A novel form of the RelA nuclear factor κ B subunit is induced by and forms a complex with the proto-oncogene c-Myc

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Members of both Myc and nuclear factor κ B (NF- κ B) families of transcription factors are found overexpressed or inappropriately activated in many forms of human cancer. Furthermore, NF- κ B can induce c-Myc gene expression, suggesting that the activities of these factors are functionally linked. We have discovered that both c-Myc and v-Myc can induce a previously undescribed, truncated form of the RelA(p65) NF- κ B subunit, RelA(p37). RelA(p37) encodes the N-terminal DNA binding and dimerization domain of RelA(p65) and would be expected to function as a *trans*-dominant negative inhibitor of NF- κ B.

Surprisingly, we found that RelA(p37) no longer binds to κ B elements. This result is explained, however, by the observation that RelA(p37), but not RelA(p65), forms a high-molecular-mass complex with c-Myc. These results demonstrate a previously unknown functional and physical interaction between RelA and c-Myc with many significant implications for our understanding of the role that both proteins play in the molecular events underlying tumourigenesis.

Key words: cancer, proteolysis, Ras, repression, transcription.

INTRODUCTION

The control of cellular proliferation and differentiation is a complex process requiring the co-ordination of growth factor-induced signalling pathways and the genes regulating both pro- and anti-apoptotic pathways. To a large extent, these pathways are controlled by the expression and interaction of families of DNA-binding transcription factors, such as nuclear factor κ B (NF- κ B) and c-Myc.

Members of the NF- κ B family of transcription factors are expressed in most cell types, where they can regulate diverse cellular processes such as inflammation, proliferation and apoptosis. NF- κ B itself can take a number of different forms and is composed of dimeric complexes formed from five distinct subunits, RelA(p65), RelB, c-Rel, NF- κ B1 (p105/p50) and NF- κ B2 (p100/p52) [1]. All these subunits contain a region of approx. 300 amino acids within their N-termini, termed the Rel Homology Domain (RHD), which mediates DNA binding together with dimerization and interactions with heterologous transcription factors. p105 and p100 share a high degree of homology and remain in the cytoplasm until proteolytic processing yields the DNA-binding isoforms p50 and p52. In contrast, RelA, RelB and c-Rel do not require proteolytic processing and contain transactivation domains within their C-termini. Distinct combinations of subunits determined by the specificity of transcriptional activation and gene knockouts in mice have revealed that all have distinct, but non-overlapping functions [2,3].

NF- κ B activity is rapidly induced by a wide range of stimuli, including ionizing radiation, cytokines such as tumour necrosis factor (TNF)- α and interleukin-1, growth factors, bacterial lipopolysaccharides, phorbol esters and hypoxia [4]. In most of the unstimulated cell types, NF- κ B is retained within the cytoplasm in an inactive form, bound to its inhibitor protein I κ B. Activators of NF- κ B initiate a kinase cascade which results in the phosphorylation, ubiquitination and subsequent degradation by the

proteasome of one of the members of the I κ B family [1]. This releases NF- κ B, allowing it to translocate to the nucleus where it can induce the expression of a diverse array of gene products, including cytokines, growth factors, adhesion molecules and immunoreceptors [4].

Consistent with its regulation of cytokines and immunoreceptors, aberrant activation of NF- κ B is often associated with inflammatory diseases such as rheumatoid arthritis and asthma [5]. An increasing body of data however, demonstrates that NF- κ B also has a critical role in tumourigenesis [1,6,7]. The molecular basis for this is due to both the anti-apoptotic and proliferative effects of NF- κ B. First, the proliferative drive resulting from oncogene overexpression in normal cells also activates a strong apoptotic signal [8], which prevents uncontrolled amplification of cell numbers. Malignant cancer cells therefore must evolve mechanisms that counteract this effect and, in many cases, NF- κ B provides the anti-apoptotic signal. Secondly, NF- κ B itself has the ability to induce cellular proliferation through the up-regulation of cyclin D1 [9]. Both aberrant activation of NF- κ B and loss of NF- κ B function has now been reported in a wide range of tumour types [6,7], thereby illustrating the critical role that this protein plays in oncogenesis.

c-Myc is a member of the basic/helix-loop-helix/leucine zipper family of transcription factors, has a molecular mass of 64–67 kDa and is found conserved in organisms ranging from *Xenopus* to humans [10,11]. The N-terminus of the protein contains two domains of approx. 20 residues, termed Myc boxes 1 and 2 (MB1 and MB2). The N-terminus is also the site of the transcriptional activation domain. The C-terminal region contains the basic, helix-loop-helix and leucine zipper domains, which mediate DNA binding and protein oligomerization. Disruption of either the N- or C-termini abrogates the activity of c-Myc *in vivo* [10]. To function as a transcription factor, c-Myc must form heterodimers with a second, smaller protein termed Max [12]. c-Myc and Max share extensive homology throughout their DNA-

Abbreviations used: DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; MB, Myc box; NF- κ B, nuclear factor κ B; RHD, Rel homology domain; TNF, tumour necrosis factor; Tos-Lys-CH₂Cl, tosyl-lysylchloromethane; Z-VAD-FMK, benzyloxycarbonyl-valylalanyl-DL-aspartylfluoromethane.

