Glutamate substitutions at a PKA consensus site are consistent with inactivation of calpain by phosphorylation

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Abstract Regulation of calpain by phosphorylation has often been suggested, but has proved difficult to detect. Calpains extracted from mammalian tissue are reported to contain 2–4 mol phosphate/mol of enzyme distributed over multiple sites, but phosphate groups are not detectable in the X-ray structures of recombinant calpain. Some serine and threonine residues in the large subunit of rat m-calpain were converted to aspartic or glutamic acid residues, at sites suggested by previous studies, to assess the probable effects of phosphate groups on the enzyme. Expression of the mutant calpains in *Escherichia coli*, and their heat stabilities, did not differ from those of the wild-type enzyme. m-Calpains with the mutations Ser50Asp, Ser50Glu, Ser67Glu, and Thr70Glu had the same specific activity and Ca^{2+} requirement as the wild-type enzyme. In contrast, Ser369-Asp-, Ser369Glu-, and Thr370Glu-m-calpain were inactive. This result is consistent with the recent report that phosphorylation at position 369 or 370 in vivo reduced m-calpain activation.

Key words: Calpain phosphorylation; Calpain regulation; Extracellular signal-regulated kinase; Cyclic adenosine monophosphate-dependent kinase

1. Introduction

The two calpains, μ- and m-calpain, are intracellular cysteine proteases, widely distributed in most tissues, and are heterodimers composed of a large subunit (Capn1 and Capn2, respectively) bound to a common small subunit (Capn4) [1–4]. It seems likely that most of the μ- and m-calpain in normal tissue is inactive at any given time, and that some fraction of one or both enzymes is activated when required, and presumably in specific subcellular locations. Activation of calpain, at least as studied in vitro, is initiated by Ca^{2+} and involves limited autolysis of both subunits, conformational change, and probably also subunit dissociation [5–7]. The activated calpain is destroyed by further autolysis, which is presumed to have the effect in vivo of limiting the duration of its activity. Although not yet well understood, some mechanisms must exist to regulate the rate of calpain activation, and to permit it to be active at physiological intracellular Ca^{2+} concentrations in the range of 0.1–1 μM.

The properties of purified natural calpains isolated from mammalian tissues, and of recombinant calpains recovered from *Escherichia coli* or insect (Sf9) cells [8,9] are apparently identical. Their in vitro Ca^{2+} requirements are approximately 5–50 μM for μ-calpain, and 325 μM for m-calpain, but these values can be reduced to approximately 1–5 μM for μ-calpain and 150 μM for m-calpain, by factors including autolysis, or binding to phospholipids, membranes, or activator proteins [1]. It has also been proposed that subcellular zones of high Ca^{2+} concentration [10] may be sufficient to cause calpain activation, either alone or in combination with other factors.

It is not clear that any of these explanations is sufficient to account for calpain activity in vivo. Alternatively, it has frequently been suggested that calpain could be activated by phosphorylation, which might conceivably reduce its Ca^{2+} requirement, affect its subcellular localization, or regulate its binding to specific targets. However, phosphate groups were not detected in the X-ray crystal structures of recombinant m-calpain purified either from *E. coli* or from insect cells [11,12]. These recombinant calpains are fully active and have the same Ca^{2+} requirement as natural calpain, so that phosphorylation is not essential for calpain activity.

Several laboratories have studied calpain phosphorylation, but the results have mostly been inconclusive, and therefore unpublished. The difficulties arise from two sources. In the first place, working with calpain isolated from natural sources, it is very difficult to prove that an observed phosphate group is not associated with a contaminant, particularly on immunoblots. Secondly, Ca^{2+}, added to activate a kinase, or to activate calpain, causes rapid autolysis, or causes aggregation of recombinant inactive calpain [6], so that the products are again very difficult to analyze.

