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# DETECTION OF A FAST ISOFORM OF C-PROTEIN WITH AN ANTISERUM DIRECTED AGAINST THE N-TERMINAL PORTION OF DYSTROPHIN

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**SUMMARY**: An antiserum raised against the N-terminal actin-binding portion of dystrophin cross-reacted with a 130-kDa protein in fast skeletal muscle. The results of purification and two-dimensional gel electrophoresis and its immunological properties demonstrated that this protein is identical to a 130-kDa basic isoform of fast-C-protein. These results suggest that the actin-binding domain of dystrophin shares one or more antigenic determinants with those of C-protein. ©1990 Academic Press, Inc.

Dystrophin is a protein product of the X-linked Duchenne muscular dystrophy (DMD) gene (1,2). The gene is the largest one ever known, comprising about 75 exons over the 2.3 megabase of the short arm of the X-chromosome. Koenig *et al.* determined the complete sequence of human dystrophin complementary DNA (cDNA) and proposed that dystrophin consists of four domains : "actin-binding domain", "triple helical segments", "cysteine-rich domain" and "C-terminal domain" (2). We have recently reported that one of the antisera against the synthetic peptide fragment of the N-terminal actin-binding domain of dystrophin cross-reacted with a 130-kDa protein which is indistinguishable from myofibrillar C-protein (3). C-protein is a structural protein, with a molecu-

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lar weight of 130-150 kDa, that is present in the A-band of vertebrate striated muscle myofibrils (4,5). It is known that this protein binds not only to myosin (6,7) but also to actin (8-10). However, its precise role remains enigmatic. To date, no homologues of this protein have been detected in any other contractile system. It is regarded to occur as three isoforms, the fast-, slow- and cardiac-types, based on differences in size, charge, antigenicity and sarcomere distribution (11-17). Polyclonal and monoclonal antibodies raised against one of these C-protein isoforms do not always cross-react with the other isoforms (11-13,17-19). This suggests that there would be major sequence differences among the various C-proteins.

In skeletal muscle, at least two isoforms, based on differences in size, were defined by immunoblot analysis with an anti-C-protein antiserum. The lower-molecular-weight form cross-reacted with an anti-dystrophin peptide antiserum against residues 215 to 264 of the dystrophin molecule.

## MATERIALS AND METHODS

Materials--- The peptide fragment used for immunization was predicted from amino acid positions 215-264 of the human dystrophin sequence deduced from dystrophin cDNA (2,20). This peptide, referred to as peptide I in this report, was supplied by Central Research Laboratories, Ajinomoto Co., Inc., Japan. It consists of 50 amino acids, PEDVDTTYPDKKSILMYITSLFQVLPQQV-SIEAIQEVEMLPRPPKVTKEE-$NH_2$, and has a molecular weight of 5,775 Da (3).

Human muscles were obtained from gastrocnemius (fast) and soleus (slow) muscles obtained from cadavers of subjects free from neuromuscular diseases.

Immunization--- Polyclonal antibodies were produced according to the previously described method (3).

Fc-18, a monoclonal antibody against C-protein, was prepared as described (21).

Polyacrylamide Gel Electrophoresis--- Polyacrylamide gel electrophoresis (PAGE) was carried out in the presence of sodium dodecyl sulfate (SDS) according to the method of Laemmli (22). Proteins were stained with Coomassie brilliant blue R250 (CBB). Two-dimensional gel electrophoresis, a combination of isoelectric focusing (IEF) and SDS-PAGE, was carried out according to the method of O'Farrell (23); and non-equilibrium pH gradient gel electrophoresis (NEpHGE) and SDS-PAGE were also performed according to the modified method of O'Farrell *et al.* (24) described by Mikawa *et al.* (25), except for the miniature scale of the system (16).

Immunoblot Analysis--- Proteins in the SDS-PAGE gel were electrophoretically transferred to a nitrocellulose sheet (0.45 $\mu$m) according to Towbin *et al.* (26), and then detected with antiserum, using an ABC kit (Vector Laboratories, Inc., USA).

C-protein preparation--- Partially purified C-protein was prepared from porcine leg muscle as described before (3).

Immunoprecipitation--- 2.8, 28 or 280 ug of peptide I was dissolved in 200 $\mu$l of distilled water. To 100-$\mu$l aliquots of crude rabbit anti-peptide I antiserum were added the diluted antigen solutions, followed by incubation for 12 h at 4°C. The immune complexes were removed by centrifugation at 10,000 g for 10 min. The resultant supernatant was used for immunoblotting as the primary antiserum.

## RESULTS

Polyclonal antibody against peptide I detected a 130-kDa protein in the partially purified C-protein (Fig. 1), and the mobility of the protein cross-reacting with anti-peptide I antibody was the same as that of the protein which reacted with Fc-18, an antibody that can detect both the fast- and the cardiac-types of C-protein (3). To clarify the fiber type specificity of the 130-kDa protein, the antiserum was reacted with adult rat soleus (slow), EDL (fast), cardiac and gastric (smooth) muscles (Fig. 2). Only EDL muscle gave a corresponding 130-kDa protein band on immunoblotting, suggesting that the cross-reactive protein is a fast type C-protein.

