The β- and γ-CH_2 of B27-WT's Leu^{11} and Ile^{18} Side Chains Play a Direct Role in Calpain Inhibition†

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ABSTRACT: Uncontrolled activation of calpain has been linked to tissue damage after neuronal and cardiac ischemias, traumatic spine and brain injuries, and multiple sclerosis and Alzheimer's disease. In vivo, the activity of calpain is regulated by its endogenous inhibitor calpastatin. The pathological role of calpain has been attributed to an imbalance between the activities of the protease and its inhibitor. Thus, it is possible that by reimposing functional control on the protease, the progression of calpain-mediated diseases could be slowed or eliminated. B27-WT is a 27-residue peptide (DPMSSTYIEELGKREVTIPPKYRELLA) derived from calpastatin that was previously shown to be a potent inhibitor of μ- and *m*-calpain. Recently, we identified two hot spots (Leu^{11}-Gly^{12} and Thr^{17}-Ile^{18}-Pro^{19}) within which the amino acid residues that are key to B27-WT's bioactivity are clustered. In the work described here, the most critical residues of B27-WT, Leu^{11} and Ile^{18}, were further probed to determine the nature of their interaction with calpain. Our results demonstrate that the side chains of both residues interact with hydrophobic pockets in calpain and that each of these interactions is indispensable for effective inhibition of calpain. Direct interactions involving the β- and γ-CH_2- of the Leu^{11} and Ile^{18} side chains, respectively, rather than the degree of side chain branching or hydrophobicity, seemed to play a significant role in the peptide's ability to inhibit calpain. Furthermore, the minimum peptide sequence that still retained the calpain-inhibitory potency of B27-WT was found to be MSSTYIEELGKREVTIPPKYRELL.

One of the pathological events that contribute to the progression of multiple sclerosis, Alzheimer's disease, cardiac and brain ischemia, and cataract formation is uncontrolled activation of the calcium-activated neutral cysteine protease calpain (*1*, *2*). Under physiological conditions, calpain plays a tightly regulated role in signal transduction, cell proliferation, differentiation and migration, and apoptosis (*3*−*7*). The two most extensively studied isoforms of calpain, μ- and *m*-calpain,[1] require micro- and millimolar amounts of Ca^{2+}, respectively, to reach half-maximal activity in vitro. The conventional calpains exist as an inactive heterodimer consisting of a large (catalytic) subunit and a small (regulatory) subunit that are divided into domains DI-DIV and DV-DVI, respectively. It has been suggested that upon binding Ca^{2+} to the EF− hands of DIV and DVI, a conformational change occurs aligning the catalytic machinery of the active site and probably dissociating the two subunits (*8*−*10*). Although there is to date no crystal structure of Ca^{2+}-bound heterodimeric calpain, Moldoveanu et al. have been successful in solving the crystal structure of Ca^{2+}-bound minicalpain (a calpain mutant consisting of DI and DII only). Their studies have revealed two additional non-EF-hand Ca^{2+}-binding regions in DI and DII of minicalpain that are required for activation of the protease (*11*).

The activity of calpain is regulated in vivo by the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) and the enzyme's endogenous inhibitor calpastatin (*12*, *13*). Calpastatin's primary structure is divided into four repeating inhibitory domains (domains 1−4) and an NH_2-terminal domain (domain L) that has no inhibitory activity toward calpain (*14*−*19*). Inside each repeating domain are three conserved subdomains known as A, B, and C (Figure 1). Subdomains A and C are noninhibitory binding regions that act to improve the inhibitory efficiency of subdomain B by promoting tight binding of calpastatin to calpain (*20*). A synthetic inhibitor peptide that corresponds to subdomain B in domain I of human calpastatin (B27-WT, DPMSSTYIEELGKREVTIPPKYRELLA) has been synthesized and shown to inactivate calpain (*21*−*24*). An interesting structural feature of B27-WT is the absence of regular secondary structures such as α-helixes or β-sheets. However, it has been determined, using ^{1}H NMR studies, that B27-WT has a type I β-turn in the Pro^{20}-Lys^{21}-Tyr^{22}-Arg^{23} region and possibly a second less

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[1] Abbreviations: μ- and *m*-calpain, the micromolar and millimolar Ca^{2+}-requiring Ca^{2+}-dependent proteinase, respectively; AMC, aminomethylcoumarin; DIPEA, *N*,*N*′-diisopropylethylamine; ESI, electrospray ionization; Fmoc, *N*-(9-Fluorenyl)methyloxycarbonyl; HBTU, *O*-benzotriazole-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization.

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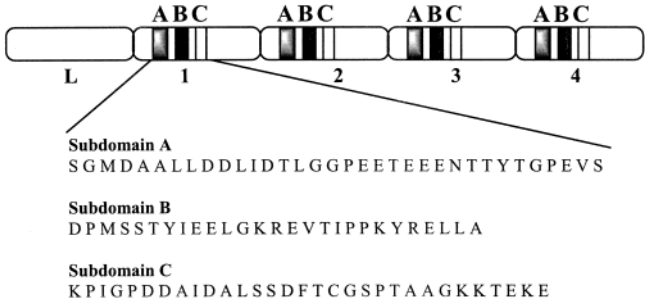

FIGURE 1: Schematic representation of human calpastatin domains. The repetitive domains (1−4) are illustrated with the l-domain at the NH_2 terminus. The amino acid sequences of the binding subdomains A (grey) and C (white) and the inhibitory subdomain B (black) are shown.

defined β-turn in the Glu[10]-Leu[11]-Gly[12]-Lys[13] region (*22*). These turns have been speculated to play a role in the inhibition of calpain.

In a recent publication, we reported that the two most critical amino acid residues in B27-WT are Leu[11] and Ile[18]. These residues are located in hot spots that consist of Leu[11]-Gly[12] and Thr[17]-Ile[18]-Pro[20] (*25*). In the work described here, we further probed the Leu[11] and Ile[18] positions to provide a better understanding of the type of interaction taking place between calpain and the hot spots. By varying the structures of the Leu[11] and Ile[18] side chains, we were able to show that these residues interact with hydrophobic pockets in calpain. As a part of this study, we carried out a stepwise truncation of the NH_2 and CO_2H termini of B27-WT in order to determine the minimum peptide sequence that still retains the calpain inhibitory function.

