Calpain cleavage of integrin β cytoplasmic domains[1]

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Abstract We showed previously that the calcium-dependent protease, calpain, cleaves the cytoplasmic domain of the integrin β3 subunit. To investigate whether susceptibility to calpain is a common feature of all integrin β subunits, and to map calpain cleavage sites in different integrin β tails, we treated recombinant cytoplasmic domains of integrin β1A, β1D, β2, β3 and β7 subunits with purified calpain in vitro. We found that the cytoplasmic domains of all these integrin chains were cleaved by calpain. HPLC followed by mass spectrometry was used to identify calpain cleavage sites. These sites were clustered in the C-terminal half of the integrin β cytoplasmic domains in regions flanking the two NXXY motifs, suggesting the possibility that the structural framework provided by these motifs is recognized by calpain. We used the knowledge of these cleavage sites to develop cleavage site-specific antibodies and to demonstrate cleavage of the β1A cytoplasmic domain in intact platelets stimulated with calcium ionophore or thrombin. Thus susceptibility to calpain cleavage is common to integrin β subunits, can be induced in intact cells, and appears to favor regions surrounding two conserved NXXY motifs.

Key words: Integrin; Calpain; Cell adhesion; Cytoskeleton

1. Introduction

Calpains, a family of intracellular cysteine endopeptidases (EC 3.4.22.17), are ubiquitously distributed in animal cells and tissues (for review, see [1–4]). The two classical members of this family, m- and μ-calpain, present a highly similar, if not identical substrate specificity, but differ in the amounts of calcium required for their activation in vitro [1,4]. In addition to intracellular calcium levels, the activity of these calpains is tightly regulated by a natural cytosolic inhibitor, calpastatin, as well as by plasma membrane-binding activator proteins and certain phospholipids indicating an important role of membrane association in calpain activation [3,5]. Cleavage sites generated by these enzymes are characterized by a high variation in the amino acids adjacent to the scissile bond [1,4]. Therefore, susceptibility to cleavage by calpains is not primarily determined by the linear amino acid sequence at one scissile bond, but rather by other structural features, which are not fully understood.

Many cellular substrates of calpains are cytoskeletal proteins [1] and regulatory proteins implicated in the formation of focal adhesions, e.g. pp125FAK [6], pp60src [7] or paxillin [8]. Together with the earlier finding of calpain immunolocalization in focal adhesions of certain cell lines [9], this has led to the concept that calpains could play a regulatory role in the turnover of focal adhesions [10]. Furthermore, fibrinogen binding to platelet integrin αIIbβ3 triggers rapid μ-calpain translocation to the membrane [11], where it is activated and cleaves cytoskeletal proteins such as filamin, talin, and spectrin [3]. Moreover, our laboratory recently provided evidence that platelet aggregation coincides with calpain-dependent proteolysis of the integrin β3 cytoplasmic domain [12]. Cleavage occurred in membrane-distal regions of the integrin β3 tail at sites flanking the two NXXY motifs. These motifs are conserved in most integrin β cytoplasmic domains and are crucial for integrin-mediated signal transduction and for their interactions with the cytoskeleton [13–15]. We therefore asked whether calpains can cleave other integrin cytoplasmic domains as well. Here we report that calpains can cleave integrin β1A, β1D, β2, β3 and β7 tails between and adjacent to their NPXY/NXXY motifs. Furthermore, using cleavage site-specific anti-β1A antibodies, we detect calpain-dependent proteolysis in intact platelets. Thus, calpains recognize and cleave a common structural framework in integrin β tails in vivo.

2. Materials and methods

2.1. Synthetic peptides and purified proteins

Purified m-calpain (80 kDa subunit) from rabbit skeletal muscle was purchased from Sigma (P4533). Alternatively, an m-calpain preparation from rabbit skeletal muscle kindly provided by Dr. T. Saido [16] was used in some experiments with identical results. Peptides NAKWDTGENPIYKSAVT, CEKEKM, CKWDTG, CSAVTT, and CVTTVV corresponding to regions in the integrin β1 cytoplasmic domain were synthesized using an Applied Biosystem Model 430A automated peptide synthesizer and purified by reverse phase HPLC. Masses of all synthetic peptides were verified by ion-spray mass spectrometry.

