Activation of Nuclear Factor κB in Single Living Cells

DEPENDENCE OF NUCLEAR TRANSLLOCATION AND ANTI-APOPTOTIC FUNCTION ON EGFPRELA CONCENTRATION

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We have studied the dynamics of nuclear translocation during nuclear factor κB activation by using a p65(RELA)-enhanced green fluorescent protein (EGFP) fusion construct. Quantitation of expression levels indicates that EGFPRELA can be detected at physiological concentrations of about 60,000 molecules per cell. Stimulation of transfected fibroblasts with interleukin (IL)-1β caused nuclear translocation of EGFPRELA, typically resulting in a 30-fold increase in nuclear protein at maximum induction and a concomitant 20% decrease in cytoplasmic levels. The response of individual cells to IL-1β was graded, and the kinetics of nuclear translocation were dependent on the dose of IL-1β and the level of EGFPRELA expression. The rate of nuclear uptake was saturable, and the time lag for uptake increased at higher EGFPRELA expression levels. Furthermore, nuclear translocation was reduced at less than saturating doses of IL-1β suggesting that the pathway is limited by incoming signals. The response to IL-1β was biphasic, demonstrating a decline in nuclear import rate at expression levels above three to four times endogenous. This correlated with the anti-apoptotic function of EGFPRELA which was more prominent at low expression levels and demonstrated successively less protection at higher levels. In comparison, transfection of p50 had no effect on the level of apoptosis and demonstrated some toxicity in combination with EGFPRELA.

Inflammatory responses involve the rapid and coordinated activation of a diverse set of genes. This activation is initiated by a variety of agonists including bacterial lipopolysaccharide, phorbol esters, IL-1, and tumor necrosis factor-α (1–6) and in addition by stress (7, 8) and cell adhesion (9, 10). These agents dynamically regulate cytoplasmic signal transduction networks dependent on developmental stage- and cell type-specific expression of extracellular receptors and intracellular adaptor proteins. A major downstream target of these networks is the NF-κB family of transcription factors (11–15) that has been shown to possess both pro- and anti-apoptotic functions dependent on cell type (16, 17).

NF-κB transcription factors are hetero- or homodimers of a family of related proteins characterized by the Rel homology domain. They form two subfamilies, those containing activation domains (p65–RELA, RELB, and c-REL) and those lacking activation domains (p50, p52) (18). The prototypical NF-κB is a heterodimer of p65 (RELA) and p50 (NF-κB1). Additionally, a family of at least seven inhibitory subunits, IκB, characterized by multiple ankyrin-like repeats has been described (19, 20). The paradigm for NF-κB activation is nuclear translocation of p50 (NF-κB1)/p65 (RELA) heterodimers following IκB degradation. Specifically, IκB binds to these dimers, thereby masking their nuclear localization signals and preventing nuclear import. Activation of cytoplasmic signal transduction pathways results in the phosphorylation and then ubiquitination and subsequent degradation of IκBα, allowing the dimers to move to the nucleus where they activate genes bearing cognate binding sites. Other events, including phosphorylation prior to or after entering the nucleus, have been shown to be involved in regulating p65-RELA-containing dimers (21–25).

Signaling in response to IL-1 is of particular interest as this cytokine is a central mediator of inflammatory processes. The signal transduction pathway from IL1-R1 resulting in nuclear translocation of p65-RELA has been elucidated in some detail. In brief, IL-1 binding at the IL1-R1 induces the formation of a heterodimer with the IL-1 receptor accessory protein (26) and subsequent recruitment of MyD88 and the kinases IRAK1 and IRAK2 (27–29). This complex activates NF-κB-inducing kinase following recruitment of TRAF6 (30, 31). The subsequent activation of IKKα and IKKβ results in phosphorylation and degradation of IκBα (32–36) and activation of the transcription factor (37–41).

