



# Cardiac-specific Abrogation of NF- $\kappa$ B Activation in Mice by Transdominant Expression of a Mutant I $\kappa$ B $\alpha$

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B. DAWN, Y.-T. XUAN, M. MARIAN, M. P. FLAHERTY, S. S. MURPHREE, T. L. SMITH, R. BOLLI AND W. K. JONES. Cardiac-specific Abrogation of NF- $\kappa$ B Activation in Mice by Transdominant Expression of a Mutant I $\kappa$ B $\alpha$ . *Journal of Molecular and Cellular Cardiology* (2001) 33, 161–173. Nuclear factor-kappaB (NF- $\kappa$ B) is a pleiotropic oxidant-sensitive transcription factor that is present in the cytosol in an inactive form complexed to an inhibitory kappaB (I $\kappa$ B) monomer. Various stimuli, including ischemia, hypoxia, free radicals, cytokines, and lipopolysaccharide (LPS), activate NF- $\kappa$ B by inducing phosphorylation of I $\kappa$ B. Phosphorylation of serine residues at positions 32 and 36 is critical for ubiquitination and degradation of I $\kappa$ B $\alpha$  with consequent migration of NF- $\kappa$ B to the nucleus. Although NF- $\kappa$ B is thought to contribute to numerous pathophysiologic processes, definitive assessment of its role has been hindered by the inability to achieve specific inhibition *in vivo*. Pharmacologic inhibitors of NF- $\kappa$ B are available, but their utility for *in vivo* studies is limited by their relative lack of specificity. Targeted ablation of genes encoding NF- $\kappa$ B subunits has not been productive in this regard because of fetal lethality in the case of p65 and functional redundancy in the Rel family of proteins. To overcome these limitations, we have created a viable transgenic mouse that expresses a phosphorylation-resistant mutant of I $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup>) under the direction of a cardiac-specific promoter. Several transgenic lines were obtained with copy numbers ranging from one to seven. The mice exhibit normal cardiac morphology and histology. Total myocardial I $\kappa$ B $\alpha$  protein level is elevated 3.5- to 6.5-fold with a concomitant 50–60% decrease in the level of I $\kappa$ B $\beta$ . Importantly, expression of I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> results in complete abrogation of myocardial NF- $\kappa$ B activation in response to tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and LPS stimulation. Thus, novel transgenic mice have been created that make it possible to achieve cardiac-specific and selective inhibition of NF- $\kappa$ B *in vivo*. These transgenic mice should be useful in studies of various cardiac pathophysiological phenomena that involve NF- $\kappa$ B activation, including ischemic preconditioning, heart failure, septic shock, acute coronary syndromes, cardiac allograft rejection, and apoptosis.

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KEY WORDS: Transgenic mouse; Myocardium; I $\kappa$ B $\alpha$ ; NF- $\kappa$ B; TNF- $\alpha$ ; LPS.

## Introduction

Nuclear factor-kappaB (NF- $\kappa$ B) is a ubiquitous dimeric transcription factor that exists in the cytosol complexed to an inhibitory kappaB (I $\kappa$ B) monomer. NF- $\kappa$ B complexes consist of either a p50 (NF- $\kappa$ B1)

or a p52 (NF- $\kappa$ B2) subunit complexed to another protein from the Rel group [which includes p65 (RelA), c-Rel and RelB].<sup>1,2</sup> Various stimuli, including ischemia, cytokines, hypoxia, free radicals and oxidants, lipopolysaccharide (LPS), activators of protein kinase C, ultraviolet radiation, and viruses

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activate NF- $\kappa$ B.<sup>1</sup> Activated NF- $\kappa$ B translocates to the nucleus and binds to specific DNA binding sites, called  $\kappa$ B, present within the promoter regions of various genes including those encoding p65, I $\kappa$ B $\alpha$ , tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-2, IL-6, IL-8, interferon (IFN)- $\beta$ , inducible nitric oxide synthase (iNOS), and several adhesion molecules.<sup>1,2</sup> Activation of NF- $\kappa$ B is controlled by site-specific phosphorylation and ubiquitination of I $\kappa$ B proteins that target I $\kappa$ B for proteasome-mediated degradation.<sup>3</sup> There are currently seven known I $\kappa$ B isoforms: I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\gamma$ , Bcl-3, I $\kappa$ B $\epsilon$ , I $\kappa$ B-R, and I $\kappa$ B-L,<sup>2</sup> and each is capable of binding all possible NF- $\kappa$ B dimers, though with variable avidity.<sup>2</sup> Of these different I $\kappa$ B proteins, I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  have been studied most extensively, and are thought to be the key regulators of NF- $\kappa$ B nuclear translocation. Phosphorylation of serine residues at positions 32 and 36 is critical for ubiquitination and degradation of I $\kappa$ B $\alpha$ <sup>4</sup> with consequent release and nuclear migration of NF- $\kappa$ B. I $\kappa$ B $\beta$  is also regulated in an analogous manner.<sup>5</sup> However, the signaling events leading to degradation of I $\kappa$ B in the myocardium and its role in myocardial NF- $\kappa$ B activation are virtually unknown.

Mounting evidence indicates that NF- $\kappa$ B plays a major role in various cardiac pathophysiological processes, including ischemic preconditioning,<sup>6</sup> congestive heart failure (CHF),<sup>7</sup> unstable angina pectoris,<sup>8</sup> atherogenesis and expression of adhesion molecules,<sup>9</sup> cardiac allograft rejection,<sup>10</sup> and apoptosis.<sup>11</sup> Although the etiologies of these diverse clinical entities are varied, the signal transduction cascades activated during these processes intersect at the activation of NF- $\kappa$ B. Furthermore, experimental evidence suggests that activation of NF- $\kappa$ B by signaling pathways that involve TNF- $\alpha$  and LPS occurs during the genesis of a wide spectrum of cardiac pathophysiological states, including congestive heart failure and apoptosis. Unequivocal assessment of the functional contribution of NF- $\kappa$ B to these processes, however, has been impeded by the lack of means to achieve selective inhibition of this transcription factor *in vivo*.

