The sensitivity of c-Jun and c-Fos proteins to calpains depends on conformational determinants of the monomers and not on formation of dimers

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Milli- and micro-calpains are ubiquitous cytoplasmic cysteine proteases activated by calcium. They display a relatively strict specificity for their substrates which they usually cleave at only a limited number of sites. Motifs responsible for recognition by calpains have not been characterized yet, and recently a role for PEST motifs in this process has been ruled out. c-Fos and c-Jun transcription factors are highly sensitive to calpains *in vitro*. They thus provide favourable protein contexts for studying the structural requirements for recognition and degradation by these proteases. Using *in vitro* degradation assays and site-directed mutagenesis, we report here that susceptibility to calpains is primarily determined by conformational determinants of the monomers and not by the quaternary structure of c-Fos and c-Jun proteins. The multiple cleavage sites borne by both proteins can be divided into at least two classes of sensitivity, the most sensitive ones being easily visualized in the presence of rate-limiting amounts of calpains. One site located at position 90–91 in c-Fos protein is extremely sensitive. However, efficient proteolysis did not have any strict dependence on the nature of the amino acids on either side of the scissile bond in the region extending from P2 to P′2. The structural integrity of the monomers is not crucial for recognition by calpains. Rather, sensitive sites can be recognized independently and their recognition is dependent on the local conformation of peptide regions that may span several tens of amino acids and maybe more in the case of the identified c-Fos hypersensitive site.

Key words: oncogene, protein degradation, proteolysis, transcription factor.

INTRODUCTION

The c-*fos* and c-*jun* genes define multigenic families of transcription factors (for reviews and references, see [1,2]). In the context of the activator protein-1 transcription-factor complex, c-Fos family members can heterodimerize with those of the Jun family. In contrast to Jun proteins, however, they are not able to homodimerize. Moreover, c-Fos can dimerize with other transcription factors such as Nrl-1, Maf-1 and Fos-interacting protein (FIP), whereas Jun proteins can dimerize with members of the cAMP-response-element-binding protein/activating transcription factor (CREB/ATF) family (see [1,2]).

c-Fos and c-Jun are unstable proteins [1]. Multiple proteolytic mechanisms have been suggested to operate on each protein. Both c-Jun and c-Fos can be degraded by the proteasome, which is the major enzymic machinery responsible for their breakdown [3]. Ubiquitinylation is clearly involved in the proteasomal degradation of c-Jun *in vivo* [3–6], although this protein is a highly sensitive ubiquitin-independent substrate for the 26 S proteasome *in vitro* [7], and is very likely to be necessary for that of c-Fos [8], although no ubiquitinylated form of c-Fos has been detected *in vivo* yet. Two cytoplasmic proteolytic systems have also been suggested to operate on c-Fos and/or c-Jun. First, selective uptake and degradation of c-Fos by lysosomes has been observed *in vitro* and a fraction of c-Fos was found associated with lysosomes *in vivo* [9]. Second, two lines of evidence support the idea that ubiquitous calpains, which are abundant calcium-dependent cysteine proteases called micro (μ)- and milli (m)-calpain, according to the calcium concentration necessary for their maximal activation *in vitro* (for reviews, see [10–12]) could contribute to the degradation of c-Fos and c-Jun: (i) both proteins have been shown to constitute highly sensitive substrates for both calpains *in vitro* [13–17] and (ii) specific modulation of calpain activity *in vivo* modifies c-Fos- and c-Jun-dependent activator protein-1 transcription-complex activity in a transient transfection assay [13]. It can be speculated that, under conditions of maximal repression of c-*fos* and c-*jun* genes, the putative calpain and lysosomal pathways participate together with the proteasome in the degradation of c-Fos and c-Jun in the cytoplasm, where these proteins are unstable [18,19].

The motifs recognized and cleaved by calpains on their substrates have not been identified yet. It is not even clear whether cleavage sites are contained within recognition motifs. PEST motifs are hydrophilic peptide motifs of varying length, rich in proline, glutamic acid, aspartic acid, serine and threonine, which have been proposed to constitute both instability motifs for short-lived proteins and target sites for calpains ([20], reviewed in [21]). However, (i) site-directed mutagenesis of the c-Fos protein [17], together with (ii) an extensive survey of PEST-containing and -non-containing proteins for their sensitivity to calpains [17] and (iii) the analysis of mutants lowering the PEST score of domains surrounding the calmodulin-binding region of the plasma Ca^{2+}-ATPase [22], have ruled out any role for PEST motifs in susceptibility to calpains. Further strengthening this notion, the mutated viral Fos protein transduced by the osteosarcomagenic retrovirus Finkel–Binkis–Reilly murine sarcoma virus (FBR-MSV) is highly resistant to calpains, despite numerous high-score PEST sequences carried by an exogenous Gag

Abbreviation used: m-calpain, milli-calpain.

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motif from the parental retrovirus fused to it [16]. Cleavage sites for calpains have essentially been studied using synthetic peptides. However, (i) these studies failed to reveal any clear consensus cleavage sequences, although preferential amino acid usage was observed on either side of scissile bonds, (ii) these preferences extend poorly to many proteins for which cleavage sites have been identified, and (iii) affinity for synthetic peptide substrates was usually 10–100-fold weaker than for proteins [23]. Taken together, these observations suggest, but do not demonstrate formally, that conformational motifs, the nature of which remains to be elucidated, rather than sequence determinants are responsible for sensitivity to calpains.

To gain information on the motifs responsible for sensitivity of c-Fos and c-Jun to calpains, various mutants of both proteins have been compared with the wild-type proteins in degradation assays *in vitro*. Our data show that sensitivity to calpains does not depend on the quaternary structure of c-Fos and c-Jun but rather on local conformational motifs of the monomers. These motifs may be as long as several tens of amino acids and probably more than 100 amino acids in the case of one hypersensitive site found in c-Fos. Furthermore, site-directed mutagenesis of this site revealed no strict dependence upon specific amino acids on either side of the scissile bond for cleavage by calpain.

MATERIALS AND METHODS

Chemicals

Bovine m-calpain (calcium-activated neutral protease) was from Sigma. Solid-phase synthesis of the calpastatin peptide inhibitor [24] was performed on a Miligen 9050 peptide synthesizer using fluoren-9-ylmethoxycarbonyl (Fmoc) group as temporary amino protection.

