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Impairment of NF-κB activation and modulation of gene expression by calpastatin

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Chen, Fei, Laurence M. Demers, Val Vallyathan, Yongju Lu, Vincent Castranova, and Xianglin Shi. Impairment of NF-κB activation and modulation of gene expression by calpastatin. *Am J Physiol Cell Physiol* 279: C709–C716, 2000.—To address the involvement of the calpain system in both basal and silica-induced nuclear factor (NF)-κB activation, several human bronchial epithelial cell lines were established in which an intracellular inhibitor of calpain, calpastatin, was stably expressed. Reduced basal and silica-induced inhibitor (IκBα) degradation and NF-κB activation were observed in cells stably overexpressing calpastatin. In addition, the cells in which calpain was constitutively inhibited by the overexpression of calpastatin exhibited a notable morphological change. Whereas empty vector-transfected cells displayed a morphology indistinguishable from that of parental cells, cells overexpressing calpastatin exhibited a mosaic morphological change with reduced formation of lamella 30 min after the cells were seeded. Gene-filter microarray experiments, in which 3,965 human genes can be evaluated for their expression at the same time, showed that calpastatin downregulated genes encoding several membrane-associated proteins or nuclear proteins and upregulated genes of collagen α2, DAZ, and mitochondrial capsule selenoprotein. These results suggest that, in addition to their proteolytic activities on cytoskeletal proteins and other cellular regulatory proteins, calpain-calpastatin systems can also affect the expression levels of genes encoding structural or regulatory proteins.

nuclear factor-κB; calpain; silica; epithelial cells

NUCLEAR FACTOR (NF)-κB proteins regulate transcription of a number of cellular genes that are critical for the maintenance of normal immune functions as well as the initiation or progression of human diseases (3, 11, 28). This transcription factor can also govern the expression of several viral genes, such as genes of human immunodeficiency virus and human T-lymphocyte leukemia virus (1, 6, 15). Unlike many other transcription factors that are constitutively localized in the nucleus, NF-κB is mainly sequestered in the cytoplasm by the binding of its inhibitor, i.e., IκB family members, in unstimulated cells. The most abundant and best-characterized inhibitor of NF-κB is IκBα, which prevents activation, translocation, and DNA binding of NF-κB transcription factor. Upon activation of cells with a variety of agents, such as the inflammatory cytokines interleukin-1 and tumor necrosis factor (TNF), bacterial endotoxins, and environmental or occupational particles, IκBα is phosphorylated by activated IκB kinases in collaboration with mitogen-activated protein kinases. The phosphorylated IκB is then ubiquitinated by an F-box/WD40 protein called β-TrCP (β-transducin repeat-containing protein) and rapidly degraded by a variety of proteolytic enzymes (7, 30). The degradation of IκB leads to the activation and translocation of NF-κB into the nucleus to regulate the transcription of target genes.

Two major proteolytic pathways have been studied with regard to their role in signal-induced NF-κB activation: proteasomes and calpains. Accumulating evidence suggests that the ubiquitin-proteasome pathway plays a major role in the degradation of IκBα protein and, consequently, in the activation of NF-κB transcription factor (2, 10, 22). Several recent studies have shown that the calpain system might be involved also under certain circumstances in basal or signal-induced degradation of IκBα and the activation of NF-κB (4, 8, 14, 20, 29). Whereas a potent proteasome inhibitor, MG132, failed to abrogate silica-induced IκBα degradation in macrophages, transient overexpression of calpastatin, a specific endogenous inhibitor for calpain, resulted in an appreciable inhibition of IκBα degradation induced by silica (8). In vitro digestion of recombinant IκBα by purified calpain or cytosolic extracts from silica-stimulated cells demonstrated further that calpain was capable of degrading IκBα protein by the cleavage of several leucine-rich domains. In an independent study, Han et al. (14) showed that calpain provides a parallel proteolytic pathway to the ubiquitin-proteasome pathway for TNF-α-induced IκBα degradation in human HepG2 cells (14). In WEHI231 immature B cells, a rapid degradation of IκBα was insensitive to proteasome inhibitors but was substan-

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tially inhibited by calpain inhibitors (20). More recently, Baghdiguian and associates (4) provided direct evidence demonstrating that patients with an autosomal muscular dystrophy caused by calpain 3 deficiency exhibited a substantial impairment of IκBα degradation and NF-κB activation in muscular cells (4).

