

Essential Role of Human Leukocyte Antigen-encoded Proteasome Subunits in NF- κ B Activation and Prevention of Tumor Necrosis Factor- α -induced Apoptosis*

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The multisubunit proteasome complex is the principal mediator of nonlysosomal protein degradation. The proteasome subunit varies minimally between cells with the exception of LMP2, LMP7, and LMP10 subunits in rodent and human cells. LMP2 and LMP7 subunits are encoded by the human lymphocyte antigen region, and they optimize proteolytic mediated antigen presentation. The proteasome is also important for the function of transcription factor nuclear factor- κ B (NF- κ B). It is required for NF- κ B subunits p50 and p52 generation and catalyzes degradation of phosphorylated I κ B α . These proteasome-mediated reactions have now been shown to be defective in T2 cells, a human lymphocyte cell line that lacks both LMP2 and LMP7. Although T2 cells contain normal expression of p100 and p105, the abundance of p50 and p52 was greatly reduced. Tumor necrosis factor- α (TNF- α) induced normal phosphorylation of I κ B α but failed to induce degradation of phosphorylated I κ B α . Both DNA binding assays and luciferase assays revealed that TNF- α -induced NF- κ B activation is defective in T2 cells. Unlike parental cells, T2 cells were susceptible to TNF- α -induced apoptosis. These data indicate human leukocyte antigen-linked proteasome subunits are essential for NF- κ B activation and protection of cells from TNF- α -induced apoptosis.

Nuclear factor- κ B (NF- κ B)¹/Rel superfamily is a transcription factor that contributes to the ability of the immune system to respond rapidly to foreign antigens (1–6). NF- κ B is activated in response to various extracellular stimuli, including interleukin 1 (IL-1), tumor necrosis factor- α (TNF- α), lipopolysaccharide, and phorbol esters (2, 7–12). NF- κ B is implicated in the regulation of genes that contribute to cytokine generation, expression of cell surface adhesion molecules, and processing and presentation of major histocompatibility complex (MHC) or human leukocyte antigen (HLA) class I-restricted antigens (1–

6). NF- κ B also plays an important role in preventing apoptosis and in activating signaling pathways that contribute to cellular transformation and development (12–20).

NF- κ B exist predominantly as heterodimers composed of subunits with molecular masses of 50, 52, or 65 kDa, known as p50 (NF- κ B1), p52 (NF- κ B2), and p65 (RelA), respectively (21–24). These proteins all contain a highly conserved region known as the Rel homology domain that is responsible for both protein dimerization and binding to DNA. In mammalian cells, the NF- κ B family of proteins can be divided into two classes as follows: one class includes p50 and p52, both of which are produced constitutively by the proteasome-mediated removal of the COOH termini of the precursor proteins p105 and p100, respectively (22, 25–27). The ubiquitin-proteasome pathway is also required for p50 generation in yeast (28). The second class of NF- κ B proteins contains RelA and the related proteins c-Rel and RelB (24, 29, 30). These proteins do not undergo proteolytic processing and contain transcriptional activation domains. The generation and characterization of corresponding knockout mice (14, 18, 31–35) have recently provided insight into the specific biological functions of p50, p52, RelA, RelB, and c-Rel.

In unstimulated cells, NF- κ B heterodimers are associated with an I κ B family molecule in the cytosol (7, 36, 37). Cellular stimulation results in the phosphorylation and subsequent proteolytic degradation of phosphorylated I κ B α (7, 36, 38), which allows NF- κ B to enter the nucleus where it regulates the expression of its target genes. The degradation of phosphorylated I κ B α , like the generation of p50 and p52, requires ubiquitination and the proteasome processing pathway (7). The COOH terminus of p105 (p105C) contains ankyrin repeats and bears a striking resemblance to I κ B α (39–41). The lymphoid cell-specific I κ B γ protein is identical to p105C; however, this protein is generated by either alternative splicing or promoter usage (39–41). Mature p50 is generated by a unique co-translational or post-translational processing event involving the ubiquitin-proteasome degradation pathway (7, 28, 42–46).

Thus, the ubiquitin-proteasome pathway plays an important role in two distinct aspects of NF- κ B activation. It mediates the generation of the p50 and p52 subunits, thereby allowing them to compose heterodimers with p65, and it degrades the phosphorylated inhibitory subunit, I κ B α . Both proteasome steps thereby allow the active NF- κ B heterodimers entry into the nucleus (47–49). The sites of phosphorylation and of ubiquitination of I κ B α have been identified, and the kinase complex responsible for I κ B α phosphorylation contains IKK α , IKK β , and IKK γ and has been isolated and characterized (36–38).

NF- κ B activation plays an important role in suppression of TNF- α -induced apoptosis. Mice lacking p65 die before birth on day 14 to 15 of gestation, apparently because of massive and accelerated liver cell death (14). Mouse embryonic fibroblasts derived from these p65^{-/-} animals undergo apoptosis when

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¹ The abbreviations used are: NF- κ B, nuclear factor- κ B; IL, interleukin; TNF- α , tumor necrosis factor- α ; MHC, major histocompatibility complex; HLA, human leukocyte antigen; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; PAGE, polyacrylamide gel electrophoresis; IL-2R α , interleukin-2 receptor α -chain; LTR, long terminal repeat; RSV, Rous sarcoma virus; CDK, cyclin-dependent kinase; HIV-1, human immunodeficiency virus-type 1; wt, wild type; mut, mutant.

exposed to TNF- α (13), due to a defect in the activation of NF- κ B in response to this cytokine (13, 14). Furthermore, introduction of a dominant-negative I κ B α into cells or overexpression of I κ B α prevents NF- κ B activation and results in cell death on exposure to TNF- α (14, 19, 50–52). Mouse embryos lacking IKK β also die on day 12 to 13 of gestation due to massive liver apoptosis (53–55). Embryonic fibroblasts derived from IKK $\beta^{-/-}$ mice fail to exhibit activation of the IKK complex or NF- κ B in response to TNF- α or IL-1 and undergo apoptosis in the presence of these agents (53–55). Culturing intact cells with various proteasome inhibitors also results in a marked potentiation of TNF- α -induced cell death (56).

We have now investigated proteasome function, NF- κ B activation, and susceptibility to TNF- α -induced apoptosis in T2 cells, a human lymphocyte cell line in which the genes for the proteasome subunits LMP2 and LMP7 have been genetically deleted. The activity of NF- κ B in T2 cells was markedly impaired, a result of a virtual lack of p50 and p52 caused by defective proteasomal processing of the corresponding precursor proteins. Furthermore these cells were defective in their ability to degrade phosphorylated I κ B α and showed a marked susceptibility to TNF- α -induced cytotoxicity. Our results indicate that, in human lymphocytes, LMP2 and LMP7 are required for the generation and activation of NF- κ B as well as for prevention of TNF- α -induced apoptosis.

EXPERIMENTAL PROCEDURES

Cells, Antibodies, and Mice—Lymphocyte cell lines used in this study included Molt-4, Jurkat, T1, and T2 cells. All were purchased through ATCC (Manassas, VA) except T2 cells, which were a kind gift from Dr. Peter Cresswell (New Haven, CT). T2 cells are a mutant derived from T1 cells; they lack a large segment of chromosome 6 that encodes HLA class II genes, the *Lmp2* and *Lmp7* proteasome genes, and the ATPase peptide transporters *Tap1* and *Tap2*. Fresh normal murine lymphocytes from spleens were harvested from 6-week-old Balb/c or *Lmp2^{-/-}* mice. BALB/c mice were purchased from The Jackson Laboratories; *Lmp2^{-/-}* mice were a generous donation from Dr. Luc Van Kaer (Nashville, TN). All antibodies were purchased through Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) or Oncogene Research Laboratories (Cambridge, MA).

