Table 3. Identity and similarity among 12 vertebrate PLEIAD sequences

% Identity	Mammals				Birds			Reptile	Amphibian	Fish		
% Similarity	*H. s.*	*M. m.*	*B. t.*	*M. d.*	*G. g.*	*T. g.*	*M. g.*	*A. c.*	*X. t.*	*O. n.*	*T. n.*[a]	*D. r.*
H. s.		93.1	92.9	70.4	51.0	47.2	48.8	46.7	41.2	33.2	*43.2*	36.1
M. m.	99.0		89.7	70.4	51.0	46.9	50.0	46.2	41.0	33.5	*35.9*	36.6
B. t.	99.5	99.0		69.4	50.2	46.5	49.5	45.9	40.5	33.5	*37.5*	36.1
M. d.	91.0	91.5	91.0		50.3	47.3	50.5	44.6	38.4	34.3	*38.5*	34.5
G. g.	84.4	83.9	84.2	84.5		65.9	91.3	50.6	41.1	33.9	*35.8*	34.0
T. g.	82.0	82.0	81.8	81.8	90.0		66.8	47.0	40.7	33.3	*30.3*	35.0
M. g.	82.2	83.2	83.2	83.5	98.0	89.8		49.6	41.4	33.9	*37.9*	35.0
A. c.	79.7	79.4	78.9	81.0	82.0	80.5	82.5		39.7	31.5	*33.9*	32.6
X. t.	75.0	75.8	75.3	78.0	76.1	76.7	76.1	75.6		31.3	*29.0*	30.4
O. n.	73.1	73.4	73.4	72.6	70.8	70.5	72.0	70.8	76.5		*46.8*	53.2
T. n.	*77.3*	*72.6*	*77.3*	*74.0*	*70.5*	*68.1*	*72.6*	*71.4*	*71.0*	*79.9*		*39.5*
D. r.	72.8	73.5	72.0	75.5	73.0	74.1	72.9	74.7	79.3	85.8	*81.5*	

Species names are abbreviated as in Table 2.
[a] The values for the *T. nigroviridis* sequence are shown in italics since this sequence contains three gaps.

the prey plasmid and were not competent for the analysis using YTH system.

Unlike the interaction between CAPN3 and PLEIAD, the interaction between CTBP1 and PLEIAD and/or CAPN3 was not detectable using protein expression in COS7 cells. To ensure the presence of excess amount of CTBP1, we performed *in vitro* cotranslation of PLEIAD and CAPN3:CS in the presence of CTBP1 using cell-free protein expression system (Fig. 3c). Both PLEIADa and PLEIADf together with CTBP1 were coimmunoprecipitated by anti-CAPN3 (Fig. 3d, lanes 5 and 7). In contrast to PLEIAD, the amount of CTBP1 in immunoprecipitate was very small and the efficiency was quite low considering its abundant presence in "Input" (lanes 1–4).

Unexpectedly, the expression of CAPN3:WT in COS7 cells caused a significant decrease in endogenous CTBP1 (Fig. 4a, lane 3, gray arrowhead). This decrease was even more pronounced when the triple-tagged CTBP1 construct, MYC-CTBP1-EGFP-FLAG, was coexpressed with CAPN3:WT (Fig. 4a, lane 1). The detected major proteolytic fragment size (open arrow), ca 35 kDa, was larger than that calculated for EGFP with FLAG, ca 30 kDa, indicating that proteolysis by coexpressed CAPN3 occurred within CTBP1 and not in the tag regions. A splicing variant of mouse CAPN3, CAPN3:ΔIS1,[35] also resulted in the proteolysis of coexpressed CTBP1 (Fig. 4b, lane 4). As summarized at the bottom of Fig. 4b, the proteolysis of CTBP1 occurred in parallel with that of other potential CAPN3 substrates identified in COS7 cells, such as calpastatin and fodrin. The C-terminal proteolyzed fragment was purified by anti-FLAG immunoprecipitation, and its N-terminal sequence was determined to be G^{410}LPPVA by N-terminal sequencing (Fig. 4b, open arrow; data not shown).

We next examined if the proteolysis of endogenous CTBP1 in differentiated mouse skm primary cultured cells was induced upon the activation of CAPN3 by ouabain treatment, which increases the intracellular Na^+ and (indirectly) Ca^{2+} concentrations.[32] The result was under our detection level (Fig. 4c, lane 2), suggesting that, unlike CAPN3 expressed in COS7 cells, substrate proteolysis by endogenous CAPN3 in skm is still tightly regulated even when its autolysis is detectable and/or the proteolysis of endogenous CTBP1 does not occur at a level that is artificially induced by CAPN3 overexpression in COS7 cells.

The effect of missense mutations that have been shown to compromise complex formation activities of CTBP1 on its property as a substrate for CAPN3 was also examined. Except for the mutation A52E, these mutations altered the susceptibility of CTBP1 to CAPN3-mediated proteolysis while the decrease in the amount of the proteolyzed fragment was observed.

These results suggest that PLEIAD has at least two functions mediated by different regions of the molecule: the N-terminal region recruits CTBP1, a substrate for CAPN3, and the C-terminal region is involved in suppressing CAPN3's protease activity.