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binding and dimerization domains. c-Myc plays a crucial role in a number of intracellular processes, including apoptosis, cell-cycle regulation, proliferation and differentiation [10,11,13–15], although the identity of c-Myc-regulated target genes has proved elusive [15–17]. Consistent with these functions, deregulation of c-Myc expression has been associated with approximately one-third of human malignancies including those of the breast, bowel and various lymphomas [18,19]. Oncogenic activation of c-Myc is typically associated with high levels of overexpression and can occur through a number of pathways, including chromosomal translocations, proviral insertion, retroviral transduction and gene amplification [18].

NF- κ B can also provide a direct stimulus towards proliferation via the activation of proto-oncogenes such as c-Myc and cyclin D1 [4,9,20]. c-Myc has also been shown to repress NF- κ B transactivation and induce sensitivity to TNF-induced apoptosis [21]. The functioning of NF- κ B and c-Myc is therefore intimately linked, although the extent of this relationship is yet to be fully understood. In the present study, we have investigated the functional relationship between the proto-oncogene c-Myc and the RelA(p65) NF- κ B subunit. Surprisingly, we have found that c-Myc can stimulate the truncation of RelA(p65) to a novel form RelA(p37). RelA(p37) is distinct from the degraded species of RelA, described previously, following apoptosis and activation of caspases. Critically, RelA(p37) resides in a high-molecular-mass complex which contains c-Myc. As such, RelA(p37) is transcriptionally inert and we propose that this unique mechanism could, in certain tumours, allow deregulated c-Myc to modify NF- κ B function and facilitate tumourigenesis.

MATERIALS AND METHODS

Cell culture, transient transfections and protein extracts

Calcium phosphate transient transfection of human 293 embryonic kidney cells has been reported previously [22]. All transfections contained appropriate levels of pRSV or pcDNA3 control plasmids such that each dish of cells received the same quantity of DNA. Nuclear extracts were prepared essentially as described previously [23]. All solutions used with protein extracts contained 0.5 mM PMSF, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, 1 μ g/ml pepstatin A, 1 μ g/ml *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) and 1 mM dithiothreitol (DTT). For whole-cell lysis analysis, the appropriate cells were washed twice in ice-cold PBS and then lysed directly into 200 μ l of SDS loading buffer and boiled for 10 min. This extract (2.5 μ l) was then mixed with a further 10 μ l of loading buffer, and then analysed by SDS/PAGE (10% gels).

Protease inhibitors added to cells in culture were benzyl-oxycarbonyl-valylalanyl-DL-aspartylfluoromethane (Z-VAD-FMK), tosyl-lysylchloromethane (Tos-Lys-CH₂Cl, 'TLCK') and lactacystin purchased from Biomol and Affiniti Research Products. These inhibitors were used for 24 h at the following final concentrations: 100 μ M of ZVAD-FMK, 100 μ M of Tos-Lys-CH₂Cl and 10 μ M of lactacystin.

Plasmids

The RSV RelA(p65) and p50 expression plasmids have been described previously [24]. RelA mutations and truncations used in the present study were generated by PCR using Pfu Turbo (Stratagene, Cambridge, U.K.) or Pwo polymerase (Roche) and were subcloned into the same RSV-expression plasmid backbone. Gal4 RelA was constructed by subcloning a fragment of RelA

corresponding to amino acids 304–551, generated by PCR with Pfu Turbo, into pcDNA3 Gal4, a Gal4 expression plasmid derived from pSG 424 [25]. pcDNA3 c-Myc and avian v-Myc were constructed by subcloning a 1.6 kb *Bam*HI fragment from spt19-cMyc and spt19-vMyc [26], in which the 5'-untranslated regions had been deleted. RSV human c-Myc was constructed by subcloning the insert from pcDNA3 c-Myc into an RSV expression plasmid [27]. RSV c-Myc (amino acids 1–350) and RSV c-Myc (amino acids 301–439) were constructed by PCR from the human c-Myc cDNA. pcDNA3 Max was created by subcloning the Max cDNA from pSPT-Max [26]. pcDNA3 feline c-Myc and T17 vMyc were derived by subcloning a 1.4 kb *Hind*III/*Eco*RI fragment from either SFCV-cMyc or SFCV-T17 vMyc, which originated in Professor Jim Neil's laboratory (Molecular Oncology Laboratory, Department of Veterinary Pathology, University of Glasgow Veterinary School, Glasgow, U.K.) and have been described previously [28,29]. The pEF-constitutively active H-Ras and pEF-dominant negative H-Ras were the gifts of Dr R. Marais (Institute of Cancer Research, London, U.K.).

Electrophoretic mobility-shift assay (EMSA)

EMSAs were performed using 5 μ g (except where stated otherwise) of crude nuclear extract in a 20 μ l reaction volume consisting of 20 mM Hepes (pH 7.9), 100 ng of poly(dI/dC · dI/dC) and approx. 0.1 ng of the appropriate ³²P-labelled double-stranded oligonucleotide. Samples were incubated at room temperature for 15 min and then DNA–protein complexes were resolved using 1 × Tris/glycine/EDTA [25 mM Tris (pH 8.0)/190 mM glycine/1 mM EDTA] and 4% non-denaturing polyacrylamide gels.

