Regulation of nuclear translocation of nuclear factor-κB relA: evidence for complex dynamics at the single-cell level

Kenneth SCHOOLEY*, Ping ZHU†, Steven K. DOWER‡ and Eva E. QWARNSTRÖM†[1]

*Department of Biochemistry, Immunex Research and Development Corporation, 51 University Street, Seattle, Washington 98101, U.S.A., †Department of Pathology, University of Washington, Seattle, Washington 98101, U.S.A., and ‡Division of Genomic Medicine, School of Medicine and Biomedical Sciences, University of Sheffield, Sheffield S10 2JF, U.K.

We have analysed activation of nuclear factor-κB (NF-κB) in response to interleukin-1 (IL-1) in human fibroblasts by tracking intracellular distribution and levels of endogenous relA, NF-κB1 and inhibitor of κB (I-κB) α using semi-quantitative confocal microscopy. Nuclear translocation of endogenous relA correlated with I-κBα degradation during stimulation with IL-1, whereas no effects were seen on levels or localization of NF-κB1. During pathway activation, relA was transported up a concentration gradient, resulting in a 3–4-fold increase in nuclear levels, but without any significant decrease in cytoplasmic concentration. IL-1 stimulation caused translocation of only 20 % of the relA, but resulted in degradation of up to 70 % of the cytoplasmic I-κBα. RelA nuclear translocation in fibroblasts correlated with DNA-binding activity measured by electrophoretic mobility shift assay (EMSA), both with respect to kinetics and IL-1 concentration-dependence. Clonal populations of cells demonstrated a marked degree of heterogeneity in the response to IL-1. The single-cell assay revealed the presence of responder and non-responder subpopulations, with an enhanced proportion of responder cells, and prolonged responses at higher concentrations of IL-1. Comparing different cell types demonstrated that whereas HepG2 cells, as fibroblasts, showed good correlation between nuclear translocation of relA and activation of DNA binding by relA-containing dimers, EL4 thymoma cells showed no effect on relA localization, even during induction of significant levels NF-κB activity, as measured by EMSA. The analysis shows that stimulation by IL-1 results in transient perturbation of the NF-κB system, which cycles between the resting and active states with net redistribution of a minor proportion of its DNA-binding component. In addition, it demonstrates significant cell-to-cell variations, as well as cell-type-specific differences in net relA nuclear transport in response to stimuli. The data are consistent with NF-κB constituting a dynamic and versatile system, regulated to a significant degree by binary events involving bidirectional trafficking between the cytoplasmic and nuclear compartments during pathway activation.

Key words: cytokine, inflammation, transcription factor.

INTRODUCTION

The nuclear factor-κB (NF-κB) pathway, which is central in regulation of the inflammatory response [1], is activated by cytokines such as interleukin-1 (IL-1) [2,3], by stress-initiated events [4–7], and by biomechanical insults [8–12]. The importance of NF-κB in inflammatory responses is underscored by the observations that promoter-binding sites for this transcription factor are present in many genes expressed during injury and infection [1], that active NF-κB is present in tissues during inflammatory responses [13] and that NF-$\kappa B^{-/-}$ mice show gross alterations in immune and inflammatory responses [14].

IL-1 acts through a heterodimeric receptor [type I IL-1 receptor/IL-1 receptor accessory protein (IL-1RI/AcP)] [15,16]. Both chains contain an approx. 200-residue intracellular portion, the TIR domain. Its presence in receptors and receptor-complex components such as the IL-1R, ST2/fit-1 [17], Toll-like receptors [18,19], MyD88 [20] and the IL-18 receptor [21,22] has been demonstrated to be associated with the activation of rel family transcription factors.

NF-κB acts as a dimer consisting of various combinations of the rel family of proteins, totalling about ten members. The prototypical dimer, the heterodimer NF-κB1/relA (p50/p65 NF-κB), plays a central role in inducing gene expression during inflammatory responses [1]. In an inactive state, NF-κB is retained in the cytoplasm by inhibitors of NF-κB (I-κBs), which block the nuclear-localization signal. Upon activation of the pathway, nuclear translocation of NF-κB occurs, following degradation of the inhibitor [1,23]. This is preceded by I-κBα phosphorylation by I-κB kinases ('IKKs'), ubiquitination and proteasome degradation [24]. However, more recently, IκBα degradation-independent activation of the pathway, in part relying on transport of NF-κB–IκB complexes across the nuclear membrane [25], has been demonstrated. In addition, mechanisms dependent on bidirectional nuclear/cytoplasmic trafficking have been shown to be fundamental during pathway activation [26]. Furthermore, quantitative analysis of real-time behaviour in live cells using green fluorescent protein (GFP)-tagged relA [26,27], demonstrated dependence of activation on levels of relA and the presence of rate-limiting steps in the pathway upstream of I-κBα degradation.

In the present study, we examine regulation of endogenous NF-κB at the single-cell level using immunocytochemistry and confocal microscopy. The data demonstrate a significant cell-to-cell variation, both with respect to the expression level of subunits and the magnitude of the response, within an untransfected clonal population. The experiments also show that net relA translocation in NF-κB activation is cell-type-specific, variable and input-dependent, and suggest a significant role for bidirectional trafficking in regulating amplification and sensitivity of the NF-κB pathway.

Abbreviations used: AcP, IL-1R accessory protein; BCA, bicinchoninic acid; EMSA, electrophoretic mobility shift assay; (E)GFP, (enhanced) green fluorescent protein; IL-1, interleukin-1; IL-1RI, type I IL-1 receptor; MKK, mitogen-activated protein kinase kinase; NF-κB, nuclear factor-κB; I-κB, inhibitor of NF-κB.

