Tumor Necrosis Factor-α-inducible IκBα Proteolysis Mediated by Cytosolic m-Calpain

A MECHANISM PARALLEL TO THE UBIQUITIN-PROTEASOME PATHWAY FOR NUCLEAR FACTOR-κB ACTIVATION*

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The cytokine tumor necrosis factor α (TNF-α) induces expression of inflammatory gene networks by activating cytosplasmic to nuclear translocation of the nuclear factor-κB (NF-κB) transcription factor. NF-κB activation results from sequential phosphorylation and hydrolysis of the cytoplasmic inhibitor, IκBα, through the 26 S proteasome. Here, we show a parallel proteasome-independent pathway for cytokine-inducible IκBα proteolysis in HepG2 liver cells mediated by cytosolic calcium-activated neutral protease (calpains). Pretreatment with either calpain- or proteasome-selective inhibitors partially blocks up to 50% of TNF-α-inducible IκBα proteolysis; pretreatment with both is required to completely block IκBα proteolysis. Similarly, in transient cotransfection assays, expression of the specific inhibitor, calpastatin, partially blocks TNF-α-inducible NF-κB-dependent promoter activity and IκBα proteolysis. In TNF-α-stimulated cells, a rapid (within 1 min), 2.2-fold increase in cytosolic calpain proteolytic activity is measured using a specific fluorogenic assay. Inducible calpain proteolytic activity occurs coincidently with the particulate-to-cytosol redistribution of the catalytic m-calpain subunit into the IκBα compartment. Addition of catalytically active m-calpain into broken cells was sufficient to produce ligand-independent IκBα proteolysis and NF-κB translocation. As additional evidence for calpain-dependent IκBα proteolysis and NF-κB activation, we demonstrate that this process occurs in a cell line (20b) deficient in the ubiquitin-proteasome pathway. Following inactivation of the temperature-sensitive ubiquitin-activating enzyme, IκBα proteolysis occurs in a manner sensitive only to calpain inhibitors. Our results demonstrate that TNF-α activates cytosolic calpains, a parallel pathway that degrades IκBα and activates NF-κB activation independently of the ubiquitin-proteasome pathway.

Nonlysosomal (cytoplasmic) protease systems have recently been identified as important regulators of intracellular activities including programmed cell death, protein kinase abundance, and cell-cycle progression (1-3). In viable cells, two prominent cytoplasmic protease systems have been identified. These include the ubiquitin-proteasome pathway, mediating targeted turnover of misfolded and unstable proteins, and the calcium-activated neutral protease (calpain)-calpastatin system, initially thought to be important in regulating turnover of protein kinases and key structural proteins in the cell (1). More recently, however, inducible proteolysis has also been shown to be important in hormonal control of gene expression by modulating the nuclear abundance of certain transcription factors. These processes include cholesterol-induced cleavage of the sterol-regulated element binding protein (reviewed in Ref. 2) and, of special interest to the pathophysiology of inflammatory processes, mechanisms for intracellular signaling produced by the cytokine tumor necrosis factor-α (TNF-α).1

Following binding its receptor on the plasma membrane, TNF-α initiates de novo transcription of genetic networks, in part, through activating nuclear translocation of the cytoplasmic transcription factor nuclear factor-κB (NF-κB) (4, 5). NF-κB, a multiprotein complex activated in the cytoplasm by association with its IκB inhibitor, translocates into the nucleus following dissociation of the NF-κB-IκB complex. TNF-α modifies NF-κB-IκB association through a process initiated by inducible IκBα serine phosphorylation on its amino-terminal regulatory domain, a modification coupled to IκBα polypeptidation (Ubα) on adjacent lysine residues (6). NF-κB-IκB dissociation requires IκBα proteolysis because phosphorylated and ubiquitinated IκBα still inactivates NF-κB (Ref. 6 and references therein).

Presently, the ubiquitin-proteasome system has been the only pathway identified in mediating cytokine-inducible IκBα proteolysis. Pretreatment with cell-permeant proteasome inhibitors blocks TNF-α-inducible IκBα proteolysis concomitantly with the accumulation of Ubα- and phosphorylated IκBα intermediates (6, 8). Independently, inducible IκBα proteolysis in cells harboring thermolabile ubiquitin-activating enzymes is markedly slowed at non-permissive temperatures (9).

