH 1.9 buffer (2.2% formic acid, 7.8% acetic acid) containing phosphoamino acid standards. After electrophoresis on thin-layer chromatography (TLC) plates, the samples were visualized with ninhydrin and autoradiographed (Boyle et al., 1991).

Phosphopeptide analysis was performed on DGC phosphorylated by either the endogenous protein kinase or exogenously added CaM kinase II (100 ng) and on dystrophin phosphorylated by the endogenous DGC protein kinase activity and eluted from gel slices as described above. Phosphorylation reactions were carried out as described for the phosphoamino acid analysis. The phosphorylated proteins were performic acid oxidized, trypsin digested, dried *in vacuo*, and dissolved in pH 1.9 buffer for electrophoresis on TLC plates as described (Boyle et al., 1991). Chromatography was performed in the second direction with the phosphochromatography buffer used by Boyle et al. The TLC plates were dried, and the phosphopeptides were visualized by autoradiography.

Stoichiometry of the Dystrophin Phosphorylation. The amount of dystrophin present in gels was determined by densitometric comparison with known amounts of glycogen phosphorylase and BSA applied as standards. Comparison with either standard resulted in estimates of dystrophin amount which agreed within 10% with one another, and the two values obtained were averaged. Total phosphate content of excised gel bands was determined by scintillation counting following digestion of minced gel slices at 80 °C for 2 h in 30% H_2O_2.

Construction of Dystrophin Fusion Proteins. Mouse (C57bl) skeletal muscle mRNA was isolated using guanidinium isothiocyanate and cesium chloride ultracentrifugation as described by Sambrook et al. (1989). A cDNA was then prepared using the Invitrogen cDNA Cycle kit using 1 μg of mRNA and random hexamers as primers. Two microliters of the resulting (20 μL) reverse transcriptase reaction was used as template for a 50-μL polymerase chain reaction, using 2.5 units of Taq polymerase (Perkin-Elmer), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and the Taq polymerase buffer supplied by Promega. The polymerase chain reaction primers (0.1 μM final concentration) were S7 (CAGTTGGCCAAAGACCTCC) and A7 (GGCGAATTCTAAGTGGTTTGGGTCTCGTGG), where the underlined sequences are not from dystrophin and engineer a stop codon and an *Eco*RI restriction site. The remaining sequences are from dystrophin, and polymerase chain reaction produces mouse dystrophin cDNA sequences 8072–9443. After restriction with *Eco*RI and gel purification, the cDNA was ligated into a *Stu*I and *Eco*RI restricted pMALc vector (New England Biolabs) and used to transform *Escherichia coli* strain TB1. This plasmid allows expression of the dystrophin protein sequences as a fusion protein with the maltose-binding protein. Several clones expressing the correct size protein were mapped with restriction endonucleases. The authenticity was then confirmed by DNA sequencing. Fusion proteins were then produced and purified by maltose-affinity chromatography using procedures provided by New England Biolabs. Additionally, *E. coli* TB1 transformed with unmodified pMALc was used to express the maltose-binding protein–$LacZ_\alpha$ fusion for use as a negative control.

RESULTS AND DISCUSSION

During the course of these experiments, DGC was purified at least seven separate times. In these preparations, dystrophin was always associated with the copurifying protein kinase activity (see below) and with gp156, p59 (a triplet), gp50, gp43 (a doublet), gp35, and p25. A Coomassie-stained SDS–polyacrylamide gel of the DGC is shown in Figure 1A. These proteins were found to have molecular weights consistent with those reported by other investigators (Ervasti et al., 1991) and constitute the DGC. Dystrophin appears as the major band near the top of the gel. The gp156 band is not readily visible with Coomassie staining (Figure 1) but can be detected by biotinylated wheat germ agglutinin binding (data not shown). The band identified here as glycoproteins were all shown to bind biotinylated wheat germ agglutinin (data not shown), and dystrophin was identified by monoclonal antibody binding (see Figure 2). The positions of dystrophin, p59, and gp50 are indicated in this and other figures.

