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Degradation of transcription factors, c-Jun and c-Fos, by calpain

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c-Jun protein, and API/PEA1 transcription factor component, is a typical short-lived protein, and like other short-lived proteins such as c-Fos, contains PEST regions. Calcium-dependent neutral protease (calpain), a candidate for the degradation of PEST-containing proteins, digests c-Jun and c-Fos efficiently in vitro. This is the first demonstration that transcription factors are substrates for calpain. The C-terminal portion of c-Jun is relatively resistant to calpain such that an 18kDa fragment, which includes the DNA binding domain, accumulates under moderate digestion conditions. The activity of c-Jun in cultured cells can be modified by changing the level of calpastatin, an endogenous calpain inhibitor, indicating that c-Jun is also a substrate for calpain in vivo.

c-Jun; c-Fos; Calpain; Calpastatin; PEST; Calcium

1. INTRODUCTION

The products of *jun*-related genes are known components of the human AP1 and mouse PEA1 transcription factors [1–5]. Three *jun*-related genes that encode highly related proteins, *c-jun, jun B*, and *jun D*, have been identified in mammalian cells [6,7]. Each Jun protein contains a conserved C-terminal DNA binding domain and a less conserved N-terminal transcription activation domain connected by a hinge region. The expression of *c-jun* is induced in cells by treatment with several kinds of agonists, including growth factors, phorbol esters and calcium ionophores [8–10]. In many cases, *c-jun* mRNA is produced transiently being degraded immediately. Like c-Fos protein [11], which forms heterodimers with Jun-related proteins [12], the half-life of c-Jun protein is very short (<2 h) [9]. It is generally assumed that transient stimulation of the mRNA and protein are crucial for the normal response of cells to extracellular stimuli, while constitutively high expression of c-Jun protein has often been observed in transformed cells [13,14]. Furthermore, over expression of c-Jun may be toxic to some cells [15].

The amino acid sequences of most short-lived proteins include one or more regions rich in proline, glutamic acid, aspartic acid, serine, and threonine. These regions, known as 'PEST' regions, may be recognized directly or indirectly by a specific protease(s) in animal cells [16]. A candidate for one such protease is calpain [16,17]. This Ca^{2+}-dependent neutral protease is found in various animal tissues and cells, but its substrates are relatively limited [18]. Since these include hormone-receptors, protein kinase C, and several calmodulin binding proteins, calpain is thought to modify signal transduction in animal cells through limited proteolysis [17,18].

Here, we report that two PEST containing transcription factors, c-Jun and c-Fos, are substrates of calpain.

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2. MATERIALS AND METHODS

2.1. *In vitro translation*

cDNA fragments encoding *c-jun, junD, c-fos*, μ-calpain domain II, or chloramphenicol acetyl transferase were cloned into T7-expression plasmids as described elsewhere [7,19]. c-Jun Δ 169 [7] encodes the C-terminal 169 amino acids of c-Jun protein, and CDL [19] encodes a c-Jun protein with a deletion in the leucine zipper region (aa Nos 284–311). μ-Calpain domain II contains 286 amino acids starting at Val^{64} [20].

RNA was synthesized using the T7-expression plasmid and T7-RNA polymerase. Protein was synthesized in a rabbit reticulocyte lysate system in the presence of [^{35}S]methionine [7,19]. The translation-reaction mixture (25 μl) was passed through a Sephadex G-50 column (1 ml) before digestion by calpain.

2.2. *Digestion of proteins by calpain*

μ-Calpain and m-calpain were purified from rabbit skeletal muscle as in [21].

The in vitro translated protein (10 μl) was mixed on ice with a calpain solution (5 μl), and the reaction was started by adding 5 μl of 400 mM Tris-HCl pH 7.5, 12 mM $CaCl_2$, and 2 mM DTT at 30°C. The final Ca^{2+} concentrations were 2 mM for both μ- and m-calpain. In some experiments, $CaCl_2$ was replaced with 2 mM EDTA. After incubation for 10 min, 20 μl of concentrated (2×) SDS sample buffer [22] was added to stop the reaction. Double-stranded oligonucleotide

