Journal of Cell Science 110, 2345-2357 (1997)
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JCS3614

Deregulated expression of the *RanBP1* gene alters cell cycle progression in murine fibroblasts

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SUMMARY

RanBP1 is a molecular partner of the Ran GTPase, which is implicated in the control of several processes, including DNA replication, mitotic entry and exit, cell cycle progression, nuclear structure, protein import and RNA export. While most genes encoding Ran-interacting partners are constitutively active, transcription of the *RanBP1* mRNA is repressed in non proliferating cells, is activated at the G_1/S transition in cycling cells and peaks during S phase. We report here that forced expression of the *RanBP1* gene disrupts the orderly execution of the cell division cycle at several stages, causing inhibition of DNA replication, defective mitotic exit and failure of chromatin decondensation during the telophase-to-interphase transition in cells that achieve nuclear duplication and chromosome segregation. These results suggest that deregulated RanBP1 activity interferes with the Ran GTPase cycle and prevents the functioning of the Ran signalling system during the cell cycle.

Key words: RanBP1, Ran, RCC1, Cell cycle, S phase, Mitosis, Chromatin

INTRODUCTION

We previously reported the identification, cloning and mapping to chromosome 16 of the murine *Htf9-a* gene (Lavia et al., 1987; Bressan et al., 1991), which encodes Ran-binding protein 1 (RanBP1) (Coutavas et al., 1993). RanBP1 acts as an interacting partner of the Ran GTPase (Lounsbury et al., 1994; Bischoff et al., 1995; Ouspenski et al., 1995; Schlenstedt et al., 1995).

Genetic evidence obtained from several organisms that carry mutations either in the *Ran* gene, or in genes encoding a Ran-interacting partner, indicate that the Ran GTPase network controls a variety of functions, including DNA replication, mitotic entry and exit, cell cycle progression, nuclear structure, nuclear protein import and RNA export (see the reviews by Moore and Blobel, 1994; Rush et al., 1996; Sazer, 1996). Considerable progress has recently been achieved to establish how, and in conjunction with which molecular partners, Ran controls downstream cellular functions that are apparently unrelated (reviewed by Avis and Clarke, 1996). Biochemical evidence indicates that the signalling activity of Ran is determined by its GTP- or GDP-bound state. The switch between Ran-GTP and Ran-GDP is in turn controlled by the interaction of Ran with partner molecules. The intrinsic Ran GTPase activity is low and is activated by the Ran GTPase-activating protein (RanGAP) (Bischoff et al., 1994), which catalyzes the formation of Ran-GDP. The GTP-bound state of Ran is instead favoured by guanine exchange factors, the best characterized of which is the chromatin-associated regulator of chromosome condensation (RCC1) protein (Bischoff and Ponstingl, 1991), which catalyzes the replacement of hydrolyzed GDP with GTP and thus regenerates GTP-bound Ran. Both the intracellular balance between Ran-interacting partners, and the interaction with factors capable of modulating their catalytic activity, appear to be crucial to the signalling activity of the Ran network.

RanBP1 was the first identified protein capable of interacting with Ran (Coutavas et al., 1993) through a highly conserved Ran-binding domain (Schlenstedt et al., 1995; Beddow et al., 1995) common to several Ran-binding proteins (Lounsbury et al., 1994; Dingwall et al., 1995). Although RanBP1 has no direct catalytic activity on Ran, it favours the formation of Ran-GDP in two complementary ways: by antagonizing the exchange activity of RCC1 on Ran, and by increasing RanGAP activity (Bischoff et al., 1995). In the absence of RanGAP, RanBP1 does instead stabilize Ran-GTP (Bischoff et al., 1995; Lounsbury et al., 1994; Richards et al., 1995); thus, RanBP1 controls the nucleotide-bound state of Ran in different ways.

The requirement of Ran signalling activity for cell cycle-related functions was originally indicated by the finding that conditional alleles reponsible for RCC1 loss of function in

mammalian cells yielded prematurely condensed chromosomes in the absence of DNA replication at the restrictive temperature (Ohtsubo et al., 1987). RCC1 was therefore postulated to function as a sensor of ongoing replication and prevent premature mitosis/maturation promoting factor (MPF) activation prior to the completion of DNA replication (Nishitani et al., 1991; reviewed by Dasso, 1993). Nuclear injection experiments in hamster cells have recently indicated that Ran-GTP inhibits premature chromosome condensation caused by *RCC1* loss of function, further supporting the idea that the exchange activity of RCC1 on Ran during S phase is a key factor in coupling the onset of M phase to completion of DNA replication (Ohba et al., 1996). However, studies with mammalian cells and with *Xenopus* extracts have yielded contrasting results concerning the nucleotide-bound form of Ran that is effective in preventing premature mitotic entry (Ren et al., 1994; Dasso et al., 1994; Kornbluth et al., 1994; Clarke et al., 1995). In addition, a careful analysis of *S. pombe* mutant strains defective in the mitosis-to-interphase transition has suggested that RCC1 activity is primarily required for proper mitotic exit (Sazer and Nurse, 1994), and for the correct nucleo-cytoplasmic compartmentalization of post-mitotic components in daughter cells (Demeter et al., 1995). These findings, though not pinpointing which processes are primarily altered by disfunctions of the Ran signalling network, converge to indicate that the expression of either GTP- or GDP-locked mutant forms of Ran, or of mutant partners that control the nucleotide-bound state of Ran, cause arrest at specific cell cycle phases (reviewed by Rush et al., 1996), suggesting that the formation of Ran-GTP and Ran-GDP must be controlled during the cell cycle.

RanBP1 has been shown to cooperate with Ran in nuclear transport (Chi et al., 1996; see also Corbett et al., 1996). In contrast, the involvement of RanBP1 in cell cycle progression has not been directly investigated, although studies with mutant proteins suggest that cell cycle control by Ran required the RanBP1-interacting domain (Ren et al., 1995). We have previously shown that expression of the *RanBP1* gene is controlled in a cell cycle-dependent manner: it is repressed in quiescent cells, transcriptionally activated at the G_1/S transition and peaks in S phase (Guarguaglini et al., 1997). Antagonistic cell cycle regulators of both the E2F and retinoblastoma families of transacting factors are implicated in this control (Di Matteo et al., 1995).

In the present work, we have sought to determine whether cyclic expression of the *RanBP1* gene is functionally related to cell cycle control by the Ran network. If the timing of *RanBP1* up-regulation is relevant for Ran function, then its deregulated expression should impair cell cycle-related functions whose orderly execution requires the Ran network signalling activity. To address this question, we have investigated the effects of deregulated RanBP1 activity in cells synchronously progressing through the cell cycle.

MATERIALS AND METHODS

Cell cultures and cell cycle analysis

Murine NIH/3T3 fibroblast cultures (ATCC CRL 1658) were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS) in a 5% CO_2 atmosphere at 37°C. For G_0/G_1 synchronization, cells were cultured in medium containing 0.5% FCS to arrest proliferation; after 48 hours, medium was replaced and FCS was added to 15%; cells were harvested at regular intervals after restimulation. For G_1/S synchronization, cells were cultured in complete medium containing 0.5 mM hydroxyurea for 16 hours. G_2/M synchronization was achieved by adding nocodazole (0.1 μg/ml) to asynchronously cycling cell cultures for 18 hours. Harvested cells were incubated with 10 μg/ml RNAse for 5 minutes, then with propidium iodide (PrI, 50 μg/ml) for 30 minutes, and analysed by fluorescence-activated cell sorting (FACS) using a FACStar Plus flowcytometer (Becton Dickinson). In experiments designed to monitor DNA replication, bromodeoxyuridine (BrdUrd, 45 μM final concentration) was added to the culture medium for the last 30 minutes before harvesting the cells. Cells were collected by trypsinization, fixed in methanol, centrifuged, washed in PBS/Tween-20 (0.5%) and incubated in 3 N HCl for 45 minutes; after partial DNA denaturation, cells were exposed to an anti-BrdUrd antibody (IgG clone BU5.1, Ylem), to the secondary fluorescein-conjugated anti-IgG antibody (Ylem) and subjected to biparametric FACS analysis (WinMDI software).

Mammalian expression constructs

The pRanBP1 construct was generated by inserting the *Htf9-a* coding sequence (X46045), carrying the RanBP1 open reading frame (Bressan et al., 1991), under the cytomegalovirus (CMV) promoter/enhancer region. Molecules were ligated using a linker adaptor carrying a Kozak consensus sequence (5′-TTGGCGGCCGC-CATGGGGGCGCGCGCGACGAATTCT-3′, and its reverse complementary sequence). Post-transcriptional and translational control sequences were derived from the SV40 polyadenylation region. The pX empty vector carried the CMV promoter/enhancer ligated to the SV40 polyadenylation region and no coding sequence. The pCMV-lacZ construct carried the coding sequence for the β-galactosidase (β-gal) enzyme downstream of the CMV promoter/enhancer in the same basic vector.

