Association of the M, 58,000 Postsynaptic Protein of Electric Tissue with Torpedo Dystrophin and the M, 87,000 Postsynaptic Protein*

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Dystrophin was purified by immunoaffinity chromatography from detergent-solubilized Torpedo electric organ postsynaptic membranes using monoclonal antibodies. A major doublet of proteins at M, 58,000 and minor proteins at M, 87,000, M, 45,000, and M, 30,000 reproducibly copurified with dystrophin. The M, 58,000 and M, 87,000 proteins were identical to previously described peripheral membrane proteins (M, 58,000 protein and 87,000 protein) whose muscle homologs are associated with the sarcolemma (Froehner, S. C., Murnane, A. A., Tobler, M., Peng, H. B., and Sealock, R. (1987) J. Cell Biol. 104, 1633-1646; Carr, C., Fischbach, G. D., and Cohen, J. B. (1989) J. Cell Biol. 109, 1753-1764). The copurification of dystrophin and M, 58,000 protein was shown to be specific, since dystrophin was also captured with a monoclonal antibody against the M, 58,000 protein but not by several control antibodies. The M, 87,000 protein was a major component (along with the M, 58,000 protein) in material purified on anti-58,000 columns, suggesting that the M, 58,000 protein forms a distinct complex with the M, 87,000 protein, as well as with dystrophin. Immunofluorescence staining of skeletal and cardiac muscle from the dystrophin-minus mdx mouse with the anti-58,000 antibody was confined to the sarcolemma as in normal muscle but was much reduced in intensity, even though immunoblotting demonstrated that the contents of M, 58,000 protein in normal and mdx muscle were comparable. Thus, the M, 58,000 protein appears to associate inefficiently with the sarcolemmal membrane in the absence of dystrophin. This deficiency may contribute to the membrane abnormalities that lead to muscle necrosis in dystrophic muscle.

Duchenne muscular dystrophy (DMD)‡ is a fatal genetic disease characterized by skeletal muscle necrosis and wasting (Engel, 1986). The protein product encoded by the DMD gene is a 427-kDa protein called dystrophin which has major homologies to β-spectrin and α-actinin (Hoffman et al., 1987; Koenig et al., 1988). Dystrophin occurs as part of a complex of proteins (Ervasti et al., 1991) associated with the cytoplasmic surface of the plasma membrane in normal skeletal, cardiac, and smooth muscle (Bonilla et al., 1988). It is absent or severely reduced in amount in muscle from Duchenne patients and the mdx mouse (Bonilla et al., 1988; Hoffman et al., 1985; Hoffman and Kunkel, 1989), a dystrophin-minus animal model for DMD (Bullfield et al., 1984). The function of dystrophin is unknown for certain, although it is believed to provide mechanical stability to the membrane by analogy to erythrocyte spectrin (Mandel 1989) and/or to regulate the activities of calcium leak channels (Fong et al., 1990) or calcium permeable mechano-transducing channels (Franco and Lansman, 1990). Evaluation of these hypotheses would be greatly aided by identification of the activities of proteins with which dystrophin is associated. In addition, efficient gene replacement therapy for DMD may require shortened forms of the dystrophin gene (Ac asi et al., 1991). Construction of optimal short forms will require knowledge of dystrophin binding proteins and their binding sites on dystrophin.

We have approached the question of dystrophin-associated muscle proteins through the use of acetylcholine receptor-rich postsynaptic membranes isolated from electric tissue of electric rays (Torpedo sp.). The electricogenic cells, or electroplaque, which make up the tissue are derived embryologically from immature striated muscle cells (Fox and Richardson, 1979) and retain many morphological and biochemical similarities to mammalian skeletal muscle. In particular, they contain a very large protein which is homologous to human muscle dystrophin by immunological criteria (Chang et al., 1986; Jasmin et al., 1990; Sealock et al., 1991) and deduced partial amino acid sequence (Yeadon et al., 1991). This Torpedo dystrophin appears to be confined to the innervated face of the electroplaque (Jasmin et al., 1990; Sealock et al., 1991) along with several other cytoplasmic, peripheral membrane proteins that also have homologs in muscle (reviewed in Froehner, 1991). These include a protein of M, 43,000 which is directly involved in AChR clustering (Froehner et al., 1990), and proteins of M, 58,000, 87,000, and 270,000 or 300,000 (Froehner et al., 1987; Carr et al., 1989; Woodruff et al., 1987). The M, 270,000/300,000 protein has been shown to be identified

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The abbreviations used are: DMD, Duchenne muscular dystrophy; AChR, nicotinic acetylcholine receptor; EGTA, [ethylenebis(oxethylenenitri1o)ltetraacetic acid; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; SDS, sodium dodecyl sulfate.