Gels, when overloaded with DGC and stained with Coomassie, also showed two minor bands with approximate M_r values of 175 and 150 kDa. These two bands were seen in all DGC preparations after succinylated wheat germ

agglutinin chromatography and were diminished about 90% following DEAE chromatography, but some still remained. While these are not normally considered DGC proteins, they are also observed in the DGC prepared by others (Ervasti & Campbell, 1991; Ohlendieck & Campbell, 1991). Both are glycoproteins (data not shown). The identity of these gp175 and gp150 proteins is not known and was not investigated further.

Incubating the DGC with Mg^{2+} and [γ-^{32}P]ATP without other additives results in phosphorylation predominantly of proteins of molecular weights 50 and 175 kDa (Figure 1C), demonstrating that protein kinase activity is present in DGC preparations. Phosphorylation was completely dependent on the presence of a bivalent cation (Mg^{2+} or Mn^{2+}, with Mg^{2+} being more effective, data not shown). Heating DGC to 95 °C prevents phosphorylation (Figure 1C).

Adding Ca^{2+} and CaM causes increased phosphorylation of dystrophin, while Ca^{2+} or CaM alone has no effect, suggesting that DGC preparation lacks endogenous CaM. A similar result is observed with the protein at 59 kDa. Other DGC phosphorylations were slightly increased by Ca^{2+}–CaM. Densitometry of the autoradiogram shows that in this 30-min reaction, Ca^{2+}–CaM increases the phosphorylation of dystrophin over 4-fold (Figure 1B). Other experiments showed that the protein phosphorylations were the same from pH 6.0 to 7.4 and are not significantly affected by either 1 μM cAMP or cGMP (data not shown). These results combined with those of Luise et al. (1993), show that while Ca^{2+}–CaM-dependent protein kinase activity present in the sarcolemma is retained in the DGC, the cyclic nucleotide-dependent protein kinase activity is not; thus the Ca^{2+}–CaM-dependent activity copurifies with the DGC proteins.

The ^{32}P incorporated into the DGC under the described conditions was stable in acid but slowly hydrolyzed in alkaline (1 M NaOH) solutions (data not shown). This is characteristic of phosphate esters (Martensen, 1984); phosphoamino acid analysis demonstrated phosphorylation of serine residues in dystrophin and the phosphorylated bands at 59 and 50 kDa (data not shown, but submitted for review). Threonine and tyrosine phosphorylations were not observed.

Further experiments focus upon the phosphorylation of dystrophin and not the other phosphorylations occurring in the DGC. While dystrophin is positively identified here as a phosphoprotein and its phosphorylation is described, the identity of the 175-, 59-, and 50-kDa proteins was not addressed in these studies for reasons discussed later (see below).

The identity of dystrophin was confirmed by immunoblotting. DGC was phosphorylated for 60 min in the presence of Ca^{2+} and CaM, blotted onto PVDF membrane, and either stained for total protein of probed using a monoclonal antibody against the C-terminal region of dystrophin (Figure 2A). The total protein stain of the blot shows a pattern of the DGC similar to that seen in the gel (Figure 1), though the amount of dystrophin is reduced due to incomplete transfer to the membrane. Dystrophin appears as a single band in preparations of DGC used (Figure 1A), but a doublet is frequently seen after incubation under the phosphorylation conditions used (Figure 2). The dystrophin antibody detects both members of the doublet, albeit with less sensitivity for the lower M_r band. In other experiments where the gel blotting protocols were changed to optimize dystrophin blotting, both dystrophin bands are readily detected by the antibody; a lane showing antibody staining of the dystrophin doublet is included in Figure 2A. In all such experiments, both bands on the autoradiogram (Figure 2B) and the antibody-stained (Figure 2A) blot align precisely; the bands which bind the dystrophin antibody are both phosphorylated. These results suggest proteolysis of dystrophin during prolonged incubation.

