Multi-parameter analysis of the kinetics of NF-κB signalling and transcription in single living cells

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Summary

Proteins of the NF-κB transcription factor family normally reside in the cytoplasm of cells in a complex with IκB inhibitor proteins. Stimulation with TNFα leads to proteosomal degradation of the IκB proteins and nuclear translocation of the NF-κB proteins. Expression of p65 and IκBα fused to fluorescent proteins was used to measure the dynamics of these processes in transfected HeLa cells. Simultaneous visualisation of p65-dsRed translocation and IκBα-EGFP degradation indicated that in the presence of dual fluorescent fusion protein expression, the half-time of IκBα-EGFP degradation was reduced and that of p65 translocation was significantly increased when compared with cells expressing the single fluorescent fusion proteins. These results suggest that the ratio of IκBα and p65 determine the kinetics of transcription factor translocation into the nucleus and indicate that the complex of p65 and IκBα is the true substrate for TNFα stimulation in mammalian cells.

When cells were treated with the CRM-1-dependent nuclear export inhibitor, leptomycin B (LMB), there was nuclear accumulation of IκBα-EGFP and p65-dsRed, with IκBα-EGFP accumulating more rapidly. No NF-κB-dependent transcriptional activation was seen in response to LMB treatment. Following 1 hour treatment with LMB, significant IκBα-EGFP nuclear accumulation, but low levels of p65-dsRed nuclear accumulation, was observed. When these cells were stimulated with TNFα, degradation of IκBα-EGFP was observed in both the cytoplasm and nucleus. A normal transient transcription response was observed in the same cells using luminescence imaging of NF-κB-dependent transcription. These observations suggest that both normal activation and post-induction repression of NF-κB-dependent transcription occur even when nuclear export of NF-κB is inhibited. The results provide functional evidence that other factors, such as modification of p65 by phosphorylation, or interaction with other proteins such as transcriptional co-activators/co-repressors, may critically modulate the kinetics of transcription through this signalling pathway.

Movie available on-line.

Key words: NF-κB, Signal transduction, Fluorescent protein fusions, Firefly luciferase, Luminescence imaging, Confocal microscopy

Introduction

Nuclear factor κB proteins (NF-κB) are a family of crucially important transcription factors involved in a range of cell responses including immune and inflammatory reactions as well as the regulation of apoptosis. They consist of homo- or heterodimers assembled from a set of at least five subunits including p65 (RelA), c-Rel and RelB, which contain transcriptional activation domains, and p50 and p52, which do not contain transcriptional activation domains (the latter two are derived from proteolytic cleavage of p100 and p105 precursors respectively) (Verma et al., 1995; Ghosh et al., 1998). Normally, NF-κB proteins are retained in the cytoplasm by a family of inhibitory proteins known as IκBs, which are composed of multiple ankyrin-like repeats (Beg and Baldwin, 1993; Baeuerle and Baltimore, 1988). Three different IκBs, IκBα (Thanos and Maniatis, 1995; Baeuerle and Baltimore, 1996), IκBβ (Thompson et al., 1995) and IκBε (Whiteside et al., 1997) have been characterised. Several pathways for NF-κB activation have been identified that can be stimulated by inducers such as tumour necrosis factor α (TNFα), interleukin 1β (IL-1β), lipopolysaccharide and UV (Siebenlist et al., 1994).

TNFα-induced activation of NF-κB involves stimulation of the TNFα receptor 1 (TNFR1) through binding of TNFα, which induces trimerisation of TNFR1. This is followed by recruitment of cytosolic factors including TNF receptor-associated death domain, TRADD (Hsu et al., 1995) and TNF receptor associated factors (TRAFs) (Pomerantz and Baltimore, 1999). This leads to stimulation of a signalling pathway that acts through a multiprotein kinase complex called the signalsome, which is known to include NF-κB-inducing kinase (NIK) and IκB kinases IKKα, IKKβ (Zandi et al., 1997; DiDonato et al., 1997; Malinin et al., 1997; Mercurio et al., 1997; Woronicz et al., 1997), and IKK_i in immune cells (Shimada et al., 1999). Two scaffold proteins, IKKγ and IKK complex associated protein, IKAP (Rothwarf et al., 1998; Cohen et al., 1998) are also part of the complex. The critical step in the signalling pathway is activation by phosphorylation of IKKα and IKKβ, which in turn phosphorylate the IκBs at N-terminal serine residues. Other

cellular signals, such as that from p53 activation (Ryan et al., 2000), activate NF-κB through IKK phosphorylation via a different cellular signalling pathway involving Raf1, MEKK1 and p90^{RSK} (Ghoda et al., 1997). Phosphorylated IκB proteins are ubiquitinated by β-TR-CP variants (Spencer et al., 1999) leading to their degradation by the 26S proteasome (Yaron et al., 1998; Coux and Goldberg, 1998). Following phosphorylation and degradation of the IκB proteins, the NF-κB is released and its nuclear localisation sequence (NLS) becomes unmasked, allowing the translocation of the NF-κB to the nucleus. It has previously been suggested from studies in insect cells that the NF-κB-IκB complex is the preferential substrate for phosphorylation and degradation of the IκB by the IKKs, and that free IκB alone is less efficiently phosphorylated (Zandi et al., 1998). This would suggest that IκB bound to NF-κB would be preferentially degraded, and that free IκB would be less efficiently degraded when the IKKs are activated. In the nucleus, the NF-κB binds to a set of related binding sites in the promoters of target genes. Each different NF-κB complex has slightly different affinities for each specific DNA binding sequence (reviewed by Zandi and Karin, 1999). One of the genes that is activated by NF-κB is the gene encoding IκBα. It is thought that newly synthesised IκBα enters the nucleus and binds to NF-κB. The complex is then relocated to the cytoplasm by CRM1-dependent nuclear export (Arenzana-Seisdedos et al., 1995; Rodriquez et al., 1999).

Although a great deal has been learned about the individual parts of the biochemistry of this signalling pathway, it is still unclear what factors control the speed and longevity of the transcriptional response. To address this, non-invasive tools are required to measure the different stages of the signalling pathway in real-time in living cells. Recently, a number of studies have used fluorescent proteins to report on the localisation and dynamics of NF-κB signalling. GFP-p105 was shown to translocate to the nucleus in approximately 20 minutes after treatment with TNFα or hydrogen peroxide (Tenjinbaru et al., 1999). The expression of a fusion protein between IκBα and EGFP (Li et al., 1999) also demonstrated rapid degradation of the IκBα-EGFP signal in response to NF-κB activating agents such as TNFα and the phorbol ester polymyristate acetate. The use of dual fluorescent protein fusions between p65 and IκBα and different coloured GFP proteins allowed the analysis of the biophysical basis of the interaction between these proteins using fluorescent resonance energy transfer (FRET) (Schmid et al., 2000). To investigate the kinetics of these processes in living cells, Carlotti and colleagues first used a p65-EGFP fusion construct to show that in response to IL-1β stimulation, the kinetics of the response were sensitive to p65-EGFP levels of expression. The levels of p65-EGFP were also critical for the