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tical to Torpedo dystrophin, with the discrepancy in reported molecular weights of the muscle and Torpedo proteins resulting from difficulties in gel calibration for very large proteins (Sealock et al., 1991). In previous work, we showed that the detailed distributions of dystrophin and the muscle homolog of the M, 58,000 protein are very similar in cultured Xenopus muscle (Kramarcy and Sealock, 1990), as in adult muscle (Carr et al., 1989; Froehner et al., 1987), and raised the possibility that dystrophin and the M, 58,000 protein may be associated. In this study, we demonstrate their association in detergent extracts of AChR-rich membranes from Torpedo and show that the association of the M, 58,000 protein2 with muscle sarcolemma is altered in dystrophin-minus muscle. We also provide evidence for a distinct complex containing the M, 58,000 and 87,000 proteins.

MATERIALS AND METHODS

Antibodies and Other Materials—The mAbs used in this work have been described in previous publications as follows: anti-dystrophin mAb 1351 (Froehner et al., 1987), anti-43,000 mAb 1234 (Peng and Froehner 1985), anti-AChR mAb 88B (Froehner et al., 1983), anti-87,000 mAb 20H2 (Carr et al., 1989), anti-spectrin mAb 4F8 (Bloch and Morrow, 1988), and rabbit anti-vinculin (Bloch and Hall, 1983). Torpedo nobiliana electric organ, frozen in liquid nitrogen, was obtained from Biofish, Inc. (Georgetown, MA).

Immunofluorescence Purification of the Dystrophin Complex—Torpedo acetylcholine receptor-enriched membranes were purified according to Porter and Froehner (1980) or with additional proteinase inhibitors (Sealock et al., 1991). Membranes (2 mg/ml) were solubilized in buffer A (150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM MgCl2, 2.5 μg/ml leupeptin, 2.5 μg/ml phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 mM sodium phosphate, pH 7.4, plus the proteinase inhibitor mix listed above) containing 1% Triton X-100, incubated on ice for 20 min, and clarified by centrifugation. MAb IgG-Affigel columns were prepared as previously described (LaRocchelle and Froehner, 1987). MAb IgG-Sepharose columns were prepared according to recommendations of Pharmacia LKB Biotechnology Inc. The extracts were incubated in batch with immunoaffinity resin (1–2 ml) for 1–2 h. The resins were transferred to columns and washed extensively with buffer A containing 1% Triton or buffer A/Triton followed by buffer A alone. Columns were eluted with elution buffer (0.1 mM NaHAc or with 0.1 M triethylammonium plus the proteinase inhibitor mix) at pH 11.5. Neutralized fractions were precipitated with trichloroacetic acid prior to gel electrophoresis. The different solution compositions used gave very similar results, but the yields of dystrophin complex appear to be consistently higher in the Chapel Hill laboratory (second set of conditions in each pair above).

In the experiment shown (see Fig. 2), the extract was incubated sequentially with anti-43,000 mAb 1234 IgG-Affigel 10, anti-AChR mAb 88B IgG-Affigel 10, and anti-dystrophin mAb 1351, and the columns were washed and eluted as described above.

SDS-Gel Electrophoresis and Immunoblotting—Gel electrophoresis in a nondenaturing system was performed as previously described (LaRocchelle and Froehner, 1987). For immunoblotting of dystrophin, proteins were transferred to nitrocellulose using the Tris glycine system (Sealock et al., 1991) in most cases. For all other proteins, transfer was carried out in 25 mM sodium phosphate, pH 6.5, at 250 mA for 2 h.