To analyze IL-1-induced activation of NF-κB in living cells, we have constructed a fusion of p65-RELA with the enhanced green fluorescent protein (EGFP). This protein is efficiently expressed in fibroblasts and smooth muscle cells and active with respect to DNA binding. We present an analysis by confocal fluorescence microscopy of its IL-1β-induced nuclear translocation in living cells. From a series of single cell recordings, we have determined the kinetics of nuclear uptake in response to IL-1 stimulation at various doses. The experiments show a biphasic response in the rate of nuclear translocation of EGFPRELA at increasing expression levels which correlates with protection against apoptosis at low expression levels. Furthermore, we demonstrate that co-transfection of p50 does not restore the defect in anti-apoptotic function of EGFPRELA expressed at high levels.

EXPERIMENTAL PROCEDURES

Materials—Polyclonal antibody to GFP, pEGFP.N2 and pEGFP.C1 vectors, and purified EGFP protein were from CLONTECH (Palo Alto, CA). Polyclonal antibodies to RELA (sc-109, sc-372-G), p50 (sc-1191X),
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IxBa (sc-371X), TRAF1 (sc-874), TRAF2 (sc-876), biotinylated goat anti-rabbit Ig (sc-2040), biotinylated donkey anti-goat Ig (sc-4024), horse-radish peroxidase-conjugated anti-goat Ig (sc-2090), and horseradish peroxidase-conjugated anti-rabbit Ig (sc-2040) were from Santa Cruz Biotechnology (Santa Cruz, CA); streptavidin-Texas Red and Prolong Antifade (Molecular Probes, Eugene, OR). IL-1β was a gift of the Immunix Corp. (Seattle, WA).

Plasmid Constructs — The plasmid pEGFPRELA contains the p65-RELA cDNA fused in frame to the carboxyl terminus of EGFP downstream of the CMV promoter. It was constructed by subcloning a HindIII to BamHI fragment from pBlueScript-RELA (42) into pEGFP-C1 followed by in-filling of the vector-derived XhoI site using Klenow enzyme. The plasmid pCMV-IκBα was constructed by first cloning the IxB cDNA from BomHI plus EcoRI-digested pGEX-IκBα (43) into pEGFPN2 digested with BglII plus EcoRI then EcoRI plus BamHI digestion, Klenow in-filling, and religation of the resulting plasmid to remove the EGFP coding sequence. The plasmid pCMV-p50 was constructed by subcloning a HindIII plus BglII-digested fragment from pRSV-NfB1(p50) (42) into pCMV.

Mammalian Cell Transfection — Human gingival fibroblasts (three lines) and monkey smooth muscle cells (one line) were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal calf serum. Cells were transfected by calcium phosphate coprecipitation with glycerol shock (60 s, 20% glycerol in PBS) 4 hours after plating. Typically yielding 10–20% transfection efficiency. Briefly, cells were plated on 8-chamber coverslips (Nunc) at 10,000 cells per transfection (44) typically yielding 10–20% transfection efficiency.

Confocal Fluorescence Microscopy — GFP fusion proteins were visualized using a Molecular Dynamics confocal laser scanning microscope with a 37°C stage incubator. Standard settings for analysis of EGFP were laser power = 10 milliwatts (split equally between 488 and 568, 647 emission lines), band selection = 488 nm, photomultiplier tube voltage = 667, and attenuation varied to maintain pixel density below 200 which is within the linear range of the instrument. Kinetic data were collected with a 60X Plan Apo oil immersion objective (NA 1.4) and a 50-μm aperture generating an optical section of 0.54 μm. For quantification of nuclear and cytoplasmic protein levels, horizontal sections were scanned through the nucleus of transfected cells. Images were acquired with optimal settings with no discrepancies noted between the fibroblasts and the EGFPReduced cellular image. Nuclear localization was calculated by measuring the mean intensity of representative areas of nucleus and cytoplasm and dividing by the attenuation setting. When multiple images of the same cell were analyzed, the readings were corrected for fade by comparison with measurements made using unstimulated cells.