Preparation of Nuclear and Cytosolic Extracts—Cells (1×10^7) were harvested, collected by centrifugation for 15 min at 3000 rpm, washed with 10 ml of ice-cold phosphate-buffered saline (PBS), and again collected by centrifugation. The resulting pellets were resuspended in 4 ml of solution A (10 mM Hepes-NaOH (pH 7.8), 10 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride) and then incubated for 15 min at 4 °C. After addition of 250 μ l of 10% (v/v) Nonidet P-40, the cell suspension was vigorously mixed, incubated for 30 min at 4 °C, and centrifuged for 15 min at 3,000 rpm. The resulting supernatant was saved as the cytosolic extract (protein concentration, 35 μ g/ μ l), and the pellet was resuspended in 1.5 ml of solution C (50 mM Hepes-NaOH (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, and 10% (v/v) glycerol). The suspension was mixed for 30 min at 4 °C and centrifuged for 15 min at 3,000 rpm. The supernatant from this centrifugation was saved as the nuclear extract (protein concentration, 20 μ g/ μ l).

Oligonucleotides and Electrophoretic Mobility Shift Assay (EMSA)—Double-stranded oligodeoxynucleotides were prepared by the phosphoramidate method with a DNA synthesizer and purified on an OPC cartridge (Life Technologies, Inc.). They correspond to wild-type κ B (5'-GATCTAGGGACTTT CCGCTGGGGACTTTCCAG) and mutant (mutant 1, 5'-GATCTACT CACTTTCCGCTGCTCACTTTCCAG; mutant 2, 5'-GATCTAGTCACTTTCCGC TGGTCACTT TCCAG) κ B binding motifs of the human immunodeficiency virus type 1 (HIV-1 κ B) enhancer. The oligonucleotides were end-labeled with [α -³²P]dCTP using the Klenow polymerase. For EMSA analysis, nuclear extracts were incubated for 30 min at 37 °C in a total volume of 10 μ l containing 10 mM Hepes-NaOH (pH 7.9), 50 mM KCl, 5 mM Tris-HCl (pH 7.0), 1 mM DTT, 15 mM EDTA, 10% glycerol, 1.0 μ g of poly(dI-dC), and 4 ng of ³²P-labeled wild-type HIV-1 κ B oligonucleotide. The resulting DNA-protein complexes were resolved by electrophoresis on nondenaturing 8% polyacrylamide gels with 0.5 \times Tris borate-EDTA buffer at 4 °C. For

competition experiments, the nuclear extracts were incubated for 15 min at 4 °C with a 100-fold molar excess of unlabeled HIV-1 κ B oligonucleotide before addition of the radioactive probe. For supershift assays, the nuclear extracts were incubated with specific antibodies for 1 h at 4 °C before addition of the DNA probe. Cytosolic extracts were exposed to final concentrations of 1.2% (v/v) Nonidet P-40 and 0.8% (w/v) deoxycholate to induce dissociation of I κ B from NF- κ B before incubation with ³²P-labeled probe (21, 57, 58). AP1 and SP1 binding activities were examined by EMSA analysis as described (59, 60).

Immunoblot Analysis—Nuclear or cytosolic extracts were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 12.5% gels under nonreducing conditions. The separated proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane, which was then incubated for 2 h at room temperature with TBS-T (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.05% (v/v) Tween 20) containing 8% (w/v) bovine serum albumin. The membrane was subsequently incubated for 12 h at 4 °C with TBS-T containing the appropriate polyclonal antibodies, washed four times with TBS-T for 15 min each time at room temperature, incubated for 2 h at room temperature with TBS-T containing alkaline phosphatase-conjugated secondary antibodies, washed five times with TBS-T, and subjected to the alkaline phosphatase color reaction by standard method.

In Vitro Processing Assay for p50 Generation—The *in vitro* p50 processing was assayed as described previously (44). In brief, the pcDNA1p105 construct was subjected to transcription and translation *in vitro* with wheat germ extract (Promega, WI) in the presence of [³⁵S]methionine. The ³⁵S-labeled p105 protein was then immunoprecipitated with polyclonal antibodies to p50 and purified. The substrate protein was incubated for 90 min at 30 °C with cytosolic extract (20 or 40 μ g of protein) in a final volume of 25 μ l in the absence or presence of 10 mM ATP (46). The proteasome inhibitor MG115 (Sigma) was preincubated with cytosolic extracts before the substrate protein was also added to the reaction mixture. The proteolytic products were separated by SDS-PAGE on a 10% gel and visualized by autoradiography.

Luciferase Assays—The reporter plasmids, IL-2R α - κ B wt, IL-2R α - κ B mut, and RSV-LTR were used in the luciferase experiments. In these constructs, the luciferase gene is driven by the interleukin-2 receptor- α -chain (IL-2R α) promoter or the Rous sarcoma virus-long terminal repeat (RSV-LTR). The reporter plasmid (10 μ g), IL-2R α - κ B wt, IL-2R α - κ B mut, or RSV-LTR were co-transfected into T1, T2, Molt-4, or Jurkat cells by the DEAE-dextran standard method using 1 μ g of a RSV-galactosidase expression vector as an internal control. In IL-2R α - κ B wt, the κ B sequence GGGGAATCTCCC was substituted by GCTCAATCTCCC. The cells were cultured in RPMI 1640 medium containing 10% fetal calf serum for 48 h after transfection and then TNF- α (final concentration, 10 ng/ml) was added to each plate. After an additional 4 h of culture with TNF- α , the cells were harvested, and luciferase assays were performed. The luciferase activities of equal amounts of extracted proteins were measured by standard methods. To adjust the transfection efficiency, quantitation of the β -galactosidase was carried out by the standard method. Values are shown as the mean \pm S.E. of three independent transcription experiments.

Cell Survival Assay—Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and exposed to TNF- α for various times. The number of viable cells was determined by trypan blue exclusion as described (13).

RESULTS

Impaired Activation of NF- κ B in T2 Cells—We first investigated the effect that deletions of the HLA-linked *Lmp2* and *Lmp7* proteasome subunit genes might have on NF- κ B function by EMSA with the nuclear extracts prepared from T2 cells. These cells were cultured in the absence or presence of TNF- α (10 ng/ml) for 4 h. Nuclear extracts from parental T1 cells and from human T cell lymphoma Molt-4 and Jurkat cells were also assayed for comparison. Whereas T1, Molt-4, and Jurkat cells all showed marked increases in nuclear κ B binding activity after exposure to TNF- α , T2 cells exhibited no such response (Fig. 1A). The specificity of the observed κ B binding in the nuclear extracts of TNF- α -treated cells was confirmed by binding activity assessed with ³²P-labeled probe. The DNA binding activity was completely inhibited by preincubation of the extracts with a 100-fold molar excess of the corresponding (wild type) unlabeled κ B oligonucleotide; similar preincubation with two different oligonucleotides in which the κ B binding motif