For analysis of M, 58,000 protein in normal and mdx mouse skeletal and cardiac muscle, lower leg muscles and the heart were dissected, frozen in liquid nitrogen, and pulverized with a cold mortar and pestle. Samples were then dissolved in 1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 mM EDTA, 2 units/ml trasyol, at a concentration of 250 μg of tissue/ml, boiled for 4 min, and centrifuged 2 min in a microcentrifuge to remove insoluble material. The protein concentration of the supernatant was determined by the Lowry method. Aliquots (75–150 μg) were subjected to gel electrophoresis and then transferred to nitrocellulose in 25 mM sodium phosphate, pH 6.5.

MABs were typically used at concentrations of 25 nm IgG. Second antibody was goat anti-mouse IgG conjugated to alkaline phosphatase (Jackson Immunoresearch; 1:500) or 125I-rabbit anti-mouse IgG (Froehner et al., 1983).

Results and Discussion—Identification of dystrophin-associated proteins in AChR-rich membranes was accomplished by Triton X-100 solubilization of the membranes followed by immunofluorescence purifications using monoclonal antibodies. After incubation with the Triton extracts, antibody columns were washed extensively, and proteins were eluted at pH 11.5. In this experiment shown in Fig. 1, dystrophin was purified from one portion of an extract using the anti-dystrophin mAb 1808, and the M, 58,000 protein was purified from a second portion using the anti-58,000 mAb 1351. When subjected to SDS-polyacrylamide gel electrophoresis, the eluate of the anti-dystrophin column contained a major protein corresponding to dystrophin, a doublet of proteins having mobility very similar to the M, 58,000 protein (Froehner et al., 1987), and weaker bands.
at mobilities corresponding to \( M_1, 140,000, 87,000, 45,000, \) and \( 30,000 \) (Fig. 1, lane 2). The two bands near \( M_2, 200,000 \) were presumably degradation products of dystrophin, since similar bands reacted with anti-dystrophin antibodies on immunoblots in previous experiments (Sealock et al., 1991). The material purified on an anti-58,000 column from the second portion of the extract contained proteins of \( M_1, 58,000 \) and \( M_2, 87,000 \) (both of which ran as doublets) and presumptive dystrophin (Fig. 1, lane 3). None of these proteins was detectable in eluates from control mouse IgG columns incubated with detergent-solubilized membranes and then washed and eluted as described above (data not shown).

Immunoblotting and additional purifications on columns containing antibodies against other Torpedo postsynaptic proteins were used to identify eluted proteins and to test the specificity of our methods. In a series of control experiments, a Triton X-100 extract of postsynaptic membranes was passed sequentially through three monoclonal antibody columns derivatized with an anti-AChR, an anti-43,000, and an anti-dystrophin mAb, respectively. Each monoclonal used (see “Materials and Methods”) was of the IgGl subclass. After extensive washes, the columns were eluted at pH 11.5, and the eluates were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting. Dystrophin and the \( M_1, 58,000 \) protein were specifically recognized by mAbs 1808 and 1351, respectively, on blots of the eluate from the third, anti-dystrophin column (Fig. 2A, lanes 2 and 5). The \( M_1, 58,000 \) protein copurified with dystrophin even when the column was washed thoroughly with \( 1 \mathrm{M} \) NaCl prior to elution at pH 11.5 (data not shown). In contrast, no AChR (Fig. 2A, lane 4) and only very small amounts of the \( M_2, 43,000 \) protein (lane 3) were detected in this fraction. The latter may not be significant, since similar amounts of the \( M_2, 43,000 \) protein were detected on immunoblots of the eluate from the anti-AChR column (Fig. 2B, lanes 3), and the \( M_2, 43,000 \) protein and AChR are known not to be associated in detergent extracts. In contrast, the \( M_1, 58,000 \) protein was not visible in the eluates from the anti-AChR or anti-43,000 columns (Fig. 2, B and C, respectively), either by Coomassie Blue staining (lane 1 in each panel) or by immunoblotting (lane 2). Both of these columns gave efficient purification of their respective antigens, however (Figs. 2B, lane 4 and 2C, lane 3). These results establish that one or both of the proteins in the copurifying doublet at \( M_1, 58,000 \) was identical to the \( M_1, 58,000 \) protein described previously (Froehner et al., 1987).

