Characterization and Quantitation of NF-κB Nuclear Translocation Induced by Interleukin-1 and Tumor Necrosis Factor-α

FREE DEVELOPMENT AND USE OF A HIGH CAPACITY FLUORESCENCE CYTOMETRIC SYSTEM* (Received for publication, May 19, 1998)

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A new quantitative cytometric technique, termed the ArrayScan™, is described and used to measure NF-κB nuclear translocation induced by interleukin (IL)-1 and tumor necrosis factor-α (TNFα). The amount of p65 staining is measured in both the nuclei defined by Hoechst 33342 labeling and in the surrounding cytoplasmic staining is measured in both cell types. In contrast, a polyclonal anti-TNF receptor 1 antibodies on the other hand could be shown to respectively inhibit IL-1 and TNFα stimulation in both cell types. In contrast, a polyclonal anti-TNF receptor 1 antibodies on the other hand could be shown to respectively inhibit IL-1 and TNFα stimulation in both cell types.

IL-1 and TNFα are two master cytokines that induce an almost identical proinflammatory response, including the production of chemotactic cytokines, adhesion molecules, and enzymes such as cyclooxygenase, nitric-oxide synthetase, and matrix metalloproteinases (1, 2). Many of these effects are a result of the activation by both IL-1 and TNFα of the NF-κB transcription factor pathway, which is associated with the activation of many cellular defense genes (3, 4). Composed of p65 (RelA) and p50 proteins, NF-κB is normally present in the cytoplasm in an inactive state in a complex with members of the IκB inhibitor protein family, chiefly the 37-kDa IκBα form. In this complexed form, a nuclear localization sequence found on NF-κB is masked by the IκBα, preventing nuclear translocation of NF-κB, DNA binding, and subsequent transcriptional activation (5–12). IL-1 or TNFα receptor activation induces within several minutes the specific phosphorylation of Ser32 and Ser36 on IκBα, the destruction of the phosphorylated IκBα protein by proteasomes, and the translocation of NF-κB to the nucleus (13–17).

Recent reports have identified an IL-1- and TNFα-activated Ser/Thr kinase cascade containing at least four kinases that serially phosphorylate each other prior to the phosphorylation of IκBα (reviewed in Refs. 18 and 19). The inhibition of proteasome activity by specific inhibitors as LLL-H (MG132) prevented both the destruction of IκBα as well as the subsequent activation of NF-κB as measured by the production of NF-κB-dependent proteins such as leukocyte adhesion proteins (20, 21).

Cellular assays of the early signaling events leading up to NF-κB activation are difficult because of the rapidity and complexity of the protein interactions. To show activation of NF-κB, electrophoretic mobility shift assays are typically performed to look at the specific binding of activated NF-κB to DNA (5, 7, 22), but this technique requires relatively large numbers of cells and is laboriously quantitative, and the assay is not performed in intact cells. In contrast, the use of gene reporter constructs in transfected cells measures a response occurring hours after cell activation, and the resulting gene transcription is influenced by other transcription factors that act cooperatively to activate individual genes (12, 23).

Because protein translocation from the cytoplasm to nucleus can be readily visualized by immunocytolocalization (see e.g. Refs. 9, 10, 24, and 25), a computerized cytometric fluorescence system, termed the ArrayScan™, has been developed to analyze translocation of cytoplasmic proteins in cells grown in 96-well plates (26). We have used this ArrayScan™ system to quantitate the rate and extent of NF-κB translocation following stimulation of varying IL-1 and TNFα concentrations. We show that in human chondrocytes and HeLa cells complete translocation of NF-κB occurs within 10–20 min, with a half-time several minutes following that of IκBα destruction. Furthermore, the effects of receptor agonists and antagonists and kinase and proteasome inhibitors can be differentially quantitated.
EXPERIMENTAL PROCEDURES

Cells—Human TH-igfl chondrocyte cells were obtained from M. B. Goldberg (27) and passaged weekly in Dulbecco’s modified Eagle’s medium (low glucose; Life Technologies, Inc.) with 10% FBS. HeLa cells were obtained from the ATCC collection and passaged weekly in Dulbecco’s modified Eagle’s medium with high glucose. For experiments, cells were removed with 0.25% trypsin/EDTA, plated in 96-well plates (Polyfiltronics, Rockland, MA) at 10,000 cells/75 μl/well, and grown for 20 h.