In calpain isolated from mammalian cells in culture, phosphorylation could not be detected by [^{32}P]-labeling and autoradiography [13]. In contrast, on the basis of measurements with matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF), it has been reported that both μ- and m-calpain isolated from mammalian tissues are heterogeneously phosphorylated at eight or nine different serine, threonine, and tyrosine residues, at a level of 2–4 mol phosphate/mol of calpain [1]. Calpain phosphorylation by several kinases in vitro (cyclic adenosine monophosphate (AMP)-dependent kinase (PKA), protein kinase C (PKC), PKG) was reported, but the positions of the phosphate groups, and the effects on activity, were not described [14]. Preliminary work

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Abbreviations: DSC, differential scanning calorimetry; ERK, extracellular signal-regulated kinase; PKA, cyclic adenosine monophosphate-dependent kinase

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suggests that calpain may be phosphorylated in vivo at Ser50 in response to extracellular signal-regulated kinase (ERK) signaling [15,16]. The m-calpain large subunit was phosphorylated in vitro at Ser369 by Ca^{2+}/calmodulin-dependent kinase II, doubling the activity of calpain [17]. Conversely, it has more recently been reported that phosphorylation of recombinant human m-calpain by PKA inhibited calpain activation in vivo [18]. Control experiments suggested that the PKA target site was at Ser369 or Thr370, in a PKA consensus sequence, Arg^{366}-Arg-Gly-Ser-Thr^{370} [19].

It was therefore of interest to study further the effects of phosphorylation of calpain on its activity and Ca^{2+} requirement. In view of the difficulties described above, we have chosen not to repeat incubations of kinases with recombinant calpains, but have relied on mutagenesis. Some serine and threonine residues in rat m-calpain, chosen on the basis of the above reports, were therefore converted to aspartate or glutamate residues, to mimic the presence of phosphate groups. The mutant calpains were expressed in *E. coli*, and their expression levels, activities, and Ca^{2+} requirements were compared with those of wild-type m-calpain. In interpreting our results, we recognize that this work does not address the possible interactions of phosphorylated calpains with regulatory factors or substrates in eukaryotic cells.

2. Materials and methods

The methods of mutagenesis, bacterial expression, purification, and Ca^{2+} titration of recombinant rat calpain have been described previously [20,21]. Rat m-calpain is obtained by coexpression of the 80 kDa large subunit and a truncated form (21 kDa) of the small subunit lacking domain V, since omission of domain V greatly increases expression levels and does not discernibly affect the relevant properties of the enzyme. The mutagenic primers are listed in Table 1. The mutations were confirmed by restriction enzyme digestion and by sequencing.

Casein zymography was performed in HEPES/imidazole buffer, which is preferred for analysis of m-calpain [22].

Heat stability of recombinant calpains was measured by differential scanning calorimetry (DSC) in a VP-DSC calorimeter (Microcal), according to the manufacturer's instructions. Calpain samples, purified in the presence of 2-mercaptoethanol, were dialyzed extensively at 4°C against 50 mM Tris–HCl, pH 7.8, 0.2 M NaCl, 5 mM ethylenediamine tetraacetic acid (EDTA), purged with N_2 and protected from air. Protein concentrations were measured with the Bradford reagent. The protein sample in one cell and the dialysis buffer in the control cell, after degassing under vacuum, were heated simultaneously from 10 to 90°C at a heating rate of 90°C per hour.

3. Results

The activities of mutant calpains were compared by casein zymography. Fig. 1A shows that the mutations Ser50Asp, Ser50Glu, Ser67Glu, and Thr70Glu did not affect the activity of calpain, while the mutations Ser369Glu, and Thr370Glu abolished its activity. Ser369Asp-m-calpain was also inactive (data not shown). Of the inactive mutants, only Ser369Glu-m-calpain showed a very faint hint of activity at higher gel loading. However, all these mutated calpains, active and inactive, were expressed as soluble heterodimeric proteins in approximately equal amounts (Fig. 1B). To permit valid comparisons, the amounts of total *E. coli* extract protein loaded per well were equal in both gel systems, and were intentionally low, and on the immunoblot the color development was stopped before saturation of the system. The immunoblot shows that the amount of m-calpain large subunit in each lane was essentially equal for all constructs.

