Proteolytic processing of nuclear factor κB by calpain in vitro

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Abstract Nuclear factor κB (NF-κB) is a transcription factor that is critical for the inducible expression of multiple cellular and viral genes. Using the electrophoretic mobility shift assay, we demonstrated that DNA binding activity of NF-κB was abolished by proteolysis with μ- and m-calpains in vitro. The proteolysis of NF-κB by calpains and hence the abolition of its DNA binding was prevented by calpastatin, calpain inhibitor I and proteasome inhibitor. We also provided evidence that calpains degrade the C-terminal domain of NF-κB by Western blot using anti-NF-κB (p65) C-terminal antibody. These observations indicate that calpains regulate gene expression through processing of NF-κB.

Key words: Nuclear factor κB; DNA binding; EMSA; Calpain; Protease inhibitor; Western blot; Gene expression

1. Introduction

Calpains (EC 3.4.22.17) are intracellular calcium-dependent cysteine proteases ubiquitously distributed in animal tissues [1,2]. There are at least two types of calpains, μ- and m-calpains, which require a micromolar and a millimolar concentration of Ca^{2+} for activation, respectively. Enzyme activity is physiologically regulated by an endogenous calpain specific inhibitor, calpastatin [1,2]. Presence of both types of calpains in mammalian tissues and cells was demonstrated by measurement of proteolytic activity [3], immunological detection [4-6], and molecular cloning [7–9]. Both calpains have distinct large catalytic subunits and an identical small regulatory subunit [10]. Calpains hydrolyze various endogenous proteins [11,12], calmodulin binding proteins [13,14] and components of receptor signaling pathways [15,16]. Additionally, various transcription factors, including AP1 (c-Fos/c-Jun), AP 2, Pit-1, Oct-1, CP 1a and b, c-Myc, ATF/CREB and AP 3, were cleaved by m-calpain to produce specific partial digestion products [17,18]. These properties of calpains raise the possibility that they are involved in regulation of turnover of transcription factors in vivo.

Nuclear factor κB (NF-κB) is one of the transcription factors and regulates a wide variety of cellular and viral genes including several cytokines, cell adhesion molecules, inducible NO synthase, and human immunodeficiency virus (HIV) [19,20]. Although NF-κB is by no means the sole determinant of inducible expression of these genes, it has been shown to play a significant role [20]. Previous studies demonstrated that NF-κB binding to the specific DNA sequence (called κB motif) is essential for the transcriptional activity. The induction of cell function by NF-κB is independent of new protein synthesis and involves dissociation and translocation of NF-κB to the nucleus where it activates the target genes [19]. However, the regulating pathway of protein turnover of NF-κB is largely unknown. In this study we investigated whether calpain is involved in turnover of NF-κB.

2. Materials and methods

2.1. Reagents

DEAE-Sephacel, heparin-Sepharose CL-6B and poly(dI-dC) were purchased from Pharmacia (Uppsala, Sweden). Calpain inhibitor I (*N*-acetyl-leucyl-leucyl-norleucinal) was supplied by Nakarai (Kyoto, Japan). Proteasome inhibitor (carbobenzoxy-L-leucyl-L-leucyl-norvalinal) was supplied by Peptide Institute, Inc., Japan and calpastatin domain I was the product of Takara Shuzo Co., Ltd. (Otsu, Japan) [21]. Western blot was performed with the affinity-purified rabbit polyclonal antibody against the C-terminal peptide of the NF-κB p65 subunit [22] using a Mini-Protean II slab gel SDS-PAGE system and immunoblot kit (BioRad, Hercules, CA).

2.2. Preparation of NF-κB and calpain

μ-Calpain was purified from human red blood cells and m-calpain was from human placenta as described previously [23,24]. NF-κB was prepared from the nuclei of human lymphocyte as reported previously [25,26]. Briefly, the crude nuclear extract was prepared by the method of Dignam et al. [27] and was fractionated on heparin-Sepharose and DEAE-Sephacel column [28,29]. NF-κB was eluted from the DEAE-Sephacel column by a buffer containing 0.3 M KCl. The fractions containing NF-κB were pooled, dialyzed against the DNA binding buffer and concentrated using Centricon 30 (Amicon, Beverly, MA).