Material purified from an extract using a column derivatized with anti-58,000 mAb 1351 was subjected to immunoblotting analysis using mAb 1351, anti-dystrophin mAb 1808, and anti-87,000 mAb 20H2, which was one of the mabs used by Carr et al. (1989) to identify the \( M_2, 87,000 \) protein. The presumed \( 58,000 \) and \( 87,000 \) proteins and dystrophin were each specifically recognized by the appropriate antibody (Fig. 3, lanes 5–7). The two proteins recognized by mAb 1351 and 20H2, respectively, had identical mobilities to the proteins recognized by these mAbs in the starting membranes (Fig. 3, lanes 2 and 3). MAb 1351 failed to recognize the \( M_2, 87,000 \) protein in both the membranes and the immunopurified preparations, confirming the evidence of Carr et al. (1989) that the \( M_1, 58,000 \) and \( 87,000 \) proteins are unrelated proteins.

These data establish that the \( M_1, 58,000 \) protein can occur in a complex with dystrophin. Not all the dystrophin in the extracts was in a complex with the \( M_1, 58,000 \) protein, however, since substantial amounts of dystrophin remained in extracts which had been treated with an excess of mAb 1351 resin. Upon affinity purification using mAb 1808, this remaining dystrophin contained only very small amounts of both the \( M_1, 58,000 \) and \( 87,000 \) proteins relative to the amount of dystrophin (SDS-polyacrylamide gel electrophoresis data not shown).

**Fig. 2.** Specificity of copurification of dystrophin and \( M_1, 58,000 \) protein. Torpedo postsynaptic membranes were solubilized in Triton X-100 and the detergent extract was passed sequentially through three antibody columns derivatized with mAbs to AChR (mAb 88B), the \( M_2, 43,000 \) protein (mAb 1234) and dystrophin (mAb 1808), respectively. The columns were washed, eluted at pH 11.5, and the eluted proteins were analyzed by SDS-gel electrophoresis (lanes 1 and immunoblotting (A, lanes 2–6 or B and C, lanes 2–4). A, lane \( M_2, \) Torpedo membranes; lane 1, proteins eluted from the antidyrophin column; dystrophin, and proteins of \( M_2, 87,000, 58,000, 45,000, \) and 30,000 (arrows, top to bottom). The same sample was immunoblotted with mAbs to the \( M_1, 58,000 \) protein (lane 2), \( M_2, 43,000 \) protein (lane 3), AChR \( \gamma \) and \( \delta \) subunits (lane 4), dystrophin (lane 5), and control mouse IgG (lane 6). B, lane 1, proteins eluted from the anti-AChR column: \( \alpha, \beta, \gamma, \delta \) subunits (arrows, bottom to top); lanes 2–4, immunoblotting with same antibodies as in A. C, lane 1, proteins eluted from anti-43,000 column. Arrow indicates the \( M_2, 43,000 \) protein; lanes 2–4, immunoblotting with the same antibodies as in A.

**Fig. 3.** Immunoblotting of Torpedo membranes and the purified \( M_1, 58,000 \) protein complex. Torpedo postsynaptic membranes (lane 1) or \( M_2, 58,000 \) protein complex purified on a mAb 1351 IgG column (lane 4) were subjected to immunoblotting with anti-58,000 mAb 1351 (lanes 2 and 5), anti-87,000 mAb 20H2 (lanes 3 and 6) or anti-dystrophin mAb 1808 (lane 7). Lane 8 was incubated with control mouse IgG. Lanes 1 and 4 are Coomassie Blue stained; the lanes 2, 3, and 5–8 are immunoblots. Differences in mobility of the \( M_1, 58,000 \) and \( 87,000 \) proteins, when compared to the gels in Fig. 1, are due to different SDS gel conditions. The relatively small amount of dystrophin in the membranes could not be detected by anti-dystrophin mAb (data not shown) because the conditions used for electrophoretic transfer (25 mM NaPO₄, pH 6.5, for 2 h at 250 mA) were inefficient for large proteins.
shown). Similarly, quantitative removal of dystrophin from extracts using mAb 1808 did not remove all the M, 58,000 protein. When the remaining M, 58,000 protein was captured using mAb 1351, it contained substantial amounts of M, 87,000 protein (data not shown). Hence, the M, 58,000 and 87,000 proteins can apparently exist in a complex independently of dystrophin. The presence of small amounts of M, 87,000 protein in preparations of dystrophin (Fig. 1, lane 2) would be compatible with a ternary M, 58,000/87,000/dystrophin complex. However, quantitative gel scanning showed that the ratio of M, 87,000 to 58,000 protein was 2- to 3-fold lower in dystrophin preparations than in M, 58,000 preparations (starting with initial extracts in both cases). Much of the dystrophin-bound M, 58,000 protein is therefore not accompanied by the M, 87,000 protein, and the origins of the small amounts of M, 87,000 protein in the dystrophin preparations are not clear.