EXPERIMENTAL PROCEDURES

Materials. Porcine erythrocyte μ-calpain (calpain I) was purchased from Calbiochem (San Diego, CA). Suc-Leu-Tyr-AMC was obtained from Sigma (St. Louis, MO). Fmoc-β-Homo-Leucine was obtained from PepTech, Inc. (Cambridge, MA). Fmoc-Norvaline, Fmoc-d-Leucine, all proteinogenic Fmoc-protected amino acids, Fmoc-Ala-Wang resin, piperidine, DIPEA, HBTU, and trifluoroacetic acid were purchased from Advanced ChemTech (Louisville, KY). Dimethylformamide and tetrahydrofuran were purchased from Burdick & Jackson (Muskegon, MI) and used without further purification. All other reagents were of analytical grade.

Peptide Synthesis. All peptides were synthesized, purified, and analyzed as described previously (*25*).

Steady-State Kinetics of Calpain Inhibition by B27-WT Its Analogues. All steady-state kinetic experiments were performed as described previously (*25*). The dose-response curve for the inhibition of calpain over varying concentrations of B27-WT and mutants was modeled to a three-parameter sigmoidal equation 1, and the IC_{50}'s were calculated using the Sigma Plot graphing software (SPPS Inc., Chicago, IL)

$$\% \text{ Inhibition} = 100/(1 + ([I]/IC_{50})^s) \quad (1)$$

[I] is the inhibitor concentration, IC_{50} is the inhibitor concentration at 50% inhibition, and *s* is the Hill coefficient that describes the steepness of the sigmoid dose-response curve.

The equilibrium dissociation constant, K_i, for the reversible inhibition of calpain was determined as described by Knight (*26*) and could be best fit to eq 2:

$$v_i/v_o = 1/(1 + [I]/K_i^{app}) \quad (2)$$

where v_i and v_o are the rates of substrate cleavage in the presence and absence of inhibitor, respectively. [I] is the inhibitor concentration and K_i^{app} is the apparent inhibitor constant. K_i is obtained from K_i^{app} after correcting for substrate competition and is described by eq 3:

$$K_i = K_i^{app}/(1 + [S]/K_m) \quad (3)$$

The Michaelis constant (K_m) for calpain-catalyzed hydrolysis of Suc-LY-AMC (3.29 ± 0.18 mM) was determined experimentally using Lineweaver−Burk plots with substrate concentrations of 0.1−4 mM (data not shown).

Gel Electrophoresis. The effect of B27-WT and its analogues on Ca^{2+}-induced autolysis of μ-calpain was investigated and analyzed by SDS-polyacrylamide gel electrophoresis as described previously (*25*).

RESULTS

Synthesis and Characterization of the Calpastatin Peptides. The 27-residue human calpastatin subdomain 1B (162−188) peptide (named B27-WT, D-P-M-S-S-T-Y-I-E-E-L-G-K-R-E-V-T-I-P-P-K-Y-R-E-L-L-A) (*23*) was selected for this study. The wild-type peptide, B27-WT, and a library of B27-WT analogues, containing specific positional modifications or truncations at the NH_2 and CO_2H termini are shown in Figure 2. The synthetic peptides were purified to near homogeneity by HPLC, and their molecular weights were confirmed by ESI and/or MALDI mass spectrometry (Table 1). No contamination by modified or incompletely deprotected peptides was detected after final purification.

Nature of Interaction between Calpain and B27-WT Hot-Spot Positions Leu[11] and Ile[18]. In our recent studies, using both β-Ala and conventional L-Ala scanning mutagenesis for the structure−function analysis of B27-WT, we identified two hot spots in B27-WT that are absolutely critical for the inhibitory activity of the peptide (*25*). The positions of the hot spots are indicated in the primary structure of B27-WT as illustrated at the top of Table 1. Leu[11] and Ile[18] were found to be the most important contributors to the inhibition of calpain. It appeared that the side chains of these residues interact with hydrophobic binding pockets in calpain. Furthermore, the Leu[11]-Gly[12] backbone seemed to play a crucial role in B27-WT's bioactivity. To further examine the nature of the calpain-calpastatin interaction at these positions, the Leu[11]-Gly[12] backbone and the side chain structures at positions 11 and 18 were modified, followed by a functional analysis of the effect of various changes in side chain branching and hydrophobicity, and amide backbone structure on the peptide's biological activity.

At position 11, it was necessary to delineate between the contributions of the side chain and the amide backbone. Therefore, two types of structural modifications were carried out. First, Leu[11] was replaced with β-Homo-Leu. This mutation modified the Leu[11]-Gly[12] backbone by inserting a methylene ($-CH_2-$) group between the α-carbon and the carbonyl group of Leu[11] while maintaining the structure of the side chain functionality (see Figure 4A). A K_i of 1462 nM was obtained for B27-β-Homo-Leu[11] (Table 1). B27-β-

