Functional characterization of NF-κB inhibitor-like protein 1 (NFκBIL1), a candidate susceptibility gene for rheumatoid arthritis

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Several studies have implicated the NF-κB inhibitor-like protein 1 (NFκBIL1) gene located in the class III region of the major histocompatibility complex (MHC) as a possible susceptibility locus for rheumatoid arthritis (RA). Based on limited homology, it has been suggested to be a member of the inhibitor of NF-κB (IκB) family of proteins, but a role in mRNA processing has also been proposed. We have investigated the expression of NFkBIL1 in RA synovial tissue and characterized its function. Real-time PCR showed the two NFkBIL1 mRNA splice variants are expressed in a tissue-specific manner. Dual immunofluorescent staining of human RA synovium with polyclonal anti-NFkBIL1 antibodies and anti-CD68, anti-CD3 or anti-factor VIII showed that NFkBIL1 was expressed in the rheumatoid synovial lining and sub-lining layers and co-localized in CD68⁺ and CD3⁺ cells. Confocal microscopy of cultured synovial fibroblasts revealed expression in speckled nuclear and homogenous cytoplasmic distributions, suggesting shuttling between the cytoplasmic and nuclear compartments. Functional tests showed that NFkBIL1 isoforms were incapable of associating with NF-κB and did not inhibit it, thus disproving the hypothesis that NFkBIL1 functions as an IκB. Affinity purification of endogenous NFkBIL1 proteins and co-immunoprecipitation experiments showed that NFkBIL1 can associate with mRNA and with three protein partners, identified by mass spectrometry as leukophysin, translation elongation factor 1 α and CTP synthase I. These data support a potential role for NFkBIL1 in the pathogenesis of RA and indicates that it may be involved in mRNA processing or the regulation of translation.

INTRODUCTION

The genetic contribution to rheumatoid arthritis (RA) has been estimated at 30–50% of total risk (1,2). Approximately one-third of this is believed to arise from the major histocompatibility complex (MHC) at 6p21.3, a gene-dense region that encodes a large number of proteins involved in the innate and adaptive immune systems (3,4). The DRB1 gene within this region has been reproducibly implicated (5) in both RA susceptibility and severity (6,7).

Recent studies have however revealed that the MHC contribution to RA is polygenic with additional susceptibility arising telomeric of DRB1. A large US family-based study reported two non-DRB1 risk loci, one in a 497 kb interval in the central MHC and a second in a class I region in a subset of DRB1*0404 haplotypes (8). We also reported DRB1-independent association with several single-nucleotide polymorphisms (SNPs) in the telomeric class III region in British RA families (9) and another British study reported differences of tumour necrosis factor (TNF) haplotypes between DRB1*04-matched RA patients and healthy controls (10). A large Japanese case–control study typed 88 SNPs and the DRB1 locus and detected an independent association of markers located around the class III and I junction (11).

The telomeric class III region bordering the class I region is particularly gene-dense containing at least 10 genes in...
addition to TNF within an 82 kb interval; BAT1, ATP6V1G2, NFKBIL1, LTA, TNF, LTB, LST1, NCR3, AIF-1, BAT3 and BAT2 (12). The function of most of these molecules remains poorly characterized, although evidence for a role in immune and inflammatory responses does exist for several (13,14). Despite the undoubted importance of TNF in RA pathogenesis there is little convincing evidence linking genetic variation at this locus to RA susceptibility (15,16).

The NFKBIL1 gene is located 30 kb telomeric of TNF and owes its name to limited homology with members of the inhibitor of nuclear factor κB (IκB) family of proteins (17). These proteins are critical components of the NF-κB signaling pathway, which regulate the transcription of many important mediators of inflammation and tissue destruction in the rheumatoid joint including TNF, IL-1, and IL-6 (18). In unstimulated cells, NF-κB exists as homo- or heterodimers complexed with IκB proteins. Cellular activation leads to the rapid phosphorylation of N-terminal serine residues of IκB-α by the IκB kinases resulting in the ubiquitination of IκB-α and subsequent degradation by the proteasome. Heterodimers of NF-κB subsequently translocate to the nucleus and activate target genes. Although on the basis of the presence of ankyrin repeat sequences it was suggested that NFKBIL1 may be a novel member of the IκB family, there is no functional data to support this, and indeed it is now appreciated that ankyrin repeats represent a versatile linear scaffold and do not imply any specific function (19).