2.3. Digestion of NF-κB by calpain

NF-κB solution (2 μl) was premixed with a 1 μl aliquot of a calpain solution (0–5 μg/ml) diluted to various protein concentrations with buffer containing 20 mM HEPES, 20% glycerol, 60 mM KCl, 0.5 mM PMSF and 0.2 mM EDTA, pH 7.9. The reaction was started by adding 1 μl of $CaCl_2$ (final concentration of 1 mM in a typical experiment) at 30°C. After incubation, EGTA was added at a final concentration of 3 mM to terminate the enzyme reaction before adding the radiolabeled DNA probe for EMSA.

2.4. Electrophoretic mobility shift assay (EMSA)

The DNA binding activity of NF-κB was examined by EMSA using α-^{32}P labeled double-stranded κB oligonucleotide as a probe [25,26,28]. The NF-κB binding sequence, κB sequence, was taken from human immunodeficiency virus type 1 (HIV-1) [26]. The sequences (plus strand) of the wild-type and mutant-type κB oligonucleotides are as follows:

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Abbreviations: NF-κB, nuclear factor κB; EMSA, electrophoretic mobility shift assay; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol bis (2-aminoethyl-ether) tetraacetic acid.

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wild type κB 5′ TTTCTAGGGACTTTCCGCCTGGGGACTTTCCAG 3′

mutant κB 5′ TTTCTACTCACTTTCCGCCTGCTCACTTTCCAG 3′.

Three thymidine residues were added to each oligonucleotide sequence by end-labeling with klenow DNA polymerase (Takara Shuzo Co., Ltd., Otsu, Japan) and [α-^{32}P]dATP (3000 Ci/mmol, ICN Biomedicals, Inc., Costa Mesa, CA). Binding reactions of the DNA probe with protein were performed at 30°C for 10 min in a total volume of 10 μl of buffer containing 22 mM HEPES-KOH (pH 7.9), 60 mM KCl, 0.2 mM EDTA, 5% (v/v) glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol (DTT), 1.0 μg poly(dI-dC), 1.0 μg tRNA and 0.1 ng (20000–30000 cpm) of the labeled 'κB' DNA probe. The labeled DNA probe was purified by passing through a gel filtration column (Quick Spin Column Sephadex G-25, Boehringer, Mannheim, Germany) according to the instructions provided by the manufacturer. After electrophoresis, gels were dried under vacuum and autoradiographed with Kodak T-Mat films (Eastman Kodak Co., Rochester, NY) at −80°C.

2.5. Western blot analysis

To confirm the digestion of NF-κB by calpains, Western blot analysis was performed using specific polyclonal antibody to the C-terminal peptide of the p65 subunit of NF-κB [26]. NF-κB was incubated with various concentrations of μ-calpain and m-calpain in the presence of a final concentration of 1 mM Ca^{2+} at 30°C for 30 min before running on a 5–20% gradient SDS-PAGE gel and was electro-transferred at 4°C to polyvinylidene difluoride (PVDF) membrane in a buffer containing 20 mM Tris-glycine (pH 8.2) and 10% methanol. After washing with Tris-HCl (pH 7.4) buffer, the membrane was blocked with 1% casein in PBS for 8 h at 4°C, and subsequently incubated with 5 μg protein per ml of the affinity purified anti-NF-κB (p65) C-terminal antibody for 3 h at 30°C. After washing, the membrane was incubated with alkaline phosphatase conjugated anti-rabbit IgG antibody (1:3000) (Bio-Rad) for 3 h at 30°C and 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium as substrate.

3. Results

3.1. Preparation and identification of NF-κB

NF-κB was partially purified from the nuclei of human lymphocytes as described in section 2. The DNA binding activity of NF-κB was measured by EMSA using the radiolabeled κB DNA probe. The specificity of NF-κB DNA binding activity was confirmed by DNA competition experiments by adding 50-fold excess of the unlabeled wild-type or mutant-type double-stranded κB oligonucleotide DNA and by DNA cross-linking experiments as reported previously [25,26,28] (data not shown). The specificity of the DNA-protein complex for NF-κB was also confirmed by the anti-NF-κB antibody that showed the supershift of the complex on EMSA (for examples see Figs. 2 and 3).