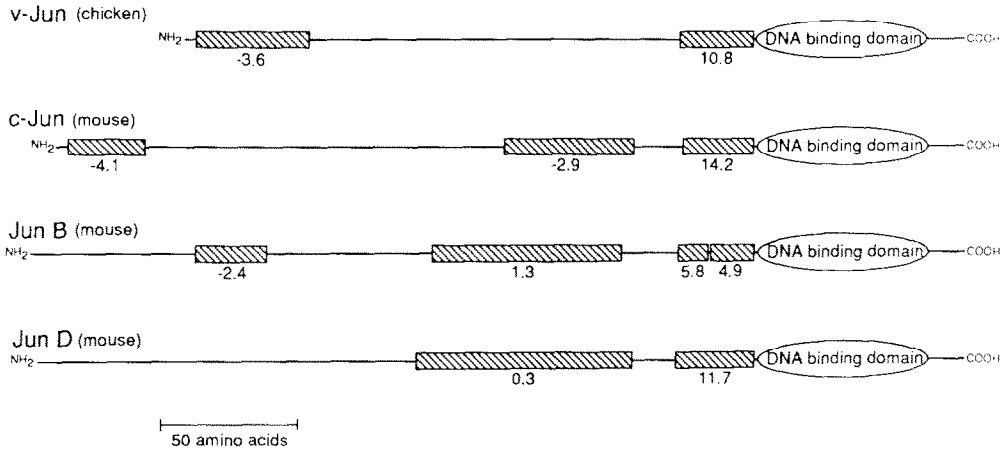

Fig. 1. PEST regions in Jun-related proteins. The PEST regions are indicated by shadowed boxes with the PEST scores underneath.

