Functional Defects of a Muscle-specific Calpain, p94, Caused by Mutations Associated with Limb-Girdle Muscular Dystrophy Type 2A*

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Calpain (EC 3.4.22.17), Ca²⁺-dependent cysteine protease, is a major intracellular protease thought to regulate the cellular function by hydrolyzing substrates in a highly limited manner (1). Previously identified μ- and m-calpains exist as heterodimers consisting of a unique large catalytic subunit and a common small regulatory subunit. The large subunit can be divided into four structural domains; the second and the fourth domains (domains II and IV) are cysteine protease and Ca²⁺ binding domains, respectively. The small subunit is composed of an NH₂-terminal Gly-rich hydrophobic region (domain V) and a Ca²⁺ binding domain similar to domain IV of the large subunit (domain IV or VI).

In addition to these two species, which are ubiquitously expressed in almost all mammalian tissues, several novel species of the calpain large subunit family expressed in a tissue-specific manner have been cloned (2, 3). Thus, it is now established that calpains, at least in mammals, can be classified into two groups, ubiquitous and tissue-specific species, and that the latter should have tissue-specific functions that cannot be accomplished by the former.

p94 (calpain3), a muscle-specific member of the calpain family, has been shown to be responsible for limb-girdle muscular dystrophy type 2A (LGMD2A), a form of autosomal recessive and progressive neuromuscular disorder. To elucidate the molecular mechanism of LGMD2A, we constructed nine p94 missense point mutants found in LGMD2A and analyzed their p94 unique properties. All mutants completely or almost completely lose the proteolytic activity against a potential substrate, fodrin. However, some of the mutants still possess autolytic activity and/or connectin/titin binding ability, indicating these properties are not necessary for the LGMD2A phenotypes. These results provide strong evidence that LGMD2A results from the loss of proteolysis of substrates by p94, suggesting a novel molecular mechanism leading to muscular dystrophies.

p94 (calpain3) has three specific insertion sequences, NS, IS1, and IS2. Contributions of these sequences, especially IS2 which includes a nuclear translocation signal, to p94 specific function are presumed.

The recent discovery that the p94 gene is responsible for limb-girdle muscular dystrophy type 2A (LGMD2A) supports the idea that p94-specific function is indispensable for normal muscular function (5). LGMD2A is a form of autosomal recessive LGMD, a genetically heterogeneous group of inherited progressive neuromuscular disorders (6, 7). In LGMD2C, -2D, -2E, and -2F, a deficiency in one of the sarcoglycan molecules results in the down-regulation of the whole sarcoglycan complex and the subsequent destabilization of membrane structure (8–13). This is consistent with the fact that deficiencies in structural proteins around the sarcolemma are commonly observed in all muscular dystrophies so far identified except LGMD2A (14).

To investigate the correlation between p94 function and LGMD2A pathology, we tried to evaluate the effects of p94 mutation in LGMD2A focusing on the following p94-specific features, which suggest its pivotal role in protein turnover and/or in function of muscle. First, although p94 is expressed abundantly at the mRNA level in skeletal muscle, little is detectable as protein because of its very rapid autolysis. Moreover, p94 autolyses even in the presence of excess EGTa, E-64, leupeptin, or calpastatin, a specific proteinaceous inhibitor of conventional calpain (4). This rapid and complete autolysis discriminates p94 from other cysteine proteases including calpains. Second, p94 associates with connectin/titin. Connectin/titin is a gigantic muscle protein in charge of muscle elasticity and structure of myofibrils (15–17). Using the yeast two-hybrid system, we found that p94 binds to the C terminus and the N2A region of connectin/titin (18), suggesting the regulation mechanism of p94 function and myofibril turnover (19).

In this study, we report that all the p94 point mutants found in LGMD2A examined so far completely or almost completely lose the fodrin proteolysis activity and that all the mutants show changes in autolytic activity and/or connectin/titin binding activity. These results indicate that the loss of p94 protease activity rather than its autolytic activity underlies the molecular mechanism of LGMD2A.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Site-directed Mutagenesis—Wild type human p94 cDNA was constructed into pSRD or pAS2–1 (20–22) for protein expression. Polymerase chain reaction-based mutagenesis using the following primers was performed.