CTBP1 is also a substrate for conventional calpains

To obtain insight into the role of calpains in the proteolysis of CTBP1, we examined the proteolysis of recombinant CTBP1 by recombinant conventional calpains *in vitro*. CTBP1 was proteolyzed by CAPN1 + CAPNS1 (also called μ-calpain, abbreviated as "CAPN1/S1" in Fig. 5a, lanes 3 and 4) and CAPN2/S1 (m-calpain; data not shown). The specificity of the reaction was shown by the suppression

of most of the proteolysis in the presence of calpastatin (Fig. 5a, lane 5) or in the absence of Ca^{2+} (lane 6). In parallel, reactions carried out in the presence (sample in lane 4) or absence (sample in lane 6) of Ca^{2+} were analyzed by mass spectrometry to determine the sequences of proteolyzed

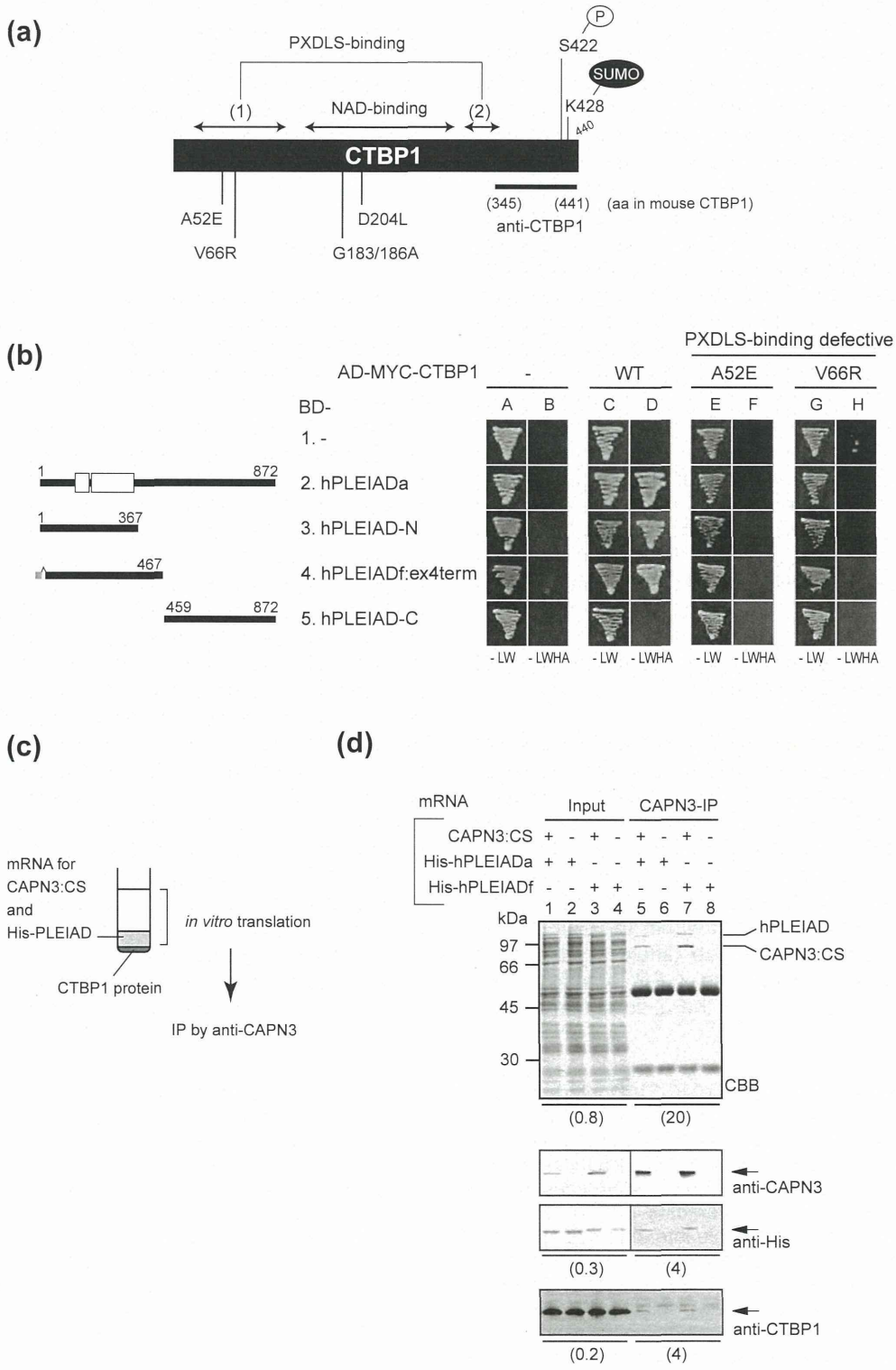


Fig. 3 (*legend on next page*)

fragments. With >99% confidence, four peptides were identified in the [+Ca^{2+}] sample [Fig. 5b, (i) to (iv)], two of which [Fig. 5b, (iii) and (iv)] were also detectable in the [−Ca^{2+}] sample. These findings suggested that CTBP1 is cleaved at the N-termini of G376 (Fig. 5b, [1]) and V388 (Fig. 5b, [2]) by CAPN1 + S1 in the presence of Ca^{2+}.

As mentioned above, CAPN3 proteolyzed CTBP1 at Gly410 at the N-terminus (Fig. 4c). Therefore, the same position detected here (Fig. 5b, [4]) is very likely to be a proteolytic site for conventional calpain, as well. However, the possibility of nonspecific proteolysis by non-calpain contaminating proteases cannot be completely eliminated due to the detection of the same peptides in the [−Ca^{2+}] sample, although their intensity was much lower than that detected in the [+Ca^{2+}] sample. In addition, analysis of the CTBP1 sequence using our cleavage site predictor for calpain[36] recognized the three positions [1], [2], and [4] by three different algorithms (Fig. 5c). Collectively, these data indicate that sites [1], [2], and [4] are probably cleaved by conventional calpain and that site [3] may represent nonspecific proteolysis. It remains unclear whether cleavage sites [1] and [2] are recognized by CAPN3. However, the small amount of a fragment detectable above the major proteolyzed fragments in Fig. 4b (lanes 2 and 4, gray arrow) may indicate that the cleavage by CAPN3 also occurred at sites [1] and/or [2].