Immunofluorescence Staining — To enable comparison of transfected EGFPRELA with endogenous p65-RELA or induced IxBα/TRA2F, fibroblasts were transfected as above and images of EGFP fluorescence recorded at 24 h. These cells were then fixed/permeabilized in methanol (5 min, −10°C) and then blocked overnight at 4°C in PBS + 5% normal goat or donkey serum (according to secondary antiserum). Immunostaining was performed with primary antisera (2 μg/ml), washed 3 times in blocking buffer, incubated with biotinylated secondary antiserum (sc-2040, Santa Cruz Biotechnology), washed 3 times in blocking buffer, incubated with streptavidin/Texas Red (Molecular Probes), washed 3 times in blocking buffer and twice in PBS, and then mounted using prolong anti-fade reagent (Molecular Probes). Previously scanned cells were identified and re-scanned as for EGFP fluorescence except using excitation at 668 nm and a photomultiplier tube at 750 V.

Electrophoretic Mobility Shift Assay — To prepare nuclear and cytosolic extracts, cells were washed in ice-cold PBS and then resuspended in buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin) (200 μl/10 cm plate). After incubation for 15 min on ice, 12.5 μl of Nonidet P-40 was added; cells were vortexed briefly and nuclei pelleted by centrifugation for 10 min at 5000 rpm. The supernatant was removed (cytoplasmic extract), and the pellet was resuspended in 100 μl of buffer B (20 mM Hepes, pH 7.9, 0.4% salt, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin). After incubation for 30 min on ice, the samples were clarified by centrifugation for 5 min at 13000 rpm at 4°C, and the supernatant (nuclear extract) was removed. A probe for NF-κB binding activity was prepared by annealing the complementary oligonucleotides TTTGGA-CCTTTCCGCAGTGACCTCTTCGGGAAGTCTCCCTTCG and then in-filling the single-stranded overhangs using [α-32P]dATP. The probe (2 fmol) and nuclear extract were mixed in binding buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, 0.5% Nonidet P-40, 50 μg/ml salmon sperm DNA), incubated at room temperature for 20 min, and then separated on 0.5 × TBE, 4% polyacrylamide native gels. The specificity of the complexes was confirmed by competition with unlabeled NF-κB oligonucleotides (data not shown). Supershifts were performed by preincubation of antibody with the nuclear extract in binding buffer for 15 min, 4°C. Dried gels were analyzed by PhosphorImager (Bio-Rad).

Immunoblotting — Total cell extracts were prepared by lysis in 1× SDS sample buffer or nuclear and cytoplasmic protein extracts prepared as above. Extracts were separated by SDS-polyacrylamide gel electrophoresis and electroblotted to polyvinylidine difluoride membrane (Amersham Pharmacia Biotech). Immunoblotting was performed using primary anti-GFP (1:1000), anti-RELA (1:2000), anti-IκBα (1:1000), anti-TRA1F (1:1000), or anti-TRA2F (1:200) and secondary horseradish peroxidase-conjugated anti-rabbit/goat Ig at 1:2000 dilution in blot (5% milk powder, 10 mM Tris, pH 8, 150 mM NaCl, 0.05%Tween 20) with ECL detection according to manufacturer’s recommen-
dations (Amersham Pharmacia Biotech). Calibration was obtained by importing scanned films into NIH Image.

Quantitation — We have estimated the absolute expression levels of transfected proteins based on fluorescence and immunoblot comparison with purified EGFP standard. For fluorescence-based quantitation of protein concentrations, the fluorescence of a series of dilutions of purified EGFP spotted on a coverslip was determined. A relative fluorescence of 1 corresponds to an EGFP concentration of 67 μg/ml (in other units: 2.47 μM or 1500 molecules/μm³). The average volume of a fibroblasts is 2000 μm³, so if the purified EGFP has similar fluorescent properties to that expressed in the fibroblasts, a relative fluorescent fluorescence of 1 corresponds with approximately 3 × 10⁶ molecules of fluorescent protein per cell. We then estimate an expression level based on immunoblot analysis, cells were transfected with EGFP, and the mean fluorescence was determined by confocal microscopy to be 13, suggesting an average EGFP expression level per cell of 39 ± 10⁶ molecules. However, the same cells analyzed by immunoblot against EGFP standards (Fig. 3C) indicates a mean expression level of 6.8 ± 10⁶ molecules per cell, i.e., approximately 6-fold lower than by fluorescence alone. The most likely cause for this discrepancy was fluorescence “bleed-through” from adjacent nuclei. Quantitation of confocally detected EGFP so we have used the lower estimate throughout this paper.