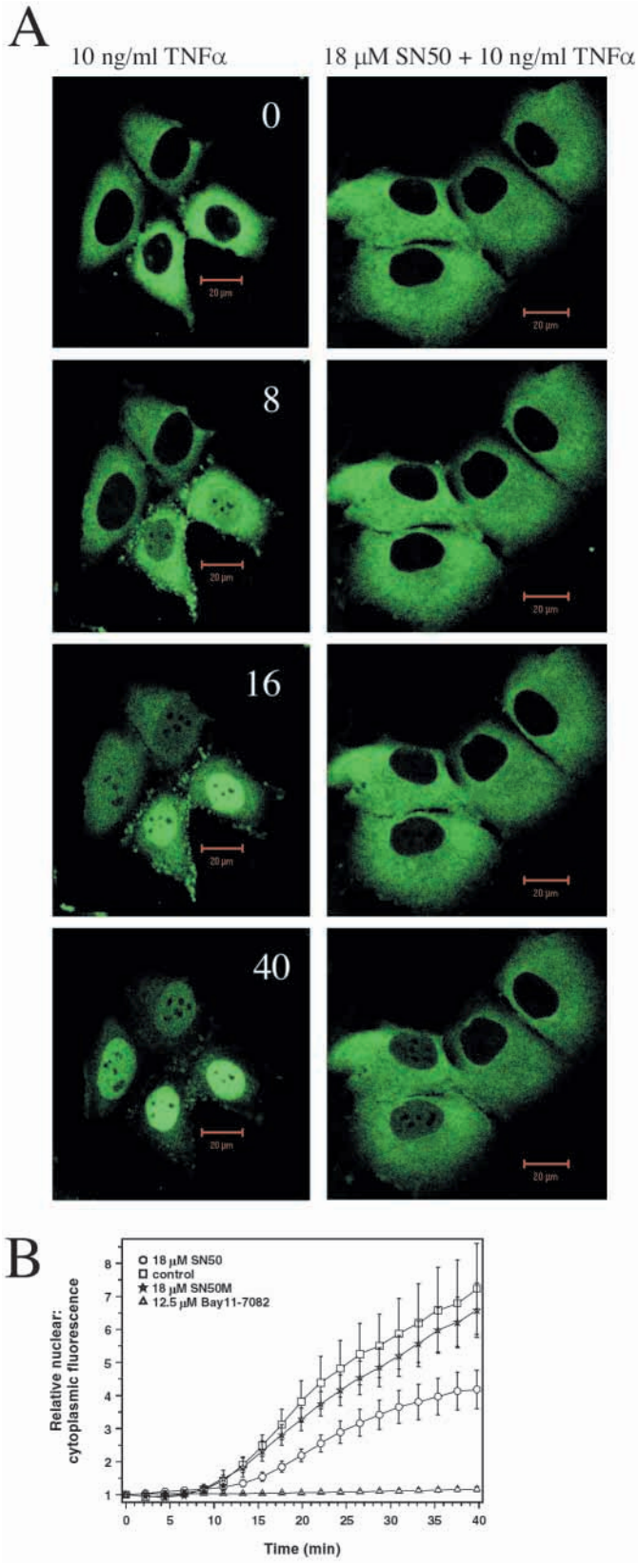

Fig. 1. Nuclear accumulation of p65-EGFP in response to TNFα stimulation. (A) Time series images of p65-EGFP fluorescence at stated times (in minutes) after addition of TNFα. Inhibition of translocation was observed in cells treated with 18 μM SN50 for 15 minutes prior to addition of TNFα. (B) Quantification of p65-EGFP translocation in response to TNFα. Mean nuclear and cytoplasmic fluorescent intensities were determined for fluorescent cells at each time point and plotted as a ratio relevant to the initial ratio at t=0 minutes. Translocation is shown in the absence of inhibitor or in the presence of 18 μM SN50, 18 μM SN50M or 12.5 mM Bay11-7082. Confocal microscopy was carried out in cells transfected with p65-EGFP 24 hours prior to their use. Cells were treated with appropriate inhibitor for 15 or 30 minutes (see text) prior to stimulation with 10 ng/ml TNFα at t=0 minutes. Results show mean values±s.d.$_{(n-1)}$ from two experiments with at least six cells per experiment plotted for each treatment.

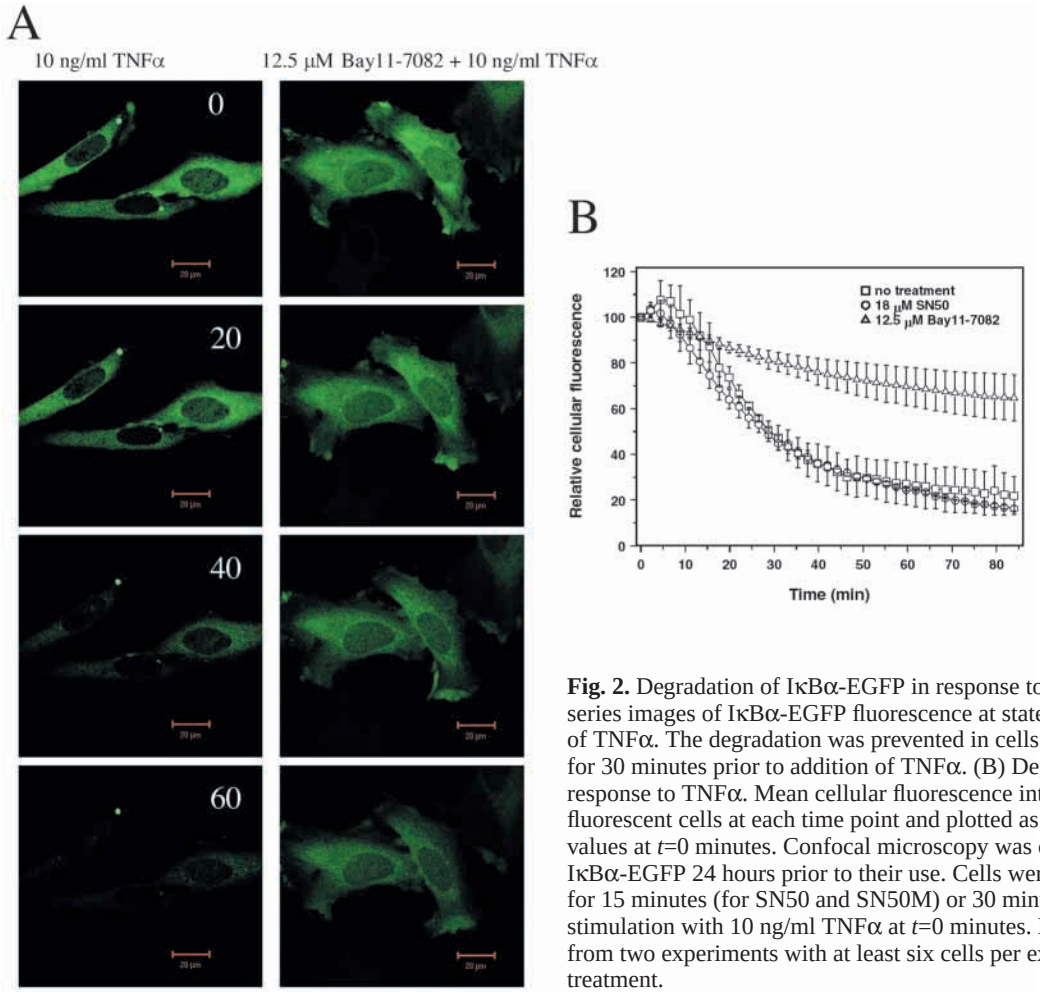

Fig. 2. Degradation of IκBα-EGFP in response to TNFα stimulation. (A) Time series images of IκBα-EGFP fluorescence at stated times (in minutes) after addition of TNFα. The degradation was prevented in cells treated with 12.5 μM Bay11-7082 for 30 minutes prior to addition of TNFα. (B) Degradation of IκBα-EGFP in response to TNFα. Mean cellular fluorescence intensities were determined for fluorescent cells at each time point and plotted as a percentage of the fluorescence values at t=0 minutes. Confocal microscopy was carried out in cells transfected with IκBα-EGFP 24 hours prior to their use. Cells were treated with appropriate inhibitor for 15 minutes (for SN50 and SN50M) or 30 minutes (for Bay11-7082) prior to stimulation with 10 ng/ml TNFα at t=0 minutes. Results show mean values±s.d.$_{(n-1)}$ from two experiments with at least six cells per experiment plotted for each treatment.

NF-κB-derived anti-apoptotic effect (Carlotti et al., 1999). In a further study they used fluorescent fusion proteins with both p65 and IκBα to confirm the previous suggestion that these proteins undergo dynamic shuttling between the nucleus and cytoplasm, which is associated with dissociation of the transcription factor and the inhibitor within the cytoplasm (Carlotti et al., 2000).

In the present study, we have applied fluorescence imaging of p65 and IκBα together with luminescence imaging of NF-κB-dependent transcription to study the real-time kinetics of the processes underlying NF-κB-regulated transcription in living cells. We have used green fluorescent protein and red fluorescent protein (dsRed) (Matz et al., 1999) chimeras of IκBα and p65 together with firefly luciferase as a reporter gene to investigate the timing of IκBα degradation, p65 translocation and NF-κB-dependent transcriptional activation in single living cells. Studies of the kinetics of p65 translocation and IκBα degradation in single or dual-transfected cells provided functional kinetic data to support the hypothesis that degradation of IκBα in the cytoplasm (or its targeting for degradation) occurs preferentially when the IκBα is bound to NF-κB. Moreover, we show that the timing of the translocation of p65 into the nucleus, in response to TNFα stimulation, is critically dependent on the ratios of these proteins. We show that LMB inhibition of nuclear export leads to rapid nuclear accumulation of IκBα and slower nuclear accumulation of p65. Treatment of cells with TNFα 1 hour after LMB addition gives rise to rapid and stable nuclear accumulation of p65 and normal transient TNFα-activation-dependent kinetics of NF-κB-dependent transcription. These results suggest that nuclear localisation of p65 is not itself sufficient for stable NF-κB-dependent transcription and that a further factor other than IκB concentration in the nucleus may be required for the inhibition of NF-κB-dependent transcription.

