INTRODUCTION

RING3, a mammalian protein kinase that participates in signal transduction and regulated transcription, shows increased autophosphorylation and substrate-directed phosphorylation after mitogenic stimulation of responsive cells (Denis and Green, 1996) or whole animals (Ostrowski et al., 1998). Its activity is greatest in primary isolates of peripheral blood lymphocytes from patients with leukaemia and in leukemic cell lines (Rachie et al., 1993). The presence of a RING3-like protein in transcription complexes purified from cell extracts, such as the mouse Mediator complex (Jiang et al., 1998) is consistent with the observation that RING3 is a transactivator of some transcriptional units such as cell cycle-regulated promoters (Denis et al., 2000). Taken together, these data support a hypothesis that RING3 plays a contributing role in oncogenesis, although the target genes through which it promotes transformation are unknown.

In exponentially growing HeLa cells, RING3 is constitutively nuclear as assayed by biochemical fractionation of cell extracts and by indirect immunofluorescence of fixed cells (Denis and Green, 1996). However, we report that in serum-starved fibroblasts, RING3 is delocalised throughout the cell. Mitogenic stimulation of these fibroblasts induces RING3 to localise to the nucleus. Nuclear compartmentalisation is widely employed in eukaryotic cells as a mechanism to activate transcription. There are numerous reports of signal transduction-induced nuclear translocation of several different types of molecules, including a diverse set of transcription factors such as Smad3 (Sun et al., 1999), Stats (Darnell et al., 1994; Wu and Bradshaw, 1996; Onishi et al., 1998), the glucocorticoid receptor (Stocklin et al., 1996), p53 (Jimenez et al., 1999) and Forkhead (Brunet et al., 1999); kinases such as pp90RSK and ERKs (Chen et al., 1992; Khokhlatchev et al., 1998), diacylglycerol kinase-ζ (Topham et al., 1998) and phosphatases such as calcineurin (Musaro et al., 1999); and cell cycle regulators such as E2Fs (Müller et al., 1997), cyclins (Gallant and Nigg, 1992) and p21Cip1/Waf1 (Levkau et al., 1998). This stimulus-induced physical translocation is believed to be an essential feature of the mechanism by which signal transduction information is conveyed to transcriptional target genes, and the field has been reviewed recently (Kaffman and O’Shea, 1999). However, some proteins do not contain localisation motifs and require a co-transported factor for proper nuclear localisation, others function as transcriptional repressors upon arrival in the nucleus, and still others are constitutively nuclear. We show that RING3 is a monopartite classical nuclear localisation sequence. These observations refine and extend the mechanism by which RING3 contributes to E2F-regulated cell cycle progression. Deregulation of this mechanism may be leukaemogenic.

SUMMARY

RING3 is a novel protein kinase linked to human leukaemia. Its Drosophila homologue female sterile homeotic is a developmental regulator that interacts genetically with trithorax, a human homologue of which is also associated with leukaemia. The RING3 structure contains two mutually related bromodomains that probably assist in the remodelling of chromatin and thereby affect transcription. Consistent with this hypothesis, a RING3-like protein has been identified in the mouse Mediator complex, where it is associated with transcription factors. We show that, whilst RING3 is constitutively localised to the nucleus of exponentially growing HeLa cells, it is delocalised throughout serum-starved fibroblasts. We use immunostaining and confocal microscopy to demonstrate that RING3 translocates to the fibroblast nucleus upon serum stimulation. After translocation, RING3 participates in nuclear protein complexes that include E2F proteins; it transactivates the promoters of several important mammalian cell cycle genes that are dependent on E2F, including dihydrofolate reductase, cyclin D1, cyclin A and cyclin E. We use site-directed mutagenesis of a putative nuclear localisation motif to show that the activation-induced nuclear localisation and consequent transcriptional activity of RING3 depends on a monopartite, classical nuclear localisation sequence. These observations refine and extend the mechanism by which RING3 contributes to E2F-regulated cell cycle progression. Deregulation of this mechanism may be leukaemogenic.

Key words: RING3, Nuclear translocation, Immunolocalisation, Confocal microscopy, Leukaemia

ACTIVATION-INDUCED NUCLEAR TRANSLLOCATION OF RING3

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MATERIALS AND METHODS

Tissue culture and DNA constructs

BALB/3T3 mouse fibroblasts were cultured in Dulbecco’s modified minimal essential medium with 4.5 g/l glucose (Gibco-BRL), buffered with sodium bicarbonate and supplemented with penicillin, streptomycin, glutamine and 10% (v:v) donor calf serum (Gibco-BRL), at 37°C in 100% humidity and 5% CO2. Monolayers about 40% confluent were transfected overnight in 100 mm dishes with 30 μg DNA by the calcium phosphate precipitate method, re-fed with complete medium the next day, trypsinised the following day and plated on fibronectin-coated glass coverslips. Cells were then cultured in 0.5% serum overnight and stimulated with 10% serum.