Translocation Experiments—The plates were washed once in Dulbecco’s modified Eagle’s medium without FBS, and 90 μl of fresh medium was added. Various compounds were added in Me2SO, and up to 1% Me2SO could be added without any effects on NF-κB translocation (data not shown). The compounds used together with their source included E2020 (Alexis Biochemicals, San Diego, CA), carbobenzoxy-Leu-Leu-Leu-aldehyde (Peptide Institute, Osaka, Japan), and clastolactacystin β-lactone (Affiniti Research Products, Manheim, Exeter, United Kingdom). Protein antagonists were added in Dulbecco’s modified Eagle’s medium and included IL-1 receptor antagonist (IL-1RA; prepared at Merck), neutralizing anti-TNFα and monoclonal anti-TNFFR1 and anti-TNFFR2 antibodies (E & D Systems, Minneapolis, MN). Polyconal IL-1R1, anti-TNFFR1, and anti-TNFFR2 antibodies were prepared at Covance (Denver, PA) from soluble IL-1R1, TNFR1, and TNFR2 (E & D Systems). All of the systems were preincubated for 20 min at 37°C, and then 10 μl of stimuli (typically 10 ng/ml [final concentration]) IL-1α or TNFα; R&D Systems) was added for each well. The cells were repipetted twice for mixing and then incubated for typically 20 min more at 37°C. Experiments were ended by washing the plates twice in ice-cold phosphate-buffered saline followed by fixation.

Cell Fixation and Staining—The cells were fixed with 100 μl of 4% formaldehyde in phosphate-buffered saline for 20 min at room temperature, permeabilized with 100 μl of 0.1% Triton X-100 in phosphate-buffered saline for 5 min at room temperature, and then washed twice with 300 μl of 0.1 M Tris-HCl buffer, pH 7.8. To block nonspecific antigenic sites, the wells were incubated for 20 min with 100 μl of 3% nonfat dry milk in 0.1 M Tris-HCl buffer, pH 7.8, at room temperature. After washing twice in 0.1 M Tris wash buffer, the cells were incubated for 1 h with 100 μl of rabbit anti-p65 NF-κB antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:2000 in 0.1 M phosphate buffer, pH 7.8, with 0.1% bovine serum albumin (fraction V; Sigma). The plates were washed three times in Tris wash buffer and incubated 30 min, room temperature, with 100 μl of a 10 μg/ml solution in water of bitionylated antirabbit IgG (Vector Laboratories, Burlingame, CA). The plates were washed three times in Tris wash buffer and incubated 30 min, room temperature, with 100 μl of a 2.5 μg/ml solution of Texas Red avidin (Vector) in the phosphate/bovine serum albumin buffer. The cells were washed three times in Tris wash buffer and stored in 100 μl of 0.1 M Tris. Two hours prior to analysis 100 μl of a 1:1 mixture of Hoechst 33342 (Molecular Probes, Inc., Eugene, OR) in phosphate-buffered saline was added to each well at room temperature, and the wells were scanned in the ArrayScan™ instrument.

Data Acquisition and Analysis—The ArrayScan™ cytometer (Cellomics, Inc., Pittsburgh, PA) is an automated fluorescent imaging microscope for extracting information about the spatial and temporal distribution of fluorescently labeled components in cells grown in the microtiter plates (26). The system was used to scan multiple fields from well to well and to acquire and analyze each of the cells in the images according to the algorithm described in Fig. 2. Within each well, multiple cellular images/well were acquired by moving the position of the plate the width of one image field (350 μm) in a square pattern of location centered on the center of the well. In each well, images were acquired until a preselected number of cells had been imaged and analyzed. The ArrayScan™ system consists of an optical system with a spatial resolution of 0.68 μm (Carl Zeiss, Inc., Thornwood, NY); a triple band fluorescence emission filter set with matched single band excitation filters for selectively imaging Hoechst, FITC, or Texas Red (model XF57, Omega Optical, Brattleboro, VT); a CCD camera with frame grabber; and a Pentium PC computer and applications software.

Immunoblotting and Densitometry—Cells grown on 96-well plates were lysed in 30 μl of 2× SDS-PAGE sample buffer, combining eight wells into one sample (2.4 × 106 cells). Alternatively, cells were grown in 35-mm clusters at the same density (106 cells/well) and solubilized in 50-μl sample buffer. SDS-PAGE was performed on 10% Novex gels (San Diego, CA) using 6.4 × 105 cell equivalents/lane. Immunoblotting was performed with rabbit anti-6xHis antibody (Santa Cruz Biotechnology) followed by incubation 30 min, room temperature, with 100 μl of 10% swine anti-rabbit IgG, followed by washing the plates twice in ice-cold phosphate-buffered saline followed by fixation.

Immunoblotting and Densitometry—Representative fields of p65 fluorescence are shown in HeLa cells (A–C) and chondrocytes (D–F) that were unstimulated (A and D), IL-1α-stimulated (B and E), or TNFα-stimulated (C and F). Stimulation was for 20 min with 10 ng/ml cytokine as described under “Experimental Procedures.” Images are those obtained with the ArrayScan™ cytometer before image analysis defining nucleus and cytoplasm. The magnification as printed is × 80.