The yields of calpain protein for all constructs were 5–10 mg from 4 l of *E. coli* expression culture, in the range expected for wild-type rat m-calpain, when purified by three column steps on diethylaminoethyl (DEAE) Sepharose, Ni-nitrilotriacetic acid (NTA) agarose, and Q25 (quaternary anion exchange). The observed specific activities were: Ser50Asp-m-calpain, 1900 U/mg; Ser50Glu-m-calpain, 2200 U/mg; Ser67-Glu-m-calpain, ~1500 U/mg; these values are all close to

Table 1
Mutagenic oligonucleotide primers

Mutation	Oligonucleotide	RE[a]
Wild-type at Ser50	GCG CTC TTC CAG GAT CCT TCC TTC CCC GCC CTG CCG Ala Leu Phe Gln Asp Pro SER Phe Pro Ala Leu Pro	BamHI
Ser50Asp	GCG CTC TTC CAG GA**C** CCT **GA**C TTC CCC GCC CTG C	BamHI[b]
Ser50Glu	CTC TTC CAG GAT CCT **GAA** TTC CC**T** GCC CTG CCG	EcoRI
Wild-type at Ser67-Thr70	G TTG GGG GCC TAC TCC AGC AAA ACT CGG GGC ATC GAA TG Leu Gly Pro Tyr SER Ser Lys THR Arg Gly Ile Glu Trp	
Ser67Glu	G TTG GGG CCC TAC **GAG** AGC AAA AC**C** CGG GGC ATC GAA TG	SmaI
Thr70Glu	TTG GGG CCC TAC TC**G** AGC AAA **GAG** CGG GGC ATC GAA TG	XhoI
Wild-type at Ser369-Thr370	AAC TGG AGG CGA GGC TCC ACT GCA GGG GGC TGC AGG AAT TAC Asn Trp Arg Arg Gly SER THR Ala Gly Gly Cys Arg Asn Tyr	
Ser369Asp	C TGG AGG CGA GGC **GA**C AC**C** GC**G** GGG GGC TGC AGG A	SacII
Ser369Glu	AC TGG AGG CGA GGC **GAA** AC**C** GC**G** GG**C** GGC TGC AGG AAT TAC	SacII
Thr370Glu	G AGG CGA GGC TCC **GAA** GC**C** GG**C** GGC TGC AGG AAT	NgoMIV

The primers are shown in alignment with the wild-type sequence, and altered bases are shown in bold. Restriction enzyme sites are underlined. The primers were made as antisense 5′-phosphorylated oligonucleotides (because of the orientation of the f1 element in pET24), but are shown here as sense, since this is much easier to understand.

[a]Restriction enzyme used for screening of mutants.

[b]The mutation was designed to cause the loss of the BamHI site.

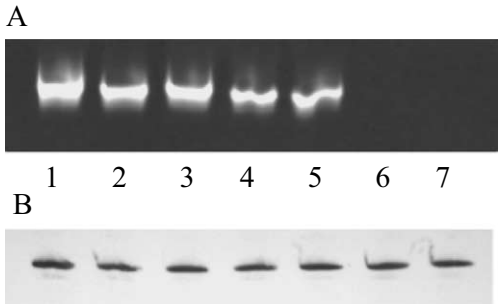

Fig. 1. Casein zymography and immunoblotting of recombinant calpains. A: Casein zymography. Equal amounts (approximately 4 μg of protein) of each sample were loaded onto a non-denaturing polyacrylamide gel. The lanes contained extracts of *E. coli* expressing heterodimeric (80+21 kDa) rat calpains with the following mutations: 1, wild-type m-calpain; 2, Ser50Asp; 3, Ser50Glu; 4, Ser67Glu; 5, Thr70Glu; 6, Ser369Glu; 7, Thr370Glu. The gel was run in the HEPES–imidazole system [22], and incubated overnight in the presence of 5 mM Ca^{2+}, followed by staining and destaining. B: Immunoblotting. Samples (4 μg of protein) of the same extracts were run on an sodium dodecyl sulfate (SDS)–polyacrylamide gel in the Tris/Tricine system, followed by blotting using standard procedures. The blot was exposed to a polyclonal antibody to rat m-calpain large subunit, followed by an alkaline-phosphatase-linked goat anti-rabbit IgG and color development.

that of 1800 U/mg for wild-type m-calpain [21]. The specific activity of Thr70Glu-m-calpain was not accurately determined, but was of the same order of magnitude. No activity could be detected in the Ser369 and Thr370 mutant calpains by the standard casein assay [20].