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§ The abbreviation used is: LGMD2A, limb-girdle muscular dystrophy type 2A.
RESULTS

Connectin/titin Binding Activity of Point Mutants—As shown in Fig. 1, various point mutations of p94 have been identified in LGMD2A patients (5, 26), and these are expected to abolish one or two but not all of various p94 characteristics described in the Introduction. To clarify which p94 properties are most related to LGMD2A, we constructed 9 of these missense mutations, L182Q, G234E, P319L, H334Q, and V354G in domain II, R490W and R572Q in domain III, and S744G and R769Q in domain IV by site-directed mutagenesis.

One question is whether the association with connectin, a unique property of p94, is affected in LGMD2A. Thus, we examined the binding activity of p94 mutants to the clones, pCNT-N2 and pCNT-52 (18), which correspond to the N2A region and the C terminus of human connectin, respectively, using the yeast two-hybrid system.

Wild type and C129S human p94 bound to both pCNT-N2 and pCNT-52, as previously reported (Fig. 2, B and D, 12 and 11). As for the mutants, P319L bound to pCNT-52 but not to pCNT-N2 (Fig. 2, B and D, 4), whereas G234E, R572Q, S744G, and R769Q bound only to pCNT-N2 (Fig. 2, B and D, 3 and 8–10). L182Q and V354G bound to neither pCNT-52 nor pCNT-N2 (Fig. 2, B and D, 2 and 6), but H334Q and R490W associated with both pCNT-N2 and pCNT-52 (Fig. 2, B and D, 5 and 7) as in the case of wild type. These results unexpectedly showed that the inability to bind connectin is not necessarily required for LGMD2A.

Expression of Point Mutants Found in LGMD2A in COS7 Cells—To examine whether LGMD2A arises from the loss of p94 autolytic activity, we used a COS expression system previously described (4). In this system, wild type p94 is hardly detected as a 94-kDa band because of its rapid autolysis but, instead, as a 55-kDa autolyzed product, whereas an active-site mutated inactive p94, C129S, is clearly detected as a stable 94-kDa translated product (Fig. 3, lanes 2 and 3). Thus, it is concluded that p94 is degraded by itself but not by other endogenous proteases in COS7 cells as we previously reported (4).

In the same system, 94-kDa protein products were also observed for the mutants, L182Q, G234E, P319L, H334Q, V354G, R490W, and R572Q (Fig. 3, lanes 4–10). For R572Q, a faint
55-kDa fragment was detected in addition to the clear 94-kDa band, indicating its residual autolytic activity (Fig. 3, lane 10). Thus, these mutants have lost their rapid autolyzing activity as in the case of C129S.

On the other hand, the remaining mutants, S744G and R769Q, were not detected even as degradation fragments (Fig. 3, lanes 11 and 12). To confirm the expression of these two mutants, we introduced a second mutation, C129S, into these constructs. The artificial double mutants, S744G/C129S and R769Q/C129S, expressed the 94-kDa product as much as C129S (Fig. 3, lanes 13 and 14), indicating that both S744G and R769Q disappeared because of autolysis that is possibly even more rapid than wild type p94.

These results showed that the loss of very rapid autolytic activity unique to p94 is not a necessary condition leading to LGMD2A but, at the same time, that none of these mutants possess the autolytic activity identical to that of wild type p94.

Reduced Autolytic Activity by the Mutations R490W and R572Q—It looks strange that R572Q mutant, whose mutation localizes in domain III not in the protease domain, showed severely reduced autolytic activity. To clarify the mechanism of the reduction of activity by mutation of domain III, autolysis profiles of R572Q as well as R490W were examined in detail in comparison with wild type and C129S. As our previous studies showed (4), wild type p94 expressed in COS7 cells continues to degrade autocatalytically in the presence of 10 mM EDTA (Fig. 4A). That is, the 55-kDa fragment of wild type p94 disappeared depending on time (Fig. 4A, wild type, lanes 1–4). A similar degradation of the 55-kDa fragment was observed in the presence of Ca\(^{2+}\) (Fig. 4B, wild type, lanes 1–4). These results showed apparent Ca\(^{2+}\)-independent autolytic activity of wild type p94. On the other hand, the 94-kDa product of C129S does not undergo remarkable degradation in the presence of EDTA or Ca\(^{2+}\) (Figs. 4A and B, C129S, lanes 1–4), indicating that endogenous proteases can be ignored in this system.