PLEIAD may regulate CAPN3 that is not bound to sarcomere components

To gain further insight into PLEIAD's physiological relevance, we examined PLEIAD's cellular localization in primary cultures of chick skeletal myotubes expressing EGFP-hPLEIADf (Fig. 6). In total, 200 transfected cells expressing EGFP-hPLEIADf were compared to 150 cells expressing EGFP alone. In ~10% of the transfected cells, hPLEIADf was localized in a striated pattern within the sarcomeric I band, but most cells showed diffuse cytoplasmic staining (Fig. 6d, inset: see GFP staining, i.e., hPLEIADf, on each side of the Z-line, marked by staining for α-actinin). These results suggested that PLEIAD's primary target may be a population of CAPN3 that exists in the cytosol. For unknown reasons, it was more difficult to express EGFP-hPLEIADa as efficiently as EGFP-hPLEIADf. These two isoforms also showed different expression/degradation patterns in COS7 cells, independent of CAPN3's protease activity (Fig. 1d, lanes 4 and 5 and lanes 8 and 9, anti-FLAG). Such a difference might be enhanced in skeletal myotubes, resulting in a more severe degradation of hPLEIADa than of hPLEIADf.

Discussion

The unique properties of CAPN3 include its very rapid and exhaustive autolysis *in vitro* in protein expression systems and nonmuscle cultured cells (CAPN3 is stable in primary skm cells[21]) and its dependence on both Na^+ and Ca^{2+} for its activation.[32] These phenomena have been studied mostly with respect to causative factors; for example, it has been shown that CAPN3-specific insertion sequences, IS1 and IS2, are required for autolytic events.[21,35,37] In contrast, it is unclear why CAPN3 remains intact in skm cells without undergoing autolysis. Surprisingly, in CAPN3:CS knockin mice, in which the endogenous wild-type CAPN3 (CAPN3:WT) is replaced by a protease-inactive mutant CAPN3:CS, the amount of CAPN3:CS protein detected in the skm cells and tissues is the same as in wild-type mice. Moreover, the presence of intact CAPN3 protein, that is, of either WT or the CS mutant, is sufficient to fulfill its function at the triad region, even when its protease activity is lacking.[18,19] These findings strongly suggest that, in addition to connectin/titin, which is a strong candidate for stabilizing and regulating CAPN3 in muscle sarcomeres, another mechanism is present to stabilize CAPN3, especially when it is not associated with sarcomeres.

As an approach to understanding the mechanism by which CAPN3 conducts diverse functions in

Fig. 3. The N-terminal region of PLEIAD interacts with CTBP1. (a) YTH screening using a human skm cDNA library identified CTBP1 as a binding protein for PLEIAD. The functional annotation for human CTBP1 is shown. Among previously validated missense mutants, four different mutants that compromised complex formation activities of CTBP1 were selected. The mouse sequence has an insertion of 1 aa at the C-terminal region and therefore consists of 441 aa. Horizontal bar indicates the antigenic polypeptides for anti-CTBP1. (b) The N-terminal region, hPLEIAD-N or hPLEIADf: ex4term, was sufficient for the interaction (3D and 4D), while the C-terminal region, hPLEIAD-C, shared by both hPLEIADa and f, failed to undergo detectable interaction (5D). Two different mutations in PXDLS-binding domain of CTB1 showed negative effect on its interaction with PLEIAD (2F to 4F and 2H to 4H). (c) Complex formation of CAPN3, PLEIAD, and CTBP1 was examined using wheat-germ cell-free expression system. In addition to the layer of mRNA and other components for translation reaction, solution of CTBP1 protein was set on the bottom of the tube. After translation, layers were mixed and subjected to immunoprecipitation using anti-CAPN3 antibody. (d) Both PLEIADa and PLEIADf were coimmunoprecipitated with CAPN3:CS (lanes 5 and 7, CBB and anti-His). In these samples, CTBP1 was also detected (anti-CTBP1). Numbers in parentheses indicate the percentage of the total amount. Nonspecific degradation or precipitation of CTBP1 during translation reaction was not detected (data not shown).