2.2. Recombinant cytoplasmic domain constructs

Cloning and recombinant production of model peptides representing the cytoplasmic domains of integrins is described in detail elsewhere [15]. Briefly, synthetic oligonucleotides were used in polymerase chain reactions (PCR) to create a cDNA for the heptad repeat protein sequence KLEALEGRLDALEGKLEALEGKLDALEG (=[heptad]$_4$). This cDNA was ligated into a modified pET15b vector (Novagen) and joined to cDNAs coding for cytoplasmic integrin domains. The final constructs coded for the N-terminal sequence GSSHHHHHHSSGLVPRGSHMCG[heptad]$_4$ linked to the cytoplasmic domains of human integrin chains. Recombinant expression in BL21(DE3)pLysS cells (Novagen) and purification of the recombinant products were performed according to the pET System manual (Novagen) with an additional final purification step on a reverse phase C18 HPLC column (Vydac).

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Abbreviations: HPLC, high performance liquid chromatography; mAb, monoclonal antibody; DTT, dithiothreitol; E64, *N*-[*N*-(L-3-*trans*-carboxyoxirane-2 carbonyl)-L-leucyl]-agmatine; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction

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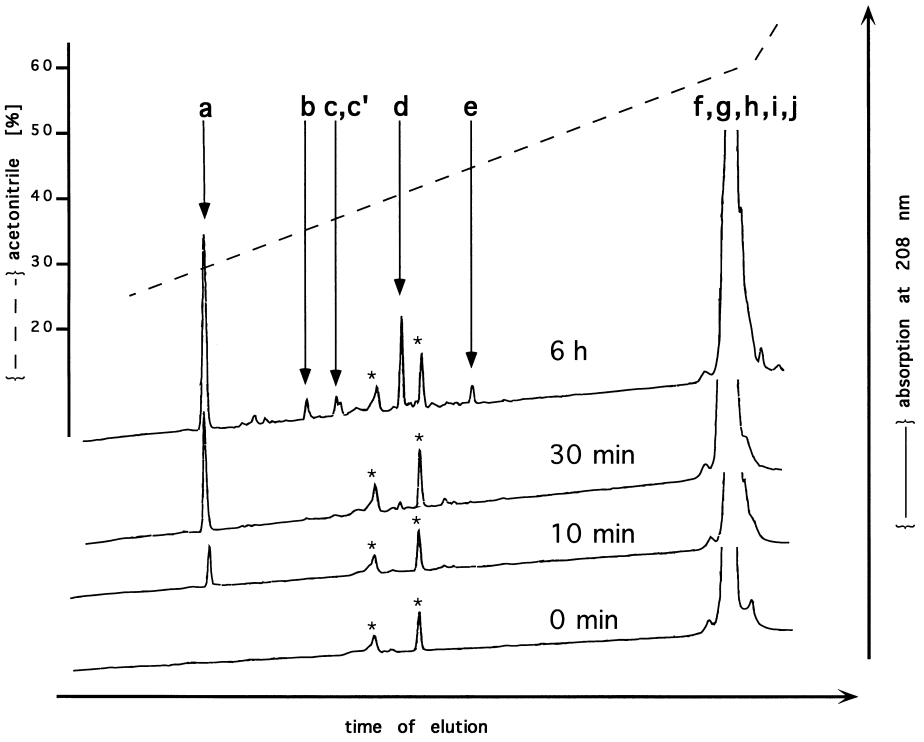

Fig. 1. Reverse phase HPLC of calpain digests obtained with the integrin β1 tail model protein obtained 0, 10, 30, and 360 min after calpain addition. UV absorption at 208 nm was monitored during elution with a 5–60% acetonitrile/water gradient. Peptides identified in the peaks a–j are listed in Table 1 and Fig. 2. The small peptide NPKYEGK, which must have been generated during the proteolysis, probably eluted in the flow-through (not shown), which was not analyzed by mass spectrometry because of its high salt content. The corresponding cleavage reaction was identified by detection of the corresponding larger polypeptide fragment (GS..AVTTVV) (j) eluting at the end of the gradient in the large peak shown on the right. This peak was collected into several fractions to allow the identification of large cleavage products (f–j) and uncleaved recombinant protein. The peptide found in peak b (GSSHHHHHH-SSGLVPRG) resulted from cleavage in the N-terminal His-tag region. Asterisks mark peaks corresponding to unidentified components, which were present in the digestion buffer prior to calpain addition.

2.3. Antibodies

A polyclonal goat antiserum against the human integrin β1 chain (Goat# 172), and rabbit sera against a synthetic integrin β1 cytoplasmic domain peptide (Rabbit# 60) and the integrin β3 calpain cleavage fragment AKWDT [12] have been described. To produce antibodies against specific calpain cleavage sites in the integrin β1 cytoplasmic domain we followed the protocol described in [12]. Briefly, synthetic hexapeptides corresponding to the C-termini of cleavage products were conjugated via N-terminal cysteines to keyhole-limpet hemocyanin (Sigma) using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce). Conjugates were used to immunize rabbits and resulting sera were affinity purified using these peptides coupled to Sulfolink coupling gel (Pierce). In ELISA-type assays we confirmed the immunoreactivity and specificity of these antibodies for the corresponding hexapeptides, as well as for peptide fragments generated in calpain digests of the integrin β1 model protein (data not shown).