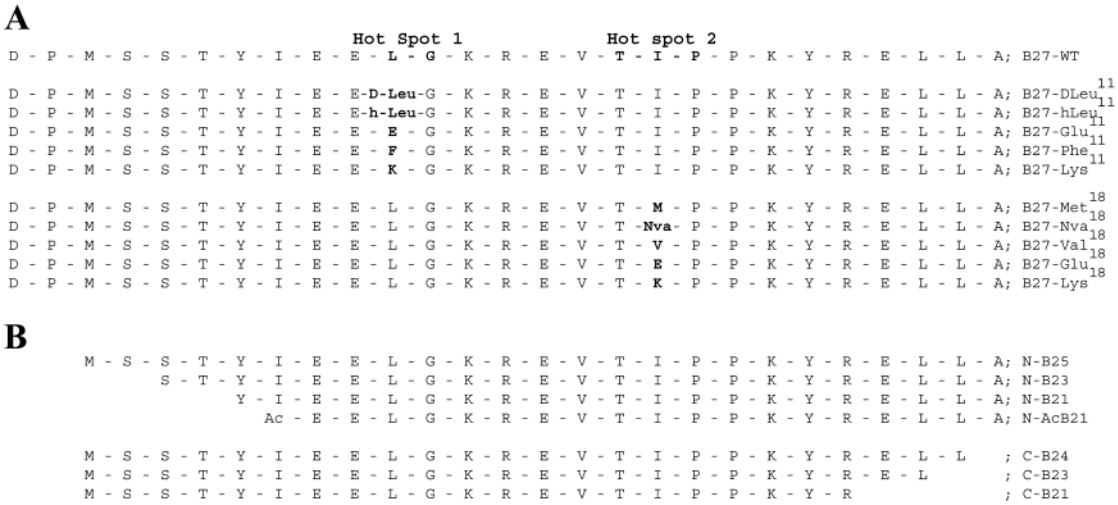

FIGURE 2: (A) Alignment of amino acid sequences of B27-WT and its position 11 and 18 mutants. The nomenclature for the library is specified on the *right* of the figure. L-Leu[11] was mutated to D-Leu and Ile[18] was replaced with Met, Nva or Val. The peptide with no structural modifications was called B27-WT and, for example, a peptide in which Ile[18] was replaced with Val was called B27-Val[18]. (B) NH_2- and CO_2H-terminal truncated fragments of B27-WT. The amino acid sequences of the NH_2-terminal truncated peptides starting with a 25-residue and ending with a 19-residue peptide are shown. The 25-residue peptide was named N-B25, where the N represents an NH_2-terminal truncation and the 25 represents the number of amino acid residues remaining in the primary sequence. The CO_2H-terminal truncated peptides are shown. The Asp[1]−Pro[2] segment was eliminated from all CO_2H-terminal truncated peptides because it was found not to be important for calpain inhibition. The CO_2H-terminal truncated peptides were named in a fashion similar to the NH_2-terminal truncated peptides.

Table 1: Data for the Synthetic Peptides

Hot spot 1 *Hot spot 2*

B27-WT: D[1]-P[2]-M[3]-S[4]-S[5]-T[6]-Y[7]-I[8]-E[9]-**E[10]-*L[11]*-*G[12]*-K[13]**-R[14]-E[15]-V[16]-*T[17]*-*I[18]*-*P[19]*-**P[20]-K[21]-Y[22]-R[23]**-E[24]-L[25]-L[26]-A[27]

Type II β-turn region **Type I β-turn region**

name	expected mass (g/mol)	observed mass (g/mol)	IC_{50} (nM)	K_i (nM)	category of inhibitory activity[a]	residue replaced (removed)
B27-WT	3134.6	3136.0	12 ± 1	8.7 ± 2	+++	none
B27-βAla[11]	3094.6	3094.8	>1000	17000 ± 5500	− − −	Leu[11]
B27-Ala[11]	3094.6	3094.7	250 ± 1	200 ± 53	+	Leu[11]
B27-DLeu[11]	3134.6	3136.0	560 ± 1	670 ± 84	− − −	Leu[11]
B27- βHomoLeu[11]	3148.6	3150.1	>1000	1500 ± 110	− − −	Leu[11]
B27-Glu[11]	3152.6	3151.9	930 ± 1	1000 ± 110	− − −	Leu[11]
B27-Phe[11]	3170.7	3107.5	620 ± 1	590 ± 30	− − −	Leu[11]
B27-Lys[11]	3151.7	3151.9	830 ± 1	870 ± 78	− − −	Leu[11]
B27-βAla[18]	3094.6	3094.5	>1000	2400 ± 220	− − −	Ile[18]
B27-Ala[18]	3094.6	3094.2	>1000	1400 ± 110	− −	Ile[18]
B27-Met[18]	3154.7	3154.0	13 ± 1	9.2 ± 1.5	+++	Ile[18]
B27-Nva[18]	3122.6	3123.6	12 ± 1	12 ± 3	+++	Ile[18]
B27-Val[18]	3120.6	3121.8	21 ± 1	13 ± 3	+++	Ile[18]
B27-Glu[18]	3152.6	3152.1	870 ± 5	930 ± 110	− − −	Ile[18]
B27-Lys[18]	3151.7	3151.4	44 ± 1	40 ± 7	+++	Ile[18]
N-B25	2922.6	2924.3	21 ± 1	n.d.	+++	D[1]P[2]
N-B23	2704.5	2706.7	76 ± 1	n.d.	++	D[1]-P[2]-M[3]-S[4]
N-B21	2516.4	2518.1	360 ± 1	n.d.	− −	D[1]-P[2]-M[3]-S[4]-S[5]-T[6]
N-AcB19	2282.3	2283.8	1800 ± 16	n.d.	− − −	D[1]-P[2]-M[3]-S[4]-S[5]-T[6]-Y [7]-I[8]
C-B24	2851.5	2852.4	25 ± 1	n.d.	+++	D[1]-P[2]; A[27]
C-B23	2738.4	2740.8	59 ± 1	n.d.	++	D[1]-P[2]; L[26]-A[27]
C-B21	2496.3	2498.5	150 ± 1	n.d.	+	D[1]-P[2]; E[24]-L[25]- L[26]-A[27]
DPMSSTYIEELG + KREVTIPPKYRE LLA	1340.5 1813.1	1341.3 1813.9	>1000	n.d.	− − −	

[a] +++, strong inhibition; ++, moderate inhibition; +, very moderate inhibition; −, weak inhibition; − −, very weak inhibition; − − −, no inhibition.

