Pulsatile Stimulation Determines Timing and Specificity of NF-κB–Dependent Transcription

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The nuclear factor κB (NF-κB) transcription factor regulates cellular stress responses and the immune response to infection. NF-κB activation results in oscillations in nuclear NF-κB abundance. To define the function of these oscillations, we treated cells with repeated short pulses of tumor necrosis factor–α at various intervals to mimic pulsatile inflammatory signals. At all pulse intervals that were analyzed, we observed synchronous cycles of NF-κB nuclear translocation.

Lower frequency stimulations gave repeated full-amplitude translocations, whereas higher frequency pulses gave reduced translocation, indicating a failure to reset. Deterministic and stochastic mathematical models predicted how negative feedback loops regulate both the resetting of the system and cellular heterogeneity. Altering the stimulation intervals gave different patterns of NF-κB–dependent gene expression, which supports the idea that oscillation frequency has a functional role.

Eukaryotic cells interpret multiple signals to coordinate the activity of transcription factors, which modulate the expression of target genes. Nuclear factor κB (NF-κB) signaling in many mammalian cell types regulates responses to pathogens and stresses (1). NF-κB, most commonly comprising a dimer of RelA and p50, is bound in the cytoplasm of unstimulated cells by inhibitor of nuclear factor κB (IκB) proteins. Stimulation by cytokines such as tumor necrosis factor–α (TNFα) activates the IκB kinase (IKK) complex that phosphorylates IκB proteins, leading to IκB degradation and NF-κB translocation into the nucleus. Activated NF-κB regulates transcription from promoter regions of approximately 300 genes, including those encoding cytokines and several NF-κB family members that can feedback to regulate the system (2). Signaling through NF-κB can regulate diverse cellular outcomes, including cell death or division (3). How such a diversity of responses is generated has remained unclear.

Real-time fluorescence imaging and mathematical modeling have shown that the activity of the NF-κB system can be oscillatory (4). This raised the possibility that, such as with calcium (5), this signaling pathway might use oscillation frequency as one component of the cellular signal that controls innate immunity and cell fate. After stimulation with TNFα, target gene expression can be regulated by negative feedback loops that modulate the cytoplasmic-nuclear translocation of NF-κB (4). One of these feedbacks is mediated by IκBα, which upon binding to NF-κB in the nucleus shuttles the NF-κB protein complex back to the cytoplasm. These oscillations have been observed in single cells expressing the fluorescently labeled NF-κB subunit RelA and IκBα (4, 6, 7) and also through bulk cell electrophoretic mobility shift assay (EMSA) analysis in IκBε and IκBε-IκBβ knockout mouse embryonic fibroblasts (MEFs) (8, 9).

Single-live-cell fluorescent imaging has demonstrated NF-κB oscillations in various cell types (4, 6). SK-N-AS neuroblastoma cells showed particularly robust oscillations in response to TNFα stimulation after transient (4) or stable transfection with a vector expressing RelA fused to the Discosoma sp. red fluorescent protein dsRed-Express (RelA-dsRedxp) (Fig. 1, A and B). Oscillations were unlikely to be the result of RelA overexpression, because stably transfected cells expressed nearly physiological amounts of the fusion protein [relative level of 0.91 ± 0.04 (SD, n = 6 replicates) compared with endogenous protein in untransfected control (7)]. In contrast to the conclusions of other reports (9, 10), we observed oscillations in the translocation of RelA-dsRedxp fusion protein in single transiently transfected MEFs (Fig. 1, C to E). These data [as well as bulk cell chromatin immunoprecipitation (ChIP) assays (fig. S1)] suggest that oscillations are a normal response to TNFα stimulation.

In an inflammatory tissue, cells receive pulsatile signals such as TNFα from neighboring cells and...
To mimic this, we exposed cells to 5-min pulses of TNFα at various intervals, followed by a wash-off. When stimulated at 200-min intervals, RelA-dsRedxp fusion protein expressed in SK-N-AS cells showed synchronous translocations from the cytoplasm to the nucleus and back of equal magnitude in response to each successive pulse (Fig. 2, A and B). These data indicate that the system completely resets between 100 and 200 min after each stimulus. Western blot analysis of synchronized cell populations supported the hypothesis that the cycles of RelA-dsRedxp fusion protein translocation were associated with cycles of phosphorylation at Ser12 and degradation of IκBα (Fig. 2C and fig. S5). Analysis of the amounts of Ser32-phospho-IκBα, relative to total IκBα levels, confirmed that the failure to reset was quantitatively reflected by the phosphorylation of IκBα (Fig. 2, C and D, and fig. S6). Cycles of phosphorylation and dephosphorylation of RelA at Ser336 were consistent with RelA being phosphorylated in the cytoplasm and dephosphorylated in the nucleus (4).