2.4. Calpain cleavage site mapping using synthetic and recombinant peptides of integrin cytoplasmic domains

The recombinant model proteins of the cytoplasmic domains of integrins (Fig. 1) were digested at 12 μM with purified m-calpain (80 kDa subunit) (75 nM; 31 U/mg) for 0, 10, 30 and 360 min at

Table 1
Calpain cleavage products of the recombinant integrin β1 cytoplasmic domain

Cleavage site[a]	Time (min)[b]	Measured mass	Predicted	Assigned sequence[c]
V[791]	10	10 026	10 026.5	(j) GS—VTTVV
T[789]	10	9 828	9 828.2	(i) GS—SAVTT
	10	1 034	1 034.2	(a) VVNPKYEGK
G[778]	> 30	8 623	8 623.9	(h) GS—KWDTG
	> 30	1 223	1 223.4	(c′) GENPIYKSAVTT
T[777]	> 30	8 566	8 566.8	(g) GS—AKWDT
	> 30	1 223	1 223.4	(c) ENPIYKSAVTT
M[771]	30	7 852	7 852.1	(f) GS—EKEKM
	30	3 011	3 011.4	NAKW—KYEGK
	> 30	2 193	2 194.4	(e) NAKW—VTTVV
	30	1 995	1 996.2	(d) NAKW—SAVTT

The recombinant model protein of the integrin β1 cytoplasmic domain was digested with calpain (E/S = 1/160) at room temperature for 10, 30 and 360 min. Reaction products were separated by reverse phase HPLC and their masses measured by electrospray mass spectrometry as described in the methods.

[a]β1 residue; numbering of amino acids relates to the human integrin chain sequence including its signal peptide.

[b]Time of first appearance of the peptide.

[c]Letters in parentheses relate to the peptide identification as depicted in Fig. 2. The dashes indicate intervening sequence omitted for clarity.

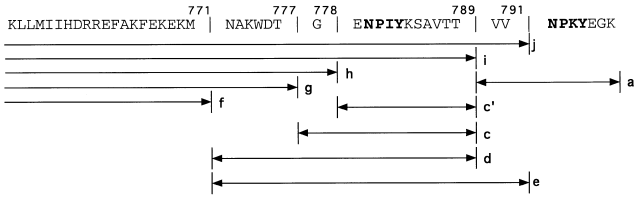

Fig. 2. Calpain cleavage sites and fragments of the recombinant integrin β1 cytoplasmic domain model protein generated in vitro. Single lower case letters refer to the identified peptides as shown in Fig. 1 and Table 1.

room temperature in 50 mM HEPES, pH 7.2, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM DTT. Reactions were stopped by addition of 5 mM EDTA and 12 μg/ml leupeptin. Each sample was separated by reversed phase HPLC and peak fractions were analyzed by electrospray mass spectrometry on an API-III quadrupole spectrometer (Sciex, Toronto, Ont., Canada). Sequences were assigned to proteolytic fragments using the program MacProMass (Terry Lee, City of Hope, Duarte, CA, USA) on the basis of the known sequence of the undigested peptide and the determined molecular masses of the fragments. For some of the smaller peptides, two to three possible peptide sequences were calculated. In these cases correct sequences could be assigned by identification of the corresponding larger cleavage products detected in the protein peak eluting at the end of the acetonitrile gradient. In the case of the β1 integrin cytoplasmic domain, additional calpain cleavage analysis was performed using the synthetic peptide NAKWDTGENPIYKSAVT and a recombinant β1 peptide with the sequence GIHPAHHKRGMCG[heptad]$_4$KLLMIIHDRREFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK. In these experiments we detected identical major cleavage sites as shown in Table 1.