Ala[11], in which the Leu[11] side chain and the Leu[11]-Gly[12] backbone functionalities were modified, was found to exhibit a K_i value of 16 660 nM. This stepwise modification of the Leu[11]-Gly[12] backbone followed by the removal of the Leu[11] side chain (i.e., Leu → β-Homo-Leu → β-Ala) resulted in ~160- and ~2000-fold increases, respectively, in K_i. B27-Ala[11], with a shortened side chain at position 11, has a K_i of 200 nM. These results demonstrate the importance of the Leu[11] side chain relative to the Leu[11]-Gly[12] peptide bond. Next, we wanted to change the enantiomeric configuration

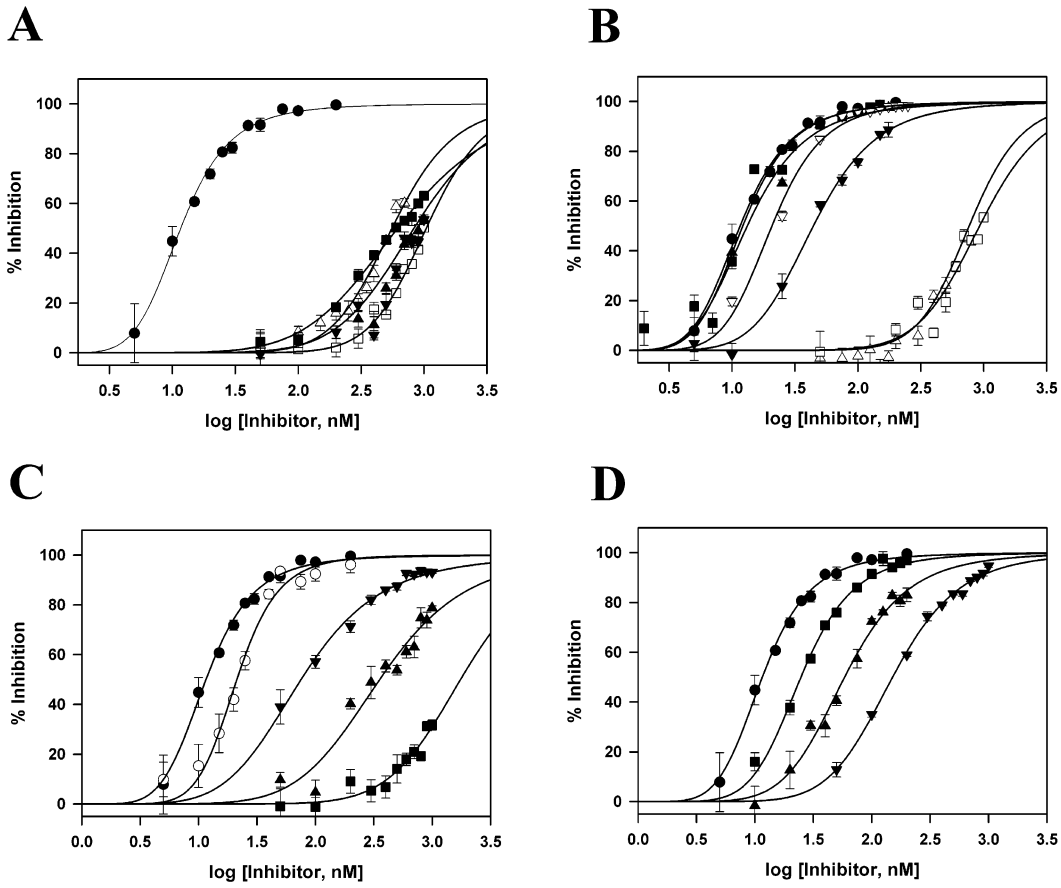

FIGURE 3: Inhibition of calpain by B27-WT analogues containing specific positional modifications or NH_2- and CO_2H-terminal truncations. Various concentrations (0−1 μM) of the inhibitor peptide were preincubated with 0.76 μg (6.8 nM final concentration) of calpain in an assay buffer containing 1 mM $CaCl_2$, followed by the addition Suc-Leu-Tyr-AMC to a final concentration of 300 μM as described under the Experimental Procedures. Residual calpain activity in the enzyme/ inhibitor complex was measured in a fluorescence microtiter plate reader, and the percent enzyme inhibition was determined by comparing the activity measured in the absence of inhibitor. Plots of percent inhibition, measured between 0 and 300 nM vs the log of inhibitor concentration are shown in the graphs. (A) A dose-response curve of calpain inhibition by a peptide mutated at position 11 in the first hot spot, Leu[11]-Gly[12]; ●, B27-WT; □, β-Homo-Leu; △, B27-d-Leu[11]; ▼, B27-Glu[11]; ▲, B27-Lys[11]; ■, B27-Phe[11]. (B) A dose-dependent curve of calpain inhibition by a peptide mutated at position 18 in the second hot spot, Thr[17]-Ile[18]-Pro[19]; ●, B27-WT; △, B27-Ala[18]; □, B27-Glu[18]; ▼, B27-Lys[18]; ▲, B27-Met[18]; ■, B27-Nva[18]; ▽, B27-Val[18]. (C) A dose-response curve of calpain inhibition by NH_2-terminal truncated peptides; ●, B27-WT; ○, N-B25; ▼, N-B23; ▲, N-B21; ■, Ac-N-B19. (D) A dose-response curve of calpain inhibition by CO_2H-terminal truncated peptides; ●, B27-WT; ■, C-B24; ▲, C-B2; ▼, C-B21. Each data point at a specific inhibitor concentration is the mean of 5−8 repeated experiments; the uncertainty of this average is represented by a standard error bar. The solid line in these plots represents the fit of the data to eq 1 described in the Experimental Procedures section.