The inability of available model structures (7, 8, 13, 14) to provide a single parameter set that could simulate the observed behavior for all

Fig. 2. Response of SK-N-AS cells to various TNFα pulse frequencies. (A) Time course of RelA-dsRedxp N:C ratio in transiently transfected cells pulsed three times with TNFα for 5 min at intervals of 60, 100, or 200 min (five typical cells shown for each). RelA-dsRedxp N:C ratio was normalized to peak 1 intensity. (B) Amplitude of successive peaks of RelA-dsRedxp localization after pulses or continuous exposure of cells to TNFα. Results were normalized to the amplitude of peak 1 (+SD). Asterisks indicate P values for a one-sample Wilcoxon test for peak amplitude equal to 1. (C) Western blot of Ser12 phospho-1xIκBα (p-1xIκBα), 1xIκBα, Ser336 phospho-RelA (p-RelA), RelA, and cyclophilin A (cyclo A) amounts in cells stimulated with TNFα pulses 200 min apart. (D) Ratio of p-1xIκBα/total IκBα (relative to that recorded at t = 5 min) in cells stimulated 60, 100, and 200 min apart (+SD) [data based on (C)] (fig. S4). p1 and p2 indicate time after pulse 1 or 2 for each stimulation protocol. (E) Two-feedback NF-κB signaling pathway showing IκK and the base module. (F and G) Computational analysis of existing (F) (2) and proposed (G) IκK structures. Heat maps [poor (red) to good (green)] represent the ability of the model to quantitatively fit the experimental data for a range of selected parameter values (table S5). A20 degradation rate (c4) was varied on a logarithmic scale two orders of magnitude above and below 0.0009 s⁻¹. The best fit is highlighted and the corresponding simulated N:C ratio (Fig. 2C) and proposed (G) IκK structures. Heat maps [poor (red) to good (green)] represent the ability of the model to quantitatively fit the experimental data for a range of selected parameter values (table S5). A20 degradation rate (c4) was varied on a logarithmic scale two orders of magnitude above and below 0.0009 s⁻¹. The best fit is highlighted and the corresponding simulated N:C ratio (Fig. 2C) and proposed (G) IκK structures.
the tested TNFα stimulation conditions demonstrated a need for model refinement (Fig. 2, E to G). We used the experimentally observed limited IκBε phosphorylation, which is associated with reduced NF-κB translocation level at higher pulse frequencies (Fig. 2, B and D), to constrain simulated IKK activity. The core NF-κB–IκBε network used a similar structure to that of previous models (Fig. 2E), although multiple model parameters were modified partly on the basis of cell-specific measurements (table S1). Model simulations using the existing IKK network structure (14) (Fig. 2F) failed to recapitulate experimental conditions because the repeated pulse stimulation required sufficient IKK activity to degrade almost all IκBε; however, persisting oscillations observed with continual TNFα were sensitive to high IKK activity (figs. S8 and S9). Using a scoring function to compare model performance with all experimental data (table S5), we composed a new deterministic model with a modified network structure of IKK and A20 NF-κB inhibitory protein interactions (Fig. 2G and fig. S10). The chosen model represented the simplest structure that included IKK state recycling (15) and was able to use a single parameter set to reproduce all of the experimental data. The A20 feedback loop was assumed to include other related negative feedback inhibitors, such as Cezanne (16) and Cylindromatosis (CYLD) (17), which together may limit the reactivation of IKK. The model predicted a low stability of A20, which could be compatible with a key regulatory factor being the ubiquitin-editing activity of A20 rather than its protein concentration. A20 activity may be linked to processes upstream of IKK (18), or IKK-mediated phosphorylation of A20 may inhibit IKK activity (19).

Persistent bulk cell oscillations have been observed after continuous TNFα by using EMSA assays of MEFs from IκBε-deficient or combined IκBε–IκBβ-deficient animals (8, 9). Although deterministic simulation was appropriate for the work described above, the heterogeneous nature of single-cell responses to continuous TNFα stimulation in wild-type cells cannot be elucidated in this manner. Stochastic models (20, 21) have been used to propose that cell-to-cell heterogeneity arises through intrinsic, stochastic, transcriptional variability because there are only two copies of the IκBε and A20 feedback genes (21). We developed a new hybrid, stochastic, three-feedback model on the basis of the deterministic model structure described above, which considered delayed stochastic transcription from the IκBε gene and stochastic transcription of the IκBε and A20 genes (Fig. 3, A and B). ChIP analysis confirmed that RelA binds to the IκBε and IκBε promoters in SK-N-AS cells within 20 min after TNFα stimulation (Fig. 3C). In contrast, RNA polymerase II was bound to the IκBε promoter before stimulation, whereas binding to the IκBε promoter was delayed (Fig. 3D), perhaps as a result of chromatin remodeling. The three-feedback stochastic model predicted persistent oscillations of similar amplitude in both wild-type (Fig. 3E) and IκBε-deficient (Fig. 3F) cells after stimulation. Furthermore, stochastic variation from delayed IκBε feedback may generate enhanced cell-to-cell heterogeneity.