2.5. Calpain cleavage in platelets and Jurkat cells

Platelets were separated from freshly drawn blood by centrifugation at 200×*g* for 20 min. Platelet-rich plasma was centrifuged at 1400×*g* for 15 min and sedimented platelets were washed twice in 0.12 M NaCl, 0.013 M Na_3citrate, 0.033 M D-glucose, pH 6.5. Washed platelets were washed once more in HEPES-saline (3.8 mM HEPES, pH 7.3, 137 mM NaCl, 2.7 mM KCl, 5.6 mM D-glucose, 3.3 mM sodium phosphate) and resuspended in this buffer with 1 mM $CaCl_2$ at 1×10^9 platelets/ml. In an aggregometer after equilibration to 37°C, calcium ionophore A23187 (1 μM) (Sigma), thrombin (1 U/ml) and other agents were added under stirring conditions for various lengths of time. Platelets were solubilized by the addition of 1 volume 2-fold concentrated sample buffer for SDS polyacrylamide electrophoresis containing 0.1 mM E64, 10 μg/ml leupeptin, 1 mM PMSF1 and 5 mM EDTA and heated to 110°C for 5 min. Samples were run on 4–20% SDS polyacrylamide gels (NOVEX) and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA). To ensure efficient transfer of proteins, membranes were routinely stained with Ponceau S prior to blocking with 5% non-fat dry milk in 50 mM Tris-Cl, pH 7.4, 0.15 M NaCl (TBS milk). Blots were washed with TBS, 0.05% Tween-20 and antibodies added in TBS milk. Detection of bound antibodies was performed with peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies (BIOSOURCE), which were visualized with an enhanced chemiluminescence kit (ECL, Amersham Corp.).

Jurkat cells were washed twice with PBS and then incubated in 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mg/ml D-glucose, 1 mg/ml BSA, 10 mM $CaCl_2$ at 4×10^7 cells/ml with or without 10 μM calcium ionophore A23187 for 10 min at 37°C. After short centrifugation, cells were resuspended in the same buffer and lysed with sample buffer for SDS polyacrylamide gel electrophoresis as described above for platelets.

3. Results

After our initial demonstration of calpain-mediated proteolysis of integrin β3 cytoplasmic domains in vitro and in vivo [12], we asked whether susceptibility to cleavage by calpains is a general property of integrin cytoplasmic domains. We used the purified catalytic subunit of m-calpain to digest recombinant protein models of integrin tails in vitro. These model proteins consist of human integrin cytoplasmic tails linked at their N-termini to a His-tag sequence and heptad repeat

