Characterization of the Recombinant C-Terminal Domain of Dystrophin: Phosphorylation by Calmodulin-Dependent Protein Kinase II and Dephosphorylation by Type 2B Protein Phosphatase†

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Received December 5, 1994; Revised Manuscript Received January 31, 1995⊗

ABSTRACT: We report that the C-terminal domain of skeletal muscle dystrophin expressed as a fusion protein with glutathione *S*-transferase (designated GST-CT-1) is a substrate for Ca^{2+}/calmodulin-dependent phosphorylation and dephosphorylation. GST-CT-1 and GST-CT-1_F (GST-CT-1 truncated by 20−25 residues) were phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II). The stoichiometries of phosphorylation by CaM kinase II were 1.65 mol of P_i/mol of GST-CT-1 and 0.39 mol of P_i/mol of GST-CT-1_F, respectively, suggesting that the principal site(s) of phosphorylation is (are) located in the C-terminal 20−25 residues that are missing from GST-CT-1_F. The GST-CT-1 fusion protein was phosphorylated on both serine and threonine residues, whereas GST-CT-1_F was phosphorylated only on serine. CaM kinase II-phosphorylated GST-CT-1 and GST-CT-1_F were efficiently dephosphorylated by calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase (type 2B protein phosphatase). Importantly, calcineurin was found to be associated with a purified sarcolemmal membrane preparation enriched in dystrophin. Type 2A protein phosphatase isolated from smooth muscle (SMP-I) and its catalytic subunit (SMP-i_c) also dephosphorylated GST-CT-1, but were less active toward these substrates than was calcineurin. Type 2C phosphatase (SMP-II) and type 1 protein phosphatases [SMP-III, SMP-IV, and myosin-associated phosphatase (PP1M) of smooth muscle and skeletal muscle protein phosphatase 1c] were ineffective in dephosphorylating the C-terminal region of dystrophin. We conclude that calmodulin-dependent phosphorylation−dephosphorylation of the C-terminal domain of dystrophin may play a role in regulating dystrophin−membrane interactions and/or transducing signals from the extracellular matrix *via* the dystrophin molecule to the cytoskeleton.

Dystrophin is the 427 kDa protein product of the Duchenne muscular dystrophy (DMD)[1] gene absent from, or defective in, the muscle of DMD patients [for reviews, see Ahn and Kunkel (1993), Matsumura and Campbell (1994), and Tinsley et al. (1994)]. The function of dystrophin is not known, and therefore, its contribution to the pathology of DMD is unclear. The protein displays some similarity to the cytoskeletal proteins β-spectrin and α-actinin and has been localized to the plasma membrane in muscle tissue and in some neurons. The functional properties of many cytoskeletal proteins are controlled by phosphorylation (Bennet, 1990), and in keeping with this, dystrophin recently was shown to be an *in vivo* substrate for protein kinase(s) in rat skeletal muscle primary cell culture (Milner et al., 1993) and was shown to be phosphorylated in the sarcolemmal vesicles isolated from either rabbit fast-twitch skeletal muscle or canine cardiac muscle (Milner et al., 1993; Luise et al., 1993) and in the isolated dystrophin glycoprotein complex (Madhavan & Jarrett, 1994). In skeletal muscle, dystrophin is associated, probably *via* a portion of its C-terminus, with a complex of integral membrane glycoproteins (Suzuki et al., 1992, 1994). Madhavan and Jarrett (1994) showed that purified dystrophin glycoprotein complex contains an endogenous, Ca^{2+}/calmodulin (CaM)-dependent protein kinase [most likely Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II)], which phosphorylates dystrophin. Furthermore, they demonstrated that dystrophin and an expressed fragment of dystrophin (residues 2618−3074) fused to maltose-binding protein were phosphorylated *in vitro* by CaM kinase II at one or more serine residues and that the dystrophin glycoprotein complex also contains dystrophin phosphatase activity.

In an effort to understand the physiological role of dystrophin, one of our laboratories has been involved in studies on multisite phosphorylation of the protein, with particular emphasis on the C-terminal domain (Milner et al.,

† This work was supported by grants to M.P.W. and M.D.P. from the Medical Research Council of Canada and to M.M. from the Muscular Dystrophy Association of Canada. M.P.W. is an Alberta Heritage Foundation for Medical Research Medical Scientist. S.Y.F. is a Postdoctoral Fellow of the Alberta Heritage Foundation for Medical Research. M.M. is a Medical Research Council Scientist and Alberta Heritage Foundation for Medical Research Scholar.

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⊗ Abstract published in *Advance ACS Abstracts,* March 15, 1995.

[1] Abbreviations: CaD, caldesmon; CaM, calmodulin; CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; DMD, Duchenne muscular dystrophy; DTT, dithiothreitol; GST, glutathione *S*-transferase; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

0006-2960/95/0434-5561$09.00/0

1993), since this is an important region of the protein: loss or alteration of the highly conserved C-terminus of dystrophin results in more severe phenotypes in DMD patients (Ahn & Kunkel, 1993; Matsumura & Campbell, 1994; Tinsley et al., 1994). This region of the protein shows sequence similarity to four other proteins, all of which are closely related to dystrophin: the autosomal dystrophin-related protein (Tinsley et al., 1992); an 87 kDa phosphoprotein cloned from *Torpedo* electric organ (Wagner et al., 1993); and two alternative products of the DMD gene, one of 71 kDa (Bar et al., 1990) and one of 116 kDa (Byers et al., 1993). Within the C-terminal 201 amino acids of skeletal muscle dystrophin, there are clusters of consensus sites for phosphorylation by different protein kinases, some of which are not found in the alternative products of the DMD gene (Milner et al., 1993).

In this study, we report that the C-terminal domain of dystrophin (201 amino acid residues) is phosphorylated *in vitro* (to almost 2 mol of P_i/mol of protein) by CaM kinase II at threonine and serine residues and dephosphorylated by calcineurin, a Ca^{2+}- and CaM-dependent protein phosphatase (type 2B protein phosphatase).