A number of studies have examined genetic variation in NFKBIL1 and RA susceptibility. One case–control study involving 116 Japanese RA patients and 97 controls reported association with a promoter SNP at position -62 (rs2071592) in a consensus motif for the transcriptional repressor δEF1, however, this study did not report on linkage disequilibrium (LD) with DRB1 alleles (20). Another much larger Japanese study reported DRB1-independent association of another NFKBIL1 promoter SNP at -293 (rs3219185) (21). Interestingly, no association of rs2071592 was detected in either our RA family-based study (9) or a Spanish case–control study (22). Hence, genetic susceptibility linked to NFKBIL1 may vary in different ethnic groups.

In this study, we examined the both the function of NFKBIL1 and expression in RA synovium and synovial fibroblasts. We demonstrate that NFKBIL1 is expressed in T cells and macrophages in the rheumatoid synovium and is mainly distributed in a nuclear speckled pattern. We also show that it does not bind NF-κB proteins or down-regulate inflammatory signalling but binds mRNA, suggesting a role in mRNA processing.

RESULTS

Detection of endogenous NFKBIL1 mRNA variants in cell lines, differentiated tissues and biopsies from inflamed tissues

Two splice variants of the NFKBIL1 mRNA called NFKBIL1-α and NFKBIL1-β have been identified previously (23). They encode proteins of 381 and 366 aminoacids, respectively, which differ by the presence in variant α of a 15-aminoacid sequence (GELEDEWQEVFMGRFE) that is absent from NFKBIL1-β. We measured the levels of expression of endogenous mRNAs for NFKBIL1-α and NFKBIL1-β in commercially available cDNA preparations from several differentiated tissues by real-time PCR. We also measured the expression of NFKBIL1 mRNA variants in skin biopsies from two donors and in peripheral blood mononuclear cells (PBMCs) prepared from three healthy donors and three RA patients. The results showed that at least one NFKBIL1 mRNA variant was expressed in all tissue tested (Fig. 1). However, there were significant differences in the expression levels of individual isoforms. Only the ovary, prostate and PBMCs were found to express both NFKBIL1 splice variants at similar levels. All the other tissues tested expressed preferentially or exclusively a specific variant. NFKBIL1-α was the only variant expressed in brain and endothelial tissue, whereas NFKBIL1-β was the preferred variant in muscle and the only detected variant detected in small intestine, placenta and skin samples. Finally, comparison of the expression levels of the NFKBIL1 variants in PBMCs from healthy donors and RA patients showed no significant difference between the two groups (Fig. 1).

Localization of NFKBIL1 protein in rheumatoid synovium

We generated three polyclonal antibodies to synthetic NFKBIL1 peptides. The first antibody, Ab1, was designed to detect both NFKBIL1 variants, whereas the other two were raised against peptide sequences specific to each individual variant. Immunoblotting tests carried out with lysates of transfected cells expressing each individual variant showed that all three antibodies specifically detected their intended antigens (data not shown). The antibodies were then used to stain synovial membrane biopsy samples from five RA patients and three knee joint osteoarthritis (OA) synovial samples. Intense staining was found in both the synovial lining layer and the sublining synovium, in biopsy samples from all five patients (Fig. 2B and C). Antibodies to both NFKBIL1 variants gave positive results. However, those directed against NFKBIL1-α, Ab2, consistently stained more intensely and were used in all immunohistochemistry or immunofluorescence experiments shown. Staining was much less intense in the OA samples (Fig. 2E and F).

To determine the cellular phenotype of cells expressing NFKBIL1, dual immunofluorescence staining was performed with the Ab2 antibody, anti-CD68 (macrophage), anti-CD3 (T cell) and anti-factor VIII (endothelial cell). Staining for NFKBIL1 co-localized with CD68+ cells in both the synovial lining and sublining layers (Fig. 3A–C). Expression was also observed on a proportion of T cells as shown in a perivascular CD3+ lymphoid aggregate (Fig. 3D–F), but minimal or no expression was detected in factor VIII-expressing synovial endothelial cells (Fig. 3G and H).