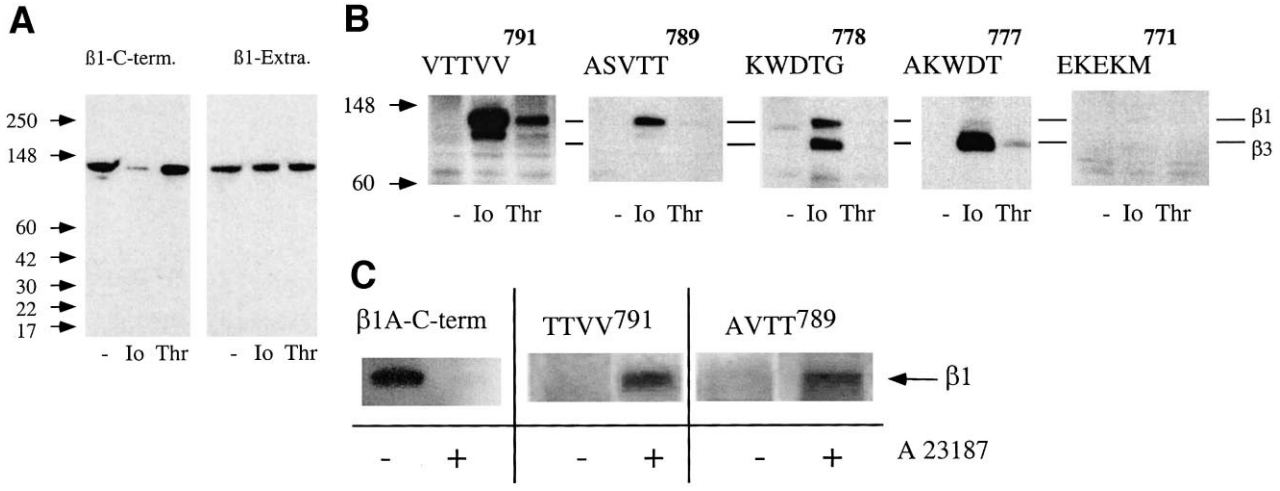

Fig. 3. A: Western blot analysis of cell lysates obtained from human platelets stirred in the absence (−) or presence of either 1 μM calcium ionophore A23187 (Io) for 2 min or of 1 U/ml thrombin (Thr) for 10 min at 37°C. A: Loss of the β1 C-terminus was detected with polyclonal antibodies reactive with the intact C-terminus of the β1 cytoplasmic domain (β1-C-Term). The presence of the extracellular domain of β1 was also verified with an antibody against this region (β1-Extra). B: Detection of cleavages with polyclonal, affinity-purified antibodies recognizing individual cleavage sites in the β1 cytoplasmic domain (see Fig. 2). Due to the sequence similarity in β1 and β3 integrin tails (Fig. 5) the anti-AKWDT^{777} antibody detected cleavage in both β1 and β3 cytoplasmic domains. Similarly, the serum raised against peptide KWDTG^{778} cross-reacted with a homologous β3 fragment with the C-terminus KWDTA^{768}. Although not reported previously [12], we now consistently observed cleavage at this site after calpain digestion of recombinant integrin β3 cytoplasmic domain peptides in vitro (data not shown). An additional band migrating slightly faster than β1 was observed with the antibodies that reacted exclusively with β1. It probably does not represent cleavage in the extracellular domain because it was also detected in undigested samples by antibodies against the C-terminus (A, Fig. 4). C: Analysis of Jurkat cells treated with 10 μM calcium ionophore for 10 min.

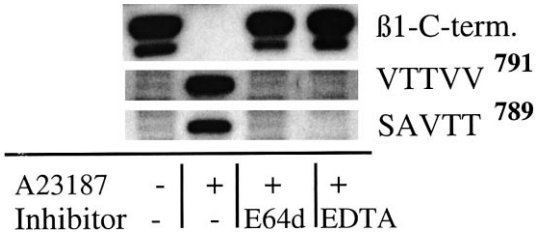

Fig. 4. Calcium ionophore-induced calpain cleavage in platelets in the absence and presence of the membrane-permeable calpain inhibitor E64d (1 mM) or of EDTA (5 mM). Detection was performed with sera recognizing cleavage fragments VTTVV^{791} and ASVTT^{789} or the C-terminus of the intact β1 cytoplasmic domain. Note that the latter serum recognized the band migrating slightly faster than the major integrin β1 chain protein. That band was also present in the anti-VTVV and anti-SAVTT blots, but was cropped from the images for clarity.

motifs forming a parallel dimeric coiled-coil (see Section 2) [15]. They appear to mimic the native cytoplasmic domains [15]. We digested the integrin β1 tail with calpain for 0, 10, 30 and 360 min and separated each digest by reverse phase HPLC on an analytical C18 column (Fig. 1). Peak fractions were analyzed by electrospray mass spectrometry. In addition, we collected the major protein peak eluting in each digest at the end of the 5–60% acetonitrile/water gradient (Fig. 1) into several fractions, which were separately analyzed by mass spectrometry. This allowed us to monitor larger peptide fragments co-eluting with uncleaved protein in this peak. Table 1 lists the fragments obtained from the β1 integrin tail protein model, which are schematically depicted in Fig. 2. Initial rapid cleavage occurred at two sites in the membrane-distal region (P1 residue = T^{789} and V^{791}) as evidenced by the appearance of peptides identified as a, i, and j in Figs. 1 and 2 and Table 1. After 30 min of digestion cleavage occurred at a more proximal site (P1 = M^{771}) as evidenced by the appearance of peptides d and f (Figs. 1 and 2, Table 1). In late (6 h) digests, there was not intact protein remaining and two additional minor cleavages were observed (P1 = T^{777}, G^{778}) as indicated by the appearance of peptides c, c′, g, and h (Figs. 1 and 2, Table 1). The 6 h digestion resulted in fragmentation in the N-terminal His-tag region of the recombinant protein (Fig. 1, peak b), however, the coiled-coil structures and the membrane-proximal integrin sequences of the recombinant peptides were not cleaved.

To analyze calpain cleavage of the β1 integrin tail in living cells, we prepared affinity-purified polyclonal antibodies against each of its cleavage sites and used them on Western blots of platelet lysates aggregated by treatment with the calcium ionophore A21387 or with thrombin (Fig. 3). Ionophore treatment resulted in nearly complete degradation of the β1 integrin cytoplasmic domain (Fig. 3A), and in the generation of truncated fragments containing the C-termini VTTVV^{791}, ASVTT^{789}, and KWDTG^{778}. In contrast, those ending with AKWDT^{777} or EKEKM^{771} were not detected (Fig. 3B). Thrombin-induced aggregation resulted in partial degradation of the β1 tail. Addition of E64d, a membrane-permeable specific calpain inhibitor [1], or of EDTA to chelate calcium, completely prevented the proteolysis (Fig. 4) confirming that intracellular calpain was the responsible protease. We also detected calcium-dependent proteolysis of β1 integrin tails in ionophore-treated Jurkat cells (Fig. 3C).

