PEST motifs are not required for rapid calpain-mediated proteolysis of c-fos protein

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Cytoplasmic degradation of c-fos protein is extremely rapid. Under certain conditions, it is a multi-step process initiated by calcium-dependent and ATP-independent proteases called calpains. PEST motifs are peptide regions rich in proline, glutamic acid/aspartic acid and serine/threonine residues, commonly assumed to constitute built-in signals for rapid recognition by intracellular proteases and particularly by calpains. Using a cell-free degradation assay and site-directed mutagenesis, we report here that the three PEST motifs of c-fos are not required

for rapid cleavage by calpains. Testing the susceptibility of PEST motif-bearing and non-bearing transcription factors including GATA1, GATA3, Myo D, c-erbA, Tal-1 and Sry, demonstrates that PEST sequences are neither necessary nor sufficient for specifying degradation of other proteins by calpains. This conclusion is strengthened by the observation that certain proteins, reportedly known to be cleavable by calpains, are devoid of PEST motifs

INTRODUCTION

The c-fos protein is a transcription factor involved in the regulation of key steps of cell division and differentiation (for reviews, see [1–3]). It defines a multi-gene family which includes fra-1, fra-2 and fos-b. fos family members dimerize with those of the jun family, which includes jun-b, c-jun and jun-d, within the AP-1 transcription complex for binding to specific DNA motifs [1–3]. However, c-fos can also interact with other transcription factors such as fip, c-maf, nrl, NFκB, NF-AT, myo D and nuclear hormone receptors [2,3]. The regulation of c-fos gene is complex and involves numerous intricate transcriptional and post-transcriptional mechanisms (see [2]). Particularly, c-fos half-life is very short *in vivo*, both in the nucleus [4] and in the cytoplasm [5]. *In vitro* and *in vivo* lines of evidence support the notion that, at least in certain situations, cytoplasmic breakdown is likely to be a multi-step process initiated by two ATP-independent and calcium-dependent proteases called calpains [6–8].

The two calpain isoenzymes display comparable substrate specificity [9] but differ in their requirement for calcium *in vitro*. Accordingly, they have been termed micro(μ)- and milli(m)-calpain since they require 2–75 and 200–800 μM of calcium for half-maximum activity respectively. Interestingly, fos-b, jun-b, c-jun and jun-d are also sensitive to both calpains, albeit to different degrees, whereas fra-2 is sensitive to m-calpain only and fra-1 is resistant to both. This observation suggests that cytoplasmic degradation contributes to the control of the activity of the AP-1 transcription complex by differentially affecting the abundance of its components available for nuclear transport [8].

Computer analysis has shown that regions rich in proline (P), glutamate (E) and/or aspartate (D) and serine (S) and/or threonine (T), bounded by basic amino acids, are found in most proteins known to be metabolically unstable or expected to be so (nearly 100 tested), but rarely in stable proteins. These regions, named PEST, were thus proposed to be built-in signals re-

sponsible for rapid protein degradation [10]. PEST sequences vary in length (from 12 to 100 amino acids). They can reside anywhere along the molecule and are often represented in multiple examples. In addition, they are also mostly solvent-exposed and negatively charged, because of the presence of glutamate and aspartate, or they can become so upon phosphorylation of serines and threonines. Calpains are puzzling in that their requirement for calcium *in vitro* is considerably higher (2 μM–1 mM) than most estimates of cytosolic calcium levels (0.1–0.4 μM). It has thus seemed possible that PEST regions could sequester calcium because of their negative charge and thereby present to calpains not only susceptible bonds but the required co-factor as well [10]. Although the situation is not yet clearly resolved (see Discussion section), several observations are consistent with the hypothesis that PEST sequences are targets for calpains: (i) calpains cleave protein kinase C [11] and amyloid precursor protein [12] within PEST sequences; (ii) cleavage sites for calpains and most PEST motifs of the proteins are both located within the C-terminal region of the plasma membrane Ca²⁺-ATPase [13]; and (iii) their preferred cleavage site resides near the first PEST sequence in hydroxymethylglutaryl-CoA reductase [14].

Using site-directed mutagenesis, we report here that PEST motifs are not indispensable for rapid degradation of c-fos by calpains. A survey of PEST-sequence-bearing and -non-bearing proteins extends this observation and clearly indicates that PEST sequences are neither required nor sufficient for susceptibility to calpains.