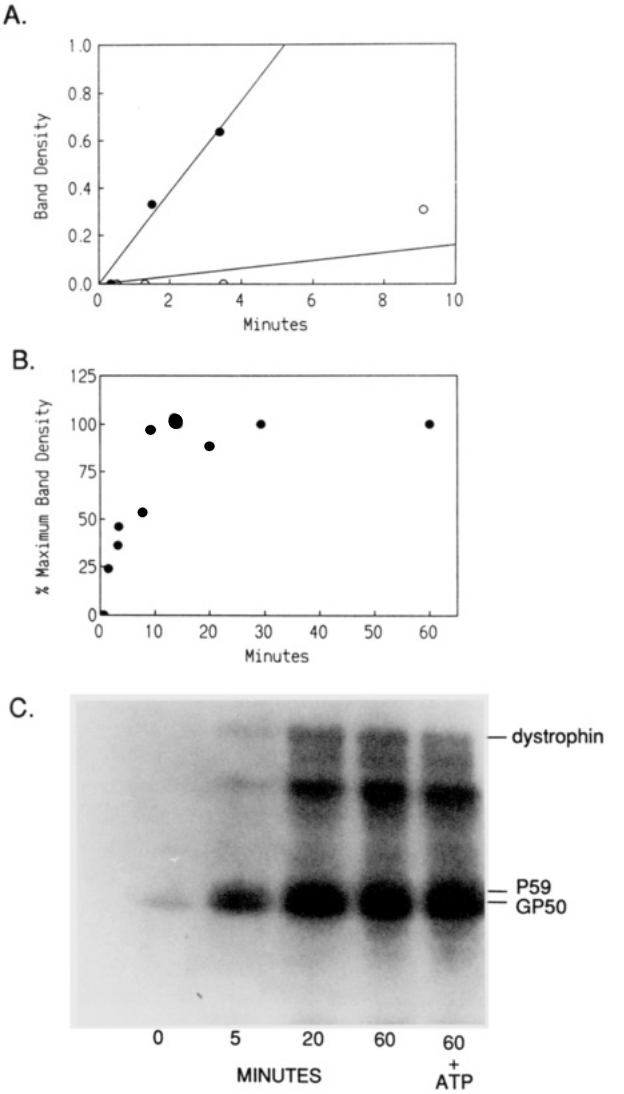

FIGURE 3: Time course of dystrophin phosphorylation and a pulse–chase experiment. (A) Plot of dystrophin's phosphorylation versus time. The data points were obtained by densitometry of the autoradiogram of the dystrophin band. Phosphorylation was in the presence of Ca^{2+}–CaM (●) or Ca^{2+}–CaM–EGTA (○) as described in Materials and Methods. (B) Time course of dystrophin's phosphorylation over a longer time period. Data from different experiments are combined here and show the phosphorylation leveling off over longer times. (C) Autoradiogram of a time course and pulse–chase experiment. The first four lanes show phosphorylation for up to 60 min in the presence of Ca^{2+}–CaM, and the last lane shows phosphorylation for 60 min, followed by addition of a 100-fold excess of unlabeled ATP, and further incubation for 60 min prior to electrophoresis.

In a 30-min reaction, Ca^{2+}–CaM increased dystrophin's phosphorylation by greater than 4-fold (Figure 1). However, Ca^{2+}–CaM causes a 12-fold enhancement in the initial phosphorylation rate of dystrophin (Figure 3A) relative to the EGTA control. The extent of phosphorylation levels off at longer times (Figure 3B).

Ca^{2+}–CaM activates several protein kinases and also activates a phosphoprotein phosphatase (phosphoprotein phosphatase 2b, calcineurin) (Klee et al., 1988). Pulse–chase experiments were carried out to determine if phosphatase activity was also present in the DGC preparation. When phosphorylation with [γ-^{32}P]ATP was followed by addition of unlabeled ATP (100-fold excess) and further incubation

for 1 h prior to electrophoresis, there was an approximately 40% decrease in the phosphorylation of dystrophin (Figure 3C). Thus a phosphoprotein phosphatase activity is present in the DGC preparation but was not characterized further in this study.