EXPERIMENTAL PROCEDURES

Materials. [γ-^{32}P]ATP (>5000 Ci/mmol) was purchased from Amersham Corp. Electrophoresis reagents were purchased from Bio-Rad Laboratories. TPCK-treated trypsin (225 units/mg) was purchased from Worthington Biochemical Corp. Restriction endonucleases and DNA-modifying enzymes were obtained from Boehringer-Mannheim, Bethesda Research Laboratories, and Bio/Can Scientific. General laboratory reagents used were of analytical grade or better and were purchased from CanLab.

Protein Purification. Calmodulin (CaM) was purified from bovine brain as described by Walsh et al. (1984). Chicken gizzard caldesmon containing endogenous CaM kinase II was purified as described by Scott-Woo and Walsh (1988). The concentration of CaM kinase II in the preparation was determined by activity measurements using phosphorylated caldesmon as substrate and comparisons with the activity of purified chicken gizzard CaM kinase II, also using phosphorylated caldesmon as substrate (Ikebe & Reardon, 1990). Calcineurin was purified from bovine brain by the procedure described by Sharma et al. (1983). Calcineurin and rabbit anti-bovine brain calcineurin A subunit antibodies were generously provided by Dr. J. H. Wang (University of Calgary). Smooth muscle protein phosphatases [SMP-I, -II, -III, and -IV, the catalytic subunit of SMP-I (SMP-I_c), and the myofibril-associated phosphatase (PP1M)] and rabbit skeletal muscle type 1c phosphatase were isolated by methods previously described (Pato & Adelstein, 1983a,b; Pato & Kerc, 1985). SMP-I is a type 2A phosphatase; SMP-II is a type 2C phosphatase; and SMP-III, SMP-IV, PP1M, and skeletal muscle protein phosphatase 1c are all forms of type 1 protein phosphatases. The C-terminal domain of skeletal muscle dystrophin, designated CT-1, was expressed in *Escherichia coli* as a GST fusion protein (GST-CT-1) (Milner et al., 1992, 1993).

Phosphorylation of the C-Terminal Domain of Dystrophin. The recombinant proteins were phosphorylated in 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM NaCl, 0.2 mM $CaCl_2$, 2.5 μM CaM, 0.2 mg/mL caldesmon, 0.5 μg/mL CaM kinase II, 0.2 mg/mL GST (control) or GST-CT-1, and 0.5 mM [γ-^{32}P]ATP (100−250 cpm/pmol) at 30 °C. Reactions were started by the addition of ATP. Samples (50 μL) were withdrawn at selected times for SDS−PAGE followed by autoradiography. In some experiments, duplicate samples were withdrawn for the quantification of protein-bound phosphate (Walsh et al., 1983).

To determine the stoichiometry of protein phosphorylation, total phosphate incorporation into TCA-precipitable protein was quantified by Cerenkov counting, and the incorporation of phosphate into caldesmon, GST-CT-1, and GST-CT-1_F (a proteolytic fragment of GST-CT-1) was determined by laser densitometry of autoradiographs following SDS−PAGE and by phosphorimaging of the dried gels. Results obtained by the two methods were very similar. The following is an example to illustrate how phosphorylation stoichiometry was calculated: In the 60 min sample shown in Figure 2C, total phosphate incorporation (determined by Cerenkov counting of the TCA-precipitated protein) was 354 pmol, and of this, 129.5 pmol was incorporated into caldesmon (determined by Cerenkov counting of TCA-precipitated protein from caldesmon control in the absence of GST-CT-1_F; see Figure 2C, lane C). From densitometric scans of a Coomassie Blue-stained gel of 2, 4, and 6 μg of the protein loaded onto the gel, GST-CT-1 represented 48.1% and GST-CT-1_F represented 51.9% of the protein. The concentrations of caldesmon and the recombinant C-terminal domain of dystrophin in the reaction mixture were 0.2 mg of each/mL, and samples of 50 μL were counted at each time point. The amount of GST-CT-1 in the sample counted was, therefore, 0.2 mg/mL × 48.1% × 0.05 = 4.81 μg or 106.9 pmol. Of the 224.5 pmol of P_i, 78.8% was incorporated into GST-CT-1 (determined by densitometric scanning of the autoradiogram), i.e., 176.9 pmol. The stoichiometry of phosphorylation of GST-CT-1 was, therefore, 176.9/106.9 = 1.65 mol of P_i/mol of protein. In similar fashion, the stoichiometry of phosphorylation of GST-CT-1_F was determined to be 0.39 mol of P_i/mol and for caldesmon 1.13 mol of P_i/mol. M_r values of 87 000, 45 000, and 42 000 were used for caldesmon, GST-CT-1, and GST-CT-1_F, respectively.

Phosphoamino Acid Analysis. GST-CT-1 was phosphorylated by CaM kinase II for 60 min in a reaction volume of 250 μL. Reaction was quenched by addition of an equal volume of SDS gel sample buffer followed by boiling. The sample was loaded in four lanes (125 μL/lane) of an SDS gel for SDS−PAGE. Radiolabeled bands were identified by brief autoradiography. The bands corresponding to GST-CT-1 and GST-CT-1_F were separately cut out of the gel, lyophilized, and incubated at 37 °C with 400 μL of 1 mg/mL TPCK-treated trypsin in 50 mM NH_4HCO_3. The trypsin solution was replaced twice during overnight incubation to ensure complete digestion and extraction of all of the ^{32}P-labeled peptides from the gel slices. The extracts were lyophilized, and the residue was dissolved in 125 μL of 6 N HCl and transferred to a Pierce Reacti-Vial. Any remaining residue was dissolved in an additional 125 μL of 6 N HCl. Acid hydrolysis was carried out on a heating block at 110 °C for 2 h. The hydrolysate was transferred to a microfuge tube, the Reacti-Vial was rinsed with an additional 125 μL of 6 N HCl, and the hydrolysate was dried in a SpeedVac for 2 h. The residue was dissolved in 20 μL of H_2O. A mixture of unlabeled phosphoserine, phosphothreonine, and phosphotyrosine (2 μL) was added, and 10 μL of sample