EXPERIMENTAL

Cells and cytoplasmic cell extracts

Daudi (human B lymphoma) and Jurkat (human T lymphoma) cells are available from the American Type Culture Collection

Abbreviation used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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and were grown in RPMI medium (Gibco/BRL) supplemented with 10% fetal calf serum. S100 cytoplasmic extracts (5–12 mg/ml final concn.) were prepared and stored as described previously [8].

Plasmids, cloning and mutagenesis

Plasmids used for transcription *in vitro* were: (i) FH2, which encodes the human *c-fos* [15]; (ii) pSC1, which encodes the hamster glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [8]; (iii) pSC2, which encodes the mouse dihydrofolate reductase [8]; (iv) pGEM-29B8, which encodes the human Tal-1 [16]; (v) pSG/*c-erbA*, which carries the chicken *c-erbA* coding sequence cloned in pSG5 [17]; (vi) pBS-GATA1 and p7.1, which encode the human GATA1 and GATA3 transcription factors [18,19]; (vii) pMyo D which encodes the mouse Myo D protein [20]; and (viii) pSry, which encodes the human sry protein [21]. Clonings were performed according to the standard procedures described by Sambrook et al. [22]. PEST mutants were generated by the site-directed mutagenesis method of Kunkel as described by Sambrook et al. [22]. Single-stranded DNA was prepared from phagemid EKL8, a derivative of FH2 [15] bearing a nearly full-length human *c-fos* cDNA, the last 150 nucleotides of the 3' UTR having been deleted, using the M13 K07 helper phage. Δ PEST1 and Δ PEST2 are deletion mutants obtained using oligonucleotides 5'-CAGAGCATTGGCAGGAGGGGGAGG-AGAATCCGAAGGGAAAGG-3' and 5'-GCAGCTCACCG-ACCTGCCTGCGTGGAACCTGTCAAGAGCATC-3' respectively. Δ PEST3 was obtained by inserting two in-frame stop codons after amino acid 360 using oligonucleotide 5'-GCC-CACCGAAAGGGCTGATGAAGCAGCAGCAACGAG-3'. For the construction of Δ PEST1,2,3, all three mutagenesis nucleotides were used in the same DNA polymerization reaction. Mutations were verified by sequencing.

Transcription and translation *in vitro*

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

Degradation assay and protein analysis

Degradation experiments were carried out at 37 °C in a final volume of 30 μ l. For degradation in cytoplasmic extracts, 1 μ l of the translation mixture and 28 μ l of S100 extract (Daudi and Jurkat cell extracts were used interchangeably) were mixed and pre-warmed at 37 °C. CaCl₂ (100 μ M final concn.) and/or protease inhibitors were then added at a time taken as t_0 . When one component was omitted or when diluted S100 extracts were used, the volume was adjusted with PBS (150 mM NaCl/10 mM Na-phosphate, pH 7). For degradation kinetics, reaction mixtures were sampled at different time points and stopped by addition of electrophoresis loading buffer containing 1% SDS. Proteins were then electrophoresed through 15% gels using the method of Laemmli [23] and electrotransferred onto nitrocellulose for autoradiography. When required, EGTA (1 mM), calpastatin peptide (0.5 mg/ml), leupeptin (0.005 mg/ml; Boehringer), PMSF (1 mM; Boehringer) and aprotinin (0.02 mg/ml; Sigma) were added. Solid-phase synthesis of the calpastatin peptide inhibitor [24] was done in-house on a

Miligen 9050 peptide synthesizer using the fluorenyl-methoxycarbonyl group as temporary amino protection.

Degradation of wild-type and mutant *c-fos* proteins by purified calpains was carried out for 30 min at 37 °C under the same experimental conditions in the presence of 50 μ g/ml of bovine m-calpain (calcium-activated neutral protease; Boehringer) and 1 mM CaCl₂. Heat denaturation of proteins was performed for 10 min at 60 °C using a 100-fold dilution of translation mixture in PBS.

Quantification of data

For quantification of data, either autoradiographs were scanned using the BioImage image analyser from Millipore or degradation kinetics were directly analysed using the 445 SI PhosphorImager from Molecular Dynamics.

Protein sequence analysis

Protein and nucleotide sequences were recovered from GenBank. PEST motifs were identified using the PEST-FIND algorithm obtained from Dr. Rechsteiner [10]. Additional details on the latter can be found in [25].