Under the conditions used (30-min incubation with Ca^{2+}–CaM), the stoichiometry of dystrophin phosphorylation ranged from 0.05 to 0.31 mol phosphate/mol dystrophin (mean ± standard deviation, 0.17 ± 0.12, $n = 9$). Stoichiometries should be integer values; however, fractional values are frequently found experimentally (King et al., 1983). This could occur if dystrophin were isolated in a partially phosphorylated state, as has been observed with other proteins (Stull et al., 1972), or if some fraction of it were unsuitable as a substrate for the protein kinase (e.g., because of denaturation or inaccessibility, etc.). Whatever the cause of the fractional values measured, it is apparent that a sizable fraction of dystrophin becomes phosphorylated under the reaction conditions used. An analysis of the cDNA-deduced amino acid sequence of dystrophin (Koenig et al., 1988) reveals several potential phosphorylation sites, which fit substrate consensus phosphorylation sequences used by different protein kinases (Pearson & Kemp, 1991). The results obtained by Luise et al. (1993) show that dystrophin is indeed phosphorylated by several sarcolemmal protein kinases, at least one of which is CaM activated. Further, using immunoprecipitation with an antidystrophin antibody, the authors found, in addition to dystrophin, phosphorylated protein bands in the 40–60-kDa molecular weight range, which is consistent with our observation of phosphorylations in the same molecular weight range. However, our results show that of the different protein kinase activities endogenous to the sarcolemma, the CaM-activated protein kinase copurifies with the DGC, while the other protein kinase activities observed by Luise et al. were not retained during DGC purification (see above, 1 μM cAMPor cGMP were without effect).

The DGC protein kinase activity clearly has several substrates within the DGC and is therefore multifunctional. Ca^{2+}–CaM activates a multifunctional protein kinase, CaM kinase II (Hanson & Schulman, 1992). Syntide-2 is a substrate for CaM kinase II (Hashimoto & Soderling, 1987). As shown in Figure 4, the DGC protein kinase activity phosphorylated syntide-2. Furthermore, a similar rate of syntide-2 phosphorylation was observed with 1 ng of baculovirus-expressed CaM kinase II-α315 (Figure 4A). The amount of DGC used in this experiment contained about 400 ng of dystrophin (data not shown) and somewhat lesser amounts of the other DGC proteins (Ervasti & Campbell, 1991). Either the DGC protein kinase phosphorylates syntide-2 with a much lower catalytic efficiency than CaM kinase II or it is a minor constitutent of the purified DGC.

Furthermore, when CaM kinase II (100 ng) is added to the DGC phosphorylation assay, it phosphorylates dystrophin to high stoichiometry. The DGC phosphorylation without added CaM kinase II was observable with 16-h exposure autoradiograms and proceeded much as shown in the other figures (data not shown). With added CaM kinase II, after 1-h exposure of the autoradiogram (Figure 4B), the phosphorylation of dystrophin is easily observed. The figure shows only the results with dystrophin, as it was the major phosphorylated band; however, p59 and gp50 are also phosphorylated, albeit to a lesser extent (data not shown). Thus, the added CaM kinase II in this experiment increases the amount of phosphate incorporated into dystrophin over that previously measured with the copurifying kinase activity alone.

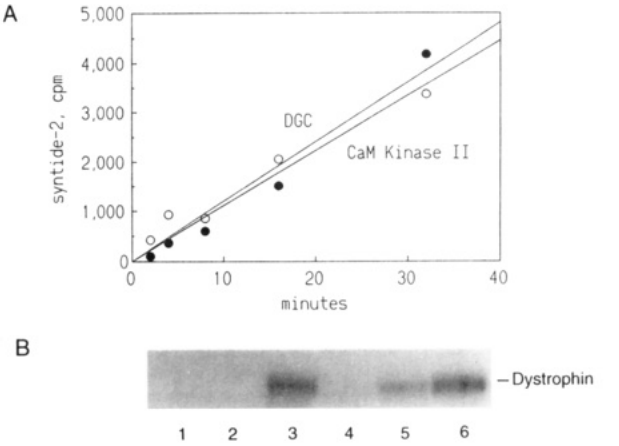

FIGURE 4: Phosphorylation of syntide-2 by the DGC protein kinase activity and the phosphorylation of dystrophin by CaM kinase II. (A) Time-dependent phosphorylation of syntide-2 by the DGC protein kinase activity and baculovirus-expressed CAM kinase II. The protein kinase activity present in DGC, containing approximately 400 ng of dystrophin, phosphorylates syntide-2 at nearly the same rate as 1 ng of purified CaM kinase II. (B) Autoradiogram showing dystrophin phosphorylated by CaM kinase II. Baculovirus-expressed CaM kinase II was added to the DGC phosphorylation mixture (20 μL, see Figure 1) to a final concentration of 0.15 μM, and the reaction was carried out for 30 min. Lanes 1 and 2 were without exogenously added kinase, lanes 3 and 4 were with the kinase, and lanes 5 and 6 were with kinase that had undergone autophosphorylation (Takeuchi-Suzuki et al., 1992). Excess EGTA was added to lanes 4 and 6. In these experiments (panels A and B), the ATP concentration was 0.1 mM (2000 cpm/pmol).