Although various pharmacologic inhibitors of NF- $\kappa$ B are available for *in vivo* use, none of these has sufficient specificity to provide conclusive evidence of NF- $\kappa$ B function.<sup>12,13</sup> A number of investigators have accomplished gene knockouts of several of the NF- $\kappa$ B encoding genes.<sup>14–17</sup> However, NF- $\kappa$ B signaling was not completely abrogated in these mice,<sup>14–16</sup> probably due to the functional redundancy of NF- $\kappa$ B subunits. Furthermore, RelA (p65) knockout mice die *in utero* due to widespread liver apoptosis.<sup>17</sup> Subsequent to the identification of

the specific phosphorylation sites of I $\kappa$ B $\alpha$ ,<sup>4</sup> another approach to repress NF- $\kappa$ B activity has been the expression of transdominant mutant I $\kappa$ B $\alpha$  proteins defective in phosphorylation. A mutant of human I $\kappa$ B $\alpha$  having the serine residues at positions 32 and 36 substituted by glycine and alanine, respectively, was shown to repress activation of all NF- $\kappa$ B signaling in NTera-2 cells.<sup>18</sup> Similarly, a cDNA with both serines replaced by alanines (I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup>) was used to abrogate NF- $\kappa$ B activation in Jurkat T-cells.<sup>19,20</sup> Transgenic expression of a truncated I $\kappa$ B $\alpha$  lacking these phosphorylation sites in a T-cell-specific manner resulted in profound inhibition of NF- $\kappa$ B activity in thymocytes.<sup>21</sup>

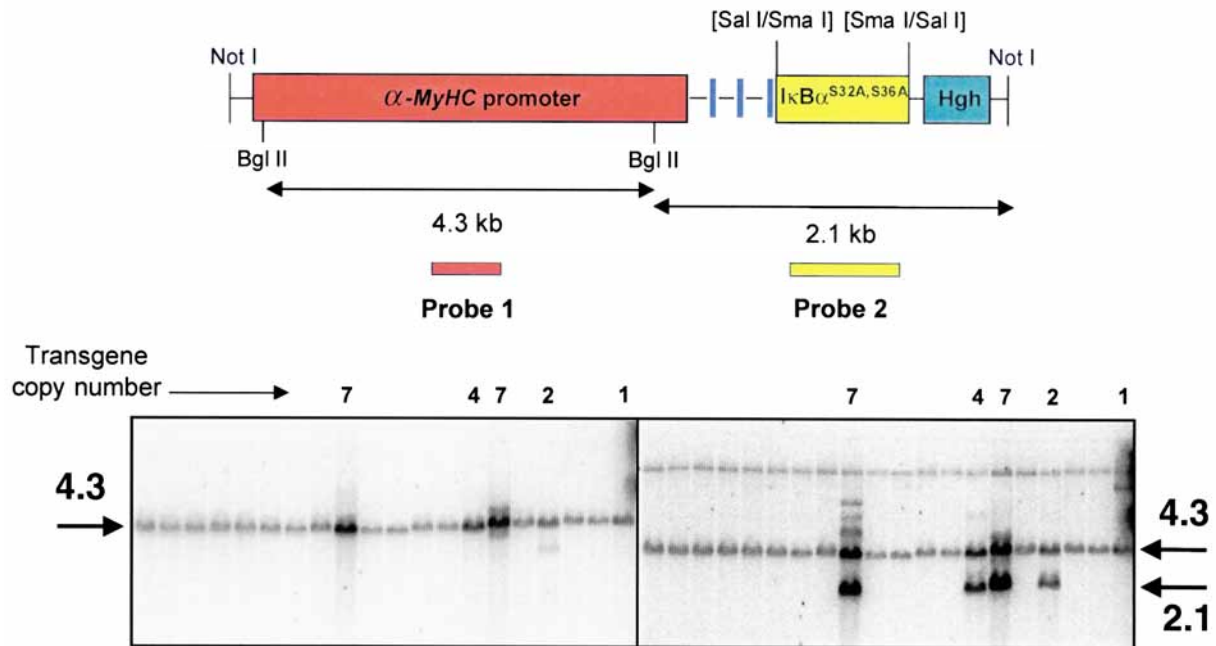
In order to determine whether expression of a transdominant mutant I $\kappa$ B $\alpha$  abrogates NF- $\kappa$ B activation specifically in the heart, we created transgenic mice with cardiac-specific expression of I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup>. The  $\alpha$ -*MyHC* promoter was chosen to drive expression of I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> since this promoter directs high level, cardiac-specific gene expression in a position-independent, copy-number-dependent manner.<sup>22,23</sup> TNF- $\alpha$  and LPS were selected as stimuli for the present study because of their potency in eliciting NF- $\kappa$ B activation as well as their physiological relevance in orchestrating NF- $\kappa$ B signaling.<sup>24–26</sup> The results demonstrate that I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> transgenic mice exhibit normal cardiac morphology and histology and that the expression of the mutant I $\kappa$ B $\alpha$  completely blocks NF- $\kappa$ B activation by both TNF- $\alpha$  and LPS. These mice provide an *in vivo* system, for the first time, to study and conclusively determine the role of NF- $\kappa$ B in specific cardiac pathophysiological states.

## Materials and Methods

The present study was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Louisville School of Medicine and with the *Guide for the Care and Use of Laboratory Animals* (Department of Health and Human Services, Publication No. [NIH] 86-23).

### Generation of I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> transgenic mice

We constructed a transgene for the expression in the murine heart of a transdominant human I $\kappa$ B $\alpha$  cDNA (with serine residues at positions 32 and 36 replaced by alanine, I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup>). The human I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> cDNA (a kind gift from Dr John Hiscott, Department of Biochemistry, McGill University, Montreal, Canada) was released from the original



**Figure 1** Diagrammatic representation of the transgenic promoter construct (upper panel). The 1.1 kb human  $I\kappa B\alpha$  cDNA ( $I\kappa B\alpha^{S32A,S36A}$ ) was subcloned between the 5.5 kb  $\alpha$ -MyHC promoter and the 736 bp human growth hormone (Hgh) polyadenylation signal. The cDNA was released from plasmid pSVK3 by excision with *Sma* I and cloned into the *Sal* I restriction site of the  $\alpha$ -MyHC vector.<sup>22</sup> The transgene was excised from the plasmid by restriction with *Not* I and used for pronuclear microinjection. The 4.3 kb fragment from the  $\alpha$ -MyHC promoter and the 2.1 kb fragment containing a portion of the  $I\kappa B\alpha^{S32A,S36A}$  cDNA were identified by Southern analysis following digestion with *Bgl* II and using, as probes, an 800 bp complementary sequence from the  $\alpha$ -MyHC promoter (probe 1) and the entire  $I\kappa B\alpha^{S32A,S36A}$  cDNA (probe 2), respectively (lower panel). Five founders with 1, 2, 7, 4, and 7 copies of the transgene were identified. We were unable to obtain any offspring from the single copy number founder. The results of the Southern analysis were consistent with the results of PCR screening (Materials and Methods).

plasmid (pSVK3)<sup>20</sup> by restriction digestion with *Sma* I and subcloned between the 5.5 kb  $\alpha$ -MyHC promoter and the 736 bp human growth hormone polyadenylation sequences<sup>22</sup> (Fig. 1). Transgenic mice were made by pronuclear microinjection of the isolated purified linear transgene into the male pronuclei of fertilized mouse oocytes (FVB/N strain) at the transgenic core facility at the University of Cincinnati according to established methods.<sup>27</sup> The founder transgenic mice were genotyped by PCR, using an  $\alpha$ -MyHC-specific primer (5'-AAGCCT-AGCCACACCAGAAATGACAGACA-3') and an  $I\kappa B\alpha$ -specific primer (5'-AGTAGCCGCTCCTTC-TTCAGCCCGTC-3'). PCR was performed using the following steps: 94°C for 7 min, followed by 94°C for 1 min, 63°C for 30 s, and 72°C for 30 s for 35 cycles, with a final extension at 72°C for 10 min.