If the M, 58,000 protein is associated with dystrophin in skeletal muscle, as has been suggested by indirect evidence (Kramarcy and Sealock, 1990; Sealock et al., 1991), it could be present in reduced quantities or even absent in dystrophin-minus muscle compared to normal muscle. To test this, skeletal and cardiac muscle from mdx mice and age-matched normal mice were probed for the M, 58,000 protein by immunofluorescence and immunoblotting. As shown previously (Bonilla et al., 1988; Chang et al., 1989; Sealock et al., 1991), immunofluorescent staining for dystrophin was confined to the sarcolemma of normal skeletal muscle (Fig. 4A), with particularly strong staining at the neuromuscular junction, but was undetectable in muscle from mdx mice (Fig. 4B). The distribution of the M, 58,000 protein in normal muscle is indistinguishable from that of dystrophin (Fig. 4C). In mdx muscle, however, the intensity of anti-58,000 staining on the sarcolemma outside the neuromuscular junction was on average markedly reduced (Fig. 4D); it ranged from almost completely negative in many mdx fibers to moderately strong but noticeably less intense than in normal fibers. Similar results were obtained in comparing normal and mdx cardiac muscle (Fig. 4, E and F) and normal and Duchenne human muscle (not shown).

A consistent and notable exception to this pattern was the neuromuscular junction in skeletal muscle. The postsynaptic region of the junction (identified by staining with rhodamine-α-bungarotoxin) was consistently brightly stained by anti-58,000 mAb in both normal and mdx samples (Fig. 4, C and D).

The decrease in anti-58,000 staining in mdx muscle was not a consequence of the general loss of cytoskeletal proteins associated with the membrane, or of the physical state of the membrane. Staining of mdx muscle with antispectrin was only slightly reduced and somewhat more diffuse than in normal muscle (Fig. 5, A and B). Similar results were obtained with anti-α-actin antibodies (not shown). In double label immunofluorescence for the M, 58,000 protein and vinculin in mdx muscle, antivinculin staining was essentially unaffected (Fig. 5, C and D), even in regions that were almost completely negative for the M, 58,000 protein (Fig. 5, E and F). These results indicate that the M, 58,000 protein is selectively lost in dystrophic muscle.

On immunoblots of normal and mdx mouse muscle, mAb 1351 specifically recognized a single protein in both skeletal and cardiac muscle which migrated slightly more slowly than the Torpedo M, 58,000 protein (Fig. 6). The amounts of this muscle M, 58,000 protein in normal versus skeletal (hind leg) muscle (Fig. 6, upper panel) and in normal versus cardiac muscle (Fig. 6, lower panel) appeared in most experiments to be very similar or indistinguishable by this method. In the experiment which showed the greatest divergence between normal and mdx muscle (shown in Fig. 6), the amounts of M, 58,000 protein were markedly reduced.

**FIG. 4.** Distribution of M, 58,000 protein in mouse skeletal and cardiac muscle. Immunofluorescence of diaphragm muscle (A–D) and cardiac muscle (E, F) from normal mouse (A, C, E) or mdx mouse (B, D, F). Cryostat sections were incubated with anti-dystrophin mAb 1808 (A, B), or with anti-58,000 protein mAb 1351 (C, D) followed by biotinylated anti-mouse IgG and fluorescein-avidin. For cardiac sections (E, F), FITC-mAb 1351, followed by rabbit anti-FITC and FITC-goat anti-rabbit IgG was used. Arrows in A, C, and D indicate neuromuscular junctions. Bar in F, 12 μm.