Transfections

On day 1, NIH/3T3 cells were passaged from one large culture flask (175 $cm^{2)}$ either in 25 cm^2 flasks, or in Petri dishes onto sterile coverslips, in order to obtain cycling cultures with the same proliferation index. On day 2, cells were lipofected using the DOTAP reagent (Boehringer) and a mixture containing 2, 5 or 10 μg of pRanBP1 or pX DNA, 2 μg of β-gal construct, and pUC DNA to bring the total DNA amount to 12 μg. The medium was replaced with fresh medium 6 hours after lipofection. Cells were either collected after 24 hours, or subjected to cell cycle synchronization protocols as described above. Transfection efficiencies were assessed by immunoenzymatic measurements of the β-gal enzyme (β-gal ELISA kit) from the cotransfected pCMV-lacZ construct, and by microscope scoring of β-gal stained cells.

Northern blot experiments

To assess *RanBP1* mRNA expression, total RNA was extracted from cells transfected with either vector or pRanBP1 construct, using the guanidinium isothiocyanate/acid phenol protocol and electrophoresed through formaldehyde-agarose gels (see Di Matteo et al., 1995 for details). The *RanBP1* mRNA transcript was analysed in northern blot experiments using the *Htf9-a/RanBP1* cDNA clone as the probe; hybridization signals were quantified by processing the filters in a ^{32}P instant-imager (Canberra Packard).

Anti-RanBP1 antibody and immunoblotting assays

The *Htf9-a/RanBP1* coding sequence was inserted in the pQE30 bacterial expression vector (Qiaexpress, Qiagen), which carries the ATG start codon in frame with six histidine codons downstream of the polycloning site (see map in Fig. 1). The chimaeric 6×His-RanBP1 protein was induced in the *E. coli* M15 strain in the presence of 30 mg/ml IPTG for 5 hours at 37°C and was affinity-purified on Ni-NTA

resin, which binds the six-histidine tag with high affinity. The purified protein was electrophoresed using SDS-PAGE; the induced band was excised from the gel and homogenized in saline solution. Mice were intraperitoneally injected with the homogenate for 5 weeks (200 μl/mouse per week); animals were then ether-anaesthesized to collect peripheral blood and spleens. The polyclonal serum was separated from the blood by centrifuging at 2,500 rpm for 10 minutes.

To prepare filters for western blotting, trypsinized NIH/3T3 cells were washed three times in PBS, resuspended in 2-4 ml of 10 mM Tris-HCl, 15 mM NaCl, 1 mM EDTA and 1% NP40 buffer and incubated on a rotary shaker for 30-45 minutes at 4°C; breakdown of the cytoplasmic membrane and separation of whole nuclei was monitored under the microscope. After centrifuging at 2,500 rpm for 10 minutes, the nuclear pellet and the cytoplasmic fraction in the supernatant were separated and stored at −70°C. Protein extracts were electrophoresed through SDS-PAGE as above, then transferred onto nitrocellulose membranes in 20% methanol, 0.3% Tris and 1.44% glycin buffer. After the transfer, filters were equilibrated in PBS containing 3% BSA and incubated for 16-18 hours with the polyclonal anti-RanBP1 antibody (1:5,000 dilution in PBS containing 3% BSA). After two washes in PBS containing 3% BSA and 0.1% Triton-X, filters were incubated for 3 hours with a peroxidase-conjugated anti-mouse IgG antibody (1:1,000 dilution in PBS containing 3% BSA). Filters were then washed and equilibrated in 50 mM Tris-HCl, pH 6.8, for 30 minutes, then incubated in peroxidase reaction mixture (0.014% H_2O_2 and 0.7 mg/ml 5-Cl-1-naphtol); the reaction was finally blocked in H_2O.

Cytological preparations and microscopy

Cells transfected either with pRanBP1 or with pX vector were grown onto glass coverslips in Petri dishes. To assess the effectiveness of transfection, expression of the cotransfected pCMV-lacZ construct was assessed in duplicate sets of cell spreads which were fixed with 4% para-formaldehyde, washed twice in PBS-0.5% Triton and incubated in staining solution for 1 hour at 37°C; the staining solution is made up by equal volumes of solution A (50 mg/ml of X-gal in dimethylformamide) and solution B (1.64 mg/ml $K_3Fe(CN)_6$ and 2.1 mg/ml $K_4Fe(CN)_6$ in PBS/0.25% Triton-X). Immunofluorescence assays using the monoclonal MPM-2 antibody (Dako) were carried out on cells grown on coverslips and fixed in cold methanol for 30 minutes. After washing in PBS, coverslips were incubated for 1 hour with the MPM-2 antibody (100-fold dilution in PBS/5% goat serum). After two washes in PBS, coverslips were incubated for 45 minutes with FITC-conjugated (Vector Laboratories) anti-mouse IgG antibody (100-fold dilution in PBS/5% goat serum). Coverslips were washed again in PBS and DNA was counterstained in 0.25 μg/ml PrI. Photographic images were taken using a CCD camera (Photometrics) on a Zeiss Axiophot microscope and processed using Adobe Photoshop software. Cells to be examined under light microscopy were fixed in methanol for 15 minutes; coverslips were then stained with 10% Giemsa for 10 minutes, extensively washed and mounted in Eukit.

RESULTS

Cell cycle analysis of the endogenous RanBP1 protein

In previous work, we found that the *Htf9-a* gene, encoding RanBP1, is expressed in a cell-cycle dependent manner (Di Matteo et al., 1995; Guarguaglini et al., 1997). It was important to assess whether regulated *RanBP1* transcription was actually reflected by a differential abundance of the RanBP1 protein in different cell cycle phases. To ask that question, we generated an anti-RanBP1 polyclonal antiserum as described in Materials and Methods. To analyse the distribution of the RanBP1 protein during the cell cycle, NIH/3T3 cell cultures were brought to growth arrest by serum starvation and subsequently allowed to synchronously resume the cell division cycle by adding fresh serum to the growth-arrested cultures; cells were collected immediately before serum addition (G_0), and after 12 and 15 hours of the cell cycle reentry, which respectively correspond to the G_1/S boundary and to S phase in the experi-

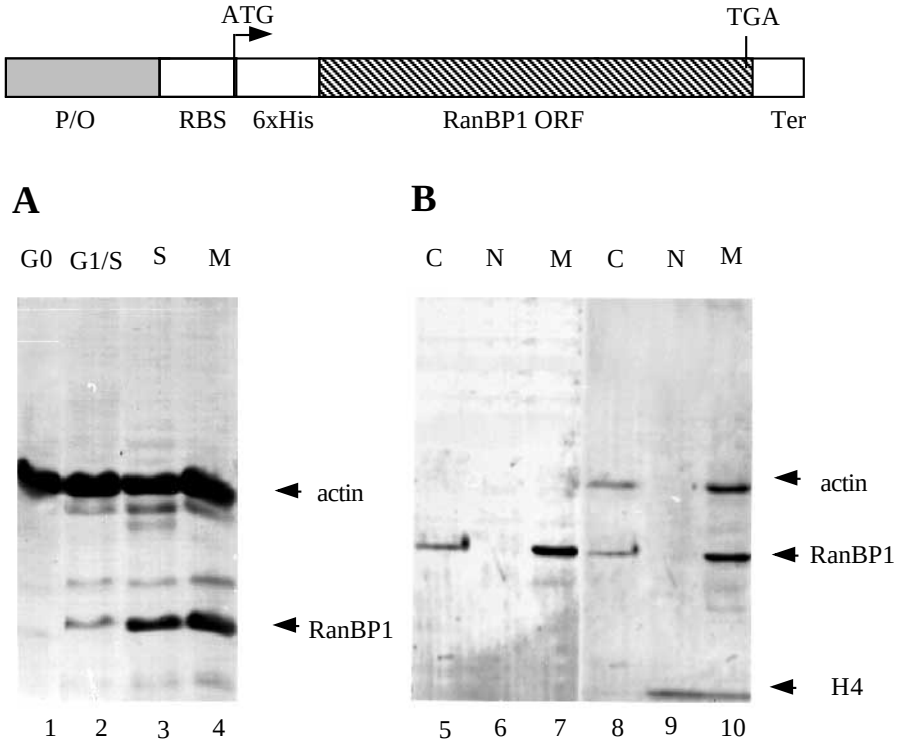

Fig. 1. The RanBP1 protein in cycling cells. The pQE30-RanBP1 construct used to induce the anti-RanBP1 antibody is shown at the top; P/O, promoter/operator; RBS, ribosome-binding site; 6×His, six repeated histidine codon tag; Ter, termination region. (A) The RanBP1 protein in growth-arrested (G_0) cells (lane 1), and in cells synchronized at the indicated stages (lanes 2-4). Approximately 40 μg of extract were loaded. Filters were incubated with both anti-RanBP1 and anti-actin antibodies. (B) Subcellular localization of RanBP1 in S-phase cells: C, cytoplasmic extract (lanes 5 and 8), N, nuclear extract (lanes 6 and 9); M, mitotic extracts are shown for control (lanes 7 and 10). Approximately 30 μg of extract were loaded. The membrane was firstly reacted with anti-RanBP1 (lanes 5-7) and subsequently incubated with both anti-actin and anti-H4 histone antibodies (lanes 8-10) to control fractionation.

