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Ribavirin-induced resistance to heat shock, inhibition of the Ras–Raf-1 pathway and arrest in G_1

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Abstract

Ribavirin [1-(β-D-ribofuranosyl)1,2,4-triazole-3-carboxamide (virazole)], a specific inhibitor of inositide 5′-monophosphate dehydrogenase (IMPDH), induces a strong depletion of GTP pools in IGR39 cells. After a 3-day treatment, the cell cycle was reversibly arrested in G_0/G_1, suggesting the involvement of GTP in the cell cycle process. The reduction of the GTP cell content modified the appearance of the microtubule network, as examined using immunofluorescence. However, the dynamics of repolymerisation were not altered. When arrested in G_0/G_1, cells displayed a surprising resistance to a 3-h period of heat shock at 45°C. Considering the lack of coimmunoprecipitation of p21ras with Raf-1, the reduction of the level of GTP-associated p21ras and the decrease of the activation of the extracellular signal-regulated protein kinases (ERK), also known as mitogen-activated protein (MAP) kinase, in ribavirin-treated cells, we suggest a possible relationship between the expression of heat-shock proteins and the change, in GTP-depleted cells, of the regulation of Raf kinase by ras protein.

Keywords: Ribavirin; Ras; Raf-1; Cell cycle; Heat shock; Extracellular signal-regulated protein kinase; GTP

1. Introduction

Ribavirin or 1-(β-D-ribofuranosyl)1,2,4-triazole-3-carboxamide (virazole) is known as an inhibitor of GTP renewal (Streeter et al., 1973) and this effect provides this drug with its antiviral properties (Potter et al., 1976). Indeed, ribavirin is a specific inhibitor of inositide 5′-monophosphate dehydrogenase (IMPDH) (Streeter et al., 1973; Potter et al., 1976), the rate-limiting enzyme of the de novo GTP biosynthesis.

Ribavirin was used for its antiguanosine properties in order to study the consequences of GTP storage depletion (Hata et al., 1993; Weber et al., 1991). Analogues of ribavirin have also been described, including tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide), which is a strong inhibitor of IMPDH (Weber et al., 1991) through its conversion to the active metabolite, thiazole-4-carboxamide adenine dinucleotide (TAD). The anticancer activity of IMPDH inhibitors was first studied with tiazofurin, which induced erythroid differentiation (Olah et al., 1988), and ribavirin-induced megakaryocytic leukaemia cell differentiation has also been reported (Majumdar et al., 1995). Generally, an increase in differentiation markers has been described, and this is accompanied by a reduction of the proliferative capacity of cells such as, for example, the megakaryocytic leukaemia cell line (Majumdar et al., 1995). Tiazofurin-induced apoptosis in myeloid lineage and lymphoblastoid cells is linked to a decreased GTP level (Catapano et al., 1995; Jayaram et al., 1993; Knight et al., 1987; Olah et al., 1996; Tricot et al., 1990; Weber et al., 1999). Depending on the cell type, ribavirin and tiazofurin induce either a delayed progression of the cell cycle (Peavy et al., 1980) or apoptosis (Jayaram et al., 1999; Luchetti et al., 1998). Some results suggest that ribavirin modifies signal transduction involving p21ras (Hata et al., 1993).

The effects of tiazofurin have been studied on chemotherapy-induced differentiation and down-regulation of Ki-

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ras and c-*myc* oncogenes (Olah et al., 1990). Tiazofurin was synergistic in causing differentiation with ribavirin, retinoic acid, and difluorodeoxycytidine (Weber et al., 1994).

As expected, in view of the leading role of GTP in this mechanism, ribavirin was found to inhibit protein synthesis. More recently (Weber et al., 1999), tiazofurin has been reported to reduce the inositol triphosphate (IP_3) concentration through both a reduction of phosphatidylinositol (PI) and phosphatidylinositol phosphate (PIP) kinase activities and a decrease of the concentration of GTP, which is required for phospholipase C (PLC) action. This result shows that IMPDH inhibitors can influence a variety of GTP-involved cellular processes, including some signal transduction pathways.

