Introducing Spatial Information into Predictive NF-κB Modelling – An Agent-Based Approach

Mark Pogson[1], Mike Holcombe[1], Rod Smallwood[1], Eva Qwarnstrom[2].

[1] *Department of Computer Science, University of Sheffield, Sheffield, S1 4DP, UK*

[2] *Department of Genomic Medicine, University of Sheffield, Sheffield, S10 2JF, UK*

Nature is governed by local interactions among lower-level sub-units, whether at the cell, organ, organism, or colony level. Adaptive system behaviour emerges via these interactions, which integrate the activity of the sub-units. To understand the system level it is necessary to understand the underlying local interactions. Successful models of local interactions at different levels of biological organisation, including epithelial tissue and ant colonies, have demonstrated the benefits of such 'agent-based' modelling[1-4]. Here we present an agent-based approach to modelling a crucial biological system – the intracellular NF-κB signalling pathway. The pathway is vital to immune response regulation, and is fundamental to basic survival in a range of species[5-7]. Alterations in pathway regulation underlie a variety of diseases, including atherosclerosis and arthritis. Our modelling of individual molecules, receptors and genes provides a more comprehensive outline of regulatory network mechanisms than previously possible with equation-based approaches. The method also permits consideration of structural parameters in pathway regulation; here we predict that inhibition of NF-κB is directly affected by actin filaments of the cytoskeleton sequestering excess inhibitors, therefore regulating steady-state and feedback behaviour. The spatial detail of the model permits explicit modelling of structural components of the cell such as the cytoskeleton, which is partly composed of actin filaments, to which IκB can bind[19].

NF-κB-IκB complex formation at equilibrium was assessed in the presence of actin-IκBα interactions. With actin present, maximal NF-κB-IκB complex formation was achieved at a 1:3 NF-κB:IκBα ratio, in agreement with levels measured in live cells during optimal activation Without actin, the maximum is reached at a 1:1 concentration, corresponding to model data. The model therefore predicts a key role for IκBα-actin interactions in sustaining optimal pathway regulation by adjusting NF-κB-IκB complex formation at the steady-state, and controlling negative feedback following activation. Biological experiment strongly supports these findings, demonstrating a ratio of actin-bound to free IκBα of 2:1±10% in unstimulated cells; the results one of three of these experiments is presented.

Activation of the NF-κB pathway is controlled by inhibitors of NF-κB (IκB) proteins, which sequester the majority of NF-κB in the cytoplasm as complexes by masking their nuclear localisation signals[8]. During activation, IκB is phosphorylated by IκB kinases (IKK), causing its destruction[9, 10]. The newly freed NF-κB is consequently transported into the nucleus, inducing inflammatory genes, including those encoding IκB, thus regulating the pathway through negative feedback[11, 12].

Pathway activation is tightly controlled at multiple levels. Detailed information of the parameters regulating specific steps and their impact on activation is of fundamental importance for understanding the pathway as a whole. Recently, modelling of regulation at the level of the inhibitor has been performed using differential equations[13, 14]. Our agent-based model provides a more complete appreciation of the regulatory mechanisms within the signalling network as a whole, demonstrating predictive behaviour at all steps from initiation at the level of the cell-surface receptor (TIR) to resultant gene regulation. The model extends the capabilities of reaction kinetics and stochastic simulation models by including explicit spatial and structural parameters such as localisation, transport,

and complex formation of signalling intermediates, thus relating directly to real time single cell analysis. Any number (within computational limitations) and distribution of molecules can be modelled, time delays in key processes are properly accounted for, and individual interactions of agents are characterised by stable and well-defined parameters. The model reflects the discrete stochastic nature of interactions, and provides a realistic description of subcellular events.

Computational modelling is a rapidly developing methodology for investigating the organisation of complex biological systems. Such modelling allows *in virtuo* experiments to complement the *in vitro* and *in vivo* methods that are already well-established in biology. The flexible, intuitive and extensible nature of agent-based modelling makes it well-suited to modelling biological systems. It requires the identification of an appropriate level to model (in our case the cell) and the entities to be modelled (here individual molecules); the complete system is derived from considering interactions of the individual components with the environment and neighbouring components, and the behaviour of the complete system is an emergent property. The behaviour of an individual component is determined by the dynamics of its internal characteristics (state), its physical location, and its relationships with those components around it (communication). Modern computing power and experience of modelling complex systems composed of many interacting autonomous parts have provided the foundation for a new approach to understanding complex biological systems.