RESULTS

Deletion of PEST motifs does not render *c-fos* resistant to calpains

c-fos (380 amino acids) contains three PEST sequences spanning residues 128–139 (PEST1), 205–250 (PEST2) and 360–380 (PEST3) (Table 1; [10]). To determine whether one or several of these motifs are responsible for calpain-mediated degradation, individual (Δ PEST1, Δ PEST2, Δ PEST3) or multiple deletion mutants (Δ PEST1,2,3) were generated using site-directed mutagenesis and then tested for degradation by calpains. To ensure that bringing together regions previously distant in *c-fos* does

Table 1 PEST motif in AP-1 family members and sensitivity to calpains

AP-1 family member nucleotide sequences were recovered from GenBank and converted into amino acid sequences. PEST motifs (see text for details) were identified using the PEST-FIND algorithm [10,25]. Positive scores define PEST motifs (for details, see [25]). The sensitivity to μ - and m-calpain is indicated (see text for details).

Protein	PEST sequence (amino acids)	Score	Reference	Sensitivity to calpains
<i>c-fos</i>	PEST1: 128–139	+10.1	[10]	μ (+)
	PEST2: 205–250	+5.7		m (+)
	PEST3: 360–380	+4.4		
<i>fra-1</i>	95–107	+12.6	This work	μ (–)
	179–197	+13.3		m (–)
	250–272	+8.8		
<i>fra-2</i>	115–127	+16.3	This work	μ (–)
	152–164	+9.3		m (+)
	223–241	+13.6		
	263–303	+4.6		
	307–335	+7.9		
<i>fos-b</i>	35–46	+23	This work	μ (+)
	208–228	+4		m (+)
<i>jun-b</i>	237–247	+5.8	This work and [6]	μ (+)
	249–265	+4.9		m (+)
<i>c-jun</i>	229–255	+14.2	This work and [6]	μ (+)
<i>jun-d</i>	224–262	+11.7	This work and [6]	m (+)

Figure 1 Calcium-dependent degradation kinetics of wild-type and mutant human c-fos in Jurkat-cell S100 cytoplasmic extracts

(A) Degradation kinetics of c-fos, ΔPEST1, ΔPEST2, ΔPEST3, ΔPEST1,2,3 and GAPDH were conducted using undiluted Jurkat cell S100 cytoplasmic extracts (5 mg/ml) as described in the Experimental section. (B) Comparable experiments were conducted with diluted Jurkat cell extracts (300 mg/ml final concn. in the assay).

not create new PEST motifs, the sequence of each mutant was processed using the PEST-FIND algorithm [10]. The PEST-FIND algorithm also indicated that neither the wild-type c-fos nor any of the c-fos mutants bear any degenerate PEST motifs (i.e. lacking either one of the contributing amino acids), thus excluding the possibility that such sequences might affect the interpretation of our experiments.

Degradation experiments were carried out as described previously [8] (see also the Experimental section). Briefly, the human c-fos was produced by translation in the rabbit reticulocyte lysate, which is devoid of any detectable level of calpain activity, then mixed with S100 Jurkat cell cytoplasmic extract, which contains the calpain activity, and finally degradation reactions were started by adding 1 mM calcium to activate calpains. Importantly, calpains are the only proteases that display proteolytic activity on c-fos under these conditions (i.e. in the presence of calcium) [8]. It is worth noting that degradation intermediates are easily visualized (see Figure 1) and may be stable for several hours in certain degradation experiments [8] (see also see Figure 1B for examples).

As a first step in our investigations, we wished to determine whether the PEST mutants retained sensitivity to calpains. For this purpose, five independent degradation-kinetics experiments of wild-type and mutant fos proteins were conducted using undiluted Jurkat cell extracts (see legend to Figure 1). GAPDH and dihydrofolate reductase were included as invariant controls, since they have already been shown to be resistant to calpains [8].

One typical experiment is presented in Figure 1(A). It shows that all PEST mutants are susceptible to calpains, since these proteins were quantitatively degraded in less than 2–5 min in conditions under which GAPDH and dihydrofolate reductase (results not shown) were not detectably cleaved. Emphasizing the specificity of the calcium-induced degradation, proteins like fra-1 [8], c-erbA (see below) and v-fos^{br} (see Discussion section), were resistant to degradation under the same experimental conditions.