Gel filtration

All chromatographic procedures were performed at 4 °C. First, 200 μ g of nuclear extract 293 was concentrated by PEG-6000 precipitation. Precipitated proteins were harvested by centrifugation at 18 300 *g* for 30 min at 4 °C. The supernatant was discarded and the residual PEG was removed. The protein pellet was then resuspended in an appropriate volume of buffer D containing 20 mM Hepes (pH 7.9), 50 mM KCl, 1.5 mM MgCl₂, 83 mM NaCl, 0.1% (v/v) Triton X-100, 1 mM DTT, 0.5 mM PMSF, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin and 1 μ g/ml E-64 and the insoluble material was removed by centrifugation as above. The supernatant (50 μ l) was then applied to a 2.4 ml Superose-6 PC gel-filtration column (Amersham-Pharmacia Biotech) equilibrated in buffer D. The column was run at a flow rate of 25 μ l/min, samples were collected as 50 μ l volumes and then 25 μ l of the sample was examined for the presence of c-Myc–RelA complexes by SDS/PAGE and Western-blot analysis. The column was calibrated with 1 mg/ml of high-molecular-mass markers (Amersham-Pharmacia Biotech), including Blue Dextran (2 MDa), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa) aldolase (158 kDa) and BSA (67 kDa).

Co-immunoprecipitation and Western-blot analysis

Further analysis of the interaction between c-Myc and RelA was performed by co-immunoprecipitating RelA and c-Myc using anti-c-Myc rabbit polyclonal antiserum (06–340; Upstate Biotechnology). Briefly, 50–200 μ g of nuclear extract 293 was diluted 5 times in an incubation buffer containing 20 mM Hepes (pH 7.9), 75 mM KCl, 2.5 mM MgCl₂, 1 mM DTT, 0.1% (v/v) Nonidet P40, 0.5 mM PMSF, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin and 1 μ g/ml E-64 and then 1 μ g of either anti-c-Myc or control IgG

was added to each sample. Samples were then incubated at 4 °C for 90 min with constant stirring. After this time, 5–10 μ l of bed volume of protein-G Sepharose 4B Fast Flow was added and the samples were incubated as before. Finally, the beads were washed in 3 $\times$ 500 μ l volumes of incubation buffer and then resuspended in 10 μ l of SDS loading buffer. Samples were then resolved by SDS/PAGE (10 % gels).

Following SDS/PAGE, the resolved proteins were electroblotted on to nitrocellulose (Sigma). The gel was first briefly soaked in toubin transfer buffer [TTB; 25 mM Tris, 192 mM glycine and 20 % (v/v) methanol] and then blotted for 40 min at 18 V (90 mA; constant current). The membrane was washed briefly in Tris-buffered saline (TBS; 20 mM Tris and 500 mM NaCl) and then blocked in a 10 % blocking buffer [TBS, including 0.05 % (v/v) Tween-20 and 10 % (w/v) of low-fat dried milk powder] for two sessions spanning 30 min each. The membrane was then washed in TTBS (blocking buffer without milk powder) with two 15 min washes.

RelA and c-Myc immunoblots were performed with antibodies that recognize the N-terminus of RelA(p65) (amino acids 3–19, sc-109, Santa Cruz; amino acids 136–224, N67620, Transduction Laboratories), the C-terminus of RelA (sc-372; Santa Cruz), the C-terminus of κ B α (PC142; Oncogene Research Products) or with the anti-c-Myc 9E10 monoclonal antibody (Sigma). Other antisera used were Egr-1, Wilms' tumour suppressor protein WT1 (-KTS isoform) (sc-110x and sc-846x respectively; Santa Cruz) and the DO-1 anti-p53 monoclonal antibody. All incubations were performed overnight in TTBS. After incubation with an appropriate secondary antiserum, the membrane was developed according to the ECL® detection method described by the manufacturer (Amersham-Pharmacia Biotech).

RESULTS

c-Myc induces proteolytic cleavage of RelA

It has been documented that both c- and v-Myc can repress transcriptional activation by NF- κ B [21,30]. In keeping with this finding, we have also observed that both c- and v-Myc can repress transcriptional activation by RelA (results not shown). Surprisingly, when performing experiments in 293 cells, to determine whether this repression resulted from changes in protein level, we observed that a novel truncated form of the protein, RelA(p37), accumulated with increasing levels of c-Myc (Figure 1A). The same result was also observed when RelA and c-Myc were expressed in COS cells (results not shown). No corresponding changes in c-Myc were observed (Figure 1B), and no truncated form of RelA was seen with an antibody recognizing its C-terminus (Figure 1C).

Constitutively, active NF- κ B and c-Myc have been described in a number of tumours and lymphomas [18,31–34]. Furthermore, cell lines derived from such tissues have also been shown to possess constitutively active forms of these transcription factors. One particular cell line is the Raji B-cell lymphoma line. Since this line has been documented to have constitutive expression of NF- κ B and c-Myc (although not necessarily overexpressed levels) [18,31–33], we sought to determine whether RelA(p37) was generated in such cell lines. When a Raji nuclear extract was blotted for RelA(p37), using two different antisera that recognize different epitopes within the N-terminus of RelA (sc-109, Santa Cruz; N67620, Transduction Laboratories), a 37 kDa band was again detected with both antibodies, although in the latter case additional intermediate bands were also seen (Figure 1D). The specificity of this interaction was confirmed when it was shown that a competing RelA peptide, but not a control p300-

derived peptide, could abolish the cross-reactivity between the Santa Cruz RelA antiserum and both Raji-derived and c-Myc-induced RelA(p37) (Figure 1E; results not shown). Interestingly, a second constitutively active oncogene H-Ras was seen to induce p37 formation, although to a lesser extent than c-Myc, whereas a dominant-negative mutant of this H-Ras had no effect (Figure 1F). Furthermore, the tumour-suppressor protein p53 did not induce such degradation (Figure 1G). Since the effect of Ras on RelA was less pronounced than c-Myc, the remainder of the present study examined the effects of c-Myc on the function of RelA(p37).