Materials and Methods

Materials

Human recombinant TNFα and the inhibitors SN50, SN50M and Bay11-7082 were supplied by Calbiochem (UK). Tissue culture medium was supplied by Gibco Life Technologies (UK) and fetal calf serum from Harlan Seralab (UK). All other chemicals were supplied by Sigma (UK) unless stated otherwise.

Plasmids

All plasmids were propagated using *E. coli* DH5α and purified using Qiagen Maxiprep kits (Qiagen, UK). pNF-κB-Luc (Stratagene, UK) contains five repeats of an NF-κB-sensitive enhancer element upstream of the TATA box, controlling expression of luciferase. p65-EGFP contains a 1.6 kb p65 cDNA cloned into the *Hin*dIII-*Bam*HI site of pEGFP-N1 (kindly donated by M. Rowe, UWCM, Cardiff). This expresses a C-terminal p65-EGFP fusion protein under the control of the human CMV immediate early (hCMV-IE) promoter. pIκBα-EGFP (Clontech, UK) contains a fusion of IκBα to EGFP under the control of the hCMV-IE promoter. p65-dsRed was produced by inserting a 1.6

kb p65 *Hin*dIII-*Bam*HI fragment from p65-EGFP into the respective sites in the multiple cloning site of pdsRed1-N1 (Clontech), producing an in-frame C-terminal fusion of p65 to dsRed under the control of the hCMV-IE promoter.

Cell culture and transfection

HeLa Cells (ECACC No. 93021013) were grown in Minimal Essential Medium with Earle's salts, plus 10% fetal calf serum, and 1% nonessential amino acids at 37°C, 5% CO_2. For confocal microscopy and fluorescence microscopy, cells were plated on 35 mm Mattek dishes (Mattek, USA) at 2.4×10^4 cells per plate in 2 ml medium. After 24 hours, cells were transfected with appropriate plasmid(s) using Fugene 6 (Boehringer Mannheim/Roche, Germany) following the manufacturer's recommendations. The optimised ratio of DNA:Fugene 6 used for such transfections was 1 μg DNA with 2 μl Fugene 6. This DNA concentration was maintained for single and dual transfections with fluorescent protein expression vectors (i.e. 0.5 μg of each plasmid). For triple transfections with two fluorescent protein expression vectors and the NF-κB-Luc reporter vector, 0.1 μg of each fluorescent protein expression vector was used together with 0.8 μg of the NF-κB-Luc expression vector DNA.

For microtitre plate-based luminescence assays of luciferase expression from the pNF-κB-Luc reporter plasmid, 1.4×10^4 cells were seeded in 1 ml of medium into each well of a 24-well plate (Falcon, Becton Dickinson, USA) and grown for 24 hours prior to transfection. Cells were transfected for 24 hours using Fugene 6, at an optimised ratio of 0.5 μg pNF-κB-Luc to 1 μl Fugene 6 per well.

Fluorescence microscopy

Confocal microscopy was carried out on transfected cells in Mattek dishes in a humidified CO_2 incubator (at 37°C, 5% CO_2) using a Zeiss LSM510 with a 40× phase contrast oil immersion objective (numerical aperture=1.3). Excitation of EGFP was performed using an Argon ion laser at 488 nm. Emitted light was reflected through a 505-550 nm bandpass filter from a 540 nm dichroic mirror. dsRed fluorescence was excited using a green Helium Neon laser (543 nm) and detected through a 570 nm long-pass filter. Data capture and extraction was carried out with LSM510 version 2.1 software (Zeiss, Germany). For p65-EGFP and p65-dsRed fusion proteins, mean fluorescence intensities were calculated for each time point for both nuclei and cytoplasm. Nuclear:cytoplasmic fluorescence intensity ratios were determined relative to the initial ratio at t=0 minutes. For IκBα-EGFP fusion proteins, mean cellular fluorescence intensities were calculated at each time point per cell, and fluorescence intensity relative to starting fluorescence was determined for each cell.

Widefield fluorescence microscopy (Fig. 5A) was carried out on a Zeiss Axiovert S100 TV microscope under the same conditions as described for confocal microscopy, except that fluorescence excitation was carried out using a monochromator (Kinetic Imaging, UK) at 558 nm (±2 nm) for dsRed. Fluorescence emission was captured through a Texas Red filter block using a Hamamatsu C4742-98 CCD camera (Hamamatsu, Japan). Data acquisition and analysis were carried out with AQM2000 software (Kinetic Imaging). Analysis involved determination of mean fluorescence intensities for each nucleus and cytoplasm at each time point.

Microplate luminescence assays of living cells

For microtitre-plate-based living cell luciferase assays, 1 mM luciferin was added to the medium 12 hours after addition of transfection reagent. Luminescence was assayed a further 12 hours after luciferin addition to the cells. Measurements of luminescence from 24-well plates were performed using a Lumistar luminometer (BMG Labtechnologies, UK), using an integration time of 10 seconds per well per time point. In between successive time points the microtitre plates were replaced in a tissue culture incubator at 37°C, 5% CO_2.

Combined fluorescence and luminescence microscopy

For triple-parameter imaging, the cells were transfected as described above with 0.1 μg of each fluorescent protein expression vector and 0.8 μg of NF-κB-Luc reporter vector. Twenty-four hours later, firefly luciferin (Biosynth, Switzerland) was added to the medium to a final concentration of 0.5 mM. Cells were used after a minimum luciferin incubation of 4 hours. Confocal microscopy was performed as described above for LMB treatment and 40 minutes of TNFα incubation. The confocal microscope was then switched off to allow luminescence imaging.

Luminescence imaging was carried out using a Hamamatsu 4880-65 liquid nitrogen cooled CCD camera attached to the top port of the confocal microscope. AQM2000 software was used for image acquisition and analysis. Images were acquired using 30 minute integration times. Successive luminescence images and control blank images were used to automatically remove dark noise and noise from random cosmic events. For dual confocal and luminescence microscopy, the same cells were identified from bright field images.

Treatment of cells with TNFα, inhibitors and leptomycin B

Treatment of cells with TNFα (10 ng/ml final concentration) was carried out immediately before microscopy by replacing one tenth of the medium volume in the dish with the appropriate solution. Inhibitors were added prior to TNFα treatment in the same manner. Each experiment was carried out at least twice with at least four cells obtained per replicate. Leptomycin B (Sigma, UK) was dissolved in methanol and added to a final concentration of 10 ng/ml (18 nM).

Results

Visualisation of TNFα-induced NF-κB signalling

To visualise the single parameter translocation of NF-κB from the cytosol to the nucleus, we used a fusion between p65 and EGFP and studied its localisation in transfected HeLa cells by confocal microscopy. In unstimulated cells expressing low levels of p65-EGFP, cytoplasmically located p65-EGFP fluorescence was observed (Fig. 1A). In agreement with previous studies of a similar fusion protein (Carlotti et al., 1999) we found that expression of low levels of the fusion protein were capable of regulating NF-κB-dependent transcription, but did not lead to inappropriately high basal levels of transcriptional activity, or aberrant nuclear localisation of the fluorescent protein (data not shown). Following the addition of 10 ng/ml TNFα to the medium of the transfected cells, nuclear fluorescence began to increase approximately 11 minutes later, followed by a rapid rate of translocation for approximately 30 minutes. Virtually all cellular fluorescence was located in the nucleus after 40 minutes (Fig. 1A). Quantitative analysis of these data showed that translocation occurred with a half-time, $t_{½}$, of 19±2.9 minutes (n=12; Fig. 1B). These observations are in general agreement with those made previously for the endogenous protein (Ding et al., 1998), a p105-GFP fusion protein (Tenjinbaru et al., 1999) and a similar p65-EGFP fusion protein (Carlotti et al., 1999; Carlotti et al., 2000).