Several lines of evidence indicate the presence of alternative

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† The abbreviations used are: TNF-α, tumor necrosis factor-α; AMG, 7-amino-4-methylcoumarin; E64, trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane; IκBα, inhibitor of NF-κB; NF-κB, nuclear factor-κB; PMSF, phenylmethylsulfonyl fluoride; Ubα, polyubiquitination; Z, benzyloxycarbonyl; Z-LLF, benzyloxycarbonyl-Leu-Leu-phenylalaninal; Z-LLL, Z-Leu-Leu-Leucinal (Z-LLL); Z-LnL, Z-Leu-norleucinal (calpeptin); Z-LLY, Z-L-leucyl-L-leucyl-L-tyrosine diazomethyl ketone; IL, interleukin; CAT, chloramphenicol acetyltransferase; Boe, t-butoxycarboxybenzoyl; CMAC, 7-amino-4-chloromethylcoumarin; FACS, fluorescence-activated cell sorter; Lacta, lactacystin; E1, ubiquitin-activating enzyme; Succ, succinyl.
(nonproteasome-dependent) processing pathways for IkB protein degradation. First, in pre-B lymphocytes, c-Rel:NF-κB is constitutively nuclear as the consequence of a calcium-dependent proteolytic activity that preferentially affects IkBα (rather than IkBβ (10)). Second, we have observed a non-proteasome-dependent pathway mediating inducible IkBα proteolysis (and NF-κB activation) following respiratory syncytial virus infection of human airway epithelial cells (11). However, whether additional nonproteasome-dependent pathways participate in cytokine-inducible NF-κB activation have not been explored.

These studies prompted us to examine whether nonproteasome-dependent pathways participate in cytokine-inducible IkBα degradation. Here we investigate the proteolytic mechanism involved in a well characterized model of NF-κB activation in TNF-α-stimulated HepG2 hepatocytes, where NF-κB activation mediates the expression of acute phase reactants (12, 13). By using calpain and proteasome-selective inhibitors, we demonstrate that inducible IkBα proteolysis is partially blocked following inhibition of either pathway and completely blocked following inhibition of both. By using a specific fluorescent assay in intact cells, we describe for the first time that TNF-α rapidly activates cytosolic calpain proteolytic activity. In subcellular fractions of TNF-α-stimulated cells, the catalytic m-calpain subunit translocates from the particulate into the cytosolic fraction (the latter containing IκBα) coincidentally with IkBα proteolysis. Moreover, TNF-α-inducible IkBα proteolysis occurs in cells conditionally deficient in the ubiquitin-proteasome pathway, and in cells expressing IkBα mutations deficient in proteasome-dependent processing. Together, these data implicate calpains are a parallel pathway in mediating IkBα proteolysis and NF-κB activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Purified phosphorylated bovine casein (sodium salt) and rabbit skeletal muscle m-calpain (specific activity 30 units/mg protein, >90% 80- and 30-kDa subunits by SDS-polyacrylamide gel electrophoresis) were from Sigma and Aldrich. Lactacytin (Lacta), was a generous gift of E. J. Corey (Harvard University). Benzyloxycarbonyl-Leu-Leu-phenylalaninal (Z-LLL) was a gift of Mark Suto (signal Pimoters, San Diego, CA). Z-Leu-norleucinal (peptin, Z-LnL), Tris-epoxy succinyl-l-leucyl-l-leucylamide (4-guanido)butane (E64), and phenylmethylsulfonyl fluoride (PMSF) (Dynabeads, Dynal Inc.) as described (7). In experiments using ubiquitin pathway-defective ts20b cells and their controls, H38–5, cells were pretreated with 32 °C (permissive temperature) to 39 °C (restrictive temperature) for a 6-h period to inactivate the temperature-sensitive E1 ubiquitinating-enzyme (14). Cells were stimulated with 30 ng/ml TNF-α at 37 °C. Identically treated wild-type E1-corrected cells were taken as control. By using calpain and proteasome-selective inhibitors, 6-h temperature-restricted ts20b cells were pretreated for 1 h at 37 °C without or with 50 μM Z-LnL, 100 μM E64, 100 μM Z-LLL, 10 μM Lacta, and 100 μM PMSF prior to TNF-α stimulation.

**Antibodies and Western Immunoblots**—Antibodies were used to IκBα (sc-203, recognizing amino acids 6–20, and sc-571, recognizing amino acids 39–53). IκBα (sc-945, recognizing amino acids 39–53), Rel A (sc-109), Rel B (sc-226) from Santa Cruz Biotech and m-calpain (Research Diagnostics, Inc.). Subunit-specific rabbit polyclonal antibodies to recombiant Methanosarcina thermophila α and β subunits, corresponding to the human proteasome subunit 90 kDa and 30 kDa, respectively, were obtained from Calbiochem. 200 μg of cytosolic or nuclear extracts were fractionated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene membranes (12). Following incubation with primary antibody, antigens were detected in the enhanced chemiluminescentence assay (Amersham Pharmacia Biotech) following the manufacturer’s recommendations.