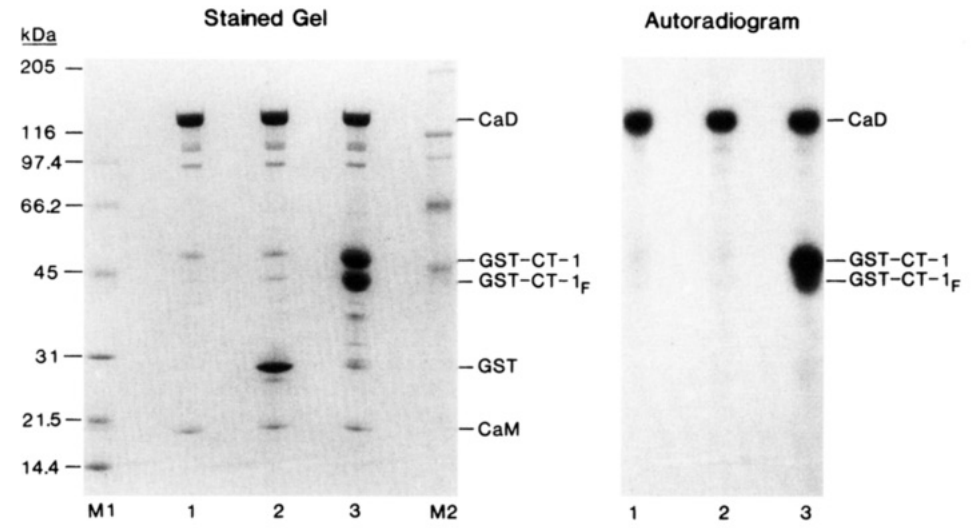

FIGURE 1: Phosphorylation of the C-terminal domain of dystrophin by CaM kinase II. Phosphorylation by CaM kinase II (60 min reaction time), SDS−PAGE, and autoradiography were carried out as described under Experimental Procedures. Lanes: M1 and M2, M_r marker proteins (masses given in kDa); 1, control, no GST-CT-1; 2, control, GST; 3, GST-CT-1. Results are representative of four independent experiments.

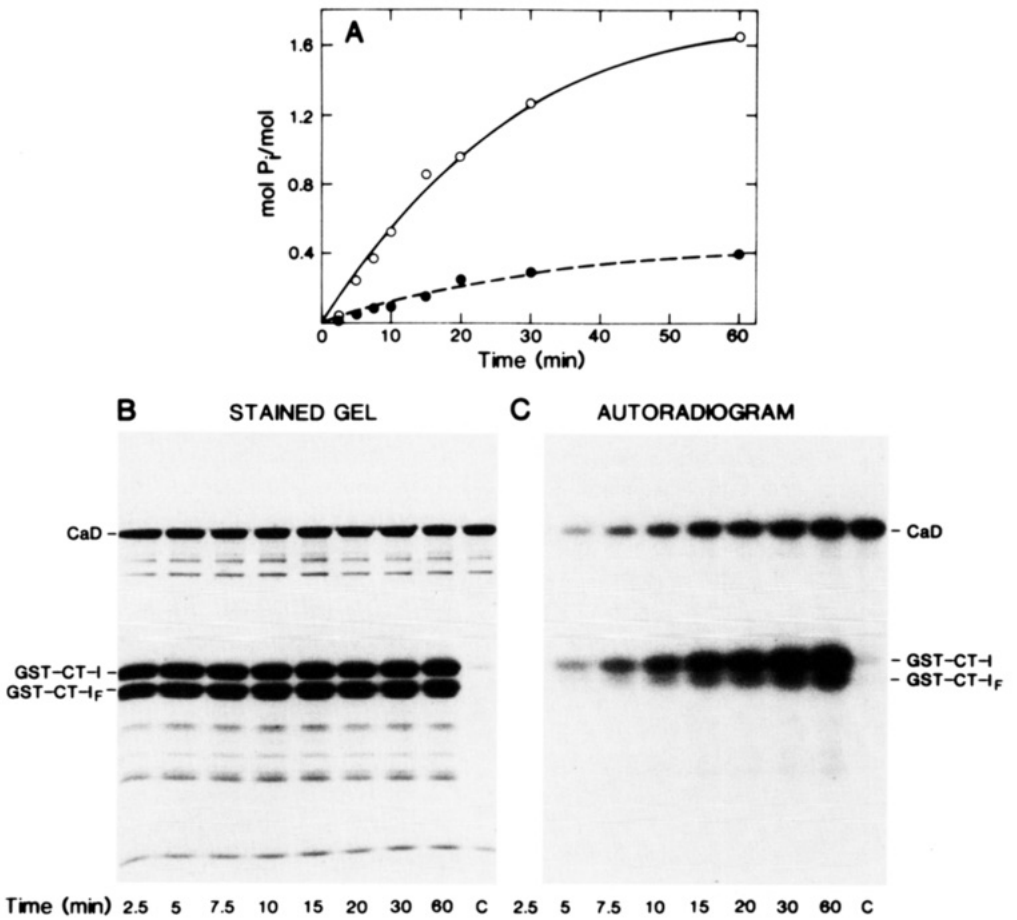

FIGURE 2: Stoichiometry and time course of phosphorylation of the C-terminal domain of dystrophin by CaM kinase II. Phosphorylation by CaM kinase II was carried out as described under Experimental Procedures. Samples were withdrawn at the indicated times for the quantification of total phosphate incorporation, SDS−PAGE, and autoradiography. (A) Time courses of phosphorylation of GST-CT-1 (○) and GST-CT-1_F (●). Stoichiometries were calculated as described under Experimental Procedures. (B) Coomassie Blue-stained gel. (C) Autoradiogram. Lane marked C is a 60 min control incubation in the absence of GST-CT-1. Results are representative of three similar but independent experiments.

was applied to thin-layer plates. Two-dimensional thin-layer electrophoresis was carried out at pH 1.9 in the first dimension and at pH 3.5 in the second dimension, as described by Cooper et al. (1983). Separated phosphoamino acids were visualized with ninhydrin, and radiolabeled phosphoamino acids derived from GST-CT-1 and GST-CT-1_F were identified by autoradiography.