In the model, molecular agents diffuse through the cell, binding and dissociating from other molecules, receptors and cell structures in accord with signals they send and receive from surrounding agents. Every agent is represented by a complete computational model – the communicating stream X-machine – which provides an intuitive and rigorous basis to model the functional behaviour of systems in a flexible and extensible manner[15, 16]. An important feature is the memory of each agent's X-

machine, which contains its physical location, meaning that the number of states required to model the system is manageably small. It is essential that the agents are both biologically plausible as entities and that their behaviour is based on experimental measurements. In the model, as in reality, molecular interactions are local events that depend only on the position and current state of the molecules involved, where the state of a molecule is whether or not it is already bound. The physics of a molecule is modelled according to specific agent-based characteristics, including which types of interaction are possible. If two molecules may interact according to the rules, they must satisfy criteria on their state and proximity, derived from standard rate constants[17]. If interaction occurs, the state of each agent changes to a 'bound' state, which can be reversed through random thermal separation. The model agrees with reaction kinetics in homogenous situations (Fig. 1).

TIR mediated activation is considered. Each NF-κB, IκB and IκB-kinase (IKK) molecule is an individual agent, as are the importing and exporting nuclear and cell surface receptors (Fig. 2a). Soluble extracellular agonists are not modelled as agents but treated as a whole chemical entity whose fluctuating local concentrations at the cell surface must rise above a certain level to initiate signal transduction in nearby receptor agents. Similarly, in relation to the cytoplasmic TIR domain, the local concentrations of certain molecules in the cytoplasm must be above a defined level in the vicinity of an active receptor to complete the process. Following this, a temporary agent with an internal time delay is created to account for the cascade that triggers the IκB kinases (IKKs). At the final steps of activation, an analogous temporary agent method is used to account for the translation of IκB.

The agents are contained within a spherical cell consisting of a cytoplasm and concentric spherical nucleus (Fig. 2b). Space is continuous and time is discrete in the model. The rules that govern agent movements incorporate cell structure by defining

spatial boundaries. This allows investigation of the impact of cell shape and biomechanical effects[18].

The model is in good agreement with biological data obtained by real time single cell analysis (Fig 2c-e). Continuous monitoring of signal transduction events in live cells was performed using GFP-tagged regulatory intermediates and confocal microscopy[5-7]. Simultaneous observations of the NF-κB subunit relA and IκBα were carried out using cyan (ECFP) and yellow (EYFP) variants of GFP respectively, as previously[7]. Complex-formation was assessed by measuring fluorescence resonance energy transfer (FRET), with ECFPrelA the donor and IκBαEYFP the acceptor.

Unlike an ODE model, the model's robustness allows it to be used to assess effects across the pathway as a whole, such as to determine the impact of alterations at the level of the cell surface receptor on downstream signalling events and gene regulation (Fig. 3a), here demonstrating an expected positive correlation between TIR levels and nuclear translocation of NF-κB.

The model is not as restricted as experiment in the duration or detail that can be observed. Results obtained over an extended periods demonstrate the expected system switch-off (Fig. 3b), in agreement with induction of negative feedback mechanisms following activation. In addition, the model demonstrates distinct regulation by IκB isoforms, and control of gene activity (Fig 3b).

The model permits investigation of various pathway features, such as the effects of changing levels of signalling proteins at specific regulatory steps. The model displays a narrow range of IKK levels within which subsequent pathway activity is affected, measured at the level of nuclear transport of NF-κB (Fig. 3c left panel) and IκBα phosphorylation and complex dissociation (Fig. 3c right panel). The impact of relative

levels of NF-κB and IκBα on cellular localisation is demonstrated in Fig. 3d, showing a concentration dependent decrease in relative nuclear NF-κB levels with enhanced inhibitor levels, reflecting complex formation and cytoplasmic retention, mimicking the resting state, as in previous biological experiments[5-7]. Further analysis demonstrates, as expected, a maximal increase in NF-κB nuclear translocation at a 1:1 NF-κB:IκB ratio (Fig 3e). This, together with a more potent effect of changing inhibitor levels, is in agreement with the significance of sequestering excess inhibitor in pathway control.