#### Southern blot analysis

To confirm genotypes and to determine copy number of the transgene in founder mice, approximately

30  $\mu$ g of genomic DNA was digested with *Bgl* II, run overnight on 0.8% agarose gel, transferred onto nitrocellulose membrane and probed with DNA fragments, one complementary to a portion of the  $\alpha$ -MyHC promoter (Probe 1), and the other complementary to  $I\kappa B\alpha$  (Probe 2). The transgenic founders were identified by the presence of the transgene-specific 2.1 kb band (Fig. 1). The copy number of the transgene in each line was determined by comparing the intensity of endogenous bands of known copy number with bands of similar size derived from the transgene, using a STORM 840 image analyzer and Imagequant software (Molecular Dynamics, Sunnyvale, CA, USA). Founder mice positive for the transgene were bred to derive four (a fifth founder did not produce  $F_1$ ) independent lines, formally designated as TgN(IKBABD)17-Kykj (two copies of transgene), TgN(IKBABD)44-Kykj (seven copies of transgene), TgN(IKBABD)-151Kykj (four copies of transgene), and TgN(IKBABD)159Kykj (seven copies of transgene).

### Histologic analyses

Heart wt to body wt ratio (mg/g) was assessed in age- (22–28 wk) and sex (male)-matched transgenic and non-transgenic (NTg) mice ( $n = 4$  in each group). Mice were euthanized by CO<sub>2</sub> inhalation and the hearts were excised while still beating. Following excision of all extracardiac structures, hearts were incised to expose the cavities, drained of all intracavitary blood, washed in  $1 \times$  PBS, blotted to remove PBS, and weighed. For histologic analyses, following euthanasia, hearts were perfused *in situ* under pressure (80 mmHg) through the apex with ice-cold  $1 \times$  PBS using a 20-gauge needle. This was immediately followed by perfusion with ice-cold cardioplegic solution (25 mM KCl, 5% dextrose in  $1 \times$  PBS) to ensure myocardial relaxation. Fixation was done by perfusion under pressure (80 mmHg) using 4% paraformaldehyde in  $1 \times$  PBS, and the hearts allowed to fix further overnight. Next day, the hearts were dehydrated through a graded series of alcohols and paraffin-embedded with apex down, with care taken to orient them similarly. Step serial sections were taken every 75  $\mu$ m from apex to base and mounted on slides. Adjacent sections were stained with hematoxylin and eosin and with Masson's trichrome and examined microscopically. The entire procedure, including examination by an experienced pathologist, was done in a blinded manner. Photomicrographs were taken using a Nikon Microphot-FXA microscope (Nikon Inc., Melville, NY, USA) and a digital camera (Kodak DCS 420, Eastman Kodak, Rochester, NY, USA).

### Western immunoblotting

#### *Tissue preparation*

Murine ventricular tissue samples were homogenized in buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, 10 mM benzamide, 50  $\mu$ g/ml PMSE, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml pepstatin A, and 0.3%  $\beta$ -mercaptoethanol. Protein concentration was determined by the Bradford method<sup>28</sup> (Bio-Rad, Hercules, CA, USA). Homogenates were aliquoted and stored at  $-80^{\circ}\text{C}$ .

#### *Western blotting*

Total myocardial I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  protein contents were assessed using standard SDS-PAGE Western

immunoblotting. Briefly, 100  $\mu$ g of protein was electrophoresed on an 8% denaturing SDS gel for 7 h and transferred to nitrocellulose membranes (Amersham, Piscataway, NJ, USA) overnight at  $4^{\circ}\text{C}$ . The membranes were stained with Ponceau S and computer densitometry was used to determine relative loading. The filters were then blocked with 5% non-fat dry milk in TBS [10 mM Tris-HCl (pH 7.2), 0.15 M NaCl] containing 0.1% Tween 20 (TTBS buffer). The blot was incubated with primary antibodies against I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a 1:2000 dilution for 1 h at room temperature. Blots were washed four times (5 min each) with TTBS buffer at RT and incubated with HRP-conjugated goat anti-rabbit secondary antibody (Bio-Rad) at a dilution of 1:5000. Signal detection was by enhanced chemiluminescence (ECL) using a NEN Renaissance kit (NEN Life Science Products, Boston, MA, USA). Immunoreactive proteins were visualized by exposure of the membrane to X-ray film (X-OMAT-AR, Eastman Kodak), and quantitated using an image scanning densitometer and Imagequant software (Personal Densitometer SI, Molecular Dynamics). The I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  signals were normalized to the Ponceau stain signal.

### Electrophoretic mobility shift assay

#### *Preparation of nuclear extracts*

Nuclear extracts from ventricular tissue samples were prepared using a modification of a previously described method.<sup>29</sup> The samples were homogenized (Bessman Tissue Pulverizer, Fisher Scientific Co., Pittsburgh, PA, USA) in buffer A [10 mM Hepes (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 1 mM DTT, 20  $\mu$ M leupeptin, and 1 mM PMSF]. After a 10-min incubation on ice, the samples were centrifuged at  $1850 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The pellets were dissolved in buffer B (Buffer A + 0.1% Triton X-100), incubated on ice for 10 min, and then centrifuged as above. The crude nuclear pellets, washed once with buffer A, were resuspended in buffer C [20 mM Hepes (pH 7.9), 25% glycerol (v/v), 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM DTT, 20  $\mu$ M leupeptin, and 1 mM PMSF] for 30 min at  $4^{\circ}\text{C}$ . The supernatants resulting from centrifugation at  $25\,000 \times g$  for 30 min were dialyzed against 100 volumes of buffer D [20 mM Hepes (pH 7.9), 4% glycerol (v/v), 50 mM NaCl, 0.5 mM EDTA, 1 mM MgCl<sub>2</sub>, 0.5 mM DTT, 20  $\mu$ M leupeptin, and 0.5 mM PMSF] for 6 h at  $4^{\circ}\text{C}$ . The dialysates were centrifuged again at  $25\,000 \times g$  for 30 min and the

resulting supernatants designated as the nuclear protein extracts. The nuclear extracts prepared in this manner were previously shown to be free of significant cytoplasmic contamination as judged by assay of LDH, a cytosolic marker.<sup>29</sup>