c-Myc overexpression specifically induces truncation of RelA

We next investigated whether c-Myc overexpression would induce truncation of other transcription factors. To address this issue, we prepared whole-cell lysates from 293 cells co-transfected with c-Myc, and p53, Egr-1 or WT1 (-KTS isoform) in addition to RelA. Western-blot analysis of these extracts demonstrated that only c-Myc induced truncation of RelA. No effects were observed on the other transcription factors, suggesting that RelA(p37) formation was a specific function of c-Myc (Figure 2). It cannot be ruled out, however, that truncated forms of these proteins might be observed with antibodies recognizing different epitopes.

Full-length c-Myc is required for RelA(p37) formation

To characterize this effect further, c-Myc expression plasmids were created encoding either their N- or C-termini. Both truncated proteins were expressed at equivalent levels to full-length c-Myc (results not shown). Both fragments of c-Myc were severely compromised in their ability to induce RelA(p37) (Figure 3A), suggesting that the formation of the truncated fragment is a property solely of the intact c-Myc protein. This is identical with the results of previous studies [19], demonstrating that disruption to either the N- or C-terminal domains cause deactivation of c-Myc. Consistent with this observation, Max, which is similar only to the c-Myc C-terminus, also failed to induce RelA(p37) (Figure 3B). Avian v-Myc, feline c-Myc and feline T17-vMyc were also capable of inducing RelA(p37), demonstrating that this is a conserved function within these proteins (Figure 3C).

RelA(p37) generation is not prevented by the NF- κ B p50 subunit

We have used RelA homodimers to examine the effect of c-Myc-induced NF- κ B transcriptional repression. Whereas such homodimers exist in nature and are active on promoters such as those controlling expression of GM-CSF and interleukins-2,-3 and -8 [35], the paradigm for NF- κ B, however, is a heterodimer of the RelA and p50 subunits. It was therefore important to investigate whether RelA, within such heterodimeric complexes, was resistant to c-Myc-induced degradation. When 293-cell nuclear extracts were prepared from cells expressing RelA, p50 and c-Myc and then subjected to Western-blot analysis it was clear that RelA within an RelA–p50 complex was still susceptible to c-Myc-induced degradation (Figure 4A). To ensure that RelA–p50 complexes were formed, EMSA was performed. EMSA confirmed that under these conditions, RelA existed predominantly as a heterodimer with p50 (Figure 4B). No effect on p50 itself, or other Rel family members such as RelB and c-Rel, was observed in the presence of c-Myc (results not shown).

Figure 1 Expression of c-Myc and H-Ras but not p53 results in the truncation of RelA(p65) to RelA(p37)

(A) Expression of c-Myc results in the truncation of RelA(p65) to a smaller 37 kDa protein. The 293 cells were transfected with 5 μ g of RSV RelA(p65) and 2, 5 and 10 μ g of pcDNA3 human c-Myc as indicated. After 48 h, nuclear protein extracts were prepared, resolved by SDS/PAGE and immunoblotted for RelA. (B) c-Myc is unaffected by co-expression of RelA. The experiment was performed essentially as in (A) except that 10 μ g of RSV human c-Myc was utilized. Protein extracts were immunoblotted for RelA or c-Myc as indicated. (C) A C-terminal-containing fragment of RelA is not detected upon co-expression of c-Myc. The experiment was performed essentially as in (A) except that immunoblotting was performed with an antibody recognizing the RelA C-terminus. With this antibody, endogenous RelA can be detected. (D) A protein similar to RelA(p37) can be detected in Raji Burkitt's lymphoma cell extracts. The experiment was performed essentially as in (A) except that the cells were lysed directly in SDS/PAGE loading buffer before immunoblotting. Extracts were blotted as indicated either with rabbit polyclonal antibody (sc-109; Santa Cruz), which recognizes amino acids 3–19 of RelA, or mouse monoclonal antibody (67620; Transduction Laboratories), which recognizes an epitope between amino acids 136 and 224 of

Figure 2 Expression of c-Myc specifically induces truncation of RelA

The 293 cells were transfected with 10 μ g of pcDNA3 human c-Myc and either 5 μ g of RSV RelA(p65), 10 μ g of RSV human p53, 10 μ g of pcDNA human Egr-1 or 10 μ g of pcDNA human WT1 (-KTS isoform) as indicated. After 48 h, the whole-cell lysates were prepared and extracts were resolved by SDS/PAGE and immunoblotted either for RelA, p53, Egr-1 or WT1.