[1] To whom correspondence should be addressed (e-mail e.qwarnstrom@sheffield.ac.uk).

MATERIALS AND METHODS

Materials

Human gingival diploid fibroblasts [28], HepG2 cells and a T cell line EL4 were maintained as described previously [29], being cell lines that have all been demonstrated to respond to IL-1 by activation of inflammatory genes [9,29,30]. Carrier-free [^{32}P]P_i was obtained from Dupont/New England Nuclear, whereas [γ-^{32}P]ATP (3000 Ci/μmol) was from Amersham Biosciences. Purified polyclonal rabbit anti-peptide antibodies specific for NF-κB relA, NF-κB p50/p105 (NF-κB1), c-Rel and I-κBα/MAD3, and corresponding immunizing peptides, were purchased from Santa Cruz Biotechnology, Inc. Recombinant human IL-1β was expressed and purified as described previously [29].

Immunofluorescent confocal microscopy

Cells were grown to subconfluency on multi-well cover-glasses (Nunc) and fixed with 2% (w/v) paraformaldehyde containing 0.1% (v/v) Triton X-100. Autofluorescence was reduced by treatment with 50 mM NH_4Cl for 10 min. Cells were incubated for 1 h in blocking/permeabilization buffer [PBS containing 5% (v/v) normal goat serum and 0.1% Triton X-100] and subsequently with primary antibody against NF-κB subunits relA, NF-κB1 and I-κBα (2 μg/ml in blocking/permeabilization buffer) for 1 h. Following washing (three times in blocking/permeabilization buffer), cells were incubated with biotinylated goat anti-rabbit immunoglobulins (Molecular Probes; 2 μg/ml in blocking/permeabilization buffer) for 1 h, washed (3×) with buffer and incubated with a streptavidin–Texas Red conjugate (Molecular Probes, Eugene, OR, U.S.A.) at 0.2 μg/ml in the same buffer for 30 min. Final washes, successively with the same buffer (3×) and with PBS (2×), preceded mounting in 100 mg/ml DABCO (1,4-diazadicyclo[2.2.2]octane) in 50% (v/v) glycerol/PBS.

Data were acquired with a Multiprobe 2010 laser-scanning confocal microscope (Molecular Dynamics, Sunnyvale, CA, U.S.A.), interfaced with a Silicon Graphics workstation, using the 568 nm line of a Kr/Ar mixed-gas laser, a 50 μm pinhole and a 60× Plan Apo oil immersion objective (NA 1.4), generating a 0.54-μm-thick optical section. After acquisition, data files were exported to a Macintosh, converted into 8-bit TIFF images and imported into the NIH Image program for analysis. Staining intensity was determined by measuring the average intensity per pixel within a fixed-area circle placed over the relevant area of the cell. Data from 100 cells were collected, and means and S.D. were calculated for each data set using Excel. Further numerical analysis was performed using MLAB (Civilized Software, Silver Spring, MD, U.S.A.). Histograms were analysed by fitting linear and log-Gauss, Cauchy and skew distributions. The simplest model that fitted all the data was a sum of 2 log-Gauss distributions. The lag times were estimated by means measured against time analyses, and by fitting a piece-wise linear function to data up to 20 min.

In a series of control experiments, by imaging solutions of the detection reagent (streptavidin–Texas Red conjugate) used in the immunofluorescence experiments, we established that the intensity recorded in the images was linearly proportional to the concentration of the fluorescent probe (Figure 1), showing that fluorescence intensity in the confocal images can be equated with the relative concentration of the fluorescent dye. In the case of relA and IκBα, this has also been confirmed by comparing the level of green (515–545 nm) fluorescence detected in enhanced GFP (EGFP)–relA-transfected cells with the intensity of red fluorescence (> 590 nm) after staining with anti-relA or anti-IκBα antibodies, using a Texas Red developing reagent [26] (L. Yang and E. E. Qwarnström, unpublished work), as above.

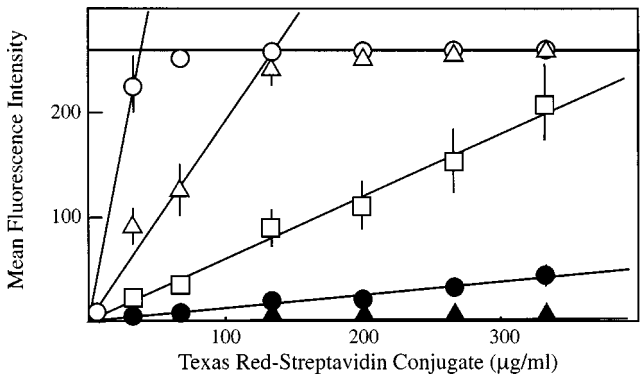

Figure 1 Quantitative detection of fluorescent probes by confocal microscopy

Analysis of signal intensity from solutions of Texas Red–streptavidin conjugates, showing a linear relationship between probe concentration and signal. Laser power 10 mW, 568 nm line, voltages 400 (▲), 500 (●), 600 (□), 700 (□) and 800 V (○).