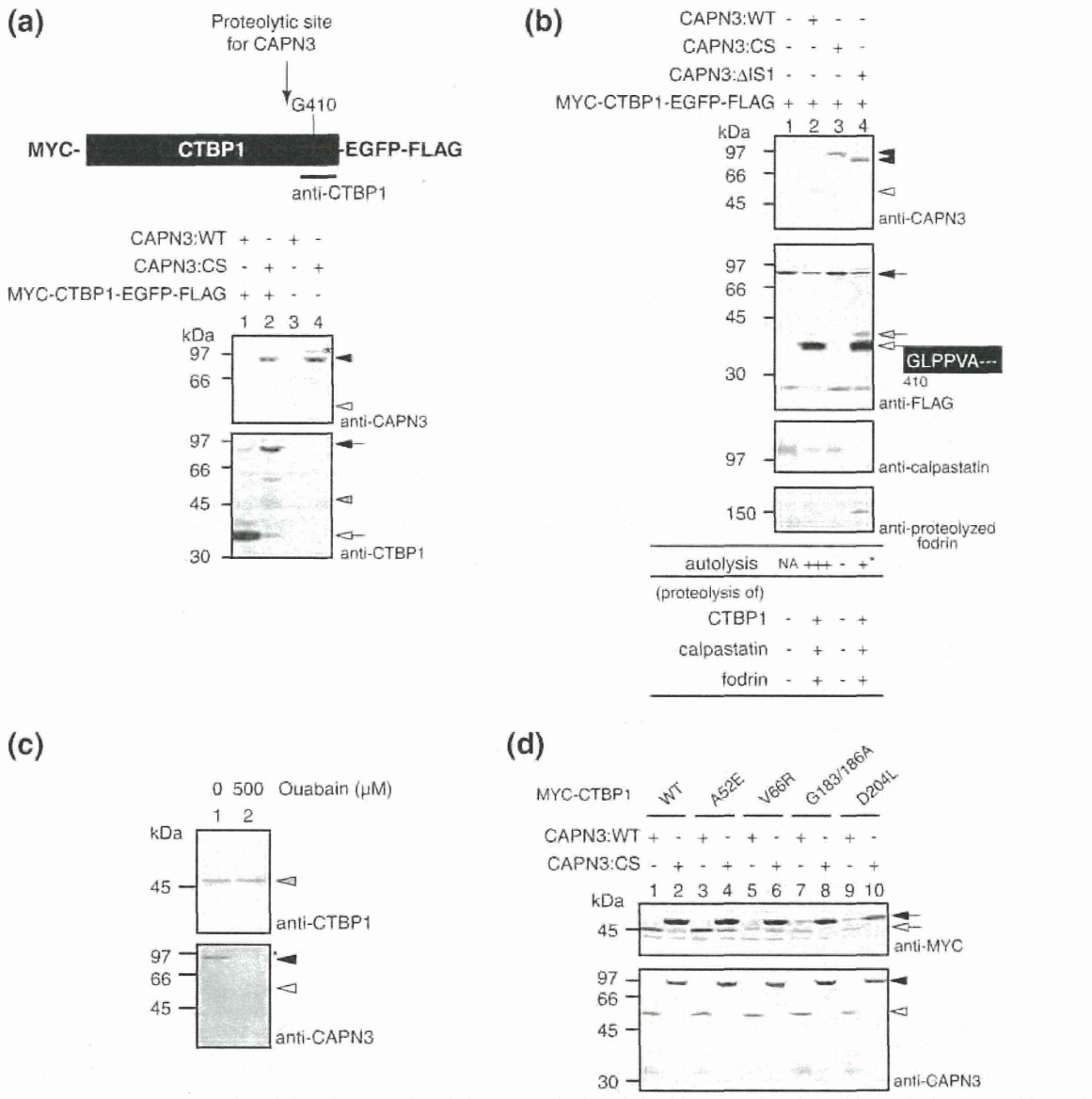
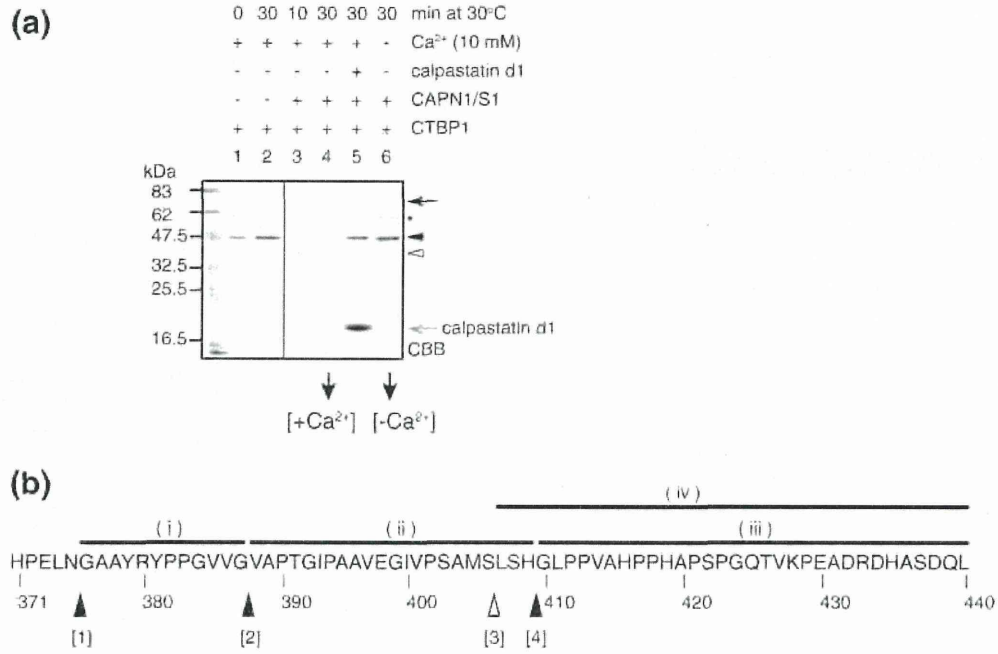


Fig. 4. CTBP1, a binding protein for PLEIAD, is a potential CAPN3 substrate. (a) Triple-tagged CTBP1 (MYC-CTBP1-EGFP-FLAG) expressed in COS7 cells was proteolyzed when CAPN3:WT was coexpressed (lane 1). This CTBP1 construct was susceptible to proteolysis (lane 2), but the difference between lanes 1 and 2 was still significant. In the cells expressing CAPN3:WT, the endogenous CTBP1 was undetectable (compare lanes 3 and 4). (b) Proteolysis of the CTBP1 construct was also observed in COS7 cells coexpressing mouse CAPN3:ΔIS1 (lane 4, anti-FLAG); CAPN3:ΔIS1 is a splicing variant that shows protease activity comparable to that of WT but lacks the autolytic sites encoded by exon 6. The C-terminal proteolyzed fragment of the CTBP1 construct was immunoprecipitated by anti-FLAG, and its N-terminal sequence was determined. (c) In cultured myotubes, the activation of CAPN3 by ouabain treatment did not cause significant proteolysis of endogenous CTBP1 protein (lane 2). (d) Proteolysis of CTBP1 by CAPN3 depends on functional structure of CTBP1. Four different CTBP1 mutants (see Fig. 3a) were coexpressed with CAPN3:WT or CAPN3:CS. In contrast to wild-type CTBP1, three of the mutants showed altered susceptibility to proteolysis (lanes 5, 7, and 9). For the mutant A52E, no significant change was observed (lane 3). Closed and open arrowheads indicate the full-length and autolytic fragments of CAPN3, respectively, detected by Western blotting using an anti-CAPN3 antibody. Gray arrowheads indicate endogenous CTBP1. Closed, open, and gray arrows indicate the full-length, the main, and the minor proteolyzed fragment of the CTBP1 construct expressed in COS7 cells, respectively.

different subcellular compartments, we sought to identify molecular interactions that affect its protease activity. Here, we showed that PLEIAD/SIMC1/C5orf25, a novel CAPN3-binding protein, moderates the protease activity of CAPN3 and possesses the potential to function as a scaffold protein.