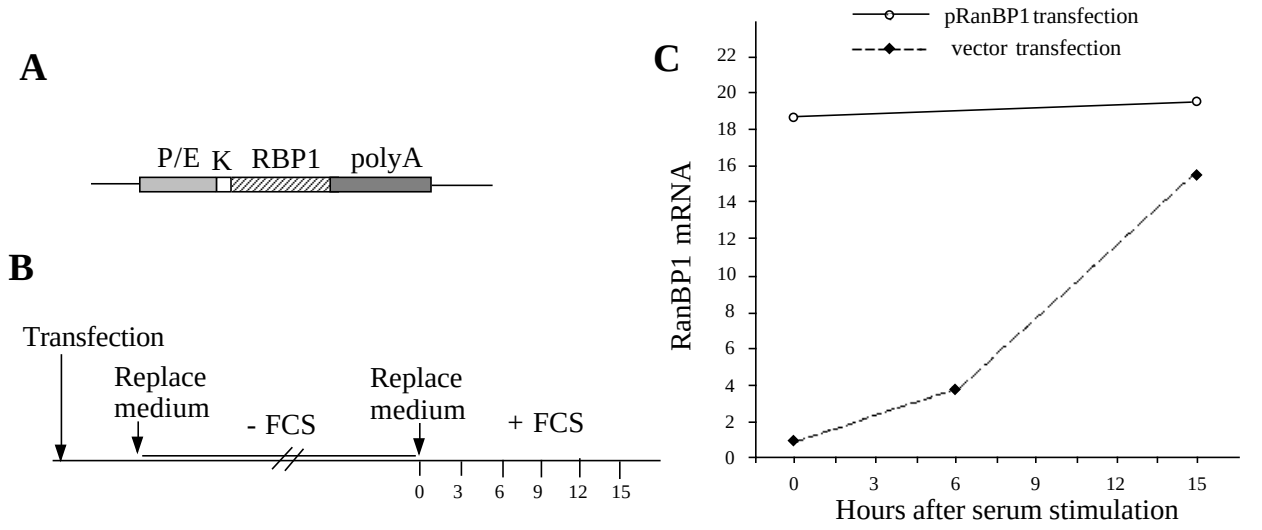

Fig. 2. Induction of *RanBP1* deregulated expression. (A) The RanBP1 construct used for transfection experiments in NIH/3T3 fibroblasts: P/E, CMV promoter/enhancer; K, Kozak consensus oligonucleotide; RBP1, *RanBP1* coding sequence; polyA, SV40 polyadenylation region. (B) Transfection and culture conditions: cells were transfected either with vector or with pRanBP1 DNA; after 6 hours, cells were placed in low serum (- FCS) for 48 hours to arrest proliferation, and subsequently stimulated to reenter the cell cycle by adding 15% serum (+ FCS). Cells were analysed at the indicated times. (C) The *RanBP1* mRNA transcript in transfected cells. The graph shows the relative levels of *RanBP1* mRNA in cells transfected either with pRanBP1 (solid line) or with vector (broken line) (10 μg of DNA). Total RNA from transfected cells was hybridized to a radioactive *RanBP1* cDNA probe; hybridization signals were quantified in a ^{32}P Instant Imager and are expressed in arbitrary units. Counts corresponding to target RNA sequences in vector-transfected cultures at time 0 were taken as 1.

mental conditions routinely used in our laboratory (see below). In addition, asynchronous cell cultures were grown in the presence of nocodazole, an effective spindle poison whose addition in the medium yields cultures that are predominantly arrested in metaphase. The effectiveness of synchronization in each cycle stage was determined by FACS analysis as will be discussed in detail below. Protein extracts from staged cells were analysed in western blot assays using the anti-RanBP1 antibody; an anti-actin antibody was also used to ensure that similar amounts of protein extract were loaded. As shown in Fig. 1A, no RanBP1 protein was detected in G_0 cells; the RanBP1 protein could be first appreciated in cells collected at the G_1/S boundary, consistent with the timing of the *RanBP1* gene transcriptional activation, and its abundance increased in S-phase cells. However, at variance with the results obtained from northern blot experiments that had shown that *RanBP1* mRNA transcription virtually ceased in metaphase cells (Guarguaglini et al., 1997), the endogenous protein clearly accumulated in those cells. Thus, the RanBP1 protein product is indeed expressed in a cell cycle-dependent manner; the protein abundance faithfully reproduces the pattern of mRNA transcription until S phase is reached, and accumulates thereafter.

Since Ran is thought to shuttle between the cytoplasm and the nucleus and is exposed to the interaction with RanGAP in the cytoplasm and with RCC1 in the nucleus (reviewed by Koepp and Silver, 1996), we next examined the subcellular localization of RanBP1 in S-phase cells, in which the protein is highly expressed. Proteins were extracted from both the nuclear pellets and the cytoplasms; the effectiveness of the fractionation procedure was controlled using anti-H4 and anti-actin antibodies. Unfractionated extracts from metaphase cells were also examined for control. The results in Fig. 1B show that virtually all synthesized RanBP1 protein was localized in the cytoplasm. A weak signal was occasionally detected in nuclear extracts, which could not be stabilized by increasing the amount of loaded protein extract in the gel (data not shown), suggesting that what little amount of RanBP1 protein may enter the nucleus is rapidly exported out, consistent with the identification of a nuclear export signal in the RanBP1 protein (Richards et al., 1996). In conclusion, high expression of RanBP1 in S phase correlates with its cytoplasmic location.

Forced *RanBP1* expression in cells subjected to serum starvation and restimulation induces dose-dependent abnormalities in cell cycle distribution

Previous experiments with stable *RanBP1* transfectants had detected no obvious effect in asynchronously growing mammalian or yeast cultures, unless the cells were simultaneously mutated at the *RCC1* locus, in which conditions viability was significantly reduced (Hayashi et al., 1995). These results suggest that the effects of RanBP1 are linked to alterations in the molecular balance among components of the Ran network. Since the RanBP1 protein is not expressed in growth-arrested or early G_1 cells, we decided to induce its deregulated expression. To achieve this, the *RanBP1* coding sequence was placed under the control of the CMV regulatory sequences (Fig. 2A), which are not subjected to cell cycle control; NIH/3T3 fibroblast cultures were transfected either with pRanBP1 construct or with vector, then brought to proliferation arrest by serum starvation and subsequently stimulated to reenter the cycle by adding high serum as outlined in Fig. 2B. Microscopical examination of cells simultaneoulsy transfected with pCMV-lacZ construct revealed that 70 to 85% of all cells were positive to β-gal staining in each experiment. Northern blot experiments were carried out to assess *RanBP1* mRNA transcription in cells transfected with the expression

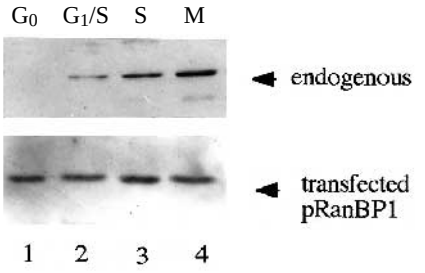

Fig. 3. Constitutive production of the RanBP1 protein in pRanBP1-transfected cell cultures. Cells were transfected with pRanBP1 construct or with empty vector as described in legend to Fig. 2, cultured in low serum for 48 hours and collected after growth-arrest (G_0, lane 1), or restimulated to cycle by adding fresh serum and harvested at the indicated stages (lanes 2-4). Approximately 30 μg of protein extract were loaded in each lane. Filters from vector- (top panel) or pRanBP1- (bottom panel) transfected cell cultures were hybridized with anti-RanBP1 antibody.

construct compared to control cultures transfected with empty vector. The results in Fig. 2C confirm that transcription of the endogenous *RanBP1* gene was extremely low until 15 hours of the cell cycle reentry. In contrast, the CMV control region directed constitutive *RanBP1* expression: the level of *RanBP1* mRNA was nearly 20-fold higher in pRanBP1-transfected cells compared to cells transfected with the vector alone during early G_1. We also compared protein levels in pRanBP1-transfected cells and in control cultures: western blot analysis confirmed that pRanBP1 transfection yielded unregulated production of the RanBP1 protein in all cell cycle phases, whereas the endogenous gene product was negligible or absent in arrested and early G_1 cells in control cultures (Fig. 3).