Intracellular localization of NFKBIL1 in synoviocytes

The intracellular distribution of NFKBIL1 was examined in rheumatoid fibroblast-like synoviocytes (FLS) between passages 5 and 8. Immunofluorescence confocal microscopy
revealed discrete patterns of expression being either cytoplasmic (Fig. 4A and B), nuclear (Fig. 4C) or both (Fig. 4D).

**NFkBIL1 lacks IκB-like functions**

Because NFkBIL1 owes its name to its structural similarity to IκB, we started our investigation of NFkBIL1 function by assessing whether or not it was involved in the regulation of NF-κB. We first investigated whether or not the NFkBIL1 proteins were capable of associating physically with the p50 and p65 subunits of NF-κB. Co-immunoprecipitations experiments showed that in marked contrast to IκBa, which was used as positive control, neither NFkBIL1-α nor NFkBIL1-β were detected in p50 or p65 immunoprecipitates (Fig. 5A). We also determined if NFkBIL1 proteins were capable of regulating the expression of an NF-κB-driven promoter-reporter construct, pIL8-Luc. The results showed that unlike IκBa, whose expression in transfected cells strongly inhibited IL-1-induced activation of the reporter construct, the NFkBIL1 proteins had no significant effect (Fig. 5B). Similar results were obtained when the pIL8-Luc construct was activated by co-transfection of the signal transduction pathway components, IRAK-1 (data not shown) and TRAF6 (Fig. 5B). Altogether, these results show that NFkBIL1 is not involved in the regulation of NF-κB.

**Association of NFkBIL1 with CTP-synthase I, elongation factor 1 α and leukophysin**

To identify NFkBIL1 possible partners, the endogenous NFkBIL1 was immunoprecipitated from 35S labelled human embryonic kidney (HEK) 293 cells in non-denaturing conditions and the immunoprecipitated material was analysed by SDS–PAGE. The immunoprecipitates were found to
contain a prominent 45-kD band, presumed to be NFkBIL1, and three weaker bands of ~60, 28 and 18 kD, designated, respectively, P1, P2 and P3. These bands were detected in immunoprecipitates obtained by all three anti-NFkBIL1 antibodies used either alone or in combination (Fig. 6A). When immunoprecipitations were carried out on a larger scale from unlabelled cells, the same co-precipitating bands were also detected by Coomassie blue staining (data not shown). The proteins present in these bands were identified by in-gel trypsin digestion followed by liquid chromatography and electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). The results (Table 1) showed that the 60-kD P1 band contained CTP synthase 1 (CTPS) and two other proteins whose identifications were not secure. The 28-kD band, P2, corresponded to leukophysin (LKP). The 18-kDa band contained two components that were identified by mass spectrometry as translation elongation factor 1-α (EF1α) and, less securely identified, the hsp90 co-chaperone, cdc37. Finally, mass spectrometry of the main 45-kD band confirmed its identity as NFkBIL1 and detected no other protein in that band (Table 1). The predicted molecular masses of CTPS and LKP, 66 and 25 kD respectively, are in agreement with the masses estimated from the migration positions of the P1 and P2 bands. However, both EF1α and cdc37 have predicted molecular masses that are much in excess of 18 kD (Table 1), which indicates that the 18-kD P3 band contained proteolytic fragments of the two proteins rather than their native functional forms.

To verify whether or not CTPS, EF1α, LKP and cdc37 were indeed capable of direct association with NFkBIL1, we expressed them as T7-tagged recombinant proteins and carried out co-immunoprecipitation experiments. The results showed that NFkBIL1-α can bind directly to EF1α, LKP and CTPS, but not to cdc37 (Fig. 6B). The same results were observed when NFkBIL1-β was used instead of NFkBIL1-α (data not shown). This evidence thus confirms the ability of both NFkBIL1 variants to associate with CTPS, EF1α and LKP.