Cells and cytoplasmic cell extracts

Jurkat cells were obtained from the A.T.C.C. They were grown in RPMI 1640 supplemented with 10 % fetal calf serum. After harvesting, they were washed twice in PBS (150 mM NaCl/10 mM sodium phosphate, pH 7) and then incubated for 5 min on ice in a hypotonic lysis buffer (20 mM Hepes, pH 7.5/10 mM potassium acetate/1.5 mM magnesium acetate; 2.5×10^8 cells $\cdot$ ml^{-1}) without any detergent. Lysis was completed using a Dounce homogeneizer and verified by microscopic examination. Nuclei were removed by low-speed centrifugation (2000 ***g*** for 15 min) and supernatants were centrifuged at 100000 ***g*** for 1 h in a Beckman SW60 rotor. Cytoplasmic extracts (S100; 5–12 mg $\cdot$ ml^{-1} protein) were then divided into portions and frozen at -80 °C until used [14].

Purification of recombinant c-Fos and c-Jun proteins

Recombinant rat c-Fos and c-Jun proteins [25] were purified from *Escherichia coli* and renatured as described in [7]. Proteins were stored in 50 % glycerol/50 mM NaCl/25 mM Tris/HCl, pH 7.5/1 mM dithiothreitol/1 mM EDTA/0.05 % Nonidet P40 at -20 °C until use.

Plasmids, cloning and site-directed mutagenesis

PCR experiments and DNA cloning were carried out as described in Sambrook et al. [26]. Rat c-Jun and c-Fos cDNAs [25] were cloned in the pT3/T7 phagemid (Pharmacia). Site-directed mutagenesis was carried out according to standard protocols [26] using single-stranded DNA prepared in Dut$^-$/Ung$^-$ CJ236 *E. coli* using the MK07 helper M13 virus. Mutations and deletions were checked by nucleotide sequencing using the T7 sequencing kit from Pharmacia. The FH2 plasmid was used for translation and mutagenesis of the human c-Fos cDNA [27]. Human Fra-1 (plasmid MM505) is described in [28]. The mouse ΔLZ c-Jun cDNA (c-junCDL) is described by Hirai et al. [29].

Transcription and translation *in vitro*

For transcription, plasmids were linearized with appropriate restriction enzymes and subsequently phenol-extracted. Transcription using T3 and T7 RNA polymerases was performed according to the supplier's specifications (Promega), as was translation in the rabbit reticulocyte lysate in the presence of [^{35}S]methionine (Amersham). Alternatively, the TNT® transcription/translation kit from Promega was used with circular plasmids.

Degradation assays and protein analysis

Degradation assays were carried out at 37 °C in a final volume of 40 μl. For degradation in cytoplasmic extracts, 1 μl of the translation mixture and 35 μl of pure or diluted S100 extract were mixed and prewarmed at 37 °C. $CaCl_2$ (usually 1 mM final) and, when necessary, EGTA (10 mM final) or the calpastatin peptide (0.5 mg/ml final) were then added in a volume of 40 μl at a time taken as t_0. When one component was omitted, the volume was adjusted to 40 μl with PBS. When degradation kinetics were carried out in the presence of m-calpain, the latter was added at the concentration indicated in the legends of the Figures. For degradation kinetics, aliquots of reaction mixtures were sampled at various time points and the reaction was stopped by addition of electrophoresis loading buffer containing 1 % SDS. Samples were then electrophoresed through 15 % gels according to Laemmli [30] and electrotransferred on to nitrocellulose for autoradiography.

Recombinant protein degradation for micro-sequencing

Recombinant proteins (20 μg) were incubated with 3 mM calcium and increasing amounts of purified m-calpain at 37 °C for 30 min. An aliquot from the degradation experiments (2 μg) was loaded on to SDS/PAGE Tris/glycine gel for electrophoresis, in order to check the degradation pattern. Large-scale proteolysis products were loaded on to SDS/PAGE Tris/borate gel. The fractionated peptides were transferred on to a PVDF membrane and submitted to Edman degradation.

RESULTS

Dimerization of c-Fos and c-Jun proteins is not required for degradation by calpains

The degradation assay *in vitro* used for testing the sensitivity of various proteins to calpains has been described elsewhere [14,16,17,31]. Briefly, proteins are produced by translation into rabbit reticulocyte lysate which is devoid of any detectable calpain activity. In a second step, either purified bovine m-calpain or a S100 cytoplasmic extract from human Jurkat cells is added in degradation kinetics experiments. Calpains are activated by addition of calcium at a time taken as t_0. The specificity of proteolysis is tested either by addition of EGTA, for chelating calcium, or by addition of a 27-mer synthetic peptide, called here calpastatin peptide [24], which is derived from the physiological protein inhibitor of calpains [10–12], calpastatin, and which inhibits specifically calpains [24].