of the Leu[11] side chain while leaving the amide backbone as unperturbed as possible. This was accomplished by substituting the D-enantiomer of Leu in place of the L-enantiomer. The L-Leu to D-Leu mutation would place the side chain of the Leu residue in the opposite orientation in space compared to L-Leu. It was expected that this structural modification would provide information on the contribution of the side chain to calpain inhibition, with minimal perturbation of the amide backbone. A K_i of 674 nM was obtained for B27-D-Leu[11]. The observation that B27-D-Leu[11] was a weak calpain inhibitor confirmed that a proper orientation of the amino acid side chain at this position of the peptide contributes significantly to the mechanism of inhibition of calpain (Table 1 and Figure 3A). It should be noted that B27-Ala[11] (K_i = 200 nM) was still a very moderate inhibitor of calpain even though the long-branched hydrophobic side chain of Leu {$-CH_2CH(CH_3)_2$} was replaced with the shorter side chain of Ala ($-CH_3$). The hydrophobic nature of the interaction involving position 11 of B27 was further analyzed by replacing Leu[11] with an amino acid carrying a negatively charged (Glu[11]) or a positively charged (Lys[11]) side chain. B27-Glu[11] and B27-Lys[11] exhibited K_i values of 1030 and 873 nM, respectively. Finally, Leu[11] was replaced with Phe[11] to probe the possibility of a hydrophobic interaction involving an aromatic ring. B27-Phe[11] (K_i = 593 nM) was almost as ineffective as B27-Glu[11] and B27-Lys[11] in inhibiting calpain. These results clearly demonstrate that the side chain in position 11 interacts with a hydrophobic pocket on the surface of calpain, and that this interaction is critical for the efficient inhibition of calpain. Furthermore, the aliphatic hydrophobic Leu[11] side chain is more favorably accommodated than the bulky aromatic ring of Phe[11].

The interaction between calpain and the amino acid side chain at position 18 of B27-WT was investigated by generating five new peptides in which Ile[18] was replaced with Nva, Met, Val, Glu, or Lys (Figure 4B). These Ile[18]-mutant peptides were then evaluated for their ability to inhibit the Suc-Leu-Tyr-AMC hydrolytic activity of calpain, using a

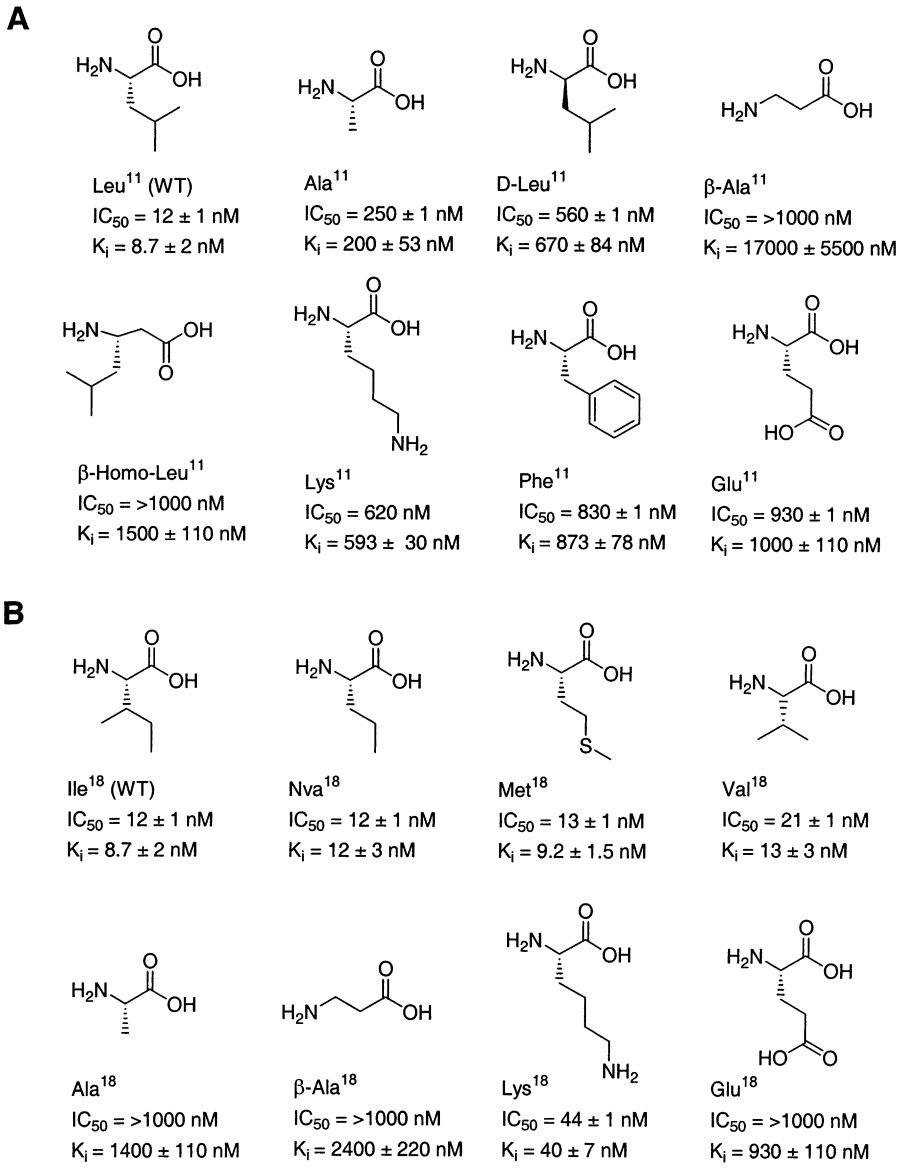

FIGURE 4: Comparison of the chemical structures of amino acid side chains that were used to replace Leu at position 11 and Ile at position 18 of B27-WT. The wild-type peptide, B27-WT, has Leu at position 11 and Ile at position 18. (A) The side chain structures of the amino acid residues (*β*-Homo-Leu, D-Leu, Glu, Lys, and Phe) that were substituted for Leu[11], together with the corresponding IC_{50} and K_i values are shown. (B) The side chain structures of the amino acid residues (Nva, Met, Val, Ala, Lys, and Glu) that were inserted at position 18 of the mutants, together with the corresponding IC_{50} and K_i values are shown.

previously described steady-state fluorescence assay (*25*). B27-*β*-Ala[18] and B27-Ala[18], which had previously been evaluated for calpain-inhibitory activity, were also included in this study for comparison. As summarized in Table 1 and illustrated in Figure 3B, B27-WT, B27-Nva[18], B27-Met[18], B27-Val[18], and B27-Lys[18] were potent inhibitors that exhibited K_i values of 8.7, 13, 9.2, 22, and 40 nM, respectively. On the other hand, B27-Glu[18] and B27-*β*-Ala[18], with K_i values of 925 and 2389 nM, respectively, were ineffective in inhibiting calpain. The observation that B27-WT, B27-Nva[18], B27-Met[18], and B27-Val[18] have similar K_i values demonstrates that the degree of side chain branching and hydrophobicity at position 18 does not play a significant role in the peptide's ability to inhibit calpain. However, a residue with a side chain consisting of at least $-CHCH_3$ is required at this position for efficient calpain inhibition. Furthermore, the results demonstrate that the negatively charged side chain of Glu[18] is not tolerated by the corresponding binding pocket in calpain whereas the positively charged side chain of Lys[18] is very well accommodated by the same binding pocket.