For reporter assays, transfections typically contained 30 μg DNA per 100 mm tissue culture plate at 40% confluence, comprising 10 μg reporter plasmid, 10 μg expression vector, 5 μg MEKK, (to provide a constitutive activation signal; Denis et al., 2000) and 5 μg β-galactosidase expression vector. The enhancer-promoter of the immediate early gene of human cytomegalovirus drove all RING3 expression constructs, as described (Denis and Green, 1996). The dihydrofolate reductase (dhfr) reporter was a gift from J. Xiao and was confirmed by sequencing. After transfection, cells were grown to confluence, which required two days, and then harvested for luciferase assay. Monolayers were washed twice with phosphate buffered saline (PBS), then cells were scraped into 0.4 ml luciferase lysis buffer (Promega) and passed through three freeze/thaw cycles. Extracts were cleared of debris by microcentrifugation, then 0.02 ml were mixed with luciferase assay reagent (Promega) to give a 0.1-ml reaction. Emitted light was quantified for 60 seconds in a TD-20/20 luminometer (Turner). The polymerase chain reaction (PCR) was used to mutate the putative NLS of RING3 with forward primer 5’-GCACGGGCAGCCAAGGCAGAGAAGCATCGA-3’.

RESULTS

We have previously shown that endogenous RING3 protein and activity are co-localised to the nucleus of exponentially growing HeLa cells (Denis and Green, 1996). These cells have a highly transformed phenotype and are not representative of cells derived from normal tissues. We chose the untransformed BALB/3T3 mouse fibroblast to investigate the subcellular distribution of RING3. These cells also have a well-defined nucleus and ratio of nuclear:cytoplasmic volume that is satisfactory for this type of localisation study; they are also mitogenically responsive to serum. Fibroblasts were grown on fibronectin-coated glass coverslips and starved of serum overnight. Under these conditions, confocal microscopy with anti-RING3 antibody revealed that the protein was ubiquitously distributed throughout the nuclear and cytoplasmic compartments of starved cells, in a punctate pattern. There was also detectable perinuclear staining and apparent nucleolar exclusion (Fig. 1A). Cells were stimulated either rhodamine- or fluorescein-conjugated secondary antibodies was detected. For double label experiments, emitted signals from the two fluorophores were acquired sequentially. Controls were performed to ensure that there was no antibody cross-reactivity. Control tests also confirmed that there was no significant cross-over of fluorescein fluorescence into the rhodamine detector channel, for example, so that photons acquired in the rhodamine channel were an accurate representation of HA-tagged epitopes. Visible cellular architecture was recorded with differential interference contrast (dic) microscopy. Images were handled in Adobe Photoshop 5.5.

Fig. 1. Nuclear translocation of endogenous RING3 protein after serum stimulation. RING3 protein was visualised with rabbit anti-RING3 primary antibody and goat anti-rabbit IgG-FITC secondary. (A) Starved BALB cells; (B) differential interference contrast (dic); (C) serum-stimulated; (D) dic. Bars, 10 μm.
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with serum for four hours, at which time nuclear localisation was maximal. Under these conditions, as shown in Fig. 1C, almost all the RING3 signal had translocated to the nucleus. The nuclear and cytoplasmic punctate pattern was replaced by more intense staining restricted to the nucleus with continued nucleolar exclusion and with little or no staining of cytoplasm or membranes.

RING3 displays intrinsic kinase activity upon post-translational activation (Rachie et al., 1993; Denis and Green, 1996; Ostrowski et al., 1998) and is an efficient transactivator of the promoters of cell cycle-regulated genes (Denis et al., 2000). We sought to confirm that the recombinant protein (Denis and Green, 1996) displayed the same localisation behaviour as the endogenous protein. To perform this experiment, we transfected BALB cells with an expression vector for wild-type RING3 cDNA under the control of the cytomegalovirus promoter. The construct was tagged at the amino-terminus with the HA antigen to distinguish it from endogenous protein, which did not alter the function of the gene product (see Fig. 5). Transfected cells were starved of serum, stimulated as before, and doubly stained with rabbit polyclonal antibody against RING3, which was visualised with anti-rabbit IgG secondary antibody conjugated to FITC; and with mouse monoclonal antibody against the HA tag, which was visualised with anti-mouse Cy3 antibody conjugated to rhodamine. These parameters permitted discrimination between endogenous material, which was visualised in green during confocal microscopy, and recombinant material, which was visualised in red. The endogenous protein (Fig. 2A) stained similarly to the recombinant protein (Fig. 2B); starved cells displayed a punctate pattern of staining in both cytoplasm and nucleus, with strong perinuclear localisation (Fig. 2A-C). Nucleolar exclusion was particularly well visualised in the starved cells. After serum stimulation as in Fig. 1, the punctate pattern was replaced with strong nuclear localisation. Both endogenous (Fig. 2E) and recombinant (Fig. 2F) proteins displayed this behaviour.