We initially tested the effect of introducing increasing amounts of pRanBP1 construct in NIH/3T3 cells. Transfected cells were brought to proliferation arrest, subsequently stimulated to enter the cycle and collected after 3 and 15 hours, respectively, corresponding to the G_0/G_1 transition and to S phase. The DNA content of the cells was estimated by FACS determination of the fluorescence intensity associated to propidium iodide incorporation in nuclei. The results of three independent experiments are summarized in Table 1. Control cells transfected with vector essentially had a G_1 DNA content during the first 3 hours of release from the proliferation block, as expected. In cultures transfected with pRanBP1, however, cells with a G_2/M DNA content were also seen. Substantially similar results were obtained at time 0 of the cell cycle reentry, i.e. using growth-arrested cells (see below). 15 hours after release of the starvation block, the majority of vector-transfected cells were in S phase. In contrast, the highest proportion of pRanBP1-transfected cells still showed a G_1 DNA content. The data in Table 1 show that the abnormalities in cell cycle distribution were more severe in the presence of increasing doses of pRanBP1 construct. Together, the results suggest that forced expression of *RanBP1* in growth-arrested cells altered the subsequent progression through the cell division cycle after restimulation, and depict two major abnormalities: on one hand, a fraction of cells maintained a G_2/M DNA content early after cell cycle reentry; on the other hand, a block or significant delay in exiting G_1 was apparent after 15 hours of cell cycle reentry, when control cells were traversing S phase.

Cell cycle progression is significantly altered following deregulated expression of the *RanBP1* gene

We wished to establish whether the abnormal cell cycle distribution depicted in Table 1 reflected a stage-specific inhibitory effect upon the cell division cycle following deregulated expression of *RanBP1*. Since the abnormalities increased with the amount of transfected pRanBP1 construct, experiments were carried out using 10 μg of DNA. To resolve the actual progression through an entire cell division cycle, transfected cells were harvested at regular intervals after serum stimulation and stained with propidium iodide.

As can be seen in Fig. 4, control cultures transfected with vector alone showed a typical cell cycle progression (Fig. 4a): the DNA began to increase above the G_1 content in a proportion of cells 12 hours after the cell cycle reentry; at 15-18 hours, the majority of the cells had a DNA content higher than G_1 and were traversing S phase; the relative ratios began to reverse again at 24 hours, indicating that at that time most cells had completed the G_2 and M phases and were in the G_1 phase of the next cycle. In contrast, pRanBP1-transfected cultures showed a significantly altered profile (Fig. 4b). A fraction (15%-20%) of pRanBP1-transfected cells showed a G_2/M DNA content at time 0 of the cell cycle reentry. In addition, the appearance of cells whose DNA content increased above G_1 was delayed by several hours compared to control cells; as a result, only a minor fraction of the cell population actually progressed from G_1 to S phase.

S phase is impaired or significantly delayed following forced pRanBP1 expression

In order to characterize the cell cycle defects depicted thus far, pRanBP1 transfection experiments were repeated in growth-arrested cells that were restimulated with serum and subjected to in vivo labeling with BrdUrd for the last 30 minutes before

Table 1. Dose-dependent effect of pRanBP1 transfection on the cell cycle

		Vector	pRanBP1		
Hours after serum stimulation		10γ % cells ± s.d.	10γ % cells ± s.d.	5γ % cells ± s.d.	2γ % cells ± s.d.
3	G_1	88.4±0.1	72.6±3.4	81.0±2.7	84.4±1.9
	S	2.9±0.1	7.9±0.3	4.7±2.6	5.7±2.2
	G_2/M	6.9±0.4	18.5±3.3	14.2±5.3	9.8±0.3
15	G_1	37.5±5.6	64.6±6.7	59.8±1.9	52.6±1.7
	S	54.3±0.6	22.1±5.9	26.6±4.1	35.3±5.5
	G_2/M	8.1±0.2	13.3±0.7	16.9±2.1	12.1±3.9

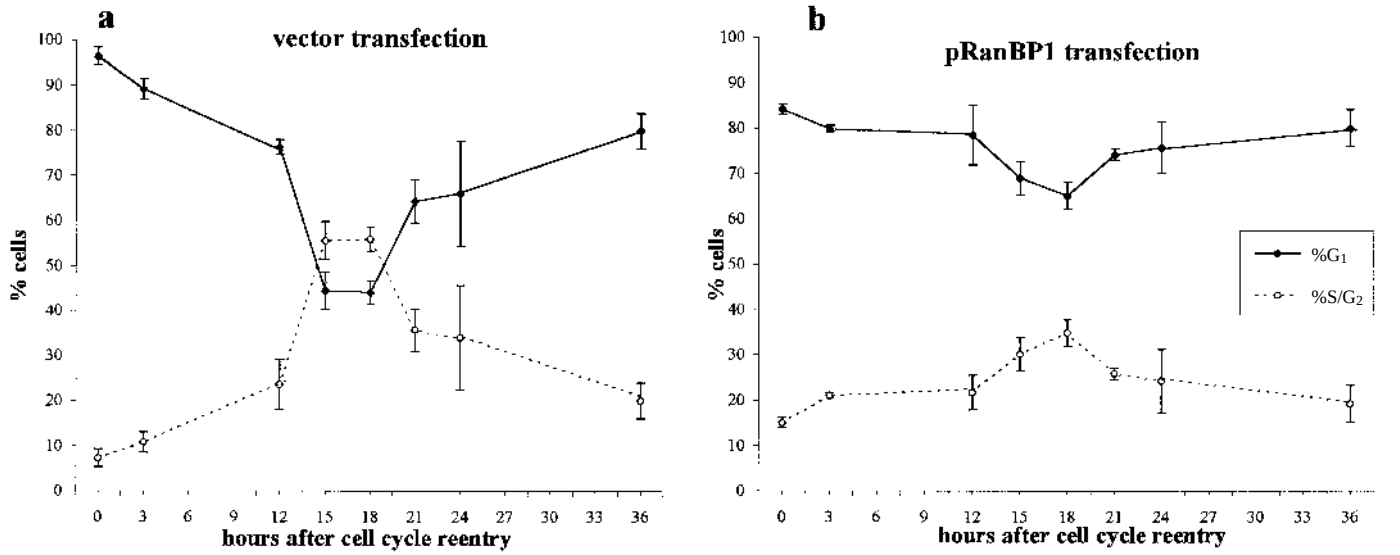

Fig. 4. Cell cycle progression in cells transfected with vector (a) or with pRanBP1 (b) construct. Transfected cells were serum-starved for 48 hours to arrest proliferation, then restimulated to cycle and harvested at the indicated times. The DNA content was analysed by FACS. Means and standard errors were calculated from four experiments. Full lines show the percentage of cells with a G_1 content, broken lines show the pooled fraction of S and G_2 cells.

harvesting. Cells were then processed for simultaneous determination of the BrdUrd incorporation in the newly synthesized DNA strands, to assess the extent of DNA replication, and of the DNA content, as revealed by the PrI fluorescence intensity. The results of FACS biparametric analyses are shown in Fig. 5.

Vector-transfected cells were effectively synchronized in the G_0/G_1 phase and showed a homogeneous FACS profile at time 0 after release of the proliferation block (Fig. 5a). At the same time, a fraction (15 to 30% in different experiments) of cells transfected with pRanBP1 showed a G_2/M DNA content, yet no BrdUrd incorporation (Fig. 5b), indicating that these cells had not completed the previous G_2/M phase during the starvation period.

Analysis of the cell population after 15 hours of the cell cycle reentry showed that most vector-transfected cells were indeed traversing S phase, as they had an intermediate DNA content between G_1 and G_2 and effectively incorporated BrdUrd (Fig. 5c). The FACS profile was instead heavily altered in cell cultures transfected with pRanBP1 (Fig. 5d), because the highest proportion of cells were still found in the G_1 region; the fraction of G_2 BrdUrd-negative cells depicted in growth-arrested cells did still persist; and, as a result of these combined blocks, only a minor fraction of the cells were found in the S phase region.