Preparation of nuclear and cytosolic extracts

Cells (5×10^6) were washed with ice-cold PBS and re-suspended in 400 μl of buffer [10 mM Hepes (pH 7.9)/10 mM KCl/0.1 mM EDTA/0.1 mM EGTA/1 mM dithiothreitol/1 mM PMSF/1 mM leupeptin]. After 15 min at 4 °C, 25 μl of 10% Nonidet P40 was added. Cells were vortex-mixed briefly, nuclei were pelleted, and supernatants were removed (cytoplasmic extracts). Pellets were re-suspended in 200 μl of a buffer [composition: 20 mM Hepes (pH 7.9)/0.4 mM NaCl/1 mM EDTA/1 mM EGTA/1 mM dithiothreitol/1 mM PMSF/1 mM leupeptin]. After 30 min at 4 °C, lysates were centrifuged and the supernatant was removed (nuclear extract). Protein concentration of extracts was measured using the bicinchoninic acid (BCA) assay (Pierce).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts (10 μg of total protein, as determined by the BCA assay) were mixed with 0.02 unit of poly(dI-dC) (Pharmacia), and an end-labelled NF-κB oligonucleotide probe [9,12]; $\{(1–2.5) \times 10^4$ c.p.m. per reaction$\}$ in binding buffer (2.5 mM Hepes/50 mM KCl/0.1 mM EDTA/1.8 mM dithiothreitol/10% glycerol), and then incubated for 20 min at room temperature. For supershift assays, 0.2 μg of anti-(NF-κB subunit) antibodies [9] were included in the reaction mixture. Samples were separated on native 6% polyacrylamide gels (Novex, San Diego, CA, U.S.A.) in low-ionic-strength buffer (0.25× TBE buffer, where 1× TBE buffer is 45 mM Tris/borate/1 mM EDTA). Dried gels were exposed to a storage phosphor screen overnight, and quantitative autoradiograms were read using a PhosphorImager (Molecular Dynamics). Control experiments demonstrated that NF-κB band intensity was a linear function of oligonucleotide probe or nuclear extract concentration over a 10-fold range. The data were saved as 8-bit TIFF files, exported to a Macintosh computer and analysed with the NIH Image program.

RESULTS

Immunostaining showed the expected staining pattern of NF-κB pathway components in both unstimulated cells and cells with a

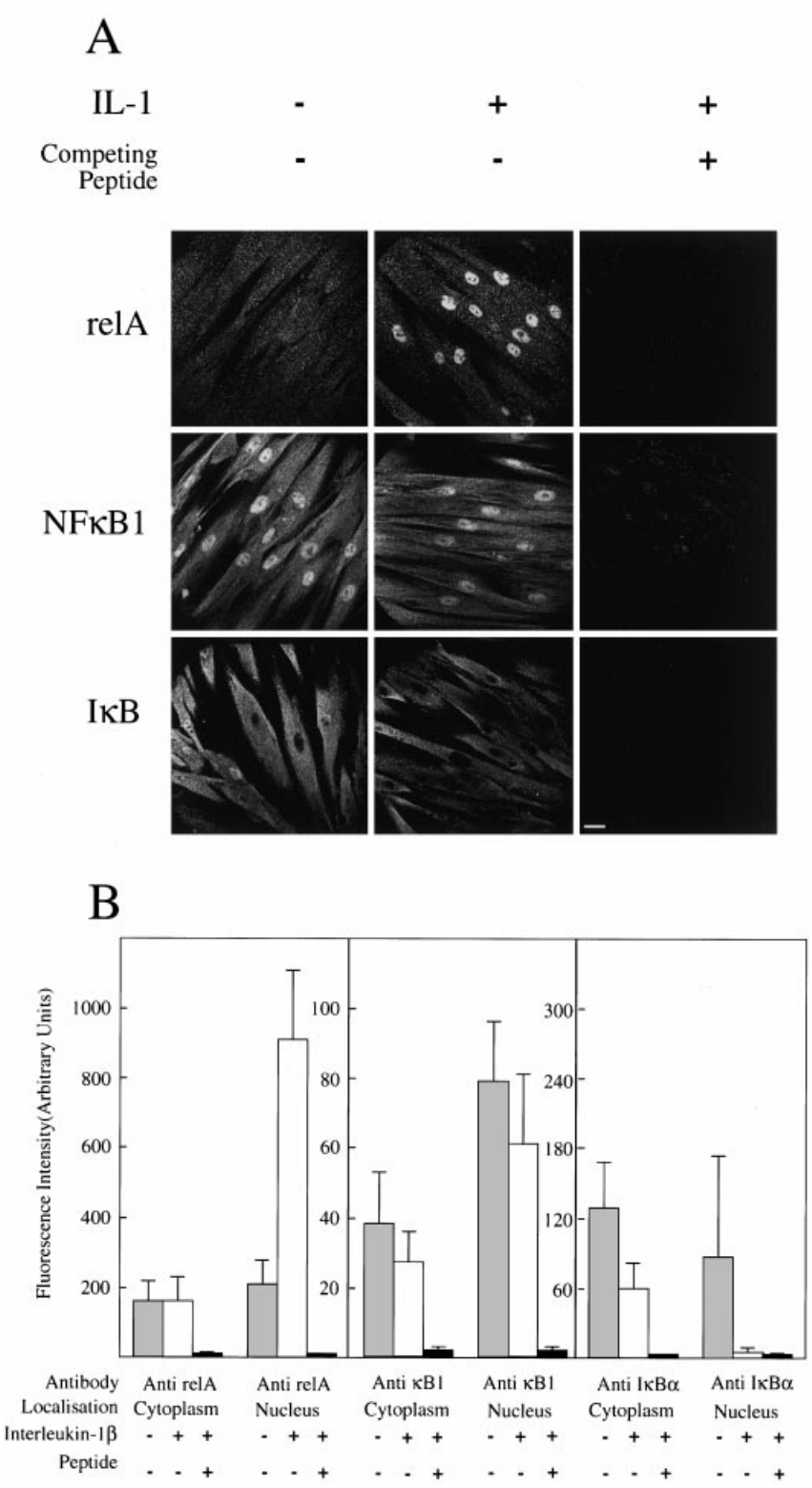

Figure 2 Quantitative immunofluorescence detection of NF-κB components in human diploid fibroblasts