To confirm the functional significance of these observations, the effects of the Bay11-7082 and SN50 inhibitors on translocation of the p65-EGFP fusion protein were investigated using concentrations previously shown to significantly inhibit TNFα-induced expression from an NF-κB-Luc expression

vector (data not shown). The cell-permeable peptide SN50 contains the sequence of the NLS of NF-κB and thus competitively inhibits NF-κB nuclear translocation (Lin et al., 1995). Bay11-7082 irreversibly inhibits TNFα-induced phosphorylation of IκBα (Pierce et al., 1997), thereby preventing the subsequent ubiquitin-mediated IκBα degradation. No visible effects of inhibitor addition prior to stimulation with TNFα were observed (data not shown). Preincubation of cells with 18 μM SN50 for 15 minutes prior to stimulation with TNFα gave rise to partial inhibition (approximately 50%) of nuclear translocation of p65-EGFP (Fig. 1), indicating that import of the fusion protein p65-EGFP is effected by the NLS-mediated import pathway. The related control peptide, SN50M, which has two amino acid substitutions in the NLS relative to SN50, showed a slight inhibitory affect on p65-EGFP translocation (Fig. 1B). An ANOVA analysis of these data showed that after 40 minutes of TNFα treatment, both the control and SN50M treated cells were significantly different from SN50, but not from one another (P=0.006). Cells treated with 12.5 μM Bay11-7082 30 minutes prior to stimulation with TNFα also showed very significant inhibition of p65-EGFP translocation, indicating that p65-EGFP is bound to IκBα and held in the cytoplasm, only to be released upon phosphorylation of IκBα. Thus, the fusion protein p65-EGFP appears to possess the functional characteristics of an NF-κB protein.

To observe the kinetics of IκB degradation following TNFα treatment, HeLa cells were transiently transfected with an IκBα-EGFP fusion protein. Cells expressing the IκBα-EGFP fusion protein showed mainly cytoplasmic fluorescence 24 hours post transfection (Fig. 2A). A rapid decay in fluorescence was observed upon stimulation with TNFα. The half-time of degradation of this protein was determined by quantitative fluorescence analysis to be 26.7±2.8 minutes (n=14; Fig. 2B). The degradation of the IκBα-EGFP fusion protein following TNFα stimulation was inhibited by treatment with 12.5 μM Bay11-7082 (Fig. 2A,B), but not with 18 μM SN50 (Fig. 2B). This observation confirms that the inhibitory action of SN50 on the translocation of p65 (Fig. 1) is downstream of IκBα phosphorylation. The inhibitory effect of Bay11-7082 (the inhibitor of IκBα phosphorylation), but not SN50 (the inhibitor of p65 translocation), supports the hypothesis that the observed fluorescence decrease is due to TNFα-induced degradation of the IκBα-EGFP fusion protein via phosphorylation of IκBα.

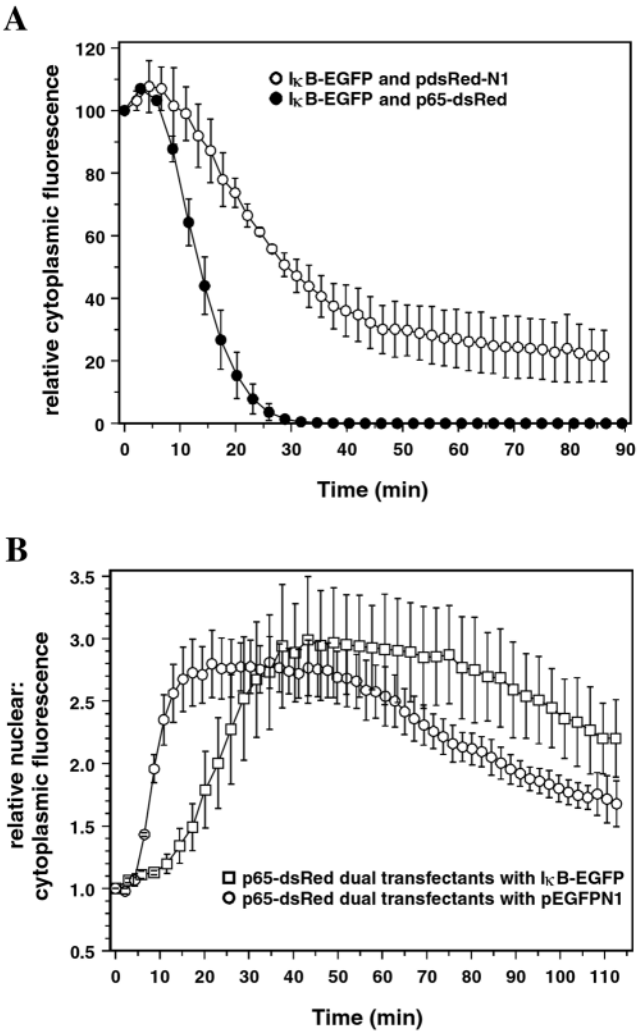

Fig. 3. Combined imaging of p65-dsRed localisation and IκBα-EGFP degradation following TNFα stimulation of dual-transfected cells. (A) Quantification of IκBα-EGFP degradation in dual-transfected cells also expressing either p65-dsRed or the control expression vector pdsRedN1. IκBα-EGFP fluorescence was determined as described in Fig. 2. (B) Quantification of translocation of p65-dsRed in dual-transfected cells also expressing either IκBα-EGFP or the control EGFP-N1. The nuclear:cytoplasmic ratio of p65-dsRed fluorescence was determined as described in Fig. 1. Results show mean values±s.d.$_{(n-1)}$ from two experiments, using at least four cells from each experiment.

Increased expression of p65-dsRed enhances the rate of degradation of IκBα-EGFP following TNFα treatment

We investigated whether extended kinetics of IκBα degradation following IκBα-EGFP overexpression could be modulated by increased levels of p65 expression. To test this hypothesis, we constructed a fusion protein between the red fluorescent protein, dsRed and p65. This allowed independent measurement of p65-dsRed translocation and IκBα-EGFP degradation as well as quantitative measurement of the starting levels of expression of both fusion proteins in each cell. Addition of TNFα to the cells produced the expected overall response from both fusion proteins, indicating that the two processes could be independently measured in the same cell (Figs 3, 4). In dual transfected cells expressing IκBα-EGFP and p65-dsRed, IκBα-EGFP degradation reached 50% of the initial cellular fluorescence in 13.5±1.7 minutes (n=9; Fig. 3A). This was significantly shorter than the $t_{½}$ for IκBα-EGFP degradation in single transfected cells (see above) and in dual transfected cells expressing IκBα-EGFP and control dsRed protein ($t_{½}$=18.2±2.2 minutes, n=12; Fig. 3A). The rate of IκBα-EGFP degradation in dual transfectants with p65-dsRed therefore more closely resembled the rate of native IκBα degradation in untransfected cells (Sun et al., 1993). The observation that the rate of IκBα degradation is accelerated in the presence of p65-dsRed fusion protein provides the first functional evidence from mammalian cells to support the hypothesis originally proposed following work in insect cells (Zandi et al., 1998) that IKK can efficiently phosphorylate only NF-κB-IκB dimers, as opposed to unbound IκB.

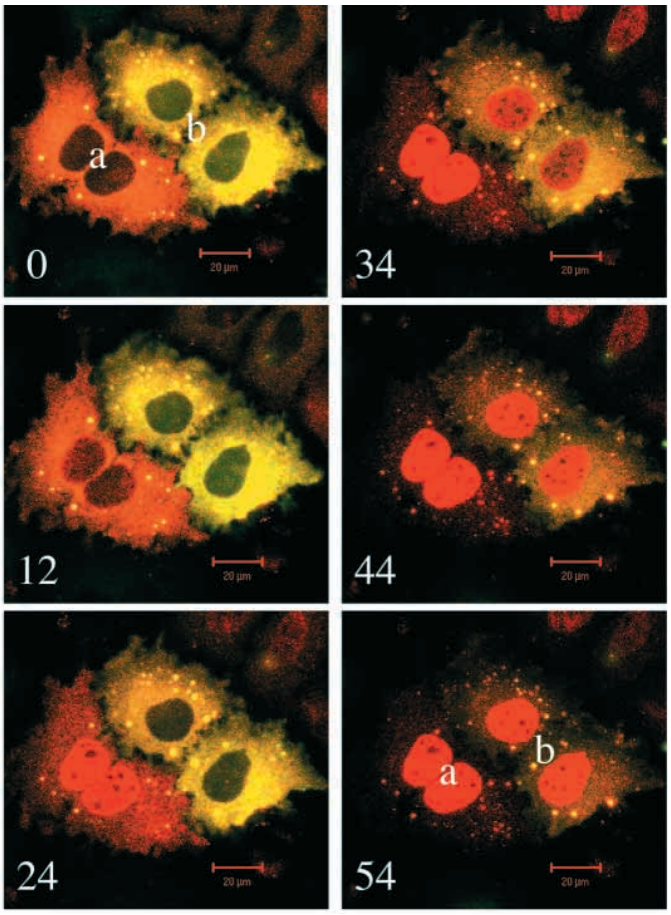

Fig. 4. Confocal microscopy of p65-dsRed and IκBα-EGFP in living cells. Time series images of IκBα-EGFP and p65-dsRed fluorescence at stated times (in minutes) after addition of 10 ng/ml TNFα. Green and red fluorescence were recorded as separate images and then merged for visualisation. Green IκBα-EGFP and red p65-dsRed co-localisation are represented as yellow in the presence of higher IκBα-EGFP:p65-dsRed ratios, and orange in the presence of higher p65-dsRed:IκBα-EGFP ratios.