To examine S phase progression in more detail, transfected cells were cultured in the presence of hydroxyurea, which prevents initiation of DNA replication and thus arrests the cell cycle at the G_1/S transition; subsequent removal of the drug allows resumption of DNA replication. Hydroxyurea-synchronized and released cells were pulse-labeled with BrdUrd before harvesting and subjected to biparametric analysis as above. No significant differences between cell cultures transfected with vector (Fig. 5e) or with pRanBP1 (Fig. 5f) were observed at time 0: both cultures were arrested at the G_1/S transition; thus, *RanBP1* overexpression did not affect the block imposed by hydroxyurea over initiation of DNA replication. However, 4 hours after the block release, when most control cells had recovered from the G_1/S arrest and had resumed DNA replication, a high proportion of pRanBP1-transfected cells were significantly delayed in S phase progression, as assessed by evaluating the proportion of cells in early and in late S phase respectively. Cells in early and in late S phase indicated, respectively, as S1 and S2 in Fig. 5g-h, were distinguished among BrdUrd-incorporating cells on the basis of the fluorescence intensity associated with propidium iodide incorporation, which was respectively below, or above, the mean value: while S1 represented 37.2% and S2 62,8% of S phase-cells in the vector-transfected population (Fig. 5g), the proportions were reversed (57.4% S1 and 42.6% S2) in pRanBP1-transfected cells (Fig. 5h).

Together, the results indicate that deregulated expression of *RanBP1* significantly delays, yet does not prevent, S phase progression when overexpression is induced in cycling cells. In contrast, S phase onset is severely impaired when overexpression is induced in cells that are stimulated to reenter the cycle from the G_0/G_1 transition.

Cultures overexpressing *RanBP1* show impaired mitotic exit and aberrantly condensed nuclei

The experiments in Fig. 5a,b had indicated that completion of the mitotic division had been impaired upon forced *RanBP1* expression during growth arrest. To further substantiate that indication, asynchronously cycling cell cultures transfected either with pRanBP1, or with vector, were exposed to nocodazole for 18 hours to obtain cultures highly enriched in metaphase cells; progression of the mitotic division from metaphase arrest to the following interphase was monitored after removal of the drug. Microscopical examination of nocodazole-exposed cells revealed a similar proportion of metaphase-arrested figures in both pRanBP1- and vector-transfected cultures, indicating that deregulated RanBP1 activity

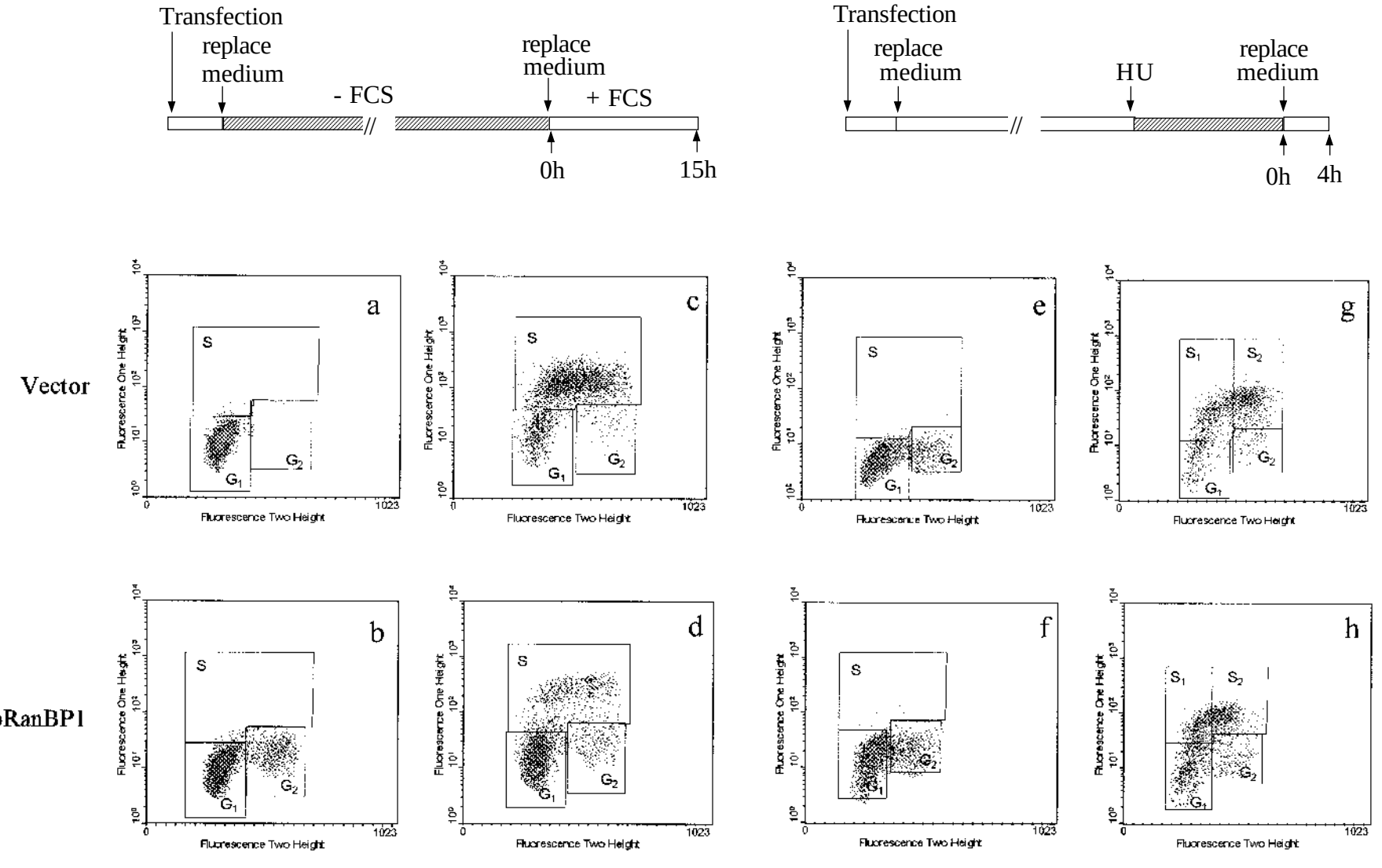

Fig. 5. G_1/S and G_2/M arrest following deregulated *RanBP1* expression. Transfected cells were either serum starved for 48 hours and then restimulated with fresh serum, or cultured in complete medium for 30 hours prior to the addition of hydroxyurea (HU), as outlined at the top. Left panels: biparametric distribution of cells transfected with vector (a,c) or with pRanBP1 (b,d) collected after 48 hours of serum starvation (– FCS) (a,b) and 15 hours after restimulation (+FCS) (c,d). Right panels: biparametric distribution of cells transfected with vector (e,g) or with pRanBP1 (f,h), 0 (e,f) and 4 hours (g,h) after hydroxyurea removal. *y*-axis, BrdUrd incorporation; *x*-axis, PrI fluorescence.