Subsequently, it was important to determine whether the susceptibilities of the different mutants significantly depart from that of c-fos. To this end, four independent degradation-kinetics experiments were conducted with extracts from Jurkat cells appropriately diluted to slow down the degradation process. Precise determination of degradation rates by normalized cell extracts was however not possible because of variation in calpain activity during the experiment: the rates are much higher at the early stages than later on. Although the point has not been studied in detail, the main reason for this is probably the fact that after rapid and efficient activation by calcium, calpains self-inactivate by autoproteolysis (see [9] for further details). A typical example of degradation kinetics is presented in Figure 1(B), whereas decay curves corresponding to the four degradation experiments are shown in Figure 2 (experiment A corresponds to that presented in Figure 1B). Taken together, these data show that the decay of c-fos and PEST mutants occur roughly in parallel, thus indicating comparable susceptibilities to calpains for the different proteins. Careful analysis of decay curves

Figure 2 Comparison of degradation rates of wild-type and mutant c-fos in Jurkat cell extract

Degradation kinetics were conducted as described in Figure 1(B). Experiment A corresponds to that presented in Figure 1(B). The key given for Experiment B applies to all experiments shown.

Figure 3 Calpain-dependent degradation of ΔPEST1,2,3 mutant in Daudi-cell S100 cytoplasmic extracts

Degradation reactions were carried out for 1 h at 37 °C in the presence of protease inhibitors using Daudi cell extracts as described in the Experimental section. MW, molecular-mass markers.

however suggests that PEST2-deleted mutants (ΔPEST2 and ΔPEST1,2,3) may be slightly less rapidly degraded than the other proteins. Although we cannot formally rule out that this may be due to experimental deviation, it is possible that this small

difference is linked to a subtle mutagenesis-linked conformational change or to a very slight contribution of PEST motifs (or of other motifs residing within them) to the susceptibility to calpains.

Since cleavage sites for other proteases might have been created or exposed due to structural alterations resulting from deletion mutagenesis, it was important to show that degradation of mutants was actually due to calpains. Therefore cleavage of ΔPEST1,2,3 was tested in the presence of a set of protease inhibitors allowing identification of the overall calpain activity (without any discrimination between μ - and m-calpain; for a review, see [26]). Figure 3 shows that, as in the case of the parental c-fos (see Figure 3 in [8]), degradation of the mutant protein is not inhibited in the presence of aprotinin, is partially inhibited in the presence of PMSF and is fully inhibited in the presence of EGTA, leupeptin and, above all, a 27-mer peptide (called calpastatin peptide herein) derived from calpastatin [24] which is a highly specific physiological inhibitor of calpains [9]. It was also necessary to rule out the possibility that cleavage of PEST mutants by calpains might occur at aberrant sites due to incorrect folding of the proteins. Calpains essentially recognize conformational determinants and not primary structure determinants [9] and can thus be used as topological probes for investigating the tertiary structure of their substrates. Accordingly, wild-type and mutant fos proteins were heat-denatured and then tested for their susceptibility to calpains. As shown in Figure 4, all denatured proteins are no longer degraded. This observation is consistent with the idea that degradation by calpains depends on the presence of specific conformational motifs, and consequently suggests that a number of the latter (if not all) are conserved in PEST deletion mutants.

Figure 4 Resistance to calpains of heat-denatured wild-type and mutant c-fos

Proteins were heated for 10 min at 60 °C and degradation reactions were carried out for 30 min at 37 °C using a Jurkat-cell S100 extract (5 mg/ml) as described in the Experimental section. HD, heat-denatured proteins.

In conclusion, deletion of either individual or all PEST motifs does not stabilize c-fos protein to any great extent in our degradation assay, indicating that these sequences are not required for the action of calpains.

The presence of PEST motifs in various transcription factors does not correlate with sensitivity to calpains

Although all members of the jun and fos families possess high-score PEST motifs (Table 1), not all of them are susceptible to μ - and m-calpain: fra-2 is cleavable by m-calpain only and fra-1 is resistant to both proteases [8]. The latter observation, coupled

to the fact that deletion of PEST motifs from c-fos does not confer resistance on calpains, already suggests that PEST sequences are neither necessary nor sufficient signals for recognition of proteins by calpains. To bring stronger experimental support to this hypothesis, we screened a series of PEST-bearing and PEST-lacking transcription factors for their susceptibility to calpains.