Dephosphorylation Reaction. GST-CT-1 was phosphorylated by CaM kinase II as described earlier for 90 min. Phosphorylated samples were dialyzed against 20 mM Tris-

HCl (pH 7.4), 500 mM KCl, and 1 mM DTT (3 × 500 mL), followed by dialysis against 20 mM Tris-HCl (pH 7.4), 50 mM KCl, 1 mM DTT, and 1 mM PMSF (3 × 500 mL). Samples (4 × 10 μL) were withdrawn, spotted onto Whatman P81 phosphocellulose paper, washed, and counted. Dephosphorylation was carried out at 30 °C in the presence of the following phosphatases: SMP-I, SMP-I_c, SMP-II, SMP-III, SMP-IV, myofibril-associated phosphatase, and rabbit skeletal muscle type 1 phosphatase, in 50 mM Tris-HCl (pH 7.4), 1 mM DTT, and 35 μL of phosphorylated proteins in a total volume of 100 μL. The reaction was started by the addition of the appropriate phosphatase (5 μL/100 μL) diluted with bovine serum albumin (1 mg/mL). Samples (40 μL) were removed at specified times, and protein was precipitated by the addition of 100 μL of 17.5% TCA and 100 μL of bovine serum albumin (6 mg/mL). Precipitated protein was removed by centrifugation, and radioactivity in samples (0.2 mL) of the supernatants was quantified by scintillation counting. Additional samples (25 μL) were removed for SDS−PAGE analysis. In the case of dephosphorylation by SMP-II, the reaction mixture also contained 10 mM $MgCl_2$.

For dephosphorylation with calcineurin, GST-CT-1 was first phosphorylated by CaM kinase II as described earlier followed by extensive dialysis against 20 mM Tris-HCl (pH 7.0) containing activated charcoal. Activation of calcineurin (12 μg/mL) was carried out for 20 min at 30 °C in a buffer containing 1 mM $MnCl_2$, 24 μg of CaM/mL, and 80 mM Tris-HCl (pH 7.0) in a total volume of 200 μL. Activated calcineurin was added to an equal volume of dialyzed, phosphorylated GST-CT-1 and GST-CT-1_F. Samples (60 μL) were withdrawn at specified times for SDS−PAGE, followed by autoradiography.

Isolation of Membrane Vesicles from Rabbit Skeletal Muscle. Heavy microsomes were isolated from rabbit skeletal muscle as described by Ohlendieck et al. (1991). To purify sarcolemmal vesicles, the heavy microsomes (1 mg of protein/mL) were agglutinated with 1 mg/ml wheat germ lectin in a buffer containing 50 mM sodium phosphate (pH 7.4) and 160 mM NaCl, centrifuged for 90 s at 12000*g*, and deagglutinated with 200 mM *N*-acetyl-D-glucosamine (Charuk et al., 1989; Ohlendieck et al., 1991). Membrane vesicles were pelleted by centrifugation for 60 min at 120000*g*. The pellet, containing purified sarcolemmal vesicles, was suspended in 50 mM sodium phosphate (pH 7.4) and 160 mM NaCl and used for SDS−PAGE analysis.

Electrophoresis. SDS−PAGE was performed in 5%, 10%, or 7.5−20% polyacrylamide gradient slab gels (1.5 mm thick) with a 3% or 5% acrylamide stacking gel, in the presence of 0.1% SDS at 36 mA using the discontinuous buffer system of Laemmli (1970). Gels were stained with Coomassie Blue, destained, and sealed in plastic bags or dried and autoradiographed using Kodak X-Omat AR film in DuPont-Cronex cassettes fitted with DuPont Quanta III intensifying screens. Densitometry of destained gels and autoradiographs was carried out in a Pharmacia Image Master desktop scanning system. The relative degree of phosphate incorporation into protein bands was also determined using a Fujiex BAS1000 phosphorimager. For immunoblotting, membrane proteins were separated by SDS−PAGE and transferred electrophoretically onto nitrocellulose membrane (Towbin et al., 1979). Immunoblotting was carried out as described by Milner et al. (1992). Rabbit anti-dystrophin synthetic peptide antibodies (Milner et al., 1992) and rabbit anti-bovine brain calcineurin antibodies were used at 1:300 dilution. Antibody binding was detected with peroxidase-conjugated second antibodies and an ECL detection reaction (Amersham).

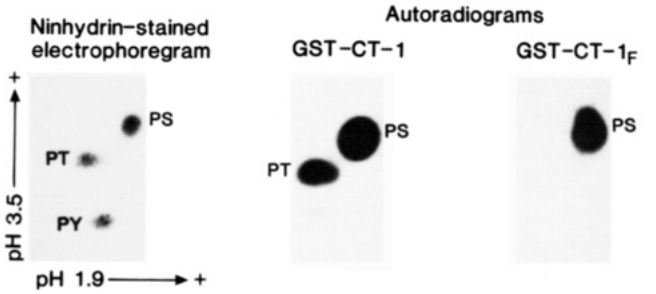

FIGURE 3: Phosphoamino acid analysis of the C-terminal domain of dystrophin. Phosphorylation by CaM kinase II followed by phosphoamino acid analysis was carried out as described under Experimental Procedures. Left panel: Ninhydrin-stained, two-dimensional thin-layer electrophoretogram to show the separation of phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine (PY). Middle panel: Autoradiogram showing both PS and PT in phosphorylated GST-CT-1. Right panel: Autoradiogram showing only PS in phosphorylated GST-CT-1_F.

Protein Determination. Protein concentrations were determined by the method of Lowry et al. (1951) or that of Bradford (1976).