Apoptosis Assay — 16 h after transfection, a grid of fluorescence images of cells at ×20 were recorded (t = 0), and then TNFα (10 ng/ml) plus cycloheximide (10 μg/ml) were added. Subsequent fluorescence images of the same area were recorded at 2-h intervals for 18 h. While the fibroblasts migrate between time points, the use of relatively low magnification and a grid of adjoining images allows them to be tracked during the experiment. The images were analyzed to determine cell death (evident as rounding and subsequent fragmentation). To determine the variation of cell death with expression level, the cells were ranked according to initial nuclear fluorescence, and the percentage survival for a 31-cell sliding window was plotted. The window size was chosen to reduce stochastic fluctuations in the data. Whereas a precise correlation with the translocation assay is not possible due to the different magnification, the range of expression levels over which the anti-apoptotic function of EGFPRELA is strongest broadly correlates with the range in which nuclear translocation is not impaired (i.e., less than 10-fold overexpression) and the loss of pro-apoptotic function correlates with constitutive nuclear expression.

RESULTS

Time and Expression Level Dependence of EGFPRELA Localization in Transiently Transfected Fibroblasts — Analysis of transiently expressed EGFPRELA by confocal fluorescence microscopy demonstrated that expression of both the quantity and distribution of protein (Fig. 1A). Quantitative analysis of transfected cells showed that the total amount of EGFPRELA varied by over 100-fold between transfected cells and demonstrated increased nuclear localization in high expressing cells (Fig. 1B). Cells expressing high nuclear levels of EGFPRELA were more numerous at early times while the ratio of

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nuclear to cytoplasmic concentration approached 1:10 between 24 and 72 h in the majority of cells. Furthermore, regulation of EGFPRELA localization by IκBα was shown to be similar to that of the endogenous protein by co-transfection with an expression plasmid (pCMV:IkBa) for IκBα, showing a dose-dependent reduction of nuclear EGFPRELA (Fig. 1C). The time-dependent reduction of nuclear EGFPRELA localization is most likely a consequence of activation of the endogenous IkBa gene by nuclear EGFPRELA as indicated by immunoblot (see Fig. 8).

Immunostaining of EGFPRELA-transfected cells with RELA antiserum was carried out to correlate the relative expression levels of EGFPRELA and endogenous RELA (Fig. 2A). There was a linear relationship between EGFP fluorescence and immunostaining measurements at moderate expression levels. The data indicate 8-fold overexpression of EGFPRELA compared with endogenous RELA at relative fluorescence 1. Calibrating the system using fibroblasts transfected with EGFP and comparison of cell extracts with purified EGFP protein by Western blot (Fig. 2B) indicates that relative fluorescence 1 corresponds to expression of approximately 500,000 molecules per cell. Thus the endogenous level of RELA expression is ~60,000 molecules per cell.

To verify the integrity and functionality of EGFPRELA, ex-
transfected EGFP. Lanes are as follows: 1, EGFPRELA-transfected cells; 2, clear localization of EGFPRELA is not a consequence of transfection. EGFPRELA-transfected cells both demonstrate that the nuclear localization of endogenous RELA but not significantly by p50 antisera demonstrating that, in contrast to endogenous RELA, transfected EGFPRELA is comparably to that of endogenous p65-RELA in stimulated but untransfected cells, and this may underlie the observed impairment of nuclear translocation at high EGFPRELA expression levels.

A. HGF cells were transfected with pEGFP-RELA. EGFP fluorescence determined then immunostaining was performed for total RELA expression. Each point represents a single cell analyzed by both methods. The ratio of EGFPRELA to endogenous p65-RELA at relative fluorescence = 1 is the gradient divided by intercept of the best-fit-line (i.e. 6.99:0.89). B. immunoblot quantitation of transfected EGFP. Lanes are as follows: 1, extract from 30,000 transfected cells; 2, extract from 7500 transfected cells; 3, 30-kDa marker; 4-8, 1, 2, 5, 10, and 20 ng of purified EGFP, respectively. The mean relative fluorescence of the transfected fibroblasts was estimated to be 13.