#### Electrophoretic mobility shift assay

A double-stranded 22 bp oligonucleotide (5'-AGTTGAGGGGACTTTCCAGGC-3') (Promega, Madison, WI, USA) containing the consensus sequence for NF- $\kappa$ B binding was end-labeled using [ $\gamma$ -<sup>32</sup>P]dATP and T4 polynucleotide kinase (Promega), and purified using a G-25 Sephadex column (Pharmacia Biotech, Piscataway, NJ, USA). The binding reactions were performed in a final volume of 10  $\mu$ l containing 8  $\mu$ g of nuclear protein, 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol (v/v), and 1  $\mu$ g Poly(dI-dC)·(Poly(dI-dC)). After a 10-min preincubation at 4°C, the labeled probe (approximately 100 000 cpm/reaction) was added to each reaction mixture and incubated for an additional 20 min at 22°C. The DNA-protein complexes were then separated on 4% non-denaturing polyacrylamide gels in 0.5  $\times$  Tris borate-EDTA buffer. Gels were vacuum-dried and exposed to X-ray film at -70°C using an intensifying screen or imaged using a phosphorimager (STORM 840, Molecular Dynamics). Gel supershift and competition assays were performed to ensure that the signal was specific for NF- $\kappa$ B. For the supershift assays, 0.3  $\mu$ g of anti-p65 antibody (Santa Cruz Biotechnology Inc.) was added to the reaction mixture immediately after addition of radiolabeled probe. For the competition assays, 100-fold molar excess of unlabeled NF- $\kappa$ B consensus oligonucleotide or an unlabeled mutated NF- $\kappa$ B oligonucleotide (Promega) were added into separate reaction mixtures. The specificity of a particular band representing NF- $\kappa$ B DNA-binding activity was verified by the ability of the consensus NF- $\kappa$ B oligonucleotide to reduce or abrogate that band (specific competition), and the inability of the mutated oligonucleotide to do so while competing several non-specific complexes (non-specific competition) (Fig. 4).

#### Studies of NF- $\kappa$ B activation

In order to determine whether transdominant expression of I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> abrogates myocardial NF- $\kappa$ B activation *in vivo*, mice received i.p. injection of vehicle (sterile water), recombinant murine TNF- $\alpha$  (rm-TNF- $\alpha$ , Peprotech, Rocky Hill, NJ, USA) or LPS

(Sigma Chemical Co., St. Louis, MO, USA). Five groups of transgenic and NTg mice (10–14 weeks old, body weight 22–25 g) were used. Mice in group I (control, NTg,  $n=4$ ) received i.p. injection of 0.4 ml sterile water. Mice in groups II (NTg,  $n=4$ ) and III (transgenic,  $n=4$ ) received i.p. injection of rm-TNF- $\alpha$  (2.5  $\mu$ g in 0.4 ml sterile water). Mice in groups IV (NTg,  $n=2$ ) and group V (transgenic,  $n=2$ ) received i.p. injection of LPS (6 mg/kg in approximately 0.4 ml sterile water). All mice were killed 30 min after the i.p. injection and ventricular tissue samples were harvested.

The concentration of TNF- $\alpha$  used in previous studies conducted both *in vivo* and *in vitro* to examine the activation of NF- $\kappa$ B in cultured cells and tissues varies over a wide range (10 ng/ml to 100 ng/ml).<sup>30–32</sup> Since calculated *in vivo* concentrations (derived from total dose used) as low as 40 ng/ml have been shown to activate NF- $\kappa$ B,<sup>31</sup> we selected a dose of 2.5  $\mu$ g/mouse of rm-TNF- $\alpha$  administered i.p. (expected to produce a serum concentration of approximately 145 ng/ml in a 25 g mouse) to activate NF- $\kappa$ B. A dose of 4 mg/kg of bacterial LPS has been shown to be adequate to activate NF- $\kappa$ B *in vivo* in several previous studies.<sup>33,34</sup> In the present study, we selected a dose of 6 mg/kg of LPS to ensure activation of NF- $\kappa$ B while avoiding potential lethality.

#### Time-course of NF- $\kappa$ B activation

The time-course of NF- $\kappa$ B activation was determined in six groups ( $n=2$  in each group) of transgenic mice (groups VII, IX and XI) and NTg littermates (groups VI, VIII and X). All mice received an i.p. injection of rm-TNF- $\alpha$  (2.5  $\mu$ g in 0.4 ml sterile water). Mice were killed 1 h (groups VI and VII), 3 h (groups VIII and IX) and 6 h (groups X and XI) after the rm-TNF- $\alpha$  injection, and ventricular tissue samples were harvested.

## Results

#### I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> transgenic mice

Of the 20 live-born mice obtained after pronuclear microinjection and embryo transplantation, five were identified as transgenic founders by PCR and confirmed by Southern blot analysis. The transgene copy numbers in these five lines were 1, 2, 4, 7 and 7. There were no obvious anatomical defects in the heart or other organs as revealed by pathologic examination. We observed no macroscopic evidence of

cardiac hypertrophy or cardiac morphologic anomaly in transgenic mice. The heart weight to body weight ratio (mg/g, mean  $\pm$  s.e.m.) in transgenic mice ( $4.10 \pm 0.28$ ;  $n=4$ ) was not significantly different from that in NTg mice ( $4.55 \pm 0.11$ ;  $n=4$ ,  $P=NS$  by Student's *t*-test).<sup>35</sup> There was no abnormality in size of cardiac chambers. We observed no unusual pre- or post-natal lethality attributable to the transgene.

#### *Histologic examination*

Microscopic examination of ventricular tissue sections stained with hematoxylin and eosin did not reveal any abnormality in the transgenic relative to NTg mice (Fig. 2). There was no evidence of right or left ventricular dilatation or wall thickening, including the interventricular septum. Similarly, we observed no cellular disarray, myocyte hypertrophy, abnormal size or shape of nuclei or abnormal cardiac vasculature. Examination of tissue sections stained with Masson's trichrome stain revealed no evidence of pathologic fibrosis in the myocardium or endocardium of hearts from transgenic mice (Fig. 2).

#### **Myocardial I $\kappa$ B protein content**

Quantitative analysis of Western blots demonstrated that the total amount of myocardial I $\kappa$ B $\alpha$  protein was increased 3.5- to 6.5-fold in transgenic relative to NTg mice (Fig. 3). There was a concomitant 50–60% decrease in the levels of myocardial I $\kappa$ B $\beta$  in all lines (Fig. 3).

#### **Abrogation of myocardial NF- $\kappa$ B activation**

Two different agents (TNF- $\alpha$  and LPS) known to be potent activators of NF- $\kappa$ B were used to determine whether transgenic expression of the mutant I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> blocks activation of NF- $\kappa$ B in the myocardium.

#### *TNF- $\alpha$ stimulation*

EMSA performed using nuclear extracts of ventricular tissue from NTg mice after i.p. injection of sterile water (group I) failed to demonstrate any detectable NF- $\kappa$ B DNA binding activity, confirming that the vehicle or the i.p. injection itself does not activate NF- $\kappa$ B in the heart (Fig. 4). A robust activation of NF- $\kappa$ B was observed in the myocardium of NTg mice after TNF- $\alpha$  administration (group II). In contrast, in I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> transgenic mice (group

III), TNF- $\alpha$  completely failed to activate myocardial NF- $\kappa$ B (Fig. 4). Competition assays using probes containing the consensus and mutated NF- $\kappa$ B binding sites confirmed the specificity of the DNA binding activity for the NF- $\kappa$ B site (Fig. 4).