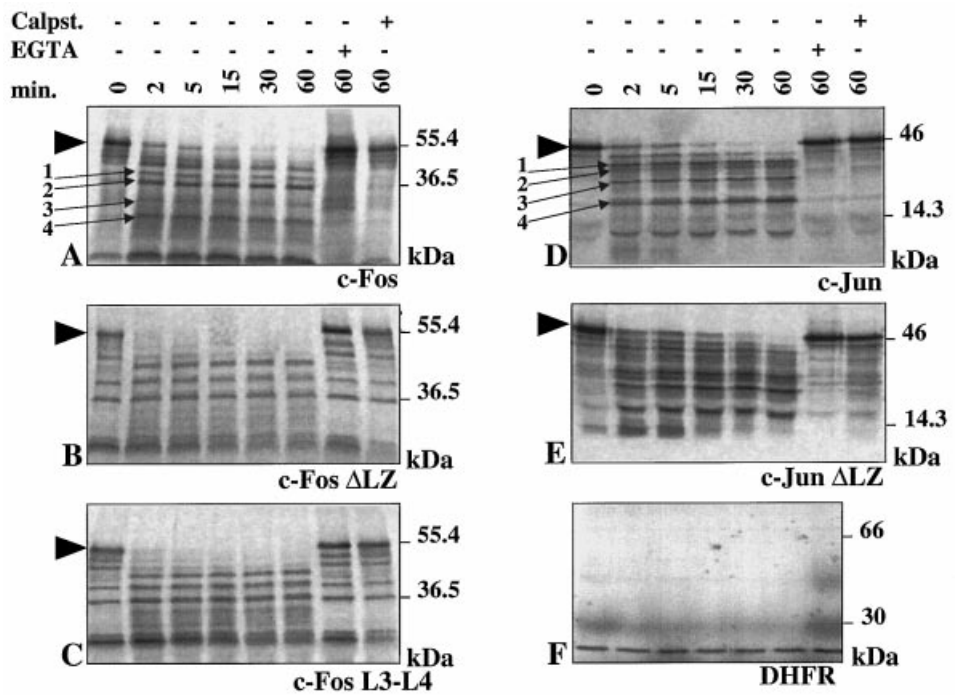

Figure 1 Sensitivity to calpains of c-Fos and c-Jun leucine-zipper mutants

Wild-type and leucine-zipper mutants of human c-Fos and mouse c-Jun were produced in rabbit reticulocyte lysate. Degradation kinetics experiments were conducted using a S100 Jurkat-cell extract (5 mg/ml). Time 0 (min) corresponds to addition of 1 mM $CaCl_2$ for activating calpain activity. When necessary, EGTA and the calpastatin peptide (Calpst.) were added at concentrations of 10 mM and 0.5 mM, respectively. Proteins were electrophoresed by SDS/PAGE (15% gel) before autoradiography. Full-length proteins (which co-migrate with *E. coli*-produced c-Fos and c-Jun proteins; [25]) are indicated by arrowheads. Although 380 amino acids long, the unmodified c-Fos displays an apparent molecular mass of 55 kDa when electrophoresed through SDS-containing polyacrylamide gels. Depending on the batch of reticulocyte lysate used, translation products can display a relatively complex pattern with higher-molecular-mass products, due to post-translational modifications, and/or lower-molecular-mass products due to internal initiation and/or abortive translation. However, whatever the state of *in vitro*-translated c-Fos and c-Jun, the final outcome of degradation experiments was similar. Numbered arrows correspond to N-terminally sequenced proteolytic products (see text and Figure 5). DHFR, dihydrofolate reductase. Molecular-mass markers are shown on the right of the panels.

c-Jun can form homodimers and can also heterodimerize with a variety of partners, including c-Fos, through leucine zipper–leucine zipper interactions. Similarly, c-Fos can also dimerize with a variety of partners. However, it cannot homodimerize. Importantly, it has never been investigated whether c-Fos and c-Jun are associated with partners that are possibly present in the reticulocyte lysate and/or the Jurkat cytosolic extract in degradation assays aiming at studying their sensitivity to calpains. To determine whether dimerization is required for confering sensitivity to calpains, we thus compared leucine-zipper mutant proteins with parental proteins in a degradation kinetics assay. In the case of c-Jun, the leucine zipper of the mouse protein was completely deleted (c-Jun-ΔLZ). In that of c-Fos, two human mutants were analysed. One carries a deletion of the whole leucine zipper (c-Fos-ΔLZ), the other is mutated only on leucines 3 and 4 (c-Fos-L3-L4), which is sufficient to completely inhibit heterodimerization [32]. Experiments conducted with a Jurkat-cell cytosolic extract (Figures 1A–1E), or in the presence of purified bovine m-calpain added to the reticulocyte lysate (results not shown), showed wild-type and mutated proteins to have comparable sensitivities. Moreover, inhibition of proteolysis by EGTA or the calpastatin peptide (Figures 1A–1E) as well as resistance of dihydrofolate reductase in a parallel experiment (Figure 1F) show the specificity of the process. This indicates that dimerization of c-Fos and c-Jun is not a prerequisite for

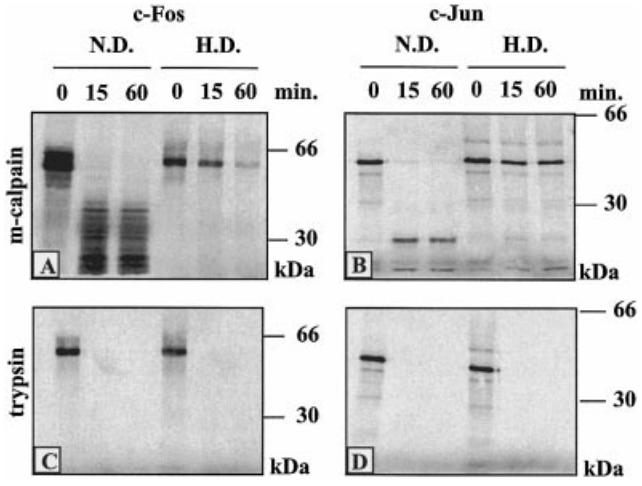

Figure 2 Sensitivity to calpains of heat-denatured c-Fos and c-Jun

Rat c-Fos- and rat c-Jun-containing rabbit reticulocyte samples were incubated at 65 °C for 5 min. Purified bovine m-calpain (50 μg/ml) was added to half of each sample in the presence of 1 mM $CaCl_2$ and various concentrations of trypsin (50 μg/ml in the experiment presented) were added to the other half. Mixtures were incubated at 37 °C for the indicated periods of time. Protein analysis was carried out as in Figure 1. N.D., not denatured; H.D., heat-denatured.

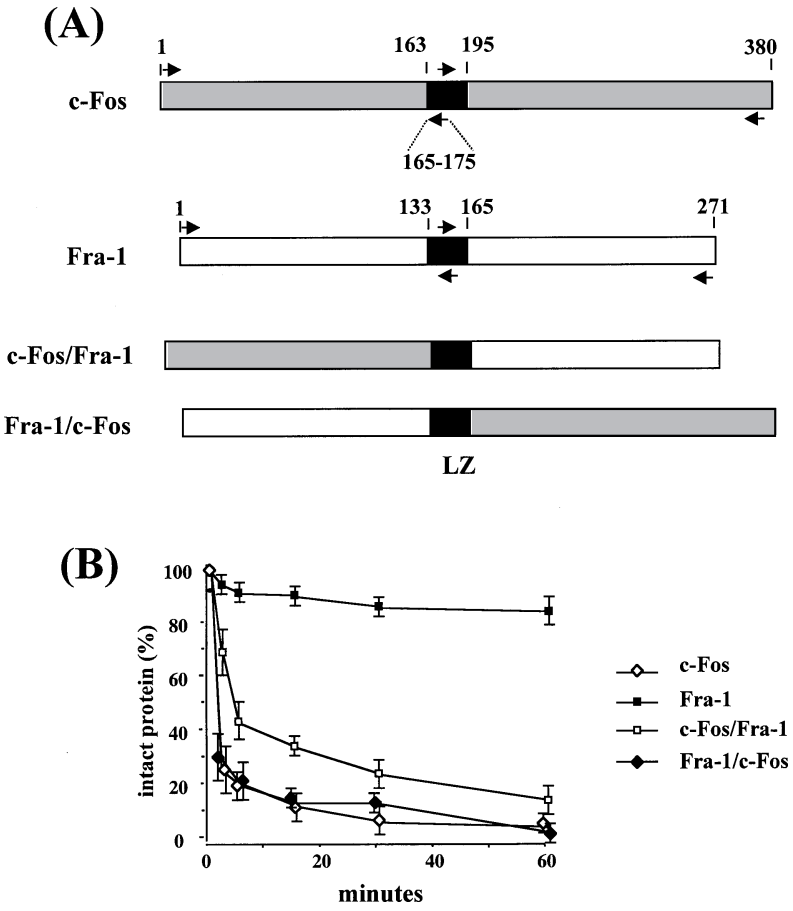

Figure 3 Sensitivity to calpains of c-Fos/Fra-1 and Fra-1/c-Fos chimaeras

(**A**) Structure of the various proteins. Numbers correspond to amino acid positions. Black boxes correspond to the leucine zippers of c-Fos and Fra-1, which are nearly identical. The arrows at either end indicate the positions of the oligonucleotides which were used for PCR amplification of the cDNA fragments carrying the N- and C-terminal moieties of both proteins. Oligonucleotides complementary to both strands of the c-Fos leucine zipper (amino acids 165–175) were used for amplifying both c-Fos and Fra-1 sequences. (**B**) Kinetic degradation experiments. The various proteins were produced in the reticulocyte lysate and degradation experiments were performed using a Jurkat-cell S100 cytoplasmic extract as in Figure 1 in the presence of 1 mM $CaCl_2$ or 1 mM $CaCl_2 + 10$ mM EGTA. Autoradiographs of electrophoresis gels were then subjected to densitometer analysis. Values are means $\pm$ S.D. of experiments performed in triplicate.