**In Vitro Protease Assays**—For protease activity, 200 μg of HepG2 lysates from control or TNF-α-treated (30 ng/ml, 15 min) were incubated in 1 ml of ATCase-containing reaction buffer (15 mM Tris-HCl, pH 8.0, the presence or absence of indicated protease inhibitor (30 min, 30 °C)). AMC release was quantitated by measuring fluorescence emission intensity at 440 nm (Iex, 365 nm) normalizing to standards. Results are mean ± S.D. of three experiments. To confirm Suc-LLVY-AMC hydrolyzing activity was dependent on proteasome activity, HepG2 lysate was proteasome-depleted by ultracentrifugation (150,000 × g, 26 h, 4 °C). Western blots before or after ultracentrifugation were done to detect the core proteasome subunits, RING 10 and 9, both RING 10 and 9, present in the crude supernatant, were lost in the S100 supernatant and recovered in the 150,000 × g pellet. By contrast, the 80-kDa m-calpain catalytic subunit remained in the S100 supernatant. Ninety percent of the pangen degradation was lost in the S100 supernatant, indicating that proteasome activity was selectively being measured by this assay. For measurement of m-calpain caseinolytic activity, 0.025 μg of plasmid CMV-driven m-calpain (not shown).

**Calpain Assay in Intact Cells**—For HepG2 and ts20b cells, calpain activity was measured by the rate of generation of fluorescent product, AMC, from intracellular thiol-conjugated Boc-Leu-Met-CMAC (17). Cells were dispersed, grown on glass coverslips, continuously superfused with physiologic saline solution at 37 °C, and sequentially imaged with a quantitative fluorescent imaging system (18). At t = 0, 162 b of pericentromere CMAC was added to the superfusion solution, and mean fluorescence intensity (excitation 350 nm, emission 470 nm) of individual cells was measured at 60-s intervals. At 10 min, TNF-α (30 ng/ml) was added to the superfusion solution with 10 μM Boc-Leu-Met-CMAC. The slope of the fluorescence change with respect to time represents the intracellular calpain activity (17). Hydrolysis of the thiol-conjugated substrate was rate-limiting for the generation of fluorescent product as shown by comparing the initial rate of cell fluorescence increase after exposure to Boc-Leu-Met-CMAC with that produced by CMAC. CMAC requires only the thiol conjugation step, not hydrolysis, for fluorescence. There was a 35.8-fold increase in the AMC fluorescence rate compared with Boc-Leu-Met-CMAC, demonstrating that hydrolysis and not conjugation was rate-limiting. For calpain assays in whole cell populations, suspension cultures of HepG2 cells were loaded with 10 μM Boc-Leu-Met-CMAC, and changes in intracellular fluorescence was measured prior to and after TNF-α addition at 37 °C using a FACS Vantage system. Cellular fluorescence of AMC was measured using a 360-nm excitation filter and a 405-nm long-pass emission filter.

**Calpain-dependent IkBα Proteolytic Activity in Cytosolic S100 Extract**—Turbo yeast with 100,000 g supernatant (S100) was incubated with 150 ng of recombinant human IkBα for indicated times in Reaction Buffer (RB, 25 mM HEPES, pH 7.2, 65 mM KC1, 2 mM MgCl2, 1.5 mM CaCl2, 2 mM dithiothreitol) at 32 °C in a final volume of 60 μl. IkBα degradation was quantitated by Western immunoblot.

**IkBα Proteolysis and NF-κB Activation Assay in Broken Cells**—Indicated amounts of purified rabbit skeletal muscle m-calpain was added

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*IkBa Proteolysis by m-Calpain*
to a mixture of 200 µg of HepG2 cytosol and 1 x 10^6 sucrose cushion-purified nuclei (12) in 10% glycerol-containing RB (100 µl, 32°C). IκB proteolysis in cytoplasmic extract and nuclear Rel A was assayed by Western immunoblot following sucrose-cushion purification of nuclei (12). Gel mobility shift assay was performed using the NF-κB-binding site from the angiotensigenon promoter as described (12).

**RESULTS**

**Non-proteasomal Component for TNF-α-inducible IκBα Proteolysis Is Sensitive to Calpain Inhibitors**—We have previously shown that administration of TNF-α (30 ng/ml) to HepG2 cells induces rapid IκB (α and β) proteolysis and NF-κB translocation, maximally detectable 15 min following addition of hormone (12). To examine initially if proteasome-independent pathways for IκB proteolysis exist, IκB abundance was assayed by Western immunoblots in protease inhibitor pretreated cells harvested 15 min after TNF-α stimulation. A battery of previously characterized proteasome (lactacystin, Z-LLL, Z-LLL (8, 15, 19)), calpain (Z-LnL (20), E64), and nonspecific serine protease (PMSF) inhibitors were used (Fig. 1a). In data not shown, pretreatment with these agents had no effect on constitutive (nonstimulated) levels of either IκB isoform. In the absence of protease inhibitors, TNF-α produced rapid proteolysis of both 37-kDa IκBα and 49-kDa IκBβ (compare lanes 1 and 2). Pretreatment with lactacystin, Z-LLL, or Z-LLL blocked IκBβ processing (compare lane 2 with 3–5), whereas Z-LnL, E64, and PMSF had no effect. These observations are consistent with a predominant role of the proteasome pathway mediating IκBβ proteolysis (6).