In contrast, R490W and R572Q showed Ca\(^{2+}\)-dependent degradation (Figs. 4A and B). Although the velocity of the degradation is different, the 94-kDa product degraded efficiently to the 55-kDa and/or the smaller fragments only in the presence of Ca\(^{2+}\), but not in the presence of EDTA (Fig. 4A, and B, R490W and R572Q, lanes 1–4). These results suggest that the R490W and R572Q mutations reduce the autolytic activity by lowering their Ca\(^{2+}\) sensitivity. In other words, R490W and R572Q mutations possibly affect interaction between domain II and domain IV, resulting in alteration of the Ca\(^{2+}\) sensitivity and the reduction of the autolytic activity.

Other mutants, L182Q, G234E, P319L, H334Q, and V354G, which lost rapid autolyzing activity (Fig. 3, lanes 4–8), were...
examined on the alteration of the Ca$^{2+}$-sensitivity by the same method. The presence of Ca$^{2+}$ did not compensate for the loss of rapid autolyzing activity for any of these mutants (data not shown).

**Enhanced Autolytic Activity by the Mutations in Domain IV, S744G and R769Q**—To further confirm the existence of the proteolytic activity of S744G and R769Q, intermolecular C129S p94 processing activity was examined by coexpression of these mutants with C129S. Wild type p94 has been shown to proteolyze other C129S p94 molecules when coexpressed with C129S in COS7 cells as previously reported (18). When C129S and wild type p94 were coexpressed in COS7 cells and extract of the cells cultured for 60 h were immediately subjected to Western blotting without further incubation, intermolecular cleavage of C129S and production of the 55-kDa proteolyzed fragment were detected by anti-IS2 antiserum (Fig. 5, lane 3). In the same manner, when S744G or R769Q was coexpressed with C129S, similar intermolecular cleavage was observed (Fig. 5, lanes 4 and 5), indicating that these two mutants possess protease activity comparable with that of the wild type. Thus, the absence of the autolytic 55-kDa fragment for S744G and R769Q (Fig. 3, lanes 11 and 12) is interpreted as meaning these mutants autolyze more rapidly than wild type p94, with the degradation fragments disappearing almost completely.

Because the NH$_2$ terminus of the 55-kDa fragment was recently determined to be in the IS1 region, it is predicted that the mutant P319L in IS1 lost rapid autolytic activity (Fig. 3, lane 6) because of the altered susceptibility of IS1 as a target for autotaxic cleavage but retains protease activity. Coexpression of C129S with P319L generated the 55-kDa proteolyzed fragment showing intermolecular cleavage of C129S by P319L (Fig. 5, lane 7). R572Q, for which residual autolytic activity was demonstrated (Figs. 3 and 4), were also subjected to coexpression with C129S. R490W also has intermolecular C129S processing activity (Fig. 4, lane 7). R572Q generates the 55-kDa fragment when expressed by itself (Fig. 3, lane 10), and thus it was impossible to evaluate its effect on the intermolecular processing activity in this system.

**Deficient Proteolysis of the Fodrin a Subunit**—In contrast to our expectations, the nine missense mutants examined showed diverged properties concerning autolytic activity and connectin/titin binding ability as discussed below, although none had properties identical to those of the wild type. This is apparently contradictory to the fact that all nine mutations result in a common phenotype. To seek a property that is altered in all nine mutants, we further analyzed the protease activity of p94 against possible substrates.