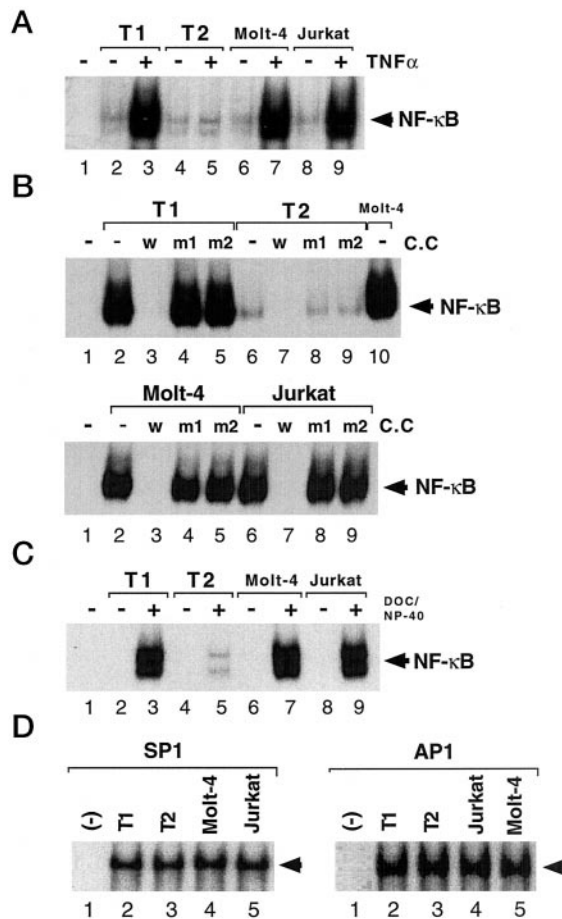


FIG. 1. EMSA of the effect of TNF- α on the DNA binding activity of NF- κ B in T2 cells. *A*, effect of TNF- α on nuclear κ B binding activity. Nuclear extracts were prepared from T1, T2, Molt-4, and Jurkat cells after incubation for 4 h in the absence (-) or presence (+) of TNF- α (10 ng/ml). The extracts were then assayed for DNA binding activity with a 32 P-labeled (wild type) κ B oligonucleotide by EMSA analysis. The arrowhead indicates the position of specific DNA-protein complexes. *B*, specificity of the nuclear DNA binding activity for the κ B binding motif. Nuclear extracts prepared from TNF- α -treated T1, T2, Molt-4, and Jurkat cells were preincubated in the absence (-) or presence of a 100-fold molar excess of unlabeled competitor oligonucleotide (wild-type (*w*), mutant 1 (*m1*), or mutant 2 (*m2*)) before exposure to the 32 P-labeled (wild type) κ B oligonucleotide. *C.C.*, cold competitor. *C*, κ B binding activity in cytosolic extracts. EMSA was performed with the 32 P-labeled κ B oligonucleotide and with cytosolic extracts of T1, T2, Molt-4, and Jurkat cells that had been treated (or not) with Nonidet P-40 (NP-40) and deoxycholate (DOC). *D*, DNA binding activities of SP1 (left panel) and AP1 (right panel). The DNA binding activities of SP1 and AP1 in nuclear extracts of T1, T2, Molt-4, and Jurkat cells were examined by EMSA with specific oligonucleotide probes. Arrowheads indicate the specific DNA-protein complexes. In all panels, lanes 1 correspond to negative controls in which the extract protein was not added to the reaction mixture.

was mutated did not inhibit binding activity measured with the labeled probe (Fig. 1B).

The κ B binding activity of cytosolic extracts was also examined by EMSA after treatment of the extracts with detergents (Nonidet P-40 and deoxycholate) to induce dissociation of I κ B from NF- κ B (21, 57, 58). Again, the κ B binding activity of cytosolic extracts from T2 cells was greatly inactivated relative to the κ B binding activity detected in cytosolic extracts from T1, Molt-4, or Jurkat cells (Fig. 1C). The nuclear DNA binding activities of the transcription factors SP1 and AP1 did not differ among the four cell types studied (Fig. 1D). Together, these observations suggest that NF- κ B activation in response to TNF- α is defective in T2 cells.

Defective Generation of NF- κ B Subunits p50 and p52 and

Defective Degradation of I κ B α in T2 Cells—To identify the NF- κ B subunits responsible for the observed κ B binding activity, we performed supershift assays. Preincubations of nuclear extracts from T1, Molt-4, or Jurkat cells with polyclonal antibodies to p50 resulted in a shift in the DNA-protein complex to a position of lower mobility; however, no such shift in mobility was detectable with the DNA-protein complex formed by nuclear extracts from T2 cells (Fig. 2A). In contrast, pretreatment of the nuclear extracts from all four cell lines with polyclonal antibodies to p65 reduced the mobility of the DNA-protein complex in every case (Fig. 2A). As a negative control, polyclonal antibodies to the transcription factor C/EBP had no effect on the mobility of the DNA-protein complex formed by nuclear extracts of each of the four cell lines (Fig. 2A).

Aberrant p52 proteins are found in lymphocytes, as a result of chromosome rearrangements at the NFKB2 locus (27); p52 is normally produced from p100, an inactive precursor protein harboring I κ B-like ankyrin-containing sequences in the COOH-terminal half, thus p52 is generated by ubiquitin-proteasome processing of the p100 precursor. To demonstrate initially whether p52 binds κ B oligonucleotide probe, we similarly studied κ B oligonucleotide probe (HIV-1 κ B) binding to nuclear extracts in a supershift assay with polyclonal antibody to p52. TNF- α -treated Molt-4, Jurkat, T1, or T2 cell nuclear extracts did not bind this κ B oligonucleotide probe (data not shown). Recent data demonstrate p52-p52 homodimers and p52-p65 heterodimers can specifically recognize and bind H2TF1 κ B (GGGGATTCCCCA) but do not bind HIV-1 κ B (GGGGACTTTC CC) (27). Further studies confirmed anti-p52 antibodies selectively reduce the mobility of the DNA-protein complex formed by the nuclear extract of TNF- α -treated control Molt-4 cells with an oligonucleotide probe to H2TF1 κ B corresponding to the κ B binding motif of the MHC class I gene enhancer (data not shown).

The basal expression of NF- κ B subunits in cytosolic and nuclear extracts from T1, T2, Molt-4, and Jurkat cells was examined by immunoblot analysis (Fig. 2B). In cytosolic extracts, the basal expression of p65, the precursors p105 and p100, and I κ B α , as well as that of the cyclin-dependent kinases CDK2, CDK7, and CDK8 (assayed as controls) did not differ among the four cell types (Fig. 2B). However, the amount of p50 and p52 in cytosolic extracts of T2 cells was markedly reduced relative to those in cytosolic extracts of T1, Molt-4, and Jurkat cells (Fig. 2B). In nuclear extracts, the abundance of p65 and c-Rel was similar for all four cell types; however, the amount of p50 and p52 was greatly reduced in T2 cells (Fig. 2B). Northern blot analysis revealed that the abundance of both p65 and p105 mRNAs in cytosolic extracts did not differ among the four cell types (data not shown).

We also examined the dynamics of I κ B α phosphorylation during TNF- α stimulation of T1 and T2 cells by immunoblot analysis of cytosolic extracts with the appropriate antibodies (Fig. 2C). Phosphorylated I κ B α was detected as the upper band of I κ B α double bands that appear after incubation of either T1 or T2 cells with TNF- α for 20 min (Fig. 2C). However, whereas I κ B α had virtually disappeared in T1 cells after incubation with TNF- α for 40 min, no such decrease in I κ B α abundance was apparent after 40 or 240 min of cytokine treatment of T2 cells (Fig. 2C). The amount of I κ B α had increased to original levels after incubation of T1 cells with TNF- α for 240 min (Fig. 2C). The abundance of CDK2, CDK7, or CDK8 was not affected by TNF- α treatment of T1 or T2 cells. Results obtained using Molt-4 and Jurkat cells were similar to those observed for T1 cells (data not shown). These data suggest that, whereas TNF- α -induced phosphorylation of I κ B α appears normally in T2 cells, the subsequent degradation of phosphorylated I κ B α ap-