(c)

Cleavage sites (between)	Prediction by MKL	SVM (Gaussian)	SVM (Linear)	PSSM
[1] 375 and 376	+ (p<6.2x10^{-4})	+	+	-
[2] 387 and 388	+ (p<5.7x10^{-3})	+	+	-
[3] 406 and 407	- N/A	-	-	-
[4] 409 and 410**	+ (p<6.2x10^{-4})	+	+	+

Fig. 5. Proteolysis of CTBP1 by classical calpain, μ-calpain (CAPN1/S1), revealed additional cleavage sites. (a) Recombinant CTBP1 protein was incubated under the conditions indicated at the top. CTBP1 was proteolyzed by CAPN1/S1 in the presence of Ca^{2+} (lane 4), and this reaction was inhibited by calpastatin peptide (lane 5). Peptide fractions generated in the presence (lane 4, [+Ca^{2+}]) or in the absence (lane 6, [−Ca^{2+}]) of Ca^{2+} were further analyzed by mass spectrometry. Closed and open arrowheads indicate the full-length and the main proteolyzed fragment of recombinant CTBP1. Closed and open arrows indicate CAPN1 and recombinant calpastatin domain 1. *, unidentified signal. (b) Four peptides were identified in the [+Ca^{2+}] sample [horizontal bars (i) to (iv)], and four cleavage sites by CAPN1/S1 at the C-terminus of CTBP1 were revealed (vertical arrowheads, [1] to [4]). Two sequences, (iii) and (iv), were also detected in the [−Ca^{2+}] sample, suggesting that the peptide bonds at positions [3] and [4] have a propensity to be hydrolyzed. (c) Calpain cleavage sites in CTBP1 were predicted using our predictor available at http://www.calpain.org. Three cleavage sites, [1], [2], and [4], coincided with those predicted by at least three different algorithms. +, predicted; −, not predicted; **, biochemically identified as the cleavage site for CAPN3 [Fig. 4, (c)].

PLEIAD is a novel CAPN3-regulating protein

So far, this is the first report on the functional properties of PLEIAD. In addition, we have shown that, in skm, a *Pleiad* transcript that lacks exons 2 and 3 is the predominant variant. Notably, this PLEIAD variant has not been identified in other human tissues, such as brain or testis, strongly suggesting that it is a low-abundance isoform that may be muscle specific. Unlike CAPN3, the ubiquitous expression of PLEIAD has been shown in databases at both transcript[†] and protein levels.[‡] The differential expression of PLEIAD has been detected by the transcriptional profiling of many diseases, including muscular dystrophies,[38] but it is not clear if these changes are related to CAPN3's functions. YTH screening revealed that PLEIAD also interacts with CTBP1, and it may interact with other cytosolic and/or cytoskeletal proteins. Therefore, further studies are necessary to address the

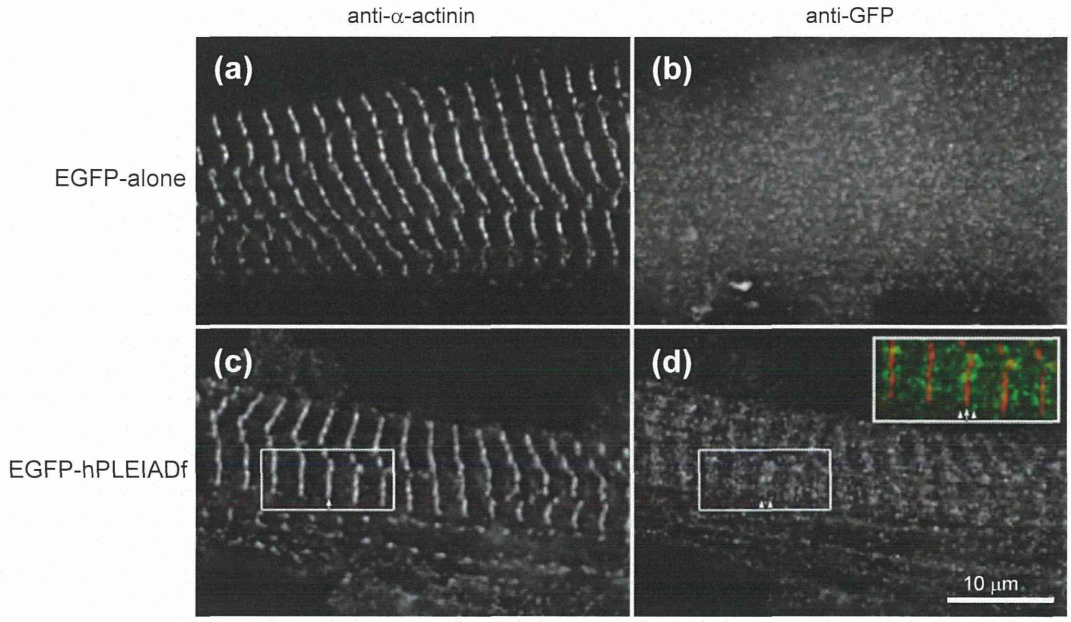


Fig. 6. PLEIAD shows diffuse cytoplasmic localization in skeletal myotubes. Localization of expressed EGFP-hPLEIADf was examined in cultured chick skeletal myotubes. Cells were stained with anti-sarcomeric α-actinin (a and d) and anti-GFP antibodies (b and d). The primary localization of hPLEIADf was diffusely cytoplasmic (data not shown). Occasionally, in about 10% of transfected cells, EGFP-hPLEIADf was localized in a striated pattern within the sarcomeric I band, on each side of the Z-line (d, inset). Arrow and arrowheads indicate a Z-line labeled by α-actinin and EGFP-hPLEIADf, respectively. The scale bar represents 10 μm.

possibility that PLEIAD has many functional links to other proteins, not only in skm but also in other tissues. The two recently identified tandem SUMO-interacting motifs at PLEIAD's N-terminus also indicate its potential roles in signaling pathways regulated by sumoylation. Another interesting finding is that humans have one PLEIAD pseudogene (*PLEIAD-ps1*, originally called *LOC202181*) close to *PLEIAD* (5q35.3 and 5q35.2, respectively).