RESULTS

Cytometric Measurements of p65 NF-κB Localization in Cyttoplasmic and Nuclear Cellular Compartments—Comparable with previous reports (10, 24), immunofluorescence staining of p65 in unstimulated HeLa cells and human chondrocytes shows largely a cytoplasmic location with significantly less nuclear fluorescence (Fig. 1, A and D, respectively). Following a 20-min stimulation with either IL-1 (Fig. 1, B and E) or TNFα (Fig. 1, C and F), large amounts of the p65 are found in nuclei, although significant amounts of p65 still remain in the cytoplasm. To quantitate this shift in p65 immunofluorescence, a technology needed to be developed to measure fluorescence separately in both the cytoplasm as well as the nucleus and to be able to do so in large numbers of cells in multiple wells. This was achieved with the development of the ArrayScan™ cytometer as is outlined in Fig. 2. On an individual cell basis, the cells were stained not only with anti-p65 antibodies to identify NF-κB but also with Hoechst 33342 to identify the nucleus of all of the cells in the well. The ArrayScan™ cytometer focused on the stained nucleus and determined the nuclear boundary by the application of a dynamic thresholding method that identifies a change in fluorescence above the background (Fig. 2, Step 2). To delineate a representative nuclear region relatively free of contaminating cytosol, the nuclear boundary to be analyzed was eroded by 2 pixels (Fig. 2, Step 3). The resultant nuclear ring is shown in Fig. 3, where in unstimulated and IL-1-stimulated HeLa cells (Fig. 3, A and C, respectively) it is superimposed on the p65 immunofluorescence image (Fig. 3, B and D). The nuclear boundary was identified by the area lying between a 2-pixel-wide annular ring drawn around the nuclear boundary itself (Fig. 2, Step 3). In images of unstimulated (Fig. 4, A–D) and IL-1-stimulated HeLa cells (Fig. 4, E–H), the cytoplasmic boundary is denoted by the two red rings.

Quantification of the amount of p65 found in each cell is shown in 16 representative unstimulated and IL-1-stimulated HeLa cells (Fig. 5). The amount of p65 fluorescence found in the nucleus is a composite of background fluorescence plus a small amount of cytoplasm surrounding the nucleus, the result averaged across all of the pixels within the nuclear area (see Fig. 2,
Side View). As is seen visually in Figs. 1, 3, and 4, the calculated p65 nuclear fluorescence increases after stimulation (Fig. 5A). The cytoplasmic fluorescence is a composite of background fluorescence within the cell as well as some background fluorescence observed in the annular ring that might fall outside of the cells (see Fig. 4). The occasional placement of the cytoplasmic ring in part outside of the cells often led to a reduced mean cytoplasmic intensity because of averaging in noncytoplasmic regions. After stimulation, the cytoplasmic staining was only partially reduced, suggesting only a partial translocation of NF-κB (Fig. 5B). Because of the low signal/noise (stimulated versus unstimulated cell values) observed in both the nuclear (Fig. 5A) and cytoplasmic regions (Fig. 5B) following stimulation, the individual cellular mean cytoplasmic staining was subtracted from its corresponding nuclear staining. This nuclear/cytoplasmic difference yielded a much more sensitive signal-to-noise ratio (Fig. 5C). Consequently the nuclear/cytoplasmic difference was typically used as a measure of NF-κB translocation.

Time Course of NF-κB Translocation—To determine how long the assays needed to be run to achieve maximal NF-κB translocation, HeLa and chondrocyte cells were incubated for up to 45 min following stimulation by a 25-ng/ml dose of IL-1 or TNFα. As is shown in Fig. 6, the HeLa cells responded slightly faster than the chondrocytes with a time for 50% maximal response being reached by 7–8 min for TNFα and IL-1a stimulation, whereas the chondrocytes were slightly slower with 50% maximal responses at 12–13 min. For both cells, maximal response was achieved by 20 min. Hence, for the translocation assays, a 20-min incubation was typically used for both cell types.

Because NF-κB translocation follows IkBa phosphorylation and degradation, the corresponding effects on IkBa in the cell types were also studied using IL-1a as a stimulus. As is shown in Fig. 7, IkBa was almost totally destroyed by 10 min after stimulation. The time for 50% maximal response was about 4 min for the HeLa cells and about 7.5 min for the chondrocytes, indicating that IkBa proteolysis preceded NF-κB nuclear translocation by 4–5 min.

IL-1 and TNFα Dose Response—Titration of the concentration of IL-1 and TNFα used for cell stimulation indicated that maximal stimulation of each cytokine occurred at about 10 ng/ml in each cell type (Fig. 8). The dose response for IL-1 was steeper than that of TNFα in both cell types; for IL-α stimulation the ED₅₀ for both cell types was about 1–2 μg/ml, whereas for TNFα stimulation the ED₅₀ was about 0.03–0.05 μg/ml.