Caspases do not generate RelA(p37)

c-Myc expression can result in the induction of apoptosis, which leads to the activation of caspases [10]. These proteases have previously been shown to have the ability to degrade RelA [36,37]. We observed no indication of cell death in the c-Myc-transfected 293 cells used in our study, possibly due to the presence of adenoviral oncogenes in the 293 cells [38], which inhibit the function of p53.

The putative caspase sites within RelA do not coincide with the region of RelA containing the p37 truncation [36,37]. Nonetheless, it remained possible that proteolytic degradation by caspases could provide an explanation for RelA(p37) formation. We found that incubation of 293 cells co-transfected with RelA and c-Myc, in the presence of the broad-range caspase inhibitor Z-VAD-FMK, however, had no effect on RelA(p37) formation (Figure 5A). Furthermore, although proteolysis is the most likely explanation for the generation of RelA(p37), we have not observed any effect upon treating cells with other protease inhibitors such as tosylphenylalanylchloromethane ('TPCK'), Tos-Lys-CH₂Cl, pepstatin and calpeptin (results not shown).

Surprisingly, treatment with lactacystin, a specific inhibitor of the proteasome, which has previously been shown to inhibit processing of p105 to p50 [39], resulted in the induction of RelA(p37) and enhanced the level of c-Myc-induced proteolysis (Figure 5B). Similar effects were also seen with other selective proteasome inhibitors such as ALLN and MG132 (results not shown).

Mapping the site of c-Myc-induced degradation of RelA

We next determined the domains within RelA required for the generation of RelA(p37). Truncation of RelA at amino acid 323 produced a protein with a slightly faster mobility than RelA(p37) (Figure 6A). Interestingly, another truncated protein RelA(Δ 368)

was still processed to RelA(p37), indicating that the C-terminal transactivation domain of RelA(p65) is not required for this effect (Figure 6A). A Gal4 fusion with the C-terminus of RelA, which includes the site of the RelA(p37) truncation, is no longer processed, however, suggesting that the N-terminal RHD is required (Figure 6B). Since one of the C-terminal domain mutants RelA- Δ 323 was seen to migrate with a molecular mass slightly less than that of RelA(p37) (Figure 6A), it suggested that the scissile-peptide bond was located in a proximal proline-rich sequence that contained three arginine residues (³²¹P-T-D-P-R-P-P-P-R-R-I³³¹; Figure 6C). To assess the contribution of this motif, full-length RelA mutants were generated in which these arginine residues were mutated to alanine. Significantly, either single (Arg³²⁵→Ala, R325A)- or double (R329A/R330A)-point mutations completely abolished the production of RelA(p37) (Figure 6D). Both mutants bound DNA and stimulated transactivation of an endogenous gene (I κ B α) to exactly the same level as wild-type RelA (Figure 6E). Their ability to transactivate was still reduced by c-Myc (results not shown), indicating that other mechanisms of inhibition, such as competition for co-activators, may also occur.

Truncated RelA fails to bind canonical κ B sites in DNA

The inhibition of NF- κ B transcriptional activity through dominant-negative effects of the RHD has been well documented [40]. In such cases, wild-type RelA undergoes proteolytic degradation to generate an RHD which is then free to bind the κ B elements. Since this truncated fragment lacks an activation domain, transcription is effectively inhibited. Thus since RelA-(p37) contains the N-terminal RHD of RelA it would also be expected to bind κ B elements and potentially function as a competitive inhibitor of NF- κ B function. To test this hypothesis, EMSA was performed on nuclear protein extracts. As expected, RelA- Δ 323 bound the consensus HIV-1 κ B element with high affinity (Figure 7, upper panel). Surprisingly, no equivalent complex was observed from an extract containing the same level of RelA(p37), although the RelA(p65) complex was clearly present. Furthermore, no complex was seen that might correspond to an RelA(p65)-RelA(p37) heterodimer. All protein levels were seen to be equivalent (Figure 7, lower panel).

RelA(p37) is associated with a high-molecular-mass complex

The failure of RelA(p37) to bind canonical κ B sites suggested that it might be interacting with other proteins that were either inhibiting its ability to bind κ B elements or were modifying its DNA-binding specificity. To test this hypothesis, nuclear protein extracts were fractionated by gel-filtration chromatography. Significantly, the majority of RelA(p37) eluted in a complex with a much higher apparent molecular mass than RelA(p65) (Figure 8A, panel [i]). This complex is not an intrinsic property of a RelA protein of this size since RelA- Δ 323 did not form this complex and eluted with a lower apparent molecular mass than RelA(p65) (Figure 8A, panels [ii] and [iv]). Interestingly, co-expression of c-Myc with RelA- Δ 323 did not result in the formation of a high-molecular-mass complex, suggesting that the properties of RelA(p37) on this column arise from the

RelA. (E) Both the RelA(p65) and RelA(p37) bands detected in Raji cells are specific for the Santa Cruz sc-109 antibody. The experiment was performed essentially as in (D) except that upon antibody incubation, parallel samples were either left untreated, incubated with 1 mg of RelA peptide (amino acids 3–19) or 1 mg of control p300 peptide. (F) Oncogenic H-Ras also induces RelA(p37) formation. The 293 cells were transfected with the indicated RSV RelA, pcDNA3 c-Myc, pEF-dominant-negative H-Ras (DN-H-Ras) or pEF-constitutively active H-Ras (CA-H-Ras) expression plasmids. Protein extracts were resolved by SDS/PAGE and immunoblotted for RelA. (G) p53 does not induce RelA(p37). The 293 cells were transfected with the indicated combinations of expression plasmids and analysed as in (A).