(**A**) Fibroblasts in monolayer culture were treated with 10^{-10} M recombinant human IL-1β for 30 min at 37 °C, and subsequently prepared for immunofluorescence microscopy by staining with anti-(NF-κB) subunit antisera (Santa Cruz Biotech) and examined with a Multiprobe 2001 Laser Scanning Confocal Microscope (Molecular Dynamics). Control incubations included competing immunizing peptide (Santa Cruz Biotechnology; 10-fold excess, w/w), in addition to the subunit-specific antiserum. One representative experiment of three is shown. Scale bar = 10 μm. (**B**) Quantification of data was carried out as in (**A**), by converting images to 8-bit TIFF files and analysing with the NIH Image program, as described in the Materials and methods section. Results, collected at 550 V (p50), 650 volts (IκBα) and 750 volts (relA) and corrected on the basis of calibrations (Figure 1), are expressed as the mean fluorescence, with error bars (as S.D.) showing the cell-to-cell variation within a clonal population.

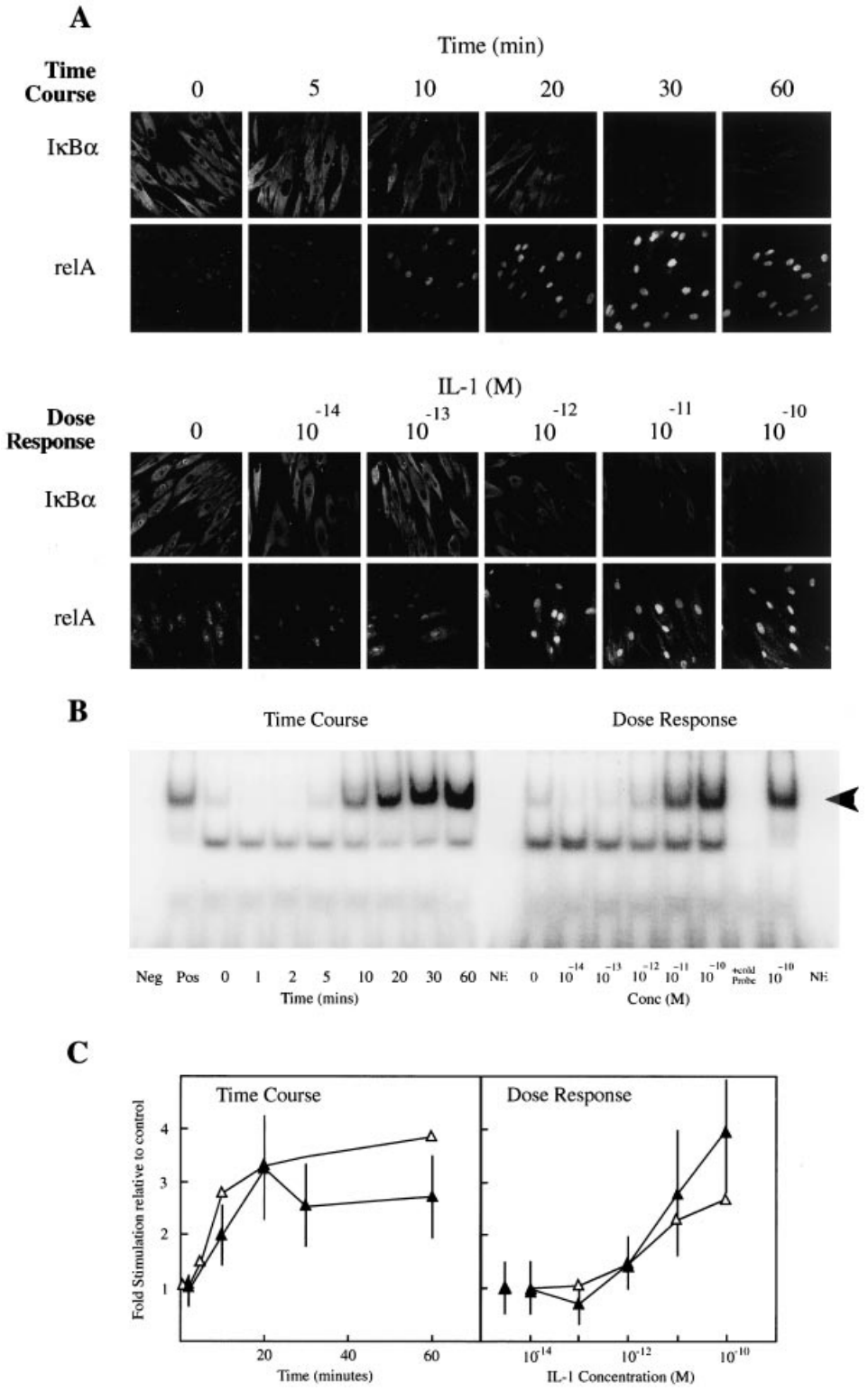

Figure 3 Quantitative analysis of the effect of IL-1β on NF-κB distribution in human diploid fibroblasts measured by confocal microscopy and nuclear DNA-binding activity measured by EMSA

(**A**) Human diploid fibroblasts in monolayer culture, treated with recombinant human IL-1β at 0.1 nM for various times at 37 °C or with various concentrations for 20 min, were prepared for immunofluorescence microscopy and labelled with antibodies specific for relA or IκBα, as described in the Materials and methods section. Representative results are presented in (**A**). Settings: 568 nm line, 10 mW total laser power and 700 V PMT voltage (relA), 600 V PMT voltage (IκBα). One of three experiments is shown. (**B**) Parallel cultures were treated identically with IL-1 and nuclei were isolated, extracted and analysed for NF-κB activity by EMSA. Gels were dried and autoradiograms acquired using a PhosphorImager (Molecular Dynamics). One of three experiments is shown. Neg, negative control (unstimulated cells); Pos, positive control (PMA-stimulated cells). (**C**) The data from experiments shown in (**A**) and (**B**) were image-analysed after export to a Macintosh and conversion into 8-bit TIFF files. Data from the microscopy measurements (▲) are calculated using 100 individual nuclei per point, and are expressed as mean fluorescence, with error bars (as S.D.) showing the cell-to-cell variation within a clonal population. Results from the EMSA assay (△) are representative of one of three experiments, as in (**B**).