The function of GTP-regulated proteins is ubiquitous (Hall, 1990). A range of cellular processes is dependent on the activity of these proteins, some of which are known as 'small G proteins' because they display intrinsic GTPase activity. Different stages of protein synthesis involve accessory ribosomic factors, which regulate themselves, their conformation and activity owing to GTP/GDP exchange and GTP hydrolysis. The Ran factor, belonging to the small G protein family, is involved in the nucleocytoplasmic transport of RNA from nucleus to cytoplasm. Other small G proteins such as Rho and Rab are involved in the perpetual remodeling of the actin cytoskeleton and in the intracellular transport occurring during protein recycling, respectively (Zerial and Stenmark, 1993). The mechanism of transduction of various messages, such as signaling from epidermal growth factor (Kaziro et al., 1991; Thomas et al., 1992; Wood et al., 1992) or extracellular matrix (Clark and Hynes, 1996) involves the first described small G protein: Ras. Polymerisation or depolymerisation of tubulin subunits and the dynamics of the microtubular cytoskeleton also involve GTP (Drechel and Kirschner, 1994).

As the cell cycle is regulated through extracellular signals which induce, within the cell, activation of GTP-regulated proteins (Satoh et al., 1992), it is conceivable that IMPDH inhibitors interfere with various stages of the cell cycle owing to depletion of the cellular GTP content.

Previously, we have established, using the IGR39 cell line, that ribavirin induces a strong reduction of the GTP level in these cells (Fouchier et al., 1996). However, this diminution appears not to be sufficient to modify the signal transduction induced via trimeric G-proteins which follows either the binding of the neurotransmittor vasoactive intestinal peptide (VIP) to its receptor in cells of neural origin or the binding of thyrotropin and subsequent activation of thyroid cells (Aublin et al., 1986).

Since GTP is also required for microtubule dynamics, another potential target for IMPDH inhibitors is the cytoskeleton. To our knowledge, no information concerning this possible target is available. As in tumour cells, the centrosome, which organises the microtubule network, has been reported to have a low sensitivity to heat shock, which may account for the relative refractoriness of some tumour cell lines to heat shock. Consequently, we speculated that the ribavirin-induced reduction of GTP level and growth inhibition may abolish this refractoriness and led to cell death. Since the result appeared opposite to that expected, i.e. ribavirin protected IGR39 melanoma cells against heat shock, the relationship between ribavirin-induced GTP diminution, cell cycle arrest, cytoskeleton redistribution, modulation of heat-shock proteins and alteration of the transduction of growth signals was examined and a possible mechanism is discussed.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco/BRL (Cergy-Pontoise, France) and foetal calf serum from Interchim (Montluçon, France). Ribavirin was a gift of ICN Pharmaceuticals (Syntex, Puteaux, France). Vinblastine, phosphate-buffered saline (PBS), phenylmethylsulfonyl fluoride, iodoacetamide, aprotinin, leupeptin, peptatin, *o*-phenanthroline, RNAse (Type I-A), propidium iodide were obtained from Sigma (L'Isle d'Abeau, France). The bicinchoninic acid (BCA) protein assay reagent was from Pierce (Interchim, Montluçon, Fr.). [^{35}S]-Translabel was from ICN (Orsay, France) and carrier-free [^{32}P]-orthophosphate from Amersham (Buckinghamshire, UK).