Consecutive measurements of IL-1β-stimulated cells (Fig. 5A) show nuclear import of the fusion protein (Fig. 5B) which, when averaged (Fig. 5C), reveals kinetics at the population level that are in accord with those of endogenous RELA as determined by immunoblot (data not shown). Three aspects of the kinetics of nuclear import, namely lag time, nuclear to cytoplasmic ratio, and the rate of nuclear import, were analyzed (Fig. 6). The lag time for nuclear import (Fig. 6A) reveals two phenomena. First, a decrease with increasing doses of IL-1β, correlating with faster ligand-receptor interaction. Second, a moderate increase at higher EGFPRELA expression levels suggesting a threshold in the upstream activation mechanism or reflecting a feedback mechanism through enhanced levels of IκBα.

Variation of the maximum rate of nuclear uptake with initial cytoplasmic EGFPRELA allows investigation of the saturability of nuclear import (Fig. 6B). The maximal increase of approximately 1 relative fluorescence unit in 10 min corresponds to 40 molecules/s entering the nucleus based on calibration as in Fig. 2 and assuming a nuclear volume of 100 μm³. However, it is notable that rather than plateauing with increasing EGFPRELA concentration, the rate of nuclear import actually peaks at a relative cytoplasmic expression level of approximately 2 and subsequently declines. Furthermore, by replottting the nuclear import data as relative rates (i.e. adjusted for the level of fusion protein expression, Fig. 6C), it is clear that even at moderate EGFPRELA levels there is a reduction. Analysis of the maximum nuclear:cytoplasmic ratios attained on stimulation with IL-1β also reveals expression level dependence; at higher levels of EGFPRELA expression, the ratios are reduced even though the absolute levels of nuclear accumulation increase (Fig. 6D).

In summary, several characteristics of the import process are
demonstrated by these results. First, the response at the single cell level is a continuum. Second, initiation of nuclear import does not lead rapidly to a new steady state. Third, although some cells are fully stimulated at the lowest dose, others are only partially stimulated by the highest dose. Fourth, nuclear import is a continuous process that does not display rapid rate changes. Fifth, down-regulation of the stimulated state is slow; although the nuclear concentration of EGFPRELA peaks in most of the cells analyzed, none of them returned to the basal state within 90 min. Finally, continuing nuclear accumulation of EGFPRELA is observed throughout the time course in some cells, indicating that the signal transduction pathway is capable of remaining in an activated state for a prolonged period.

**EGFPRELA Protects Fibroblasts from TNFα Plus Cycloheximide-induced Apoptosis at Low Expression Levels**—Our data show that the expression level of EGFPRELA is critical for IL-1β induction, suggesting that biological effects such as anti-apoptotic function may also be sensitive to RELA levels in the cells. To investigate this possibility, we have analyzed the effect of EGFPRELA transfection on TNFα plus cycloheximide-mediated killing of fibroblasts. The addition of cycloheximide is necessary in these cells to render them TNFα-sensitive. Although it will block further expression of protective genes, it is added 16 h after transfection to allow induction of the protective state by EGFPRELA. We find that this treatment induces the rapid apoptosis of control, EGFP-transfected fibroblasts, consistently yielding greater than 90% cell death within 18 h compared with approximately 20% cell death of control cells. By contrast, cells transfected with EGFPRELA are less sensitive to this treatment, and approximately 70% cell death is observed at 18 h compared with 30% in untreated cells (Fig. 7A, left). In light of these data, we investigated whether cell survival was influenced by the level of EGFPRELA expression. We find that the anti-apoptotic function is strongest in the cells expressing relatively low levels of nuclear EGFPRELA, cell survival being approximately 4-fold higher for low than high level expressors (Fig. 7B, column 4). At high levels of EGFPRELA expression, there is an increased death of untreated cells (Fig. 7B, column 3). By contrast, no expression level dependence is seen for the survival of cells transfected with EGFP (Fig. 7B, columns 1 and 2).