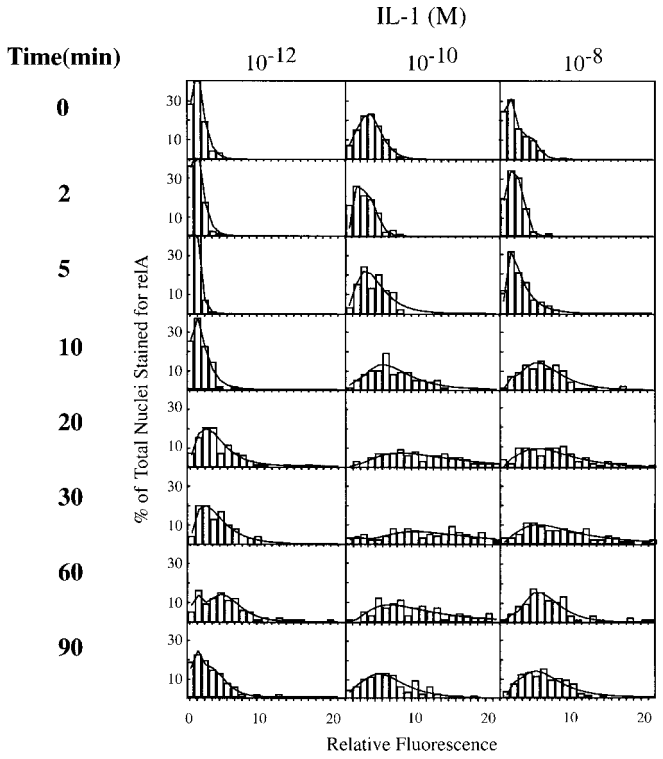

Figure 4 Time course of change in relA levels in nuclei of human fibroblasts following treatment with IL-1β

Cells were stimulated with IL-1 for the times and at the concentrations indicated, and labelled for relA by immunocytochemical staining. Images of 100 nuclei per time point were subjected to image analysis with NIH Image and the data were converted into histograms using Excel. The data were analysed with MLAB (Civilized Software). The continuous curves are calculated from the best-fit means and S.D. for a sum of two log-Gaussian distributions, as described in the Materials and methods section. Settings: 568 nm line, 10 mW total laser power, PMT 700 V. Data are representative of five experiments, all showing the same distribution.

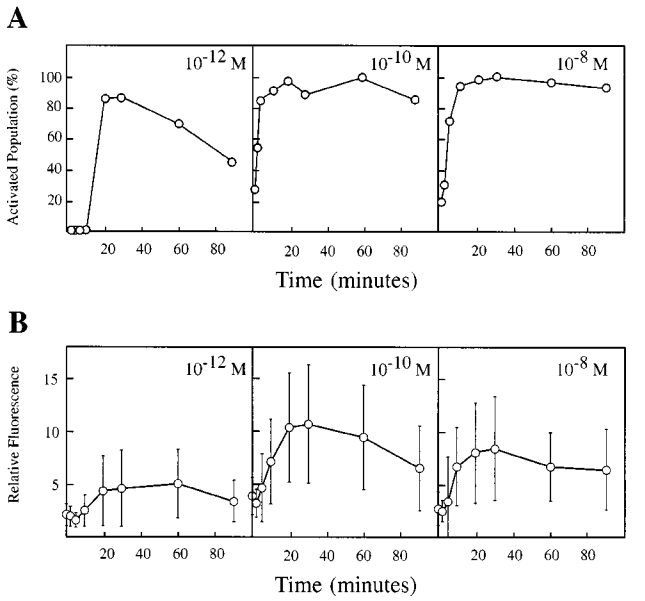

Figure 5 Analysis of change in nuclear relA levels in human fibroblasts following treatment with IL-1β

(**A**) Linear mean values for relative nuclear relA concentrations estimated by analysing the data in Figure 4, as described, are plotted against time. The error bars are the linear S.D. calculated from the best fit dual log normal distribution, estimated by non-linear leas-squares fitting of the standard form of the Gaussian distribution to log transformed data. (**B**) Percentage of cells in the responder subpopulation from the data shown in Figure 4 and determined by curve fitting, as described in the Materials and methods section.

concentration of IL-1 that results in 100% receptor occupancy by 5 min [28] (Figures 2A and 2B). RelA appeared to be distributed uniformly between the two compartments in unstimulated cells and underwent nuclear accumulation in the presence of IL-1. The level of NF-κB1 (p50) was, as expected, significantly higher than that of relA. It showed a higher concentration in the nucleus than in the cytoplasm in unstimulated cells, with no effect on the relative distribution following IL-1 treatment. I-κBα showed a wide variation in distribution, agreeing with variations in basal cycling [25,26]. Despite these variations, the inhibitor was excluded from the nucleus in 90% of both unstimulated cells and IL-1-treated cells and showed a marked reduction in levels in the cytoplasm (up to 70%) after stimulation. Image analysis revealed that relA accumulated in the nucleus to a final nuclear/cytoplasmic ratio of approx. 4:1 (Figure 2B). No significant change in cytoplasmic levels occurred, agreeing with results obtained using an EGFP–relA fusion protein, where only a small net decrease (20%) was observed in cells followed individually over time, due to the difference in nuclear/cytoplasmic volume [26].