proteolysis by calpains and suggests that recognition motifs are borne by each monomer.

Higher-order structure determinants are responsible for the sensitivity of c-Fos and c-Jun to calpains

A number of cleavage sites for calpains have been identified in various protein substrates (see [10,23]). However, the motifs that actually confer sensitivity to calpains are still unknown. It has, however, been suggested that calpains recognize structural rather than sequence motifs [10]. To test whether this also holds true for c-Jun and c-Fos, both proteins, produced in the reticulocyte lysate, were heat-denatured at 65 °C for 5 min and their sensitivity to bovine m-calpain was compared with that of native proteins. Heat-denatured c-Fos and c-Jun proteins showed dramatically decreased susceptibility to calpains (Figures 2A and 2B). Importantly, resistance to degradation was not due to heat-induced inaccessibility for proteases since denatured proteins retained their sensitivity to trypsin (Figures 2C and 2D). This supports the notion that higher-order structure determinants are responsible for the sensitivity of both c-Fos and c-Jun proteins to calpains.

The overall structure of c-Fos protein is not indispensable for degradation by calpains

A limited number of bands, the sum of molecular masses (> 200 kDa) of which far exceeds that of c-Fos (apparent molecular mass of 55 kDa), reproducibly accumulated in our degradation assay *in vitro* ([14]; see Figure 1 and below). This indicates that, on one hand, c-Fos bears multiple cleavage sites for calpains and, on the other hand, that certain proteolytic fragments, even though they still carry cleavage sites for calpains, are not digested to completion under the experimental conditions used. The latter observation raised the possibility that the overall structure of c-Fos may be necessary for recognition and cleavage by calpains. As a first step for testing this possibility, we asked whether each half of c-Fos can be proteolysed by calpains independently of the other. To this aim, we took advantage of the fact that the Fos-related Fra-1 protein is poorly sensitive to degradation by calpains [14] and constructed chimaeric c-Fos/Fra-1 (mutant MAL19) and Fra-1/c-Fos (mutant MAL20) proteins by swapping domains upstream and downstream of the leucine zipper, which occupies a central position in both proteins (Figure 3A). Because c-Fos conformation is important for recognition by

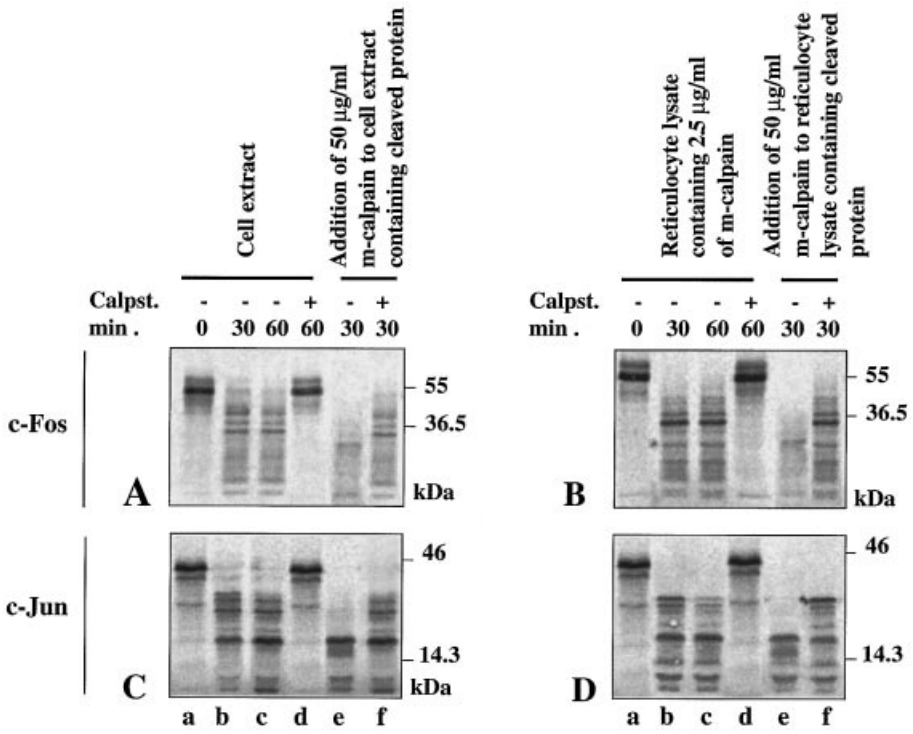

Figure 4 Sensitivity of c-Fos and c-Jun to high concentrations of calpain

Proteins were produced in rabbit reticulocyte lysate (lanes a) and subjected to degradation in a Jurkat-cell cytoplasmic extract, as described in Figure 1 (**A** and **C**), or in reticulocyte lysate containing 2.5 μg/ml m-calpain (**B** and **D**) for 60 min (lanes c). m-Calpain (50 μg/ml) was then added in the absence (lanes e) or in the presence (lanes f) of calpastatin peptide (Calpst.) and reactions were allowed to proceed for another 30 min. A 30-min time point is presented to show the accumulation of stable proteolytic products in the presence of low calpain activity (lanes b) as well as a 60-min incubation in the presence of calpastin peptide to show the specificity of the initial proteolysis reaction (lanes d).

calpains (see above), this approach was preferred in a first step over the use of deletion mutants (see below) for providing a structurally related, but calpain-resistant, protein context that would minimize conformational alteration.