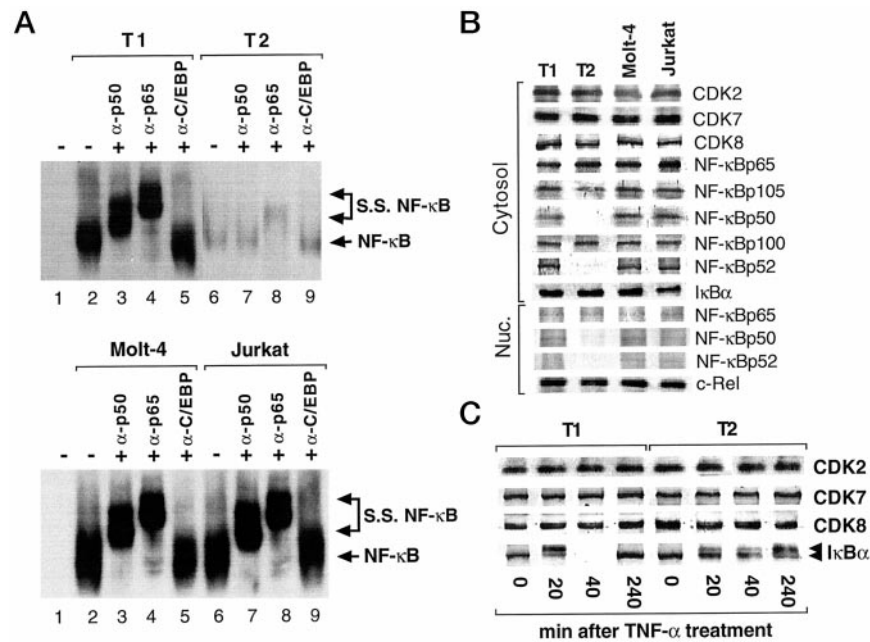


FIG. 2. Impaired p50 and p52 generation, no degradation of phosphorylated I κ B α in T2 cells. *A*, supershift analysis of κ B binding activity. Nuclear extracts prepared from TNF- α -treated T1, T2, Molt-4, and Jurkat cells were incubated in the absence (–) or presence (+) of polyclonal antibodies to p50, p65, or C/EBP before EMSA analysis with the 32 P-labeled κ B oligonucleotide. Original DNA-protein complexes (NF- κ B) and supershifted complexes (S.S. NF- κ B) are indicated by arrows. Lanes 1 represent negative controls in which nuclear extract was not added to the reaction mixture. *B*, immunoblot analysis of NF- κ B subunits and precursors, I κ B α , and cyclin-dependent kinases. Cytosolic and nuclear (Nuc.) extracts of T1, T2, Molt-4, and Jurkat cells were subjected to immunoblot analysis with antibodies to the indicated proteins. *C*, immunoblot analysis of the effects of TNF- α on the phosphorylation and degradation of I κ B α . T1 and T2 cells were incubated for the indicated times with TNF- α (10 ng/ml), after which cytosolic extracts were prepared and subjected to immunoblot analysis with antibodies to I κ B α , to CDK2, to CDK7, or to CDK8. Arrowheads indicate phosphorylated (upper) and nonphosphorylated (lower) I κ B α .

parent in T1 cells is defective in TNF- α -treated T2 cells.

NF- κ B Inactivation in TNF- α -treated *Lmp2*^{-/-} Lymphocytes—To verify that the apparent proteasome dysfunction and NF- κ B inactivation in T2 cells is caused by the down-regulation of the 20 proteasome β subunits, we examined the DNA binding activity of NF- κ B in lymphocytes lacking *Lmp2* that were derived from *Lmp2*^{-/-} mouse spleen. The effect of TNF- α on NF- κ B activation was investigated using Molt-4 cells and compared with activation in spleen cells from BALB/c and *Lmp2*^{-/-} mice. Incubation of Molt-4 cells or BALB/c mouse spleen cells with TNF- α (10 ng/ml) for 4 h resulted in a marked increase in nuclear NF- κ B DNA binding activity as determined by EMSA (Fig. 3A, left panel). In contrast, TNF- α at concentrations of 10 ng/ml had no significant effect on the nuclear NF- κ B activity in *Lmp2*^{-/-} lymphocytes (Fig. 3A, left panel). The specificity of the DNA binding activity in nuclear extracts from TNF- α -treated lymphocyte cells from both BALB/c and *Lmp2*^{-/-} mice was confirmed by cold competition assays with unlabeled wild-type κ B and mutant κ B oligonucleotides. NF- κ B binding to the κ B probe was prevented by preincubation of the nuclear extracts with a 100-fold molar excess of unlabeled wild-type κ B oligonucleotide but not by preincubation of the nuclear extracts with mutant κ B oligonucleotide (data not shown). We concluded that the DNA binding activity we measured is due to the activity of NF- κ B.

Cytosolic NF- κ B-I κ B complexes in *Lmp2*^{-/-} lymphocytes were similarly tested by EMSA. NF- κ B DNA binding activity in detergent-treated cytosolic extracts from *Lmp2*^{-/-} mouse spleen cells was markedly reduced compared with binding observed in cytosolic extracts from BALB/c mouse spleen cells (Fig. 3A, right panel). Again, the DNA binding activities of SP1 and AP1 did not differ between nuclear extracts of BALB/c and *Lmp2*^{-/-} mouse spleen cells (data not shown). Furthermore, antibodies to p50 or to p65 reduced the mobility of the DNA-protein complexes formed in the nuclear extracts of TNF- α -

treated BALB/c spleen cells incubated with the κ B1 oligonucleotide as described by supershift assay. In contrast, antibodies to p65, but not those to p50, had an effect on mobility in the nuclear extracts of TNF- α -treated *Lmp2*^{-/-} spleen cells (Fig. 3B). Antibodies to C/EBP had no effect on the DNA-protein complexes formed by the nuclear extracts of either mouse strain (Fig. 3B). To investigate whether p52 binds to the κ B1 oligonucleotide probe, we performed supershift assays with polyclonal antibodies to p52. These antibodies had no effect on the mobility of the DNA-protein complexes formed in nuclear extracts of TNF- α -treated spleen cells from either BALB/c or *Lmp2*^{-/-} mice or by those of TNF- α -treated Molt-4 cells with κ B1 oligonucleotide probe (data not shown).

The basal expression of NF- κ B subunits in the cytosolic and nuclear extracts of BALB/c and *Lmp2*^{-/-} mouse spleen cells was examined by immunoblot analysis (Fig. 3C). The abundance of p65, the precursor protein p105, and the precursor protein p100, as well as the amount of the I κ B α and the cyclin-dependent kinases CDK8, CDK7, and CDK2 (assayed as internal controls) did not differ markedly between BALB/c and *Lmp2*^{-/-} mice (Fig. 3C). However, the expression of p50 and p52 in the cytosolic extracts prepared from *Lmp2*^{-/-} spleen cells was markedly reduced relative to their expression in extracts from BALB/c spleen cells (Fig. 3C). Northern blot analysis also revealed that the abundance of both p65 and p105 mRNAs in cytosolic extracts of spleen cells did not differ between BALB/c and *Lmp2*^{-/-} mice (data not shown).

We also investigated the dynamics of I κ B α protein degradation during TNF- α -induced lymphocyte activation in spleen cells from BALB/c and *Lmp2*^{-/-} mice. I κ B α virtually disappeared from the cytosol of BALB/c spleen cells after exposure to TNF- α for 40 min (Fig. 3D). This decrease in cytosolic I κ B α was not accompanied by an increase in the amount of the protein in the nucleus (data not shown). The abundance of I κ B α in the cytosol of BALB/c spleen cells began to recover after treatment