The spatial detail of the model permits explicit modelling of structural components of the cell such as the cytoskeleton, which is partly composed of actin filaments, to which IκB can bind[19] (Fig 4a). NF-κB-IκB complex formation at equilibrium was assessed in the presence of actin-IκBα interactions (Fig 4b). With actin present, maximal NF-κB-IκB complex formation was achieved at a 1:3 NF-κB:IκBα ratio, in agreement with levels measured in live cells during optimal activation Without actin, the maximum is reached at a 1:1 concentration, corresponding to data in Fig 3d. The model therefore predicts a key role for IκBα-actin interactions in sustaining optimal pathway regulation by adjusting NF-κB-IκB complex formation at the steady-state, and controlling negative feedback following activation. Biological experiment strongly supports these findings, demonstrating a ratio of actin-bound to free IκBα of 2:1±10% in unstimulated cells; the results one of three of these experiments is presented in Fig 4c.

The formal agent-based modelling paradigm illustrated here provides a useful technique for understanding many aspects of biological systems. It is a suitable model for cellular regulatory events such as the NF-κB pathway, providing a clear and intuitive mechanism to determine and explore the key features of the system. The model provides predictions about regulation of the pathway, which are unachievable by other modelling methods, and displays an appropriate robustness, allowing future investigation of the

variability of cells in nature. The detailed mathematical structure of the model should allow the use of automated reasoning techniques such as model checking to properly understand the system in all possible circumstances within which it operates[20]. This is an important capability that has not yet been exploited in biological modelling.

Methods

Cell culture

Hela cells were kept at 37°C in 5% CO_2 and maintained in DMEM (Gibco) supplemented with 10% foetal calf serum (Gibco).

Immunoprecipitation and Cross linking

Whole cell lysates were prepared from 1 x 10 cm dishes transfected using the calcium phosphate method with IkBα-EGFP chimera. Transfected cells were cross linked with 5 mM DSS (Pierce) in PBS pH 8.0 for 30 minutes at room temperature. The solution was quenched with 1 m TrisHCl pH 7.5 to a final concentration of 10 mM and left for 15 minutes. Cross linked cell lysate was prepared using a RIPA buffer kit (Santa Cruz) per the manufacturer's instructions. Supernatant lysate (S1) was precleared with Protein A/G PLUS-Agarose Immunoprecipitation Reagent (Santa Cruz) according to the manufacturer's instructions and incubated with rocking at 4°C for 30 minutes with appropriate IgG (Santa Cruz). 1 ml of lysate was incubated with rocking at 4°C overnight with rabbit polyclonal anti-Actin antibody (Santa Cruz, sc-1616) and the A/G agarose beads. The P1 was reserved for Western analysis. The S1 was then used a second time to carry out a second immunoprecipitation under the same conditions but substituting the Actin antibody with an anti- IκBα mouse monoclonal antibody (Santa Cruz sc-1643). The resultant P2 was analysed by Western blotting.

SDS-PAGE analysis

Immunoprecipitates were boiled in 1 x Laemelli sample buffer for 3 minutes and analysed by 10% Laemelli SDS-PAGE. Gels were transfered onto PVDF membrane (Hybond-P, Amerham) and blocked with 5% milk powder in TBS/Tween for 1 hour at room temperature. Membranes were incubated for 2 hours with rocking at room temperature with IκBα primary antibody (Santa Cruz sc-371). Following 2 x 5 minute TBS/Tween washes and 1 x TBS wash, membranes were incubated in blocking solution with either Anti-rabbit HRP secondary or anti-mouse HRP secondary (Cell Signalling) for one hour with rocking. Membranes were incubated with Luminol Reagent (Santa Cruz) for 1 minute before detection by ECL.