For the active mutants, the K_d values for Ca^{2+} were not significantly different from that of wild-type calpain (Table 2).

The question arises whether the absence of activity in the Ser369 and Thr370 mutants is due to misfolding of the proteins. Our experience with expression of mutant rat m-calpains suggests that a high yield of soluble heterodimeric protein which can be purified by standard procedures, is itself an indication that the protein is correctly folded. In previous work, the structural integrity of active calpains was assessed by comparing their rate of loss of activity on incubation at 45°C [21]. In the present work, the thermal transitions (T_m) of several active and inactive calpains were measured by DSC.

Table 2
K_d values of mutant calpains

Calpain mutant	K_d (μM Ca^{2+})
Wild-type rat m-calpain	325
Ser50Asp	347
Ser50Glu	368
Ser67Glu	314
Thr70Glu	350

K_d is defined as the concentration of Ca^{2+} required for 50% of maximum activity under the given conditions [21]. The values were measured once only, always in parallel with a wild-type m-calpain. The values have been normalized to the wild-type value of 325 μM. The differences between these values are not considered to be significant.

All the calpains tested, both active and inactive, showed an initial thermal transition close to 50°C, and another close to 60°C (Fig. 2). These values suggest that the folded structure of all these calpains is the same, although differences in the peak ratios suggested more subtle differences in structure. The data could be fitted to models with two or three T_m values, but extensive aggregation at 60–65°C made it difficult to define a base line, and therefore precluded more detailed analysis.

4. Discussion

4.1. Mutations at Ser50

Preliminary work has suggested that m-calpain may be phosphorylated at Ser50 in a pathway downstream of ERK [15,16]. This point requires further study, but the in vitro Ca^{2+} requirements of Ser50Asp- and Ser50Glu-m-calpains were essentially equal to that of wild-type m-calpain. The crystal structure of m-calpain shows that Ser50 is an exposed surface residue, with no apparent interactions with residues so far considered important for Ca^{2+} activation [7,11].

4.2. Mutations at Ser67 and Thr70

These residues were found to be partially phosphorylated in calpains isolated from bovine tissue [1]. The replacement of these two residues by glutamic acid did not affect the activity or Ca^{2+} requirement of the mutant products. These residues, like Ser50, are also surface exposed and devoid of obvious interactions. It cannot yet be excluded that phosphate groups