It is well established that fodrin is a good substrate for conventional calpains. When calpain digests the fodrin alpha subunit, the 230-kDa full-length molecule is proteolyzed to 150-kDa fragment (27, 28). When wild type p94 is expressed in COS7 cells, the 150-kDa fragment is detected by an antibody to the NH$_2$-terminal sequence of the calpain-catalyzed proteolytic fragment of the fodrin alpha subunit (Fig. 6, lane 2, closed arrowhead), showing significant fodrinolysis in the cytosol (25). In contrast, COS7 cells transfected by C129S or vector only showed no proteolytic fodrin fragment (Fig. 6, lanes 1 and 3). These results clearly indicate that in vivo fodrinolysis is greatly promoted by the expression of wild type p94, if not by p94 itself. In other words, p94 is involved either directly or indirectly in the proteolysis of fodrin by calpain in cultured cells.

The expression of other mutant molecules does not generate the 150-kDa proteolyzed fragment of fodrin as efficiently as that of wild type p94 (Fig. 6, lanes 4–12). Surprisingly, cells transfected by S744G or R769Q, which retains autolytic activity and C129S-hydrolyzing activity comparable with wild type (Fig. 3, lanes 11 and 12, Fig. 5, lanes 4 and 5), showed almost undetectable 150-kDa fodrin fragment as shown in Fig. 6, lanes 11 and 12. The result clearly shows that all nine mutants are almost, if not completely, inactive as to fodrinolysis like the active-site mutant, C129S (Fig. 6, lane 3). It is concluded that mutations found in LGMD2A commonly affect the proteolysis by p94.

**DISCUSSION**

In this study, we examined the properties of p94 point mutants found in LGMD2A (5, 26), and identified a complete or nearly complete loss of in vivo fodrinolysis as a common property of these nine mutants. None of the mutants were identical to wild type p94 as summarized in Table I. Namely, all mutants show a deficiency in autolytic activity and/or binding ability to the N2A and/or C-terminal regions of connectin/titin. Based on the fact that only COS7 cells transfected with wild type human p94 produce substantial amounts of the 150-kDa proteolyzed fodrin fragment, proteolysis of p94 substrates, if not fodrin, is certainly altered in all mutants.

The involvement of p94 activity in the proteolysis of fodrin observed in our experiments is either direct or indirect. If p94 cleaves fodrin in the same manner as $\mu$- and m-calpains (25),
the complete or nearly complete loss of this proteolysis observed for all mutants provides direct evidence that insufficient proteolytic reaction mediated by p94 causes LGMD2A. Alternatively, p94 may regulate fodrinolysis indirectly, i.e. proteolysis of unidentified p94 substrates finally results in the activation of endogenous calpain(s), possibly m-calpain in COS7 cells. In this case, the reduction in the amount of final product, the 150-kDa fragment, can be attributed to a defect in the step catalyzed by p94. Either case is consistent with the idea that a loss of proper protease function is a mechanism for the etiology of LGMD2A, which has been proposed from the existence of mutant species lacking the protease domain.

Our results provide evidence that unique characteristics of p94 distinct from ordinary calpain are functionally important. Our finding that R490W and R572Q can autolyze efficiently even at very low concentrations such as the sub nanomolar level. It is interesting that these mutations in do- cause a 9-fold increase in the Ca2+ requirement of calpain show association with the small subunit (18). The cause a 9-fold increase in the Ca2+ requirement of as efficiently as wild type p94, means that a too rapid autolysis causes functional defect. This is not unrelated to rapid autolysis as efficiently as wild type p94, but cannot catalyze autolysis of unidentified p94 substrates finally results in the activation of endogenous calpain(s), possibly m-calpain in COS7 cells. In this case, the reduction in the amount of final product, the 150-kDa fragment, can be attributed to a defect in the step catalyzed by p94. Either case is consistent with the idea that a loss of proper protease function is a mechanism for the etiology of LGMD2A, which has been proposed from the existence of mutant species lacking the protease domain.