Ability of Each Hot Spot To Independently Block Calpain Activity. The inhibition of calpain relies on the simultaneous interaction of B27-WT's hot spots with hydrophobic pockets on calpain. However, a question that remains is whether the hot spots need to be covalently connected in order to efficiently inhibit calpain. To investigate this possibility, two peptides containing either the NH_2-terminal (DPMSSTYIEELG) or CO_2H-terminal (KREVTIPPKYRELLA) hot spots were synthesized and evaluated together for their ability to inhibit calpain. The design of this assay involved incubating equimolar amounts of the two peptides together with calpain in the presence of Ca^{2+}, to mimic the conditions that would be present in the assay had the full-length B27-WT peptide been employed. There was no inhibition of calpain even when μM concentrations of the two peptides were incubated with the protease. This demonstrates that the hot

spots of B27-WT have to be covalently connected in order to obtain a functional calpain inhibitor.

Contribution of the NH_2- and CO_2H-Terminal Regions of B27-WT to Bioactivity. The need for the hot spot regions of B27-WT to be covalently connected in order to efficiently inhibit calpain has led us to carry out a more detailed examination of the importance of the NH_2- and CO_2H-terminal regions. We, therefore, determined whether the inhibitory potency of B27-WT could still be maintained following amino acid deletions from the extremities of the peptide. This possibility was investigated by generating a series of peptides that contain NH_2- and CO_2H-terminal truncations.

As shown in Table 1 and Figure 3*C*, residues $D^{1-}P^2$ (N-B25, $IC_{50} = 21$ nM) could be deleted from B27-WT without any significant loss of inhibitory potency. However, upon deletion of $M^{3-}S^4$ (N-B23, $IC_{50} = 84$ nM) and $S^{5-}T^6$ (N-B21, $IC_{50} = 365$ nM) the inhibitory effect of the peptide decreased significantly, and upon deletion of residues $Y^{7-}I^8$ (N-AcB19, $IC_{50} = >1000$ nM) the inhibitory activity was completely lost (Figure 3*C*). Interestingly, a β-Ala scan of the NH_2-terminal region of the peptide did not reveal any major loss in inhibitory activity following the single residue point mutations.

The importance of the CO_2H terminus of B27-WT was examined in a manner similar to that employed for the NH_2 terminus except that, in addition to truncating the CO_2H-terminal residues, two NH_2-terminal residues ($D^{1-}P^2$) were deleted because they were found in the previous experiments to be nonessential for calpain inhibition. Therefore, Ala^{27} (C-B24, $IC_{50} = 24$ nM), Leu^{26} (C-B23, $IC_{50} = 65$ nM), and Glu^{24}-Leu^{25} (C-B21, $IC_{50} = 165$ nM) were deleted in order to establish the absolute minimum sequence required for the efficient inhibition of calpain (Figure 3D). The minimum sequence that still retained the calpain-inhibitory potency of B27-WT was found to be MSTYIEELGKREVTIPPKYRELL. The CO_2H-terminal portion of B27-WT was probed more closely because it has been suggested previously that the Arg^{23}-Glu^{24}-Leu^{25}-Leu^{26} region was important for efficient inhibition of calpain (*24*). It is interesting to note that the stepwise deletion of residues from the NH_2- and CO_2H-terminal regions of B27-WT had a dramatic effect on inhibition, whereas replacement of residues in the same regions with β-Ala had minor effects on the peptide's bioactivity. This could be due to the fact that the overall inhibitory effect depends, in a large measure, on the sum of a certain number of discrete interactions between calpain and B27-WT's NH_2- and CO_2H-terminal regions. A stepwise reduction in the number of discrete interactions, through peptide truncation, would result in the steady loss of inhibitory activity.

Effect of NH_2- and CO_2H-Terminal Truncated B27-WT Analogues on Calpain Autolysis. Calpain is activated by a Ca^{2+}-mediated conformational change that aligns the catalytic machinery of the active site. It has been proposed that, as a result of this conformational change, the prosegment (anchor) of the catalytic subunit is released from its interaction with the regulatory subunit (*9*) and the catalytic and regulatory subunits are autolyzed from 80- to 76-kDa and 28- to 18-kDa, respectively (*27*). Autolysis of the prosegment is a two-step process that involves cleavage of the 80-kDa catalytic subunit to a 78-kDa intermediate, and finally to a 76-kDa fragment. In our previous studies on the effect of B27-WT and its β-Ala mutants on calpain autolysis, it was demonstrated that B27-WT-β-Ala-X mutants with moderate to strong inhibitory activities ($IC_{50} \leq 100$ nM) slowed both steps of autolysis (80 to 78 kDa and 78 to 76 kDa) with an efficacy comparable with what was obtained with 50 μM B27-WT. Mutants that were weakly effective ($IC_{50} = 100-300$ nM) blocked only the second step (78 to 76 kDa) of the autolytic process. Noninhibitory mutants ($IC_{50} > 1000$ nM) failed to inhibit any of the early steps of autolysis (*25*). Our findings suggested that, at least in vitro, the calpastatin peptides inhibited calpain's ability to proteolyze its substrates by blocking the formation of the 76-kDa form of the catalytic subunit. A similar trend in the ability of the mutant peptides to block both steps of autolysis was observed for the CO_2H-terminal truncated peptides C−B24, C−B23 and C−B21 with IC_{50} values of 24, 65, and 165 nM, respectively (Table 1 and Figure 5B). Interestingly, N-AcB19, which was not effective at inhibiting the catalytic activity of calpain ($IC_{50} > 1000$ nM), did block the second step (78 to 76-kDa) of the autolytic process while N-B23 ($IC_{50} = 84$ nM) blocked the second step but was not effective in slowing the first step as would have been predicted from the IC_{50} values (Table 1 and Figure 5A). As mentioned previously, the apparent discrepancies between the qualitative autolysis data and quantitative substrate kinetic results for some of the mutants seem to be within an allowable margin of experimental error. However, it is tempting to deduce from the data on N-AcB19 that the deletion of 8 amino acid residues from the NH_2 terminus of B27-WT allowed entry of the small molecular weight fluorogenic substrate (Suc-Leu-Tyr-AMC) to the active site of calpain while entry of a large protein substrate (intermolecular autolysis of calpain) was hindered by the presence of the truncated N-AcB19 peptide.