To study possible calpain cleavage of other integrin cytoplasmic domains, we digested model proteins of integrin β1D, β2, β3 and β7 tails with calpain in vitro (Tables 2–4, Fig. 5). Cleavage of the recombinant integrin β3 cytoplasmic domain occurred at sites reported previously [12] in addition to one novel site (KWDTA^{768}) (Fig. 5), which could be detected in ionophore-treated platelets due to a cross-reaction of the antibodies generated against the peptide KWDTG^{778} in the integrin β1 tail (Fig. 3B). Cleavage sites obtained from the other three β tails are listed in Tables 2–4 and the deduced cleavage sites of all analyzed integrin cytoplasmic domains are summarized in Fig. 5. In each case, cleavages were observed around the two conserved NXXY motifs. These cleavage sites manifested no other obvious conserved consensus sequence.

4. Discussion

The central finding of this study is that calpains have the potential to cleave most if not all integrin β tails at sites flanking their conserved NPXY/NXXY motifs. Mutational analysis of these motifs has established their importance in regulating the affinity of integrins for their ligands as well as their capacity to localize to focal adhesion complexes [13,14,17]. They are expected to introduce a unique structural pattern into the integrin β tail due to their predicted tendency

Table 2
Major calpain cleavage products of recombinant integrin β1D cytoplasmic domain

Cleavage site[a]	Time (min)[b]	Measured mass	Predicted mass	Assigned sequence[c]
Y^{795}	30	10 728	10 729.2	GS—KNPNY
	30	896.5	987.0	NFKNPNY
K^{791}	30	10 239	10 240.1	GS—INNFK
	30	1 090	1 090.2	NPNYGRKAGL
F^{790}	30	10 111	10 112.5	GS—PINNF
	30	1 218.5	1 218.4	KNPNYGRKAGL
N^{789}	10	9 964	9 965.4	GS—SPINN
	30	1 365.0	1 365.6	FKNPNYGRKAGL
N^{788}	10	9 850	9 851.3	GS—KSPIN
	30	1 479.5	1 479.7	NFKNPNYGRKAGL
T^{777}	30	8 566	8 566.8	GS—AKWDT
M^{771}	30	7 850	7 851.1	GS—EKEKM
	30	2 132	2 133.3	NAKW—SPINN
	30	2 018.5	2 019.2	NAKW—KSPIN

See Table 1 for annotations.
For clarity, only the major, more rapidly generated fragments are indicated.

Table 3
Major calpain cleavage products of recombinant integrin β2 cytoplasmic domain

Cleavage site[a]	Time (min)[b]	Measured mass	Predicted mass	Assigned sequence[c]
M[762]	10	10040	10039.4	GS—TTTVM
	10	792.5	792.9	NPKFAES
F[754]	30	9218	9219.4	GS—DNPLF
K[744]	30	8003	8003.2	GS—KEKLK
	30	2055.5	2055.3	SQWN—TTTVM

See Table 1 for annotations.
For clarity, only the major, more rapidly generated fragments are indicated.

to fold into tight β turns [14]. Moreover, residues in the first NPXY motif of the integrin β1 tail are crucial for its binding to the cytoskeletal proteins talin and filamin [15,18]. Thus, calpain cleavage of integrin cytoplasmic domains close to these motifs is likely to disrupt integrin function and to strongly compromise preexisting interactions with the cytoskeleton. Preliminary experiments using recombinant protein models of integrin αIIb and α4 tails indicate that calpain cleavage is a unique feature of integrin β and not α cytoplasmic domains (data not shown). This is again consistent with the concept that calpains specifically disrupt integrin cytoskeletal interactions, which are primarily established by the β tails [19].

Our in vitro calpain cleavage analysis demonstrates most rapid proteolysis of all tested β tails in at least one site located between these motifs. Interestingly, the slight sequence variations between β1A, β2, β3 and β7 tails as well as the more obvious sequence divergence between the β1A and β1D tails in this short stretch do not alter its high sensitivity to calpain cleavage (Fig. 5). Therefore, the susceptibility of integrin cytoplasmic domains to calpain cleavage is not primarily determined by the linear amino acid sequence at the scissile bonds, but rather by the adjacent structural framework provided by the NXXY motifs in the integrin β tails.