The figure also shows that the added CaM kinase II activity was dependent on Ca^{2+}–CaM (Figure 4B, lanes 3 and 4); however, upon autophosphorylation of CaM kinase II, the activity was Ca^{2+} (and presumably CaM)-independent (lanes 5 and 6). This is a characteristic trait of this enzyme (Hanson & Schulman, 1992). Interestingly, the autophosphorylated CaM kinase II phosphorylated dystrophin to a 2-fold greater extent in EGTA (determined by scintillation counting of the excised bands). These results suggest there are at least two effects of Ca^{2+}–CaM on the DGC phosphorylations: Ca^{2+}–CaM binding to dystrophin (Madhavan et al., 1992) prevents some of dystrophin's phosphorylation while also activating dystrophin phosphorylation by its action on the protein kinase. These two effects of Ca^{2+}–CaM are separable when autophosphorylation is used to activate CaM kinase II. In this regard, it should be pointed out that Ca^{2+}–CaM binding to dystrophin has recently been disputed (Ervasti & Campbell, 1993). The results in Figure 4B suggest that Ca^{2+}–CaM does bind to dystrophin.

The identity of the protein kinase(s) responsible for the DGC copurifying activity is unclear, although at least one component of this activity has substrate specificity (Figure 4) which is similar to that of CaM kinase II. The DGC phosphorylations observed could be accounted for by a single, copurifying protein kinase similar to CaM kinase II isolated in a partially phosphorylated and therefore partially CaM-independent form. This CaM-independent activity would represent about one-twelfth of the total activity (Figure 3) and could cause the phosphorylations at 50 and 175 kDa observed in the absence of Ca^{2+}–CaM (Figure 1). These proteins could be preferential or more accessible substrates, accounting for their phosphorylation at this low activity. Significant dystrophin phosphorylation (and of the band at 59 kDa) occurs only when Ca^{2+}–CaM increases the activity of this single protein kinase 12-fold (Figure 3). If this single kinase model is correct, then this kinase may represent one

of the recognized components of the DGC or another protein which copurifies with the DGC. The sequence of dystrophin, gp156, and gp43 has been reported, and no homology to known protein kinases was reported, suggesting that the kinase is either one of the other four DGC proteins or a minor constituent. Since the purification of the DGC involves isolation of a sarcolemma-enriched microsome fraction, solubilizing in detergent, affinity chromatography on a lectin agarose, and finally ion-exchange chromatography, it seems unlikely that a protein kinase would copurify with the DGC unless there were some specific association with one or more constituents of the DGC. The affinity of the kinase for its protein substrates in the DGC could account for this coisolation. This is also supported by the observation that some of the protein kinase activities present in the sarcolemma [e.g., the cAMP- and the cGMP-stimulated protein kinase activities observed by Luise et al. (1993)] did not remain associated with the DGC after its purification. It should also be pointed out that while this single kinase model could account for the data, so could the presence of more than one copurifying protein kinase.

Most of what is known about CaM kinase II has been learned in brain, or to a lesser extent liver, and little is known about its occurrence or distribution in skeletal muscle, though a CaM-dependent glycogen synthase kinase has been described (Woodgett et al., 1983). CaM kinase II is often found associated with cell membranes in high concentration (Hanson & Schulman, 1992). Furthermore, fodrin (also called calspectin), a cytoskeletal protein in brain which is homologous to dystrophin, is associated with and is phosphorylated by a Ca^{2+}–CaM-dependent protein kinase which has characteristics similar to CaM kinase II (Sobue et al., 1982).