References

1.   Jackson, D., Holcombe, M. & Ratnieks, F. Trail geometry gives polarity to ant foraging networks. *Nature* **432**, 907-909 (2004)

2.   Jackson, D., Holcombe, M. & Ratnieks, F. Coupled computational simulation and empirical research into the foraging system of Pharaoh's ant (Monomorium pharaonis). *BioSystems* **76**, 101-112 (2004)

3.   Walker, D. C., Southgate, J., Hill, G., Holcombe, M., Hose, D. R., Wood, S. M., MacNeil, S. & Smallwood, R. H. The Epitheliome: modelling the social behaviour of cells. *BioSystems* **76**, 89-100 (2004)

4.   Walker, D. C., Hill, G., Wood, S. M., Smallwood, R. H. & Southgate, J. Agent-based computational modelling of wounded epithelial cell monolayers. *IEEE Trans Nanobioscience* **3**,153-163 (2004)

5.   Carlotti, F., Chapman, R., Dower, S. K. & Qwanstrom, E. E. Activation of Nuclear Factor κB in single living cells. *J. Biol. Chem.* **274**, 37941-37949 (1999)

6.   Carlotti, F., Dower, S. K. & Qwanstrom, E. E. Dynamic Shuttling of Nuclear Factor κB between the nucleus and cytoplasm as a consequence of inhibitor dissociation. *J. Biol. Chem.* **275**, 41028-41034 (2001)

7.   Yang, L., Ross, K. & Qwanstrom, E. E. RelA control of IκBα phosphorylation: a positive feedback loop for high affinity NF-κB complexes. *Biol. Chem.* **278**, 30881-30888 (2003).

8.   Beg, A. A., Ruben, S. M., Scheinman, R. I., Haskill, S., Rosen, C. A. & Baldwin, A. S. IκB interacts with the nuclear localization sequences of the subunits of NF-κB: a mechanism for cytoplasmic retention. *Genes Dev.* **6**, 1899-1933 (1992) Correction *Genes Dev.* **6**, 2664-2665 (1992)

9.   Taussig, R., Ranganathan, R., Ross, E. M. & Gilman, A. G. Overview of the alliance for cellular signalling. *Nature* **420**, 703-706 (2002)

10.   Ghosh, S., May, M. J. & Kopp, E. B. NF-κB and Rel proteins: Evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* **16**, 225-260 (1998)

11.   Baeuerle, P. A. & Baltimore, D. NF-κB: ten years on. *Cell* **87**, 13-20 (1996)

12.   Kopp, E. B & Ghosh, S. NF-κB and Rel proteins in innate immunity. *Adv. Immunol.* **58**, 1-27 (1995)

13.   Hoffmann, A., Levchenko, A., Scott, M. L. & Baltimore, D. The IκB-NF-κB signalling module: temporal control and selective gene activation. *Science* **298**, 1241-1245 (2002)

14.   Nelson, D. E., Ihekwaba, A. E. C, Elliott, M., Johnson J. R., Gibney, C. A., Foreman, B. E., Nelson, G., See, V., Horton, C. A., Spiller, D. G., Edwards, S. W., McDowell, H. P., Unitt, J. F., Sullivan, E., Grimley, R., Benson, N., Broomhead, D., Kell, D. B. & White, M. R. H. Oscillations in NF-κB signalling control the dynamics of gene expression. *Science* **306**, 704-708 (2004)

15. Holcombe, M., Holcombe, L., Gheorghe, M. & Talbot, N. A hybrid machine model for rice blast fungus. *BioSystems* **68**, 223-228 (2003)

16. Balanescu, T., Holcombe, M., Cowling, A. J., Gheorgescu, H., Georghe, M. & Vertan, C. Communicating stream X-machines systems are no more than X-machines. *Journal of Universal Computer Science* **5**, 494-507 (1999)

17. Andrews, S. S. & Bray, D. Stochastic simulation of chemical reactions with spatial resolution and single molecule detail *Physical Biology* **1,**137-151 (2004)

18. Rosette. C & Karin, M. Cytoskeletal Control of Gene Expression: Depolymerization of Microtubules Activates NF-κB. *The Journal of Cell Biology.* **128**, 1111-1119 (1995)

19. Are, A. F., Galkin, V. E., Pospelova, T. V. & Pinaev, G. P. The p65/RelA Subunit of NF-κB Interacts with Actin-Containing Structures. *Experimental Cell Research.* **256**, 533-544 (2000)

20. McMinn, P. Search-Based Software Test Data Generation: A Survey. *Software Testing, Verification and Reliability.* **14**, 105-156 (2004)