Calpains are calcium-dependent cysteine proteinases present in a variety of cells (31, 33). Two major groups of calpains, termed ubiquitous calpains and tissue-specific calpains, have been identified. The ubiquitous calpains include calpain 1 (μ-calpain) and calpain 2 (m-calpain), which require micromolar and millimolar concentrations of calcium for their activation, respectively. Calpain 3 (n-calpain-1), requiring nanomolar concentrations of calcium for its activation, is skeletal muscle specific, whereas calpain 4 (n-calpain-2) was mainly found in smooth muscle. Experiments using synthetic inhibitors of calpains have shown them to be pivotal proteases participating in a limited proteolytic reaction of a number of cellular structural or regulatory proteins, such as cytoskeletal proteins (24), kinases (32), cytokines (17), and the tumor-suppressing protein p53 (12, 18, 23, 34). However, the reliability of the use of synthetic pharmacological inhibitors to delineate the role of calpains has been compromised because of the low cellular permeability and poor substrate specificity of these inhibitors. These inhibitors include calpain inhibitor I/II, E64 family compounds, and leupeptin. Recently, an intracellular calpain inhibitor, calpastatin, has been identified as a specific inhibitor of calpains, having no direct effect on other cellular proteases such as proteasome and lysosome systems (5, 33).

Silica is one of the earliest recognized inhaled occupational dusts, inducing severe and debilitating lung diseases among miners and constructional workers through damaging of bronchial epithelial cells and alveolar cells (19). The aim of the present study was to determine whether NF-κB activation by silica in human bronchial epithelial cells is mediated by a similar mechanism found in mouse macrophages cell lines as described previously. Using several cell lines derived from human bronchial epithelial cells, we investigated the effect of calpastatin, an intracellular inhibitor of calpains, on silica-induced NF-κB activation. We demonstrated that the activation of NF-κB transcription factor and the expression of a variety of cellular genes, such as cytoskeletal protein genes, are impaired or altered by the constitutive inhibition of calpains.

MATERIALS AND METHODS

Cells. Human bronchial epithelial cells, BEAS-2B, were purchased from American Type Culture Collection (ATCC, Rockville, MD). The cells were cultured in DMEM (Mediatech, Washington, DC) supplemented with 10% fetal bovine serum, 2 mM glutamine, and 1,000 U/ml penicillin-streptomycin (Sigma, St. Louis, MO).

Expressing plasmids. Human calpastatin cDNA was a gift from Dr. Masatoshi Maki (School of Agricultural Sciences, Nagoya University, Japan). An expression vector for calpastatin was constructed by inserting a full-length human calpastatin cDNA into the *Eco*R V and *Xba* I sites on the pcDNAI/Neo vector (Invitrogen, Carlsbad, CA).

Stable transfection. Lipofectin (GIBCO BRL) was used for transfection of BEAS-2B cells with an empty vector (pcDNA I/Neo) or an expression vector for full-length human calpastatin. The cells were seeded at a density of 2×10^5 cells/well in six-well tissue culture plates and cultured for 2 days before the performance of transfection. The cells, containing the empty vector or expression vector for calpastatin, were screened in geneticin (700 μg/ml, GIBCO) for 3 wk.

Electrophoretic mobility shift assay. The extraction of nuclear proteins and preparation of a ^{32}P-labeled double-stranded oligonucleotide containing the consensus κB-site was performed as described previously (9). For electrophoretic mobility shift assay (EMSA), 4 μg of nuclear protein were mixed with the labeled double-stranded probe and incubated at room temperature for 30 min. The reaction solution was subjected to electrophoresis on a native 5% polyacrylamide gel in 0.25× Tris base EDTA buffer for 2–3 h. The protein-DNA complexes, indicating NF-κB binding, and free probe were visualized in dried gels by autoradiography.

Proteolysis reaction. In vitro degradation of recombinant IκBα protein (Santa Cruz Biotechnology, Santa Cruz, CA) was performed as described previously with minor modification (8). Briefly, total cytoplasmic extracts were prepared by lysing the cells in a hypotonic buffer containing 10 mM HEPES (pH 7.4), 1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol, 50 mM NaF, 1 mM sodium orthovanadate, and 0.2 mM phenylmethylsulfonyl fluoride. After being incubated on ice for 30 min, the cells were vortexed for 10 s and centrifuged at 2,000 *g* for 5 min. The supernatant was collected and further centrifuged at 80,000 *g* for 1 h at 4°C. For the proteolysis assay, the glutathione-*S*-transferase (GST) tag in the GST-IκBα fusion protein was enzymatically removed by thrombin digestion. The resulting recombinant IκBα was incubated with various concentrations of μ-calpain or cytoplasmic extracts from the cells transfected with empty vector or a vector for calpastatin in 20 μl of proteolysis reaction buffer [30 mM Tris·HCl (pH 7.4), 30 mM NaCl, and 10 mM $CaCl_2$] at 30°C for 10 min. The proteolytic reaction was terminated by adding 8 μl of 3× SDS reducing buffer [350 mM Tris·HCl (pH 6.8), 15% SDS, 10% glycerol, 3.6 M β-mercaptoethanol, and 0.01% bromphenol blue] and subjected to SDS-polyacrylamide gel electrophoresis in 12% gels. Western blot analysis was performed to determine the degradation of IκBα protein, using anti-NH_2-terminal and anti-COOH-terminal IκBα antibodies (C-15 and C-21, respectively; Santa Cruz) and enhanced chemiluminescence immunoblot detection reagents (ECL; Amersham Life Science, Arlington Heights, IL).