RESULTS

Phosphorylation of the C-Terminal Domain of Dystrophin by CaM Kinase II. We reported earlier (Milner et al., 1993) that recombinant GST-CT-1 (the C-terminal domain of dystrophin, i.e., residues 3486−3686, fused to glutathione *S*-transferase) undergoes partial proteolysis, generating a polypeptide band (designated GST-CT-1_F) with a mobility on SDS−PAGE of approximately 42 kDa. GST-CT-1_F lacks the C-terminal 20−25 residues of dystrophin. Purified GST-CT-1 (2, 4, and 6 μg) was subjected to SDS−PAGE. Laser densitometry of the Coomassie Blue-stained gel revealed that GST-CT-1 represented 48.1% and GST-CT-1_F 51.9% of the purified recombinant protein. Figure 1 (lane 3) shows that GST-CT-1 and GST-CT-1_F were both phosphorylated by CaM kinase II. No phosphorylation of the recombinant GST was observed (Figure 1, lane 2). Caldesmon (CaD), a known substrate of CaM kinase II (Scott-Woo et al., 1990), was used as a positive control (Figure 1, lanes 1−3). In separate experiments, CaM kinase II-catalyzed phosphorylations were shown to be CaM-dependent (data not shown).

Figure 2 shows the time course and stoichiometry of phosphorylation of GST-CT-1 and GST-CT-1_F. Phosphate incorporation after 60 min of incubation with CaM kinase II was 1.65 mol of P_i/mol of GST-CT-1, 0.39 mol of P_i/mol of GST-CT-1_F, and 1.13 mol of P_i/mol of caldesmon. These results suggest that the principal site(s) of phosphorylation is (are) located in the C-terminal 20−25 residues that are missing from GST-CT-1_F.

CaM Kinase II Phosphorylates Serine and Threonine Residues in the C-Terminal Domain of Dystrophin. Phosphoamino acid analysis indicated that the GST-CT-1 fusion protein was phosphorylated on both serine and threonine residues, whereas GST-CT-1_F was phosphorylated only on serine (Figure 3). Together with the quantitative data in Figure 2, these results indicate that one of the principal

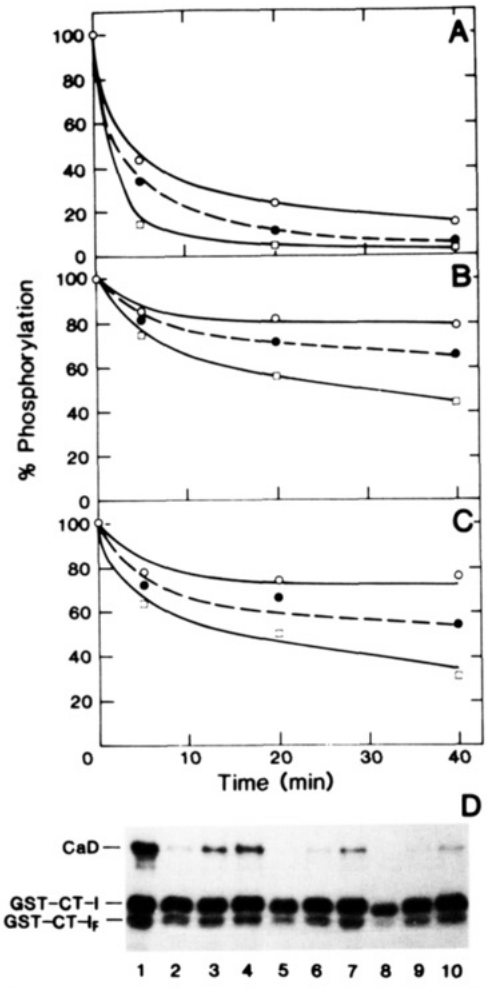

FIGURE 4: Dephosphorylation of the C-terminal domain of dystrophin by SMP-I. GST-CT-1, phosphorylated by CaM kinase II, was incubated with three different concentrations of SMP-I under conditions defined in Experimental Procedures. Samples of reaction mixtures were withdrawn at time zero and 5, 20, and 40 min after the addition of SMP-I for SDS−PAGE and autoradiography (panel D). Protein phosphorylation was quantified using a phosphorimaging system (panels A−C). (A) Caldesmon; (B) GST-CT-1; (C) GST-CT-1_F. Symbols: ○, 3.8 μg of SMP-I/mL; ●, 7.7 μg of SMP-I/mL; □, 38.3 μg of SMP-I/mL. Values are given as the percentage of the phosphorylation level of each protein at time zero. (D) Autoradiogram: lane 1, time zero; lanes 2, 5, and 8, 38.3 μg of SMP-I/mL at 5, 20, and 40 min, respectively; lanes 3, 6 and 9, 7.7 μg of SMP-I/mL at 5, 20, and 40 min, respectively; lanes 4, 7 and 10, 3.8 μg of SMP-I/mL at 5, 20, and 40 min, respectively. Results are representative of four independent experiments.

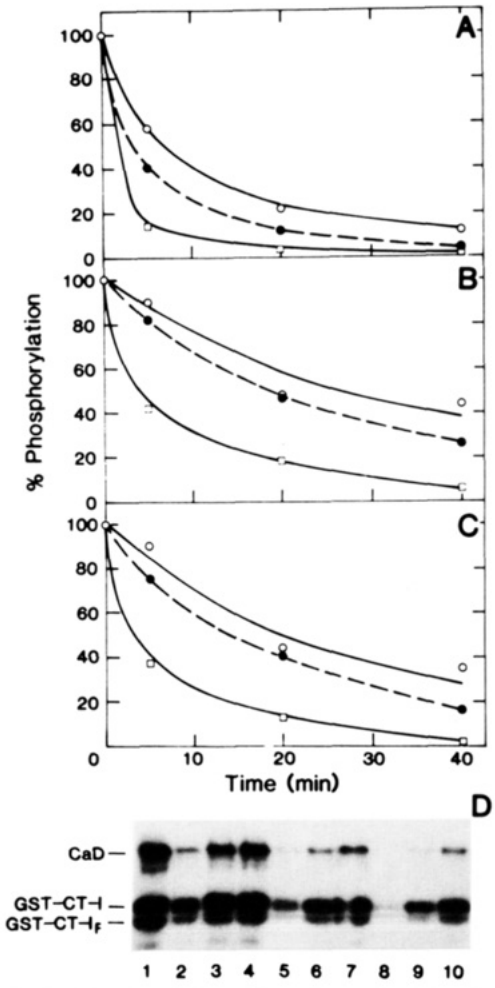

FIGURE 5: Dephosphorylation of the C-terminal domain of dystrophin by SMP-I_c. Experimental conditions and symbols are as described in the legend to Figure 4, with the exception that the concentrations of SMP-I_c used were 2.3 (○), 4.7 (●), and 23.3 (□) μg/mL. Results are representative of three similar but independent experiments.

phosphorylation sites is located very near the C-terminus of dystrophin and is probably Thr 3685, based on the site specificity of CaM kinase II.