Figure 3 Formation of RelA(p37) is specific for full-length Myc

(A) Truncation of RelA(p65) to RelA(p37) requires full-length c-Myc. The 293 cells were transfected with 5 μ g of RSV RelA and 10 μ g of RSV c-Myc expression plasmids as indicated. The C-terminal fragment of Myc contains the entire bHLHZip DNA-binding and dimerization domain (amino acids 301–439), whereas the N-terminal fragment contains the conserved MBs and transcriptional activation domains (amino acids 1–350). Protein extracts were resolved by SDS/PAGE and immunoblotted for RelA. (B) Max does not induce RelA(p37) and inhibits the ability of c-Myc to do so. The 293 cells were transfected with RSV RelA and pcDNA3 c-Myc expression plasmids. pcDNA3 Max expression plasmid (10 μ g) was also included as indicated. Co-expression of Max did not significantly alter the expression level of c-Myc (results not shown). (C) Avian v-Myc, feline c-Myc and T17-vMyc also induce RelA(p37). The 293 cells were transfected with the indicated RSV RelA and pcDNA3 Myc-expression plasmids.

process of its truncation from RelA(p65) (Figure 8A, panel [iii]). When c-Myc was analysed, it was also found to elute in the high-molecular-mass fractions, irrespective of whether RelA was present (Figure 8A, panels [v] and [vi]). This would be in keeping with the fact that exogenous c-Myc forms amorphous nuclear bodies when expressed in cultured cells [41]. Furthermore, this observation suggested that the fractionation properties of RelA(p37) might result from its recruitment to a high-molecular-mass complex containing c-Myc. Since the resolution properties of this column did not allow us to conclude this directly, co-immunoprecipitation studies were performed on non-fractionated nuclear extracts. Interestingly, RelA(p37), but not RelA(p65), co-precipitated with c-Myc (Figure 8B). No RelA(p37) bound to the appropriate IgG control (results not shown).

DISCUSSION

Generation of RelA(p37)

There is a growing body of data illustrating that transcriptional repression of NF- κ B can, under certain physiological circumstances, be mediated by c-Myc [21,30]. We have also observed this effect. In an attempt to delineate the mechanism of this repression, we examined the protein levels from 293 extracts expressing RelA and c-Myc. Surprisingly, co-expression of c-Myc, and to a lesser extent H-Ras, caused the generation of a truncated form of RelA with a molecular mass of approx. 37 kDa, which we have termed RelA(p37) (Figures 1A and 1F).

Truncation of RelA has been documented to occur during the process of extract preparation [42]. To address this possibility, cells were lysed directly in SDS/PAGE loading buffer: the generation of RelA(p37) was still observed, indicating that this effect occurs within the cell and not during the process of extract preparation (results not shown). RelA(p37) generation could also be an artifact of the overexpression system utilized in the present study. To address this concern, co-expression of c-Myc with the transcription factor Egr-1 and the tumour suppressor proteins p53 and WT1 (-KTS) was performed. No truncation of these proteins was observed in such experiments (Figure 2). Furthermore, co-expression of p53 with RelA did not induce p37 either (Figure 1G). Finally, co-expression of c-Myc with other NF- κ B subunits RelB, c-Rel and p50 did not induce any form of truncation (results not shown). Taken together, this would suggest that the generation of RelA(p37) is a specific function of c-Myc. That H-Ras also harbours this function suggests that it is a mechanism employed by specific oncogenes to circumvent the anti-apoptotic pathway in early tumour cells.

We could not detect RelA(p37) when the c-Myc-transfected 293 cells were stimulated with TNF- α (results not shown). At present, we believe that this is because the level of NF- κ B generated must still be below the threshold of detection of both the antisera used. In an attempt to identify RelA(p37) generation without using overexpression studies, nuclear extracts from the Raji B-cell lymphoma line was examined and seen to contain RelA(p37), as judged by Western-blot analysis (Figure 1D). Interestingly, the oestrogen receptor-negative breast tumour cell line, MDA MB231, has been shown to possess a 37 kDa protein that is immunologically related to RelA [32].

Generation of p37 is associated with full-length c-Myc and can be totally abrogated by co-expression of Max with c-Myc (Figures 3A and 3B). The ability of Max to block the generation of RelA(p37) strongly suggests that c-Myc plays a central role in this process. What is less clear is the role played by Ras. RelA(p37) can be induced by a constitutively active form of

Figure 4 Formation of RelA(p37) still occurs when RelA is dimerized with p50

(A) The 293 cells were transfected with 5 μ g of the RSV RelA, RSV p50 and 10 μ g of pcDNA3 human c-Myc-expression plasmids as indicated. Protein extracts were resolved by SDS/PAGE and immunoblotted for RelA. (B) The nuclear extracts from lanes 1–4 of (A) were subjected to EMSA analysis using an Ig/HIV κ B probe.