Autolysis of p94 is rapid and exhaustive but under a certain regulation. The fact that S744G and R769Q autolyze even more rapidly than wild type p94, but cannot catalyze in vivo fodrinolysis as efficiently as wild type p94, means that a too rapid autolysis causes functional defect. This is not unrelated to structural changes in the EF-hands of the Ca2+ binding domain. Recently, the crystal structure of domain IV (VI) of the calpain small subunit, which is similar to domain IV of the large subunit, has been determined (30, 31). The result shows the existence of five, rather than four EF-hands previously predicted, and reveals that the extreme C-terminal EF-hand structure is involved in the homodimer formation of domain VI. Ser744 and Arg769 corresponding to Ser189 and Arg214 in the predicted, and reveals that the extreme C-terminal EF-hand binding loop of EF-3 (Ser744) and EF-4 (Arg769) are located at the boundary of the α-helix and Ca2+ binding loop of EF-3 (Ser744) and EF-4 (Arg769). Thus, it is likely that the S744G or R769Q mutations cause changes in the structure and/or Ca2+ binding affinity of EF-3 and EF-4, resulting in the hyperactivation of p94. We have shown that p94 does not associate with the small subunit under the conditions where the large subunits of µ- and m-calpain show association with the small subunit (18). The above observation suggests that, although p94 shows apparent Ca2+-independent protease activity in vitro, Ca2+ ions play an important role in the regulation of p94 activity, presumably at very low concentrations such as the sub nanomolar level.

Further investigation is required as to how the interaction between p94 and connectin/titin is involved in LGMD2A. We have found no common alteration in connectin/titin binding ability among the nine mutants so far examined. Although the result might indicate that connectin/titin binding has nothing to do with LGMD2A, it does not necessarily exclude physiological importance of an association between p94 and connectin/titin. p94 is known to bind to the Z-line of myofibril other than the N2A and the C-terminal regions, although we have not yet identified the binding site at the molecular level (18). It is therefore, possible that mutant p94 cannot bind to the Z-line. Moreover, because the two p94 binding sites of connectin/titin, N2A and C terminus, are produced by alternative splicing, alternative splicing might regulate the interaction between p94 and connectin/titin (32, 33).

From the viewpoint of molecular mechanism underlying the disease, it is noteworthy that p94, a responsible gene product for LGMD2A, is not a membrane or cytoskeletal component as are the products of the genes responsible for other muscular dystrophies, including LGMD2C–2F. Studies using antisense oligodeoxyribonucleotides showed the involvement of p94 in myofibrillar integrity (34), and thus defects in p94 might cause high susceptibility of the sarcolemma to stress by disturbing proper myogenesis or myodifferentiation. The present work together with the further characterization of p94 function will provide a clear definition and reveal the molecular mechanism of LGMD2A (35–38).

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**TABLE I**

| Mutation | Domain | Rapid autolysis | Ca2+ requirement | In vovo C129S proteolysis | In vovo fodrinolysis | Binding to connectin/titin |
|----------|--------|-----------------|-----------------|--------------------------|---------------------|--------------------------|
| Wild type |        | +               | -               | +                        | +                   | +                       |
| C129S    | II     | -               | -               | -                        | -                   | -                       |
| L182Q    | II     | -               | -               | -                        | -                   | -                       |
| G234E    | II     | -               | -               | -                        | -                   | -                       |
| H334Q    | II     | -               | -               | -                        | -                   | -                       |
| V354Q    | II     | -               | -               | -                        | -                   | -                       |
| P319L    | IS1    | -               | -               | -                        | -                   | -                       |
| R490W    | III    | -               | -               | -                        | -                   | -                       |
| R572Q    | III    | -               | -               | -                        | -                   | -                       |
| S744G    | IV     | ++              | ND              | +                        | +                   | +                       |
| R769Q    | IV     | ++              | ND              | +                        | +                   | +                       |

* Residual autolytic activity is observed.

* Proteolysis of C129S cannot be measured due to endogenous 55-kDa band of R572Q.

* Autolysis is too rapid to measure Ca2+ requirement.
Loss of p94 Activity by LGMD2A-associated Mutation

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J. Biol. Chem. 1998, 273:17073-17078.
doi: 10.1074/jbc.273.27.17073

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