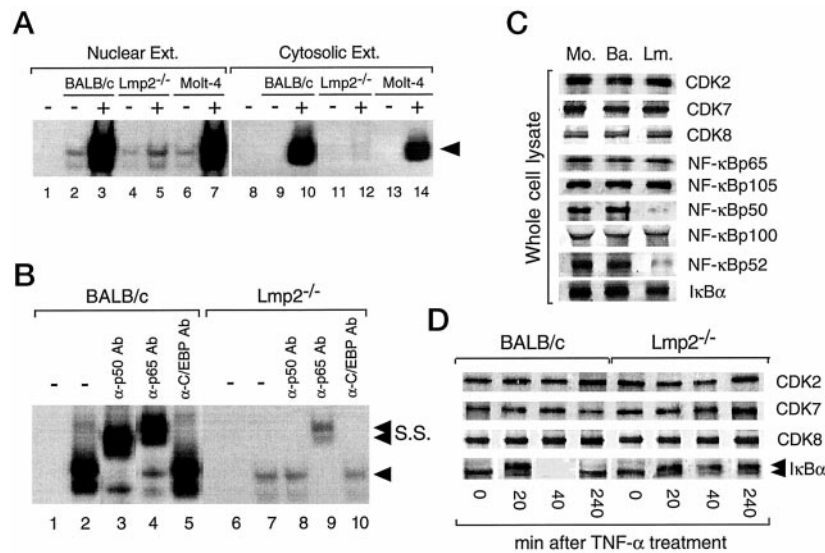


FIG. 3. Defective NF- κ B activation and degradation of I κ B α in TNF- α -treated Lmp2^{-/-} lymphocytes. *A*, NF- κ B DNA binding activity was examined by EMSA with the κ B1 oligonucleotide and nuclear extracts (*Ext*) prepared from BALB/c and Lmp2^{-/-} mouse spleen cells after incubation of cells for 4 h in the absence (-) or presence (+) of TNF- α (10 ng/ml) (*A*, left panel). The results of an identical experiment with TNF- α -treated Molt-4 cells are also shown (lanes 6 and 7). NF- κ B DNA binding activity in cytosolic extracts BALB/c and Lmp2^{-/-} mouse spleen cells or Molt-4 cells was analyzed by EMSA with the κ B1 oligonucleotide after incubation of extracts with (+) or without (-) Nonidet P-40 and deoxycholate detergent (*A*, right panel). Lanes 1 and 8 correspond to a negative control in which cytosolic extract was not added to the reaction mixture. *B*, spleen cells from BALB/c (left panel) or Lmp2^{-/-} (right panel) mice were treated with TNF- α (10 ng/ml) for 4 h. Nuclear extracts were then prepared and incubated in the absence (-) or presence (+) of polyclonal antibodies (Ab) to p50 (α -p50), to p65 (α -p65), or to C/EBP (α -C/EBP) before EMSA with the κ B1 oligonucleotide. Lanes 1 and 6 represent negative controls in which nuclear extract was not added to the reaction mixture. Original DNA-protein complexes and supershifted complexes (S.S.) are indicated by arrowheads. *C*, immunoblot analysis of NF- κ B subunits (p50, p52, p105, p65, and p100), I κ B α , and cyclin-dependent kinases in Molt-4 and spleen cells derived from BALB/c and Lmp2^{-/-} mice. Whole cell lysates of Molt-4 cells (*Mo.*) and BALB/c (*Ba.*) or Lmp2^{-/-} (*Lm.*) mice spleen cells were subjected to immunoblot analysis with antibodies to the indicated proteins. *D*, effect of TNF- α on the abundance of I κ B α in spleen cells of BALB/c and Lmp2^{-/-} mice. Spleen cells isolated from BALB/c and Lmp2^{-/-} mice were incubated with TNF- α (10 ng/ml) for the indicated times, after which cytosolic extracts were subjected to immunoblot analysis with antibodies to I κ B α or to CDKs.

with TNF- α for 4 h (Fig. 3D). In contrast, the amount of I κ B α in the cytosol of Lmp2^{-/-} mouse spleen cells was not markedly affected by TNF- α (Fig. 3D). The phosphorylated form of I κ B α was detected as the upper band of two immunoreactive bands in TNF- α -treated spleen cells from both BALB/c and Lmp2^{-/-} mice.

Impaired Processing of p50 in T2 Cell Extracts—The generation of p50 from p105 is mediated by the ubiquitin-proteasome processing pathway (28, 44, 46, 61). To investigate whether the decrease in p50 generation in T2 cells is directly attributable to defects in the proteasome processing pathway, we examined the processing of ³⁵S-labeled recombinant p105 in the cytosolic extracts of T2 cells in an *in vitro* assay (44, 46). Incubation of p105 with cytosolic extracts from T1, T2, Molt-4, or Jurkat cells in the absence of ATP did not result in the generation of p50 (Fig. 4A, upper panel). However, p50 was produced when p105 was incubated with cytosolic extracts from T1, Molt-4, or Jurkat cells in the presence of 10 mM ATP (Fig. 4A, lower panel); the generation of p50 has previously been shown to be processed by ATP-dependent pathway (44, 46). In contrast, incubation of p105 with cytosolic extracts of T2 cells even in the presence of 10 mM ATP did not result in the generation of p50 (Fig. 4A, lower panel). To confirm that the formation of p50 in this *in vitro* processing assay was mediated by the proteasome, we examined the effect of MG115 on p50 generation. MG115 is a potent inhibitor that binds to the chymotryptic site on the 20 S proteasome particle. This compound reduces the degradation of ubiquitin-conjugated proteins in cell extracts (46). The p50 processing in cytosolic extracts from T1, Molt-4, and Jurkat cells was completely inhibited by 50 μ M MG115 (Fig. 4B).

TNF- α induces phosphorylation of a PEST-rich domain downstream of ankyrin repeats in p105 (42, 62–64). We therefore examined the phosphorylation of recombinant p105 after

incubation with cytosolic extracts from T1 and T2 cells with [γ -³²P]ATP (Fig. 4C). Incubation with cytosolic extracts of T1 cells resulted in an increase in the extent of phosphorylation of p105 that reached a maximum at 30 min and decreased thereafter, presumably because the phosphorylated protein was degraded by the ubiquitin-proteasome pathway (Fig. 4C). In contrast, the phosphorylation of p105 by cytosolic extracts of T2 cells continued to increase for up to 40 min, presumably because the phosphorylated protein did not undergo proteolysis (Fig. 4C). Thus, the activity of the p105 kinase appeared to be normal in cytosolic extracts of T2 cells.

Ubiquitination of the ankyrin repeats of p105 is also required for its proteolytic processing (28, 46, 61, 62). We therefore examined the extent of ubiquitination of recombinant p105 after incubation with the cytosolic extracts from T1 and T2 cells (Fig. 4D). Cross-linking of ubiquitin-p105 complexes by glutaraldehyde treatment, followed by immunoprecipitation with anti-p50 antibodies and immunoblot analysis with antibodies to ubiquitin, revealed that the time courses for ubiquitination of p105 are similar to those for phosphorylation of p105. Whereas the ubiquitination of p105 by cytosolic extracts of T1 cells reached a maximum at 30 min and decreased thereafter, ubiquitination in cytosolic extracts of T2 cells continued to increase for up to 40 min (Fig. 4D). Ubiquitination activity therefore appeared not to be down-regulated in the cytosolic extracts of T2 cells (Fig. 4D). Overall, these data suggest that the defect in p50 generation in T2 cells is due to a failure of proteasome-mediated cleavage of p105.

We next examined the basal expression of components of the 20 S proteasome in cytosolic extracts of T1, T2, Molt-4, and Jurkat cells by immunoblot analysis (Fig. 4E). Whereas the proteasome subunits LMP2, LMP7, LMP10, and HC9 were detected as apparent bands in the cytosolic extracts from T1,