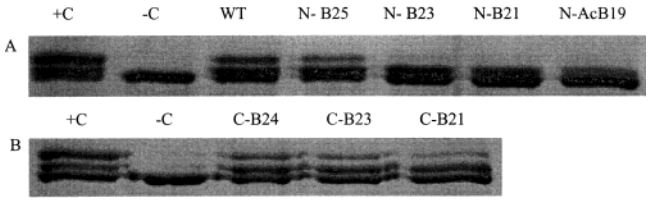

FIGURE 5: Inhibition of calpain autolysis by NH_2- and CO_2H-terminal truncated peptides. The degradation of the large subunit of μ-calpain from 80 → 78 → 76 kDa was monitored by SDS−PAGE after incubation with increasing amounts of B27-WT or its NH_2- and CO_2H-terminal truncated versions. The + control lane (+C) includes calpain in the absence of Ca^{2+} and in the absence of B27-WT. The − control lane (−C) includes calpain after a 15 min incubation in the presence of Ca^{2+} without B27-WT. 23 μg of calpain was incubated for 15 min with Ca^{2+} and 50 μM of truncated peptide. The reaction was stopped by adding SDS−PAGE reducing buffer (2X) reaction and heating at 95 °C for 5 min. 4-μg samples of truncated peptide-treated calpain samples were analyzed by 7.0% SDS−PAGE, and the gels were stained with GelCode Blue Stain Reagent. (A) Analysis of the NH_2-terminal truncated peptides' ability to inhibit the autolysis of calpain. The lanes are labeled with the name of the peptide tested. (B) Analysis of the CO_2H-terminal truncated peptides' ability to inhibit the autolysis of capain. The lanes are labeled with the name of the peptide tested.

DISCUSSION

Structure−activity analysis of B27-WT, using L-Ala and β-Ala scanning mutagenesis, has revealed the presence of two hot spots (Leu^{11}-Gly^{12} and Thr^{17}-Ile^{18}-Phe^{19}) that are

absolutely critical for calpain inhibition (*25*). Single residue mutation of any one of the hot spot residues to β-Ala resulted in the complete loss of the peptide's inhibitory activity, suggesting that a simultaneous interaction of both hot spots is required for efficient calpain inhibition. In the course of analyzing the B27-β-Ala library for inhibitory potency, it was discovered that Leu[11] and Ile[18] are the residues most critical for calpain inhibition. Therefore, to establish a better understanding of the type of interaction occurring between calpain and these critical residues, a study that further examined the role(s) of Leu[11] and Ile[18] residues was carried out.

By varying the degrees of side chain branching and hydrophobicity at positions 11 and 18, we were able to show that Leu[11] and Ile[18] are indeed involved in an interaction with hydrophobic pockets in calpain. B27-Ala[11] ($-CH_3$) was found to be a very moderate to a weak inhibitor of calpain. This indicated that a small hydrophobic side chain at position 11 can interact with a hydrophobic environment in calpain, although not as efficiently as the longer side chain of Leu. The inability of charged or polar side chains at position 11 of B27 to effectively interact with calpain further confirms the hydrophobic nature of the interaction. For example, Glu with a polarity of -10.2 and a carboxylate side chain that is almost completely ionized at physiological pH values and Lys with a polarity of -15.0 and a basic side chain which is fully protonated at neutral pH were not good replacements for Leu[11]. Residues such as Ala and Leu, with a polarity and chain length of $+1.9/1.53$ and $+2.3/4.59$ Å, respectively, were very well tolerated at position 11. It was deduced from the structure−function analysis that the ideal length of the side chain at position 11 should be between 1.53 and 4.59 Å. Phenylalanine, with a strongly apolar side chain, was not a good candidate at position 11. Leu and Phe have a van der Waals volume of 124 and 135 Å, respectively. Our results suggest that the corresponding hydrophobic binding pocket in calpain that interacts with the residue at position 11 of B27 is not flexible enough to accommodate the resonance-stabilized ring system of the Phe side chain. The introduction of polar uncharged residues such as Thr or Asn at position 11 might have revealed novel interactions. Furthermore, the choice of substitutions for Leu[11] could have included other nonpolar residues such as Ile, Nval, and Val as was done at position 18. Nevertheless, the distinct hydrophobic nature of the interactions as well as differences in the size of the binding pockets for Leu[11] and Ile[18] are clear from the present studies. Leu[11] and Gly[12] are located within a region of B27-WT, which is probably a type II β-turn, in which case Leu[11] and Gly[12] would be the $i + 1$ and $i + 2$ residues, respectively. On the basis of our new observations and previous NMR studies on B27-WT (*22*), we propose that the Glu[10]-Leu[11]-Gly[12]-Lys[13] sequence is involved in a β-turn structure with the Leu[11] side chain occupying one of the corner positions of the turn and pointing outward to serve as a site for interaction with a hydrophobic binding pocket in calpain. This hydrophobic pocket favors binding interaction with aliphatic hydrophobic side chains that are about 5 Å long. This information has important implications for further studies that are aimed at incorporating photoactivatable amino acid analogues at position 11 of B27-WT for subsequent photo-cross-linking experiments to identify this calpastatin-binding site in calpain. The large size of most photoactivatable amino acid analogues such as benzophenone (Bpa; *p*-benzoylphenylalanine) or l-4′-[3-(trifluoromethyl)-3*H*-diazirin-3yl]phenylalanine (TmdPhe) could be a disadvantage since it could change the affinity of the ligand as seen from the K_i for B27-Phe[11]. At position 18, it was discovered that a longer side chain was important for efficient calpain inhibition. B27-Ala[18] was previously shown to be a very weak inhibitor of calpain, displaying an IC_{50} of >1000 nM and a K_i of 1411 nM (*25*). However, B27-Val[18] ($IC_{50} = 24$ nM; $K_i = 22$ nM) has IC_{50} and K_i values that are in a similar range to those obtained for B27-WT ($IC_{50} = 18$ nM; K_i=8.7 nM). This is interesting because, the side chain of Val {-$CH(CH_3)_2$} is only one CH_3 group longer than the side chain of Ala ($-CH_3$) and one $-CH_2-$ shorter than the side chain of Ile ($-CH_2CH(CH_3)_2$. These observations suggest that, for position 18 of B27-WT, side chain length is more important than side chain branching, a theory that is supported by the fact that B27-Nva[18] ($-CH_2-CH_2-CH_3$) is a strong inhibitor of calpain even though Nva has no side chain branching. The results further demonstrate that the B27-WT Ile[18]-binding pocket in calpain is deeper and less restrictive than the Leu[11]-binding pocket. Croall and McGrody have suggested that the interaction between calpain and calpastatin subdomain B involves binding to DIII of the catalytic subunit of calpain (*28*). Although the results from our study cannot confirm the interaction of B27-WT with DIII of calpain, it has identified specific residues that may be in direct contact with hydrophobic pockets in calpain. This information will be exploited to perform chemical cross-linking experiments to precisely identify the specific location of the calpain/B27-WT interactions.