Genefilter microarray. The gene expression pattern in both vector- and calpastatin-transfected cells was determined by microarray experiments using Genefilter membrane gf211 (Research Genetics, Huntsville, AL). Briefly, 1 μg of total RNA extracted from cells transfected with empty vector or expression vector for calpastatin was incubated with 2 μg of oligo(dT), 1.5 μl of reverse transcriptase, 20 mM of dATP, dGTP, and dTTP, and 100 μCi [^{33}P]dCTP in 30 μl of diethyl pyrocarbonate-treated water for 90 min at 37°C. After purification through a Bio-Spin 6 Chromatography Column, labeled probe was mixed with prehybridization solution and incubated with Genefilter membranes overnight at 42°C. To minimize possible manufacturer's variations among individual membranes, the same membrane was stripped and rehybridized with the second probe after the first round of hybridization.

RESULTS

Impaired NF-κB activation in calpastatin-transfected cells. Even though the ubiquitin-proteasome system is considered to be a universal pathway mediating the activation of NF-κB transcription factor, a possible alternative pathway under certain circumstances cannot be excluded (8, 14, 16). Previous studies using a murine macrophage cell line, RAW264.7, demonstrated that inhibition of calpain by transient transfection of calpastatin resulted in a reduction of NF-κB activation in response to silica particles (8). To examine the possibility that a calpain-calpastatin system may also be involved in the activation of NF-κB in human bronchial epithelial cells, stable transfected cell lines were established in which calpastatin was constitutively expressed. EMSA showed that, in contrast to the cells transfected with an empty vector in which a time-dependent NF-κB induction by silica could be identified (Fig. 1*A*, *lanes 5–8*), calpastatin-expressing cells exhibited no or only marginal activation of NF-κB in response to silica (*lanes 1–4*). Likewise, immunoblot experiments indicated that the degradation of intracellular IκBα protein induced by silica was impaired in the cells in which calpains were constitutively inhibited by stable overexpression of calpastatin (Fig. 1*B*, *lanes 1–4*).

IκBα is a substrate for calpain both in vitro and in vivo. The ability of calpain to regulate protein turnover has suggested an important role of this protease in cellular functions. To investigate the digestion of IκBα protein by calpain in vitro, purified μ-calpain was incubated with recombinant IκBα for 10 min. As depicted in Fig. 2, *A* and *B*, digestion of IκBα by calpain was

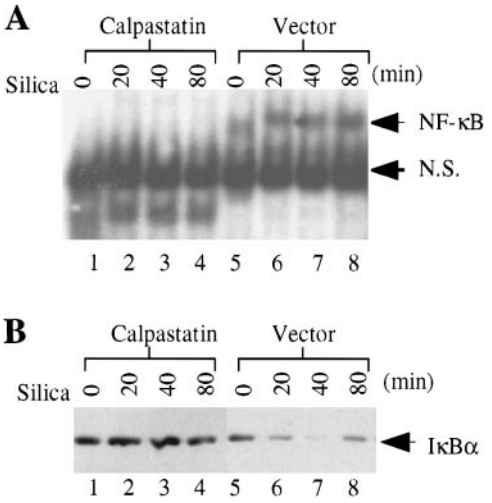

Fig. 1. Inhibition of nuclear factor (NF)-κB activation in calpastatin-expressing cells. *A*: stable calpastatin-transfected (*lanes 1–4*) and empty vector-transfected (*lanes 5–8*) human bronchial epithelial cells, BEAS-2B, were treated with 20 μg/ml silica for indicated times. Nuclear extracts were prepared and subjected to electrophoretic mobility shift assay (EMSA) with a radiolabeled double-stranded κB oligonucleotide. The arrows at *right* indicate the NF-κB binding complex and a nonspecific band (N.S.), respectively. *B*: lysates of cells as indicated in *A* were subjected to an immunoblot assay for the detection of NF-κB inhibitor (IκBα) degradation.