Cell Numbers Required for Statistical Significance—To determine the minimal number of cells required for the assay to
assure statistical significance, a series of plates containing unstimulated and IL-1α-stimulated chondrocytes were analyzed by two-way analysis of variance. In these experiments, the mean difference between nuclear and cytoplasmic p65 staining in stimulated cells was about 55 units more than in unstimulated cells. The number of cells needed for analysis to be assured that a change of 90% had occurred (i.e., a nuclear/cytoplasmic difference of 49.5 (0.9 × 55)) was 56 at the 99% confidence level (i.e., a 1% false positive, type I error; see Table I). This was true regardless of whether the change was due to the addition of varying amounts of a cytokine stimulator or to varying amounts of an inhibitor added to a 10-ng/ml cytokine stimulation. For 95% confidence that the 90% inhibition was achieved, a total of only 37 cells was required to be analyzed. For statistically significant smaller changes in the nuclear/cytoplasmic difference, correspondingly larger numbers of cells needed to be analyzed to achieve comparable confidence (Table I). If only the change in nuclear p65 staining were analyzed, rather than the nuclear/cytoplasmic difference, then about 9% more cells were required to be analyzed to give the same statistical significance (data not shown). Based upon these data, a cell number of 75 was typically analyzed in the experiments described in this paper.

Assay Variability—Several replicate plates of chondrocytes stimulated with IL-1α in the presence or absence of an IL-1RA antagonist were analyzed by the ArrayScan™ system. Reproducibility of this assay for screening purposes was assessed by calculating the coefficient of variation between IC_{50} (with IL-1RA) or EC_{50} (without IL-1RA) values generated from 11 point curves in each row (n = 8) of a 96-well microtiter plate. In this way, row to row, plate to plate, and day to day variability was measured and did not exceed 20, 30, and 22%, respectively.
The reproducibility of the detection method was evaluated by repeatedly scanning the same plate five times, yielding after analysis a coefficient of variation of 6.8% (Table II).

**Effects of Cytokine and Receptor Antagonists on NF-κB Translocation**

IL-1RA (30) inhibited the NF-κB translocation with an IC₅₀ of 20–60 nM, a concentration about 100-fold higher than the stimulatory IL-1 concentration (Fig. 9, top), but it had no effect on the activation of NF-κB translocation induced by TNFα (not shown). This IC₅₀ concentration of IL-1RA determined by the ArrayScan™ method was comparable with that shown earlier to be required to inhibit IL-1-driven cellular responses (31). For complete IL-1 antagonism, 1000-fold higher IL-1RA concentrations were required to prevent NF-κB translocation (Fig. 9, top), just as were seen in other bioassays (32).

**NF-κB Nuclear Translocation Induced by IL-1 and TNFα**

**FIG. 5.** Changes in intracellular p65 fluorescence following IL-1 stimulation of HeLa cells. A, separately eight unstimulated cells and eight cells stimulated for 20 min with IL-1α were analyzed to determine the mean amount of p65 fluorescence in the nucleus. B, the same cells as in A were analyzed for the amount of mean p65 fluorescence found in the cytoplasmic area. C, for each of the 16 cells in A and B, the nuclear minus cytoplasmic (Nuc Cyt) difference of their pixel-averaged values was determined and plotted. For each group of cells in A–C, the mean value is printed above the group.

**FIG. 6.** Time course of NF-κB nuclear translocation. Plates of HeLa (open symbols) and chondrocyte cells (closed symbols) were stimulated with 25 ng/ml IL-1α (circles) or TNFα (triangles) for the indicated times at 37 °C. The cells were fixed and stained with anti-p65 antibody and Hoechst 33342 and analyzed in the ArrayScan™ instrument as described under “Experimental Procedures.” Nuc Cyt, nuclear minus cytoplasmic.

**FIG. 7.** IκBα degradation following IL-1α stimulation of chondrocytes and HeLa cells. Cells were stimulated at 37 °C with 25 ng/ml of IL-1α or TNFα for the indicated times and lysed in sample buffer for SDS-PAGE for determination of IκBα by immunoblot (bottom). For each cell type, the immunoblot density at each time was scanned and plotted as the absorption relative to that of the unstimulated cells (top). The cells were grown in 35-mm dishes for IκBα analysis as described under “Experimental Procedures.”

**FIG. 8.** Dose response of IL-1α- and TNFα-induced NF-κB nuclear translocation. The indicated concentrations of IL-1α (top panel) or TNFα (bottom panel) were added to cultures of chondrocytes (●) or HeLa cells (▲) incubated for 20 min 37 °C and then assayed by the ArrayScan™. Nuc Cyt, nuclear minus cytoplasmic.