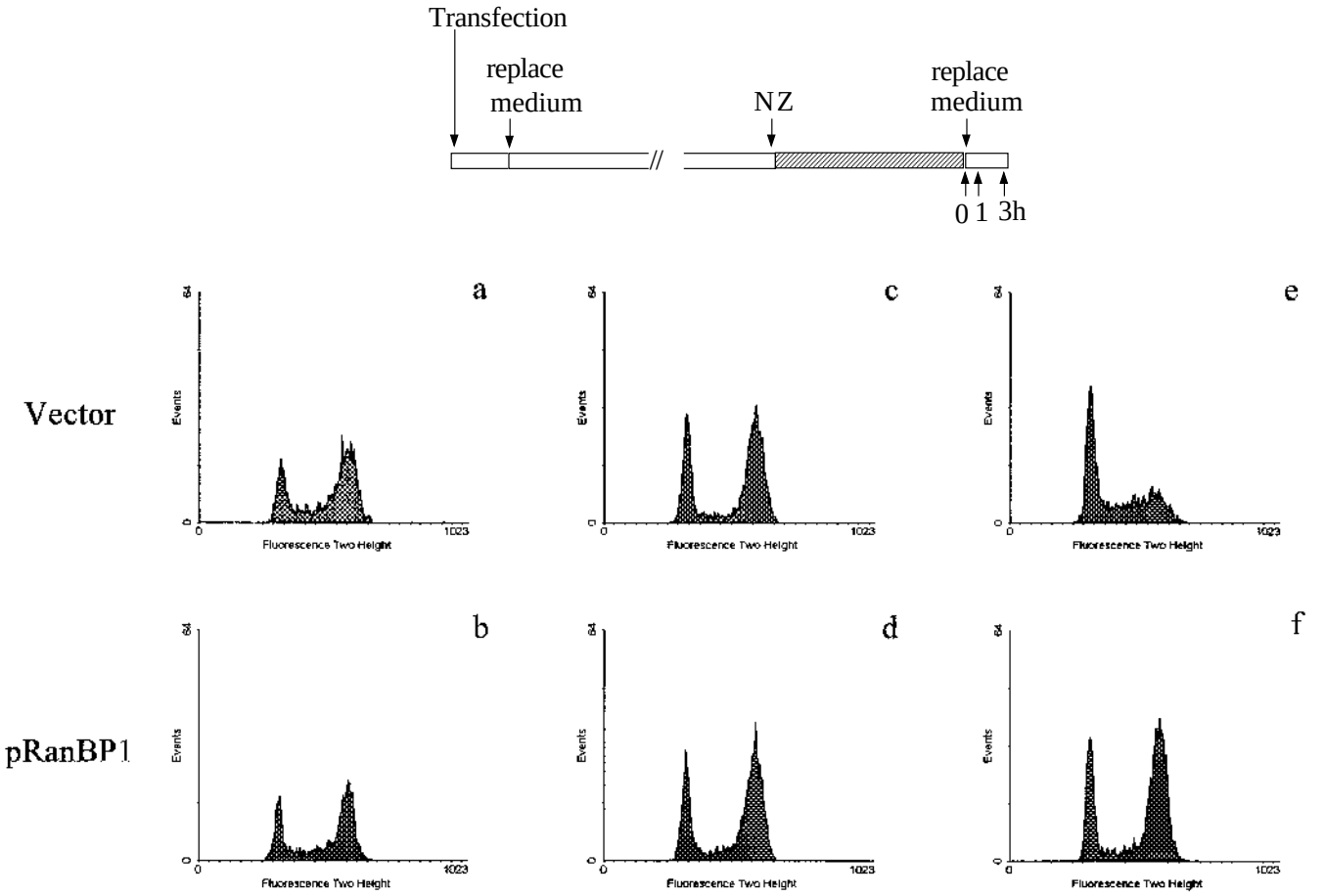

Fig. 6. G_2/M block following deregulated *RanBP1* expression in asynchronous cells. The experimental design is outlined at the top: transfected cells were cultured in complete medium for 30 hours prior to nocodazole (NZ) addition to induce metaphase arrest; cells were harvested at time 0 (a,b), or after 1 (c,d) and 3 hours (e,f) after NZ removal. Below, the FACS panels show the DNA content of the cells as determined by PrI incorporation (*x*-axis). Cell numbers are indicated on the *y*-axis.

had not significantly altered mitotic entry (data not shown). FACS analysis indicated that control cells exited mitosis and accomplished G_1 reentry within three hours after nocodazole removal (Fig. 6a,c,e). The release from nocodazole arrest was instead ineffective in pRanBP1-transfected cells: a high proportion of cells maintained a G_2/M DNA content three hours after nocodazole removal, and their distribution showed no significant variation compared to the FACS profile of nocodazole-arrested cells (Fig. 6b,d,f).

We then examined the cytological phenotype of cell cultures in which *RanBP1* expression had been forced during serum starvation and where cells with a G_2/M DNA content depicted by flow cytometry were suggestive of incomplete growth arrest. Mitotic figures were indeed present in cell cultures subjected to forced *RanBP1* expression during serum starvation (see for example Fig. 7b). In addition, a high proportion of cells with small, pyknotic nuclei were seen (Fig. 7b and c). Several control experiments were carried out to establish whether the pyknotic nuclei represented apoptotic figures which might have been induced in response to the cell cycle defects accompanying pRanBP1 expression. These experiments enabled us to rule out that the condensation was related to the induction of apoptosis, because FACS analysis did not depict a significant fraction of hypodiploid cells after pRanBP1 transfection compared to control cultures; in addition, the nuclei had an intact morphology and showed no chromatin fragmentation. Furthermore, in situ immunofluorescence detection of 3′ DNA breaks using the terminal transferase-based TUNEL assay did not stain the small condensed nuclei while giving an intense staining of apoptotic nuclei from γ ray-irradiated lymphoid cell cultures (data not shown). These results did not reflect an impaired accessibility of 3′ breaks in condensed nuclei, because extracted DNA from both vector- and pRanBP-1 transfected cells subjected to in vitro end-labeling with the Klenow polymerase and ^{32}P-deoxynucleotides migrated as high molecular mass DNA. Since DNA fragmentation may be viewed as a marker of advanced apoptosis, we also assessed the distribution of phosphatidylserine residues, whose exposure on the external cell membrane face is regarded as an early apoptotic event. In situ immunofluorescence using annexin V to react dislocated phosphatidylserine finally ruled out that pRanBP1-transfected cells differed from control cells. Thus, flow cytometric analysis, the membrane protein distribution and the DNA integrity in situ and in vitro, all converge to rule out that the pyknotic nuclei in Fig. 7 represent apoptotic figures, and thereby indicate that they derive from the truly aberrant condensation of nuclear chromatin in intact cells.

Table 2. Aberrantly condensed nuclei in pRanBP1-transfected cells

Cell cycle reentry	0 Hours				15 Hours			
Transfection	Number of experiments	Scored cells	Condensed nuclei	%	Number of experiments	Scored cells	Condensed nuclei	%
Mock	2	3,000	125	4.2	2	2,000	98	4.9
Vector	3	2,664	111	4.2	4	6,746	454	6.7
pRanBP1	4	7,112	1,076	15.1	4	7,199	1,707	23.7

Since FACS analysis depicted a significant proportion of pRanBP1-transfected cells with a G_2/M DNA content after serum starvation, and mitotic figures were actually present in cell spreads from such cultures, it was possible that the aberrantly condensed nuclei represented aberrant mitotic products. To assess that possibility, cell spreads were characterized by immunofluorescence using the MPM-2 antibody, which visualizes a set of proteins that are specifically phosphorylated at the G_2/M transition (Davis et al., 1983), and include cdc25-C, wee 1, Myt 1, microtubule-associated protein (MAP) 1 and 4, topoisomerase II and others (Ding et al., 1997, and references therein). These experiments showed that MPM-2 reacted to virtually all condensed nuclei as well as arrested mitotic figures (Fig. 8a,b). Over 96% of pyknotic nuclei were associated with MPM-2 reactivity, suggesting that the aberrant condensation represents a distinctive defect of mitotic cells in pRanBP1-transfected cultures. MPM-2 antigen expression was found to persist for some time after telophase, when nuclear chromatin started to decondense in control cells; however, pRanBP1-transfected cells showing MPM-2 reactivity failed to reestablish a normally decondensed chromatin after daughter nuclei had separated and cytokinesis had occurred (see for example Fig. 8d). The presence of pyknotic nuclei in MPM-2 reactive cells was recorded after microscopical examination; metaphase figures were not considered in this analysis. Results are shown in Fig. 9: post-mitotic cells expressing MPM-2 antigens in asynchronous control cultures showed normal chromatin decondensation; a high frequency of decondensed nuclei was also recorded among MPM-2 stained, vector-transfected cells after induction of growth arrest by serum starvation. In contrast, virtually all pRanBP1-transfected cells that were positive to MPM-2 showed failure of chromatin decondensation after serum starvation.

The appearance of hypercondensed nuclei in pRanBP1-transfected cultures was dependent on the starvation /restimulation procedure, and was not significant in asynchronously cycling cells, indicating that pRanBP1 expression during the starvation period had yielded the accumulation of cells defective in chromatin decondensation after nuclear separation. Interestingly, aberrantly condensed nuclei were also detected in cells harvested 15 hours after serum refeeding (Table 2), indicating that the defect in decondensation was not rescued by inducing cell cycle reentry. These observations indicate that deregulated expression of *RanBP1* profoundly intereferes with chromatin decondensation in post-mitotic cells.