#### *LPS stimulation*

EMSA performed using nuclear extracts from NTg mice after LPS administration (group IV) demonstrated myocardial NF- $\kappa$ B DNA-binding activity (Fig. 4). Activation of myocardial NF- $\kappa$ B in response to LPS stimulation was, within the limits of detection of the assay, abrogated in I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> transgenic mice (group V, Fig. 4).

#### *Time-course of NF- $\kappa$ B activation*

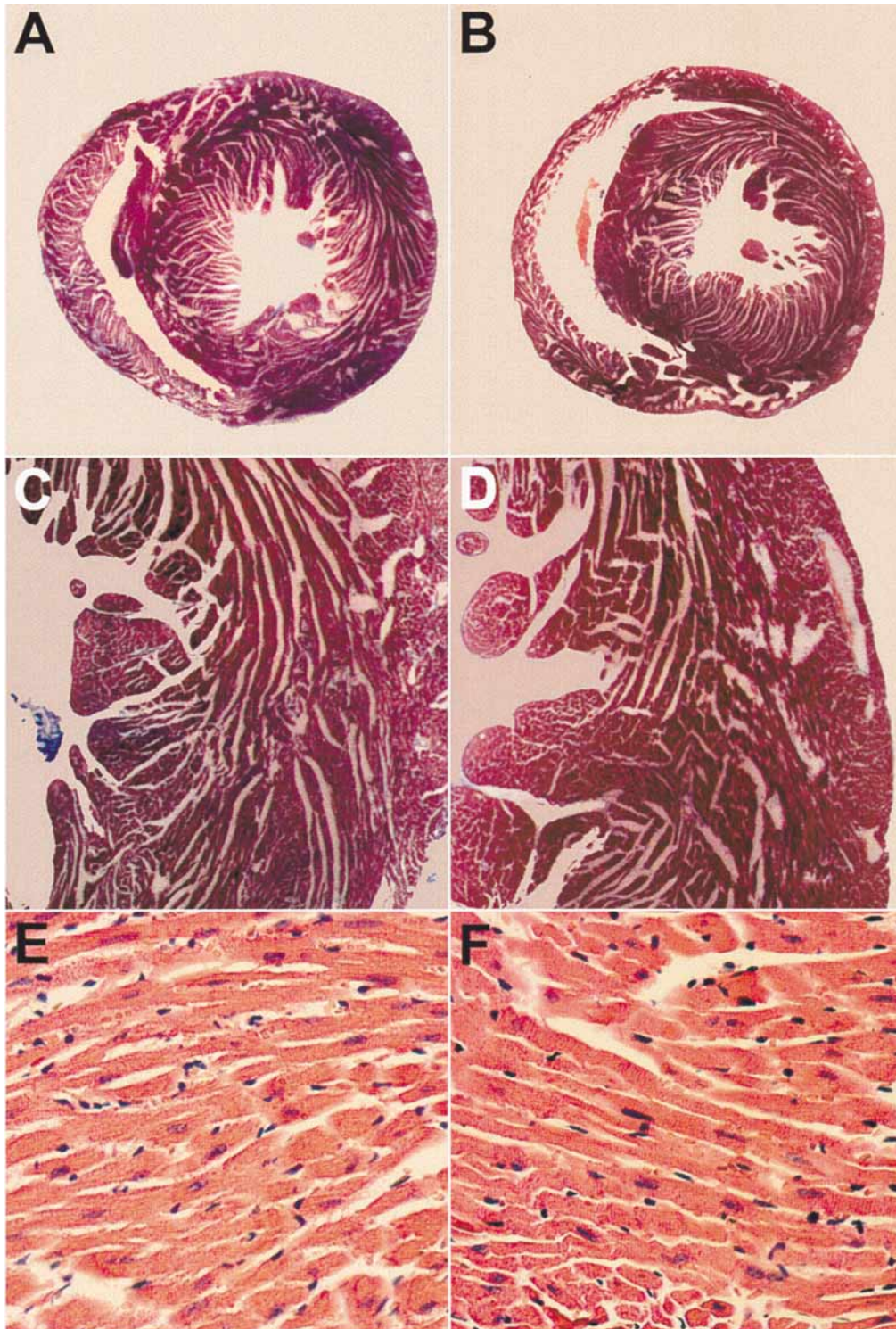
As shown above, we detected a robust induction of NF- $\kappa$ B DNA-binding activity 30 min after TNF- $\alpha$  administration (group II, Fig. 4). EMSA performed using nuclear extracts from NTg mice after similar treatment demonstrated a time-dependent decrease in the intensity of myocardial NF- $\kappa$ B activation at 1 h, 3 h, and 6 h (groups VI, VIII and X, respectively, Fig. 5). The NF- $\kappa$ B DNA binding activity was nearly undetectable in NTg mice 6 h after TNF- $\alpha$  injection (group X). There was no detectable NF- $\kappa$ B DNA-binding activity at any time-point in the I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> transgenic mice.

#### *Supershift assays*

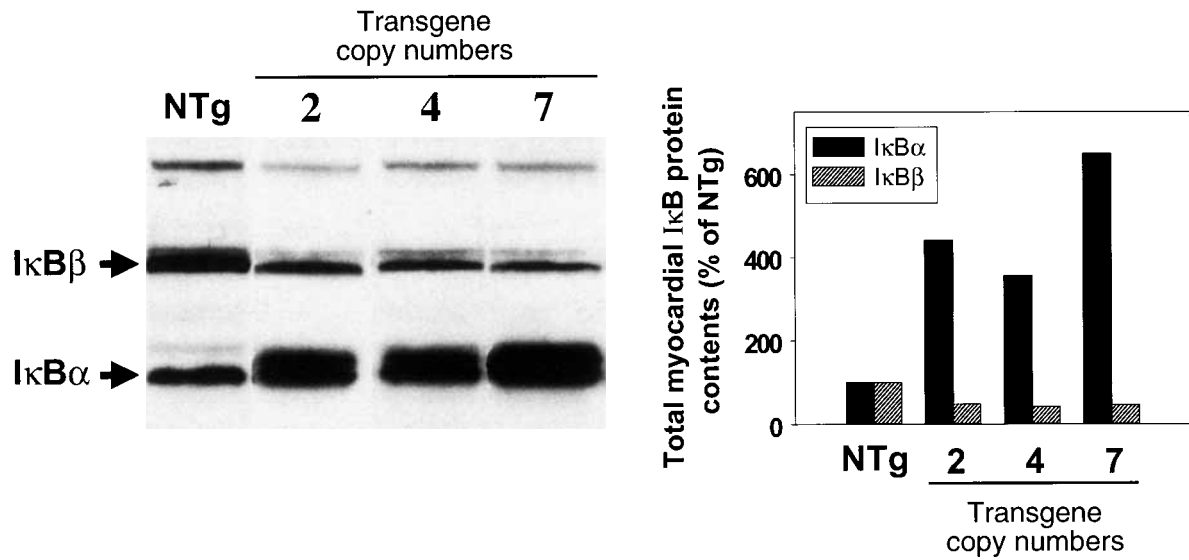
Supershift assays using antibodies against the p65 component of NF- $\kappa$ B identified p65 as one of the components of the NF- $\kappa$ B DNA-binding complex activated in response to TNF- $\alpha$  stimulation in the myocardium (Fig. 6).