Figure 5 Effect of protease inhibitors on the induction of RelA(p37)

(A) The induction of RelA(p37) by c-Myc is not blocked by the caspase inhibitor Z-VAD-FMK. The 293 cells were transfected with the indicated RSV RelA and pcDNA3 Myc-expression plasmids. After transfection for 24 h, 100 μ M of Z-VAD-FMK was added as indicated. Also at 24 h, control RelA and c-Myc-transfected cells were harvested to demonstrate that most of RelA(p65) and RelA(p37) protein is synthesized between 24 and 48 h. Protein extracts were resolved by SDS/PAGE and immunoblotted for RelA. (B) RelA(p37) is induced by lactacystin but not Tos-Lys-CH₂Cl. The 293 cells were transfected with the indicated RSV RelA and pcDNA3 Myc-expression plasmids. After transfection for 24 h, cells were treated with 100 μ M Tos-Lys-CH₂Cl or 10 μ M lactacystin as indicated and harvested 24 h later.

H-Ras, although to a lesser degree than c-Myc. At present, we believe that, in this context, Ras can activate a mutual downstream signalling pathway that is stimulated by expression of exogenous c-Myc. Data detailing c-Myc target genes are incomplete and often contradictory [16,17,43], thereby complicating studies which aim at identifying specific c-Myc-regulated gene targets that may be involved in RelA(p37) biogenesis.

Induction of RelA(p37) is a conserved function of Myc proteins (Figure 3C). Furthermore, we observed that T17-vMyc, an oncogenic form of c-Myc found in a variant strain of feline leukaemia virus [29,44], also induced RelA(p37) (Figure 3C). T17-vMyc contains a large deletion affecting amino acid chain 49–124 together with a 3 bp insertion within the basic DNA-

binding domain [29]. Although T17-vMyc is transforming *in vivo* and retains sequence-specific DNA binding, it has previously been found to lack other functions found in Myc proteins, such as an ability to transform chick embryo fibroblasts and induce apoptosis in cell culture [28,29]. That T17-vMyc shares the ability to induce RelA(p37) with other Myc proteins suggests that this is a significant function, which may well contribute towards tumourigenicity *in vivo*.

Is RelA(p37) generation a result of proteolysis?

We have been unable to characterize fully the exact mechanism by which RelA(p37) is generated. We, at present, believe that

RelA(p37) arises through proteolytic degradation of RelA. A number of lines of evidence suggest that this is a regulated enzymic process within the cell. First, the proteosome inhibitor

lactacystin can induce RelA(p37) formation in the absence of c-Myc, suggesting that the proteosome negatively regulates the activity that induces p37 (Figure 5B). c-Myc is itself negatively

Figure 6 Mapping the site of the c-Myc-induced truncation of RelA

(A) A C-terminal truncated form of RelA is still processed to RelA(p37). The 293 cells were transfected with the indicated RSV RelA and pcDNA3 human c-Myc-expression plasmids. Protein extracts were resolved by SDS/PAGE and immunoblotted for RelA. (B) Formation of RelA(p37) requires the N-terminal RHD of RelA. The 293 cells were transfected with pcDNA3-expression plasmids encoding Gal4, Gal4 RelA (amino acids 304–551) and c-Myc as indicated. Immunoblot analysis was performed with an anti-Gal4 antibody. (C) Schematic diagram indicating the structure of RelA, the site of the $\Delta 323$ truncation and the position of the point mutations that prevent formation of RelA(p37) from RelA(p65). (D) Mutation of amino acids 325 or 329/330 of RelA inhibits c-Myc-induced formation of RelA(p37). The 293 cells were transfected with the indicated RSV RelA and pcDNA3 Myc-expression plasmids. The mobility of RelA($\Delta 323$) relative to RelA(p37) is also shown. (E) RelA transactivation is not abolished by mutation of amino acids 325 or 329/330, but is still repressed by c-Myc. The 293 cells were transfected with the indicated RSV RelA and pcDNA3 Myc-expression plasmids. Protein extracts were resolved by SDS/PAGE and immunoblotted for I κ B α .

Figure 7 RelA(p37) does not bind canonical κ B-site DNA

The 293 cells were transfected with 5 μ g of RSV RelA(p65) or RSV RelA- Δ 323 and 10 μ g of pcDNA3 human c-Myc and nuclear extracts were prepared 48 h later. EMSAs were then performed as detailed under the Materials and methods section. No complex that corresponded to RelA(p37) was seen (upper panel). The same nuclear extract samples were then probed for RelA(p37) (lower panel).

regulated by the proteasome [45,46]. However, no induction of endogenous c-Myc was observed upon lactacystin treatment in these experiments (results not shown). A range of other enzymic inhibitors was used without notable effect. Interestingly, a recent study using the serial analysis of gene expression ('SAGE') technique identified a number of proteases as being either induced or repressed by c-Myc [47]. Interestingly, the proteasome subunit β type 4 was seen to be repressed greater than 6-fold in this analysis, hinting at a possible link between the effects observed here upon c-Myc overexpression and treatment with lactacystin (Figure 5). Additional support for this being a regulated process comes from the observation that an intact RHD is an obligatory requirement for RelA(p37) formation (Figure 6B). If the C-terminal domain of RelA, including the putative cleavage site, is fused with the Gal4 DNA-binding domain then degradation is also abolished. This suggests the need for a protein-protein interaction in this event. Finally, full-length mutants of RelA, where arginine residues are replaced by alanine residues, are not cleaved in the presence of c-Myc (Figure 6D). Such perturbations of the primary structure imply that an enzymic substrate has been disrupted.