Kinetic experiments showed a progressive increase in nuclear relA, peaking after 30 min (Figure 3A). In addition, there was a reciprocal relationship between relA and I-κBα levels, demonstrating both IL-1 concentration- and time-dependent reduction of inhibitor levels. The level of nuclear translocation of relA demonstrated kinetics which closely paralleled those of NF-κB activation, determined by EMSA in parallel cultures (Figures 3B and 3C). Similarly, quantification of the dose–response effect, showing a successive enhancement in band intensity from undetectable levels at 10^{-14} and 10^{-13} M of IL-1, to pronounced levels of activity at 10^{-10} M (Figure 3B) [28], demonstrated a precise correlation with the microscopy data. At saturating levels, nuclear DNA-binding activity was detectable after 5–10 min and was maximal between 30–60 min, with a half-maximal response to IL-1β at 10^{-12}–10^{-11} M at 20 min. Supershift assays (see Figure 6A) confirmed that, as shown previously [9,26,31], all DNA-binding activity in the nuclei of the cells could be accounted for by relA-containing complexes.

A more detailed assessment was carried out by quantitative single-cell analysis. The extent of the variation in relA levels, both in control and stimulated samples, was demonstrated by plotting relative nuclear fluorescence against the percentage of stained nuclei and over the time of IL-1 incubation. To examine the heterogeneity of the endogenous response systematically, we selected three concentrations of IL-1 (10^{-12} M, 10^{-10} M and 10^{-8} M) yielding a 200-fold range in receptor occupancy at 5 min [28,32]. The data show an enhancement in nuclear relA between unsaturated (10^{-12} M) and saturating (10^{-10}–10^{-8} M) levels of the cytokine (Figure 4). In addition, they show a reduction in the lag-time with increasing IL-1 concentrations, corresponding to 7–10 min at 10^{-12} M and 4 min at 10^{-10} M and 10^{-8} M (Figure 4). Furthermore, at all concentrations there is a small population of cells with very low levels of nuclear fluorescence throughout stimulation, and an active population, demonstrating an IL-1-induced increase in relA nuclear levels. The induced population shows a transient increase in fluorescence, again correlated with IL-1 concentration. A cell-to-cell variation with the same type of

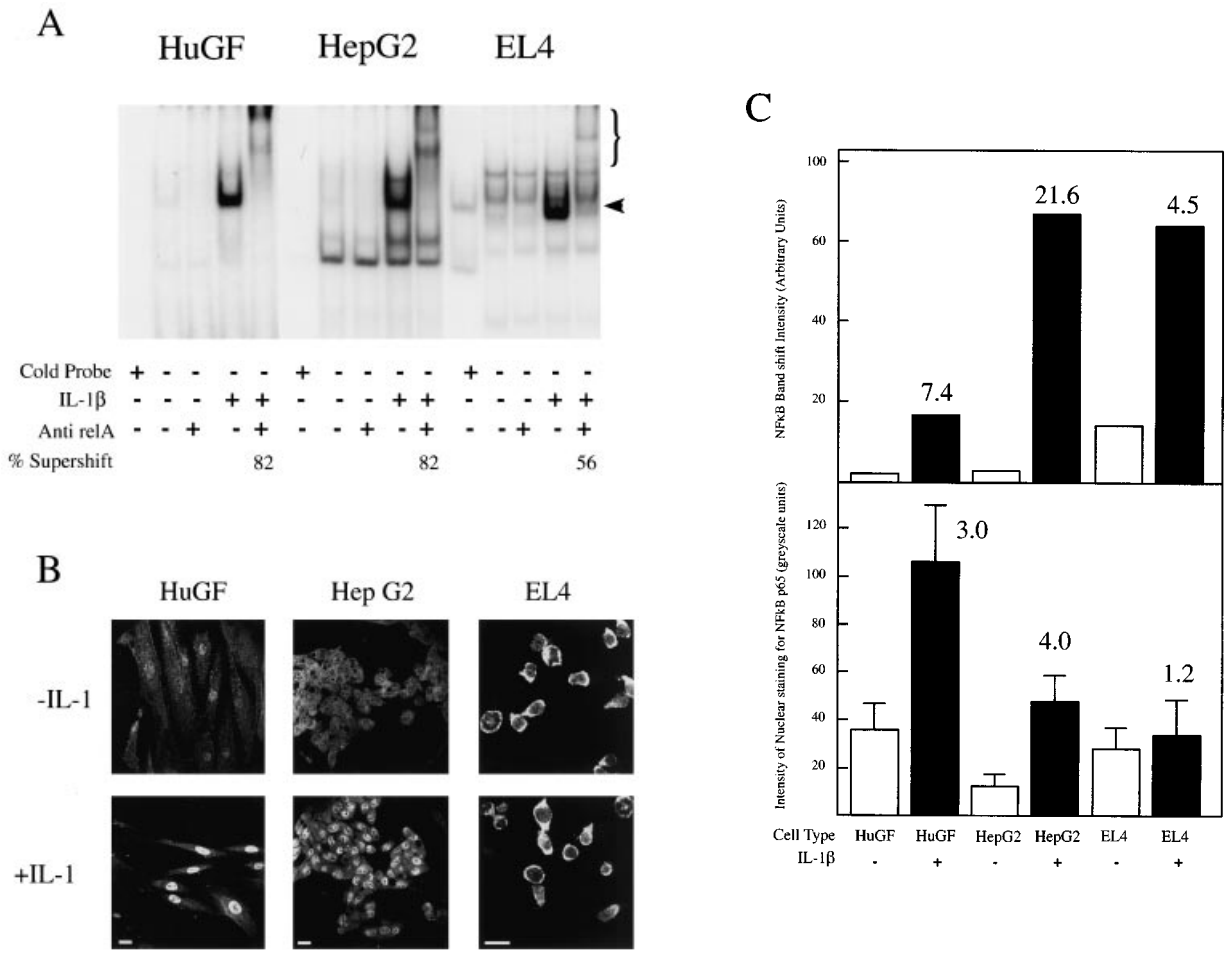

Figure 6 Correlation of the relative level of DNA-binding activity with relA translocation in different cell types