GATA1, GATA3, Tal-1, c-erbA, the myogenic transcription factor Myo D and the sex determining factor Sry were produced *in vitro* in the reticulocyte lysate and tested for their sensitivity to calpains. This was achieved either through the addition of purified bovine m-calpain directly to the translation mixture (results not shown) or through incubation of neo-synthesized proteins in Daudi cell S100 cytoplasmic extracts, as in the case of c-fos. Outcomes of degradation were similar whatever the experimental conditions used. The specificity of calpain action was demonstrated through inhibition of the reaction by the calpastatin peptide. The kinetics of protein degradation are presented in Figure 5. PEST motifs in each protein were identified using the PEST-FIND program and can be found in Table 2. From our experiments, tested proteins clearly fall into three categories. The first corresponds to proteins that do have PEST sequences and are sensitive to calpains (GATA1, GATA3, Tal-1 and Myo D). The second corresponds to proteins that have no PEST sequences but that are cleavable by calpains (Sry). The last corresponds to proteins which have PEST motifs but which are resistant to calpains, e.g. c-erbA to which fra-1 must be added (see [8]).

In conclusion, the last two groups of proteins clearly show that there is no strict correlation between the presence of PEST motifs and the susceptibility of transcription factors to calpains. This conclusion is strengthened by a survey of a number of calpain substrates which do not contain any PEST sequence (see Discussion section).

Figure 5 Degradation of GATA 1, GATA 3, Tal-1, c-erbA, Myo D and Sry by calpains

Degradation experiments were conducted using Daudi cell extracts as described in the Experimental section. Because of internal initiation and/or premature termination of elongating chains, translation patterns are most often complex. Full-length proteins are indicated by arrowheads. MW, molecular-mass markers.

Table 2 PEST motifs in a variety of proteins

Nucleotide sequences were recovered from Genebank and converted into amino acids. PEST motifs were identified using the PEST-FIND algorithm of Rogers et al. [10]. References for calpain susceptibility are to be found in the text.

Protein	Pest sequence (amino acids)	Score
Transcription factor		
c-erbA		
Chicken	118–131	+ 7.91
	228–246	+ 0.44
GATA 1		
Man	113–127	+ 7.58
	140–189	+ 0.58
GATA 3		
Man	159–177	+ 6.08
Myo D		
Mouse	9–41	+ 5.84
	191–202	+ 4.45
	257–318	+ 6.36
Sry		
Man	None	
Tal-1		
Man	95–117	+ 2.67
	170–183	+ 4.75
	258–311	+ 3.88
Other proteins		
Connexin 32		
Mouse	None	
Filamin		
Man	None	
F1-20		
Mouse	261–281	+ 0.05
	344–495	+ 4.69
	495–577	+ 4.42
	577–597	+ 0.04
	597–630	+ 0.85
	630–744	+ 1.92
IL1 α		
Man	None	
InsP ₃ kinase		
Man	62–91	+ 10.55
	285–300	+ 4.59
NF-H		
Man	502–514	+ 12.09
pTP1B		
Man	None	
pp-60 ^{c-src}		
Man	None	
Tyrosine hydroxylase		
Bovine	395–404	+ 2.27
Calpain large subunits		
μ -Calpain		
Man	295–322	+ 16.83
	517–548	+ 3.31
m-Calpain		
Man	296–312	+ 2.24
Calpain		
Chicken	285–312	+ 4.10
m-Calpain		
Rat	None	
Calpain		
<i>S. mansoni</i>	97–110	+ 0.80
	45–459	+ 17.01
Calpain small subunits		
Man	None	
Pig	None	
Ox	None	

DISCUSSION

Rogers et al. [10] have suggested that PEST sequences may target proteins for rapid intracellular degradation. They have also elegantly hypothesized that these negatively charged motifs could sequester calcium for activating calpains in the close vicinity of substrates. Although commonly accepted, none of these proposals has received strong and unambiguous experimental support so far. On one hand, the most significant observation concerning the relationship between PEST motifs and rapid protein degradation, regardless of the proteolytic system involved, is not clear-cut: rapid degradation of ornithine decarboxylase requires, in addition to a C-terminally located PEST motif, the last five amino acids of the protein, which are not part of the PEST motif and which are not sufficient to target ornithine decarboxylase for degradation [27–29]. On the other hand, cleavage sites for calpains, when they have been studied, have rarely been reported to reside in, or near, PEST motifs. To our knowledge, the only documented examples are protein kinase C [11], hydroxymethylglutaryl-CoA reductase [14], the plasma membrane Ca²⁺-ATPase [13] and the amyloid precursor protein [11]. However, in no case has any functional relationship between PEST motifs and cleavage by calpains been demonstrated by experiment. Thus, it cannot be ruled out that the close vicinity or superposition of PEST motifs and cleavage sites for calpains is just coincidental in the few proteins mentioned above.