Dephosphorylation of the C-Terminal Domain of Dystrophin. If dystrophin phosphorylation is of physiological significance, there must be a phosphatase capable of dephosphorylating dystrophin. Several phosphatases were, therefore, tested for their ability to dephosphorylate the C-terminal domain of dystrophin phosphorylated by CaM kinase II.

Figures 4 and 5 show that SMP-I (type 2A protein phosphatase isolated from smooth muscle) and SMP-I_c (the dissociated catalytic subunit of SMP-I) both dephosphorylated GST-CT-1, its proteolytic fragment, and caldesmon. Several other phosphatases [SMP-II, SMP-III, SMP-IV, and myosin-associated phosphatase (PP1M), all isolated from smooth muscle, and skeletal muscle protein phosphatase 1c] were ineffective in dephosphorylating GST-CT-1 or GST-CT-1_F. The SMP-II is a type 2C phosphatase, while SMP-III, SMP-IV, myosin-associated phosphatase, and skeletal muscle phosphatase 1c are type 1 protein phosphatases. Of these phosphatases, SMP-II, -III, and -IV dephosphorylated caldesmon, albeit at a much slower rate than did SMP-I; PP1M and PP1c, on the other hand, were virtually inactive toward phosphorylated caldesmon. The activities of all of these phosphatases were verified using known substrates (20 kDa myosin light chain phosphorylated by myosin light chain kinase for SMP-I, -II, -III, -IV, and PP1M, and phosphorylase kinase phosphorylated by cAMP-dependent protein kinase for PP1c).

Since it was apparent from the results in Figures 4 and 5 that GST-CT-1 was a relatively poor substrate of SMP-I compared with caldesmon, we considered the possibility that the C-terminal domain of dystrophin may be dephosphorylated by calcineurin, the Ca^{2+}/calmodulin-dependent type 2B phosphatase. As shown in Figure 6, calcineurin, in the presence of Ca^{2+} and CaM, dephosphorylated GST-CT-1, GST-CT-1_F, and caldesmon. The rates of dephosphorylation of all three proteins were comparable. The dependence of phosphatase activity on Ca^{2+} and CaM was verified in separate experiments (data not shown). From the initial rates