It has been reported that calpain has hydrophobic areas located on its regulatory subunit that are somewhat solvent exposed in the absence of calcium. Upon binding Ca^{2+}, calpain undergoes a conformational change that further exposes this hydrophobic pocket (*27*). Our laboratory has demonstrated, using immobilized B27-WT on CNBr-activated Sepharose 4B resin (Amersham Biosciences), that calpain binds to B27-WT only in the presence of Ca^{2+} (*29*). B27-WT bears no regular secondary structures but possesses two β-turns that are located near the hot spots of the peptide. It is conceivable that upon the addition of Ca^{2+}, calpain reveals a hydrophobic pocket(s) that would interact with Leu[11] and Ile[18] and other residues of the hot spots. This "docking" of B27-WT to Ca^{2+}-activated calpain could trigger a structural change in the inhibitor, and the structural information would then be relayed through the amide backbone of the peptide, enabling the entire peptide to form a well-defined structure. The concerted interaction of both hot spots is required for enzyme inhibition, suggesting that the hot spots and the residues ($K^{13}R^{14}E^{15}V^{16}$) between them could be acting as a "gate" that physically prevents the entrance of substrates into the active site of calpain. Covalent attachment of both hot spots, via the $K^{13}R^{14}E^{15}V^{16}$ linker, is required in order to keep the gate closed. This mechanism is supported by the fact that two peptides (DPMSSTYIEELG and KREVTIPPKYRELLA) that together make up the entire primary sequence of B27-WT could not inhibit the catalytic activity of calpain even when the concentrations of both peptides reached the micromolar range. Further experiments which would involve the deletion of at least one residue, e.g., Lys[13], from the junction of the two halves of B27 could

probably provide information on the distance between the binding sites of the two hot spots.

The proposed mechanism of inhibition suggests that the overall structure of B27 could play a role in the inhibition of calpain. This led to the question of whether the hot spots and the amino acids connecting them were all that were required for efficient inhibition of calpain. In our studies, we have found that the minimum peptide sequence that retains 100% of the inhibitory activity of B27-WT is MSSTYIEELGKREVTIPKYRELL. It was discovered that upon deletion of residues from either terminus of B27-WT the peptide began to significantly lose its potency. This demonstrates that the contributions of multiple interacting sites of B27-WT, including the hot spots, and the NH_2- and CO_2H-terminal regions are required for an effective inhibition of calpain. If B27-WT adopts an active conformation and forms a well-defined functional structure upon binding to calpain, it would follow that the ability of the peptide to form a definable structure could be impaired by deleting enough residues from its NH_2 and/or CO_2H termini. The probability of the NH_2-terminal region of subdomain B of calpastatin to adopt a well-defined structure is, however, diminished by the fact that this region is not well conserved across domains 1−4 of rabbit, human, and pig calpastatin (*25*). It appears as if the type of residues in the NH_2-terminal segments of these calpastatin subdomains is insignificant to the overall inhibition of calpain. This observation is supported by the fact that β-Ala scanning of the NH_2 terminus of B27-WT did not reveal any significant perturbation of the inhibitory function of the peptide, suggesting that the interaction of these residues with calpain could be somewhat nonselective and weak. It is likely that the NH_2-terminal residues in B27-WT are involved in multiple discrete interactions with calpain. For instance, there could be a series of hydrogen bonding occurring between residues on calpain with the hydroxyl-bearing side chains of Ser^4, Ser^5, Thr^6, and Tyr^7 that are clustered together in the NH_2-terminal portion of B27-WT. There is also potential for charged residues such as Glu^9 and Glu^{10} to interact with calpain in an electrostatic fashion. Met^3 and Ile^8 could be involved in hydrophobic interactions with residues on the surface of calpain. Therefore, removal of any one particular interaction in this region, by β-Ala mutagenesis, would not significantly perturb the inhibitory potency of the peptide because the other discrete B27-WT/calpain interactions would remain intact.

Our findings have provided an increased understanding of how B27-WT interacts with calpain to inhibit its biological activity. This information can form the basis for rational design of highly specific and nontoxic calpain inhibitors for use as a research tool for investigating the pathophysiological role(s) of calpain. These calpastatin-derived inhibitors can be further developed into therapeutic agents for treating diseases mediated by dysregulated calpain activity.

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