Mouse monoclonal anti-α-tubulin, fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody and the ECL immunodetection system were obtained from Amersham (Buckinghamshire, UK). Mouse monoclonal anti-Hsp70 (Stress Gen), anti-Hsp90 (heat-shock protein), rat monoclonal anti-H-Ras (259) and rabbit polyclonal anti-Raf-1 (C-20) and control peptides [H-Ras(259), Raf-1(C-20)] of Santa Cruz Biotechnology were obtained from Tebu (Le Perray, France). The Y13-259v-H-ras rat monoclonal antibody was obtained from Oncogene Research Products (Genzyme, Pontoise, France) and rabbit anti-active extracellular signal-regulated protein kinases (ERK) was from Promega France (Charbonnières, France). Recombinant protein A or protein G coupled to agarose beads was from Boehringer Mannheim (Meylan, France). Protein A and recombinant protein G conjugated to peroxidase were obtained from Sigma.

The ApopTag in situ apoptosis detection kit was obtained from Oncor (Appligene/Illkirch, France).

2.2. Cell culture

Human melanoma IGR39 cells (Fouchier et al., 1996) were maintained routinely in DMEM medium supple-

mented with 10% (v/v) foetal calf serum. To cells that had reached 50–70% confluence, ribavirin was added at a final concentration of 100 μM for 3 days. When tested, vinblastine (10 nM) was added for the last 18 h. The medium was removed and replenished daily. The viability of IGR39 cells was assessed for each condition by Trypan blue exclusion and at least 200 cells were counted.

2.3. Flow cytometric measurements and cell cycle analysis

Cells (50–70% confluence) were trypsinised and fixed in cold 70% methanol. Cells were then washed with PBS, centrifuged and resuspended for 30 min at 37°C in PBS containing 60 μg RNAse/ml. Before analysis, the cells were stained with 20 μg propidium iodide/ml. Measurements were performed using a FACScan flow cytometer. Percentages of cells in the G_0/G_1, S, and G_2/M cell-cycle phases, respectively, were determined using the mathematical method of Fried (1976).

2.4. Immunofluorescence microscopy

The method was as described by Garcia et al. (1994). Briefly, cells (50–70% confluence) cultured on a Nunc Lab-Tek chamber slide were fixed with formaldehyde (3.7% in PBS) for 10 min at room temperature and with cold methanol (−20°C, 70% in PBS). Then the cells were incubated with cold acetone (−20°C) for 30 s. After three washes with PBS, the cells were incubated with mouse monoclonal anti-α-tubulin (1/400 dilution with PBS-1% (w/v) bovine serum albumin (BSA)) for 1 h at 37°C. After PBS washes, the FITC-conjugated anti-mouse antibody (1/20 dilution with PBS-1% (w/v) BSA) was added for a 45-min incubation at 37°C, then the cells were rinsed with PBS, the antifading agent mowiol was added and coverslips were mounted on glass slide. The cells were viewed with a Nikon Diaphot Photomiscroscope (objective, ×40).

2.5. Heat-shock treatment and quantification of apoptotic cells

For heat-shock experiments, cells (50–70% confluence) were incubated in DMEM medium containing 10% (v/v) FCS at 45°C for a short (30 min) or a long duration (3 h) in an atmosphere of 5% CO_2. After completion of the thermic stress, the culture medium was replaced by fresh medium prewarmed to 37°C, and culture was resumed according to the different assays.

For the measurement of apoptotic cells, the 'in situ apoptosis detection kit' (APOPTAG, fluorescein version) was used for the detection of DNA fragmentation. In this case, cells (50–70% confluence) were grown on coverslips, stained according to the manufacturer-recommended procedure, and then observed by both phase-contrast and epifluorescence microscopy. Total and stained cells were counted in four fields (around 200 cells at least/field).

2.6. Cell metabolic labeling

Cells were cultured to 50–70% confluence and then incubated for 1 h in methionine and cysteine-deficient DMEM medium supplemented with 10% dialysed foetal calf serum, and radiolabeled with 100 μCi/ml [^{35}S]-Translabel for 2 h. Before lysis, cells were washed three times with culture medium containing cysteine, methionine and 10% FCS. For labeling with [^{32}P] orthophosphate, cells were incubated for 3 h in phosphate-free DMEM medium supplemented with 10% dialysed FCS, and 40 μCi/ml carrier-free [^{32}P] orthophosphate.