From the viewpoint of molecular evolution, it is noteworthy that the C-terminal region of PLEIAD, which is responsible for the CAPN3 suppressor effect, is significantly conserved from human to fish and that CAPN3 is also evolutionarily conserved among vertebrates. There are, however, some inconsistencies: (1) The mouse ortholog, mPLEIAD, is much larger than the orthologs found in most mammals. The observed increase in protein size is caused by the insertion of a highly repetitive sequence in mouse exon 2, which corresponds to human exon 4. (2) At least in human and in mouse, one splicing variant has a structurally identical exon composition, and this variant lacks exon 4 in the human and exon 2 in the mouse [Figs. 1b, (c:Δex2-4) and 2c, (b)], respectively. Although these variants were not detected in skm in our hands, the identified transcripts in lower vertebrates show more similarity to these variants and lack the N-terminal region. (3) The ortholog found in marsupials, *Monodelphis domestica* (opossum), lacks the sequence corresponding to the Pro-rich region in hPLEIAD [Fig. 2a, (4)].[39] It is a serious concern that potential sequencing errors in databases could result in our misinterpretation of the structure of PLEIAD homologs, while our sequence analyses suggested that the PLEIAD gene has been a target of evolutionary modification by repetitive sequences, such as transposable elements.[40] These findings complicate our conclusion regarding the canonical functional structure of PLEIAD. In particular, the second point described above argues against the simple explanation that the N-terminal region of PLEIAD recruits substrates while its C-terminal region suppresses the CAPN3 protease activity. It is possible that the CAPN3-suppressing activity was conserved during vertebrate evolution, whereas the functions of the N-terminal parts diverged. It is nonetheless tempting to speculate that the N-terminal substrate scaffold function is applicable to the PLEIAD orthologs found in large mammals such as human and bovines.

Relevance of the C-terminal cleavage in CTBP1 functions

Our biochemical evidence indicated that CTBP1 is a good substrate for calpains including CAPN3. CTBP1 has multiple functions in both the nucleus and the cytoplasm.[30,31,41,42] In particular, its function

as a nuclear transcriptional regulator has been well studied.[43–45] CTBP1's regulation is important for proper proliferation and differentiation.[45–49] For example, the down-regulation of CTBP1 at the protein level, which is catalyzed by the ubiquitin-proteasome pathway,[50] counteracts oncogenic gene expression.[51] In addition, phosphorylation and sumoylation at the C-terminus of CTBP1 exert regulatory effects.[42] Notably, CTBP1's cleavage by calpains removes these modification sites, suggesting that calpains also participate in the regulation of CTBP1, although we could not detect this cleavage *in vivo*.

The function of CTBP1 in skm remains unclear; therefore, it is difficult to speculate about what the outcomes of CAPN3-mediated CTBP1 proteolysis are. One possibility is that the regulation of CTBP1 by CAPN3 is functionally analogous to a previously proposed role of CAPN3 in down-regulating β-catenin in skm.[13] Since CTBP1 itself is capable of carrying out multi-directional functions, its stage-specific regulation may be involved in skm cell fusion and development into myotubes.

For the interaction between CTBP1 and PLEIAD, it was suggested that PXDLS-binding site on CTBP1 is involved at the level of YTH assay, and further analyses are required to evaluate its biological significance. It was also suggested that CAPN3 recognizes the structure of CTBP1 relevant for its PXDLS binding and NAD(H) binding. However, there is a possibility that the selected CTBP1 mutations have too strong effect on the overall structure of the molecule and the essential structure of CTBP1 as a substrate for calpain warrants further investigation.

Possible link between the CAPN3–PLEIAD and CAPN3–connectin/titin interactions

We previously proposed that the N2A region of connectin/titin supports the functions of CAPN3 by stabilizing CAPN3 while it recruits MARPs (*m*uscle *a*nkyrin *r*epeat *p*roteins) as substrates for CAPN3. Such a model is physically possible for connectin/titin, since it is located in sarcomeres, which are mostly composed of extension-competent structures, such as immunoglobulin motifs and fibronectin type III-like domains. From the primary sequence of PLEIAD, it is not clear if a robust structural change occurs upon its interaction with CAPN3 and/or CTBP1. Still, it is worth noting that the C-terminal functional region of PLEIAD has no defined structure and/or motif. The specificity and efficiency of calpastatin, a specific inhibitory protein for classical calpains except CAPN3, relies on its unstructured nature; it is classified as an intrinsically unstructured/disordered protein[52]; calpastatin binds close to, but loops out into, the side opposite calpain's active site, escaping cleavage by the calpain.[53,54] Although there is not a strong sequence similarity between PLEIAD and calpastatin, it is possible that PLEIAD functions in an analogous manner. We also hypothesize that the N-terminal region of PLEIAD is involved in the regulation of CAPN3 autolysis by increasing the affinity of the molecule to the full-length, pre-autolyzed form of CAPN3 but is susceptible to protease activity of CAPN3. On the other hand, the C-terminal region of PLEIAD is resistant to protease activity of CAPN3 but has lower ability to interact with CAPN3 by its own.

We sought to detect endogenous mPLEIAD in mouse tissues using two commercially available anti-hPLEIAD antibodies. These antibodies did not yield consistent results; thus, we suspended our analysis of mouse PLEIAD localization during the preparation of this manuscript. Instead, we used an alternative approach, the expression of hPLEIAD in chicken primary cultured skeletal myotubes. The localization of expressed hPLEIADf (see Fig. 6) showed that this new regulator of CAPN3 is primarily distributed diffusely in the cytoplasm. At the same time, the occasional localization of hPLEIADf within the sarcomeric I-band region raises the intriguing possibility that connectin/titin and PLEIAD cooperate as CAPN3 regulators. Further investigation of the spatiotemporal expression of the PLEIAD protein in skm is required in order to address this issue.