**Association of NFkBIL1 with mRNA**

Because the co-localization of NFkBIL1 with the splicing factor, Sm, suggested a role in processing or nuclear export of mRNA (23), we also tested whether or not endogenous NFkBIL1 is associated with mRNA in HeLa cells. After immunoprecipitation of NFkBIL1, the immunoprecipitates were treated with proteinase K and the released soluble material was then subjected to an oligo-dT affinity-based mRNA purification procedure as described in the ‘Materials and Methods’ section. The results showed that NFkBIL1-α can bind directly to EF1α, LKP and CTPS, but not to cdc37 (Fig. 6B). The same results were observed when NFkBIL1-β was used instead of NFkBIL1-α (data not shown). This evidence thus confirms the ability of both NFkBIL1 variants to associate with CTPS, EF1α and LKP.
in the NFkBIL1 immunoprecipitates was similar to that of the mRNA associated with PABP (Fig. 6C) and to mRNA prepared directly from HeLa cells by standard methods (data not shown). These results suggest that like PABP, which binds mRNA with apparently no preference for specific sequence motifs or secondary structures (24), NFkBIL1 isoforms are associated with bulk cellular mRNAs.

**DISCUSSION**

Phylogenetic analysis indicates that the RA candidate gene NFkBIL1 has been strongly conserved in mammalian evolution, the rat and opossum orthologues displaying 92 and 61% identity to the human amino-acid sequence. However, only distantly related homologues were found in lower vertebrates (data not shown). This strong sequence conservation in mammals, together with the localization of the NFkBIL1 gene within the MHC and its proposed association with RA, suggest that NFkBIL1 may have a conserved function in mammalian immunity. However, the biological role of NFkBIL1 is at present poorly known.

NFkBIL1 is expressed as two isoforms denoted by α and β, with the β isoform being 15 amino acids shorter than the α isoform. Real-time PCR data from human tissues shows that some tissues or cell types, such as PBMCs, express both splice variants in similar amounts, while other tissues preferentially express NFkBIL1-α (e.g., brain and endothelial tissues) or NFkBIL1-β (muscle, small-intestine, placenta and skin). These results indicate that expression of the two isoforms is regulated in a tissue-specific manner.

This study has shown that NFkBIL1 proteins are expressed in the rheumatoid, but not OA synovium suggestive of a role in the pathogenesis of RA. This is the first demonstration of NFkBIL1 proteins expressed in human cells or tissues. Protein expression was seen in both the lining and sub-lining layers, but remarkably was not uniform. The antigen was detected in CD68 positive macrophages, a proportion of T cells and cultured FLS from five patients but not at all in the endothelium. We also found that NFkBIL1 adopted predominantly a nuclear speckled distribution in cultured FLS, but was also observed in the cytoplasm of some cells. This result is in agreement with a previous report which showed that in transfected cells, fluorescent recombinant NFkBIL1 adopted the same localizations as reported here for the endogenous protein (23).

Although the partners of most MHC class III gene products have been identified by means of the yeast-two hybrid system, this approach was unsuccessful for NFkBIL1 (25). Based on limited sequence homology, it has been proposed that NFkBIL1 is a member of the 1kB family (17). However our results, including the intracellular distribution of NFkBIL1 proteins, their inability to associate with two NF-κB subunits and their lack of activity in 1kB functional tests clearly show that NFkBIL1 does not function as a inhibitor of NF-κB.

Our search for proteins associated with endogenous NFkBIL1 in HeLa cells revealed a diverse group of possible partners. Three of the candidates partners identified by mass spectrometry were then confirmed to be capable of directly binding to 1kB when expressed in transfected cells. They correspond to CTPS, EF1α and LKP. Although the functional significance of the interaction with CTPS, which catalyses the ATP-dependent amination of UTP to CTP, is unclear, the other two partners provide interesting insights into the molecular function of NFkBIL1. EF1α, which is also known as leucocyte receptor cluster member 7, is involved in the translation of mRNAs and is believed to be an autoantigen for Felty’s syndrome, which is characterized by systemic illness in a small subset of RA patients (26). LKP is a 235-residue protein of unknown function that owes its name to its initial detection in leucocyte secretory granules, but appears to be expressed in a variety of cell types. Our evidence clearly shows that it is produced in HeLa cells.

The sequence of LKP is identical to the C-terminal end of RNA helicase A (RHA), also known as DEAD box helicase 9 (DHX9). Indeed, LKP and RHA are produced by alternative
The association of NFkBIL1 with LKP thus suggests that it probably also associates with RHA. The physiological role of the helicase is much better known than that of LKP. It has an active role in transcription and has also been shown to stimulate the translation of specific messages by associating with regulatory secondary structures of their 5′-UTRs (27,28). Autoantibodies to helicase A/DHX9 are considered an early marker for systemic lupus erythematosus (SLE) (28).