A site-directed mutant of the wild-type protein in which the putative catalytic lysine has been changed to alanine (K574A) no longer has intrinsic kinase activity (Denis and Green, 1996). We have demonstrated that this mutant is no longer capable of transactivating the promoter for dihydrofolate reductase (Denis et al., 2000). It was possible that inactivation of the autoprotolytic activity of the RING3 enzyme could prevent its activation-induced nuclear accumulation, because phosphorylation is known to be essential to the regulated nuclear translocation of SWI5 (Moll et al., 1991), SV40 T-antigen (Rihs et al., 1991) and cyclin B2 (Gallant and Nigg, 1992), for example. Autophosphorylation might be necessary to unmask a cryptic nuclear localisation signal (NLS) or promote a conformational change. To test this possibility, we performed the same experiment as in Fig. 2, but with the K574A mutant of RING3. Starved cells showed diffuse nuclear and cytoplasmic localisation of both endogenous (Fig. 3A) and recombinant (Fig. 3B) proteins, whereas after serum stimulation, both endogenous (Fig. 3E) and recombinant (Fig. 3F) signals were strongly localised to the nucleus. The mutant protein behaved the same as the wild type.

We next considered a hypothesis that the putative NLS in RING3, first described as a monopartite motif at amino acid residues 507-512 (KKKKRK; Beck et al., 1992), is necessary and sufficient to achieve the observed activation-induced nuclear translocation. We therefore fused the RING3 NLS and carboxyl-terminal flanking sequences (KKKKRKAEKHR) to green fluorescent protein (GFP). These constructs were transfected into BALB cells and visualised by confocal microscopy. As shown in Fig. 4A, a GFP control protein without any fusion peptide was not localised to a particular cellular compartment and is visualised diffusely throughout nucleus and cytoplasm. GFP with a carboxyl-terminal fusion
of the RING3 NLS was localised to the nuclear compartment, as hypothesised (Fig. 4C). To confirm this result, we used the polymerase chain reaction to generate site-specific mutations in the NLS, which created the peptide KARAALKHR, which we again fused to GFP. This motif replaced key lysine residues with alanine and introduced an arginine residue into a position in that motif where it is not normally found. Fluorescence of this GFP fusion protein was now restored to its original distribution and was no longer restricted to the nuclear compartment (Fig. 4E).

Next, in an extension of this hypothesis, the same mutation was introduced into the NLS of HA-tagged, recombinant RING3, which was then transfected into BALB cells as before. Endogenous and recombinant signals were visualised with the same combination of chromophores: green for endogenous and red for recombinant. As expected, the endogenous and recombinant signals were again diffusely delocalised throughout the starved cells (results not shown), whereas after serum stimulation, the localisation of the signals diverged. The fluorescence of the endogenous RING3 protein (green) was again tightly localised to the nucleus (Fig. 5A), but the fluorescence of the recombinant RING3 protein with a mutated nuclear translocation sequence (red) remained delocalised (Fig. 5B), with punctate staining similar to that observed in starved conditions (Figs 1A, 2A, 2B, 3A and 3B). This result confirmed that the putative NLS was necessary and sufficient for proper translocation of RING3 protein in response to mitogenic stimulation. Further verification of this result was obtained when the localisation mutant was tested for its ability to transactivate a luciferase reporter construct. We have previously shown that RING3 is capable of transactivating the promoters of several cell cycle-regulated genes, such as dihydrofolate reductase (Denis et al., 2000). Addition of an amino-terminal HA peptide does not alter transactivation ability (Fig. 5E), but inactivation of the NLS resulted in a RING3 mutant that no longer transactivated this promoter.

**DISCUSSION**

Mitogenically induced nuclear translocation of proteins such as kinases and transcription factors that are normally resident in the cytoplasm has emerged as an important regulated process in signal transduction. The cytoplasmic/nuclear partitioning of several signal-transducing kinases has been shown to change
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minutes after mitogenic stimulation from primarily cytoplasmic, or delocalised, to primarily nuclear. The ERKs, pp90\textsuperscript{RSK} (Chen et al., 1992) and mitogen-activated protein kinases (Lenormand et al., 1993) respond to mitogenic signals in this manner. This mechanism is thought to be an essential part of the process in mammalian cells by which mitogenic information is carried to the key promoters that will initiate the genetic program for cell division. Regulated partitioning between cytoplasm and nucleus again becomes important hours after mitogenic stimulation as E2Fs, cyclins and cyclin-dependent kinases move in and out of the transcriptional environment to co-ordinate the progress of the cell through S, G\textsubscript{2} and M phases of the cell cycle. Because RING3 transactivates the promoters of several cell cycle regulatory genes, including dihydrofolate reductase, cyclin D1, cyclin A and cyclin E (Denis et al., 2000), we wondered whether its subcellular localisation plays a role in its transcription activation function.