It is interesting to note that a Ca^{2+}–CaM-dependent protein kinase activity was reported in the sarcolemma from porcine skeletal muscle. The sarcolemma contained several proteins phosphorylated in a Ca^{2+}–CaM-dependent manner including several high molecular weight proteins, one of which may have been dystrophin (Mickelson & Louis, 1985). A Ca^{2+}–CaM-dependent protein kinase which phosphorylates a 60-kDa protein in sarcoplasmic reticulum vesicles has also been reported (Campbell & MacLennan, 1982). This apparent molecular weight is within experimental error of that given for the 59-kDa phosphorylated protein reported here and may suggest that some of the phosphorylation observed here in the DGC may also occur elsewhere in other skeletal muscle membranes. Thus, Ca^{2+}–CaM-dependent protein kinase activity has frequently been found associated with skeletal muscle membranes.

These results taken together with those of Luise et al. (1993) and ours presented in this report strongly suggest that a Ca^{2+}-CaM-activated protein kinase activity is closely associated with the DGC. However, data showing the specific interaction of this protein kinase with one or more components of the DGC are not yet available.

To determine if the protein kinase activity observed in the DGC preparation shared similar features with CaM kinase II, purified DGC was probed using antibodies against CaM kinase II (Figure 5). The results show that the DGC cross-reacts with the CaM kinase II antibodies and further that DGC binds more of the antibody than a comparable amount of a crude rat brain extract. The comparison was made to brain extract because this tissue is a very rich source for CaM kinase II. Purified CaM kinase II and BSA provided positive and negative controls, respectively.

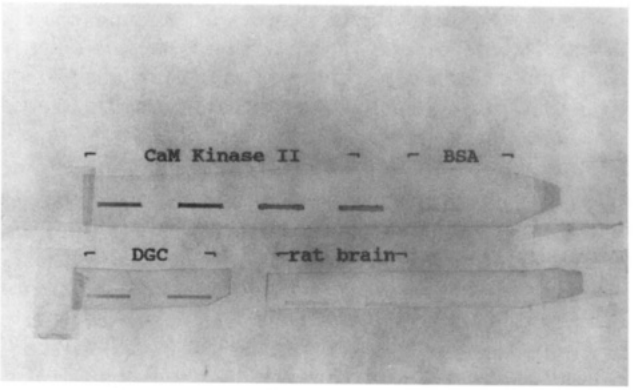

FIGURE 5: Cross-reaction of the DGC preparation with antibodies against CaM kinase II. DGC blotted onto nitrocellulose cross-reacts with antibodies raised against CaM kinase II. Two micrograms of purified CaM kinase II, DGC, a 100 000*g* supernatant of rat brain homogenate [prepared as described by Jarrett and Madhavan (1991)], or BSA was blotted onto the nitrocellulose membrane, probed using antibodies against CaM kinase II, and stained using a second antibody, horseradish peroxidase conjugate.

To learn if dystrophin is phosphorylated in a similar manner by the DGC protein kinase activity and CaM kinase II, phosphopeptide analyses were performed. Three of the peptide maps are shown in Figure 6. Panel A shows the peptide map of dystrophin eluted from a gel after phosphorylation by the DGC protein kinase activity and then trypsin digested. Panels B and C are the peptide maps of total DGC protein phosphorylated either by the endogenous protein kinase activity or purified CaM kinase II, respectively, and then trypsin digested. Of the several phosphorylated peptides observed, the one marked by the arrow is phosphorylated in all three panels. Since this peptide is derived from dystrophin (panel A), clearly dystrophin is phosphorylated at at least one site by both the DGC protein kinase and CaM kinase II. Thus, in addition to having similar substrates, the DGC protein kinase and CaM kinase II phosphorylate a common site on dystrophin. The peptide maps have large regions that are obscured in one or more maps, which makes it difficult to assess if there may also be other common sites.