Fra-1 is a poor substrate for calpains. More than 90 % of it was found to be resistant after 60 min of incubation in Jurkat-cell extract in the presence of calcium (Figure 3B). In contrast, more than 50 % of c-Fos and of the Fra-1/c-Fos and c-Fos/Fra-1 chimaeras were degraded within 5 min, c-Fos/Fra-1 being slightly less sensitive than the two other proteins (Figure 3B). Similar observations were made when degradation experiments were conducted with m-calpain added to the reticulocyte lysate. These data indicate that sub-domains of the c-Fos protein can be recognized independently by calpains. A difference in sensitivity of the two halves of c-Fos is observed, however. It is probably accounted for, in part, by the number of calpain cleavage sites, which is higher in the C-terminal moiety of the molecule (see below). However, the analysis of recurrent deletion mutants suggests that peptide motifs located within the C-terminal half of c-Fos may be useful for cleavage located at sites located upstream of the leucine zipper (see below).

At least two classes of cleavage site showing differential sensitivity to calpains in c-Fos and c-Jun proteins

As exemplified in Figure 1, a reproducible and characteristic pattern of bands, stable for several hours [14], was observed when c-Fos and c-Jun were degraded using a Daudi- or Jurkat-cell S100 cytoplasmic extract as a source of calpain. Importantly, c-Fos and c-Jun proteins added to the reaction mixture 15, 30 or 60 min after the onset of the reaction were degraded within a few minutes, indicating that accumulation of these stable proteolytic products was not due to exhaustion of the calpain activity under the experimental conditions used (results not shown; see [14]). In contrast, recombinant c-Fos and c-Jun proteins produced in *E. coli* can be degraded to much smaller peptides by bovine m-calpain, although peptide fragments, with apparent molecular masses similar to those of the fragments detected in cytosolic extracts, accumulate at low concentrations of calpains (see [14]).

Three mechanisms may account for this observation: (i) abnormal conformations of the peptide fragments generated from the *E. coli*-produced proteins reveal cryptic cleavage sites for calpains, (ii) certain calpain cleavage sites of the *in vitro*-translated proteins are protected by proteins of the cytosolic extract or of the reticulocyte lysate and (iii) calpains contained in S100 extracts are rate-limiting for permitting proteolysis of c-Fos and c-Jun at possible poorly sensitive sites. To discriminate between these possibilities, *in vitro*-translated c-Fos and c-Jun proteins were proteolysed either in Jurkat-cell extract (Figures 4A and 4C) or in the reticulocyte lysate supplemented with 2.5 μg/ml m-calpain (Figures 4B and 4D) for 1 h to produce stable proteolytic products (Figure 4, lanes a–c). Then, 50 μg/ml m-calpain was added in the presence or absence of calpastatin peptide and reactions were allowed to proceed for another 30 min. Data presented in Figure 4 show further proteolysis of c-Fos and c-Jun fragments only in the presence of a high concentration of m-calpain (Figure 4, lanes e). In addition, this enhanced proteolysis was inhibited by calpastatin (Figure 4,

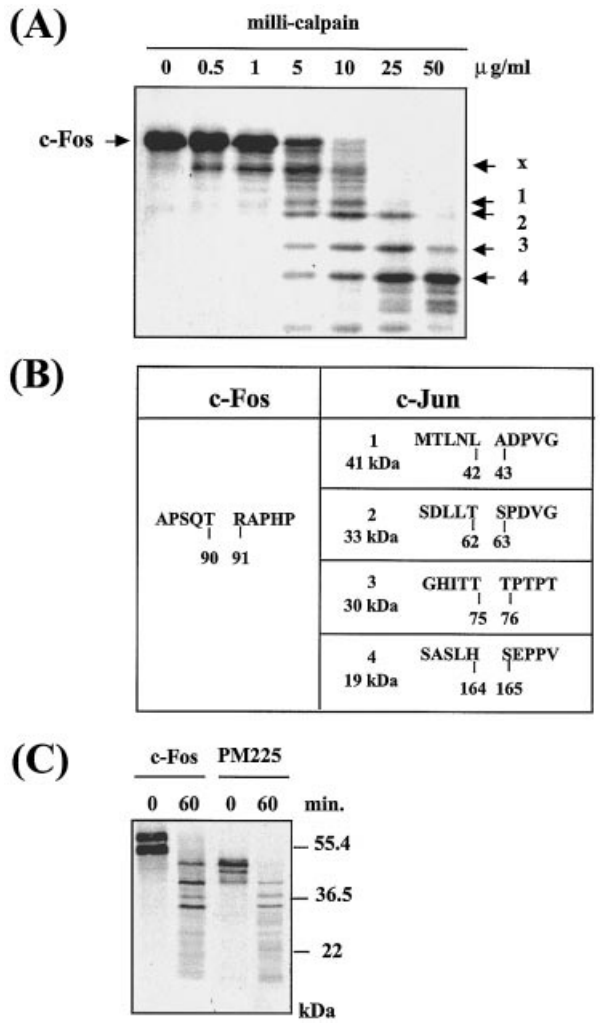

Figure 5 Identification of calpain cleavage sites in c-Fos and c-Jun

(**A**) Cleavage of rat c-Fos protein by calpains. The rat c-Fos protein was produced in *E. coli* and renatured as described in [7]. Degradation experiments were carried out as described in the Materials and methods section using bovine m-calpain at the concentrations indicated in the Figure. (**B**) The sequences surrounding the identified cleavage sites within c-Fos and c-Jun. Numbers 1–4 correspond to the numbering of c-Jun fragments, as indicated in Figure 1(D). (**C**) Wild-type c-Fos and a mutant (PM225) with the N-terminal 90 amino acids deleted were translated and degraded in a Jurkat-cell extract as indicated in the Materials and methods section.

lanes f). This observation is consistent with the idea that both proteins contain sites for calpains (maybe more than originally thought, see [14]) that can be shared among at least two classes: a class of higher sensitivity, which can be revealed in the presence of rate-limiting amounts of calpains, and a class of lower sensitivity, which can be revealed in the presence of higher concentrations of calpains and which allows further degradation (see below for further details).

A highly sensitive cleavage site for calpains at positions 90–91 in c-Fos protein

We next aimed at characterizing the most sensitive calpain cleavage sites in c-Fos. The N-termini of peptide fragments generated from the recombinant rat protein which correspond to fragments 1–4 accumulating in Jurkat-cell cytoplasmic extracts (Figure 1A) were purified from partially digested c-Fos protein (Figure 5A) and their N-terminal sequences were determined according to the Edman degradation method. Moreover, a 49-kDa fragment (fragment x), detectable only at the lowest concentrations of m-calpain (0.5–1 μg/ml) and which is also transiently detectable in degradation assays using *in vitro*-translated protein and low concentrations of either Jurkat-cell extract or m-calpain (results not shown), was also sequenced. Interestingly, all fragments showed the same N-terminal sequence beginning at position 91 (called hereafter cleavage-site 1; Figure 5B), in a region which is highly conserved among the c-*fos* genes sequenced in various species.