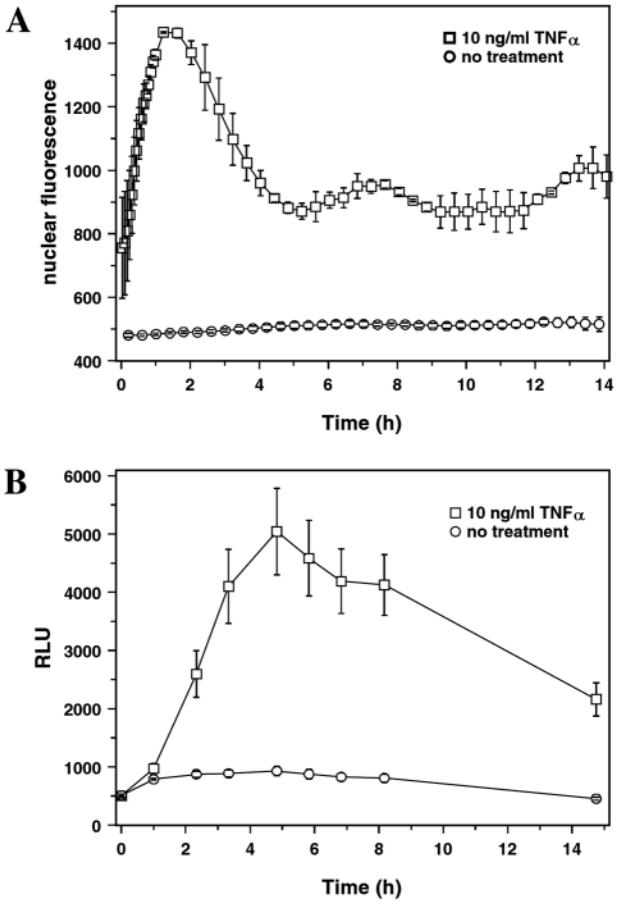

Fig. 5. Long term NF-κB response to TNFα. (A) Long term widefield fluorescence microscopy of p65-localisation in response to TNFα. Cells were treated at *t*=0 hours with 10 ng/ml TNFα or carrier (control). Mean nuclear fluorescence intensities were determined for each cell at each time point. Results show mean values obtained from two experiments. (B) Transcriptional regulation of NF-κB-sensitive promoter. Luciferase expression monitored in living cells transfected with pNF-κB-luc. Cells were transfected in 24-well microtitre plates. 1 mM luciferin was added to cells 12 hours prior to addition of TNFα (10 ng/ml final concentration) or carrier added at *t*=0 hours [*n*=4, results show means±s.d.$_{(n-1)}$].

Increased levels of IκBα-EGFP delay nuclear translocation of p65-dsRed following TNFα treatment

We next investigated whether the level of IκBα fusion protein could modulate the rate of nuclear translocation of p65-dsRed. The rate of p65-dsRed translocation was indistinguishable from that seen with p65-EGFP translocation (data not shown). Cells expressing p65-dsRed with a control EGFP expression vector gave rise to significantly more rapid p65-dsRed translocation than that observed in cells transfected with p65-dsRed together with IκBα-EGFP (Fig. 3B). The half-time for translocation of p65-dsRed in cells co-transfected with IκBα-EGFP was 24±2.9 minutes, as opposed to a much shorter half time of 11.6±1.1 minutes for dual transfectants expressing p65-dsRed with pEGFP-N1. This suggests that the ratio of p65 and IκBα in cells determines the kinetics of p65-EGFP nuclear accumulation.

To provide further evidence to support this conclusion, we studied the rate of p65 translocation in dual-transfected cells expressing markedly different levels of IκBα-EGFP. Cells expressing significantly higher levels of IκBα-EGFP showed slower IκBα-EGFP degradation and delayed p65-dsRed translocation compared with cells expressing much lower levels of IκBα-EGFP. This is illustrated in Fig. 4, which shows two pairs of cells (marked *a* and *b*) with approximately threefold variation in their levels of IκBα-EGFP, but similar expression levels of p65-dsRed (as determined by fluorescence quantification). Cell pair *a* had lower levels of IκBα-EGFP relative to cell pair *b* and showed faster translocation of p65 following TNFα stimulation compared with cells *b*, which had higher levels of IκBα. A different panel of cells are shown in the movie file (see http://jcs.biologists.org/supplemental), which shows a group of cells with widely varying initial p65-dsRed:IκBα-EGFP ratios. The rate of p65-dsRed nuclear accumulation is again seen to be inversely proportional to the level of IκBα-EGFP in each cell. These data suggest that the ratio of IκBα and p65 plays a role in determining the kinetics of the NF-κB transcriptional response to TNFα.

Monitoring of long-term p65 dynamics coupled with transcriptional activation

To investigate the longer term kinetics of activation of the NF-κB signalling pathway by TNFα, we studied the kinetics of p65 localisation over periods of several hours following TNFα treatment (Fig. 5A). Following initial nuclear translocation of p65-dsRed, the vast majority of the transcription factor was observed to move back into the cytoplasm within 5 hours. To determine whether this was caused by genuine protein export or simply degradation of the nuclear protein followed by de novo synthesis of new cytoplasmic p65, we compared the kinetics of export using p65-dsRed and p65-EGFP (data not shown). A major difference between the properties of these two fluorescent proteins lies in the time over which the fluorescent protein acquires its fluorescence. DsRed takes several hours longer than EGFP for its chromophore formation (Baird et al., 2000). The fact that the kinetics of apparent nuclear export of the p65-fusion protein were indistinguishable when using either fluorescent protein (data not shown) strongly suggests that this corresponds to the export of pre-synthesised nuclear protein rather than new fluorescent protein synthesis. These data suggest that the p65 protein is present in the nucleus for only a relatively short period of time and that the change in timing of p65 translocation due to IκBα levels (up to 12 minutes) results in a delay in transcription upregulation that is of a significant duration in comparison to the length of time that p65 resides in the nucleus.

To investigate the relationship between this transient p65 occupation of the nucleus and the timing of transcription, we investigated the timing of transcription in living cells. Luminescence