To investigate whether an enhanced anti-apoptotic activity could be obtained by increased levels of heterodimer, the above experiments were repeated in the presence of co-transfected p50. We find that death of cells co-transfected with EGFP plus p50, compared with cells transfected with EGFP alone, is increased in the absence of an apoptotic stimulus and not significantly different in the presence of TNFα plus cycloheximide (Fig. 7A, 3rd graph). Similarly to the increased cell death observed in untreated cells transfected with EGFPRELA, the effect of p50 in untreated cells is most pronounced at high expression levels (Fig. 7B, compare columns 1 and 5). When p50 is co-transfected with EGFPRELA, there is increased cell death at high expression levels compared with transfection of EGFPRELA alone both in the absence and presence of TNFα plus cycloheximide (Fig. 7B, compare columns 3 and 4 with 7 and 8). This clearly demonstrates that p50 is not a limiting component for the anti-apoptotic effect of EGFPRELA.

### Fig. 3. Characterization of EGFPRELA fusion protein.

**A,** nuclear and cytoplasmic protein fractions were isolated from fibroblasts transfected with EGFP or EGFPRELA and analyzed by immunoblotting with anti-RELA antibody. Position of endogenous p65-RELA (E) and transfected EGFPRELA (T) are indicated. **B,** analysis of DNA binding properties of EGFPRELA. Cells were transfected with EGFP or EGFPRELA. The following day cells were left untreated (−) or treated for 30 min with 1 nM IL-1β (+), and electrophoretic mobility shift analysis of NF-κB DNA binding was performed. Position of endogenous and transfected complexes are indicated. **C,** characterization of subunit composition of complexes in EGFPRELA-transfected, IL-1β-induced nuclear extract. Supershift analyses were performed using increasing amounts (1, 2, or 4 μl) of antisera to RELA, p50, or GFP as indicated. *Con* = no antibody control.
The anti-apoptotic effect of EGFPRELA in the above assay is most likely a consequence of induction of protective gene(s) during the interval between transfection and addition of TNFα plus cycloheximide. Therefore, we have investigated the induction in EGFPRELA-transfected cells of IκBα and TRAF1/2 (whose expression in RELA-deficient embryonic fibroblasts protects from TNFα-induced apoptosis (46)). IκBα is induced in cells transfected with RELA or EGFPRELA but not p50. However, there is no induction of TRAF1 or TRAF2 (Fig. 8). Since the anti-apoptotic “window” is confined to a narrow range of EGFPRELA, we have additionally analyzed transfected cells by immunofluorescence to examine induction as a function of EGFPRELA expression level. Whereas induction of IκBα is observed at a broad range of EGFPRELA expression levels, induction of TRAF is not detected at any level (data not shown).

**DISCUSSION**

To analyze NF-κB function in single, living cells we have used EGFPRELA as a fluorescent probe. The wide range of expression levels in transiently transfected cells enables analysis of NF-κB regulation as a function of intracellular concentration, both absolute and relative to endogenous RELA, and by inference to analyze the endogenous function. Quantitation of EGFPRELA expression relative to the endogenous protein (Fig. 2) shows that among the cells we have analyzed, a significant fraction expresses the fusion at or near physiological levels. At less than 10-fold overexpression, the ratio of nuclear to cytoplasmic concentrations of EGFPRELA is approximately 1:10 in unstimulated cells compared with 4:1 in maximally IL-1β-stimulated cells, indicating robust cellular regulation of the nuclear:cytoplasmic concentration gradient (Fig. 4). At higher expression levels, we find that either the protein is concentrated in the cytoplasm but impaired for nuclear translocation or it is aberrantly concentrated in the nucleus.