3.2. Proteolytic effect of NF-κB by calpains on its DNA binding activity

To examine the effects of NF-κB processing by calpains on DNA binding activity, NF-κB was first preincubated with calpain and EMSA was subsequently carried out using κB probe (Fig. 1). The DNA binding activity of NF-κB was abolished by preincubation with either type of calpain in a Ca^{2+}-dependent fashion. Fig. 1 demonstrates that the abolition of DNA binding activity of NF-κB is dose-dependent for both types of calpains (Fig. 1A) and Ca^{2+} ion (Fig. 1B). However, no effect was observed with either calpain or Ca^{2+} alone (Fig. 1A, lanes 2, 7 and 8; Fig. 1B, lanes 2 and 6). As shown in Fig. 1B, the proteolytic effect of m-calpain on

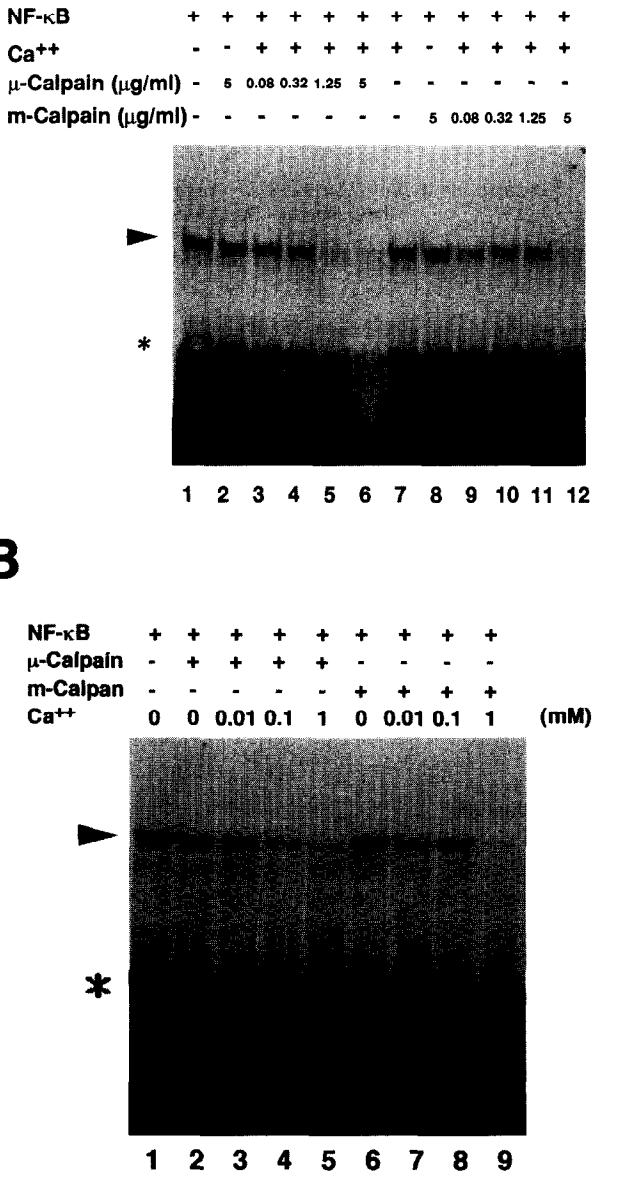

Fig. 1. Inhibition of DNA binding activity of NF-κB by calpains. The calpain-treated NF-κB samples were analyzed by electrophoretic mobility shift assay (EMSA). (A) NF-κB was preincubated with μ-calpain (lanes 3–6) or m-calpain (lanes 8–12) at the indicated concentrations in the presence or absence of 1 mM Ca^{2+} at 30°C for 60 min before adding the radiolabeled κB oligonucleotide probe. In lanes 2 and 8, NF-κB was preincubated with μ-calpain or m-calpain without Ca^{2+}. The closed triangle indicates the NF-κB-DNA complex. An asterisk indicates a non-specific band. (B) Effect of Ca^{2+} on the actions of μ- or m-calpain. The NF-κB and calpain were preincubated for 60 min at 30°C with different concentrations of Ca^{2+} as indicated before EMSA.

the DNA binding activity of NF-κB appeared from as low a concentration as 1 mM of Ca^{2+}.

3.3. Effects of protease inhibitors on degradation of NF-κB by calpain

In Figs. 2A and 3A, the time course of the action of calpains in blocking the DNA binding activity of NF-κB and the effects of protease inhibitors was examined. NF-κB was digested by μ-calpain in the presence of calcium ion for 10, 30

and 60 min. After 10 and 30 min incubation of NF-κB with μ-calpain, position of the DNA-protein complex was shifted downwards (Fig. 2A, lanes 2 and 3), suggesting the partial degradation of NF-κB by μ-calpain. After incubation of NF-κB with μ-calpain for 60 min, the DNA-protein complex disappeared completely. In contrast, incubation of NF-κB with m-calpain gradually blocked the NF-κB–DNA complex formation without affecting the mobility of the complex (Fig. 3A). The disappearance of the NF-κB–DNA complex is considered to be due to proteolysis of NF-κB by calpains in the presence of calcium, although the modes of action appear to be different.