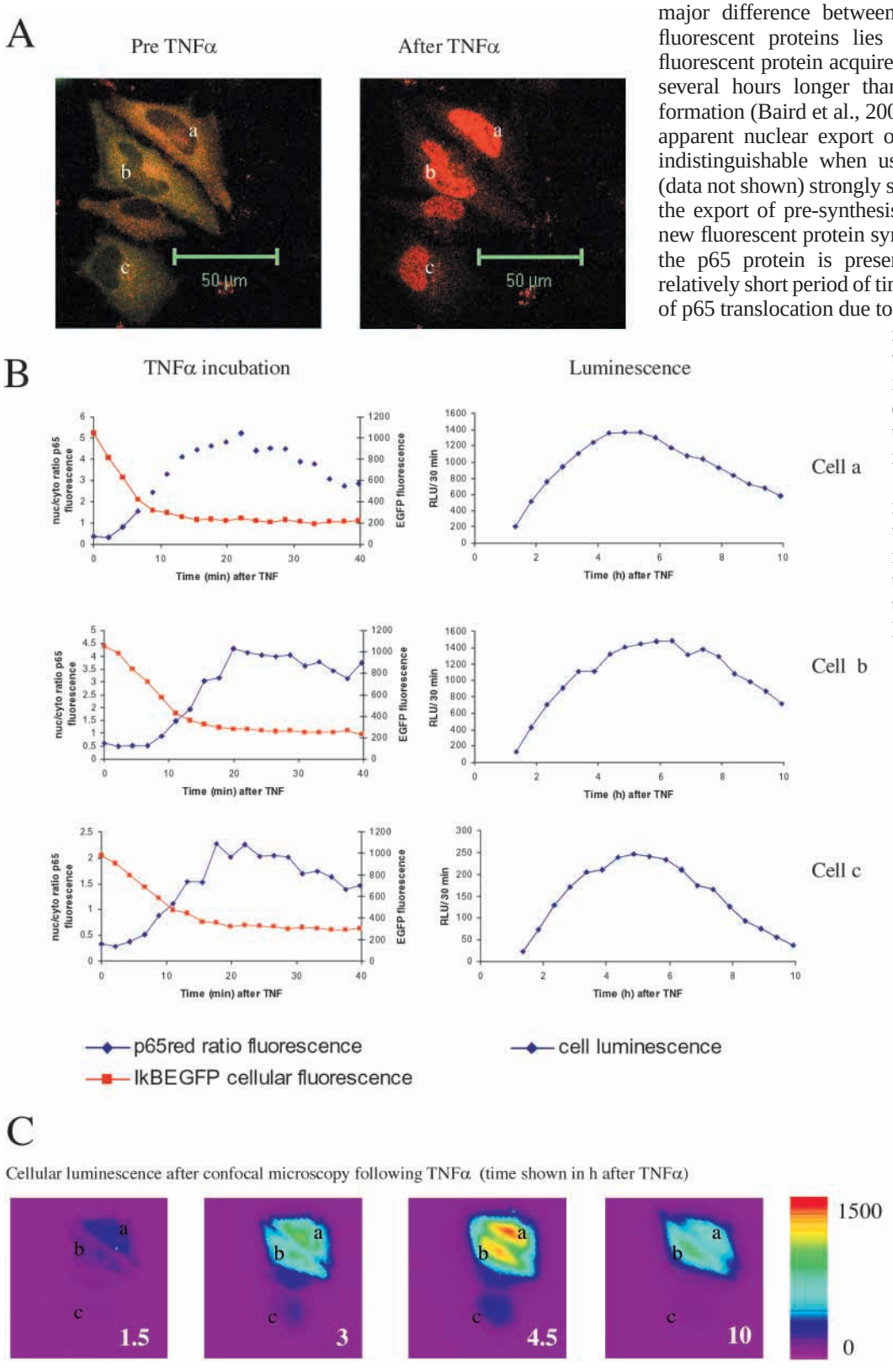

Fig. 6. Quantitative analysis of p65 translocation, IκBα degradation and NF-κB-dependent transcription in single living cells. (A) Dual confocal microscopy images of p65-dsRed and IκBα-EGFP before and 40 minutes after TNFα stimulation. (B) Quantitative analysis of the time course of p65-dsRed translocation, IκBα-EGFP degradation and cell luminescence (luciferase activity) in three cells, a-c (A,C). p65-dsRed translocation is shown as nucleo-cytoplasmic ratio and IκBα degradation is shown as EGFP fluorescence. Luminescence activity is shown as luminescence counts from successive luminescence images with background counts removed. (C) Luminescence images of cells following TNFα stimulation. Luminescence directed by NF-κB-promoter-directed luciferase activity. Cells marked a-c correspond to those marked in A and used for quantification in B.

assays of cells plated in a 24-well plate and treated with luciferin indicated that the timing of transcription was transient with a peak at around 4-5 hours after TNFα addition (Fig. 5B). Allowing for the typical 4 hour delay in synthesis of the luciferase fusion protein (data not shown), these observations suggested that the timing of transcription correlated closely with the occupancy of the nucleus by NF-κB.

Measurement of p65 translocation, IκBα degradation and NF-κB-directed transcription using combined confocal microscopy and luminescence imaging

To investigate the relationship between signalling and transcription in single cells we used a low light level camera attached to a confocal microscope. HeLa cells were transfected with p65-dsRed, IκBα-EGFP and an NF-κB-Luc expression vector. The cells were initially monitored for fluorescence after treatment with TNFα and then the resulting luminescence over the following 10 hours was measured in the same cells by luminescence imaging. Analysis of single cells indicated that similar dynamics of translocation of p65 and degradation of IκBα (Fig. 6A,B) were seen compared with those described above. In agreement with the results obtained from cell population analysis (Fig. 5B), the individual cells gave rise to a consistent transient luminescence response indicating rapid activation and repression of NF-κB-directed transcription (Fig. 6B,C). The analysis of the timing of induction in cells with widely varying levels of luminescence intensity (Fig. 6, cells a-c) suggested that this timing was maintained irrespective of the level of transfection with the luciferase reporter plasmid.

Rates of nuclear accumulation of p65 and IκBα following inhibition of nuclear export

To investigate the relationship between transcription and nuclear export of p65 we used the CRM1-dependent inhibitor of nuclear export, LMB. Treatment of cells with this inhibitor led to nuclear accumulation of both p65-dsRed and IκBα-EGFP (Rodriquez et al., 1999; Carlotti et al., 2000), supporting the hypothesis that these proteins are involved in nucleo-cytoplasmic shuttling even in unstimulated cells (Johnson et al., 1999; Huang et al., 2000). Analysis of the rate of nuclear accumulation of these proteins showed significantly more rapid accumulation of IκBα than p65-dsRed (Fig. 7A) in agreement with previous results (Carlotti et al., 2000). To show that LMB treatment led to stable localisation of p65 and IκBα in the nucleus, cells were transfected with p65-dsRed and IκBα-EGFP and treated with LMB. Both p65-dsRed and IκBα-EGFP were maintained in the nucleus for more than 8 hours (Fig. 7B, top). When cells were treated with 10 ng/ml TNFα 1 hour after LMB treatment, there was a more rapid translocation of the p65-dsRed into the nucleus followed by its maintenance in the nucleus for 8 hours (Fig. 7B, bottom).

Analysis of protein dynamics and transcription in leptomycin B-treated cells

After long periods of LMB treatment (6 hours or more) when both IκBα-EGFP and p65-dsRed were localised to the nucleus, TNFα treatment did not give rise to nuclear degradation of IκBα-EGFP or NF-κB-dependent transcription (data not shown). This suggested that some p65 must remain in the cytoplasm in order for a response to TNFα to occur. (NF-kB-independent transcription from an exogenous promoter was not inhibited, suggesting that this observation was not caused by non-specific transcriptional inhibition following long-term LMB treatment.) One hour after LMB treatment, there was significant nuclear IκBα-EGFP accumulation, but a large proportion of the p65-dsRed remained present in the cytoplasm (Figs 7, 8). When these cells were stimulated with TNFα, there was nuclear translocation of the remaining cytoplasmic pool of p65-dsRed, indicative of a normal response. Owing to the continued presence of the LMB, the p65-dsRed then remained in the nucleus, since nuclear export was inhibited (Figs 7, 8). (Note that the scale in Fig. 8B is nuclear/cytoplasmic fluorescence, rather than simply nuclear fluorescence as in Fig. 7A, making relative movement of p65 appear less significant in the first hour after LMB treatment.) There was degradation of IκBα-EGFP in both the cytoplasm and nucleus, although 40 minutes after TNFα treatment, some nuclear IκBα-EGFP fluorescence remained, whereas cytoplasmic IκBα-EGFP fluorescence was undetectable (Fig. 7B; Fig. 8A,B). These cells also displayed a normal transient stimulation of NF-κB-dependent promoter-directed transcription in response to TNFα (Fig. 8B). Cells transfected with a control promoter and treated with LMB under similar conditions indicated that the transient time course of luciferase expression was not due to non-specific effects of LMB treatment (data not shown).

Discussion

We report for the first time the real time non-invasive kinetic analysis of three steps in the NF-κB signalling pathway; IκBα degradation, p65 translocation and NF-κB-dependent transcription. We have used these tools to investigate the link between the kinetics of the NF-κB pathway, the levels of NF-κB and IκB proteins in cell compartments, and the resulting timing of transcription.

We showed that both the p65-EGFP and p65-dsRed fluorescent fusion proteins gave rise to the nuclear translocation in response to TNFα treatment, which is characteristic of the functional endogenous protein. Ding et al. previously reported an endogenous p65 nuclear translocation half time of 7-8 minutes in HeLa cells following TNFα stimulation (Ding et al., 1998). In comparison, we obtained a longer half time of 19±2.9 minutes for nuclear translocation of p65-EGFP in singly transfected cells (Fig. 1A) in agreement with other studies using a p65-EGFP fluorescent fusion protein and stimulation with IL-1β (Carlotti et al., 1999; Carlotti et al., 2000). One explanation is that high expression of the p65 fusion protein results in these differences. When we studied the translocation of p65-dsRed (or p65-EGFP) in cells co-transfected with a control fluorescent protein expression vector (Fig. 3B), the half-time of translocation was faster (11.6±1.3 minutes). In the dual transfected, compared with the single transfected cells, expression of the p65-fluorescent protein was markedly reduced (typically 70% of the single transfectant levels), perhaps due to promoter or plasmid competition. The difference between our observed translocation times and those of Ding et al. may therefore represent the effect of the level of p65 expression on the dynamics of its translocation.