The concentration dependence of EGFPRELA function may relate to its association with other cellular proteins, such as IκBα (Fig. 1). In addition to IκBα, endogenous RELA associates...
with various members of the NF-κB gene family to form homo- and heterodimers, and these interactions are also likely to be saturable. Therefore, EGFP-RELA overexpression is likely to alter the population distribution of NF-κB dimers; for example, at physiological levels it is likely that EGFP-RELA/p50 heterodimers predominate, whereas at higher levels EGFP-RELA homodimers may become more abundant as suggested by the EMSA data (Fig. 3 C), and this is likely to have functional consequences.

The anti-apoptotic function of NF-κB is dependent on the induction of protective genes, suggesting that the lower EGFP-RELA threshold for anti-apoptosis is determined by free IκBα levels. In addition, there is an upper threshold above which anti-apoptotic function of EGFP-RELA is lost (Fig. 7B) corresponding to expression levels at which nuclear translocation is impaired. Loss of anti-apoptotic function at high expression level is not due to loss of transactivation potential of EGFP-RELA since IκBα is efficiently induced. Rather, it is probably a consequence of activation of a different set of genes, either those having promoters with weaker NF-κB-binding sites or whose activation is not dependent on the interaction of RELA with a limiting cofactor.

Similar to our findings, contrasting effects of NF-κB have been reported by other groups. Strong evidence for an anti-apoptotic function of p65-RELA is provided by increased apoptosis in knockout mice, whereas other data suggest that activation is required for the completion of apoptosis in serum-starved 293 cells (11, 17). Likewise, c-REL induces apoptosis in avian bone marrow cells but extends the life span of fibroblasts in vitro (16). These data all illustrate that effects of NF-κB activation are intimately associated with other cellular factors, in line with an emerging concept that cells are protected from transformation by the requirement to activate multiple pathways to induce proliferation rather than differentiation or cell death.

Complete nuclear translocation of EGFP-RELA in response to IL-1β requires near-complete receptor occupancy, whereas at lower occupancies partial activation is attained (1 nM IL-1 gives 90% occupancy at 5 min and maximal response compared with 0.1 nM giving 30% occupancy at 5 min and partial response) (Figs. 4–6). The relatively low excess capacity in the pathway may be important in allowing the system to be rapidly reset following transient stimulation. The consequences for gene regulation of complete versus partial activation remain to be determined, but a strong possibility is that different genes will be sensitive to different levels of activation as a consequence of variations in the number or affinity of their cognate binding sites. These differences probably underlie the observation that, in contrast to NF-κB activation, certain IL-1-induced biological responses show a large spare receptor effect (47). Additionally, our data allow us to quantitate the potential amplification between binding of IL-1β and nuclear translocation of EGFP-
Gingival fibroblasts express 5,000–10,000 IL-1R1 molecules per cell, and we show that maximal activation of EGFP-RELA nuclear translocation requires near complete occupation of these, resulting in a maximum translocation rate of 40 EGFP-RELA molecules/s.

Like NF-κB, the glucocorticoid receptor is a transcription factor that undergoes cytoplasmic to nuclear translocation upon activation, but notable differences exist between the properties of EGFP-RELA and those reported for glucocorticoid receptor fusions with GFP (48–50). First, the glucocorticoid receptor fusions are not reported to display aberrant nuclear localization, probably as a consequence of intra- rather than intermolecular masking of the nuclear translocation domain. Second, the translocation of the glucocorticoid receptor is faster, significant nuclear localization being observed within three min. Finally, no loss of regulatory function is reported on overexpressing the glucocorticoid receptor, probably because there are no other limiting components involved in its nuclear translocation. We suggest that these differences are a characteristic of activation by direct ligand binding in contrast to activation by a signal transduction cascade.

In conclusion, our results indicate that the pathway transducing signals from IL-1R1 to activation of NF-κB in fibroblasts is saturable with respect to RELA concentration, limited by incoming signal, and displays modest amplification. The kinetics of this pathway are perturbed by even moderate overexpression of EGFP-RELA. Additionally, we find that impaired nuclear translocation on overexpression of EGFP-RELA correlates with the loss of anti-apoptotic function. Future studies will address the identity of rate-limiting steps and therefore critical control points within this signal transduction system.

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