In contrast, IκBα proteolysis was partially blocked by the proteasome inhibitors lactacystin and Z-LLL (Fig. 1a, lanes 3 and 4). Of the presumed proteasome-selective inhibitors, only Z-LLL was a completely effective inhibitor of IκBα proteolysis. Surprisingly, moreover, the calpain inhibitors Z-LnL and E64 also partially blocked IκBα proteolysis, even under conditions where IκBβ proteolysis was unaffected (compare lane 2 with 6 and 7). These data suggest a parallel contribution of calpain-like proteases in TNF-α-inducible IκBα hydrolysis.

To define the kinetics of proteasome-independent pathways mediating IκBα proteolysis, IκBα half-life in TNF-α-treated

of IκBα and IκBβ antibodies (indicated at left). Cells were stimulated following preincubation with indicated protease inhibitors (doses under "Experimental Procedures"). Compared with TNF-α treatment alone, IκB proteolysis is blocked by Lacta (43%), Z-LLL (46%), Z-LLL (100%), Z-LnL (56%), E64 (35%), and PMSF (11%), respectively. b, top panel, TNF-α induced IκBα degradation in Hep G2 cells. Apparent IκBα half-life is 1–3 min following TNF-α treatment, preceded by IκBα phosphorylation (IκBα'). Bottom panel, IκBα half-life in lactacystin-pretreated cells (10 µM for 1 h). TNF-α induces IκBα degradation in the time-dependent manner even in the absence of detectable 28 S proteasome activity. ~30-kDa IκBα intermediate produced is indicated with arrow (left) detected on longer exposure, c, combined effect of proteasome and calpain inhibition completely blocks inducible IκBα proteolysis. HepG2 cells pretreated for 1 h with the cell-permeant irreversible calpain inhibitor Z-LLL (21) (50 µM) and/or 10 µM lactacystin were TNF-α-stimulated (15 min). Rel B staining is used as an internal control for protein recovery. TNF-α-induced IκBα degradation is totally blocked by the combined treatment of Z-LLL and lactacystin. d, calpastatin blocks NF-κB dependent reporter activity. HepG2 cells were cotransfected with ~162-44 human IL-2/CAT reporter, internal control SV40-driven alkaline phosphatase reporter, and either empty pcDNA 1- or pcDNA I-calpastatin expression vector. Normalized CAT/alkaline phosphatase activity (± S.D.) in control and TNF-α-stimulated cells is shown. e, capastatin expression blocks IκBα proteolysis. HepG2 cells transfected with IL-2 receptor expression plasmid and pcDNA I-calpastatin expression plasmid were TNF-α-stimulated and transiently affinity purified. Shown is a Western blot of IκBα (top) and control β-actin (bottom). Lane 1, control-treated; lanes 2–4, TNF-α-treated. Lanes 1 and 2, 0 µg of pcDNA 1-calpastatin; lane 3, 0.5 µg; lane 4, 2.5 µg. Relative to control cells, normalized IκBα signal to β-actin in lane 2 is 30%; lane 3 is 35%, and lane 4 is 50%.
cells was compared in cells containing that in cells lacking proteasome activity (Fig. 1b). In cells not treated with protease inhibitors, IκBα proteolysis is rapid (t/2 of 1–3 min), occurring coincidently with the generation of phosphorylated IκBα intermediates (Fig. 1b, IκBα	extsuperscript{P}). In cells pretreated with the potent irreversible proteasome inhibitor lactacystin, IκBα proteolysis occurs with a detectably slower half-life (t/2 of 7–15 min) and is incomplete, with the appearance of a <30-kDa intermediate (Fig. 1b, bottom). To determine whether any pathway other than the combined calpain/proteasome account for IκBα proteolysis, the additive effects of the specific irreversible calpain inhibitor Z-LLY (21) and lactacystin were studied (Fig. 1c). At saturating doses, neither Z-LLY nor lactacystin alone could completely inhibit IκBα proteolysis. In the presence of both inhibitor types, IκBα proteolysis was completely blocked with accumulation of non- and phosphorylated IκBα intermediates (Fig. 1c). We note consistently that IκBα	extsuperscript{P} intermediate was detectable at the 15-min time point in the presence of proteasome inhibitors but not in calpain inhibitors (see “Discussion”).