In this study, a novel protein–protein interaction that is capable of regulating CAPN3 in the cytoplasm as well as in the sarcomere was shown. Along with the identification of possible CAPN3 functions in different cellular compartments, it has been unknown how CAPN3 avoids autolytic degradation while it is translocated. We anticipate that the present study will serve as a good starting point toward understanding how CAPN3 conducts its multiple functions. It is likely that there are other interacting molecules relevant to CAPN3's functions that are yet to be discovered.

Materials and Methods

Experimental animals

All procedures used for experimental animals were approved by the Experimental Animal Care and Use Committee of Tokyo Metropolitan Institute of Medical Science, and the animals and related materials were treated in accordance with the committee's guidelines.

cDNA constructs

The cDNA clone for PLEIAD/C5orf25, which was originally purchased from Ressourcenzentrum Primärdatenbank (German Science Centre for Genome Research), is now available from Source BioScience (IRATp970H0629D). The cDNAs for human CAPN3 and mouse CAPN3Δ1 were subcloned into the pSRD expression vector for

protein expression in mammalian cells, as described previously.[25,35] These cDNAs were also expressed as N-terminal FLAG-tagged or MYC-tagged proteins using the pSRD or pcDNA3.1 expression vector (Invitrogen, Grand Island, NY).[55] The pEGFP-C1 expression vector was also used. For the YTH assay, the pAS2-1c[25] and pACT2 expression vectors (U29899; Clontech, Mountain View, CA) were used. Enzymes used to manipulate recombinant DNA were purchased from Takara Bio (Shiga, Japan) or New England Biolabs (Ipswich, MA). The mutations described here were introduced by long PCR using *Pfu*-Turbo DNA polymerase, as described previously.[56] All the constructs were verified by DNA sequencing.

PCR and RT-PCR analyses

Endpoint PCR reactions to detect the expression of C5orf25 were performed using ExTaq DNA polymerase (Takara) and the appropriate primer pairs (Fig. 1b and Table 1). Some PCR products were sequenced for verification. The human skm cDNA library for YTH (Clontech) or mouse skm cDNA synthesized from total RNA was used as a template. Total RNA was prepared from cultured mouse muscle cells and the quadriceps femoris of 30-week-old C57BL/6J mice using TRIzol® Reagent (Invitrogen), according to the manufacturer's instructions. For RT by PrimeScript® Reverse Transcriptase (Takara), 1.5 μg and 1.1 μg of total RNA from cultured cells and tissues, respectively, were used per 10-μl reaction.

Cell culture and protein expression

Protein expression in HEK293 cells was performed as previously described.[28] COS7 cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum that had been heat inactivated at 56 °C for 30 min before use. For recombinant protein expression in COS7 cells, the plasmid was prepared at a concentration of 1 μg/μl and transfected using TransIT®-LT1 (Mirus Bio LLC, Madison, WI). As a standard, 2–4 μg of plasmid plus a 3× volume of transfection reagent were used per 1.5×10^5 cells plated in 3.5-cm dish, 12–16 h prior to transfection. The cells were harvested 40–60 h after transfection using ice-cold phosphate-buffered saline.[26]

Primary cultures of chick skeletal myotubes were prepared as described previously.[57] Cells used for immunofluorescence were grown on coverslips coated with Matrigel™ (BD Biosciences, San Jose, CA) diluted to 0.5 mg/ml in minimum essential medium. Transfection was performed 12–16 h after plating using Effectene (Qiagen, Valencia, CA), as previously described.[58] Primary cultures of mouse skm cells were prepared as described previously.[27] Cells were allowed to differentiate for 5 and 10 days and used for total RNA purification and a 2-h incubation with ouabain, respectively.

In vitro transcription and translation were performed using a wheat-germ cell-free expression system, according to the manufacturer's instructions (CellFree Sciences, Japan). The mRNAs for each construct were transcribed separately. The translation reaction was carried out at 16 °C for 18–20 h without shaking. Two micrograms of recombinant CTBP1 (1 mg/ml stock solution) was added to the bottom of 112-μl translation reaction mixture.

Immunoprecipitation

Harvested cells were suspended in lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM CsCl, 1 mM ethylenediaminetetraacetic acid-KOH (pH 8.0), and 1% Triton X-100] containing protease inhibitors [1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 0.1 mM leupeptin, 10 μg/ml aprotinin, and 10 mM iodoacetamide] and incubated on ice for 30 min with occasional mixing. The supernatant was collected after centrifugation at 20,630***g*** at 4 °C for 15 min. Incubation with anti-FLAG M2 affinity gel (Sigma-Aldrich) and elution of the immunoprecipitated proteins were performed according to the manufacturer's instructions using a modified wash buffer [50 mM Tris–HCl (pH 7.5) and 150 mM CsCl].[26] To detect the CAPN3–PLEIAD interaction, we carried out the incubation for 2–3 h. When anti-CAPN3 antibody was used for immunoprecipitation, the immunocomplex was recovered by Protein-G Sepharose (GE Healthcare). The eluate was subjected to SDS-PAGE and Western blot analysis. For N-terminal sequencing, the immunoprecipitated proteins were blotted onto a ProBlott membrane (Applied Biosystems, Carlsbad, CA) after SDS-PAGE. The target protein bands were visualized by CBB G-250 staining, excised, and submitted to APRO Science Institute Inc. (Tokushima, Japan) for sequence analysis.

Western blot analysis

Proteins were separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). The membranes were probed with the appropriate primary antibodies and horseradish-peroxidase-coupled secondary antibodies (Nichirei, Tokyo, Japan) followed by visualization using a POD immunostaining kit (Wako, Osaka, Japan) or SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).[26] Scanned images were processed for presentation using Adobe Photoshop CS6 (Adobe Systems, San Jose, CA).