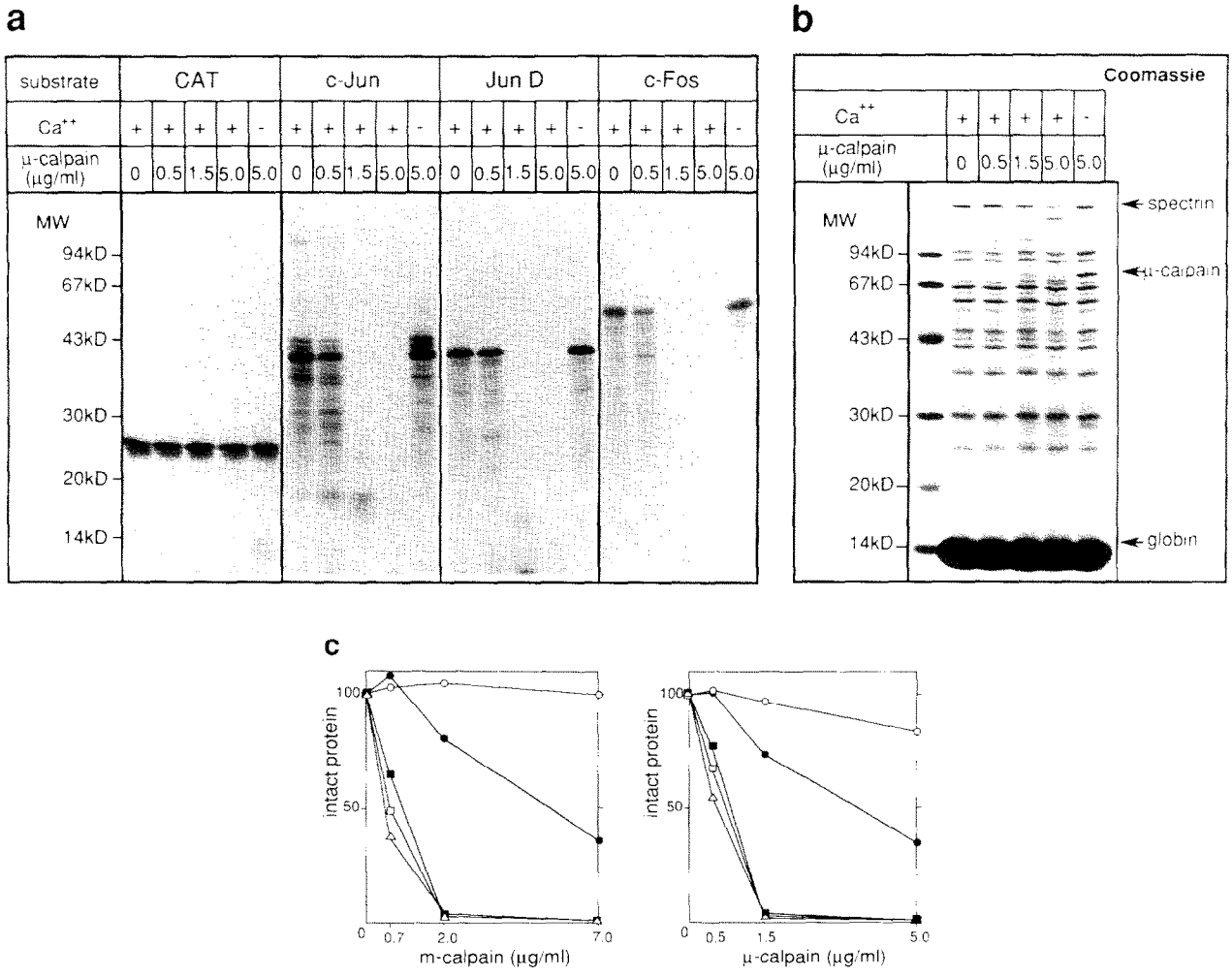

Fig. 2. Digestion of in vitro translated proteins by calpain. Proteins were translated in vitro and simultaneously labeled with [^{35}S]methionine. A mixture of labeled protein and reticulocyte proteins was incubated with different concentrations of calpain in the presence or absence of Ca^{2+} as indicated. After 10 min incubation at 30°C, proteins were separated on a 12% SDS gel, and the ^{35}S signals were detected on X-ray film (a). A Coomassie brilliant blue-stained gel is also shown (b). Radioactivities at the positions of intact in vitro translated proteins were quantified with a Fuji imaging analyzer (c). Chloramphenicol acetyl transferase (○), μ-calpain domain II (●), c-Jun (□), Jun D (■), c-Fos (△).

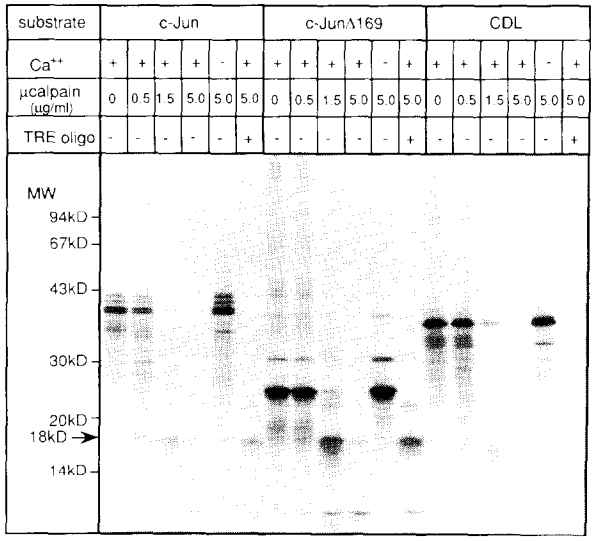

Fig. 3. Digestion of c-Jun mutants by calpain. c-JunΔ 169 encodes the C-terminal half of c-Jun starting at Glu^{166} [7]. The leucine zipper region (Glu^{284}–Leu^{311}) was deleted in CDL [19]. Double stranded oligonucleotide including a Jun/AP1 binding site (TRE-oligo) was added at a concentration of 2 μg/ml as indicated. Other methods were the same as those described in Fig. 2.

(TRE-oligo: sequence below) was pre-mixed with the in vitro translated protein when required.

TRE-oligo: 5′-CTAGGTGTCTGACTCATGCTTTA-3′
3′-CACAGACTGAGTACGAAATGATC-5′

2.3. *SDS-PAGE*

SDS-polyacrylamide gel electrophoresis was performed as in [22]. The gel was first stained with Coomassie brilliant blue, and the in vitro translated protein incorporating [^{35}S]methionine was detected by autoradiography. To quantify the radioactivity, the gel was exposed to a Fuji imaging plate and the radioactivity quantified with a Fuji image analyzer (BAS2000).