Enzymatic activity of calpains are influenced by the effect of endogenous calpain inhibitor, calpastatin. As additional evidence for the role of calpains in NF-κB activation, the effect of transiently expressed calpastatin was determined on NF-κB-dependent reporter activity in transient cotransfection assay (18, 22). We have previously shown that the human IL-8 promoter is TNF-α-inducible in a manner solely dependent on a high affinity NF-κB site (23). Cotransfection of calpastatin expression plasmid (pcDNA I-calpastatin) did not affect basal IL-8/CAT activity but significantly blocked TNF-α-inducible CAT activity (Fig. 1d). As a control, the effect of calpastatin on IκBα steady state levels was measured in transient transfectants. HepG2 cells cotransfected with IL-2 receptor expression plasmid in the absence or presence of various concentrations of pcDNA I-calpastatin were stimulated with TNF-α. Following isolation of transfected cells, a Western immunoblot was done to determine changes in IκBα in cytosolic lysates (Fig. 1c). In the presence of 2.5 μg of pcDNA I-calpastatin, IκBα proteolysis was inhibited by ~50%. Combined, these data strongly suggest a parallel contribution of the calpain system in TNF-α-inducible proteolysis of IκBα and NF-κB activation.

Specificity of Protease Inhibitors—The specificity and effect of protease inhibitors for proteasome and calpain activities were directly measured in vitro (Fig. 2). Hydrolysis of the fluorogenic substrate Succ-LLVY-AMC was used to measure proteasome activity in whole cell lysates (15, 19). As described under “Experimental Procedures,” following proteasome depletion, 94% of the Suc-LLVY-AMC hydrolyzing activity was depleted, indicating that the assay is measuring bona fide proteasome activity. As shown in Fig. 2a, in both control and TNF-α-treated cells, Succ LLVY-AMC hydrolytic activity was indistinguishable (12.5 and 13 nmol/min/mg, respectively). Also, greater than 90% inhibition of proteasome activity was seen following lactacystin, Z-LLF, and Z-LLL treatment, indicating these inhibitors potently inhibited (whereas Z-LnL, E64, Z-LLY and PMSF had no effect) cellular proteasome activity. This effect was consistent for either control or TNF-α-stimulated cells.

Caseinolytic activity of purified m-calpain was measured in the presence of the same inhibitors (Fig. 2b). Surprisingly, the presumed “selective” proteasome inhibitors Z-LLF and Z-LLL, as well as the calpain inhibitors Z-LnL, E64, and Z-LLL, were potent inhibitors of m-calpain. Only lactacystin, therefore, was able to differentiate calpain from proteasome activity, and Z-LnL, E64, and Z-LLL, conversely, were specific for caseinolytic activity of calpain, without effects on the proteasome.

**TNF-α-inducible Changes in Intracellular Calpain Proteolytic Activity**—Direct measurement of dynamic changes in intracellular calpain activity in broken cells has been hampered due to the presence of endogenous calpastatin inhibitor that rapidly associates with active calpains following cell disruption. However, the recent development of a specific fluorescent assay using a cell-permeant calpain substrate to measure changes in calpain proteolytic activity has obviated the need for broken cell assays (17). After diffusion of the substrate, Boc-Leu-Met-CMAC, into cells, it is conjugated with glutathione (GSH) to form a membrane-impermeant, nonfluorescent calpain substrate. Following its hydrolysis, the unquenched fluorescent product (AMC-GSH) accumulates, where its rate of accumulation is a measure of intracellular calpain activity (17). Specificity of this assay for calpain has been previously demonstrated by its inhibition by the specific calpain inhibitor, Z-LLL, and independence from lysosomal, serine, or cathepsin proteases (17). A basal rate of generation of AMC-GSH was observed in individual cells (Fig. 3a, 1–10 min). We further examined the assay specificity by measuring the effects of inhibitors on basal proteolytic activity. Basal generation of the fluorescent proteolysis product is calcium-dependent due to the inhibitory effects of the intracellular calcium chelator 1,2-bis(o-aminophenoxyl)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxyethyl) ester (76% inhibition, 100 μM (n = 30)) and is quantitatively inhibited by the calpain inhibitors, Z-LnL (59%
In individual cells, TNF-α increased calpain activity ~2.2-fold over the basal rate within 1 min of exposure (Fig. 3a). The TNF-α-induced stimulation of calpain activity is blocked by the calpain inhibitor Z-LnL but not lactacystin (Fig. 3b), indicating an exact parallel of inhibitor specificity for intracellular calpain activity as for purified m-calpain in vitro (cf. Figs. 3b and 2c). This assay was also applied by FACS analysis to determine the portion of TNF-α-responsive HepG2 cells. As shown in Fig. 3c, in HepG2 populations, mean cellular fluorescence intensity, as an indicator of calpain activation, increased an average of 2.5 ± 0.3-fold in greater than 95% of cells following TNF-α administration for 60–800 s. Specificity of changes in mean fluorescence intensity also follows the same inhibitor profile as shown in the single cell assay (not shown).