Fig. 1. Agent interactions. Agreement of agent-based model of chemical interactions with a model based on reaction kinetics ODEs. Reaction A + B -> D, A + C -> E. Left: agent model results. Right: corresponding reaction kinetics model results.
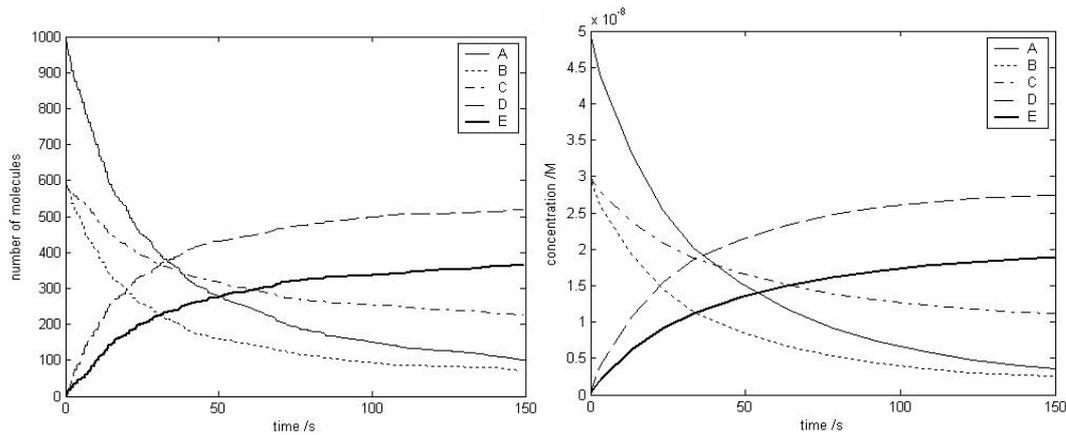
Fig. 2. Formulation and validation of the agent-based model. (a) Simplified diagram of the principal pathway agents in the model. Each agent can exist in a number of states. (b) Three-dimensional visualisation of the positions of agents in the model at a moment in time. The cell is scaled down to reduce computation, containing in the order of 1000 agents. Concentrations of molecules are based on biological data. The genes that NF-κB can activate are placed randomly along a line at the centre of the nucleus. (c) Images of single cells co-transfected with ECFPrelA and IκBαEYFP. Prior to stimulation, both components are located in the cytoplasm (top row). Following pathway activation, NF-κB translocates to the nucleus whilst IκBα and NF-κB-IκBα complex levels fall (bottom row). (d) Quantitation of single cell data as in (c). (e) Model results following TIR activation over the same time period. Results are for a single cell, and demonstrate fundamental similarities with experiment.
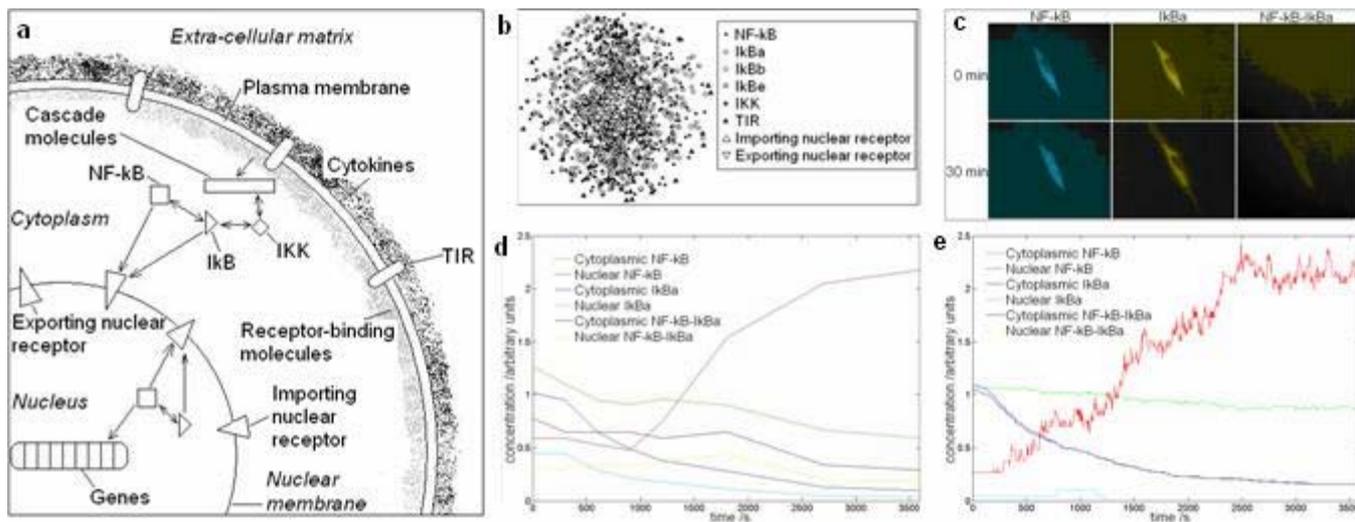
Standard error 5.4% for nuclear concentration