This observation suggests that, in fragments 1–3 accumulating in S100 cell extracts, calpain cleavage sites located downstream of site 1 are no longer recognized in the presence of rate-limiting amounts of calpains. A reasonable explanation for this would be that conformational changes are induced within peptide fragments after two proteolysis events, as reported for histone hydrolysis by calpains [33], one consequence of which would be a structural change in calpain-recognition motifs situated between the two selected cleavage sites.

Selection of the two sites could occur according to two mechanisms. On one hand, a first cleavage could occur randomly at one of the downstream sites and would then be followed by the cleavage at position 91 that is ultimately responsible for the conformational change. On the other hand, cleavage at site 1 would be kinetically favoured and cleavage at the second downstream cleavage site would entail the conformational change. It is difficult to rule out the first possibility. However, the following observations support the idea that the second mechanism, if not exclusive, predominates over the first one: (i) fragment x appears first when recombinant c-Fos is proteolysed in the presence of a low concentration of m-calpain (see lanes 0.5 and 1 μg/ml of calpain in Figure 5A), but is no longer visible at higher concentrations (lanes 25–50 μg/ml of calpain in Figure 5A) and (ii) a c-Fos protein with the first 90 amino acids deleted (mutant PM225) by site-directed mutagenesis shows the same size as fragment x, is degraded as efficiently as the wild-type protein and gives a very similar pattern of proteolytic products in a S100 cytoplasmic extract from Jurkat cells (see Figure 5C).

Cleavage between amino acids 90 and 91 of c-Fos is not strictly dependent upon the nature of the amino acids at positions P2, P1, P′1 and P′2

The hypersensitivity of site 1 and the accumulation of stable peptide fragments in the presence of rate-limiting amounts of calpains in Jurkat-cell extracts provides a favourable situation for studying whether specific amino acids are required on either sides of a calpain cleavage site in a native protein. Various mutants generated by site-directed mutagenesis were thus compared with the wild-type c-Fos (Table 1). First, a 10-amino acid fragment from Fra-1 was substituted for the homologous region of c-Fos spanning amino acids 85–95 (mutant PM 226). In this construct, the most notable change was the replacement of Thr-90 by Pro, which is a destructuring amino acid. In a second series of mutants, c-Fos was mutated at unique positions on either side of the scissile bond: Thr-90 was replaced by Pro (mutant c-FOS90PRO) and Arg-91 was replaced by Glu (because its charge is opposite to that of Arg; mutant c-FOS91GLU). Moreover, Gln-89 was changed to Glu (see the Discussion section; mutant PM303). Finally, amino acids at positions P1 and P′1 (mutant PM266), P2 and P′2 (mutant PM290) and P2, P1, P′1

Table 1 Sensitivity to calpains of site-1-mutated c-Fos variants

The arrow indicates the position of the cleavage site. Numbers indicate amino acid positions. (+), means as sensitive as the wild-type c-Fos in kinetic degradation experiments carried out when adding either Jurkat-cell extract or bovine m-calpain to c-Fos-variant-containing reticulocyte lysate. Mutated amino acids are indicated in bold.

	85	86	87	88	P2 89	P1 90	↓ P′1 91	P′2 92	93	94	95	96	Sensitivity to calpains
c-Fos	V	A	P	S	Q	T	R	A	P	H	P	Y	(+)
PM226	V	A	**S**	S	**Y**	**P**	R	—	P	**L**	**T**	Y	(+)
c-FOS90PRO	V	A	P	S	Y	**P**	R	A	P	H	P	Y	(+)
PM303	V	A	P	S	**E**	T	R	A	P	H	P	Y	(+)
c-FOS91GLU	V	A	P	S	Q	T	**E**	A	P	H	P	Y	(+)
PM266	V	A	P	S	Q	**E**	**E**	A	P	H	P	Y	(+)
PM290	V	A	P	S	**E**	T	R	**E**	P	H	P	Y	(+)
PM291	V	A	P	S	**E**	**E**	**E**	**E**	P	H	P	Y	(+)

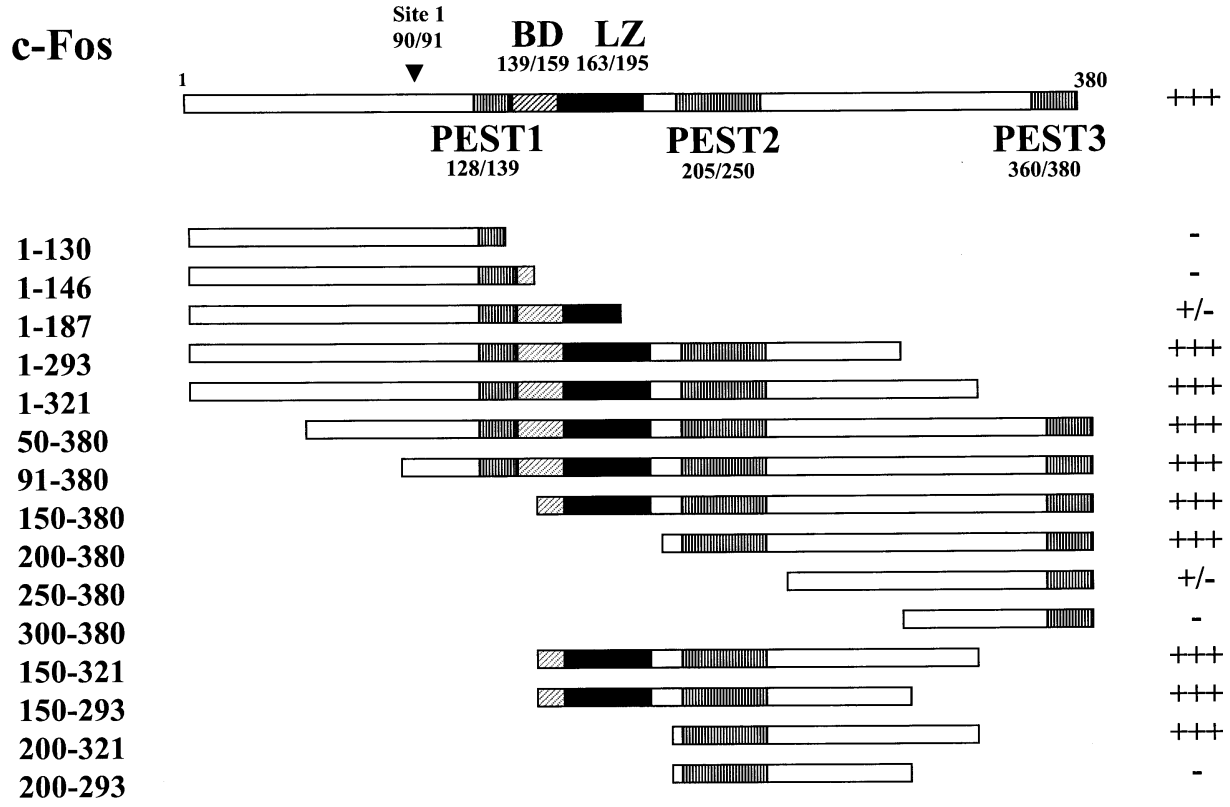

Figure 6 Sensitivity to calpains of N- and C-terminal recurrent deletion mutants of c-Fos