The IκBα-EGFP fusion protein is subject to TNFα-induced reduction of fluorescence, which can be related to the

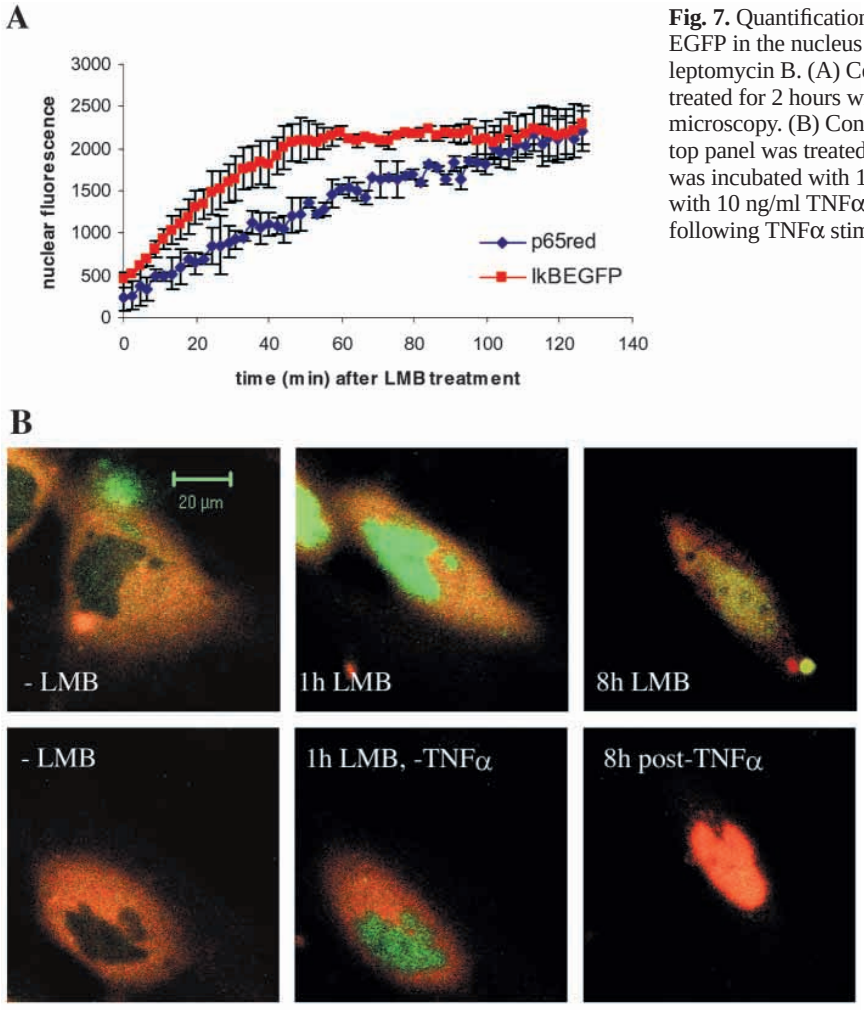

Fig. 7. Quantification of differential accumulation of p65-dsRed and IκBα-EGFP in the nucleus following treatment of dual-transfected cells with leptomycin B. (A) Cells transfected with p65-dsRed and IκBα-EGFP were treated for 2 hours with 10 ng/ml LMB and monitored by confocal microscopy. (B) Confocal microscopy of long term LMB-treated cells. The top panel was treated for 8 hours with 10 ng/ml LMB. The bottom panel was incubated with 10 ng/ml LMB followed 1 hour later by stimulation with 10 ng/ml TNFα. The cells were then analysed for a further 8 hours following TNFα stimulation.

degradation of the endogenous IκBα protein. Expression of the IκBα-EGFP fusion protein for extended periods in HeLa cells was found to induce apoptosis (data not shown). This IκBα-specific effect supports the hypothesis that the IκBα moiety is functional as a part of the fusion protein. The relationship between the relative levels of the p65- and IκBα-fluorescent proteins and the related changes in the kinetics of the response of both proteins to TNFα treatment suggest that these proteins have retained the ability to interact with each other (see below). The observation of expected inhibitory effects using the NF-κB inhibitors SN50 and Bay11-7082 further supported the hypothesis that the fluorescent fusion proteins retained endogenous protein function.

The half-time of fluorescence degradation in cells transfected with IκBα-EGFP alone (26.7±2.8 minutes; Fig. 2B) and cells co-transfected with a control dsRed expression vector (18.2±2.2 minutes; Fig. 3A) was significantly slower than that described in previous studies (Li et al., 1999; Henkel et al., 1993; Sun et al., 1993). Western blot analysis of cells transfected with IκBα-EGFP showed that this delay was not due to cleavage of EGFP from the fusion protein, as no bands smaller than the fusion protein were observed when probing with an anti-EGFP antibody, and endogenous IκBα degradation occurred at the same rate as IκBα-EGFP degradation in transfected cells (data not shown). A previous study observed a 5 minute half life of an IκBα-EGFP fusion protein after treatment with 100 ng/ml TNFα (Li et al., 1999). Expression of IκBα-EGFP in that study was under inducible control, which may have prevented accumulation of high levels of IκBα-EGFP in the cell. Single transfectants with IκBα-EGFP in our study showed approximately 40% higher expression of fluorescent IκBα-EGFP than cells co-transfected with a control dsRed expression vector. The lower expressing dual-transfected cells showed faster degradation of IκBα-EGFP than the higher-expressing single transfectants. Higher levels of IκBα expression may therefore saturate the IκB phosphorylation, ubiquitination or degradation pathways.

We observed that co-expression of p65-dsRed together with exogenous IκBα-EGFP gave rise to significantly faster degradation of IκBα-EGFP fluorescence (half life 13.5±1.7 minutes; Fig. 3A) compared with single (26.7±2.8 minutes; Fig. 2B) or control dual transfections (18.2±2.2 minutes, P=0.024; Fig. 3A). Therefore, these data suggest that higher levels of p65 expression specifically and significantly increase the rate of IκBα degradation. This suggests that NF-κB-IκB complexes may be the natural substrates for an IKK rather then IκB proteins alone, confirming, in living mammalian cells, previous results obtained from phosphorylation studies in insect cells (Zandi et al., 1998).

We also show that higher levels of IκBα-EGFP significantly delay the timing of p65-dsRed nuclear translocation (Fig. 3B; Fig. 4). For p65-dsRed+IκBα-EGFP dual transfections the half time of translocation was 24.5±2.9 minutes compared with 11.6±1.3 minutes in p65-dsRed + control EGFP dual transfections (P=0.012). These data therefore suggest that high levels of IκBα specifically delay nuclear import of p65.

The effect of the ratio of two proteins on the timing of nuclear signalling is a potentially important mechanism by which cells may respond differentially to the same signal. This may have important functional consequences in cells treated with TNFα, since TNFα is known to elicit a death response leading to apoptosis through the TNFR. However, the NF-κB response to TNFα is known to protect cells from apoptosis (Foo and Nolan, 1999). It is therefore possible that a delayed NF-κB response in cells with a high IκB:NF-κB ratio might be more likely to lead to apoptosis. This possibility remains to be investigated. We also show that the timing of p65 nuclear occupancy is approximately 2.5 hours as measured by half maximum to minimum translocation levels, or 10 minutes (or less) as measured by peak nuclear p65-dsRed fluorescence (Fig. 5A). The observed delay