**TNF-α Induces Changes in m-Calpain Abundance**—For m-calpain proteolytic activity to be relevant for IκBα proteolysis, we sought to determine the subcellular distribution of m-calpain in control and TNF-α-stimulated cells. For this, particulate (S100 pellet) and cytosolic (S100 supernatant) fractions were prepared at various times following TNF-α treatment by ultracentrifugation at 100,000 × g and analyzed for both 80-kDa m-calpain catalytic subunit and IκBα by Western immunoblot (Fig. 4a). Although m-calpain could be detected in both cytosolic and particulate fractions, normalizing each fraction per microgram of protein, the highest specific activity of m-calpain was found in the particulate fraction. In the cytosolic fraction, m-calpain abundance increased 2-fold within 2 min following TNF-α stimulation in the cytosolic fraction. In both fractions, however, m-calpain abundance fell (compare 15-min time points with control, Fig. 4a). Importantly, we noted that the cytosolic fraction contained IκBα and that changes in m-calpain subunit occurred concomitantly with IκBα proteolysis (Fig. 4a) and synchronously with calpain proteolytic activity (Fig. 3). Internal control immunostaining of inert Rel B in the same membrane was used to document equivalent protein loading (Fig. 4a, bottom).

To determine whether cytosolic fractions from TNF-α-treated cells containing translocated m-calpain catalytic subunit (determined by Western immunoblot, Fig. 4) also contain IκBα proteolytic activity, an *in vitro* protease assay was established (Fig. 4b). In this assay, purified recombinant human IκBα (rhIκBα) was added to TNF-α-stimulated cytosolic lysates (S100 supernatant), and the effect on rhIκBα proteolysis was determined by Western immunoblot. We observed a time-dependent proteolysis of rhIκBα, a proteolysis that was blocked either by calcium chelation or the addition of the calpain inhibitors, E64 or Z-LnL but not lactacystin or PMSF (Fig. 4b, bottom).

**m-Calpain Is Sufficient for Ligand-independent IκBα Proteolysis and NF-κB Translocation**—To determine whether m-calpain proteolytic activity measured before and after TNF-α addition (arrow, at 10 min), relative stimulation of single cell calpain activity in the presence of protease inhibitors. Relative calpain activity was measured after no addition (control), TNF-α (30 ng/ml), TNF-α + Z-LnL (10 μM), or TNF-α + lactacystin (10 μM, 30 min). The basal activity in each cell was measured for use as its own control (open bars) prior to TNF-α stimulation (solid bars). Increased proteolytic rate occurred only for TNF-α (p < 0.001) and TNF-α with lactacystin (p < 0.05). c, kinetics of change in intracellular fluorescence intensity in TNF-α-exposed cell populations. FACS analysis. A representative experiment is shown (n = 6). Fluorescence intensity increased 2.5 ± 0.3 fold in >95% cell population.
calpain proteolyzed native IκB within the NF-κB-IκB complex and could produce ligand-independent NF-κB activation, activated m-calpain was added to broken cell lysates (containing nuclei, Fig. 5). m-Calpain produced a time-dependent degradation of endogenously expressed IκBα (Fig. 5, top). IκBα was proteolyzed into transiently stabilized carboxyl-terminal intermediates of ~30 kDa (arrow, Fig. 5), an intermediate also seen in TNF-α-stimulated cells lacking proteasome activity (see Fig. 1b). The effect of m-calpain was dose-dependent and required m-calpain proteolytic activity (Fig. 5, bottom). Inducible phosphor- ylation is apparently not required for calpain-induced IκBα proteolysis because the phosphorylation-defective IκBα mutant, S32/36A, is inducibly degraded, and nonphosphorylated recombinant IκBα (IκBα expressed in Escherichia coli) is efficiently degraded in vitro (not shown).

To determine whether calpains could result in NF-κB activation, nuclei were purified on sucrose cushions and nuclear proteins extracted. Gel mobility shift assays showed that m-calpain induced a time- and dose-dependent increase in Rel A:NF-κB1 DNA binding activity (Fig. 6a), indicated as complex 2, a species previously characterized by supershift assay (12). To additionally demonstrate NF-κB translocation, changes in 65-kDa Rel A nuclear abundance was measured by Western immunoblot (Fig. 6b), where a 2.3-fold increase in Rel A in the m-calpain treated nuclei was seen. We therefore conclude that ligand-independent IκBα proteolysis and NF-κB activation can be effected by m-calpain.