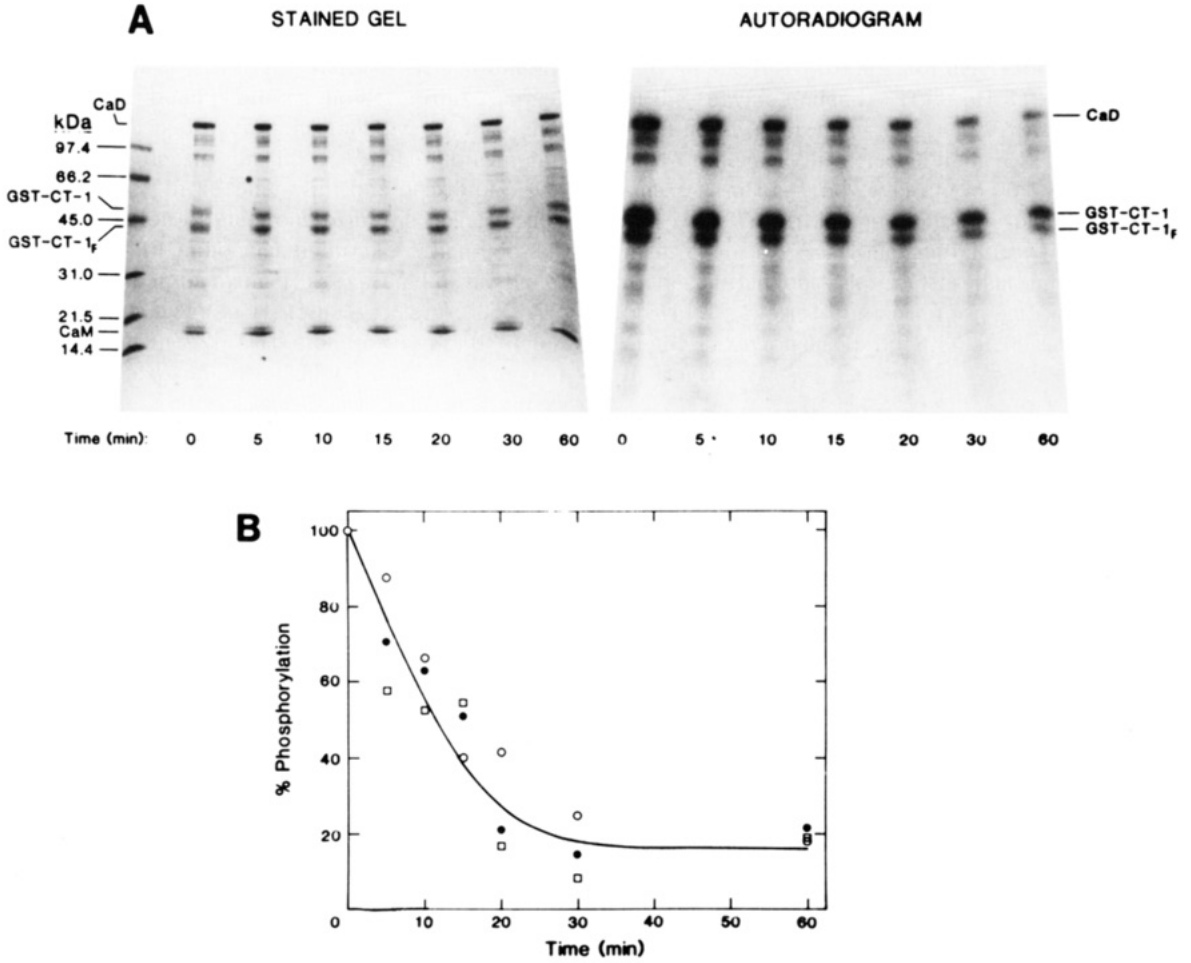

FIGURE 6: Dephosphorylation of the C-terminal domain of dystrophin by calcineurin. GST-CT-1, phosphorylated by CaM kinase II, was incubated with activated calcineurin as described under Experimental Procedures. Samples of the reaction mixture were withdrawn at the indicated times for SDS−PAGE and autoradiography (panel A). Protein phosphorylation was quantified by laser densitometry of the autoradiogram (panel B). Symbols: ○, caldesmon; ●, GST-CT-1; □, GST-CT-1_F. Values are given as the percentage of the phosphorylation level of each protein at time zero. The asterisk on the stained gel in panel A indicates the A subunit of calcineurin. Results are representative of two similar but independent experiments.

of dephosphorylation and the molar concentrations of the phosphatases used in the experiments in Figures 4−6, we calculated that the specific activity of calcineurin with GST-CT-1 as substrate is approximately 3 times that of the holoenzyme or free catalytic subunit of SMP-I.

Identification of Calcineurin in Skeletal Muscle Sarcolemmal Vesicles. Since calcineurin was the most effective phosphatase in dephosphorylating the C-terminal region of dystrophin, we investigated whether or not calcineurin was associated with muscle sarcolemma, a membrane fraction enriched in dystrophin (Ohlendieck et al., 1991). Heavy ("crude") microsomes (a mixture of sarcoplasmic reticulum, transverse tubule system, and sarcolemmal membrane vesicles) and wheat germ agglutinin-purified skeletal muscle sarcolemma were tested for the presence of dystrophin and calcineurin. Figure 7 (lanes 1 and 2) shows that both membrane preparations contained dystrophin and that the wheat germ agglutinin-purified sarcolemmal membranes contained membrane-associated calcineurin (lane 4). Considering that 100 μg of microsomal protein was loaded in lanes 1 and 3, but only 1.76 μg of purified sarcolemmal protein was loaded in lanes 2 and 4, there was a large (over 50−100-fold) enrichment in the amount of calcineurin (and dystrophin) in the sarcolemmal vesicles compared to the heavy (crude) skeletal muscle microsomes.

DISCUSSION

In this study, we have shown that the C-terminal domain of dystrophin fused to glutathione *S*-transferase (referred to as GST-CT-1) is phosphorylated by CaM kinase II and dephosphorylated by the type 2B Ca^{2+}/CaM-dependent phosphatase (calcineurin). SMP-I (a type 2A protein phosphatase) also dephosphorylated the C-terminal domain of dystrophin, but with approximately 3-fold lower activity than that observed for calcineurin. Type 2C phosphatase (SMP-II) and type 1 protein phosphatases [SMP-III, SMP-IV, smooth muscle myosin-associated phosphatase (PP1M), and skeletal muscle protein phosphatase 1c] were ineffective in the dephosphorylation of the C-terminal region of dystrophin. Phosphorylation of dystrophin *in vivo* in cultured skeletal muscle myoblasts and *in vitro* in isolated sarcolemmal vesicles, the isolated dystrophin glycoprotein complex, and purified fragments of dystrophin suggests that the protein undergoes multisite phosphorylation and that the phosphorylation of dystrophin plays an important role in the organization and function of this protein (Luise et al., 1993; Milner et al., 1993; Madhavan & Jarrett, 1994).

In skeletal muscle, dystrophin interacts *via* its C-terminal domain with a complex of integral membrane glycoproteins (Suzuki et al., 1992, 1994). Phosphorylation−dephosphor-

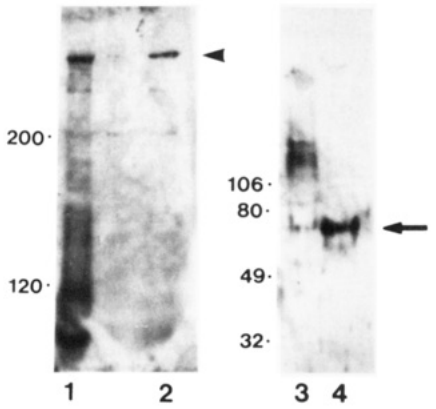

FIGURE 7: Identification of calcineurin in skeletal muscle membrane vesicles. Immunoblot analysis of heavy microsomes isolated from skeletal muscle and wheat germ agglutinin-purified sarcolemma preparations. Heavy microsomes and sarcolemmal vesicles were prepared as described in Experimental Procedures. Membrane proteins were separated by SDS−PAGE (lanes 1 and 2, 5% polyacrylamide; lanes 3 and 4, 10% polyacrylamide) followed by electrophoretic transfer to a nitrocellulose membrane and immunoblotting with polyclonal antibodies against dystrophin-specific synthetic peptide (lanes 1 and 2) and calcineurin A subunit (lanes 3 and 4). Lanes 1 and 3, heavy microsomes (100 μg of protein loaded per lane); lanes 2 and 4, wheat germ agglutinin-purified sarcolemmal membranes (1.76 μg of protein loaded per lane). The arrowhead indicates the position of dystrophin; the arrow indicates calcineurin. The positions of Bio-Rad prestained molecular weight marker proteins are indicated.