It is unknown whether RING3 has any cytoplasmic functions, like the c-Abl tyrosine kinase (Prendergast, 1996). However, the presence of two mutually related bromodomains in the RING3 structure suggests that one function of the protein is to participate in transcription regulation (Jeanmougin et al., 1997; Winston and Allis, 1999). Bromodomains have been identified in several important transcription factors and have been shown to interact with N-acetyl-lysine residues of core histones (Dhalluin et al., 1999; Ornaghi et al., 1999). This feature suggests that RING3 is required in the nucleus, an interpretation that is supported by the classical NLS in the carboxyl-terminal domain of the protein.

We first established that endogenous and recombinant proteins translocate in the same way in response to serum stimulation. Fig. 2 shows that both green and red signals are poorly localised in the starved cells, but strongly localised to the nucleus in the stimulated cells. It was possible to discriminate between the two forms because the recombinant protein has an amino-terminal HA tag and could be visualised with specific antibody. This tag does not interfere with transactivation function, as demonstrated in Fig. 5E. Both wild-type and K574A mutant proteins translocate in the same manner as endogenous RING3 protein, so the previously observed failure of the K574A mutant to transactivate the dihydrofolate reductase promoter (Denis et al., 2000) is not due to failure to translocate (Fig. 3). These data further suggest that an autophosphorylation event is not required to initiate nuclear localisation, although potential interactions with endogenous RING3 molecules cannot be ruled out.

We tested the hypothesis that a small protein motif that includes a NLS is sufficient to confer the ability to localise GFP to the nucleus. This approach has been employed successfully to study motifs from other nuclear-localised factors, such as diacylglycerol kinase-\(\zeta\) (Topham et al., 1998) and Stat5b (Herrington et al., 1999). The putative NLS did indeed prove to be sufficient to localise GFP to the nucleus and mutation of key residues within the motif is sufficient to abolish this behaviour. When the equivalent mutation is made in the putative NLS of the recombinant protein, the altered RING3 now fails to translocate under conditions where the endogenous protein does translocate. RING3 should therefore be grouped in the class of eukaryotic proteins that require only a simple, classical monopartite motif to regulate their nuclear translocation behaviour.

The observation that RING3 has the greatest activity in acute leukaemic blasts provides an intriguing link to transcription regulation. The majority of paediatric acute leukaemias derive from chromosome breaks at 11q23 and damage the ALL-1 gene, which encodes a large transcription factor that is homologous to the \textit{Drosophila} Trithorax protein (Gu et al., 1992; Tkachuk et al., 1992). Genetic evidence from \textit{Drosophila} indicates that a RING3 homologue, \textit{female sterile homeotic (fsh)} interacts with \textit{trithorax} as an upstream activator, because...
RING3 plays an important role as a mediator of signal highly regulated E2F transcriptional machinery suggests cycle (Denis et al., 2000). The mitogen-induced nuclear complexes within the nuclear environment. Indeed, RING3 and cycle regulatory proteins normally reside in large multiprotein complexes with the distribution of cyclins, cyclin-dependent kinases, E2Fs may represent a class of potentially oncogenic defects. transactivation (Fig. 5E), translocation mutants in this pathway derive from specific mutations in the NLS of either RING3 or ALL-1 have been reported. However, in view of the observation that failure to translocate to the nucleus abolishes transactivation (Fig. 5E), translocation mutants in this pathway may represent a class of potentially oncogenic defects.

The observed nucleolar exclusion of RING3 is coincident with the distribution of cyclins, cyclin-dependent kinases, E2Fs and other proteins involved in DNA replication. These cell cycle regulatory proteins normally reside in large multiprotein complexes within the nuclear environment. Indeed, RING3 and RING3-like proteins have been detected in multiprotein complexes in nuclear extracts (G. V. Denis, unpublished observations; Jiang et al., 1998), associated with factors known to be important in transcriptional regulation. We have observed E2F in association with RING3 nuclear complexes and demonstrated an E2F requirement for RING3-dependent transactivation of the promoters of genes that regulate the cell cycle (Denis et al., 2000). The mitogen-induced nuclear translocation of RING3 that precedes its participation in the highly regulated E2F transcriptional machinery suggests RING3 plays an important role as a mediator of signal transduction and transcription.

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