Degradation of IκBα in Ubiquitin-Proteasome-defective Cell

**Fig. 4.** Calpain particulate-cytosol translocation and hydrolysis of recombinant human IκBα (rhIκBα), a, Western immunoblot of m-calpain 80-kDa catalytic subunit and IκBα in particulate (100,000 g pellet, Ref. 18) and cytosolic (100,000 g supernatant) fractions of TNF-α-treated cells. Relative to control, in TNF-α-treated cytosol, m-calpain abundance rapidly increases 2.08-fold (2 min), 1.67-fold (5 min), and 1.37-fold (10 min) and thereafter decreases to 0.6-fold (15 min). In membrane extracts, m-calpain gradually decreases to 88% (2 min), 86% (5 min), 61% (10 min), and 62% (15 min). Control Rel B staining is shown (bottom). b, calpain-dependent degradation of recombinant IκBα by TNF-α-treated S350 cytosolic extract. Top panel, time course. Bottom panel, effect of calpain inhibitors. S350-induced rhIκBα proteolysis is blocked by 5 mM EGTA (40%), 50 μM E64 (50%), 50 μM Z-LnL (81%) but not 50 μM lactacystin (Lact) or 100 μM PMSF.

**Fig. 5.** Endogenous IκBα proteolysis by purified m-calpain in broken cell assay. Top panel, time course. Western blot of IκBα abundance following introduction of m-calpain in broken cell assay for indicated times. Arrow, 30-kDa IκBα intermediate (cf. Fig. 1c). Bottom panel, dose response and sensitivity to calpain inhibitors. Calpain proteolytic activity is required for IκBα proteolysis.

**Fig. 6.** Ligand-independent activation of Rel A: NF-κB1 by purified m-calpain catalytic subunit in broken cell assay. a, gel mobility shift assay for nuclear DNA binding activity. Purified m-calpain was added to broken cell assay at the indicated concentrations and times. Following incubation, nuclei were prepared by sucrose cushion centrifugation. Assays were performed using 15 μg of nuclear extract binds to radiolabeled angiotensinogen NF-κB-binding site (12). Migration of RelA:NF-κB1 heterodimer (complex C2) demonstrated previously by supershift assay is shown (12). Left panel, time course. RelA:NF-κB1 DNA-binding activity increases 1.16- (5 min) and 2.07-fold (15 min). Right panel, dose response, b, Rel A nuclear translocation by m-calpain. Following calpain treatment of broken cells (0.1 units of m-calpain as in a), Rel A was extracted from sucrose cushion-purified nuclei and detected by Western blot. Rel A increases 2.3-fold (arrow). Nonspecific band (NS) as control for protein loading is indicated.

Lines—As additional evidence for calpain-mediated, protea- some-independent pathway for IκB proteolysis and NF-κB activation, we analyzed the effect of TNF-α in Balb/c 3T3 cells conditionally defective in the ubiquitin-proteasome pathway. ts20b cells express a temperature-sensitive E1 responsible for initial ATP-dependent step in the Ub reaction (14, 18), whereas control H38-5 cells are corrected ts20b stably transfected with the wild-type E1 (14). Relative stimulation of calpain activity was observed in individual cells measuring hy-
Calpains are intracellular calcium-dependent cysteine proteases whose ubiquitously expressed subunits include milli (m)-calpain and micro (μ)-calpain. Although these heterodimeric isoforms have indistinguishable substrate affinities, m- and μ-calpain are found in distinct subcellular localizations and therefore may subserve distinct physiological roles (24, 25). Here we show for the first time that the calpain-calpastatin system is a parallel pathway partly responsible for TNF-α-inducible IκBα proteolysis and NF-κB activation. TNF-α, therefore, activates NF-κB through the participation of two distinctly regulated cytoplasmic (nonlysosomal) protease systems as follows: (i) the constitutive proteasome pathway, where IκBα proteolysis is governed by its rate-limiting post-translational modification (coupled phosphorylation/ubiquitination), and (ii) the inducible calpain-calpastatin system, where protease activity is directed modified by TNF-α.

In the past, distinguishing between the effects of calpains and the proteasome in intracellular regulatory processes has been difficult because few selective inhibitors of the two cytoplasmic protease systems were identified. Data presented herein indicate that pathway-selective probes exist that can be used to dissect the parallel function of these protease systems in cytokine signaling. A role for the calpain-calpastatin pathway mediating NF-κB activation is based on the convergence of the following observations. 1) Inducible IκBα proteolysis is only partially blocked by either calpain-selective or proteasome-selective inhibitors and completely blocked by both. 2) In TNF-α-stimulated cells, a rapid (within 1 min), 2.2-fold increase in cytosolic calpain proteolytic activity in intact cells is measured. 3) Calpain proteolytic activity occurs indistinguishably with the particulate to cytosol redistribution of the catalytic m-calpain subunit. 4) IκBα proteolysis occurs coincidentally with increases in m-calpain abundance in the cytosol. 5) Introduction of catalytically active m-calpain is sufficient to produce ligand-independent NF-κB activation. 6) Calpain-dependent IκBα proteolysis is demonstrated in cells lacking proteasome activity (ts20b cells).