Where at least one site of phosphorylation is roughly located in the dystrophin protein sequence was also investigated. CaM kinase II phosphorylates sites of the motif -R-X-X-S- (Kennelly & Krebs, 1991). There are several instances of this motif located in different regions of mouse dystrophin, but, in the sequence regions 2618–3074, there are three (beginning at 2626, 2642, and 3056). This region of dystrophin was cloned as a fusion protein and phosphorylated with either of the protein kinase activities. CaM kinase II phosphorylates this fusion protein (Figure 7), as does the endogenous DGC protein kinase activity (data not shown). The stoichiometry obtained with CaM kinase II was 0.402 ± 0.003 mol phosphate/mol DysS7 protein (mean ± standard deviation, duplicate determination). The maltose-binding sequences in a fusion with the β-galactosidase *LacZ*α peptide (i.e., "Mal-lac") is not phosphorylated and provides a negative control (Figure 7). In other experiments (data not shown), we also found no phosphorylation of a fusion protein containing dystrophin sequences 1–385. Thus, dystrophin is not phosphorylated at its amine-terminal end by CaM kinase II but is phosphorylated in the COOH-terminal end of the molecule at at least one site. It is interesting to note that of the three potential phosphorylation sites listed above, only the last two (at 2642 and 3056) are conserved in human dystrophin and are the most likely candidates for the site(s) of phosphorylation.

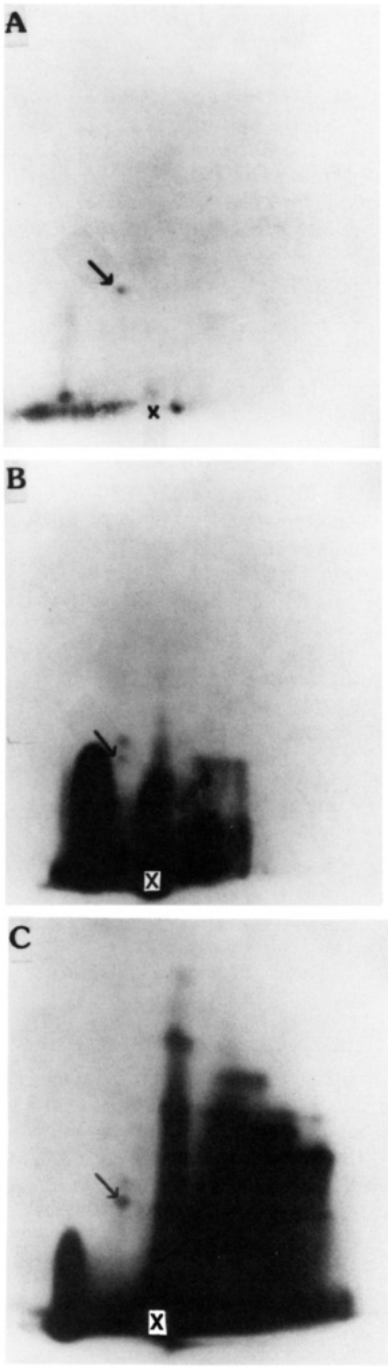

FIGURE 6: Phosphopeptide maps of dystrophin and DGC. Autoradiograms showing phosphorylated peptides derived from dystrophin and other DGC proteins. The phosphorylated proteins were digested with trypsin, and separated by electrophoresis and chromatography on TLC plates, and visualized by autoradiography. (A) Peptide map of phosphorylated dystrophin eluted from a polyacrylamide gel. The phosphorylation was carried out by the endogenous DGC protein kinase activity. (B) Phosphopeptide map of DGC phosphorylated by the endogenous protein kinase. (C) Phosphopeptide map of DGC phosphorylated by exogenously added excess CaM kinase II. In all three panels, the arrow marks a peptide derived from dystrophin and the "X" marks the origin. Phosphorylation conditions were as described in Material and Methods.

In a recent report, Milner et al. (1993) showed, using dystrophin fusion proteins, that dystrophin has phosphorylation sites for $p34^{cdc2}$ protein kinase in sequence regions 3491–3686 in its C-terminal region, and this protein kinase phosphorylates native dystrophin. Thus, sites for phosphorylation by at least two different protein kinase exist in the COOH-terminal end of dystrophin. Furthermore, they demonstrated that dystrophin phosphorylation occurs *in vivo*.