Immunofluorescence analysis

Immunofluorescence microscopy was performed as described previously.[58] At 4–5 days after transfection, the cells were incubated in relaxing buffer [150 mM KCl, 5 mM $MgCl_2$, 10 mM 3-(*N*-morpholino) propanesulfonic acid (pH 7.4), 1 mM ethylene glycol bis(β-aminoethyl ether) *N,N*′-tetraacetic acid, and 4 mM ATP] for 15 min, followed by fixation using 2% formaldehyde in relaxing buffer for 15 min. The primary and secondary antibodies were used at the concentrations described in Antibodies. Coverslips were mounted onto slides with Aqua Poly/Mount (Polysciences, Warrington, PA), and the samples were analyzed on an Axiovert microscope (Zeiss, Oberkochen, Germany) using 63× (NA 1.4) or 100× (NA 1.3) objectives. The cells were also analyzed using a Deltavision RT system (Applied Precision, Issaquah, WA) with an inverted microscope (IS70; Olympus, Tokyo, Japan), a

100× (NA 1.3) objective, and a charge-coupled device camera (CoolSNAP HQ; Photometrics, Huntington Beach, CA). The images were deconvolved using SoftWoRx 3.5.1 software (Applied Precision) and processed for presentation using Adobe Photoshop CS6 (Adobe Systems).

Antibodies

The antibodies used in this study include anti-FLAG mouse monoclonal (1:1000, M2; Stratagene, La Jolla, CA), anti-MYC mouse monoclonal (1:1000, 9E10; Developmental Studies Hybridoma Bank), anti-calpastatin mouse monoclonal (1:1000, PI-11; American Type Culture Collection), anti-CTBP1 mouse monoclonal (1:1000, 3/CtBP1; BD Transduction Laboratories™), anti-α-actinin mouse monoclonal (1:2000, EA-53; Sigma-Aldrich), anti-GFP rabbit polyclonal (1:2000; Abcam, Cambridge, MA), anti-CAPN3 goat polyclonal (1:1000; Cosmo Bio, Tokyo, Japan), anti-His mouse monoclonal (1:2000; Novagen), and anti-proteolyzed fodrin (anti-GMMPR) rabbit polyclonal (1:1000, anti-GMMPR) antibodies.[26,32] As secondary antibodies, Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1000; Invitrogen) and Texas Red-conjugated donkey anti-mouse IgG (1:600; Jackson Immunoresearch Laboratories, West Grove, PA) antibodies were used.

Protein identification by mass spectrometry

The FLAG-immunoprecipitates from HEK293 cells were analyzed using direct nanoflow liquid chromatography coupled with tandem mass spectrometry.[28] To identify the peptide sequence generated during the *in vitro* proteolysis of CTBP1, we desalted the reaction solution using ZipTip® Pipette Tips (Millipore), and tandem mass spectrometry spectra were acquired by a 4800 MALDI TOF/TOF™ analyzer (AB SCIEX, Framingham, MA). Data were analyzed by Protein Pilot™ software (version 4.5) (AB SCIEX).

Calpain cleavage assay *in vitro*

Two hundred nanograms of recombinant human CTBP1 (ProSpec, Ness Ziona, Israel) was incubated with 25 ng of recombinant human CAPN1/S1 (μ-calpain) (BioVision, Milpitas, CA) in 10 μl of incubation buffer [20 mM Tris–HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid-KOH (pH 8.0), and 1 mM DTT] with or without 10 mM $CaCl_2$ at 30 °C for 10 and 30 min. Where indicated, recombinant human calpastatin domain I (Takara) was added to a concentration of 29 mM. The reaction was stopped by the addition of SDS sample buffer, and the sample was subjected to SDS-PAGE followed by CBB G-250 staining.

YTH assay

Proteins interacting with C5orf25 were screened as previously reported[22] using *Saccharomyces cerevisiae* strain AH109. The full-length PLEIAD cDNA cloned into pAS2-1c was coexpressed in the yeast with approximately 1.3×10^6 of a human skm cDNA library in the pGAD424 vector (Clontech). The plasmids were rescued from colonies grown on SD medium that lacked Leu, Trp, His, and Ade (SD-LWHA) and were sequenced. The rescued plasmids were retransformed with a series of PLEIAD mutants using the Fast™-Yeast Transformation Kit (G-Biosciences/Genotech, St. Louis, MO), according to the manufacturer's instructions. Cotransformants were selected on SD-LW plates, and the expressions of reporter genes were assessed by growth on SD-LWHA plates.

Sequence analysis

Sequence data were retrieved from the NCBI[§] and Ensembl[‖] databases. The sequences from UniProtKB[¶] were also used to check the consistency. BLAST searches were performed with psi-BLAST and BLAST-p and tBLAST-n *versus* the nonredundant protein or nucleotide databases. For phylogenetic analysis, sequences were aligned using MAFFT[a 59] and converted to an unrooted tree diagram after manual inspection. The identity and similarity of aligned sequences were calculated using Genetyx (version 11) (Genetyx Co., Tokyo, Japan). Sequence repeats were identified with RADAR,[b 60] and designated sequences were aligned using MAFFT and Genetyx. To predict calpain cleavage sites, we analyzed the protein sequences with an online cleavage site predictor.[c 7,36,61]

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†For example, see http://www.ebi.ac.uk/gxa/gene/ENSG00000170085.
‡For example, see http://www.ebi.ac.uk/pride/searchSummary.do?queryTypeSelected=identification%20accession%20number&identificationAccessionNumber=Q8NDZ2.
§http://www.ncbi.nlm.nih.gov/
‖http://ensembl.org/
¶http://www.uniprot.org/
[a]http://www.genome.jp/tools/mafft/
[b]http://www.ebi.ac.uk/Tools/pfa/
[c]http://calpain.org/

Abbreviations used:
NCBI, National Center for Biotechnology Information; YTH, yeast two-hybrid.

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