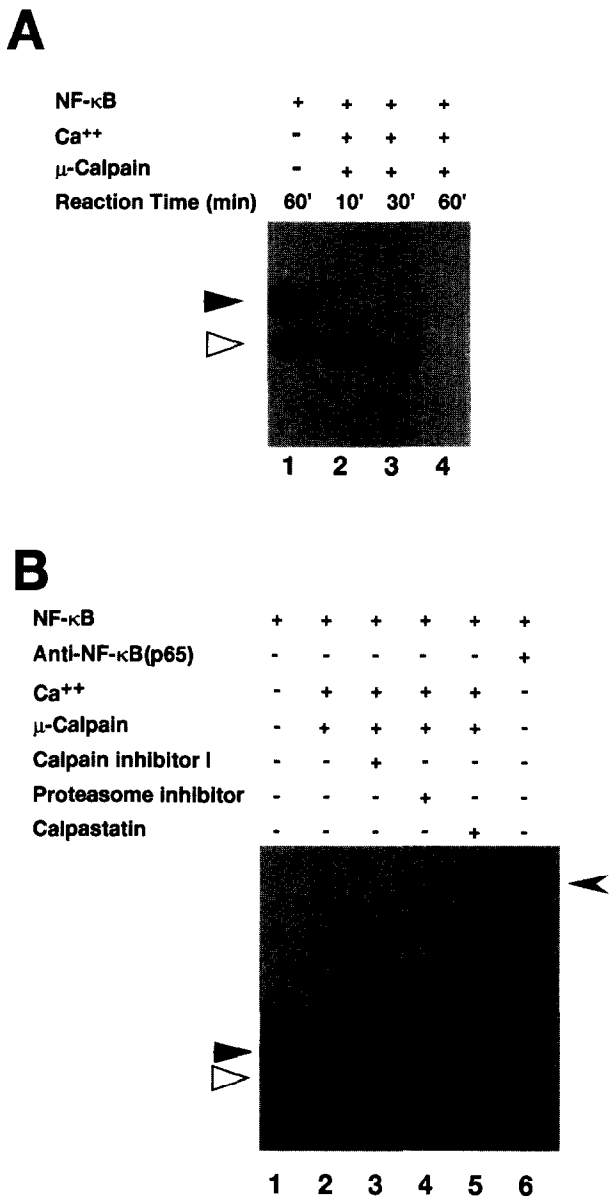

Fig. 2. Effect of μ-calpain in the DNA binding activity of NF-κB. (A) Time course of NF-κB degradation with μ-calpain (5 μg/ml, see section 2). Proteins were preincubated at 30°C for the indicated periods of time and subjected to EMSA. The closed triangle indicates the position of NF-κB-DNA complexes. The open triangle indicates the position of the NF-κB-DNA complexes after partial digestion with μ-calpain. (B) Effects of protease inhibitors. 10 μM each of calpastatin domain I, proteasome inhibitor or calpain inhibitor I was preincubated with NF-κB in the presence of μ-calpain and Ca^{2+} (1 mM) for 30 min at 30°C before adding radiolabeled κB probe. An arrowhead from the right indicates the position of the supershifted band of the NF-κB–DNA complex.