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**Fig. 3.** Role of the IκBε feedback loop. (A) Diagram of NF-κB signaling pathway, including three feedback mechanisms. (B) Stochastic NF-κB-dependent regulation of IκBε, A20 (upper) and delayed IκBε (lower) genes. (C) RelA and (D) RNA polymerase II DNA binding to the IκBε and IκBε promoters after continuous TNFα stimulation by means of ChIP analysis. (E and F) Simulations of single-cell trajectories and the 100-cell average (black line) for (E) wild-type and (F) IκBε knockdown conditions. (G and H) Time course of N:C ratio of RelA-dsRedxp in cells transiently transfected with RelA-dsRedxp and either (G) nonspecific or (H) IκBε siRNA. The average population (nonspecific siRNA, n = 57 cells; IκBε siRNA, n = 61 cells) response is shown by a black line.

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**Table S1.** Parameters used in the deterministic model, and parameter values used in the stochastic model. The table lists the parameters used in the deterministic model, along with the parameter values used in the stochastic model. The parameter values were chosen to reflect the experimental data and to capture the range of cell-to-cell variability observed in the experiments.
which contradicts the prediction that IκBα had no effect on oscillation amplitude (Fig. 3G), in wild-type cells as compared with that in IκBα-deficient cells. The lack of IκBα feedback could therefore generate increased cell-to-cell homogeneity, which was predicted to be detectable as oscillations at the average population level (Fig. 3F). Experimental small interfering RNA (siRNA) depletion of IκBα feedback in SK-N-AS cells (Fig. 3H) had no effect on oscillation amplitude (Fig. 3G), which contradicts the prediction that IκBα feedback might dampen oscillations in wild-type cells (9). Although IκBα-deficient MEFs showed homogeneous cell-to-cell oscillations, the stochastic three-feedback model predicted that this effect would not occur in cells overexpressing RelA by as little as twofold [as in our siRNA knockdown experiments (fig. S15)]. Therefore, the three-feedback stochastic model was able to simulate and predict key aspects of the available experimental data.

To assess the functional importance of oscillation timing in TNFα-induced NF-κB signaling in single cells, we used quantitative reverse transcription polymerase chain reaction (RT-PCR) to measure the time course of transcription from a set of early, middle, and late NF-κB–dependent endogenous genes in response to various durations and frequencies of TNFα treatment. ChIP analyses after repeated 100- and 200-min pulses showed cycles of DNA binding (Fig. 4, A and B, and fig. S23). Over a 430-min time course, we observed successively later peaks in the mRNA transcription of four representative genes in response to TNFα stimulation (from early to late: IκBα, IκBε, MCP-1, RANTES) (Fig. 4C) (8, 22). Although IκBε responded equally to a 5-min single pulse of TNFα and to pulses at 200-min intervals, later genes showed reduced responses, and the gene encoding the RANTES chemokine exhibited almost no response. There was an increase in late transcript abundance in which stimuli were applied at 100-min intervals, which was even more marked when the cells were stimulated at shorter intervals (Fig. 4D). Thus, varying frequencies of NF-κB nuclear entry result in the differential regulation of particular downstream genes.

In this study, we used combined experimental and computational studies to explain the source of cellular heterogeneity and show that oscillations are an important characteristic of the response of NF-κB to TNFα. Cells in inflammatory tissues may experience varying cytokine stimulation. In response to timed fluctuations in TNFα stimulation, the NF-κB response can become homogeneous and can be driven at differing frequencies. Such varying frequencies in stimulation resulted in altered gene-expression profiles, specifically affecting the abundance of late gene transcription. These results therefore support the idea of oscillatory dynamics having a key functional role in this important stress-response system. The recent observation that the yeast calcium–regulated transcription factor Crz1 also controls gene expression through nuclear translocation frequency (27) suggests that this behavior may be a property of other important signaling pathways, such as p53 (24).

References and Notes

Antibody Recognition of a Highly Conserved Influenza Virus Epitope

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Influenza virus presents an important and persistent threat to public health worldwide, and current vaccines provide immunity to viral isolates similar to the vaccine strain. High-affinity antibodies against a conserved epitope could provide immunity to the diverse influenza subtypes and protection against future pandemic viruses. Cocrystal structures were determined at 2.2 and 2.7 ångstrom resolutions for broadly neutralizing human antibody CR6261 Fab in complexes with the major surface antigen (hemagglutinin, HA) from viruses responsible for the 1918 H1N1 influenza pandemic and a recent lethal case of H5N1 avian influenza. In contrast to other structurally characterized influenza antibodies, CR6261 recognizes a highly conserved helical region in the membrane-proximal stem of HA1 and HA2. The antibody neutralizes the virus by blocking conformational rearrangements associated with membrane fusion. The CR6261 epitope identified here should accelerate the design and implementation of improved vaccines that can elicit CR6261-like antibodies, as well as antibody-based therapies for the treatment of influenza.