It has been shown that different isoforms of C-protein exist in myofibrils of different fiber types (11-17). We separated partially purified porcine C-protein by electrophoresis (Fig. 3). Two molecular species were found in the one-dimensional SDS gel

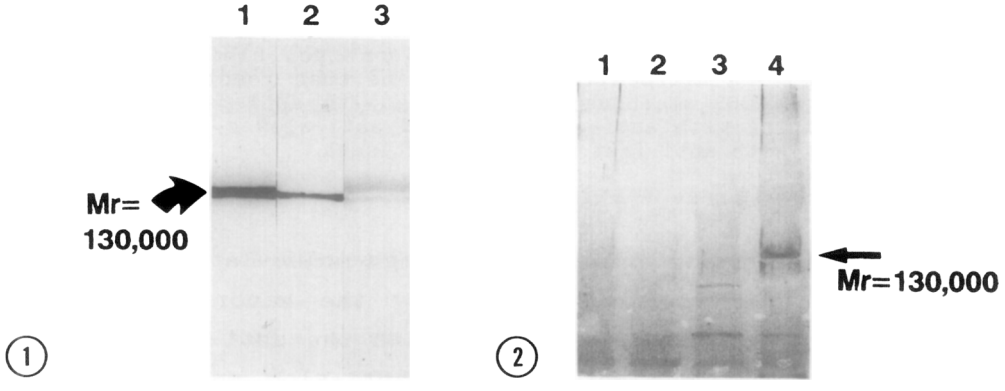


Fig. 1. **Immunoblot analysis of C-protein.** Partially purified C-protein from porcine leg muscle was electrophoresed on 6% polyacrylamide gels. The gels were blotted onto nitrocellulose sheets, followed by visualization with the anti-peptide I antibody (*Lane 1*) and Fc-18 (*lane 2*). *Lane 3* is the amidoblack staining pattern.

Fig. 2. **Specificity of the antiserum against peptide I.** Rat muscles were solubilized and analyzed by immunoblotting as described in Fig. 1. *Lane 1*, cardiac; *lane 2*, stomach; *lane 3*, soleus; *lane 4*, EDL. Several unidentified bands (Mr<100,000) can be seen in *lanes 3 and 4*.

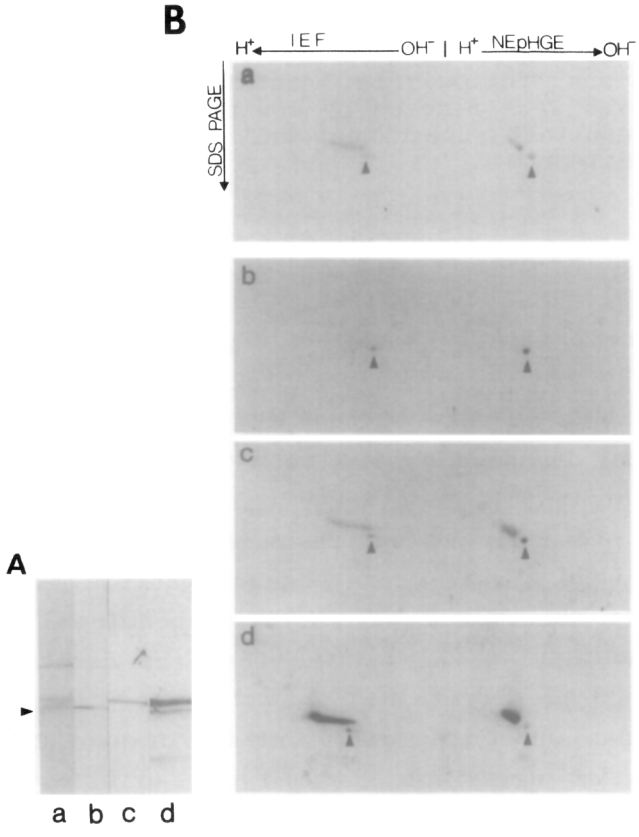


Fig. 3. **Immunoblot analysis of the proteins recognized by the antiserum against peptide I.** *A*, the partially purified porcine C-protein was electrophoresed on a 7.5 % polyacrylamide gel, and then immunoblot analysis was performed using anti-peptide I (*lane b*), anti-human slow muscle C-protein (*lane c*) and anti-human fast muscle C-protein (*lane d*) antisera. *Lane a* is the amidoblack staining pattern. *B*, the partially purified porcine C-protein was subjected to two-dimensional gel electrophoresis, transferred to a nitrocellurose sheet and then reacted with the antiserum. *a*, CBB staining; *b*, staining with anti-peptide I; *c*, double staining with anti-peptide I and anti-slow c-protein; and *d*, staining with anti-fast C-protein antisera.