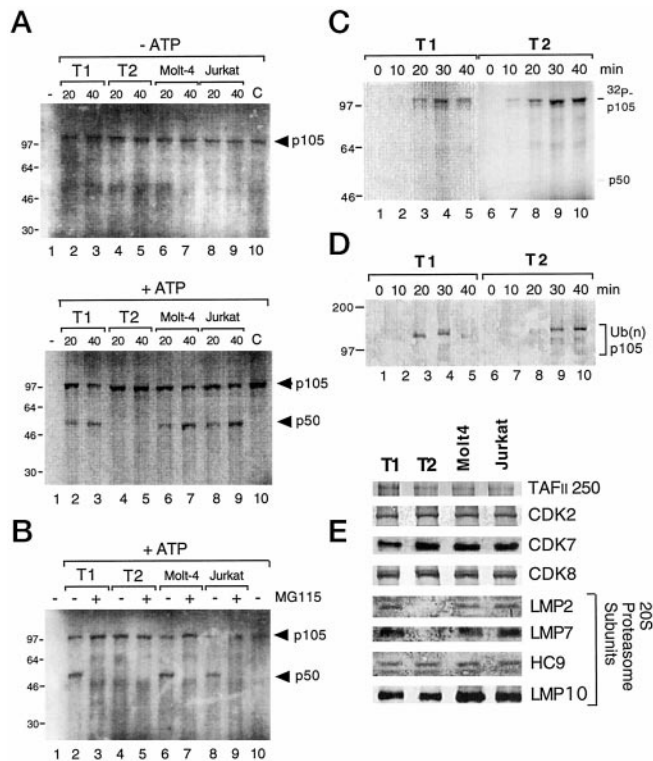


FIG. 4. *In vitro* assay of p50 processing by the ubiquitin-proteasome pathway in cytosolic extracts of T2 cells. *A* and *B*, assay of p50 generation from p105. Purified 35 S-labeled recombinant p105 was incubated for 90 min at 30 °C with cytosolic extracts (20 or 40 μ g of protein in *A* and 40 μ g of protein in *B*) of T1, T2, Molt-4, or Jurkat cells in the absence (*upper panel* in *A*) or presence (*lower panels* in *A* and *B*) of 10 mM ATP. *B*, incubations were also performed in the absence (–) or presence (+) of 50 μ M MG115. Reaction mixtures were analyzed by SDS-PAGE and autoradiography. *Lanes 1* in *A* and *B* correspond to reaction mixtures without extract and substrate; *lanes 10* correspond to reaction mixtures containing substrate but without extract. The positions of molecular size standards (in kilodaltons) are shown on the left, and those of p105 and p50 are shown on the right. *C*, phosphorylation of recombinant p105 by cytosolic extracts of T1 and T2 cells. Recombinant p105 was incubated for the indicated times at 30 °C in a reaction mixture (25 μ l) containing [γ - 32 P]ATP and cytosolic extracts (40 μ g of protein) of T1 or T2 cells, after which p105 was immunoprecipitated with antibodies to p50 and subjected to SDS-PAGE and autoradiography. The positions of phosphorylated p105 (32 P-p105) and of p50 are indicated. *D*, ubiquitination of recombinant p105 by cytosolic extracts of T1 and T2 cells. Recombinant p105 was incubated for the indicated times at 30 °C in a reaction mixture (25 μ l) containing cytosolic extracts (40 μ g of protein) of T1 or T2 cells, after which p105-ubiquitin complexes were cross-linked with glutaraldehyde, immunoprecipitated with antibodies to p50, and detected by immunoblot analysis with antibodies to ubiquitin. The position of ubiquitinated p105 (*Ub(n)-p105*) is indicated. *E*, immunoblot analysis of the expression of subunits of the 20 S proteasome in T1, T2, Molt-4, and Jurkat cells. Cytosolic extracts were subjected to immunoblot analysis with antibodies to the indicated proteins.

Molt-4, and Jurkat cell, as expected the T2 cell extracts specifically lacked basal expression of LMP2 and LMP7 (Fig. 4E). As an internal control, the basal expression of CDKs (CDK2, CDK7 and CDK8) and of TAF_{II}250 was shown not to differ among the four cell types (Fig. 4E).

Defective Transcriptional Activation by NF- κ B in T2 Cells—To examine further the transcriptional activation by NF- κ B activation *in vivo*, a transient luciferase assay was performed. T1, T2, Molt-4, and Jurkat cells were transfected with a reporter plasmid in which luciferase gene is expressed under the control of the IL-2R α promoter (IL-2R α - κ B wt) or its derivative which contains a mutant κ B binding region (IL-2R α - κ B mut) (Fig. 5A). A reporter plasmid expressing luciferase under the control of the RSV-LTR was used as an internal

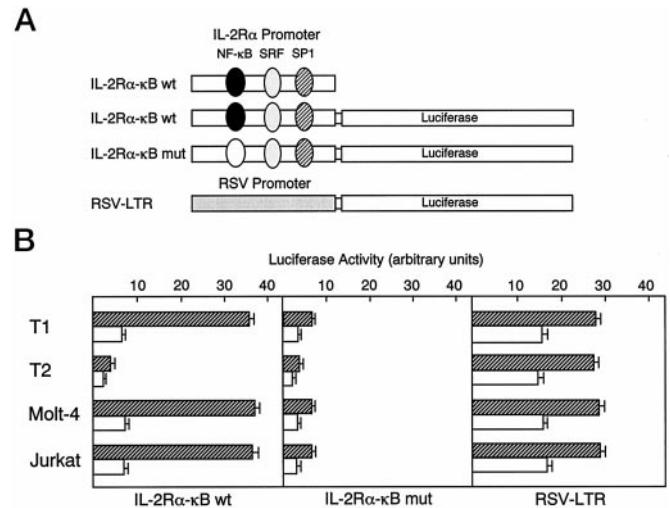


FIG. 5. Functional analysis of κ B-dependent transcriptional activation in T2 cells. *A*, schematic representations of IL-2R α luciferase reporter plasmids. The binding sites for the transcription factors NF- κ B, SRF, and SP1 located in the promoter of the IL-2R α gene are shown. The κ B binding sequence was mutated in the mutant IL-2R α promoter. *B*, effects of TNF- α on luciferase activity in transfected cells. T1, T2, Molt-4, and Jurkat cells were transfected with the indicated plasmids and an RSV- β -galactosidase expression vector, incubated for 4 h in the absence (*open bars*) or presence (*hatched bars*) of TNF- α (10 ng/ml), lysed, and assayed for luciferase and β -galactosidase activities. Differences in the efficiency of transfection were corrected based on β -galactosidase activity, and luciferase activity was expressed in arbitrary units. Data are means \pm S.E. of values obtained from three independent transcription experiments.

control (Fig. 5A). The transfected cells were cultured for 4 h in the absence or presence of TNF- α (final concentration 10 ng/ml), after which cell extracts were prepared and assayed for luciferase activity by the standard protocol (Fig. 5B). Luciferase activity under the IL-2R α promoter increased approximately 6-fold when the transfected T1, Molt-4, and Jurkat cells were treated with TNF- α (Fig. 5B, left panel). However, when T1, Molt-4, or Jurkat cells were transfected with IL-2R α - κ B mut, which no longer binds NF- κ B, little or no increase in luciferase activity occurred following treatment with TNF- α (Fig. 5B, center panel). These results indicate that in the transfected T1, Molt-4 or Jurkat cells, the luciferase gene was markedly stimulated by TNF- α -induced NF- κ B activation. However, luciferase activity was never dramatically induced in T2 cells transfected with IL-2R α - κ B regardless of whether the cells were stimulated by TNF- α treatment (Fig. 5B, left and center panels). These results indicate that TNF- α -induced activation of NF- κ B is defective in T2 cells. To verify the specificity of the impaired TNF- α -induced NF- κ B activation in T2 cells, we performed a luciferase assay with RSV-LTR plasmid, in which the luciferase gene is directly controlled under the RSV promoter (Fig. 5B, right panel). Luciferase activity was strongly induced in all cell types transfected with the RSV-LTR plasmid, including T2 cells (Fig. 5B, right panel). These results prove that T2 cells are insensitive to transcriptional activation in response to TNF- α , specifically due to the lack of sufficient NF- κ B activity.

Increased Susceptibility of T2 Cells to TNF- α -induced Apoptosis—NF- κ B activation has been shown to protect cells from TNF- α -induced cell death (13, 19, 20, 50–52). Furthermore, inhibiting the nuclear translocation of NF- κ B enhances the apoptotic effects of these agents. We therefore investigated the effect of TNF- α treatment on the viability of T2 cells and of Lmp2 $^{-/-}$ lymphocytes obtained from Lmp2 $^{-/-}$ mice. Incubation of T1, Molt-4, Jurkat cells, and normal murine lymphocytes derived from BALB/c mice with various concentrations of TNF- α for 24 h had no marked effect on cell survival. However,