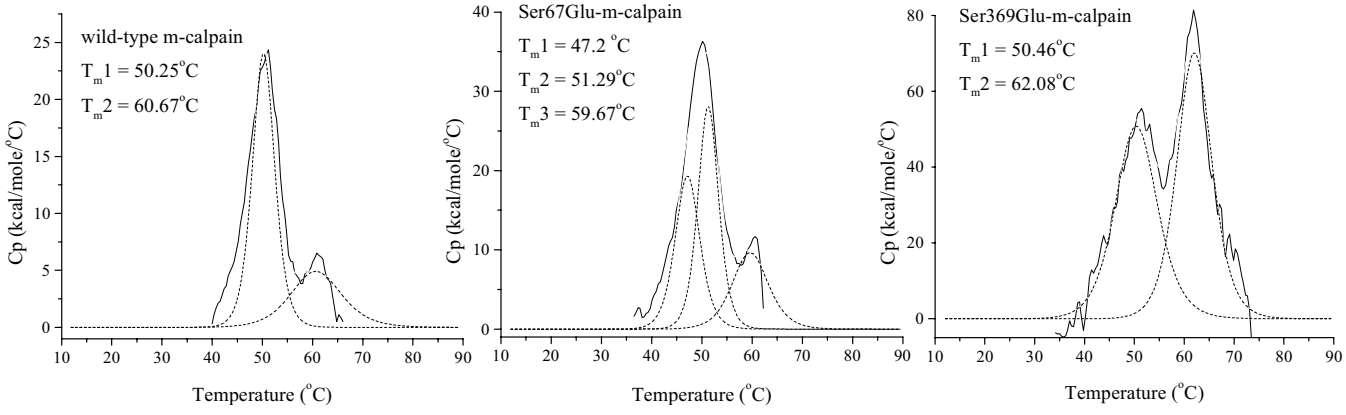

Fig. 2. DSC. Protein samples (0.3–1.8 mg/ml) were scanned as described in Section 2, and the data were processed with the software provided by the manufacturer. The continuous lines represent the raw data corrected by subtraction of the background buffer values, and the dotted lines represent the fit of the data to models for two T_m values, as shown for wild-type and Ser369Glu-m-calpain, and for three T_m values as shown for Ser69Glu-m-calpain. Aggregation of the samples occurred at ~65°C, and was dependent on both protein concentration and rate of heating.

at positions 50, 67, or 70 might affect interactions of m-calpain in eukaryotic cells with possible substrates or regulatory factors, for example with components of focal adhesion complexes where calpain activity is thought to be important [15,23].

4.3. Mutations at Ser369 and Thr370

With respect to phosphorylation at Ser369, there have been some conflicting reports. The mutation at Thr370 was performed because it was not entirely clear in previous work whether Ser369 or Thr370 had been phosphorylated [18]. As noted earlier, Ser369 is located within a PKA consensus target sequence, Arg-Arg-Xxx-Ser/Thr [19], and is one of the residues reported to be phosphorylated in natural calpain [1]. Phosphorylation at this position has been reported either to increase [17] or to decrease [18] the activity of calpain. Our results with mutants expressed in *E. coli* showed that Ser369-Asp-, Ser369Glu-, and Thr370Glu-m-calpains were inactive. This demonstrates that maintenance of these serine and threonine residues is essential to calpain activity. These results are therefore also consistent with the idea that PKA phosphorylation at these positions could down-regulate m-calpain in vivo.

It is not immediately clear why the Ser369Glu- and Thr370Glu-calpains are inactive. The normal level of protein expression of these mutants, and the T_m values of Ser369Glu-m-calpain, indicate that this glutamic acid residue does not disrupt folding of calpain during its expression. This is consistent also with the modeling prediction, which indicates that a glutamic acid residue can be accommodated at these positions without disruption of the Ca^{2+}-free structure. It has not yet been found possible to crystallize the whole calpain molecule in the presence of Ca^{2+}, so that the effect of these mutations on the response of calpain to Ca^{2+} cannot be directly investigated. It was suggested earlier that interactions of a putative Ser369-phosphate group with Arg628 and His643 could inhibit the conformational changes in the whole calpain molecule induced by Ca^{2+}, and similar arguments apply to Thr370 [18]. Many of the residues 363–374, including the PKA consensus sequence, are highly conserved, not only in μ- and m-calpain, but also in the wider family of calpains, indicating that this whole region is important for function. Position 369 is less conserved, since it is serine in both μ- and m-calpain, but it is highly variable in the other calpains. In contrast, residue 370 is almost always threonine, and only occasionally serine.

It is recognized that studies on recombinant calpains isolated from *E. coli* do not immediately provide information on their behavior in eukaryotic cells. Phosphorylation could affect both the subcellular localization of calpain in animal cells, and its interactions with substrates and putative regulatory factors, which are all still far from clear. The results reported here show that acidic residues at positions 50, 67, and 70, mimicking the presence of phosphate groups, do not affect the intrinsic properties of purified m-calpain in vitro, but they are consistent with down-regulation of m-calpain activity in vivo by phosphorylation at Ser369 or Thr370.

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