2.4. *Transfection and CAT assay*

The structures of TRE-CAT and RSV-c-jun are described in [7]. To construct RSV-CSN, RSV-R/CSN and RSV-R/213, rabbit calpastatin cDNA fragments [23] (Cf. Fig. 4a) were cloned into an expression vector [7] containing RSV-LTR and an SV40 transcription termination signal.

NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. The cells at a density 2×10^5 cells/6 cm dish were transfected by the calcium phosphate co-precipitation method with 2 μg of TRE-CAT, 0.2 μg of RSV-c-jun, and 1 μg of an expression vector derived from calpastatin cDNA. After 8 h exposure to the DNA-calcium phosphate precipitate, the culture medium was changed to DMEM containing 0.5% fetal calf serum. The cells were cultured further for 30 h, washed with phosphate buffered saline and harvested. F9 cells were grown and transfected by the calcium phosphate co-precipitation method as previously described [19]. CAT assays were carried out by standard methods [7].

3. RESULTS AND DISCUSSION

3.1. *Jun-related gene products are specific substrates for calpain*

Three PEST regions [16] were found in the c-Jun sequence (Fig. 1). One strong PEST region is adjacent to the N-terminal side of the DNA binding domain and has an amino acid sequence that is highly conserved among Jun-related proteins. Two weak PEST regions lie in less-conserved regions.

Several PEST-containing proteins are calpain substrates in vitro (for a review, see [17]). These include cytoskeletal proteins, enzymatic proteins, and hormone receptors. To determine whether c-Jun is a substrate for calpain, in vitro translated c-Jun protein was first incubated with calpain in the presence or absence of Ca^{2+} and analyzed on SDS-PAGE. As shown in Fig. 2a, c-Jun is digested quite efficiently by μ-calpain when Ca^{2+} is present. Although the reaction mixture contained large amounts (~100 μg) of reticulocyte proteins compared to ^{35}S-labeled c-Jun (<0.1 μg), no change in the Coomassie-staining pattern of the gel was observed after incubation with calpain, except for spectrin degradation at high calpain concentrations (>5.0 μg/ml) (Fig. 2b). c-Jun seems to be much more susceptible to calpain than spectrin, one of the most sensitive substrates.

To rule out the possibility that susceptibility to calpain is a common feature of in vitro translated proteins, we analyzed several other proteins including JunD, c-

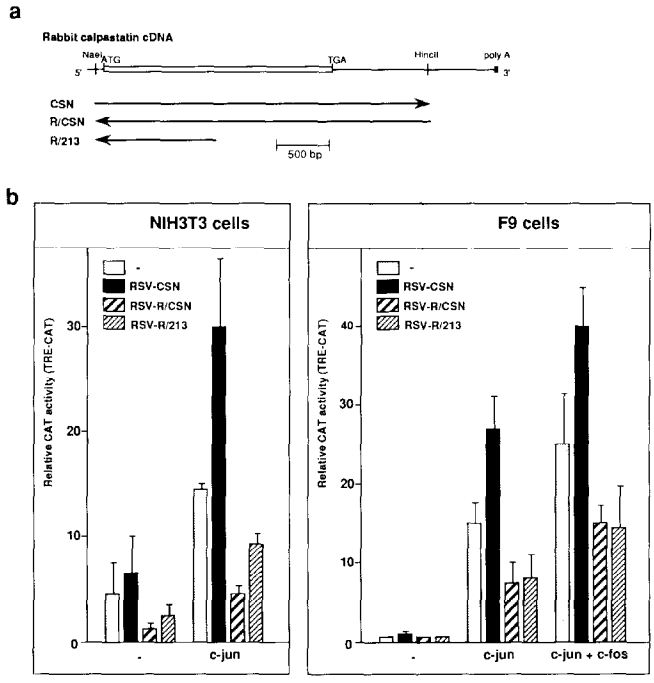

Fig. 4. The effect of calpastatin on c-Jun activity in serum-starved NIH3T3 cells or in mouse embryonal carcinoma F9 cells. (a) Fragment of calpastatin cDNA [23] cloned into the expression vector. Length and orientation of fragments cloned into the RSV-LTR expression vector [7] are shown by arrows. R213 was derived from truncated cDNA clone λC1213 [23]. (b) The effect of the expression of calpastatin or calpastatin antisense RNAs on the basal or c-Jun induced expression of TRE-CAT reporter gene. Data are the averages of three independent experiments and expressed as relative CAT activity (percent acetylation). Bars indicate standard deviation.