Our experimental approach to study calpain cleavage in vitro with recombinant model proteins of integrin tails is validated by a number of findings. Firstly, calpain digestion of synthetic peptides and of different recombinant protein models of β1 and β3 cytoplasmic domains resulted in cleavage at identical sites (this report, [12], and our unpublished observations). Secondly and more importantly, essentially all sites determined in the β1 and β3 cytoplasmic domains in vitro, were also generated by calpain proteolysis in aggregating platelets with the exception of the most membrane-proximal site (see also [12]). We have previously shown, that calpain cleavage at this latter site requires platelet treatments leading to the dissociation of integrin heterodimers [12]. This suggests, that this cleavage site is not accessible in the intact integrin in its cellular environment. Altogether, our data strongly indicate that the calpain reactivity, which we monitored in vitro, reflects the potential of calpains to digest integrin cytoplasmic domains in intact cells.

Calpain activation during platelet aggregation results in the cleavage of a variety of proteins. For example, there is cleavage of talin, filamin, pp125FAK, pp60src, p120cbl, and a 58 kDa protein cross-reacting with paxillin antibodies, which has recently been identified as the paxillin-paralogue hic-5 [20] (our unpublished observations, see also [1,6–8,21]). In contrast, thrombin-induced platelet aggregation induced only partial, often barely detectable degradation of these proteins. Thus, only limited proteolysis of specific subsets of these proteins occurs in the physiological situation rendering the detection of calpain reactivities rather difficult. On the other hand, platelets represent a relatively unique cell type, in which μ-calpains are present in excess compared to m-calpains and to its natural inhibitor calpastatin [1]. Therefore, regulation of calpain activity seems to differ in platelets and most nucleated cells, where the levels of calpastatin, and μ- and m-calpain are more balanced. This might explain why, in our hands, cleavage of integrin β1 cytoplasmic domains and cytoskeletal proteins in Jurkat cells required the presence of elevated concentrations of ionophore and calcium (data not shown).

The physiological significance of calpain-dependent proteolysis still remains largely obscure. It has been suggested that the cleavage of cytoskeleton-associated structures by calpain during platelet aggregation results in dissociation of membrane attachment sites from the actin cytoskeleton and inhibits fibrin clot retraction [22]. In nucleated cells, the physiological functions of calpains are even less clear. Our data fit well into an emerging concept, in which calpains regulate integrin-

Table 4
Major calpain cleavage products of recombinant integrin β7 cytoplasmic domain

Cleavage site[a]	Time (min)[b]	Measured mass	Predicted mass	Assigned sequence[c]
T[784]	10	9978	9981.1	GS—SAITT
	10	1598.5	1598.7	TINP—DSPTL
Y[778]	10	9378	9379.4	GS—SNPLY
	10	2191	2191.4	KSAI—DSPTL
Q[766]	30	7889	7887.9	GS—EKEQQ
	30	3677	3679.0	QLNW—DSPTL
	30	2108.5	2108.4	QLNW—SAITT
Q[765]	30	7764	7763.7	GS—FEKEQ
	30	3808	3807.2	QQLN—DSPTL
	30	2237	2236.5	QQLN—SAITT
	30	1635	1634.8	QQLN—SNPLY

See Table 1 for annotations.
For clarity, only the major, more rapidly generated fragments are indicated.

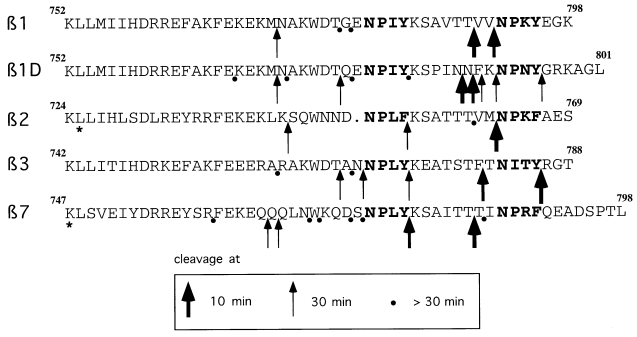

Fig. 5. Summary of the in vitro calpain cleavage sites identified in this study using recombinant integrin β cytoplasmic domains. Amino acid numbering relates to the sequences of human integrin β chains including their signal peptides. Asterisks indicate conservative modifications of the original sequences (A^{725} to L in integrin β2, and R^{747} to K in integrin β7), which have been introduced in the recombinant proteins to preserve a *Hin*dIII restriction site.

dependent cytoskeletal organization [10]. This concept is supported by the subcellular localization of calpain [9] and of calpain reactivity [23] in focal adhesion complexes, as well as by the identification as calpain substrates of a large number of proteins structurally or functionally implicated in focal adhesion formation [1,6–8,10,12,24]. Moreover, recent studies indicate that calpains are required for efficient formation and turnover of integrin-dependent focal adhesion complexes during epithelial cell spreading and migration [25,26]. These calpain activities might depend on fluctuations of intracellular Ca^{2+} levels, which have been observed in migrating neutrophils and reported to regulate the detachment of integrins from their ligands [27,28].