Most transcription factors (c-jun, jun-d, jun-b, c-fos, fos-b, Myo D, Tal-1, GATA-1, GATA-3) tested here and previously [6–8] are efficiently cleaved by calpains and harbour PEST motifs. This is also true for other proteins, such as several calmodulin-binding proteins [13], tyrosine hydroxylase [30], Ins(1,4,5)P₃ kinase [31], the neurofilament heavy subunit (NF-H) [32], the synapse-specific protein F1-20 [33] and the erythrocyte β -adducin [34] (see Table 2). However, despite this apparent correlation, some of our observations strongly argue against a strict causal relationship between the presence of a PEST sequence and susceptibility to calpains. These can be summarized as follows. (i) Deletion of PEST sequences does not significantly decrease c-fos susceptibility to calpains. (ii) We have recently characterized one of the major calpain proteolytic sites of c-fos between amino acids 89 and 90, i.e. 40 amino acids upstream of PEST1 (M. Pariat, S. Carillo, J. Derencourt and M. Piechaczyk, unpublished work). (iii) Transcription factors such as fra-1, fra-2 [8] and c-erb-A (this work) are resistant to cleavage by calpains although they bear multiple high-score PEST motifs. Along the same lines, it is worth noting that the gag-fos fusion protein, carried by the oncogenic FBR-MSV murine retrovirus, is highly resistant to calpains (A. M. Steff, S. Carillo, M. Pariat and M. Piechaczyk, unpublished work) despite the fact that several high-score PEST motifs are carried by the gag moiety of the molecule. (iv) A transcription factor such as Sry is very sensitive to calpains although it does not contain any PEST- or even PEST-degenerated sequence. (v) Proteins other than transcription factors, such as phosphotyrosine phosphatase 1B (pTPB1) [35], filamin [36], connexin [37], pp60^{c-src} [38] and interleukin 1 α [39] are sensitive to calpains despite lacking PEST motifs (see Table 2). Along the same lines, it is worth mentioning that calpains are activated through autolysis of both their long (80 kDa) and short (30 kDa) constituent polypeptide chains but that neither the rat large subunit, nor the human, pig and ox small subunits, carry PEST motifs (Table 2).

Although our observations strongly suggest that PEST motifs are neither absolute requirements nor hallmarks for calpain-mediated degradation of proteins, it is important to emphasize two points. First, in the specific case of c-fos, we cannot eliminate

the possibility that one or several of the multiple calpain cleavage sites coincidentally falls within PEST regions. Amino acid sequencing of all c-fos proteolytic products is currently underway to solve this issue. Secondly, recent data in the literature indirectly argue against a role for PEST motifs as recognition signals for calpains. The original hypothesis was initially proposed on two bases (see the Introduction): (i) an attempt has been made to find a molecular function for sequences selected through a statistical analysis of primary structures of stable and unstable proteins; and (ii) taking into account that the requirement of calcium for activating calpains *in vitro* is one to three orders of magnitude (depending on the type of calpain) higher than physiological intracellular calcium concentrations, it has been suggested that PEST motifs might chelate calcium and thus activate calpains at the substrate level. However, it has more recently been reported that both lipid [40,41] and protein [42–45] activators increase dramatically (up to 100-fold) the affinity of calpains for calcium. This thus makes unlikely the hypothesis that the concentration of calcium at the level of substrates, through chelation by PEST motifs, is a prerequisite for cleavage by calpains.

Despite the lack of a causal relationship between PEST motifs and susceptibility to calpain, we feel that the statistical support for the PEST hypothesis still remains attractive with regard to rapid protein degradation by other proteolytic systems. c-fos catabolism is interesting in this regard since different lines of evidence argue for the existence of several breakdown mechanisms operating on it. First, calpains appear to be involved in the control of c-fos abundance in the cytoplasm (see above). Secondly, using the rabbit reticulocyte extract as a degradation assay *in vitro*, experiments by Papavassiliou et al. [46] point to the existence of another pathway depending on the presence of ATP and of an appropriately phosphorylated c-jun. Thirdly, Kovary and Bravo have demonstrated that c-fos can be rapidly broken down *in vivo* even in the absence of c-jun [47,48]. It is therefore possible that PEST motifs are necessary to allow degradation of c-fos, according to the catabolic pathways described by Papavassiliou et al. [46] and Kovary and Bravo [47,48], and may also eventually be required for other proteins that remain to be characterized.

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