An earlier study has shown that recombinant NFkBIL1 proteins co-localized with the spliceosome component, Sm in transfected cells (23). Like RHA, Sm is an autoantigen associated with SLE (28). While this manuscript was in preparation, 13 other partners of NFkBIL1 were reported in the IntAct protein–protein interactions database (available on-line at http://www.ebi.ac.uk/intact/site/index.jsf). Similar to Sm, EF1α and RHA, many of these possible of partners of NFkBIL1 have functions associated with RNA processing or translation (29). Spliceosomal factors can redistribute into the cytoplasm during the cell cycle, and indeed can shuttle between nuclear speckles and the cytoplasm (30). Altogether, these findings and our observations that endogenous NFkBIL1 associates with mRNA in HeLa cells and can adopt either a nuclear speckled or a cytoplasmic distribution

mRNAs of the DHX9 gene. The association of NFkBIL1 with LKP thus suggests that it probably also associates with RHA. The physiological role of the helicase is much better known than that of LKP. It has an active role in transcription and has also been shown to stimulate the translation of specific messages by associating with regulatory secondary structures of their 5′-UTRs (27,28). Autoantibodies to helicase A/DHX9 are considered an early marker for systemic lupus erythematosus (SLE) (28).

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in synoviocytes thus strongly suggest that it is involved in mRNA processing.

In summary, we report the expression of NFkBIL1 in rheumatoid synovial tissue including T cells, macrophages and fibroblasts, but not endothelial cells supporting a potential role for this molecule in the pathogenesis of RA. While there is abundant evidence showing the presence a genetic susceptibility marker for RA telomeric of DRB1, its identification is complicated by the high gene density and large number of plausible candidate genes. The finding that NFkBIL1 is expressed in activated cells in the rheumatoid synovium lends support to a role in the pathogenesis of RA. Our evidence also indicates that NFkBIL1 proteins associate with mRNA and with proteins that are involved in transcription, processing and translation of mRNA and have been associated with SLE (RHA) or Felty’s syndrome (EFT1). These findings provide directions for future further biological and genetic studies of the mechanistic link between these proteins and chronic inflammation.

MATERIALS AND METHODS

Reagents and established cell lines

Restriction enzymes and T4 ligase were from Promega (Madison, WI, USA). RNase A and RNase-free DNase I were from Qiagen (Crawley, UK). DNA amplifications were carried out with *Pfu* polymerase (Stratagene, La Jolla, CA, USA) unless otherwise stated. Preparations of cDNAs from differentiated cells and tissues were purchased from OriGene Technologies, Inc. (Rockville, MD, USA), except the cDNAs from skin biopsies and PBMCs, which were prepared as described previously (31). The IMAGE cDNA clones were obtained from the Rosalind Franklin Centre for Genomics Research (Hinxton, Cambridge, UK). The pCMV-IRAK and plkBeaEGFP expression constructs and the pIL8-Luc promoter/reporter construct have all been described before (32–34). Recombinant human IL-1β was from R&D systems (Oxford, UK). Anti-p65 and anti-p50 antibodies were purchased from EMD Biosciences (Merck, Darmstadt, Germany), except for the RNA co-precipitation experiments, for which antibodies from Calbiochem (Merck) were used. The monoclonal anti-body to PABP and the anti-GST and anti-HA antibodies were from Sigma (St. Louis, MO, USA).

The anti-EGFP and anti-T7 antibodies were from Roche (Mannheim, Germany) and Novagen (Merck), respectively.

COS-7 and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% de-complemented foetal calf serum (FCS), penicillin G (100 ng/ml) and streptomycin (100 ng/ml) (all from Gibco, Paisley, UK). HEK 293-T cells were maintained in the same conditions except that high-glucose DMEM (Gibco, cat No 21969-035) was used.