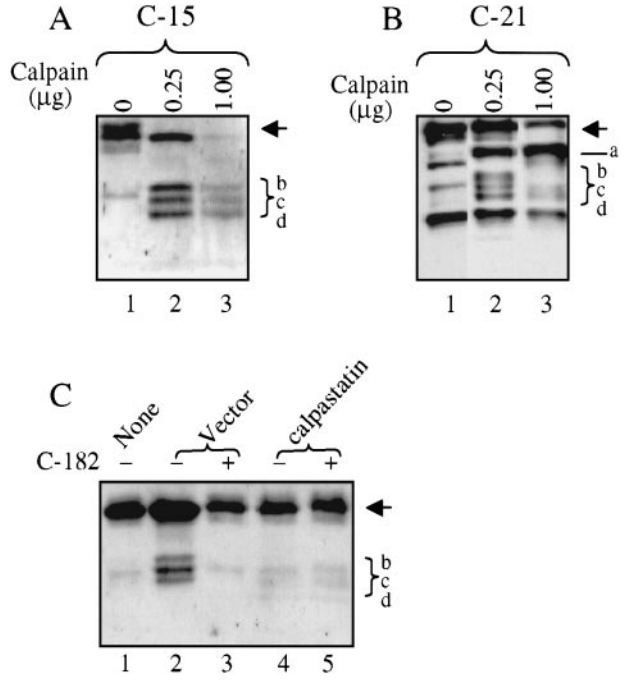

Fig. 2. IκBα is a substrate of calpains. *A* and *B*: recombinant IκBα (0.25 μg) was incubated with 0.25 and 1.0 μg of purified μ-calpain in a total of 20 μl of reaction buffer for 10 min at 30°C. The reaction was terminated by adding 8 μl of 3× SDS reducing buffer, and the solution was subjected to SDS-PAGE in 12% gel. After being transferred to a nitrocellulose membrane, the digested products were detected by using antibody C-15 against the NH_2 terminus of IκBα (*A*) and antibody C-21 against the COOH terminus of IκBα (*B*). *C*: recombinant IκBα was incubated with the cytoplasmic extracts from the silica-treated BEAS-2B cells stably transfected with an empty vector (*lanes 2* and *3*) or an expressing vector for calpastatin (*lanes 4* and *5*) in the absence (−; *lanes 1, 2,* and *4*) or presence (+; *lanes 3* and *5*) of a specific inhibitory peptide of calpain, C-182, for 10 min at 30°C. The proteolytic products were detected with antibody C-15 against the NH_2 terminus of IκBα. Arrows denote full-length IκBα protein; *a–d* denote digested fragments of recombinant IκBα protein.

dose dependent. Antibody C-15, raised against amino acids from positions 6 to 20 at the NH_2 terminus of IκBα, recognizes three closely migrated fragments with approximate molecular weights between 20 and 25 kDa (Fig. 2*A*, *lane 2*, *fragments b–d*). When an antibody directed to the amino acids from positions 297 to 317 at the COOH terminus of IκBα protein was used (antibody C-21), an additional immunoreactive fragment with an approximate molecular weight of 28 kDa was also detected (Fig. 2*B*, *lanes 2* and *3*, *fragment a*). On the basis of their apparent molecular masses and their patterns of recognition by NH_2- and COOH-terminal IκBα antibodies, the major calpain cleavage sites of IκBα should be within the NH_2 terminus and the middle of IκBα. It is also important to note that further proteolytic processing of *fragments b–d* occurs when the ratio of calpain to IκBα is increased, e.g., 1 μg vs. 0.25 μg of calpain (Fig. 2, *A* and *B*, *lanes 3*).

To address the involvement of calpain on the degradation of IκBα in vivo, proteolytic activity of cytosolic extracts from both the vector and calpastatin-overexpressed cells treated with silica was investigated by using recombinant IκBα as a substrate. Fragmentation

could not be identified after incubation of IκBα with the cytosolic extracts from nontreated cells (Fig. 2*C*, *lane 1*) or the cells constitutively expressing calpastatin, which inhibits calpain (Fig. 2*C*, *lanes 4* and *5*). In contrast, limited proteolysis, with a fragmentation pattern similar to that shown in Fig. 2*C*, *lane 2*, was observed after incubation of IκBα with the cytosolic extract from silica-treated cells transfected with an empty vector. Addition of C-182, a synthetic calpastatin peptide, into the cytosolic extract from vector-transfected cells inhibited the fragmentation of IκBα by this cytosolic extract (Fig. 2*C*, *lane 3*). This result suggests that calpain is responsible for the limited degradation of IκBα protein in this assay.

Morphological changes of calpain inhibition. The most prominent activity of calpain is its limited proteolytic effect on proteins that are closely associated with membranes, such as cytoskeletal proteins and membrane proteins (27). Consequently, structural alteration of the plasma membrane and morphological changes of cells can be anticipated when the activity of calpains is inhibited. In our initial experiments using murine macrophage cells, RAW264.7, we found that the cells transfected with calpastatin exhibited a cube-like morphology rather than a spherical shape as exhibited by the parental cells or the cells transfected with an empty vector (data not shown). In human bronchial epithelial cells, although a dramatic morphological change could not be identified by the transfection of calpastatin, the cells expressing calpastatin did exhibit an impaired spreading and an enhanced early adhesion onto tissue culture flasks. More than 50% of empty vector-transfected cells displayed a thin and smooth-edged circumferential lamella 30 min after seeding into the cell culture flasks (Fig. 3*A*, arrows). Instead of the lamella formation, cells overexpressing calpastatin showed a triangular morphological characteristic and formed a mosaic monolayer among individual cells (Fig. 3*B*). Moreover, although the lamella formation and cell spreading were absent among the cells overexpressing calpastatin, these cells exhibited a faster adhesion to the tissue culture flasks. No morphological differences could be distinguished between the cells transfected with an empty vector and the cells transfected with calpastatin 4 h after seeding.