Translocation—IL-1RA (30) inhibited the NF-κB translocation with an IC₅₀ of 20–60 nM, a concentration about 100-fold higher than the stimulatory IL-1 concentration (Fig. 9, top), but it had no effect on the activation of NF-κB translocation induced by TNFα (not shown). This IC₅₀ concentration of IL-1RA determined by the ArrayScan™ method was comparable with that shown earlier to be required to inhibit IL-1-driven cellular responses (31). For complete IL-1 antagonism, 1000-fold higher IL-1RA concentrations were required to prevent NF-κB translocation (Fig. 9, top), just as were seen in other bioassays (32). In contrast, a neutralizing anti-TNFα antibody blocked TNFα...
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A similar inhibition was performed with antibodies specific to individual cytokine receptors. IL-1 stimulation of chondrocyte or HeLa cell NF-κB translocation could be prevented by serial dilutions of an anti-IL-1R1 antiserum but not by a pre-immune bleed (Fig. 11, A and C, respectively). The antiserum on its own had no agonist activity, indicating that it was a strict antagonist. Similarly, a neutralizing monoclonal antibody against the TNFR1 blocked TNFα-induced NF-κB translocation, but it had no agonism of its own, and a monoclonal anti-TNF2 antibody was ineffective (Fig. 11, B and D). This indicated that all of the functional TNF receptors in these cell types were TNFR1. In contrast, when a polyclonal anti-TNFR1 antibody was added with chondrocytes stimulated with TNFα, it showed both partial antagonism and partial agonism (Fig.

TABLE I
Number of cells needed in the nuclear translocation assay to detect differences at specified levels of confidence

Chondrocytes stimulated 20 min with 10 ng/ml IL-1α were analyzed to determine the number of cells required for statistical significance at the 99 and 95% confidence levels for the indicated percentage change of a 55-unit nuclear/cytoplasmic difference. The false negative (Type II) error was assumed to be 20%.

| Stimulation or inhibitiona | 99% confidenceb | 95% confidencec |
|----------------------------|-----------------|-----------------|
| % | cells/well | cells/well |
| 20 | >500 | >500 |
| 30 | >500 | 388 |
| 35 | 331 | 220 |
| 45 | 213 | 141 |
| 55 | 149 | 99 |
| 65 | 111 | 73 |
| 70 | 86 | 57 |
| 80 | 68 | 45 |
| 90 | 56 | 37 |
| 100 | 47 | 31 |

a Given a mean nuclear/cytoplasmic difference of 55 units, following stimulation.

b False positive (type I) error rate assuming a false negative (type II) error rate of 20%.

c

TABLE II
Sources of variability and their relative magnitude in the ArrayScan™ nuclear translocation assay

Chondrocytes were stimulated with IL-1α.

| Source of variation | Relative variation (coefficient of variation) |
|---------------------|---------------------------------------------|
|                     | Exp. 1 | Exp. 2 | Exp. 3 |
| %                   |        |        |        |
| Row to rowd         | 20.1   | 13.1   |
| Plate to platee     | 29.6   | 6.7    |
| Day to dayf         | <1.0   | 21.8   |
| Assay to assayg     |        |        | 6.8    |

a Experiment 1; IL-1α stimulation titration.
b Experiment 2; IL-1RA inhibition titration.
c Experiment 3; one plate of IL-1α stimulation and one plate of IL-1RA inhibition.
d Variation in EC50 or IC50 of eight rows of the same plate.
e Variation in EC50 or IC50 of two plates (eight rows each).
f Variation in EC50 or IC50 of the means of two sets of two plates (eight rows each) run on different days.
g Variation in EC50 or IC50 among five successive readings of the same plate (eight rows/plate) on the same day.

stimulation of NF-κB translocation (Fig. 9, bottom) but had no effect on IL-1α stimulation (not shown). The blockage of IL-1 stimulation of NF-κB translocation by IL-1RA occurred at the same concentration as that necessary to block IκBα degradation. In an experiment in which IκBα degradation was quantitated in a similar plate in which NF-κB translocation was measured, both assays yielded the same 24 nM IL-1RA concentration for 50% inhibition (Fig. 10). This gives further evidence of the close correlation of NF-κB translocation to the degradation of IκBα.

A similar inhibition was performed with antibodies specific for individual cytokine receptors. IL-1 stimulation of chondrocyte or HeLa cell NF-κB translocation could be prevented by serial dilutions of an anti-IL-1R1 antiserum but not by a pre-immune bleed (Fig. 11, A and C, respectively). The antiserum on its own had no agonist activity, indicating that it was a strict antagonist. Similarly, a neutralizing monoclonal antibody against the TNFR1 blocked TNFα-induced NF-κB translocation, but it had no agonism of its own, and a monoclonal anti-TNF2 antibody was ineffective (Fig. 11, B and D). This indicated that all of the functional TNF receptors in these cell types were TNFR1. In contrast, when a polyclonal anti-TNFR1 antibody was added with chondrocytes stimulated with TNFα, it showed both partial antagonism and partial agonism (Fig.

FIG. 9. Inhibition of NF-κB translocation by IL-1RA and anti-TNFα antibody in chondrocytes (●) and HeLa cells (▲). Top, cells were stimulated with 10 ng/ml IL-1α for 20 min at 37 °C in the presence of the indicated concentration of IL-1RA. Bottom, cells were stimulated for 20 min at 37 °C with 10 ng/ml TNFα that had been preincubated 30 min at room temperature with the indicated concentration of a neutralizing anti-TNFα antibody. Nuc Cyt, nuclear minus cytoplasmic.