NFkBIL1 antibodies

Three rabbit polyclonal antibodies to NFkBIL1 called Ab1, Ab2 and Ab3 were generated using KLH-conjugated synthetic peptides of sequences RWHPDRFLQRC, EDEWQEVMGR and RQKLQGDASH, respectively (Harlan Sera-Lab, Loughborough, UK). The peptide used to produce Ab1 is present in both NFkBIL1 variants, but the Ab2 and Ab3 antibodies correspond to sequence motifs specific of NFkBIL1-α and NFkBIL1-β, respectively. The specificity of each antiserum was confirmed by western blotting lysates of HEK 293 cells transiently transfected with plasmids encoding each splice variant of NFkBIL1 (data not shown). The antibodies were then purified by affinity chromatography using the immunizing peptide immobilized to a 4% beaded agarose support (AminoLink Plus Immobilization Kit, Pierce, Rockford, IL).

Synovial tissue samples

Samples for immunohistochemistry and confocal microscopy were obtained from knee joints of RA and OA patients recruited at St. Vincent’s University Hospital (Dublin, Ireland), and ethical permission was obtained from the Medical Research Ethics Committee in accordance with the Declaration of Helsinki principles. Briefly, arthroscopy and synovial biopsy was performed, under local anaesthesia and sterile conditions, using a Storz 2.7-mm needle arthroscope (Storz, Tuttingen, Germany) and a 2-mm grasping forceps as previously described (35). Biopsy samples were obtained from all compartments of the knee joint, embedded in Tissue-Tek OCT compound (Sakura Finetek, Zoeterwoude, The Netherlands), snap frozen and stored in liquid nitrogen until used.
Isolation of RA FLS

Fresh synovial biopsies were placed directly into RPMI (Invitrogen Ltd, Paisley, UK) and digested using 1 mg/ml collagenase type 1 (Worthington Biochemical, Freehold, NJ, USA) in RPMI for 4 h at 37 °C in humidified air with 5% CO2. Dissociated cells were grown to confluence (~10 days) in RPMI, 10% FCS (Invitrogen Ltd), penicillin (100 units/ml), streptomycin (100 units/ml) and fungizone (0.25 µg/ml) before trypsinization and passage. Cells were used for experiments between passages 5–8. In the stimulation experiments, 200 000 FLS were plated per well and left for 48 h to recover from trypsinization. Cells were serum deprived overnight prior to stimulation with 0.1 ng/ml TNF (R&D Systems) for the times indicated. Stimulation was terminated by removal of the media and lysis of cells in Trizol (Invitrogen Ltd) prior to RNA extraction.

Quantitative determination of NFkBIL1 splice variants expression by real time-PCR

Intron-spanning primers were used in order to generate different-sized PCR products from each transcript which were resolved by PAGE. All probes and primers were synthesized by MWG-Biotech (Edersberg, Germany). Oligonucleotide probes specific for NFkBIL1-α or NFkBIL-β were designed using the manufacturer’s software. Variant-specific probes were labelled with FAM (5-Carboxyfluorescein) with TAMRA as a quencher; the nucleotide sequences specific for NFkBIL1-α and NFkBIL1-β were 5’-AGTCACTGGGGAGGTTTGAAG-3’ and 5’-ATGGAGACAGAAGGCTC CAGGGTGAT-3’, respectively. RT–PCR data were normalized against GADPH as previously described (31).

Immunohistology

Cryostat sections (7 µM) were mounted on 3-aminopropyltriethoxysilane-coated glass slides, air dried overnight, wrapped in foil and stored at −80 °C until immunohistochemical analysis was performed. Sections were fixed in acetone for 10 min. Prior to staining, endogenous peroxidase was quenched by treatment with 3% H2O2 for 5 min, followed by pre-treatment with 3% normal serum for 20 min. A routine three-stage immunoperoxidase labelling technique was used. Sections were incubated for 1 h with primary antibodies against rabbit polyclonal anti-NFkBIL1 or with an irrelevant isotype-matched monoclonal antibody as a negative control. Colour was developed in solution containing diaminobenzidine (Sigma), 0.5% H2O2 in PBS buffer (pH 7.6). Slides were counterstained with haematoxylin and mounted.