Altered gene expression in the cells expressing calpastatin. The proteolytic activity of calpains is well established and considered as a major contributor to the turnover of proteins involved in the morphological changes, spreading, and adhesion of cells (24, 27). Little is known, however, concerning the role calpains play in the regulation of cellular gene expression. The fact that the activation or functional maintenance of many transcription factors is dependent on proteolytic processes suggests that it is possible that calpains participate in the regulation of gene expression. To determine what set of genes or how many genes were affected by the inhibition of calpains, we carried out Genefilter microarray experiments in which the expression of 3,965 randomly selected human genes was compared between the cells transfected with an empty vector and the cells expressing calpastatin. We arbitrarily set a criterion of a twofold difference to judge substantial inhibition or induction of gene expression in the cells stably expressing calpastatin. A total of 12 genes satisfied this criterion. As presented in Table 1, a constitutive inhibition of calpain by overexpressing calpastatin in the cells resulted in a decreased expression of nine genes. It is intriguing to note that most of the decreased genes were those genes encoding membrane-associated proteins or ion channel-related proteins (protein phosphatase 2B and the sodium-/chloride-dependent betaine transporter). The expression of three genes was upregulated by the inhibition of calpains. These genes were collagen type Iα2, DAZ, and mitochondrial capsule selenoprotein (MCS). Both DAZ

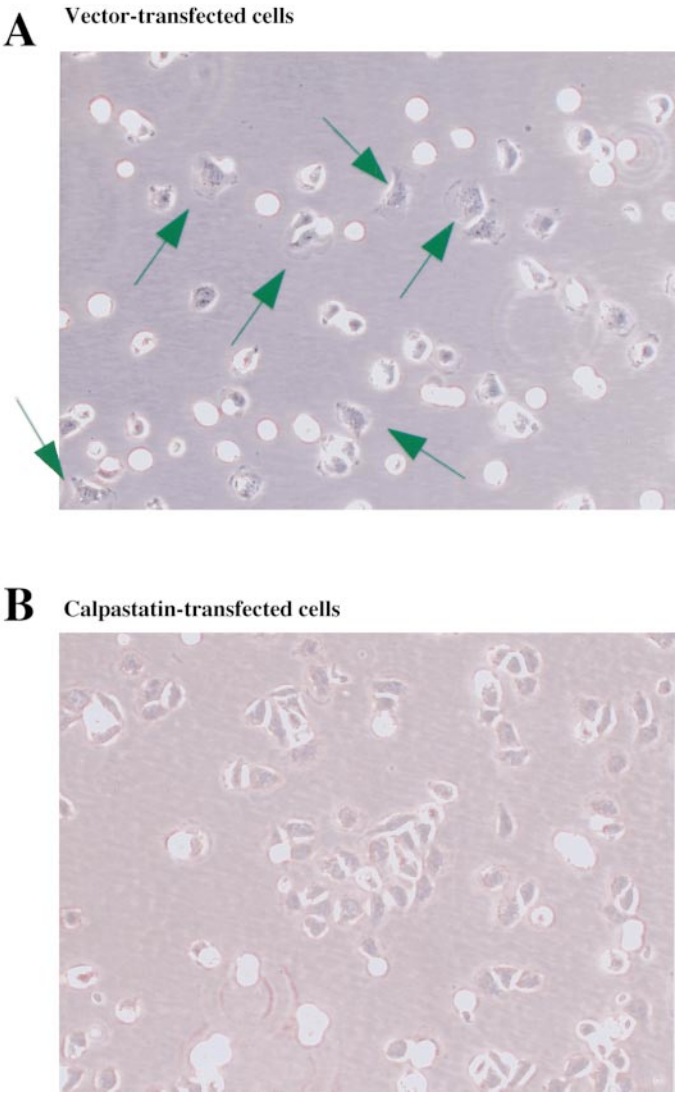

Fig. 3. Lamella formation and cell morphology are affected by calpastatin overexpression. Vector-transfected (*A*) and calpastatin-transfected (*B*) cells were seeded onto the surface of 75-cm^2 tissue culture flasks in DMEM containing 5% fetal bovine serum at 37°C. Micrographs were taken 30 min after seeding. Arrows indicate the formation of thin and smooth-edged circumferential lamella in vector-transfected cells (*A*).

Table 1. *Altered gene expression in calpastatin-expressing cells*

Gene Name	Fold Difference	Functions
Downregulated		
Protein phosphatase 2B	2.5	Ion channel regulator
Cartilage glycoprotein-39	2.23	Adhesion/migration
G protein-coupled receptor	2.05	Surface receptor
TRIP9	2.02	Adhesion plaque protein
Betaine transporter	2.18	Ion channel
Islet cell autoantigen	2.11	Surface protein
Y-linked testis-specific protein	2.06	Transcription factor
hERR1	2.58	Transcription factor
Cytokine inducible protein	2.02	Nuclear protein
Upregulated		
Type I collagen α2	2.19	Extracellular matrix
DAZ	2.0	RNA binding protein
Mitochondrial capsule selenoprotein	2.21	Sperm structural protein

and MCS are important for the spermatogenesis and fertilization of male reproductive cells.