FIG. 10. Comparison of the extent of inhibition of IL-1-induced IκBα degradation and NF-κB translocation by IL-1RA. Parallel 96-well plates of chondrocytes were stimulated for 20 min at 37 °C with 10 ng/ml IL-1α in the presence of the indicated concentration of IL-1RA in columns 1–12 of the plate (see “Concentrations for the Immunoblots”). All eight rows were replicates. For the IκBα blots, all eight replicates of each concentration from one plate were combined with SDS-PAGE sample buffer to concentrate the cells to perform the indicated SDS-PAGE and immunoblot. The scanned intensity of the IκBα blot was plotted (●, top panel) and compared with the NF-κB translocation obtained from ArrayScan™ analysis of the other plate (○, top panel). Nuc Cyt, nuclear minus cytoplasmic.

11E). This antiserum in the absence of TNFα gave roughly a maximal 50% stimulation of the NF-κB translocation, while in the presence of the TNFα stimulus, the same 50% of NF-κB translocation was seen. Titration of the antiserum down re-
sulted in either an increasing loss of NF-κB translocation (without TNFα stimulation) or a comparable increase in NF-κB translocation (samples with TNFα present; Fig. 11B). The IC50 values were the same for stimulation and inhibition, suggesting that the same antibodies that could block a TNFα-induced activation could on their own activate the cells.

**Effect of Proteasome and Kinase Inhibitors on NF-κB Translocation**—Because proteasome inhibitors prevent IκB degradation (16, 33), the potent proteasome inhibitors LLL-H (20) and clastolactacystin β-lactone (34) were tested for their potency as NF-κB translocation inhibitors in chondrocytes and HeLa cells stimulated with IL-1 and TNFα. As is shown in Fig. 12, clastolactacystin β-lactone gave complete inhibition of translocation with an IC50 of about 10 μM. In contrast, LLL-H only inhibited maximally about 3% of the total translocation, and this occurred with an IC50 of about 2 μM. The only partial inhibitory effect of LLL-H on NF-κB translocation was also seen correspondingly on IκBα degradation; concentrations as high as 25 μM LLL-H only delayed rather than blocked IκBα degradation (data not shown; see Ref. 28).

The nonspecific kinase inhibitor K252b has been shown to inhibit the phosphorylation of both the IL-1 receptor association kinase and IκBα (28). The compound K252a (which is more cell-permeable than K253b) was tested for its effects on NF-κB translocation in chondrocytes and HeLa cells stimulated with IL-1 or TNFα and analyzed by the ArrayScan™. As is shown in Fig. 13A, the IC50 for both cell types and both stimuli in the ArrayScan™ assay was about 0.4 μM. To compare the inhibition of a compound such as K252a in the ArrayScan™ assay to its effects in a traditional assay measuring inhibition of an NF-κB-up-regulated and -secreted protein, the effects of K252a on the IL-1- and TNFα-induced production of IL-6 in MRC-5 cells were determined in an overnight assay. This normal human fibroblast MRC-5 cell line is highly sensitive to both cytokines, IκBα is rapidly phosphorylated and degraded (28), and the MRC-5 cells behaved comparably with chondrocytes when tested in the ArrayScan™ technique (data not shown), factors making them an excellent choice for comparison with chondrocytes and HeLa cells. K252a was found to inhibit the MRC-5 cells at an IC50 of approximately 0.2–0.3 μM (Fig. 13B), about the same as the chondrocytes and HeLa cells (Fig. 13A).

**DISCUSSION**

In the present paper, we describe a new technique for quantification of early events in IL-1 and TNFα signal transduction in intact cells by measuring translocation of NF-κB from cytoplasm to the nucleus. Cellular analysis of NF-κB translocation is performed within 20 min following the synchronized stimulation of cells with IL-1 and TNFα. This rapid response focuses on just the process leading up to the appearance of NF-κB in the nucleus prior to any nuclear binding and expression of its transcription activity. The assay thus reduces the number of postreceptor, intracellular signaling steps affecting the measurement to those of the kinase and adaptor protein cascade producing IκBα phosphorylation, the IκBα destruction by proteasomes, and the unknown factors enabling nuclear entry of NF-κB. As is shown in this report, ArrayScan™ analysis of NF-κB translocation is highly quantitative, sensitive, and reproducible. The low coefficient of variation (6.8%) for repeatedly scanning the same wells (but different fields of cells)
indicates the ability of the system to extract statistically similar data from a number of fields in the same well. The analysis of variance indicated that this nuclear translocation technique was robust and produced only a minimal variation between rows and between plates regardless of whether they were analyzed on the same or successive days. Assay, plate, and day variability (Table II) were similar to those obtained for other cell-based assay formats currently used in compound screening. ArrayScan™ analysis is also rapid, taking less than 1 h to scan 75 cells/well in a 96-well plate. Furthermore, determination of protein distribution can be made on native, untransfected cells using fixation and staining such as that described here, or dynamic analysis can be performed in living cells in which proteins of interest are labeled with a fluorescent probe such as green fluorescent protein attached to receptors like the glucocorticoid receptor (26, 35) or to other transcription factors such as that of measurement of transcriptional activity of NF-κB (28). The half-time for nuclear NF-κB translocation during the course of the cytokine stimulation. Only a small amount of the entire pool of NF-κB was observed to translocate (11). One might consider an alternative scenario producing the same average nuclear/cytoplasmic difference in which say only 50% of the cells responded but where the amount of the NF-κB translocation in those cells was twice as much. If this were the case, however, kinetics of nuclear NF-κB may enable a clearer understanding of those events activating NF-κB in the nucleus such as phosphorylation (38).