## **Discussion**

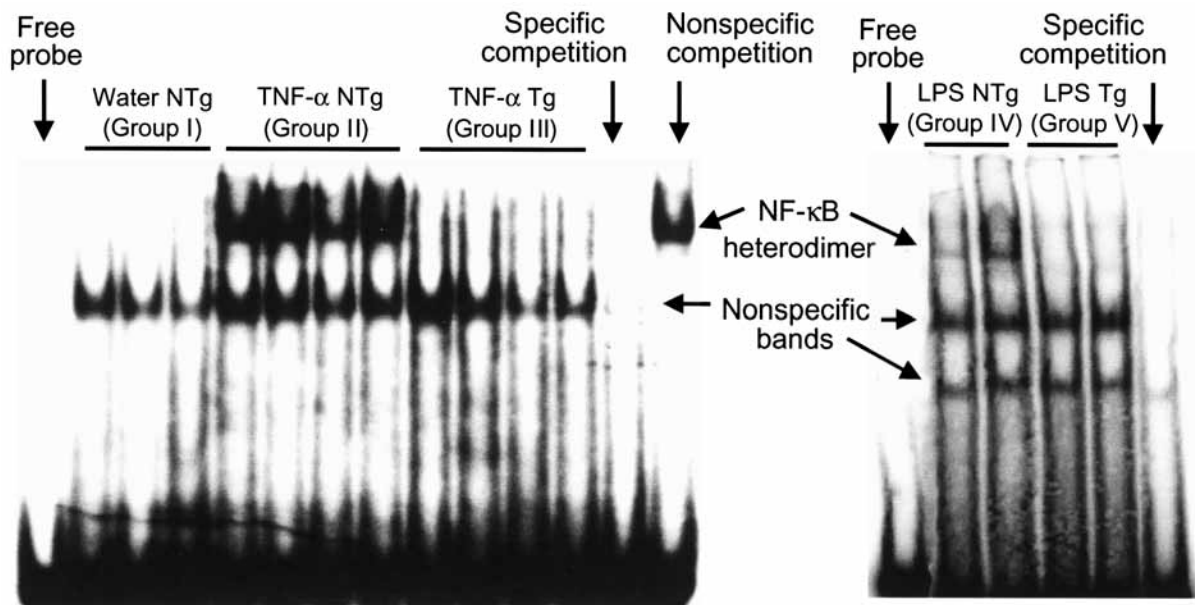
NF- $\kappa$ B, a pleiotropic transcription factor first identified as a regulator of the expression of the kappa light-chain gene in murine B lymphocytes,<sup>36</sup> has been implicated in the signal transduction pathways underlying various cardiac pathophysiologic states, including ischemic preconditioning, congestive heart failure, unstable angina pectoris, atherogenesis, and apoptosis.<sup>6–9,11</sup> However, unequivocal determination of a role of NF- $\kappa$ B in pathophysiologic processes requires specific inhibition of the transcription factor in the heart *in vivo*, which thus far has not been possible. As a result, the contribution of NF- $\kappa$ B to cardiovascular pathophysiology in the



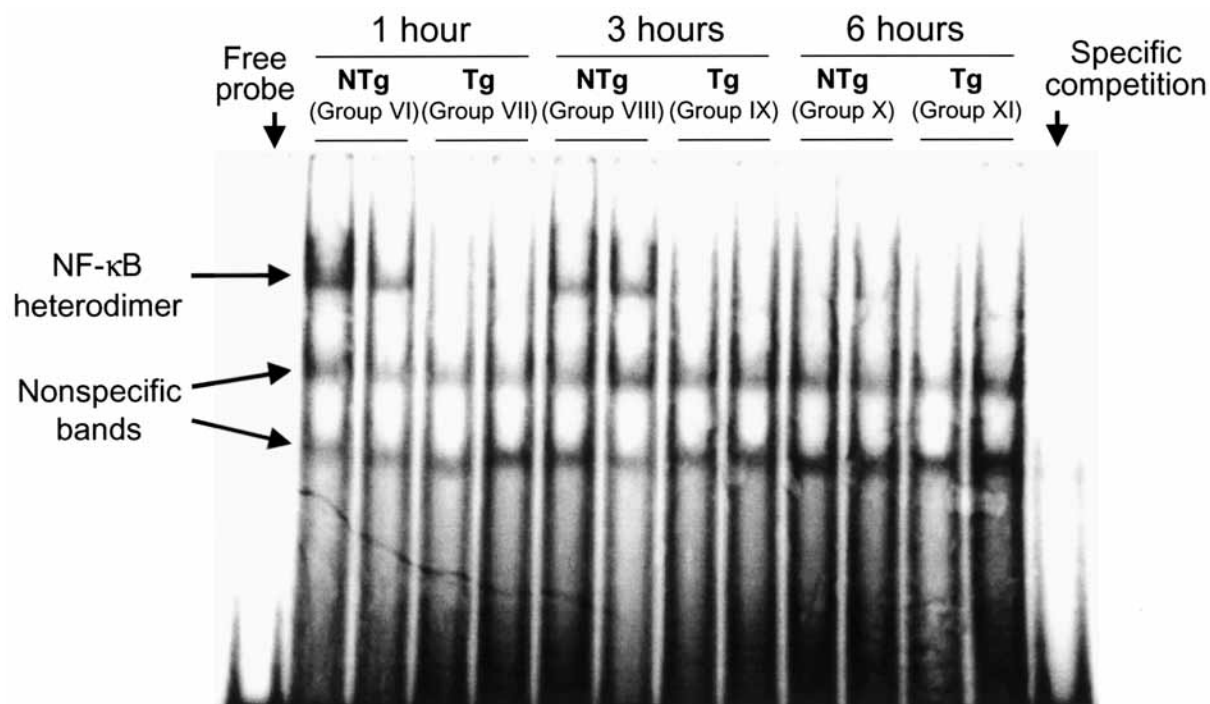
**Figure 2** Histologic examination of the  $I\kappa B\alpha^{S32A,S36A}$  transgenic mouse hearts. Cross-sections of transgenic mouse hearts (hematoxylin and eosin stain, panel B,  $\times 4$  magnification) did not show any evidence of chamber dilatation and free wall or septal thickening compared to the non-transgenic mouse heart (panel A). No cellular disarray, excessive fibrosis or abnormal vascularity was evident in the transgenic mouse heart (Masson's trichrome stain,  $\times 100$  magnification, panel D) compared to the non-transgenic heart (panel C). Nuclear shape and size, nuclear-cytoplasmic ratio, and overall cellular structure appeared normal in the transgenic hearts (hematoxylin and eosin,  $\times 400$  magnification, panel F) compared to the non-transgenic hearts (panel E).