If the stringent criterion of twofold difference of gene expression between vector- and calpastatin-transfected cells was lowered to 1.4-fold, 125 genes were downregulated and 80 genes were upregulated in the cells transfected with calpastatin (Table 2). Although chaotic patterns were observed when we used this lower criterion to judge the change of gene expression, it was apparent that only certain sets of genes were affected by the transfection of calpastatin. For example, most of the genes encoding ribosomal protein family members were downregulated in cells transfected with calpastatin, except the gene encoding ribosomal protein L34, which was increased 1.59-fold. Also, an appreciable alteration was noticed for those genes encoding cellular structural proteins such as keratin type II, troponin, and kinesin heavy chain.

DISCUSSION

Proteolysis is a pivotal physiological event required for the control of fundamental cellular processes including cytoskeletal remodeling, cell migration, cell cycle progression, transcription factor activation or inactivation, antigen presentation, and programmed cell death (21). Not surprisingly, proteolytic systems are also crucial for the initiation of disease processes. Eukaryotic cells contain two major nonlysosomal proteolytic pathways, the ubiquitin-proteasome pathway and the calpain pathway, that participate in the signal-induced proteolysis.

A vast majority of studies have indicated that ubiquitin-proteasome plays a major role in signal-induced IκBα degradation and consequent activation of NF-κB transcription factor (10, 22). However, several lines of evidence suggest the presence of alternative degradation pathways for IκBα proteins. First, in the murine macrophage cell line (RAW264.6), the inhibition of proteasome was unable to block IκBα degradation induced by the occupational dust, silica (8). Second, a proteasome-independent proteolytic pathway mediating the degradation of IκBα was considered responsible for the constitutive nuclear localization of NF-κB c-Rel/p50 complex in pre-B lymphocytes (20). Third, a pathway parallel to proteasome, calpain degradation pathway, has been identified in TNF-α- and respiratory syncytial virus-induced IκBα degradation in human liver cells and airway epithelial cells (14). Finally, calpain 3 deficiency in patients with limb-girdle muscular dystrophy type 2A showed a substantial inhibition of IκBα degradation, which was accountable for the cytoplasmic sequestration of NF-κB (4).

The findings in the present report provide further supportive and confirmational evidence to these observations. We have shown here that the inhibition of calpains by stable transfection of calpastatin in human bronchial epithelial cells led to a significant reduction of NF-κB DNA binding and IκBα degradation induced by silica. Moreover, in vitro digestion analysis revealed preferential cleavage site(s) of μ-calpain on the NH_2 terminus of the recombinant IκBα molecule (Fig. 2, *A* and *B*), which is consistent with previous reports by us and others (8, 14, 29). This implies that despite the existence of a COOH-terminal PEST domain [a sequence rich in proline (P), glutamic and aspartic acid (E), serine (S), and threonine (T)] assumed as a target region of calpain cleavage (25), the NH_2 terminus is more susceptible to calpain than the COOH terminus of IκBα. A most recent study by Reuther and Baldwin (26) indicated that IκBα truncated at the amino terminus by caspase-3 might function as a stable inhibitor of NF-κB. This appears not to be the case in calpain-mediated degradation of IκBα because the fragments of NH_2-terminal truncated IκBα are still susceptible to further degradation by calpains.

Another important observation from the present study is the alteration of the gene expression profile due to the constitutive inhibition of calpain. In agreement with others, our finding that calpastatin-overexpressing cells exhibited impaired migration and altered morphological characteristics implies a crucial role of calpains in the regulation of cell mobility and morphology by cleaving and remodeling cytoskeletal proteins in a site-specific manner. However, there was limited information on calpain regulation of gene expression. On the basis of the involvement of calpain in the activation or functional maintenance of transcription factors including NF-κB- and c-Fos-containing complexes, it should be predictable that the expression of some housekeeping and inducible genes will be affected by the inhibition of calpains. An unanticipated result from the Genefilter microarray experiments is that only a few sets of genes were downregulated substantially in calpastatin-overexpressing cells. Interestingly, five of nine underexpressed genes in calpastatin-overexpressing cells are related to membrane-associated proteins, such as surface proteins, ion channels, and Ca^{2+} channel-regulating proteins (Table

Table 2. *Altered gene expression with 1.4- to 2.0-fold difference in calpastatin expression cells*