Dual immunofluorescence staining

Synovial tissue sections were fixed in 4% paraformaldehyde and were incubated with mouse monoclonal antibodies against CD3, CD68 or Factor VIII alone or co-incubated with rabbit polyclonal anti-NFkBIL1 for 1 h. Sections were also incubated for 1 h with an irrelevant isotype-matched control. Following incubation, cells were washed three times in PBS and incubated with a goat anti-mouse secondary Cy2 antibody (1:200) (Jackson Laboratories, Cambridgeshire, UK) and a goat anti-rabbit Cy3 (1:500) (Jackson Laboratories). The sections were then washed and mounted with an anti-quenching agent, Citifluor (Citifluor Ltd, Leicester, UK). For immunofluorescence of cultured synoviocytes, the cells were plated on 8-well cell culture-treated glass chamber slides at a density of 5000 per well and allowed to adhere overnight. After washing twice with PBS, the cells were fixed in 4% paraformaldehyde for 20 min at room temperature. They were then washed with PBS an incubated with primary antibody against rabbit polyclonal NFkBIL1 (1:50) or an irrelevant isotype-matched control. Following incubation, the cells were washed three times in PBS and incubated with a goat anti-rabbit Cy3 (1:500) (Jackson Laboratories). They were then washed and mounted with Citifluor.

NFkBIL1 cDNA expression constructs

The NFkBIL1, IκBα and β-glucoronidase (Gus) expression constructs were generated by in vitro recombination using the Gateway system (Invitrogen Ltd). The IκBα, NFkBIL1-α and NFkBIL1-β entry clones were generated by amplifying the open reading frames from IMAGE clones 3161686, 544778 and 6158780, respectively (GenBank accession numbers BC002601, BM044744 and BQ425655). The forward and reverse primers used were 5’-CACCAGCCGCGG-3’ and 5’-CAGCGTCGACAGTTATGAGT GCA-3’ for IκBα, 5’-ACCCATAGTGAACCCTCCTCCCC CAG-3’ and 5’-CTAGGGTCAGGCGCCTCTG-3’ for NFkBIL1. The PCR products were ligated into the Gateway entry vector pENTR-D-TOPO (Invitrogen Ltd). The inserts of the entry constructs were fully sequenced to ensure that they did not encode mutant products. The Gus entry clone, pDNOR201-gus, was obtained commercially (Invitrogen Ltd). Mammalian expression constructs encoding GST-tagged and untagged products were then generated by in vitro recombination between the pENTR constructs and the pDEST27 or pcDNA3.2-DEST destination vectors, respectively.

Expression constructs encoding putative NFkBIL1 partners

The pCMV-relA and pCMV-p50 constructs, encoding untagged relA and p50 proteins, were generated by excising the CFP open-reading frame form pECFPrelA and pECFPp50 (33) with Bsr GI and Nhel. The recessed ends were then filled with Klenow polymerase and the plasmids were ligated. The T7-tagged constructs encoding other putative NFkBIL1 partners (CTPS, EF1α, LKP and cdc37) were then filled with Klenow polymerase and cloned into the pCMV-T7 vector. The primers used were 5’-CGC GAATTCATGAGTATCCACACTCCC-3’ and 5’-CCGGTC GACCTATAGCCGCCACCTCC-3’ for LKP, 5’-CCG GAATTCATGAGAAGAAAAAGACT-3’ and 5’-CCGGTC GACCTATAGCCGCCACCTCC-3’ for EF1α, 5’-CCG GAATTCATGAGAAGAAAAAGACT-3’ and 5’-CCGGTC GACCTATAGCCGCCACCTCC-3’ for CTPS. A. D. N. C. T. S. 1. K. E. P. A. G. 2. 3. 4. 5. 6.
GACATCAGTACTAAATAACTACCT-3' for CDC37 and 5'-CGC
GAAATCATGAAGTACATTCTGTTAC-3' and 5'-CGCGTC
GACATCAGTACTAAATAACTACCT-3' for CTPS. The
inserts of the entry constructs were fully sequenced to
ensure that they did not encode mutant products.

Immunoprecipitations and western blotting
For detection of associations between tagged constructs, HeLa
cells were seeded at 1.6 × 10^6 cells per 10 cm dish and trans-
fected the following day. At 24 h-post-transfection, cells were
lysed in 400 μl lysis buffer (50 mM HEPES, pH 7.9, 0.25%
Nonidet P-40, 150 mM NaCl, 5 mM DTT, 1 mM EDTA,
20 mM β-glycerophosphate, 10 mM sodium fluoride, 0.5 mM
sodium orthovanadate) supplemented with protease inhibitors
(Sigma). The cell lysates were then centrifuged (14 000g,
2 min) to remove cellular debris and pre-cleared by addition
of Sepharose 4B beads (Sigma) to remove proteins binding
non-specifically to the beads.