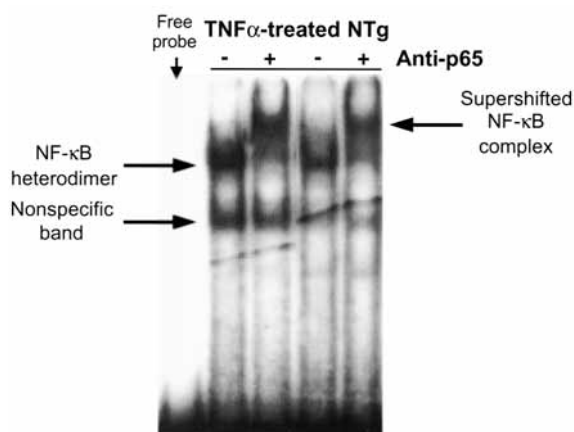
**Figure 3** Representative Western blots of total myocardial IκBα and IκBβ protein contents (left panel) using specific antibodies for IκBα and IκBβ. The total myocardial IκBα protein level was increased (+250% to +550%) with a concomitant decrease (−53% to −58%) in IκBβ protein content in transgenic (Tg) mouse hearts (second, third and fourth lanes from the left) as compared with that observed in nontransgenic mice (NTg, first lane from the left). The total amount of IκBα protein was higher in the 7 copy number line relative to the 2 and 4 copy number lines as demonstrated by densitometric analysis (right panel) of signal intensity from Western analysis of total myocardial IκBα and IκBβ protein levels. The Western analyses were performed in two mice for each line.



**Figure 4** Electrophoretic mobility shift assay analysis of NF-κB DNA binding activity. Both non-transgenic (NTg) and transgenic (Tg) mice were injected i.p. with vehicle (sterile water), rm-TNF-α (left panel) and LPS (right panel). NTg mice injected with sterile water (group I) did not show any evidence of myocardial NF-κB DNA binding activity. NTg mice injected with either TNF-α (group II) or LPS (group IV) showed robust activation of NF-κB in the heart. In contrast, both TNF-α (group III) and LPS (group V) failed to induce any NF-κB DNA binding in the myocardium of transgenic mice, indicating that transgenic expression of IκBα<sup>S32A,S36A</sup> completely abrogated activation of all NF-κB subunits in the myocardium. The specificity of the myocardial NF-κB DNA binding activity was verified by specific competition with a 100-fold excess of (NF-κB consensus sequence) probe.



**Figure 5** Time-course of myocardial NF- $\kappa$ B activation following rm-TNF- $\alpha$  stimulation by electrophoretic mobility shift assay. Mice were euthanized 1 h (groups VI and VII), 3 h (groups VIII and IX), or 6 h (groups X and XI) after rm-TNF- $\alpha$  administration. Myocardial nuclear extracts from non-transgenic (NTg) mice (groups VI, VIII and X) showed detectable activation of NF- $\kappa$ B at all time-points, though the intensity of activation decreased over time with only minimal activation detectable at 6 h (group X). In contrast, rm-TNF- $\alpha$  failed to activate NF- $\kappa$ B in the myocardium of transgenic (Tg) mice at all time-points, indicating that the transgenic blockade of NF- $\kappa$ B activation persisted for at least 6 h, and that there was no delayed activation of NF- $\kappa$ B in transgenic mice.



**Figure 6** Supershift assay using p65 antibodies following rm-TNF- $\alpha$  stimulation. Non-transgenic (NTg) mice were injected with rm-TNF- $\alpha$ , and killed 30 min later. Ventricular tissue was used for preparation of nuclear extracts and electrophoretic mobility shift assays performed, with and without the addition of p65-specific antibody for the supershift assay. The upward shift of the band specific for NF- $\kappa$ B activation indicates that one of the components of the NF- $\kappa$ B heterodimer translocated to the nucleus in the heart of NTg mice in response to TNF- $\alpha$  stimulation is p65.

intact animal has been inferred either from correlative evidence (e.g., association of NF- $\kappa$ B activation with a certain outcome) or by inhibition with pharmacologic agents of limited specificity.<sup>12,13</sup> The present study demonstrates that cardiac-specific expression of a transdominant I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> is effective in completely abrogating NF- $\kappa$ B activation in mice without apparent effects on cardiac morphology and histology, or on viability. To our knowledge, this is the first transgenic mouse with *in vivo* repression of NF- $\kappa$ B in a cardiac-specific manner.

#### Viability of I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> transgenic mice

Previous attempts to use genetic manipulation to inhibit NF- $\kappa$ B activation failed due in large part to the embryonic lethality of the p65 knockout mice.<sup>17, 37</sup> Moreover, there are at least five known NF- $\kappa$ B subunits (p50, p52, p65, RelB and c-Rel) which can form multiple heterodimers and homodimers,<sup>1,2</sup> and the constellation of NF- $\kappa$ B subunits expressed in adult murine heart is currently unknown. Thus, combining multiple specific knockouts of NF- $\kappa$ B subunits to abrogate NF- $\kappa$ B activity in the heart is not

feasible. In order to circumvent these problems, in the present study we used a different approach, i.e., cardiac-specific transdominant inhibition of NF- $\kappa$ B signaling. Our results indicate that mice bearing a cardiac-specific transgene encoding a transdominant I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> are viable and fertile, at least at the levels of expression examined here. Furthermore, while achieving what is essentially a functional knockout of NF- $\kappa$ B in the heart, there appear to be no obvious deleterious pathophysiologic effects of transgene expression. Because we did not obtain transgenic founders with transgene copy numbers higher than seven, we cannot rule out the possibility that mice having higher copy numbers were inviable. Even if high copy numbers of the I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> transgene were associated with deleterious phenotypes, this would not seriously impinge upon the utility of these mice, since our data demonstrate that the low copy number lines studied herein result in complete inhibition of NF- $\kappa$ B activation in the heart. However, further studies are needed to address whether expression of I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> results in non-specific effects on activation/inhibition of Rel homology domain transcription factors other than NF- $\kappa$ B.

#### Myocardial I $\kappa$ B $\alpha$ protein expression

Western blot analyses, using an antibody directed against a carboxy terminal epitope that is conserved between mouse and human I $\kappa$ B $\alpha$ , demonstrated an increase in the total level of I $\kappa$ B $\alpha$  in the heart. In underloaded lanes we can identify the mutant I $\kappa$ B $\alpha$  by its altered mobility relative to the endogenous I $\kappa$ B $\alpha$  (data not shown). Thus, the increase is attributed to the expression of the mutant I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> in the myocardium of transgenic mice. Quantitative analysis revealed that the total myocardial I $\kappa$ B $\alpha$  protein levels in transgenic mice were 3.5- to 6.5-fold higher than the endogenous I $\kappa$ B $\alpha$  levels in NTg mice.