As is shown here, IL-1- or TNFα-stimulated nuclear NF-κB translocation begins within 2–4 min following the phosphorylation of IκBα, which is initiated 1–2 min after stimulation (see also Ref. 28). The half-time for nuclear NF-κB translocation occurs at about 7–12 min, depending on the cell type, and occurs about 4–5 min following IκBα degradation. This time course is comparable for both IL-1 and TNFα activation despite the differences in the nature of the receptors and the different adaptor proteins involved (see Ref. 18). The NF-κB translocation occurs gradually and uniformly in the entire cell population during the course of the cytokine stimulation. Only a small amount of the entire pool of NF-κB in the cytoplasm translocates despite the proteolysis of essentially all of the associated IκBα; the cytoplasmic fluorescence decreases by only 20% following stimulation (Fig. 5). This observation is comparable with earlier observations in which only 10–20% of the cytoplasmic NF-κB was observed to translocate (11). One might consider an alternative scenario producing the same average nuclear/cytoplasmic difference in which say only 50% of the cells responded but where the amount of the NF-κB translocation in those cells was twice as much. If this were the case, however, then the S.D. in the nuclear/cytoplasmic difference values would be increased over the course of the translocation. This increase is not seen; the S.D. between cells is constant during the entire translocation (data not shown).

Both IL-1 and TNFα produce a maximal response when incubated at about 10 ng/ml (0.6 nM), but they show substantial differences in the concentrations for half-maximal response; whereas IL-1 titrates over about 1.5 logs with an ED₅₀ of about 2 ng/ml, the TNFα titrates over 3 logs with an ED₅₀ of about 0.15 ng/ml (Fig. 8). These titrations in NF-κB nuclear translo-
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8. Beg, A. A., Ruben, S. M., Scheinman, R. I., Haskell, S., Rosen, C. A., and Baldwin, A. S., Jr. (1992) Genes Dev. 6, 1899–1913
9. Henkel, T., Zabel, U., van Zek, M., Müller, J. M., Fanning, E., and Baeuerle, P. A. (1992) Cell 68, 1121–1133
10. Zabel, U., Henkel, T., dos Santos Silva, M., and Baeuerle, P. A. (1993) EMBO J. 12, 201–211
11. Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D., and Miyamoto, S. (1995) Genes Dev. 9, 2723–2735
12. Siebenlist, U., Franzeno, G., and Brown, K. (1994) Annu. Rev. Cell Biol. 10, 335–455
13. Beg, A. A., Fineo, T. S., Nanerter, P. V., and Baldwin, A. S. (1993) Mol. Cell. Biol. 13, 3301–3310
14. Brown, K., Gerstberger, S., Carlson, L., Franzeno, G., and Siebenlist, U. (1995) Science 267, 1455–1488
15. Traenckner, E. B.-M., Pahl, H. L., Henkel, T., Schmidt, K. N., Wilk, S., and Baeuerle, P. A. (1995) EMBO J. 14, 2876–2883
16. Chen, Z., Hagler, J., Palomena, V. J., Melandri, F., Schere, D., Ballard, D., and Maniatis, T. (1995) Genes Dev. 9, 1586–1597
17. DiDonato, J., Mereufo, F., Rosette, C., Wu-Li, J., Suyang, H., Ghosh, S., and Karin, M. (1996) Mol. Cell. Biol. 16, 1295–1304
18. Baeuerle, P. A. (1994) Curr. Biol. 8, R19–R22
19. May, M. J., and Ghosh, S. (1998) Immunol. Today 19, 80–88
20. Palomonbela, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. (1994) Cell 78, 775–785
21. Read, M. A., Neish, A. S., Lascinaskas, F. W., Palomena, V. J., Maniatis, T., and Collins, T. (1995) Immunity 2, 493–506
22. Garner, M. M., and Revinon, A. (1981) Nucleic Acids Res. 9, 3047–3060