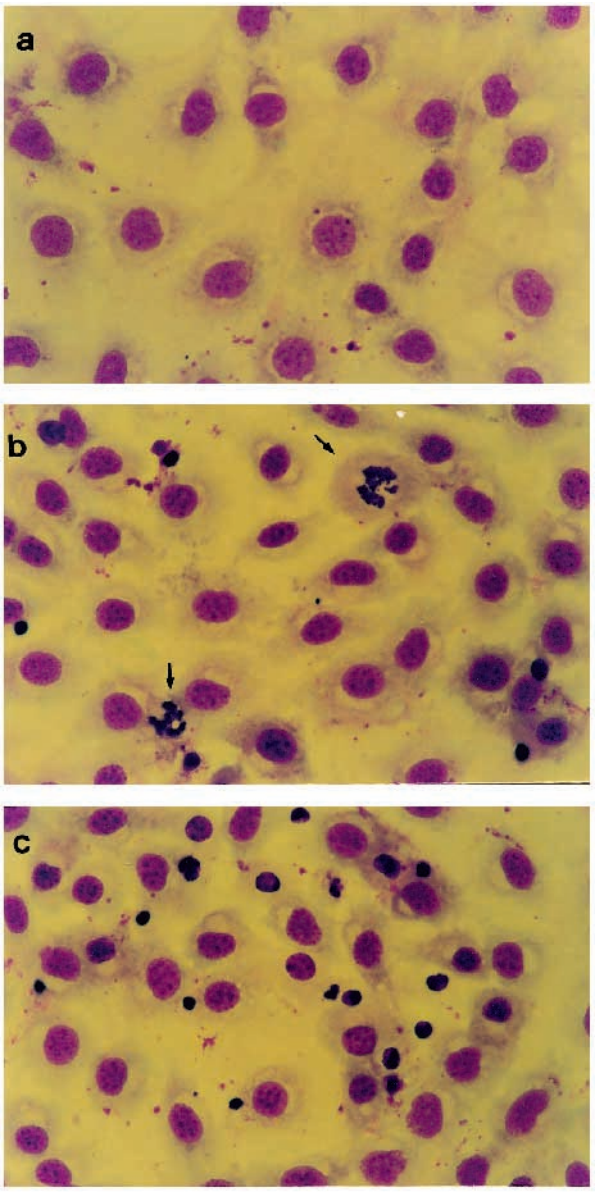

Fig. 7. Metaphase arrest and aberrantly condensed nuclei in serum starved NIH/3T3 cells. Cells transfected with vector (a) or with pRanBP1 (b,c) were cultured on coverslips in the absence of serum for 48 hours to arrest proliferation, and subsequently stained with Giemsa. Metaphase-arrested cells are arrowed (×40 objective).

DISCUSSION

RanBP1 during the cell cycle

Genetic (Ouspenski et al., 1995) and biochemical (Bischoff et al., 1995; Richards et al., 1995) experiments have shown that RanBP1 is a major molecular partner of Ran. The *RanBP1* gene is inactive in growth-arrested cells, is transcriptionally induced at the G_1/S transition and is maximally expressed in S

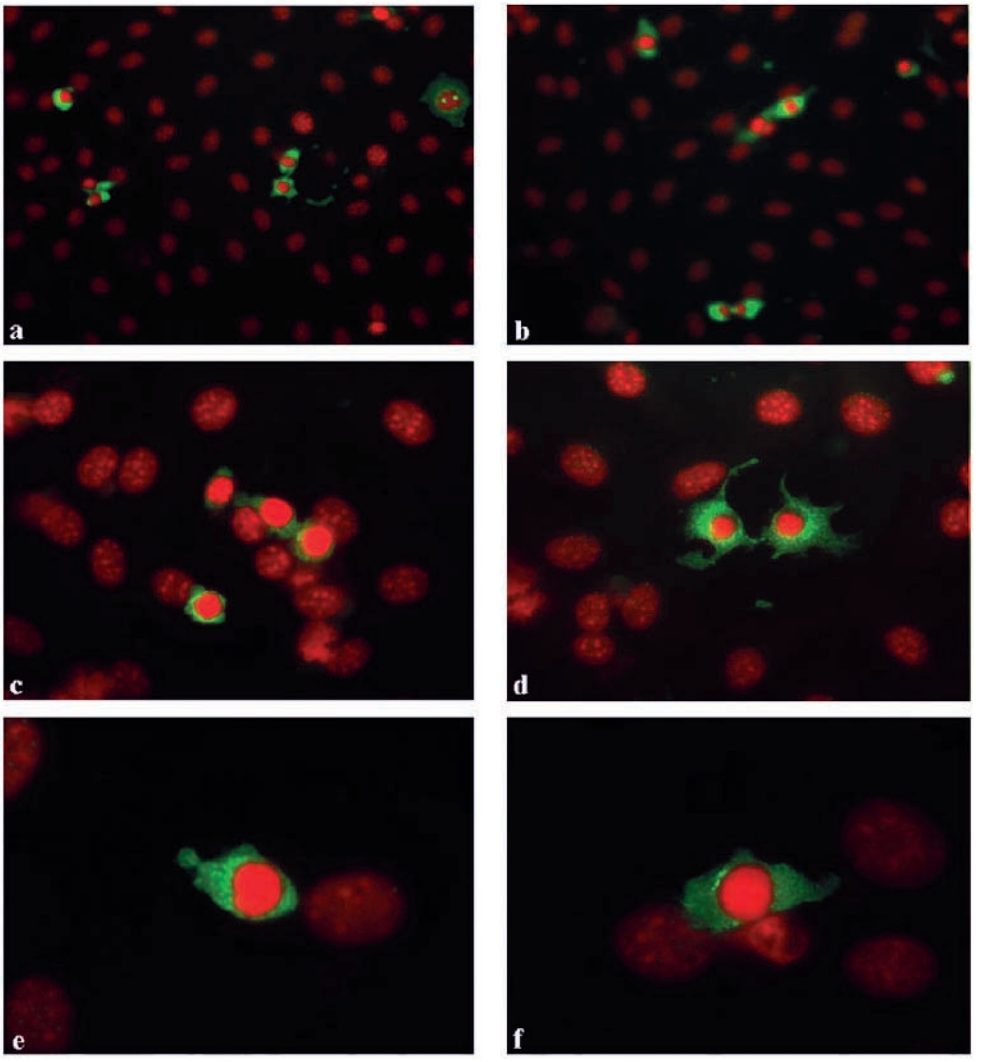

Fig. 8. Incomplete mitotic exit and defective post-mitotic decondensation following deregulated *RanBP1* expression. Transfected cells were serum-starved for 48 hours, then immunostained with MPM-2 antibody to visualize mitotic phosphoproteins (green), while the DNA was counterstained with PrI (red). (a,b) Low magnification (×20 objective) shows that the MPM-2 antibody decorates both mitotic figures and cells with aberrantly condensed nuclei. (c,d) Abnormal condensation in isolated nuclei and in cells exiting mitosis (×40 objective); (e,f) pyknotic nuclei (×100 objective); the abormal condensation is evident by comparison with the diffuse staining of surrounding nuclei.

phase; E2F and retinoblastoma-related factors are involved in this control (Di Matteo et al., 1995; Guarguaglini et al., 1997). In contrast, expression of other genes of the Ran network does not significantly vary in relation to cellular proliferation or to the cell cycle phase: *RanGAP* mRNA is expressed in early developmental stages and maintains comparable levels in both proliferating and quiescent tissues and cell types (De Gregori et al., 1994; Krebber and Ponstingl, 1996); the *Ran* gene shows growth-dependent expression, yet is rapidly induced upon entry into the cell cycle and does not vary thereafter (Coutavas et al., 1994); similarly, the *RCC1* gene is activated in proliferating cells (Tsuneoka et al., 1997) and is expressed at all cell cycle stages (Nishimoto et al., 1981). Consistently, we have found that both RCC1 and Ran proteins are rapidly synthesized upon entry in the cell cycle and their levels remain constant throughout (A. Palena, G. Guarguaglini and P. Lavia, unpublished data). Thus, *RanBP1* is so far the only gene of the Ran network showing phase-specific regulation. These observations suggest that *RanBP1* may act as a pivotal gene, linking control at the G_1/S transition by members of the E2F and retinoblastoma families of regulators to the signalling activity of the Ran network.

We presently report that cell cycle control of *RanBP1* transcription is paralleled by cell cycle-specific expression of the RanBP1 protein: the synchronization protocols employed here have enabled us to ascertain that the endogenous RanBP1 product is essentially present from S phase to metaphase. These data suggest that different interactions are established between components of the Ran network in different cycle phases: RanBP1 is virtually absent or expressed at extremely low levels in early G_1 cells; in S-phase cells, the protein is synthesized at high levels and accumulates in the cytoplasm, where RanGAP is also found; finally, all components of the network, including nuclear RCC1, potentially come in contact after nuclear envelope breakdown in metaphase cells.