#### Abrogation of myocardial NF- $\kappa$ B activation in response to TNF- $\alpha$ and LPS

TNF- $\alpha$  and LPS are two of the most potent activators of NF- $\kappa$ B known to exist. Our EMSA analysis demonstrates that both TNF- $\alpha$  and LPS activate NF- $\kappa$ B in the hearts of NTg mice and that this activation, within the limitations of the assay, is completely abrogated in the myocardium of I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> transgenic mice (Fig. 4). The failure of TNF- $\alpha$  and LPS to activate NF- $\kappa$ B at 30 min after the stimulus was not simply due to a delay in NF- $\kappa$ B activation, since our

time-course studies demonstrated no subsequent NF- $\kappa$ B activation in transgenic mice (Fig. 5). These results confirm that phosphorylation of serine residues at positions 32 and 36 of I $\kappa$ B $\alpha$  is critical for the regulation of NF- $\kappa$ B activation, and that this mechanism is operative in the murine heart. This also proves, for the first time, that blockade of activation of all NF- $\kappa$ B subunits expressed in the heart is possible *in vivo* by expressing the phosphorylation-resistant mutant I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup>. Furthermore, since the  $\alpha$ -*MyHC* promoter drives gene expression only in cardiac myocytes,<sup>22</sup> our results imply that TNF- $\alpha$  activates NF- $\kappa$ B predominantly in cardiac myocytes which comprise only approximately 25% of all myocardial cells.<sup>38</sup> This finding has profound implications for the action of TNF- $\alpha$  upon the heart.

#### Myocardial I $\kappa$ B $\beta$ protein expression

Interestingly, we noted a 50–60% decrease in the levels of myocardial I $\kappa$ B $\beta$  protein in I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> transgenic mice (Fig. 3). This would be expected if I $\kappa$ B $\beta$  is a  $\kappa$ B-dependent gene as has been demonstrated for I $\kappa$ B $\alpha$ , I $\kappa$ B $\epsilon$ , and I $\kappa$ BL.<sup>39–42</sup> Kontgen *et al.*<sup>16</sup> concluded that I $\kappa$ B $\beta$  is not a  $\kappa$ B-dependent gene based upon the observation that I $\kappa$ B $\beta$  transcript levels were not increased in cultured murine pre-B cells 12 h after NF- $\kappa$ B activation. Our results show that I $\kappa$ B $\beta$  protein levels are reduced by chronic suppression of NF- $\kappa$ B activation in the heart and are consistent with NF- $\kappa$ B regulation of I $\kappa$ B $\beta$ . Other interpretations of our observation that I $\kappa$ B $\beta$  levels are reduced in I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> transgenic mice are certainly possible, for example, enhanced degradation of I $\kappa$ B $\beta$  due to its displacement from NF- $\kappa$ B by mutant I $\kappa$ B $\alpha$ . Direct demonstration that I $\kappa$ B $\beta$  is transcriptionally regulated by NF- $\kappa$ B awaits further experimentation. Our results in transgenic mice, Kontgen's in cultured cells, as well as the persistent (late) activation of NF- $\kappa$ B that has been observed in mouse,<sup>16</sup> rat,<sup>43,44</sup> and human cells<sup>45</sup> could all be explained by an extremely slow (>12 h) transcriptional response of the I $\kappa$ B $\beta$  gene. There is evidence that differential degradation of I $\kappa$ B $\beta$  relative to I $\kappa$ B $\alpha$  is the basis of the persistent NF- $\kappa$ B activation in human and rat<sup>44,46</sup> and there is evidence that one of the two human I $\kappa$ B $\beta$  isoforms, I $\kappa$ B $\beta$ 2, mediates persistent NF- $\kappa$ B activation via its degradation kinetics (slow).<sup>47</sup> Interestingly, the single murine I $\kappa$ B $\beta$  isoform closely resembles human I $\kappa$ B $\beta$ 1, which has degradation kinetics similar to I $\kappa$ B $\alpha$  (fast).<sup>47</sup> That I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  in the mouse are functionally interchangeable is supported by recent gene replacement experiments in which the I $\kappa$ B $\alpha$  protein coding region was replaced by I $\kappa$ B $\beta$  and the

kinetics of NF- $\kappa$ B activation was found to be unaltered.<sup>48</sup> There is evidence that I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  have different association constants for NF- $\kappa$ B.<sup>49</sup> Thus, in the mouse, prolongation of NF- $\kappa$ B activation may be mediated in part by the differing association constants of individual I $\kappa$ B isoforms, and in part by transcriptional regulation of I $\kappa$ B genes. Although Chandrasekar *et al.* reported that the persistent NF- $\kappa$ B activation has a biphasic nature in postischemic rat myocardium,<sup>43</sup> our results are more consistent with an initial activation of NF- $\kappa$ B followed by re-establishment of repression over a period of hours (Fig. 4).

#### Applications of I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> transgenic mice

NF- $\kappa$ B, a major regulator of stress-induced gene transcription, is thought to play an important role in the genesis of various cardiac pathologies. TNF- $\alpha$  and LPS have also been implicated in the genesis of a wide spectrum of cardiac pathologies, including heart failure, cardiac hypertrophy, apoptosis and myocardial depression in septic shock. Although existing evidence from studies in cell lines and other tissues suggests a link between TNF- $\alpha$ , LPS and NF- $\kappa$ B activation in the genesis of disease processes, there is currently no *in vivo* model to verify the cause-and-effect relationship of these interactions in the heart. Although several pharmacologic inhibitors of NF- $\kappa$ B activation are currently available, they lack specificity, and thus, do not yield conclusive results. For example, dithiocarbamates are antioxidants and iron chelators,<sup>12</sup> and therefore would be expected to produce a multitude of effects, including inhibition of other transcription factors like AP-1<sup>50</sup> in addition to inhibition of NF- $\kappa$ B. Peptide inhibitors of NF- $\kappa$ B that have been used in isolated cells<sup>51</sup> are not effective *in vivo*. Furthermore, using pharmacologic inhibitors *in vivo*, it is impossible to localize the effect of inhibition of NF- $\kappa$ B activity to a specific organ. The transgenic mouse lines presented herein provide a novel tool to interrogate specifically and conclusively the role of NF- $\kappa$ B in cardiac pathophysiology. As a result of the cardiac-specific expression of the transgene, these mice enable assessment of the role of NF- $\kappa$ B specifically in the heart. Furthermore, this approach obviates the lack of specificity that limits pharmacologic interventions. Accordingly, the I $\kappa$ B $\alpha$ <sup>S32A,S36A</sup> transgenic mice should be useful not only for signal transduction research, but also to verify the utility and efficacy of new therapeutic approaches in modifying NF- $\kappa$ B activity and in modulating the effects of TNF- $\alpha$  and LPS in the setting of

myocardial ischemia-reperfusion injury, heart failure, septic shock, cardiac hypertrophy, atherogenesis, acute coronary syndromes, and cardiac allograft rejection.

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