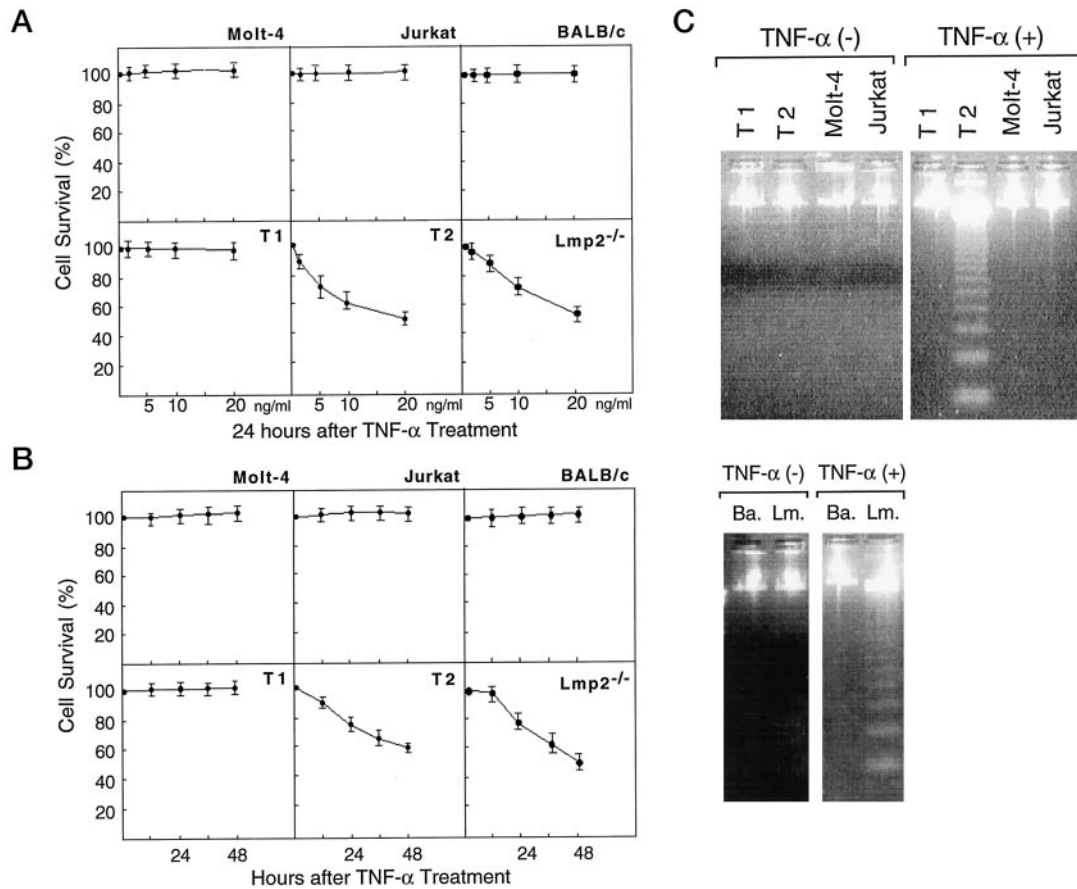


FIG. 6. Requirement of HLA-encoded proteasome subunits in preventing TNF- α -induced cell death. A and B, effect of TNF- α on the survival of T1, T2, Molt-4, Jurkat, lymphocytes from BALB/c, and Lmp2^{-/-} mice. Cells were cultured for 24 h with the indicated concentrations of TNF- α (A) or for the indicated times in the presence of TNF- α at 10 ng/ml (B). Cell viability was assessed by trypan blue exclusion. Data are expressed as percentage survival relative to that of cells not exposed to TNF- α and are means \pm S.D. of four replicates from a representative experiment. C, effect of TNF- α on DNA fragmentation. T1, T2, Molt-4, Jurkat, lymphocytes from BALB/c and Lmp2^{-/-} mice were incubated for 24 h in the absence or presence of TNF- α (10 ng/ml), after which DNA fragmentation was examined by agarose gel electrophoresis and ethidium bromide staining by standard method. Lymphocytes were obtained from BALB/c (Ba.) and Lmp2^{-/-} (Lm.) mice spleen.

TNF- α induced a dose-dependent decrease in the viability of T2 cells and Lmp2^{-/-} lymphocytes (Fig. 6A). Similarly, incubation of T1, Molt-4, Jurkat cells, and normal murine lymphocytes with TNF- α at a concentration of 10 ng/ml for up to 48 h had no effect on cell viability. The same concentration of TNF- α , however, induced a time-dependent decrease in the cell survival of T2 cells and Lmp2^{-/-} lymphocytes (Fig. 6B). This effect on cell survival was apparent as early as 12 h. DNA fragmentation assays followed by gel electrophoresis confirmed that TNF- α treatment resulted in apoptosis in T2 cells and Lmp2^{-/-} lymphocytes but not in T1, Molt-4, Jurkat cells, and normal murine lymphocytes (Fig. 6C). These results indicate that the proteasome subunits, Lmp2 and Lmp7, are required for NF- κ B activation and for the protection of cells from TNF- α -induced apoptosis.

DISCUSSION

Specific destruction of proteins *in vivo* plays a critical role in regulating diverse biological activities. So far, studies have shown that different targeting signals can lead to the degradation of proteins by the ubiquitin-proteasome pathway. The eukaryotic transcription factor superfamily, NF- κ B/Rel superfamily, is induced in response to several signals that lead to cell growth, differentiation, inflammatory responses, apoptosis, and neoplastic transformation. The NF- κ B-Rel complex is an ideal tertiary messenger for communicating signals necessary for these biological functions. The ubiquitin-proteasome signal pathway plays an essential role in two distinct steps for the

activation of NF- κ B as follows: it directs the production of the NF- κ B subunits p50 and p52 and the degradation of phosphorylated I κ B α . We now show that both of these mechanisms require the HLA-encoded proteasome β subunits LMP2 and LMP7. In cells lacking LMP2 and LMP7, the phosphorylation and ubiquitination of the precursor protein p105 appears normal in cytosolic extracts; however, the proteolytic processing of p105 to p50 by the ubiquitin-proteasome pathway was impaired (Fig. 4, A and B). Furthermore, neither p50 nor p52 was detected by immunoblot analysis in cytosolic or nuclear extracts of T2 cells and Lmp2^{-/-} lymphocytes (Fig. 2B and Fig. 3C). The ubiquitin-proteasome pathway also mediates the degradation of phosphorylated I κ B α , which leads to NF- κ B activation. Our data indicate that, whereas the phosphorylation of I κ B α in response to TNF- α appears to proceed normally in T2 cells and Lmp2^{-/-} lymphocytes, the degradation of the phosphorylated I κ B α by the ubiquitin-proteasome pathway is impaired, again indicating a requirement for the LMP2 and LMP7 and Lmp2^{-/-} lymphocytes proteasome subunits (Fig. 2C and Fig. 3D). These defects in proteasome function in T2 cells were associated with a marked decrease in the ability of TNF- α to induce NF- κ B activation, as revealed by both EMSA with cell extracts and luciferase assays on cells transfected with reporter plasmids (Figs. 1, 3, and 5).

Signaling by NF- κ B is linked to apoptosis, cellular transformation, and limb development (65). Thus, the defect in the proteasome-mediated synthesis and activation of NF- κ B in T2

cells and *Lmp2*^{-/-} lymphocytes was associated with an increased susceptibility to TNF- α -induced apoptosis (Fig. 6). We have therefore shown that the proteasome subunits LMP2 and LMP7 are required for generation of p50 and p52, for degradation of phosphorylated I κ B α , for NF- κ B activation, and for protection from TNF- α cytotoxicity in human and murine lymphocytes. This study also identified the HLA-linked LMP proteasome subunits with a new role in cell death protection.

The proteasome is a large multisubunit protease complex that is centrally involved in ubiquitin-mediated protein degradation in eukaryotic cells. Similarly LMP like complexes and biological functions are also found in bacteria and yeast (66–68). In eukaryotic cells, the 20 S-proteasome associates with a 19 S regulatory complex creating the 26 S proteasome. The 20 S proteasome is composed of two types of subunits, α and β . The α subunits are believed to form the outer structure; the inner β subunits contain the catalytic activity (69, 70). Mammalian proteasomes exhibit at least five distinct peptidase activities that are defined *in vitro* by an ability to cleave substrates at sites immediately downstream of basic (“trypsin-like” activity), hydrophobic (a “chymotrypsin-like” activity), or acidic (“peptidylglutamyl peptide hydrolyzing” activity) residues (67, 71). These activities are mediated by the 20 S proteasome β subunits and are subject to regulation by cytokines, at least in part through control of the expression of MHC region genes encoding LMP2 and LMP7 and the gene encoding LMP10 (72–74). Interferon- γ enhances antigen presentation by increasing the expression of these proteasome β subunit genes (75–78) which then replace the non-MHC-encoded β subunits X, Y, and Z. Furthermore, recent reports have demonstrated NF- κ B regulation of mammalian *Lmp2* gene expression and the essential requirement of LMP7 for the generation of mature LMP2 (77–80). Therefore in *Lmp7*^{-/-} lymphocytes, mature LMP2 protein is not produced, and NF- κ B activation is most likely not induced by TNF- α treatment. Presumably, TNF- α treatment significantly induces time- and dose-dependent decreases in viability of *Lmp7*^{-/-} lymphocytes. In *in vitro* assays, an increase in the amount of the MHC-encoded subunits is associated with an increased cleavage of peptides at sites downstream of hydrophobic or basic residues and reduced cleavage at sites downstream of acidic residues (81). A recent report has demonstrated p50 generation in yeast cells that continuously express a subunit homologous to LMP2 (28). Prior to this report, variations in mammalian proteasome subunit were known to impact antigen presentation. The present data extend the role of human HLA-encoded proteasome subunits as obligatory in transcription factor activation in eukaryotic cells (human and murine lymphocytes) and in protection from apoptosis.