Fig. 3. Investigating the pathway in further detail with the model. (a) Positive correlation between saturated TIR levels and pathway activity at the level of NF-κB nuclear translocation. Dashed lines indicate control runs. (b) Extended kinetic analysis following stimulation. System returns to non-stimulated state following activation. All error bars show standard error of the mean. (c) Effect of IκB kinase (IKK) on pathway regulation. Increasing IKK levels demonstrates concentration dependent activation and saturation of activity at key regulatory steps. Left: effect on nuclear translocation of NF-κB. Right: effect on IκBα phosphorylation and NF-κB-IκBα complex levels. (d) Relative nuclear and cytoplasmic concentrations of NF-κB with varying IκBα levels at equilibrium. Uninhibited, the cytoplasmic:nuclear concentration of NF-κB is approximately 1:1 (solid diagonal line); increasing inhibitor levels to equal those of NF-κB results in a 10:1 cytoplasmic:nuclear concentration of NF-κB (lower dashed diagonal line), in close agreement with experiment. (e) Effect of changing NF-κB to IκBα ratios on response to simulation.
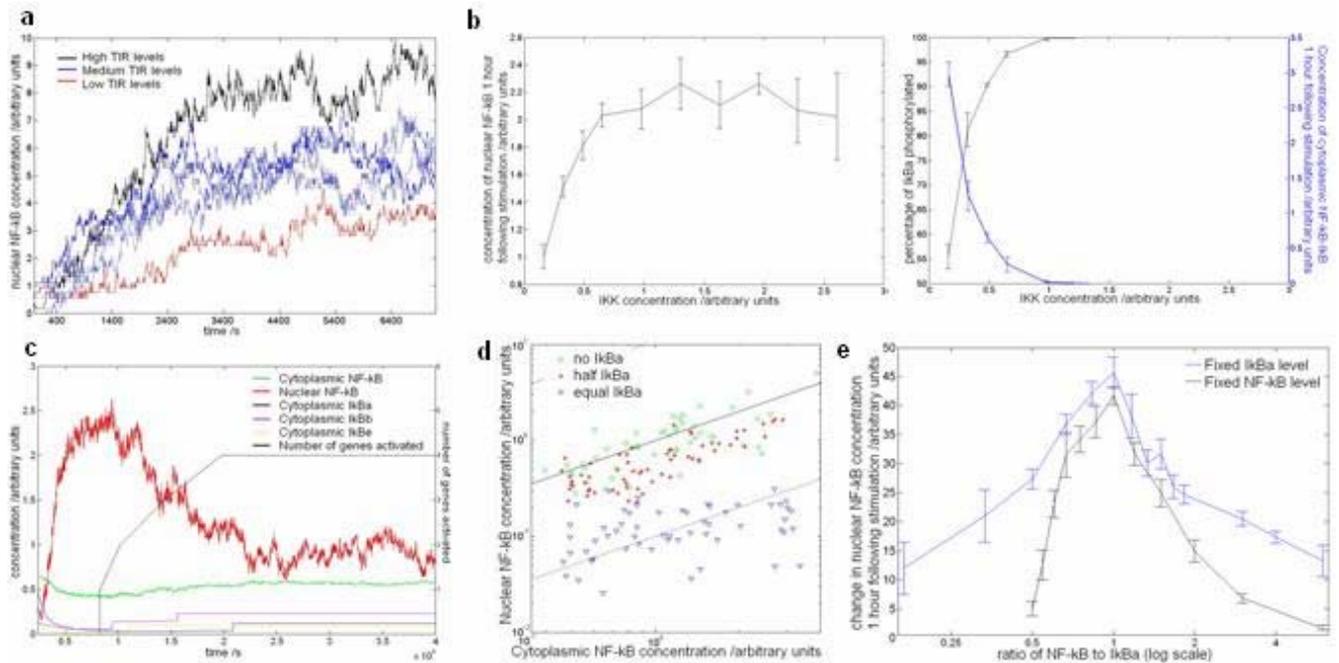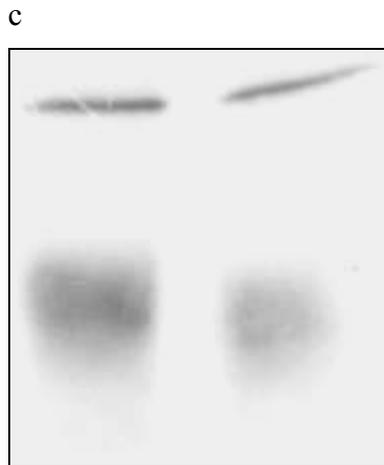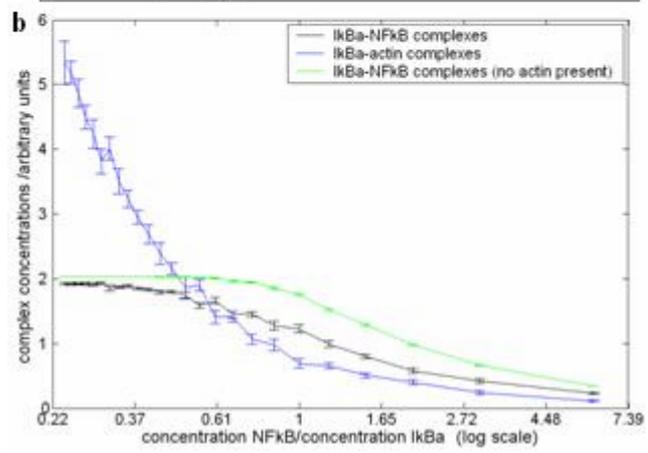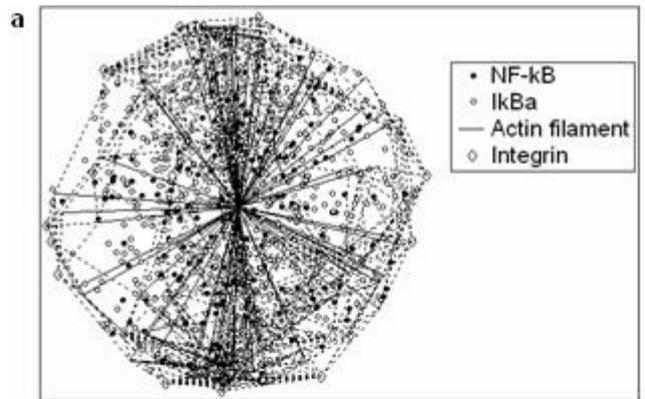
Fig. 4. Introducing actin cytoskeletal filaments to the model. (a) Three-dimensional visualisation of the model cell, with dashed lines a visual aid. (b) NF-κB-IκBα and actin-IκBα levels at equilibrium with changing relative NF-κB:IκBα concentrations (NF-κB concentration constant). With actin present, the maximum NF-κB-IκB complex level is reached at around 1:3 NF-κB:IκB concentration (black line), with the excess IκB bound to actin (blue line). The same maximum is reached at around 1:1 concentration in the absence of actin (green line). (c) Immunoprecipitation of IκBα (left column) and secondary actin immunoprecipitation (right column). (d) Graphing of (c), showing an approximately 2:1 ratio of actin-bound IκBα to free IκBα.

a

- NF-kB
- IkBa
- Actin filament
- Integrin

b

- IkBa-NFkB complexes
- IkBa-actin complexes
- IkBa-NFkB complexes (no actin present)

concentration NFkB/concentration IkBa (log scale)

complex concentrations /arbitrary units

c

d