ylation of the C-terminal domain of dystrophin may play a role in regulating dystrophin−membrane interactions and/or transducing signals from the extracellular matrix *via* the dystrophin molecule to the cytoskeleton. In skeletal muscle dystrophin is bound to an oligomeric complex of dystrophin-associated proteins [for a review, see Matsumura and Campbell (1994); Tinsley et al., 1994). Six dystrophin-associated proteins have been identified in skeletal muscle sarcolemma: a 156 kDa α-dystroglycon, 59 kDa protein, 59 kDa adhelin, 43 kDa β-dystroglycan, 35 kDa glycoprotein, and 25 kDa protein (Cambpell & Kahl, 1989; Ervasti et al., 1990; Yashida & Ozawa, 1990). The α-dystroglycon interacts with laminin and may play a role in the communication between components of the extracellular matrix and the interior of a muscle cell (Matsumura & Campbell, 1994). Furthermore, α-dystroglycan interacts with agrin, a protein implicated to play a role in contact between pre- and postsynaptic cells (Campanelli et al., 1994; Gee et al., 1994; Bowe et al., 1994). Binding of agrin to α-dystroglycan may serve as a link between the extracellular matrix and the intracellular cytoskeleton responsible for the formation of acetylcholine receptor-rich postsynaptic membrane (Campanelli et al., 1994; Gee et al., 1994; Bowe et al., 1994). CaM-dependent phosphorylation and dephosphorylation of the C-terminal domain of dystrophin (utrophin) may play a role in these interactions and transduction processes; specifically, the C-terminal domain of dystrophin may play a role in communication between the membrane-associated glycoproteins and intracellular events.

Examination of the amino acid sequence of GST-CT-1 reveals two CaM kinase II consensus sites (RXXS/T; Kennelly & Krebs, 1991): Ser 3624 and Thr 3685. Another site (Ser 3538) with the sequence KGLS may also be phosphorylated. Phosphoamino acid analysis of GST-CT-1 and a proteolytic fragment of GST-CT-1 lacking the C-terminal 20−25 residues supports the conclusion that Thr 3685 is a site of phosphorylation by CaM kinase II. Madhavan and Jarrett (1994) have recently reported the phosphorylation of dystrophin by a Ca^{2+}/CaM-dependent protein kinase. The stoichiometry of this phosphorylation was determined to be 0.17 of mol P_i/mol of protein, and phosphoamino acid analysis demonstrated, in contrast to our results, the phosphorylation of serine residue only. We have shown previously that the C-terminal domain of dystrophin is susceptible to proteolysis, leading to the formation of a fragment (referred to as GST-CT-1_F) that lacks the 20−25 C-terminal residues (Milner et al., 1993). This short fragment of dystrophin may be lost during the lengthy procedures used for the purification of the dystrophin glycoprotein complex. Alternatively, the bacterially expressed GST-CT-1 may expose a site that is not available for phosphorylation in the native dystrophin, or the muscle protein kinase detected by Madhavan and Jarrett (1994) may have different site specificity than CaM kinase II. The C-terminal 20−25 amino acids of dystrophin likely play an important role in the function of dystrophin. This domain is phosphorylated not only by CaM kinase II (this study) at Thr 3685 but also by the protein kinase $p34^{cdc2}$ at Thr 3676 (Milner et al., 1993). Interestingly, neither of these consensus sites is conserved in the dystrophin-related protein (DRP), a protein that otherwise shows up to 80% amino acid identity with dystrophin within its C-terminal domain (Tinsley et al., 1992) or in the 71 kDa alternative protein product of the DMD gene (Lederfein et al., 1992). These highly specific sequence variations may well play a role in the determination of the functional differences between these three closely related proteins.

An important finding in this study is that the C-terminal region of dystrophin is a substrate for dephosphorylation by calcineurin. Calcineurin is a Ser/Thr phosphatase with relatively narrow substrate specificity, which has been implicated in the regulation of diverse physiological functions in different organisms and cell types (Klee, 1991). For example, the immunosuppressants cyclosporin A and FK506, when associated with their respective binding proteins (immunophilins), inhibit the activity of calcineurin in lymphocytes, indicating that calcineurin is an essential component of the T-cell receptor signal transduction pathway (O'Keefe et al., 1992; Clipstone & Crabtree, 1992). Calcineurin is also required for the inactivation of Ca^{2+} channels in mammalian brain cells and of K^+ channels in plant guard cells (Armstrong, 1989; Luan et al., 1993). The protein has also been implicated to play a role in the control of gene expression (Enslen & Soderling, 1994). Here we show that calcineurin may play an important role in the transduction of signals *via* the dystrophin molecule. Importantly, we show that calcineurin is associated with wheat germ agglutinin-purified sarcolemmal membranes. The association of calcineurin with human placental membrane has also been reported (Pallen et al., 1985).

Luise et al. (1993) and Madhavan and Jarrett (1994) showed that CaM kinase activity is associated with purified sarcolemmal vesicles and the purified dystrophin glycoprotein complex. CaM kinase II exhibits relatively broad substrate specificity and is thought to prolong the effects triggered by transient increases in intracellular [Ca^{2+}] (Edelman et al., 1987; Hanson & Schulman, 1992). Calcineurin and CaM kinase II have different Ca^{2+} sensitivities and

affinities for CaM, suggesting that their activities can be independently controlled (Klee, 1991). The K_{diss} values for CaM are ~0.1 and 20−100 nM for calcineurin and CaM kinase II, respectively (Klee, 1991). The possibility of dystrophin being phosphorylated and dephosphorylated by enzymes that are activated both by Ca^{2+} and CaM is, therefore, not as surprising as it may appear at first sight. Furthermore, CaM kinase II can be rendered Ca^{2+}/CaM-independent by autophosphorylation (Hashimoto et al., 1987). CaM-dependent phosphorylation (Madhavan & Jarrett, 1994) and dephosphorylation of dystrophin may play an important role in the function of this cytoskeletal protein.

In addition to its activation of protein kinases and phosphatase, CaM plays a role in the organization of the cytoskeleton by direct interaction with cytoskeletal proteins (Klee, 1991). Dystrophin is a cytoskeletal protein (Ahn & Kunkel, 1993; Matsumura & Campbell, 1994), and it is a substrate for CaM-dependent phosphorylation (Luise et al., 1993; Madhavan & Jarrett, 1994; this study) and dephosphorylation (this study). Furthermore, direct binding of CaM to dystrophin has been reported (Madhavan et al., 1992; Bonet-Kerrache et al., 1994). It is likely, therefore, that CaM plays an important role in the organization and function of this large cytoskeletal protein *via* multiple mechanisms.

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BI942804V