In addition to the fact that the proteasome is responsible for the generation of peptides for MHC class I antigen presentation in mammals, there were reasons to suspect that the eukaryotic 20 S proteasome subunits might exhibit expanded substrate specificity based on the composition of the β subunits. The 20 S proteasome structure is highly conserved in evolution and is found in archaeobacterium *Thermoplasma acidophilum* as well as yeast such as *Saccharomyces cerevisiae* (82). Furthermore, the x-ray structure of the *Thermoplasma* proteasome verifies the assumption that the β -type subunits contain the proteolytically active site (69). Each of the β subunit peptidase activities assigned to 20 S proteasomes (basic, hydrophobic, and acid) are secondary to an NH₂-terminal threonine, and mutational analyses in yeast and archaeobacterium identified the similarly placed residues involved in proteolysis (83–86). The eukaryotic β subunits, LMP2, LMP7, and LMP10, similarly display the critical threonine residue in the NH₂

terminus region of the mature proteins. In sum, mutation of β -chain containing the NH₂-terminal threonine resulted in mutants with altered initial cleavage of protein substrates *in vivo* and varying phenotypes that often impacted growth. In total, yeast and mammalian proteasomes appear to have β subunits that contribute to specific catalytic activities linked to specific β subunits (87, 88). Regulation of β subunit composition is likely to have qualitative influences on diverse proteolytic products in eukaryotic organisms.

The T1 cell line is a cloned hybrid of a human B lymphoblastic cell line and a T lymphoblastic cell line. The T2 cell line is a variant of T1 cells in which both *Lmp2* and *Lmp7* have been deleted from the HLA class II region (89). T2 cells thus do not express the HLA class II antigens due to direct chromosomal deletion of the region, and they are indirectly HLA class I-negative due to the deletion of the antigen presentation genes in this region including *Lmp2* and *Lmp7*. T1 cells express large amounts of HLA DR7 and HLA A2 (89). Extracts of T2 cells exhibit defective proteasome activity with test substrates that reflect the lack of the LMP2 and LMP7 subunits (78, 90, 91).

The 20 S proteasome is essentially inactive because the β -catalytic sites form a narrow chamber. This requires the proteasome to bind to additional regulatory structures such as PA700 and PA28. The regulatory components of the proteasome are responsible for the ATPase activity and the ubiquitin dependence of the proteasome (92, 93). Proteasomal ATPases are thought to contribute to the unfolding of protein substrates so the substrate has access to the tight catalytic core (70). Six of the 20 subunits of the mammalian PA700 proteasome regulator contain nucleotide-binding consensus domains and belong to a large family of proteins known as AAA-type ATPases (70, 92–94). Proteasome AAA-type ATPases are highly homologous to one another and have been markedly conserved during evolution as have the proteasomes themselves. Members of this regulatory protein family are components of the proteasome in species as diverse as yeast, invertebrates, and mammals (95–97). Point mutations that impair the ATPase activity of proteasome-associated ATPases in budding yeast were recently shown to inhibit processing of p105 (28). PA28 is a proteasome activator only found in mammalian cells.

Resistance of cells to TNF- α -induced apoptosis is mediated by specific activation of NF- κ B. The role p65 plays in apoptosis became clear with the generation of p65 knockout mice. These p65^{-/-} mice die by day 15 of embryonic development, and the histologic examination reveals that this death is most likely caused by the massive apoptosis of hepatocytes in these knockout mice (14). Embryonic fibroblasts lacking p65 are susceptible to TNF- α -induced apoptosis, whereas wild-type and reconstituted cells demonstrate resistance to TNF- α toxicity (13). In contrast, cells depleted of p50 are resistant to TNF- α -induced apoptosis (13). Inhibition of NF- κ B by the overexpression of a dominant-negative I κ B α conferred a dramatic sensitivity to TNF- α -induced apoptosis in otherwise resistant cell types (19, 20, 50–52). Furthermore, expression of a catalytically inactive form of IKK- β , a component of the kinase complex that targets I κ B α for degradation, also inhibits activation of NF- κ B and renders cells sensitive to TNF- α -induced apoptosis (54). The fact that the biological function of NF- κ B in living cells to protect against the apoptotic signals mediated by a variety of agents suggests that it exerts its effects at a common distal point in the cellular response to these and other stimuli. Consistent with this conclusion, our data show that T2 cells and *Lmp2*^{-/-} lymphocytes are markedly sensitive to TNF- α -induced apoptosis and that the LMP2 and LMP7 subunits of the proteasome are required for proteasome function for NF- κ B activation.

Our data along with previously published data documents the essential role of the NF- κ B cascade for B cells when an apoptotic signal is present (51, 52). NF- κ B signaling components critical for TNF- α resistance include an intact p65 subunit with proper degradation of phosphorylated I κ B α mediated by IKK β and correct ubiquitin-proteasome function. NF- κ B thus probably functions directly as a pro-apoptotic factor as well as an indirect protector from TNF- α -induced cell death by induction of downstream protective factors. NF- κ B promotes cell cycle progression, and NF- κ B-mediated events could attenuate apoptotic signals. For instance, NF- κ B stimulation leads to an increase in the expression of the proto-oncogene product c-Myc; c-Myc protects cells from TNF- α -induced cell death by inducing gene transcription of cyclin A and D3, mediators of the cell cycle (98). Indeed, c-Myc expression was not significantly up-regulated in T2 cells treated by TNF- α (data not shown), confirming ablated downstream NF- κ B activity from the defective proteasome function. IEX-IL, another downstream protein, is also markedly up-regulated with TNF- α treatment and similarly diminished in cells with defective NF- κ B activation (99). Further analysis of the function and the transcriptional activities of *IEX-IL* gene in T1 and T2 cells and in *Lmp2*^{-/-} lymphocytes are necessary to determine the phenotypic effect of the polymorphism.

Several studies have demonstrated immune system abnormalities in knockout mice lacking NF- κ B or proteasome subunits as follows: (i) B cells derived from *p50*^{-/-} mice do not proliferate in response to CD40L or bacterial lipopolysaccharide, exhibit differentiation defects, secrete increased amounts of interferon- β , fail to undergo normal germ line CH gene transcription, and have abnormal immunoglobulin class switching (18, 34); (ii) both splenic microarchitecture and B cell responses are altered in *p52*^{-/-} mice (31); (iii) development of both B cells and osteoclasts is defective in *p50*^{-/-} and *p52*^{-/-} double knockout mice (32, 33); (iv) the development of CD8⁺ T lymphocytes and MHC class I molecule expression is abnormal in *LMP2*^{-/-} mice (100); and (v) the extent of both surface expression of MHC class I molecules and MHC class I-restricted antigen presentation is reduced in *LMP7*^{-/-} mice (101, 102). Recent reports also demonstrate down-regulation of *Lmp2* transcriptional expression in spleen cells from the nonobese diabetic mouse, which is an animal model of human type 1 (autoimmune) diabetes (78). Furthermore, down-regulation of *LMP2* and *LMP7* is associated with oncogenic progression in malignant melanoma (103). These observations indicate the importance of the proteasome and NF- κ B function for normal immune response networks.

Prior to this report, a role of HLA genes in apoptosis protection was undescribed. Our findings may have possible disease implications for human disease because most autoimmune diseases show strong statistical risk mapping to the HLA region, and diminished apoptosis protection is apparent in some disease settings (104). Furthermore, proteasome isolated from autoimmune diabetic patients and spontaneous diabetic mice (nonobese diabetic) *in vitro* demonstrated altered proteasome activity indicative of HLA-encoded proteasome subunit malfunction (78, 90). Thus, specific proteasome subunits and components of the NF- κ B signaling pathway are potential targets for the development of drugs for the treatment of immunological diseases and oncogenesis.

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