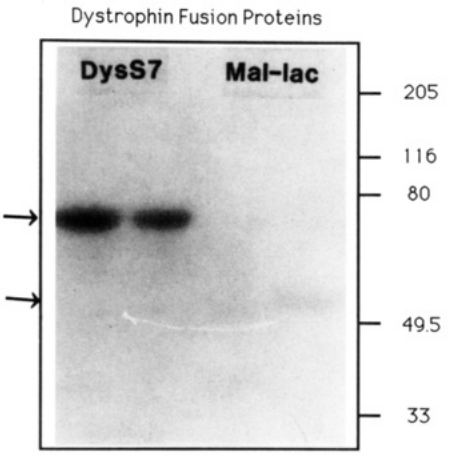

FIGURE 7: Phosphorylation of a dystrophin fusion protein by CaM kinase II. Autoradiogram of fusion proteins of the maltose binding protein and dystrophin sequences 2618–3074 (Dys S7) or *LacZα* (Mal-lac), phosphorylated by CaM kinase II. The reaction mixtures contained 2 μg of the fusion proteins, and the phosphorylation was carried out using autophosphorylated CaM kinase II for 15 min. The reaction conditions were as described previously. The M_r values of the molecular weight markers (in kilodaltons) are shown on the right side of the figure, and the arrows on the left mark the positions of the fusion proteins.

Dystrophin is homologous to spectrin and fodrin, which, like dystrophin, are cytoskeletal protein substrates for protein kinases (Reinhart et al., 1986; Sobue et al., 1982). More recently, Wagner et al. (1993) showed that an 87-kDa postsynaptic membrane protein from *Torpedo* with weak sequence homology to dystrophin's C-terminal domain is phosphorylated on tyrosine residues *in vivo* and on serine, threonine, and tyrosine residues *in vitro* and is most likely present in a complex with dystrophin. Thus, like dystrophin, its homologues are also protein kinase substrates.

The results of this study show that dystrophin is phosphorylated by a copurifying protein kinase activity in the DGC preparation which shows several similarities to CaM kinase II. Furthermore, dystrophin is phosphorylated by purified, baculovirus-expressed CaM kinase II, there is at least one phosphorylation site for this enzyme within dystrophin (Figure 6), and one or more sites in dystrophin sequences 2618–3074 are phosphorylated. In this paper, the phosphorylation of dystrophin is mainly discussed, although other phosphorylations are also observed in the DGC preparation. The identity of dystrophin as a phosphorylated protein is based upon its unique electrophoretic mobility (being a 427-kDa protein), antibody recognition of the phosphorylated protein and its proteolytic products, and the phosphorylation of its sequences produced by recombinant techniques. The 175-kDa glycoprotein is phosphorylated but was not characterized or identified. The phosphorylated bands at 59 and 50 kDa could well be the p59 and gp50 components of the DGC; however, because these are also the molecular weights of the subunits of CaM kinase II which autophosphorylate and a CaM kinase II-like activity is clearly present in the DGC preparation, some caution about their identity is warranted.

DGC components are reduced in amount or absent from dystrophic muscle tissue and in the *mdx* mouse model for Duchenne muscular dystrophy (Ohlendieck & Campbell, 1991; Ervasti et al., 1990). Other studies have shown that gp50 is deficient in severe childhood autosomal recessive muscular dystrophy (Matsumura et al., 1992). The physiological function of the DGC is not known with any certainty, but a general model has been proposed (Ervasti & Campbell, 1991). Dystrophin binds actin (Way et al., 1992), and gp156

binds laminin (Ibrahimov-Beskrovnaya et al., 1992). The DGC is thus thought to provide a link between the cytoskeleton and laminin in the extracellular matrix (Ohlendieck & Campbell, 1991; Matsumura et al., 1992).

The demonstration that dystrophin is a protein kinase substrate (this report, Luise et al. 1993, and Milner et al. 1993) and our previous report that dystrophin, gp156, and p59 bind CaM in a Ca^{2+}-dependent manner[2] strongly suggest that signal tranducing pathways function within the DGC and may play important roles in the cytoskeleton and matrix interactions of the sarcolemma of muscle.

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[2] While this manuscript was in preparation, a report was published by Ervasti and Campbell (1993), where the authors were not able to detect CaM binding to the proteins of the DGC. However, using recombinant techniques, CaM binding to dystrophin has been recently confirmed (J. L. Foster and H. W. Jarrett, personal communication).

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