N- and C-terminal mutants of c-Fos were translated in rabbit reticulocyte lysate and tested for degradation in a Jurkat-cell S100 extract in the presence of 1 mM $CaCl_2$. + + +, as sensitive as the wild-type c-Fos (half-reaction time < 5 min under the experimental conditions used). +/−, poorly sensitive (50% of input protein digested after 1 h at 37 °C). −, not susceptible to cleavage by calpains even when 50 μg/ml exogenous bovine m-calpain was added to the cytoplasmic extract. BD and LZ refer to the DNA-binding basic domain and to the leucine zipper, respectively. Numbers correspond to amino acid positions. The arrowhead represents the identified cleavage site.

and P′2 (mutant PM291) were converted to Glu. Interestingly, all mutant proteins were degraded at the same rate as the wild-type protein in kinetic degradation experiments conducted with Jurkat-cell extracts or in the presence of bovine m-calpain added to the reticulocyte lysate. Moreover, no change in the pattern of peptide fragments accumulating in the reaction mixture was observed. This supports the idea that, at least for site 1, the nature of amino acids at positions P2, P1, P′1 and P′2 is not critical for efficient cleavage by calpains.

Local conformational determinants of c-Fos protein are crucial for proteolysis by calpains at several cleavage sites at least

To gain more information concerning the structural constraints required for efficient degradation of c-Fos by calpains, a number of N- and C-terminal recurrent deletion mutants were compared with the wild-type rat protein in degradation kinetics experiments conducted using a Jurkat-cell extract. N-terminal deletions were obtained by site-directed mutagenesis whereas C-terminal ones

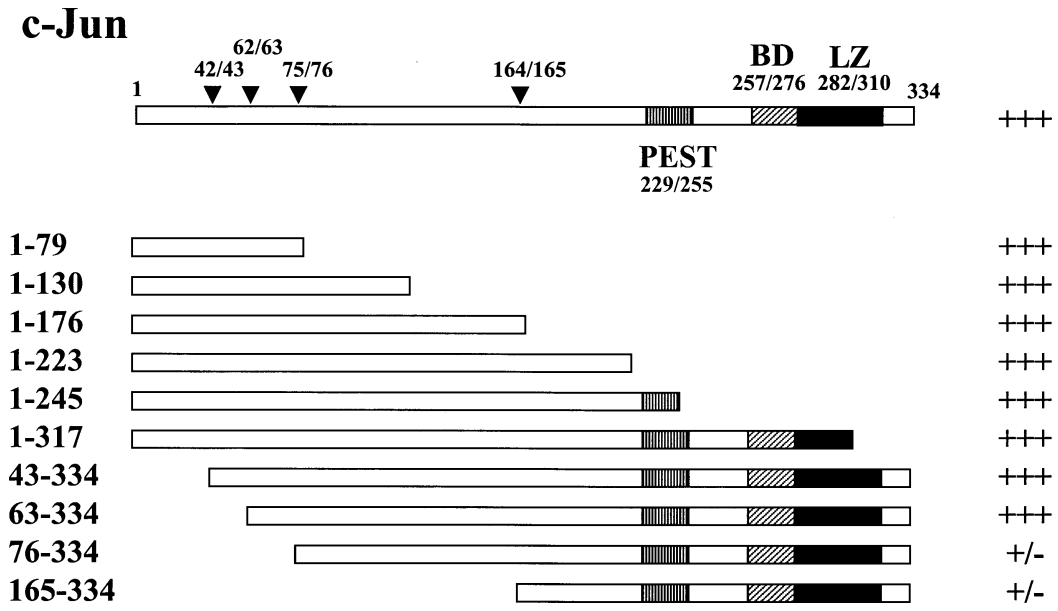

Figure 7 Sensitivity to calpains of N- and C-terminal recurrent deletion mutants of c-Jun

N- and C-terminal mutants of c-Jun were translated in rabbit reticulocyte lysate and tested for degradation in a Jurkat-cell S100 extract in the presence of 1 mM $CaCl_2$. For the meanings of + + + and +/−, see Figure 6. BD and LZ refer to the DNA-binding basic domain and to the leucine zipper. Numbers correspond to amino acid positions. Arrowheads represent identified cleavage sites.

were obtained by linearization at unique restriction sites of the vectors used for translation *in vitro* (Figure 6). The most interesting observations were the following.

(i) The C-terminal half of c-Fos can be cleaved as efficiently as the wild-type protein. This confirms the conclusion reached when analysing the Fra-1/c-Fos chimaera (Figure 3B, mutant 200–380).

(ii) In the case of C-terminal deletions, it is interesting to note that the fragment spanning amino acids 1–187 is poorly sensitive to calpains despite the presence of the most sensitive peptide bond (site 1) of c-Fos. This confirms the conclusion reached when analysing the c-Fos/Fra-1 chimaera that the N-terminal moiety of c-Fos can be cleaved independently of the other, although motifs located downstream of the leucine zipper may help in this process. It is noteworthy that fragment 1–146 is completely resistant to calpains (even when exogenous calpain is added to the reaction mixture). This suggests that the peptide region responsible for recognition of site 1 is relatively large (in the range of 100 amino acids).

(iii) In the case of N-terminal deletions, the apparent molecular masses of the fragments generated from the wild-type protein in Jurkat-cell extract indicate that several of these sites are located downstream of amino acid 250. Despite this, the peptide fragment spanning amino acids 250–380 is poorly sensitive to calpains, suggesting that peptide motifs required for cleavage at these sites are at least several tens of amino acids long.

The N-terminal fifth of c-Jun is important for the structuration of the rest of the molecule

Four of the most sensitive cleavage sites for calpains, corresponding to fragments 1–4, as indicated in Figure 4(B), lane a, were identified by N-terminal sequencing of peptides generated from the recombinant c-Jun protein (Figure 5B). As in the case of c-Fos, N-and C-terminal deletion mutants of the rat c-Jun protein were compared with the wild-type protein in degradation kinetics experiments conducted with a Jurkat-cell extract (Figure 7). Identified cleavage sites were used as limits for creating N-terminal deletion mutants and C-terminal deletions were obtained by linearization at unique restriction sites of expression vectors *in vitro*. C-terminal deletion mutants lacking the leucine zipper retained a high sensitivity to calpains, further confirming that dimerization is not a prerequisite for degradation of c-Jun by calpains. In contrast, the deletion of the first 75 amino acids drastically reduces the sensitivity to calpains despite the presence of the highly sensitive scissile bond between amino acids 164 and 165 of c-Jun. This suggests that the presence of the N-terminus of c-Jun is important for the correct structure of the rest of the protein.