(**A**) EMSA and relA supershift analysis of human diploid fibroblasts, HepG2 cells or EL4 cells treated with 0.1 nM recombinant human IL-1β for 30 min at 37 °C. One of three experiments is shown. (**B**) Confocal fluorescence micrographs of cells incubated with or without 0.1 nM IL-1β for 30 min at 37 °C and subsequently stained for relA. All three scale bars represent 10 μm. One of three experiments is shown. (**C**) Quantitative comparison between activity from band-shift (top histogram) and nuclear translocation of relA (bottom histogram) from confocal microscopy in fibroblasts, HepG2 cells and EL-4 cells, as in (**A**) and (**B**) above.

distribution was obtained similarly following activation of the pathway by cell attachment via binding of the fibronectin receptor, and following simultaneous stimulation via the cytokine and via adhesion (results not shown). Further information regarding the cell-to-cell variation was obtained by curve-fitting, using a series of models of increasing complexity. The simplest model that fitted all the data sets was a sum of two log-Gaussian distributions, underscoring the wide variation in nuclear relA levels.

The single-cell data was summarized by calculating the average fluorescence over time of stimulation with various concentrations of IL-1 (Figure 5A), with the error bars demonstrating the true spread in the readings within the population. This distribution closely resembles the variation in the NF-κB response, observed with EGFP–relA [26]. With an increase in concentrations between 10^{-12}–10^{-10} M, a reduction in lag-time prior to the increase was observed, as was an increase in the level of the plateau subsequently reached, in agreement with the data using EGFP–relA [26]. Following saturation of receptor occupancy at higher concentrations, no further reduction in lag-time or increases in plateau value were seen. In addition, the increase to saturating levels of IL-1 correlated with an enhancement of the percentage of the activated cell population (Figure 5B). Furthermore, the duration of the activation within the total responder population was extended at saturating levels of the cytokine.

To examine the significance of the relA translocation as a readout for pathway activation, we used EMSA assays to compare fibroblasts with two other IL-1-responsive cell types from different lineages: the hepatoma line HEPG2 and the thymoma line EL-4 (Figure 6), which have been demonstrated to respond to IL-1 stimulation by enhanced levels of NF-κB activity and induction of NF-κB-regulated genes [28–31]. Of these, the fibroblasts showed the simplest response, with $> 80\,\%$ of the IL-1-stimulated DNA-binding activity migrating as a single band, essentially all of which could be super-shifted with anti-relA antibody, demonstrating the presence of relA in all dimers activated by IL-1 (Figure 6A). In comparison, in IL-1-stimulated HEPG2 cells three clearly resolved IL-1-induced species on the basis of mobility were observed, in addition to a constitutive species. Of these four bands, only the two slower migrating

species were super-shifted with antibody, and hence contained relA. The EL4 cells showed four species in both unstimulated and IL-1-stimulated cells. Only one, which by super-shifting was demonstrated to contain relA, was IL-1-inducible.

Comparing these data with confocal analysis of NF-κB/relA translocation showed similarities between hepatoma cells and the fibroblasts, both of which demonstrated nuclear accumulation of relA when stimulated with IL-1 (Figure 6B). In contrast, whereas EL4 cells showed intense cytoplasmic staining and weak nuclear staining for relA, there was no effect of IL-1 on compartmental distribution. Quantification of the results from both assays showed an increase in fibroblasts of band intensity and nuclear translocation (7.5- and 3-fold increases respectively; Figure 6C). In comparison, the hepatoma cells, which demonstrated an increase in nuclear relA translocation of the same order as that of the fibroblasts (4-fold), showed a significantly greater increase in DNA-binding activity, reaching approx. 22-fold, suggesting that additional mechanisms are involved in regulating activation of the pathway. In contrast, with a relative increase in DNA-binding activity that was very similar to that induced in fibroblasts, the EL4 cells showed no changes in relA nuclear levels, suggesting a mechanism of regulation of NF-κB that is independent of net effects of relA translocation.

DISCUSSION

We have used antibody labelling and confocal microscopy in order to analyse NF-κB responses at the single-cell level, specifically as it relates to the role of nuclear translocation in the regulation of pathway activity. The experiments showed that, of three NF-κB components (rel-A, NF-κB1 and I-κBα), relA was present at the lowest concentration, and IL-1-induced nuclear translocation of relA correlated well with both concentration-dependence and kinetics of DNA-binding activity, as measured by EMSA. The relative nuclear concentration of relA during stimulation with saturating levels of the cytokine increased to up to 4:1. Cell-to-cell variation in the endogenous response, demonstrating the presence of responder and non-responder populations, showed either an 'all' or a 'nothing' effect on cytokine stimulation for the two populations respectively, with the former population showing a concentration-dependent level of nuclear translocation. Assessing the significance of net translocation of relA as a key mechanism for regulation of NF-κB activation demonstrated cell-type-specific differences.