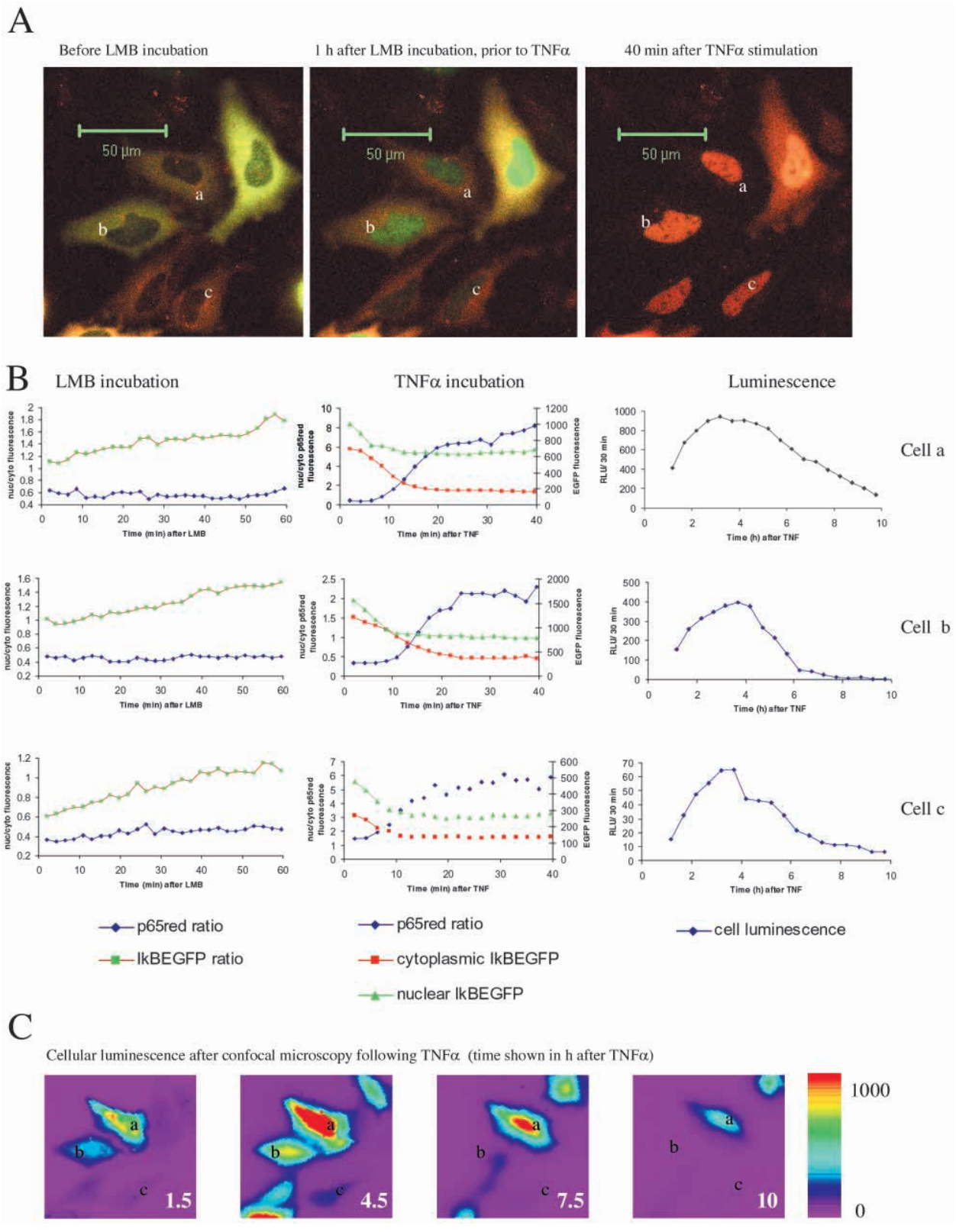

Fig. 8. Quantitative analysis of p65 translocation, IκBα degradation and NF-κB-dependent transcription in single living cells.(A) Dual confocal microscopy images of p65-dsRed and IκBα-EGFP at different time intervals following 1 hour LMB treatment followed by TNFα stimulation. Images are shown before and after the 1 hour LMB treatment and 40 minutes after TNFα stimulation. (B) Quantitative analysis of the time course of p65-dsRed translocation, IκBα-EGFP degradation and cell luminescence (luciferase activity) in three cells a-c (A,C) as described in Fig. 6. (C) Luminescence images of cells following TNFα stimulation. Luminescence directed by NF-κB-promoter-directed luciferase activity. Cells marked a-c correspond to those marked in A and used for quantification in B.

in p65 import in cells expressing high IκB:p65 ratios might therefore contribute to the overall timing of the NF-κB signal and its functional significance.

To investigate the relationship between p65 translocation, IκBα translocation/degradation and transcription in the same cells, we applied a novel technique involving two-colour fluorescence and luminescence imaging. The time-course of transcription from the NF-κB consensus promoter was found to be transient, as previously observed by cell population analysis (Arenzana-Seisdedos et al., 1995). The repression phase of the transient NF-κB transcription response has been suggested to involve the induction of endogenous synthesis of IκBα following NF-κB activation (Place et al., 2001). This may result in nuclear accumulation of the newly synthesised IκBα, which binds to NF-κB, inhibiting transcription and leading to CRM-1-dependent nuclear export of the inactive IκBα-NF-κB complex into the cytoplasm. To investigate the relationship between the timing of protein movement and transcription, we treated the cells with the inhibitor of CRM-1-dependent nuclear export, LMB. This led to import of p65-dsRed and IκBα-EGFP into the nucleus of the cells, caused by inhibition of the export component of normal nucleo-cytoplasmic shuttling. As reported previously (Carlotti et al., 2000), the rate of import of IκBα was significantly higher than that of the p65 fluorescent fusion protein, suggesting that these proteins enter the nucleus by separate pathways and as separate entities. Treatment with LMB did not activate basal NF-κB-dependent transcription. After a 6 hour treatment with LMB, the NF-κB-dependent transcription response to TNFα was blocked, but not general transcription from a control promoter (data not shown). However, cells that had been treated for only 1 hour with LMB before treatment with TNFα still showed cytoplasmic p65 and were able to elicit NF-κB-dependent transcription. Under these conditions, we observed IκBα-EGFP degradation both in the cytoplasm and to a lesser extent in the nucleus, as well as rapid and stable nuclear accumulation of the remaining cytoplasmic p65-dsRed. The demonstration of IκBα degradation in the nucleus may support the recent suggestion that this is an important component of NF-κB regulation (Renard et al., 2000), although we do not see significant IκBα degradation (or transcriptional activation) in response to TNFα at later times following LMB treatment. Despite the accumulation of p65 in the nucleus (since nuclear export was inhibited with LMB), the transcription response to TNFα stimulation was transient, with similar kinetics to those observed in cells that had not been treated with LMB. This confirms that the dynamics of transcription are not dependent simply on the nuclear localisation of p65.

Since a transient transcription response still occurs in the presence of a high concentration of remaining nuclear IκBα after 1 hour of LMB treatment, it seems likely that a further parameter may affect the rate of IκBα degradation in the nucleus and the ability of nuclear IκBα to regulate transcription. One possibility is that functional transcription requires a further cytoplasmic event to occur, such as phosphorylation of the p65 subunit of NF-κB. The functional importance of phosphorylation of serine residues on p65 has been demonstrated in a number of studies (Wang and Baldwin, 1998; Zhong et al., 1998; Anrather et al., 1999; Mercurio et al., 1997; Sakurai et al., 1999; Fognani et al., 2000; Martin and Fresno, 2000; Wang et al., 2000; Jang et al., 2001; for a review, see Schmitz et al., 2001). This might explain the observation in the present experiments that transcriptional upregulation from the NF-κB promoter is only seen at times when there is still significant p65 remaining in the cytoplasm following LMB treatment. Recently, cells from knockout mice that lack the gene encoding glycogen synthase kinase-3β protein were shown to elicit translocation of p65-p50 heterodimers to the nucleus, but did not give an NF-κB transcriptional response (Hoeflich et al., 2000). Cytoplasmic modifications of NF-κB proteins may therefore modulate transcriptional activity in conjunction with NF-κB nuclear translocation.

The present results suggest that treatment with LMB does not prevent the post-induction repression of NF-κB-dependent transcription as suggested previously (Rodriquez et al., 1999). Rather, the kinetics of the transient reporter gene response that we observe is remarkably similar in 1 hour LMB pre-treated cells and non-LMB-treated cells. This suggests that newly synthesised IκBα may enter the nucleus and rapidly repress transcription, despite the inhibition of nuclear export resulting in long-term nuclear localisation of p65. It might be expected that we should see significant inhibition of the initial phase of transcription induction by the accumulated excess of nuclear IκBα that is present at the time of TNFα induction. However, this does not seem to occur. The level of nuclear IκBα-EGFP remained significant (albeit lower due to some nuclear degradation) 40 minutes after TNFα stimulation. These results suggest that a further factor that might include p65 or IκBα interactions with other proteins [such as hnRNPA1 (Hay et al., 2001)], co-activators or co-repressors, such as silencing mediator of retinoic acid and thyroid hormone receptor [SMRT (Jang et al., 2001; Jong and Privalsky, 2000)], or phosphorylation of p65 (Jang et al., 2001) may further regulate the timing of these processes.

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