(Fig. 3A); the major one was the high-molecular weight form. Anti-peptide I antiserum stained only the minor low-molecular weight form, which was also stained with the anti-fast muscle C-protein antiserum, but not with the anti-slow muscle C-protein (Fig. 3A). In the two-dimensional gel, a single, discrete spot was stained with the anti-peptide I in basic NEpHGE gels, while it migrated as a smear of protein in IEF, which may be due to phosphorylation (Fig. 3B). When the nitrocellulose sheet was stained with anti-peptide I antiserum (Fig. 3B, b) and subsequently with the anti-slow muscle C-protein antiserum (Fig. 3B, c), several additional spots of high-molecular-weight

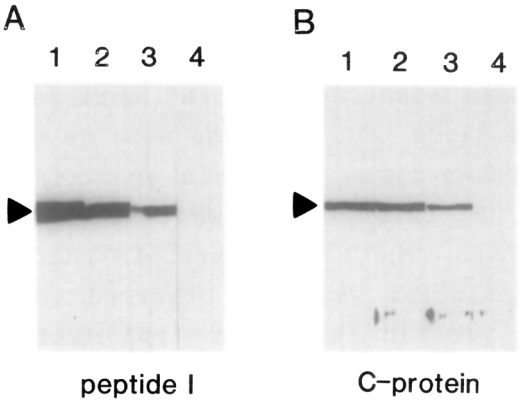


Fig. 4. Immunoprecipitation experiment. Immunoprecipitation of the anti-peptide I antiserum with peptide I antigen to determine whether or not the 130-kDa C-protein corresponds to peptide I. An adsorption experiment was performed as follows: 2.8 (*lane 2*), 28 (*lane 3*) or 280 μg (*lane 4*) of peptide I was dissolved in 200 μl of distilled water, and then 100 μl of anti-peptide I antiserum was added to each solution. This was followed by incubation for 12 h at 4°C. As a control, 100 μl of the antiserum was added to 200 μl of water, followed by incubation in the same manner (*lane 1*). After the immune complexes were removed by centrifugation, the resultant supernatants were used for immunoblotting as primary antisera. Peptide I (*A*) and partially purified C-protein (*B*) were electrophoresed on 25 % and 6 % polyacrylamide gels, respectively. After transfer to nitrocellulose sheets, immunostaining was performed with non-adsorbed antibodies.

were stained, and this staining pattern was coincident with the CBB staining pattern of total purified C-protein. These spots were also stained with anti-fast C-protein antiserum (Fig. 3B, d). This indicates that the antibody against peptide I recognizes the 130-kDa fast type C-protein. Preimmune serum did not cross-react with any myofibrillar components (data not shown). To determine whether or not this characteristic of anti-peptide I antiserum is a result of simple contamination, we performed an adsorption experiment (Fig. 4). It showed that an increase in added peptide I concentration to the antiserum caused a decrease in the staining intensity of the 130-kDa protein (B) as well as that of the antigen (A). When 280 μg of peptide I was added to 100 μl of antiserum, immunoreactivity against both disappeared.

From these results, we concluded that the peptide I antiserum actually detects one of the C-protein isoforms.

## DISCUSSION

Our preliminary report has reveald that one of the antisera against peptide I cross-reacted with a 130-kDa protein that is

indistinguishable from myofibrillar C-protein (3). When partially purified C-protein was applied to an HPLC hydroxyapatite column, the 130-kDa protein reacting with the anti-peptide I antiserum was eluted in fractions that were the same as those reacting with the fast and cardiac types C-protein specific monoclonal antibody, Fc-18 (3). In this report, we have further characterized this 130-kDa protein. The 130-kDa protein that reacted with the anti-peptide I antiserum was only observed in EDL muscle. This implies that the protein is a fast type isoform of C-protein. This possibility was supported by the results of purification and two-dimensional gel electrophoresis. We observed that the spots, which were stained with the anti-peptide I antibody, also cross-reacted with the anti-human white muscle C-protein antibody (Fig. 3A, b,d; Fig. 3B, b,d). The same spots were not stained by anti-human red muscle C-protein antibody (Fig. 3A, c; Fig. 3B, c).

As described above, we confirmed that anti-peptide I antiserum cross-reacted with a 130-kDa fast-type isoform of C-protein, but this antiserum did not cross-react with the 400-kDa dystrophin. The synthetic peptide sequence, amino acid positions 215 to 264, is located at the junction of the N-terminal "actin-binding domain" and the second "triple helical segments" of dystrophin (2). Since C-protein binds not only to myosin (6,7) but also to actin (8-10), we propose that the epitope in peptide I resides in the "actin-binding domain" of dystrophin, and that the antibody recognizes similar actin-binding sites of C-protein. This epitope may be hidden inside of the molecule by the folding of dystrophin in both the native and SDS-denatured forms, because anti-peptide I antiserum does not cross-react with the 400-kDa dystrophin. The possibility that this antibody is the result of chance contamination was ruled out by the result of an adsorption experiment. When the antiserum was adsorbed with peptide I, the resultant antiserum did not recognize C-protein in proportion to the increase in peptide I concentration (Fig. 4). These results strongly suggest that the N-terminal portion of dystrophin and one of the fast types of C-protein share antigenic determinant(s), possibly on the actin-binding domain.

In summary, we found that the actin-binding domain of dystrophin may share antigenic determinant(s) with one of the fast types of C-protein, but the physiological significance of this is not clear. Analyses of the amino acid sequences of C-protein are necessary to solve this problem.

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