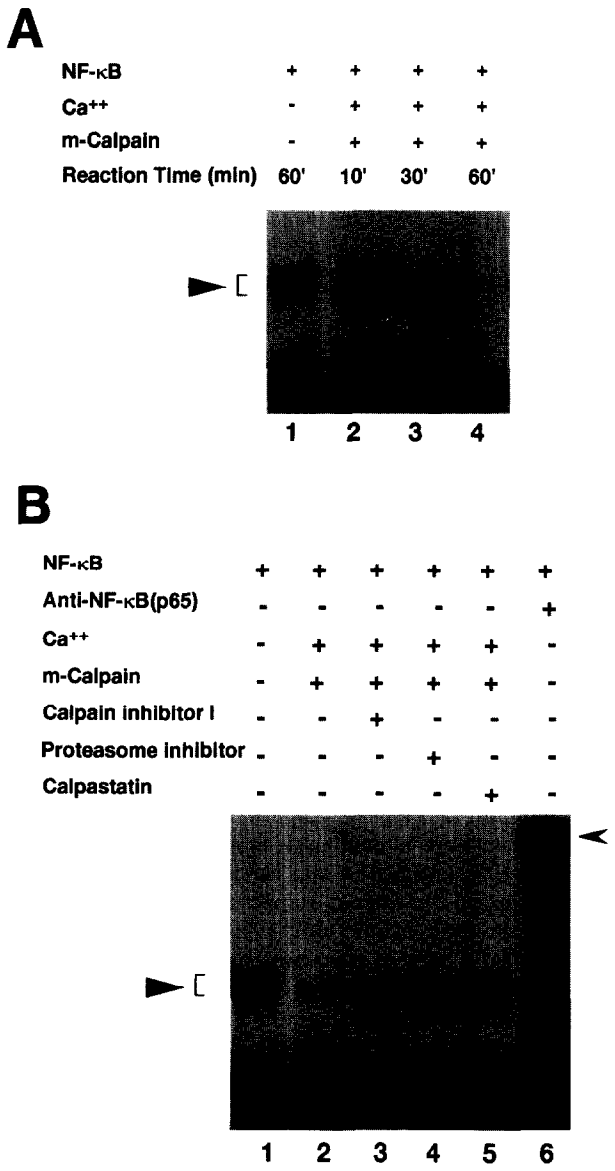

Fig. 3. Effect of m-calpain in the DNA binding activity of NF-κB. The experiments were similarly carried out as in Fig. 2 except that m-calpain was used. (A) Time course of NF-κB degradation with m-calpain (5 μg/ml, see section 2). NF-κB was preincubated with m-calpain in the presence of Ca^{2+} at 30°C for the indicated periods of time and subjected to EMSA. The closed triangle indicates the position of NF-κB–DNA complexes. In this series of experiments, the different preparation of NF-κB was utilized from that in Fig. 2. These bands were similarly retarded on a gel and supershifted by anti-p65 antibody as in Fig. 2 and were thus considered the same NF-κB–DNA complex. (B) Effects of protease inhibitors. 10 μM of calpastatin domain I, proteasome inhibitor or calpain inhibitor I was preincubated with NF-κB in the presence of m-calpain and Ca^{2+} (1 mM). An arrowhead from the right indicates the position of the supershifted band of the NF-κB–DNA complex by anti-p65 antibody.

We then examined the effects of protease inhibitors on this phenomenon with calpains. Calpastatin domain I, calpain inhibitor I and proteasome inhibitor, which are known to be

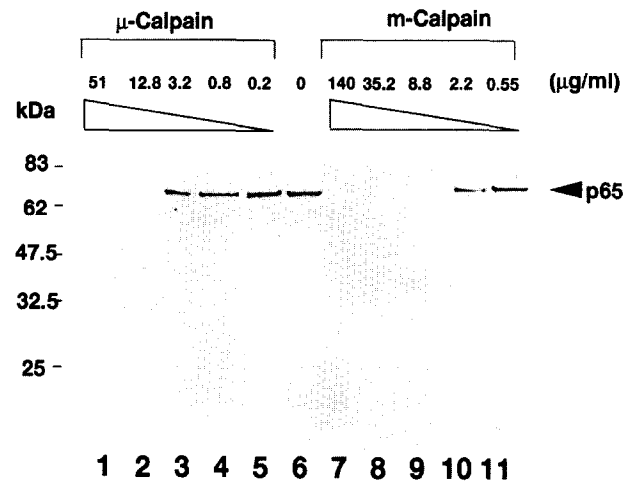

Fig. 4. Western blot analysis of p65 processing by calpains. NF-κB was incubated with various concentrations of μ-calpain (lanes 1–5) or m-calpain (lanes 7–11) at 30°C for 30 min in the presence of 1 mM Ca^{2+}. Positions of molecular weight markers (in kDa) are indicated on the left.

effective inhibitors of μ- and m-calpains [21,29,30], were added during the preincubation period of NF-κB with calpains to examine the specificity of proteolytic actions. The same molar concentration (10 μM) of either calpastatin domain I, proteasome inhibitor or calpain inhibitor I was added to the preincubation mixture containing μ- or m-calpain and NF-κB for 30 min at 30°C and EMSA was carried out (Figs. 2B and 3B, for μ-calpain and m-calpain, respectively). The results showed that the abolition of NF-κB-DNA complex formation was most efficiently prevented by calpastatin, an endogenous calpain-specific inhibitor, although it was also prevented by the calpain inhibitor I and proteasome inhibitor to lesser extents. These observations confirmed that both types of calpains were responsible for the proteolysis of NF-κB and thus abolished NF-κB DNA binding activity.