Deregulated expression of *RanBP1* affects the cell division cycle at several stages

We have asked whether perturbing the regulated pattern of *RanBP1* expression would disrupt cellular functions that are under the control of the Ran signalling network. In our experiments, expression of *RanBP1* from the CMV-driven construct yielded high protein levels during improper cycle phases; although the overall protein abundance in pRanBP1-transfected cells was not significantly higher than that of the endogenous protein in S-G_2/M phases, the pattern of phase-

specific accumulation was completely disrupted. Constitutive expression is unlikely to have altered the cytoplasmic localization of RanBP1, which is dependent upon a nuclear export signal identified by deletion mapping analysis (Richards et al., 1996). Thus, any alteration in the phenotype of pRanBP1-transfected cells can be expected to derive from loss of phase-specific activity of RanBP1, rather than reflecting gross quantitative alterations or mislocalization of the protein.

The present results show for the first time that progression of the cell division cycle is altered following deregulated expression of *RanBP1*. One obvious defect following forced *RanBP1* expression in growth-arrested cells that were subsequently stimulated to reenter the cycle was inhibition of S phase onset. In cultures released from the G_1/S hydroxyurea arrest, replication did initiate in a proportion of cells, yet its further progression was impaired. A major defect was reflected by the persistence of G_2/M cells upon forced *RanBP1* expression during serum starvation, indicating that completion of the previous division had been impaired. In addition, pRanBP1-transfected cells arrested in metaphase by nocodazole failed to complete the mitotic division after nocodazole removal. These results suggest that regulated *RanBP1* expression is important for the complex of reactions that lead to mitotic exit. We also observed aberrantly condensed nuclei, reminiscent of the aberrant nuclei observed in murine cells overexpressing the *Spa1* gene, which encodes a putative RanGAP (Hattori et al., 1995). In our experiments, aberrantly condensed nuclei were associated with the expression of MPM-2 phosphoepitopes, which are specifically generated at the G_2/M transition. MPM-2 reactivity indicates that the aberrant condensation reflects a defect occurring during mitosis. The defective chromatin decondensation persisted after the mitosis-to-interphase transition, yielding aberrantly condensed nuclei in post-mitotic cells. The defects described here, i.e. defects in DNA replication, impaired mitotic completion and failure of chromatin decondensation during the mitosis-to-interphase transition, might be interconnected: the impairment in DNA replication in pRanBP1-transfected cells might in fact reflect a structural hindrance of pyknotic nuclei to undergo replication, rather than defects in the replication machinery per se.

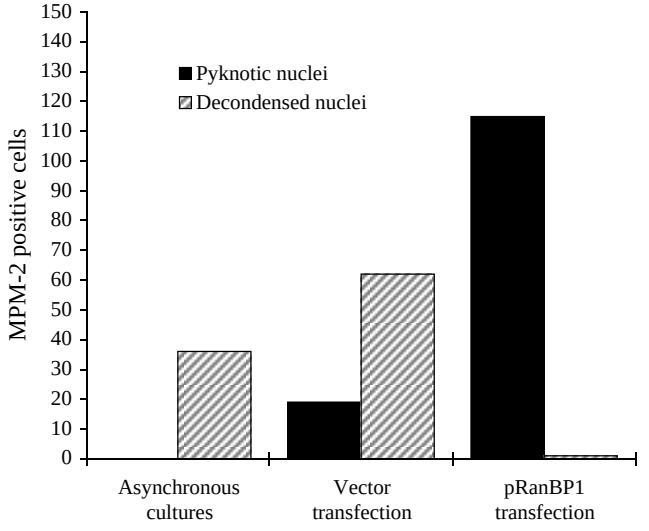

Fig. 9. Distribution of pyknotic (black histograms) and decondensed (hatched histograms) nuclei among MPM-2 reactive cells. MPM-2 immunostaining was carried out in asynchronously cycling control cultures, or in cultures transfected with vector or with pRanBP1 construct and subsequently maintained in low serum for 48 hours to induce growth-arrest. DNA was counterstained with PrI to assess chromatin condensation. 2,000 cells were scored in each sample.

Possible mechanisms underlying the defects associated with forced *RanBP1* expression

All identified mutations either in the Ran GTPase itself, or in its partners, have been found so far to inhibit the biological processes that are under Ran control. That has led to the assimilation of the Ran system with the Rab-mediated signalling system that regulates vesicular sorting, where the actual switch between the GTP- or GDP-bound form constitutes the signal: the signalling activity is therefore primarily dependent upon the rate of nucleotide turnover.

A simple interpretation of the present results is that forced expression in cells in which the *RanBP1* gene is normally inactive prevented the signalling activity by forcing the Ran molecule into the GDP-bound form at inappropriate cell cycle phases. Interestingly, expression of a non-hydrolyzable, i.e. GTP-locked, Ran mutant also caused cell cycle arrest, as expected in the framework of the Rab paradigm, though different cell cycle transitions were specifically affected: cells expressing the non hydrolyzable Ran were resistant to the hydroxyurea block, progressing towards G_2/M, and showed a predominant G_2 block with a concomitant inhibition of mitotic onset (Ren et al., 1994).

A very similar phenotype to that described here, including hypercondensed chromatin and post-mitotic arrest, was observed in yeast strains defective in the RCC1 homologous *pim-1* gene (Sazer and Nurse, 1994), and was attributed to defects in the distribution of nuclear and cytoplasmic components upon reformation of the nuclear envelope in daughter cells (Demeter et al., 1995). *RCC1* genetically interacts with genes encoding mitotic proteins and regulators, the best characterized of which include the cdc25-C protein required for MPF activation, which prematurely localizes to nuclei upon *RCC1* loss of function (Seki et al., 1992); α-tubulin, whose conditional mutants determine cell cycle arrest and are suppressed by *RCC1* overexpression (Kirkpatrick and Solomon, 1994); and NuMA, which is involved in nuclear reassembly at terminal telophase and whose overexpression rescues the micronucleation phenotype observed in $RCC1^{ts}$ cells (Compton and Cleveland, 1993). These data converge to indicate that the Ran/RCC1 network regulates several aspects of mitotic division.

Studies in mammalian (Bishoff et al., 1995) and *S. cerevisiae* (Noguchi et al., 1997) cells show that RCC1 activity is inhibited by RanBP1. It is tempting to speculate that deregulated *RanBP1* expression in our experimental conditions unbalanced the Ran network during specific cycle phases in a manner equivalent to that resulting from *RCC1* loss-of-function mutations. Further support to this hypothesis comes from the interesting finding by Matynia et al. (1996) that very similar phenotypes are generated in *S. pombe* by either *pim1* loss-of-function, yielding to loss of RCC1, or by overexpression of the *rna1* gene encoding RanGAP: since RanGAP is activated by RanBP1, it is consistent that RanGAP overex-

pression and RanBP1 deregulated expression generate similar phenotypes, both of which mimick RCC1 loss-of-function.

The present experiments do not address whether RanBP1 affects the cell cycle directly, or whether the effects are mediated by alterations in nuclear transport. Recent evidence implicate RanBP1 in nuclear import (Lounsbury et al., 1996; Chi et al., 1996). If deregulated *RanBP1* expression perturbed the transport activity and/or the subcellular localization of molecules, the cell cycle abnormalities reported here might arise as a consequence of a primary effect of RanBP1 on scheduled transport of cell cycle regulators; in this respect, it is interesting that yeast mutant strains defective in protein import arrest at the G_2/M phase, suggesting that the import of particular regulators is crucial for mitotic onset (Loeb et al., 1995).

In conclusion, the results reported here indicate that the molecular balance established among components of the Ran network at any particular cell cycle phase is crucial for proper signalling. Disruption of phase-specific control of RanBP1 levels generates cell cycle abnormalities similar to those seen in the absence of RCC1, or in the presence of abnormally high levels of Ran GAP.

We are grateful to our colleagues M. Fiore, G. Gensabella and R. Mangiacasale for help with flow cytometry analysis, to F. D'Ottavio for technical assistance and to G. Bonelli for photography. We also acknowledge S. Bonaccorsi, P. Dimitri, M. Gatti, G. Piaggio, A. Sacchi and F. Tatò for helpful discussions and suggestions on this work. The work was supported by the European Community (contract BMH4-CT96-1529), the Italian Association for Cancer Research (AIRC) and CNR Progetto Strategico 'Cell cycle and Apoptosis'. A.B. and G.G. were supported by grants from the MURST Ministry of Public Education; C.P. was supported by a fellowship from Fondazione A.Buzzati-Traverso; G.D.M. is an AIRC research fellow.

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(Received 23 May 1997 - Accepted 28 July 1997)