23. Thanos, D., and Maniatis, T. (1995) Cell 80, 529–532
24. Auspitan, N., DiDonato, J. A., Rosette, C., Helmberg, A., and Karin, M. (1995) Science 270, 286–290
25. Shibasakti, F., Price, R. E., Milan, D., and McKeon, F. (1996) Nature 382, 370–373
26. Giuliano, K. A., DeBiastio, R. L., Dunlay, R. T., Gough, A., Volosky, J. M., Zock, J., Pavlakis, G. N., and Taylor, D. L. (1997) J. Biomol. Screening 2, 249–259
27. Goldring, M. B. (1994) J. Clin. Invest. 94, 2397–2416
28. Yamin, T.-T., and Miller, D. K. (1997) J. Biol. Chem. 272, 21540–21547
29. Yamin, T.-T., Ayala, J. M., and Miller, D. K. (1996) J. Biol. Chem. 271, 13273–13292
30. Arend, W. P. (1990) in Progress in Growth Factor Research, pp. 193–205, Pergamon Press, London
31. Arend, W. P., Welsch, H. G., Thompson, R. C., and Eisenberg, S. P. (1990) J. Clin. Invest. 85, 1684–1697
32. McIntyre, R. W., Stepan, J. G., Kolinsky, K. D., Benjamin, W. R., Plocienski, J. M., Kaffka, R. L., Campen, C. A., Chizmonite, R. A., and Kilian, P. L. (1991) J. Exp. Med. 173, 931–939
33. Traenckner, E. B.-M., Wilk, S., and Baeuerle, P. A. (1994) EMBO J. 13, 5433–5441
34. Dick, L. R., Cruikshank, A. A. Destree, A. T., Grenier, L., McCormack, T. A., Melandri, P. D., Nunes, S. L., Palomena, V. J., Parent, A. L., Plamondon, L., and Stein, R. L. (1997) J. Biol. Chem. 272, 182–188
35. Hsu, H., Barsony, J., Renyi, I., Gould, D. L., and Hager, G. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4845–4850
36. Baeuerle, P. A., and Baltimore, C. (1994) Science 242, 540–546
37. Tjian, R., and Maniatis, T. (1994) Cell 77, 5–8
38. Zhong, H., SiYang, H., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1997) Cell 89, 413–424
39. Engelmann, H., Heitman, M., Brakebusch, C., Avni, Y. S., Sarov, I., Nophar, Y., Hadas, K., Leitner, O., and Wallach, D. (1990) J. Biol. Chem. 265, 14497–14504
40. Chin, J., Cameron, P. M., Rupp, E., and Schmidt, J. A. (1987) J. Exp. Med. 165, 70–86
41. Sims, J. E., and Dowker, S. K. (1994) Eur. Cytokine Netw. 5, 539–546
42. Aggarwal, B. B., Eensuul, T. E., and Hass, P. E. (1985) Nature 318, 665–667
43. Aggarwal, B. B., Kohr, W. J., Hass, P. E., Moffat, B., Schneider, S. A., Heinzel, W. J., Bringman, T. S., Nedwin, G. E., Goeddel, D. V., and Harkins, R. N. (1985) J. Biol. Chem. 260, 2345–2354
44. Smith, R. A., and Baglioni, C. (1987) J. Biol. Chem. 262, 6951–6954
45. Heiden, H.-P., Remy, R., Posch, B., and von Loun, A. P. G. M. (1990) J. Biol. Chem. 265, 15183–15188
46. Ruegg, U. T., and Burgess, G. M. (1989) Trends Pharmacol. Sci. 10, 218–229
47. Sonoda, Y., Kasahara, T., Tsumaguchi, Y., Kume, K., Matsuhashima, K., and Mukaida, N. (1997) J. Biol. Chem. 272, 15366–15372
48. Fenteany, G., and Schreiber, S. L. (1998) J. Biol. Chem. 273, 8545–8548
49. Glas, R., Bugyo, M., McMaster, J. S., Gazcynska, M., and Ploegh, H. L. (1998) Nature 392, 618–622

REFERENCES
1. Dinarello, C. A. (1992) Semin. Immunol. 4, 133–145
2. Arend, W. P., and Dayer, J.-M. (1995) Arthritis Rheumatism 38, 151–160
3. Siebenlist, U., Brown, K., and Franzeno, G. (1995) in Inducible Gene Expression (Baeuerle, P. A., ed) pp. 93–141, Birkhauser, Boston
4. Baldwin, A. S., Jr. (1996) Annu. Rev. Immunol. 14, 649–681
5. Baeuerle, P. A., and Baltimore, D. (1988) Cell 53, 211–217
6. Baeuerle, P. A., and Baltimore, D. (1988) Science 242, 540–546
7. Shirakawa, F., and Mizel, S. B. (1989) Mol. Cell. Biol. 9, 2424–2430

2 D. K. Miller and S. M. Raju, unpublished observations.