Fos, μ-calpain domain II, and chloramphenicol acetyl transferase (CAT). The CAT protein was fully resistant to calpain, in clear contrast to JunD or c-Fos, which, like c-Jun, were efficiently digested. μ-Calpain domain II was also digested, but with a significantly lower efficiency (Fig. 2c). The substrate specificity of m-calpain is essentially the same as that of μ-calpain (Fig. 2c). The ATP-dependent protease system, which may be present in reticulocyte lysates [24], is not involved in the protein degradation observed here, since free ATP was not present in the reaction, and the addition of 2 mM AMP did not affect the degradation patterns (data not shown).

These results are consistent with the identification of c-Jun as a short-lived PEST-containing protein [16], and add another example to the prevailing theory that calpain recognizes PEST proteins.

3.2. *Calpain resistant-core in c-Jun*

Although high concentrations of calpain digests c-Jun completely, significant amounts of an 18 kDa fragment accumulate under moderate digestion conditions (Fig. 3). When an N-terminal deletion mutant of c-Jun (c-JunΔ 169, MW 24 kDa) was used, the 18 kDa fragment appeared again, indicating that the fragment originates from the C-terminal DNA binding domain of c-Jun (see Fig. 1). The addition of a double-stranded oligonucleotide including a c-Jun binding site (TRE-oligo in Fig. 3), which probably stabilizes a dimer form of c-Jun, increased the accumulation of the 18 kDa fragments. CDL is a c-Jun mutant with a deletion in the leucine zipper region that cannot form dimers or bind DNA. The 18 kDa fragment was not produced from this mutant, even in the presence of the oligonucleotide (Fig. 3), further indication that the 18 kDa fragment is located in the C-terminal region of c-Jun.

Western blot analysis shows the presence of an 18 kDa fragment of the c-Jun protein in cell extracts prepared from chick embryo fibroblasts (data not shown). Thus, calpain may produce the 18 kDa fragment in vivo as it does in vitro, and the truncated c-Jun molecule could play a role in the regulation of transcription [19].

3.3. *c-Jun is a substrate for calpain in vivo*

To determine whether c-Jun is degraded by calpain in vivo also, c-Jun activity was monitored by expression of TRE-tk-CAT plasmids when the level of calpastatin, an endogenous calpain inhibitor [23], was increased by over-production or decreased by production of antisense RNA. The expression of TRE-CAT, which remained low in serum-starved NIH3T3 cells was induced by about 3-fold or more by over-expression of c-Jun [7] (see also Fig. 4). c-Jun-induced expression was further enhanced ($\sim$2-fold) by the co-expression of exogenous calpastatin (Fig. 4, RSV-CSN), which might stabilize c-Jun by decreasing the amount of active calpain in the cells. In contrast, the expression of calpastatin antisense RNAs (Fig. 4, RSV/R-CSN and RSV-R/213) neutralized or reduced the c-Jun-induced expression of TRE-tk-CAT. The results of the same kind of experiments obtained with mouse embryonal carcinoma F9 cells are also shown in Fig. 4. In this case, the induction of TRE-tk-CAT expression by c-Jun was much more significant compared with the NIH3T3 cells, since the basal level of the TRE-tk-CAT expression was quite low [19,25]. Enhancement of the c-Jun induced TRE-tk-CAT expresion by calpastatin was again observed with F9 cells, and the suppression by antisense calpastatin was also clear. c-Fos enhances the c-Jun-induced transcription by forming a heterodimer with c-Jun, which is more active than the c-Jun homodimer [5–7,12,19]. This c-Fos/c-Jun induced TRE-tk-CAT expression was also affected by over expression of calpastatin or the expression of antisense RNA of calpastatin. These results indicate that the stability of c-Jun in these cells depends at least partially on the level of calpastatin, i.e. calpain.

The present results suggest that calpain is involved in the degradation of transcription factors such as c-Jun and c-Fos, and thus in the regulation of gene expression, the final step of signal transduction in cells.

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