Data obtained from pulse-chase experiments in 293 cells overexpressing RelA and c-Myc were inconclusive (results not shown). We believe this is due to the fact that RelA(p37) is in a large protein complex, and as such, the relevant epitopes recognized by the RelA antiserum would be masked. Furthermore, whereas c-Myc antiserum can precipitate RelA(p37), this inter-

action is thought to be indirect since no c-Myc:RelA interaction was observed in glutathione S-transferase-pull down assays (results not shown).

Other possibilities to account for the generation of RelA(p37) include RNA editing, which is a biochemical process, where bases in the mRNA are chemically modified. Most commonly, C-U transitions occur (see [48] for a review) and such modifications can result in the formation of a new stop codon, thereby prematurely terminating the message. RelA contains codon CGA at amino acid position 329 (arginine). This codon falls within the putative cleavage site of RelA that generates p37. Furthermore, deamination of the C to U would give the stop codon UGA. We addressed this possibility by isolating RNA from 293 cells overexpressing RelA and c-Myc. The RNA isolated was reverse-transcribed and then ultimately sequenced. No modification of this codon was observed at all, which suggested that RNA editing is not the mechanism by which RelA(p37) arises (results not shown).

Modification of RelA DNA-binding specificity

Previous observations have illustrated the fact that the RHD of NF- κ B alone can act as a dominant-negative inhibitor of transcription from κ B-responsive genes [36,37]. Therefore we were intrigued when RelA(p37) failed to bind a HIV-1 κ B element in EMSAs (Figure 7, upper panel). This truncated fragment would be expected to interact with this DNA sequence to a similar degree as the full-length RelA and the RelA- Δ 323 truncation. The reasons for this apparent anomaly are presently unclear but we believe that it reflects the fact that RelA(p37) is localized in a protein complex, whereas RelA- Δ 323 is not (Figure 8A, panels [i]–[iii]). This suggests that while residing in this complex, the DNA-binding ability of RelA(p37) is modified. An analogy for this phenomenon is that of the complex between RelA and its inhibitor I κ B α . I κ B α induces a gross morphological change in the tertiary structure of RelA which completely abrogates RelA–DNA binding [49]. Such an allosteric change, induced by cooperation between other transcription factors and co-activators/repressors, could be envisaged for RelA(p37), whilst it resides in the high-molecular-mass complex.

Functional significance of RelA(p37) formation

At present, the functional significance of RelA(p37) biogenesis is yet to be defined. While we are cautious in not ascribing a definite role to RelA(p37), one possible implication that arises from our study is the (mis)function of RelA in tumourigenesis. The generation of RelA(p37) suggests that certain oncogenes and viral gene products (c-Myc, v-Myc, T17-vMyc and H-Ras) possess the ability to initiate a pathway that results in the degradation of RelA to RelA(p37). As such, we speculate that RelA(p37) formation and its recruitment into a high-molecular-mass complex may reflect a pathophysiological event in a given cell as it evolves towards a neoplastic phenotype.

Interestingly, our results would be consistent with studies which have highlighted that the inappropriate oligomerization of transcription factors, such as RAR and AML1, occurs in certain kinds of leukaemia [50]. Therefore our data would provide a further example whereby inappropriate or modified oligomerization of transcription factors could increase the cellular propensity towards oncogenic transformation. Furthermore, exogenous c-Myc has been documented to form large amorphous globules when expressed in CV-1 cells [41]. In the present study, c-Myc

Figure 8 RelA(p37) is associated with a high-molecular-mass complex

The 293 cells were transfected with 5 μ g of RSV RelA(p65) or the appropriate truncation mutants of RelA(p65) with or without 10 μ g of pcDNA3 human c-Myc and nuclear extracts were prepared 48 h later. **(A)** Gel filtration was performed as detailed in the Materials and methods section. RelA(p37) is seen to co-elute with c-Myc in the high-molecular-mass fractions (panel [i]). RelA- Δ 323 truncation does not reside in the high-molecular-mass fractions even in the presence of c-Myc (panels [ii] and [iii]). Full-length RelA is not observed in these fractions (panel [iv]). c-Myc elutes in the high-molecular-mass fractions both in the presence and absence of RelA (panels [v] and [vi]). **(B)** RelA(p37) exclusively associates with c-Myc in a co-immunoprecipitation assay. The same extracts utilized for gel filtration were also subjected to co-immunoprecipitation with either anti-c-Myc antiserum or control IgG and then the blot was probed for RelA(p37) with the N-terminal domain anti-RelA antiserum. Only RelA(37) is seen to co-precipitate with c-Myc and not full-length RelA.

was found to co-localize with the chaperone protein heat-shock protein 70 in the nucleus. Whether the observations of Henriksson et al. [41] represent a physiological process is unclear, although it was postulated that this would provide a mechanism by which the cell could deal with excessive levels of c-Myc. In the context of our present study, it is possible that excess RelA is recruited into these c-Myc-containing bodies. Once there, RelA(p37) could then be generated, possibly through a mechanism involving the chaperone activity of heat-shock protein 70 to unfold RelA and facilitate the exposure of the cleavage site to the protease. Why the process would be specific for RelA and not other NF- κ B subunits is unclear, although further work is in progress to define

clearly both this mechanism and the physiological significance of RelA(p37) generation.

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