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References

[1] Croall, D.E. and Demartino, G.N. (1991) Physiol. Rev. 71, 813–847.
[2] Sorimachi, H., Ishiura, S. and Suzuki, K. (1997) Biochem. J. 328, 721–732.
[3] Saido, T.C., Sorimachi, H. and Suzuki, K. (1994) FASEB J. 8, 814–822.
[4] Carafoli, E. and Molinari, M. (1998) Biochem. Biophys. Res. Commun. 247, 193–203.
[5] Suzuki, K. and Sorimachi, H. (1998) FEBS Lett. 433, 1–4.
[6] Cooray, P., Yuan, Y., Schoenwaelder, S.M., Mitchell, C.A., Salem, H.H. and Jackson, S.P. (1996) Biochem. J. 318, 41–47.
[7] Oda, A., Druker, B.J., Arioshi, H., Smith, M. and Salzman, E.W. (1993) J. Biol. Chem. 268, 12603–12608.
[8] Yamaguchi, R., Maki, M., Hatanaka, M. and Sabe, H. (1994) FEBS Lett. 356, 114–116.
[9] Beckerle, M.C., Burridge, K., DeMartino, G.N. and Croall, D.E. (1987) Cell 51, 569–577.
[10] Jockusch, B.M., Bubeck, P., Giehl, K., Kroemker, M., Moschner, J., Rothkegel, M., Rüdiger, M., Schlüter, K., Stanke, G. and Winkler, J. (1995) Annu. Rev. Cell. Dev. Biol. 11, 379–416.
[11] Fox, J.E.B., Taylor, R.G., Taffarel, M., Boyles, J.K. and Goll, D.E. (1993) J. Cell Biol. 120, 1501–1507.
[12] Du, X., Saido, T.C., Tsubuki, S., Indig, F.E., Williams, M.J. and Ginsberg, M.H. (1995) J. Biol. Chem. 270, 26146–26151.
[13] O'Toole, T.E., Ylanne, J. and Culley, B.M. (1995) J. Biol. Chem. 270, 8553–8558.
[14] Reszka, A.A., Hayashi, Y. and Horwitz, A.F. (1992) J. Cell Biol. 117, 1321–1330.
[15] Pfaff, M., Liu, S., Erle, D.J. and Ginsberg, M.H. (1998) J. Biol. Chem. 273, 6104–6109.
[16] Saido, T.C., Shibata, M., Takenawa, T., Murofushi, H. and Suzuki, K. (1992) J. Biol. Chem. 267, 2458–2459.
[17] Hughes, P.E. and Pfaff, M. (1998) Trends Cell. Biol. 8, 359–364.
[18] Tapley, P., Horwitz, A., Buck, C., Duggan, K. and Rohrschneider, L. (1989) Oncogene 4, 325–333.
[19] Williams, M.J., Hughes, P.E., O'Toole, T.E. and Ginsberg, M.H. (1994) Trends Cell Biol. 4, 109–112.
[20] Hagmann, J., Grob, M., Welman, A., van Willigen, G. and Burger, M.M. (1998) J. Cell Sci. 111, 2181–2188.
[21] Oda, A., Ozaki, K., Druker, B.J., Miyakawa, Y., Miyazaki, H., Morita, H., Ohashi, H. and Ikeda, Y. (1996) Blood 88, 1330–1338.
[22] Schoenwaelder, S.M., Yuan, Y., Cooray, P., Salem, H.H. and Jackson, S.P. (1997) J. Biol. Chem. 272, 1694–1702.
[23] Tranqui, L. and Block, M.R. (1995) Exp. Cell. Res. 217, 149–156.
[24] Raja, S., Avraham, S. and Avraham, H. (1997) J. Biol. Chem. 272, 10941–10947.
[25] Kulkarni, S., Goll, D.E., Saido, T.C., Suzuki, K. and Fox, J.E.B. (1997) Mol. Cell. Biol. Suppl. A 8, 282.
[26] Huttenlocher, A., Palecek, S.P., Lu, Q., Zhang, W., Mellgren, R.L., Lauffenburger, D.A., Ginsberg, M.H. and Horwitz, A.F. (1997) J. Biol. Chem. 272, 32719–32722.
[27] Hendey, B., Klee, C.B. and Maxfield, F.R. (1992) Science 258, 296–299.
[28] Hendey, B., Lawson, M., Marcantonio, E.E. and Maxfield, F.R. (1996) Blood 87, 2038–2048.