Gene Name	Fold Difference	Gene Name	Fold Difference
Downregulated		Downregulated	
Ribosomal protein L30	1.41	Early growth response protein 1	1.69
Ribosomal protein L24	1.42	Breast cancer-specific protein 1	1.42
Ribosomal protein S23	1.40	Double-stranded RNA adenosine deaminase	1.43
Ribosomal protein L38	1.54	L-3-Phosphoserine-phosphatase	1.92
Ribosomal protein L5	1.58	ISLR	1.59
Ribosomal protein L6	1.49	Tax interaction protein 33	1.65
Ribosomal protein L31	1.40	Zinc finger protein 148	1.46
Ribosomal protein L17	1.40	Clutathione peroxidase 3	1.43
Ribosomal protein S29	1.44	hNop56	1.50
Ribosomal protein L32	1.40	Osteoblast mRNA	1.60
Kinesin heavy chain	1.59	Retinal protein HRG4	1.68
Troponin T2	1.83	Jun B	1.41
Troponin I	1.63	PKC inhibitor-1	1.55
LDL receptor related	1.46	ITBA 1	1.93
Metaxin	1.85	Prothymosin α	1.41
MHC I-like protein	1.43	TGF-β binding protein 1	1.46
Syntrophin α	1.89	α2 Plasmin inhibitor	1.81
K^+-dependent channel	1.52	Pre-T/NK cell associated protein	1.44
Folate receptor γ precursor	1.62	HBV-X associated protein	1.59
Keratin II	1.44	IP3 kinase isoenzyme	1.42
Cleavage stimulation factor	1.44	CDEP	1.55
Cytochrome P450IIIA7	1.46	Cytoplasmic antiproteinase	1.58
2,3-bisphosphoglycerate mutase	1.60	HnRNP F protein	1.46
Cytochrome P450 IVA	1.41	RNA polymerase II	1.53
MHC class II DQ-β	1.41	Luman	1.68
Serotonin *N*-acetyltransferase	1.40	Proteasome subunit Z	1.41
Transcription factor 3	1.52	Cathepsin H	1.66
Syndecan 2	1.49	Cytochrome C oxidase polypeptide Vic precursor	1.60
CSF3R	1.59	Cytochrome C oxidase polypeptide Via-liver precursor	1.44
Guanine nucleotide-binding protein-β	1.63	Repressor protein	1.70
Peptidylglycine a-amidating monooxygenase	1.41	Coatomer β-subunit	1.57
Tumor rejection antigen gp96	1.46	Proline-rich Gla protein 2	1.45
D9 splice variant A	1.41	Sgk gene	1.82
Nuclear ribonucleoprotein A2/B1	1.41	Protein phosphatase 6	1.48
Glutathione *S*-transferase M4	1.73	S100 α protein	1.54
Coagulation factor II receptor	1.49	PK-120	1.61
CDC28 protein kinase 2	1.42	MNK1	1.43
PDGF-A	1.41	HnRNP A/B protein	1.42
APEX nuclease	1.64	O-linked GlcNAc transferase	1.57
ATP citrate lyase	1.74	CAGH1α	1.42
Annexin VI (p68)	1.74	Factor VIII intron 22 protein	1.56
Carbonic anhydrase II	1.42	RNA polymerase II subunit hsRPB7	1.55
DNA-binding protein A	1.45	RNA polymerase II subunit hsRPB4	1.41
Defender against cell death 1	1.42	Hypothetical protein A4	1.61
Pim-2 protooncogene	1.42	DNA-directed RNA polymerase II	1.44
FE65-like protein	1.96	Int-6	1.45
Tyrosine phosphatase IA-2	1.46	Heat shock cognate 71-kDa protein	1.40
Glial firacidic protein	1.42	Neuronal tissue-enriched acidic protein	1.53
hMed 7	1.46	Gu binding protein	1.58
Putative OSP-like protein	1.41	Processing a-glucosidase I	1.82
Pre-B cell leukemia transcription factor-3	1.60	TFAR19	1.44
UBA52	1.43	ATP synthase γ-subunit	1.51
TGF-β1	1.49	LDLC	1.55
Calpastatin	1.45	ATP synthase subunit e	1.43
Carboxypeptidase N 83-kDa chain	1.55	L23-related	1.83
PIP2 phosphodiesterase β3	1.52	Upregulated	
Histone H3.3	1.45	Robisomal protein L34	1.59
Glucosamine-6-phosphate isomerase	1.50	*Integrin αV*	1.43
DNA binding protein A20	1.44	*Actin α2*	1.46
Norrie disease protein	1.46	*PECAM-1*	1.43
Munc13	1.75	*TWIK K^+ channel*	1.41
Glucocorticoid receptor	1.41	*G protein β5*	1.59
Tetranectin	1.50	*Adhalin-35*	1.46
G_1 to S phase transition 1	1.48	*A-tubulin isotype H2-α*	1.40
Basic transcription factor-3	1.64	*K^+ channel* β2	1.43
Multiple endocrine neoplasia I	1.45	HMG box-containing protein	1.44
Creatine kinase B	1.81	PP2A	1.59
Endothelin converting enzyme 1	1.61	Cysteine-rich FGF receptor	1.41
Complement C3 precursor	1.48	G protein-coupled receptor (STRL22)	1.60
Cytochrome C reductase	1.82		