DISCUSSION

We have investigated the requirements for proteolysis of c-Jun and c-Fos proteins by calpains using degradation assays *in vitro* and site-directed and deletion mutagenesis. To our knowledge, this is the first time that such an approach has been used for studying the structural requirements of a substrate for hydrolysis by calpains.

Dimerization is not a prerequisite for the degradation of either of the two proteins. However, further work is necessary for determining whether association with one of their many partners modulates their breakdown rate. Thermal-denaturation experiments provide evidence that conformational determinants rather than primary structural motifs are responsible for recognition of c-Fos and c-Jun by calpains. Both c-Fos and c-Jun show multiple cleavage sites for calpains. Some of these are probably close to one another, as deduced from the N-terminal sequencing of c-Jun peptides (positions 43, 63 and 76) and from the fact that small peptides are generated from both proteins at high calpain concentrations. This raises the possibility that certain structural

determinants recognized by calpains may overlap. If this is true, the fine study of proteolysis by calpains will be complex, since the individual contributions of the different motifs may prove to be difficult to characterize. The analysis of both chimaeric proteins and various N- and C-terminus deletion mutants showed that at least some of the cleavage sites can be recognized independently of the others. At this stage of investigation, however, it is not possible to rule out the co-recognition or the interdependency between certain sites for efficient cleavage. Cleavage sites can be divided into two main classes: one class of sensitive sites, which can be revealed in the presence of limiting amounts of calpains, and a class of lower sensitivity. Moreover, in c-Fos, one scissile bond between amino acids 90 and 91 shows hypersensitivity to calpains.

Cleavage sites for calpains have been reported for a number of proteins and peptide substrates [10,23]. The amino acids adjacent to the cleaved peptide bonds are somewhat variable, albeit with preferences at certain positions. Thus at P1, the preferred residues are those with bulky or basic side chains such as Tyr, Lys and Arg and, at P2, Leu and Val are most commonly found, suggesting a preference for residues with bulky aliphatic side chains. In contrast, no marked preferences have been identified at positions P′1 and P′2. To better understand the sequence requirements for recognition of substrates by calpains, we have conducted a site-directed-mutagenesis analysis of the calpain-hypersensitive c-Fos site 1. To our knowledge, the only other mutagenesis study conducted within the context of a native protein concerns the unique calpain cleavage site of human αII spectrin [34]. In their work, Stabach et al. [34] have tested the sensitivity to calpains of αII spectrin variants displaying each one of the 20 amino acids at position P2. They observed that only replacement of the original Val by Gly, Pro, Asp and, to a lesser extent, Phe and Glu, substantially inhibited the susceptibility to calpains. Basing their analysis on the crystal structure of the *Drosophila melanogaster* α-spectrin 14th subunit, they conducted a molecular modelling study of this region in the various mutants and concluded that secondary and tertiary conformational features surrounding the cleavage sites, rather than the linear sequence itself, dominate the determinants that define αII spectrin sensitivity to calpain. In our study, no stabilization of c-Fos was detected when the P2-Gln was changed into Tyr or Glu residues. Since all three amino acids are structurally different, this extends the notion that there is no strict dependency upon specific types of amino acids at this locus. It is of note that Glu, which is stabilizing in the case of human αII spectrin, is not stabilizing in the case of c-Fos. Remarkably, the other changes made at the level of site 1 [including the following changes: (i) P1-Thr into Pro and Glu, (ii) P′1-Thr into Glu, (iii) P1-Thr + P′1-Arg into Glu + Glu, (iv) P2-Gln + P′2-Ala into Glu + Glu and (v) P2-Gln, P1-Thr, P′1-Arg, P′2-Ala] did not entail any detectable reduction in c-Fos susceptibility to calpains. Taken together, these observations indicate that the nature of the amino acids of the region extending from P2 to P′2 at the level of site 1 is not critical for efficient recognition by calpains. Because Glu is different in both structure and/or charge to all of the other original amino acids of this region, and because Pro is a destructurating amino acid, these data are consistent with the notion that the cleavage site, even if included within the region recognized by calpains, is not the primary recognition motif and is not even part of the conformational motif responsible for susceptibility to calpains.

The analysis of N- and C-terminal deletion mutants indicates that peptide regions required for optimal conformation/exposure of at least several cleavage sites span several tens of amino acids, and maybe more in the case of c-Fos site 1. This observation is consistent with the fact that short peptides are usually poor substrates for calpains (see [10,23]). Narrowing down several susceptible regions of c-Fos and c-Jun, each bearing a single cleavage site, followed by comparative structural analysis, should help in understanding the interactions between calpains and their substrates. Particularly, this should lead to the establishment of whether recognition motifs also contain cleavage sites or whether calpains recognize specific motifs at the surface of their substrates and cleave at a distinct site in the vicinity. Finally, deletion mutants of c-Jun are interesting with respect to two points. First, C-terminal mutants further confirm the conclusion reached by us [17] and others [22] that PEST motifs are not necessary for recognition by calpains, since the unique c-Jun PEST sequence, extending from amino acid 229 to 255, is absent from the 1–223 mutant, which is as sensitive as the wild-type protein. In the same vein, it is worth noting that (i) the 1–146, 200–293 and 300–380 deletion mutants, which contain c-Fos PEST1, PEST2 and PEST3 motifs respectively, are completely resistant to calpains and (ii) none of the cleavage sites identified during this work fall within any c-Fos or c-Jun PEST regions (see [17,20]). Secondly, the N-terminal fifth of c-Jun appears to be structurally important for the rest of the protein, since mutant 76–334 is very poorly susceptible to calpains (which can be used as topological probes since they recognize conformational determinants), despite the presence of a sensitive site at position 164. Strikingly, this N-terminal region bears a peptide motif of 27 amino acids, called the δ domain, which is deleted from the variant protein carried by the avian sarcomagenic retrovirus ASV17 and whose deletion is the mutation that is responsible primarily for the increased tumorigenic potential of v-*jun* (see [16]). Specific properties, such as the ability to trigger ubiquitinylation of c-Jun and of *E. coli* β-galactosidase when grafted to the latter [4], are intrinsic to the δ domain. It can however be speculated from our observation that other effects attributable to the deletion of the δ domain, such as modification in the transcription activity, might be indirectly the consequence of conformational changes induced further downstream. In this regard, it is important to consider that c-Jun is a molecule displaying some structural flexibility since it is subjected to intramolecular signal transduction, as suggested by the fact that C-terminal dephosphorylation is an indirect consequence of a separate phosphorylation event targeted to the N-terminus [35].

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