The relatively low absolute level of relA translocation compared with the marked reduction in IκB agrees with our observations using EGFP-fusion proteins [26,33], and suggests a high level of bidirectional trafficking and nuclear/cytoplasmic cycling of NF-κB. The level of relA may be regulated through a limiting component, maintaining continuous trafficking between compartments [27]. Activation of NF-κB, even by a potent stimulator such as IL-1, thus represents a perturbation of the system from its basal state, involving a net translocation of a minor fraction of the DNA-binding component and depending significantly on nuclear/cytoplasmic cycling. This type of regulation could also explain the differences between cell types, reflecting specific cytoplasm/nuclear ratios and/or cell-specific rates of translocation, and may thus account for the capacity of IL-1 to induce strong activation of relA-containing complexes, without any change in nuclear concentration.

Bidirectional trafficking of NF-κB components is supported by mathematical analysis, accounting for this parameter as an important part of regulation (S. K. Dower and E. E. Qwarnström, unpublished work). Furthermore, specific pathway-activating translocation of relA has been demonstrated to be dependent on bidirectional trafficking, destined for both direct DNA activation [27,34] and enhancing transactivation subsequent to phosphorylation of the NF-κB subunit [31]. Furthermore, the significance of diverse trafficking in regulation of the pathway is in agreement with the observation of independent nuclear translocation of IκBα and NF-κB during tumour necrosis factor induction of the pathway [25]. Negative regulation dependent either on translocation of the inhibitor alone [35,36] or of NF-κB1 homodimers [37–39] supports the involvement of complex trafficking patterns in regulation of the pathway.

The wide range of variation in the nuclear concentration of relA among cells was observed at all IL-1 concentrations and times tested. It is unlikely that the primary source of this variability is simply the receptor number, since the variation of the response is the same over a 10000-fold range in IL-1 concentration. In addition, the similarity in the cell-to-cell variation obtained following induction of the pathway via structural agonists suggests this to reflect true variations in signalling. These distributions are maintained throughout stimulation and, once detectable, the mean within the responding population alters progressively with time. This suggests that, once committed, cells respond with an all-or-none effect on DNA-binding activity and transcription of NF-κB-regulated genes. This type of binary-control mechanism has been demonstrated in stress-kinase cascades [40]. Since such cascades have been shown to lie downstream of the IL-1R and upstream of NF-κB [41,42], it is tempting to speculate that signalling elements account for the on–off switch in the response, particularly at low levels of IL-1R occupancy. The results are similar to those observed in complete time courses from individual cells using EGFP–relA [26], and show that once activated, translocation is slow and heterogeneous among the population, suggesting that steps distal to receptor activation determine the final rate and extent of nuclear uptake, and that the concentration/activity of the components mediating these steps vary widely among cells in a 'homogeneous' clonal population.

The moderate increase in relA translocation and I-κBα degradation induced by increasing concentrations, even at full receptor occupancy, agrees with the large 'spare-receptor effect' demonstrated for IL-1 [32]. In addition, it suggests that a step between IL-1 binding and inhibitor phosphorylation is rate-limiting. IL-1 binding does not constitute the rate-determining step, since it would reach 99 % receptor occupancy by 20 s [32]. Furthermore, it would probably lie upstream of I-κB degradation, which also appears to be slow and relatively uniform among the responding population. Data already published in the literature [1] suggest that IκBα phosphorylation occurs at rates comparable with the rates of its degradation and that of nuclear translocation of relA, becoming detectable within 5–10 min. This would suggest that some rate-limiting step related to the magnitude of the incoming signal is present either at the level of the IκB kinase(s) [18,24,40,43] or upstream. Recent experiments have demonstrated two levels of regulation immediately upstream of relA nuclear translocation, present at the level of IκBα phosphorylation, regulated through IκB kinases (I. Evans and E. E. Qwarnström, unpublished work), and at the level of degradation, regulated by inhibitor levels [33]. The presence of specific limitations at numerous steps in the pathway agrees with the concept of a very tightly controlled system limited at numerous steps of the pathway.

The pathway is also amplified, as suggested by the similarities of the response over a 10000-fold range in IL-1 concentrations and a 100-fold range in receptor occupancy. This could be induced at the level of the receptor itself [3,29,44], at the level of the adapter proteins MyD88 [20] and tumour-necrosis-

factor-receptor-associated factor ('TRAF') 6 [45], or through the receptor-associated kinases, IL-1R-associated kinase (IRAK)-1, IRAK-2, IRAK-M or IRAK-4 [20,46–48]. In addition, amplification could also be induced through co-activation via the mitogen-activated protein kinase cascade, with subsequent effects on the NF-κB pathway [49]. Regulation at these levels, or downstream, could be induced through activation of a second agonist and, for example, be related to structural events [9–12]. This could be initiated at the level of the receptor following attachment-induced recruitment of the receptor-associated heparan sulphate [50], or the receptor associated Rap-Gap, IL-1R-interacting protein-1 ('IIP-1'; [51]), with downstream effects on inhibitor regulation (C. J. Caunt and E. E. Qwarnström, unpublished work). In addition, it could be induced through IL-1 activation of a member of the Rac/Rho/cdc42 family [52,53]. Such events could regulate the activity at the level of the adapter protein complex [54] or directly increase the level of transactivation [55].

The results from our analysis of the NF-κB pathway are consistent with a tightly regulated complex system, responding to a variety of inputs and regulated by an all-or-none mechanism, when examined at the single-cell level. They also agree with the notion that regulation of the forward element, I-κB degradation and nuclear import of NF-κB involves several rate-limiting and amplification step(s). Further, the data are consistent with transient responses to incoming signals, which take place against a background of basal cycling, a classic futile cycle typical of critical regulatory points in metabolic networks, and with bidirectional cycling of the endogenous NF-κB subunits, constituting a major regulator during pathway activation.

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