Table 2. —*Continued.*

Gene Name	Fold Difference
Upregulated	
Aldolase C	1.41
Acyl-coA dehydrogenase	1.46
Chromosome segregation gene CAS	1.60
Carbamoyl-phosphate synthetase I	1.45
Cyclin B1	1.58
cGMP gated 2	1.41
eIF-2α	1.56
Histone H2A	1.52
NAD(P)H:menadione oxidoreductase	1.43
Na^+-K^+-ATPase	1.45
Phospholipase C	1.43
Kinesin light chain	1.48
Zinc finger protein 74	1.52
Zinc finger protein 174	1.47
RNA binding protein RNPL	1.40
Thyroxin-binding globulin	1.45
Homolog 3 of *Drosophila* large discs	1.41
Mannose receptor	1.47
Glycophorin E	1.40
Dopachrome tautomerase	1.40
Calcium modulating ligand	1.43
Carnitine palmitoyltransferase I	1.40
Sulfated glycoprotein 1	1.60
Phospholipase D	1.48
GABA solute carrier family 6	1.51
L-3-Hydroxyacyl-CoA dehydrogenase	1.55
Flavin-containing monooxygenase 4	1.40
Staf 50	1.41
Alcohol dehydrogenase 4	1.44
Sjogren syndrome antigen A1	1.41
Amyloid bata precursor-like 2	1.43
Dominant negative HLH protein	1.77
Adehyde dehydrogenase 6	1.57
Protein kinase zpk	1.47
UDP glucuronosyltransferase	1.55
Dentatorubral-pallidoluysian atrophy	1.44
Amyloid A4 protein	1.43
Mch2 α	1.52
CDK inhibitor p27kip1	1.52
Neutral calponin	1.50
FGF-1 binding protein	1.43
Prodynorphin A precursor	1.68
Bet3	1.43
Garp	1.45
Carbonic anhydrase precursor	1.50
Glycogenin-2γ	1.66
Synaptopodin	1.71
NF-H	1.53
Intestinal trefoil factor	1.43
Mch3 α	1.45
IFN-induced 54-kDa protein	1.53
DEC1	1.46
SRE-ZBP	1.41
Hc-cul-1	1.57
G-aminobutyric acid A receptor a	1.40
Desmoplakin I/II	1.43
L-Kynurenine hydrolase	1.40
Homeodomain protein	1.43
UBE2G2	1.47
Nuclear antigen H731	1.45
Vitamin K-dependent protein Z	1.65
Estrogen sulfotransferase	1.81
DNase1-like III	1.49
GCAP	1.40
Arylamine *N*-acetyltransferase	1.63
Myosin-I β	1.47
FEZ2	1.45

Genes encoding membrane-associated proteins and cytoskeleton proteins are italicized.

1). It is possible that the characteristic morphology of calpastatin-overexpressing cells may be partially attributable to the downregulated expression of genes encoding these membrane-associated proteins. Another four of nine underexpressed genes are related to nuclear localized proteins with transcription factor-like function, including hERR1, TRIP9, Y-linked testis-specific protein, and cytokine-inducible nuclear protein. It is recognized that a subtle difference of gene expression can have dramatic biological consequences. Therefore, we also evaluated genes whose repression or upregulation was between 1.4- and 2-fold of control (Table 2) in cells overexpressing calpastatin, even though such changes are usually considered insignificant in DNA microarray assays. It was interesting to note that the expression of most of the genes encoding ribosomal protein family members was inhibited.

It is unknown whether interconnected events are involved in NF-κB activation, altered gene expression, and cellular morphological changes induced by the calpain-calpastatin system. There are no previous reports indicating NF-κB-mediated expression of any of these genes listed in Table 1. Whereas the gene structural data for most of these genes are not available currently, DNA sequence analysis revealed several highly conserved NF-κB binding sites, such as 499-GGGAATTTCA-508 and 549-GGGAATTTCCC-559, located within the promoter region of the cartilage glycoprotein-39 gene (GenBank accession no. Y08374; data not shown).

It would be premature to conclude that the observed alteration of gene expression in calpastatin-overexpressing cells is the result of impaired activation of NF-κB. First, we compared the gene expression pattern in the cells cultured under a basal condition without further treatment. As shown in Fig. 1*A*, the basal level of NF-κB activation could be notably inhibited by the inhibition of calpains through constitutive overexpression of calpastatin. For the determination of gene expression mediated by NF-κB, however, an inducible condition is usually required. Second, it has been demonstrated that Cdc42, Rho, and Ras GTPases were associated with the cytoskeleton (13). The effect of calpastatin on calpain-mediated turnover and remodeling of the cytoskeleton system could conceivably lead to alteration of signal transduction cascades and gene expression, which bypasses regulation by NF-κB. Third, besides NF-κB, other transcription factors may also serve as targeting factors of calpains. Finally, certain secondary effects may exist in this nonphysiological overexpression condition.

In summary, we explored two important functional aspects of calpains that have not been well defined previously, i.e., involvement in the activation of NF-κB and regulation of gene expression. It is possible that calpains may be involved in a number of physiological and pathological processes. Several issues need to be addressed in future studies, including the mechanisms of calpain activation, consequences of calpain deficiency or overfunction, cross talk with proteasome and

other proteolytic systems, and the feasibility of targeting calpains to interfere with disease processes.

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