Immunoprecipitations were carried out by adding antibodies
to the lysates at concentrations recommended by the manufactu-

ers and the reactions were incubated for 2 h at 4°C. Protein
A or G sepharose was then added and incubations were carried
on for a further hour. The beads were then washed three times
in lysis buffer. For western blotting, the samples (cell lysates,
GST pull-downs or immunoprecipitates) were heated at 100°C
for 5 min in 40 μl SDS–PAGE sample buffer, then separated
on 10% SDS–PAGE gels and transferred to polyvinylidene
difluoride membranes. The membranes were probed with
appropriate antibodies, which were then visualized by incu-
bation with horse-radish peroxidase-conjugated antibodies
against rabbit or mouse immunoglobulin G (Cell Signalling
Technology Inc., Danver, MA, USA) followed by chemolumi-
nescent detection using the ECL detection reagents (GE
Healthcare, Little Chalfont, UK).

Promoter-reporter assays
Cells were seeded into 96-well tissue culture plates at
1.0 × 10^5/well 24 h before transfection, which was performed
using PolyFect reagent (Qiagen). Each well was transfected
with 500 ng of pIL8-Luc reporter construct, 100 ng of
pTK-rLuc control construct (Promega) and the cDNA
expression constructs under test. Stimulations by IL1-
p44/42 kinase (JNK) signalling was carried out as described before and the cleared lysates were incubated with
anti-NFkBIL1 antibodies for 2 h at 4°C. Secondary antibodies
were then added at concentrations recommended by the manu-
facturers and the reactions were incubated for a further 2 h at
4°C. Immunoprecipitates were collected with Protein G sepha-
rose and washed three times in lysis buffer. The immunopre-
cipitates were analysed by SDS–PAGE gels followed by
autoradiography.

Identification of proteins co-purifying with NFkBIL1
To identify the putative NFkBIL1 partners, NFkBIL1 comple-
exes were immunoprecipitated from unlabelled cells grown
in 175-cm2 flasks using anti-NFkBIL1 antibodies that had
been covalently conjugated to sepharose beads. The purified
proteins were then separated on SDS–PAGE gels and detected
with Coomasie blue staining. The stained bands were excised
from the gels and subjected to in-gel trypsin digestion.
Tryptic peptides were recovered and subjected to
LC-ESI-MS/MS as described by Sostaric et al. (36). Three
MS runs were performed for each sample. The MS spectra
were searched against the Mass Spectrometry Data Base in
a sequence query search using MASCOT 2.0 software
(www.matrixscience.com) (36). The taxonomy was limited
to only human matches. Tryptsin was used as the
cleavage enzyme, with one missed cleavage site allowed.
The peptide tolerance was set to 0.5 Da and the MS/MS
tolerance was set to 0.3 Da. Carbamidomethyl modification
of cysteine and oxidized methionine were set as variable
modifications.

Isolation of RNA associated with NFkBIL1
Endogenous NFkBIL1 and PABP were immunoprecipitated
from HeLa cells seeded at 1.6 × 10^6 cells/10-cm dish and
grown at 37°C for 16 h. Immunoprecipitations were carried
out as described for transfected cells. The immunoprecipi-
tates were then treated with proteinase K (30 min, 37°C).
The soluble phase was then recovered and subjected to
precipitation using phenol:chloroform:isoamyl alcohol (25:24:1)
and centrifugation at 17 000g for 30 min. After centrifugation,
the RNA pellet was washed in 70% ice-cold
ethanol and resuspended in TE buffer (10 mM Tris pH 7.2
and 1 mM EDTA) and subjected to ethanol precipitation
using 0.1 volume 3 M sodium acetate pH 5.2 and 2
volumes of ice-cold 100% ethanol. After a second centrifugation,
the pellet was dissolved in TE buffer and the recov-
ered mRNA was further purified by affinity to oligo-dT
beads (GenElute kit, Sigma).

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