**FIG. 5.** Distribution of spectrin, M, 58,000 protein, and vinculin in skeletal muscle. Immunofluorescence of control (A, C, E) or mdx (B, D, F) mouse gastrocnemius muscle is shown. Cryostat sections were incubated with anti-spectrin mAb 4F8 followed by FITC anti-mouse IgG (A and B). The two bottom panels (C–F) are double labeled with a mixture of rabbit anti-vinculin and mouse anti-58,000 protein mAb 1351 followed by rhodamine-labeled goat anti-rabbit IgG (shown in C and D) and fluorescein-labeled goat antimouse IgG (shown in E and F). Bar in D, 20 μm.
58,000 protein in mdx muscle appeared to reduced at most by about 50%. These data suggest that the abundance of the M, 58,000 protein does not depend to a large extent on the presence of dystrophin.

DISCUSSION

Using monoclonal antibodies, we have shown that Torpedo dystrophin purified by anti-dystrophin immunoaffinity chromatography is accompanied by the M, 58,000 protein. Similarly, dystrophin copurifies with the M, 58,000 protein isolated with an anti-58,000 antibody. Since neither protein was captured with control antibodies or with antibodies against other major proteins in these membranes (M, 43,000 protein and AChR), these findings constitute strong evidence that dystrophin and the M, 58,000 protein exist in a complex. The two proteins also occurred independently in our Triton extracts, since each could be captured alone after complete removal of the other. These individual entities may reflect dissociation that occurs after membrane solubilization, or may represent physiologically important states of these proteins.

Our second finding was that immunoaffinity-purified M, 58,000 protein contained large quantities of the M, 87,000 postsynaptic protein described by Carr et al. (1989). This was true whether or not dystrophin was removed from extracts prior to capture of the M, 58,000 protein. Hence, the M, 58,000 protein apparently forms a separate complex with the M, 87,000 protein, a possibility which has not been previously suspected. The M, 87,000 protein was present in most of our dystrophin preparations (i.e. dystrophin prepared using antidystrophin columns), but the amounts were low. In the absence of crossed immunoaffinity purification (the use of anti-87,000 columns to purify dystrophin), it is difficult to be certain that this copurification was due to a specific associa-

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tant to determine whether these differences arise for technical reasons or because of species, tissue, or isoform differences.

Our finding that the M, 58,000 protein can occur in Triton extracts of postsynaptic membranes independently of dystrophin may be related to the fact that it is found in tissues such as kidney that express dystrophin at only very low levels.4 Clusters of AChR in cultured muscle are also rich in the M, 58,000 protein (Froehner et al., 1987) but, at least in Xenopus muscle, contain little dystrophin (Sealock et al., 1991). In dystrophic muscle, immunofluorescent staining of the neuromuscular junction with mAb 1351 remained bright while staining of the extrajunctional sarcolemma was weak. The M, 58,000 protein in mammalian tissues therefore appears to interact with additional proteins, possibly including the M, 87,000 protein and other members of the dystrophin family. Candidates in the latter include the muscle isoform of β-spectrin described by Bloch and Morrow (1989) and the dystrophin-related protein described by Fardeau et al. (1990), both of which are concentrated at neuromuscular junctions. Dystrophin-independent activities for the M, 58,000 protein may explain the partial reduction of M, 58,000 protein content and sarcolemmal incorporation in dystrophic muscle, in contrast to the near total absence of the dystrophin-associated M, 156,000 glycoprotein studied by Ervasti et al. (1990).

The function of the M, 58,000 protein is not known, and sequence analyses of Torpedo and mouse skeletal muscle cDNAs encoding the M, 58,000 protein have not revealed homology to known proteins.5 Its diminished association with the sarcolemma in dystrophic muscle may, however, contribute to the pathology of muscular dystrophy. Abnormalities in the function or expression of the M, 58,000 protein could also potentially be the primary defect in other muscle pathologies in which dystrophin is normal.

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