3.4. Western blot analysis of NF-κB processing by calpains

We examined the proteolytic processing of NF-κB by μ- or m-calpain using Western blot with the C-terminal peptide antibody to p65. NF-κB was incubated with either μ- or m-calpain in the presence of 1 mM Ca^{2+} at 30°C for 30 min. At μ-calpain and m-calpain concentrations higher than 3.2 and 2.2 μg/ml, respectively, the specific p65 band disappeared (Fig. 4). These observations confirmed that the abolition of the DNA binding of NF-κB activity is done by similar concentrations of both calpains. Since the degraded form of NF-κB was not detected by this C-terminal peptide antibody, both calpains were considered to cleave p65 at its C-terminal region.

4. Discussion

Most of the short-lived proteins contain one or more regions rich in proline (P), glutamic acid (E) and aspartic acid (D), serine (S) and threonine (T), which are known as 'PEST' regions [31]. Known PEST proteins include oncogene products (c-Myc, c-Fos and c-Myb), enzymes (hydroxymethylglutaryl-CoA reductase and ornithine decarboxylase) and components of signal pathways (kinases and steroid receptors) [31].

These 'PEST' regions are considered to be recognized by a specific protease(s).

Particularly, calpain is a candidate for the degradation of PEST-containing proteins [14,31]. By computer analysis of the amino acid sequences using PC Gene software (Intelligenetics, Mountain View, CA), three PEST regions (P1: 279–294, P2: 316–324, P3: 510–551) were predicted in the p65 subunit of NF-κB but no typical PEST region was found in the p50 subunit of NF-κB. The PEST scores of P1 to P3 were 5.81, 3.39 and −0.81, respectively. P1 is located on the DNA binding domain, P2 is adjacent to the DNA binding domain and P3 is located in the C-terminal end of p65.

We demonstrated in this paper that DNA binding activity of NF-κB was abolished by μ- and m-calpain using EMSA (Fig. 1–3). Using Western blotting assay, we demonstrated that p65 was actually cleaved by calpains at the similar effective concentrations (Fig. 4). Degradation of NF-κB by calpain was efficiently inhibited by calpastatin domain I, proteasome inhibitor and calpain inhibitor I (Figs. 2 and 3).

Since the anti-NF-κB (p65) C-terminal antibody did not detect the degraded forms of the p65 molecule (Fig. 4), it was suggested that calpains may degrade p65 at the C-terminal region. μ-Calpain may degrade p65 at the C-terminal region and at a region other than that involved in DNA binding since in the earlier step of protein processing we detected the lower molecular weight form of the protein-DNA complex (Fig. 2A). On the other hand, m-calpain may degrade p65 at the DNA binding domain as well as the C-terminal region since the NF-κB degraded by m-calpain immediately lost the DNA binding activity (Fig. 3A). These results are consistent with the notion of NF-κB as PEST-containing proteins and this report contributes one more example to the previous postulate that calpains recognize PEST proteins and regulate the steady-state level of a certain set of cellular proteins by proteolytic processing [18,31].

Currently there are contradictory arguments with regard to the intracellular location of calpains [17,32–36]. Although there have been a number of papers demonstrating that calpains are located mainly in the cytoplasm and very scarcely in the nucleus [32,33], recent papers have indicated the possibility that m-calpain is located in the nucleus and involved in the processing of nuclear transcription factors [17,34-36]. Furthermore, Onizuka et al. have recently demonstrated selective distribution of μ-calpain in the nuclei of the central nervous system as well as peripheral nervous system neurons using specific anti-μ-calpain antibody [37].

Considering the physiological role of Ca^{2+} mobilization upon stimulation of lymphocytes by various signals, our findings of the involvement of calcium-dependent proteases in the processing of the p65 subunit of NF-κB may have biological implications. Recent studies indicated that the C-terminal region of p65 serves as a transcriptional activation domain [38,39]. The action of calpains upon the p65 molecule may have a regulatory role through specific proteolytic degradation at this region and thus are considered to be involved in regulated expression of the target genes of NF-κB. Recognition of a specific regulatory pathway in the processing of transcription factors such as shown here should provide a novel insight into gene regulation by transcription factors. Identification of effective protease inhibitors specific for the protein processing pathway would provide a novel strategy for disease control.

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