The mechanism for activation of calpains in intact cells is unknown. In vitro, calpains exposed to nonphysiological concentration of calcium acquire enzymatic activity through autoproteolysis of their constituent subunits (1). In intact cells, evidence for autolytic activation or activation following changes in intracellular calcium concentration is weak. In other studies, calpains are known to be long-lived proteins with half-lives of >5 days; this observation would not be consistent with an autolytic protease (27). Our data indicates that TNF-α-stimulated calpain activity occurs in the absence of detectable autolysis because autolytic products are not detected at times when changes in protease activity can be measured. Moreover, calpain activation in the absence of detectable changes in intracellular calcium concentrations has been described in hepatocytes (26). In data not shown, we have not observed any changes in total intracytoplasmic calcium concentrations in HepG2 cells. Nevertheless, intracellular calcium is apparently required for calpain activity in intact cells, because intracellular calcium chelators block calpain activity and IκBα proteolysis (Table I, data not shown).

One other mechanism for calpain activation could involve changes in subcellular localization. Calpains are not randomly distributed throughout the cell. In cultured cell lines, m-calpain is distributed in a fine reticular network in the cytosol, implicating an association with cytoskeletal elements (28), and in the central nervous system, m-calpain content is membrane-associated (29). In cultured HepG2 cells, we observe consistently that m-calpain redistributes into the soluble cytoplasmic fraction, a fraction containing IκBα, following TNF-α treatment. Whether redistribution is the mechanism for m-calpain activation will require additional investigation. Although m-calpain activity in the membrane fraction was previously thought to be important for proteolysis of protein kinase C (30), cytosolic calpain activity appears to be important in turnover of

![Fig. 7.](image-url)
the p53 oncoprotein (18). Based on our subcellular fractionation experiments, the intracellular site of proteolysis of IκBa probably also occurs in the cytosol.

Calpain activity is inducible following activation of other hormone receptors, including the hepatic purinergic receptor (17), and the pituitary thyrotropin-releasing hormone receptor (31), perhaps indicating a role for second messenger involvement. Others have shown that phospholipid mediators, including second messengers implicated in TNF-α signaling, can activate calpain catalytic activity through a mechanism that may involve their direct binding to the 30-kDa regulatory subunit (29, 32, 33). These lipids apparently lower calcium requirements to a range normally found intracellularly (29). Lipid mediators may be important intermediates for TNF-α-induced calpain activity for several reasons. First, TNF-α is known to increase ceramide production through its effects on acid sphingomyelinase activity in endosomal compartments (34); this second messenger has been linked to NF-κB activation (35). Second, ceramide directly stimulates intracellular calpain activity in permeabilized cells (32).

Calpains are increasingly recognized to be important regulators of intracellular signaling processes. Initially described in turnover of activated protein kinase C, erythrocyte ankyrin, and calmodulin-binding proteins (Ref. 1 and references therein), calpains have recently been implicated in mediating turnover of the c-Fos transcription factor (36, 37) and the tumor suppressor gene product p53 (18). Interestingly, both c-Fos and p53 were initially described to be proteasome substrates. Our data adds IκBa to the expanding list of key regulatory proteins acted upon by a parallel calpain-proteasome pathway. Of relevance to IκBa, erythrocyte ankyrin itself is a substrate for calpain proteolysis (18, 38).

The TNF-α-inducible calpain pathway mediating IκBa proteolysis described here is probably distinct from the two previously reported nonproteasome-dependent IκBa proteolytic pathways (10, 11). In the first report, constitutive IκBa turnover in an undifferentiated pre-B lymphocytic cell line was not inhibited by the potent calpain inhibitors, calpain inhibitors I and II, or Z-LLF, agents that interfere with IκBa proteolysis in our system (Fig. 1 and see Ref. 12). Second, potent calpain inhibitors MG132 and Z-LLF do not have significant effects on IκBa proteolysis in respiratory syncytial virus-infected epithelial cells (11). The relationship of these pathways to calpain-calpastatin pathway, therefore, seems unlikely.

In summary, we implicate the calpain-calpastatin and proteasome pathways are parallel mechanisms mediating inducible IκBa proteolysis by the cytokine TNF-α. These data indicate that calpains contribute to rapid IκBa proteolysis through a mechanism involving changes in total cytosolic calpain proteolytic activity.

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