2.7. Immunoprecipitation and immunoblotting

Cells were lysed at 4°C with (1 ml/10^6 cells) 10 mM HEPES (pH 7.3) containing 150 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 1 mM EDTA, phosphatase inhibitors (10 mM NaF, 25 mM β-glycerophosphate, 1 mM $NaVO_4$) and protease inhibitors (20 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin and 1 mM PMSF) and 20 μM $ZnCl_2$. The lysates (1 ml) were precleared with 10 μl of protein A- or G-agarose for at least 45 min at 4°C on a rocking platform. After centrifugation, supernatants were incubated in the presence or absence of control peptides (6 μg of H-Ras or Raf-1), with 1 μg of antibodies (anti-Raf-1, anti-H-Ras, anti-Hsp70 or anti-Hsp90) at 4°C for at least 18 h on a rocking platform. Then 10 μl protein A- or G-agarose was added for the last 45 min at 4°C. After five washes with the lysis buffer, the bound proteins were eluted by boiling the agarose beads in the presence of the sample buffer, resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) (8.5% acrylamide for the analysis of Raf-1, Hsp70 or Hsp90 or 20% acrylamide for Ras protein) and blotted onto nitrocellulose. Immunoblots were probed with either H-Ras, Raf-1, Hsp70 or Hsp90 antibody followed by incubation with peroxidase-conjugated protein A or G. Detection was carried out using the ECL immunodetection system.

2.8. Analysis of p21ras bound GTP / GDP

Around 15×10^6 metabolically [^{32}P] labeled cells were lysed at 4°C in 50 mM HEPES buffer, pH 7.4, 1% Triton X-100, 100 mM NaCl, 5 mM $MgCl_2$, 1 mg/ml BSA, protease inhibitors (20 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin and 1 mM PMSF). Nuclei were removed by centrifugation at $15{,}000 \times g$ for 5 min and 0.5 M NaCl, 0.5% deoxycholate and 0.05% SDS were added. Immunoprecipitation was carried out for 1 h using antibody Y13-259 and then for 45 min in the presence of

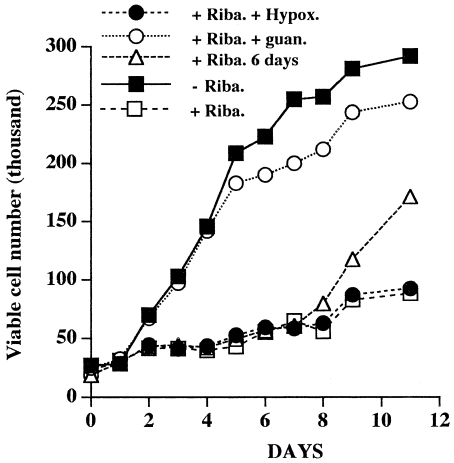


Fig. 1. IGR39 cell growth was inhibited by ribavirin. IGR39 cells were cultured for 11 days at 37°C in 24-well Petri dishes (around 20,000 cells/well) either without treatment (■) or in the presence of 100 μM ribavirin (●, ○, △, □) with 10 μM guanosine (○) or 10 μM hypoxanthine (●). Cell counting was performed every day. In some experiments, ribavirin was maintained only for 6 days (△). Each value reported is the mean of the cell count of three separate wells.

protein G-agarose. Immunoprecipitate containing Ras protein was washed seven times with 0.5 ml of buffer containing 50 mM HEPES pH 7.4, 500 mM NaCl, 5 mM $MgCl_2$, 0.1% Triton X100, 0.005% SDS. Bound nucleotides were eluted with 2 mM EDTA, 2 mM DTT, 0.2% SDS, 0.5 mM GDP, 0.5 mM GTP at 60°C for 30 min. Chromatographic separation of nucleotides was performed on PEI-cellulose plates run in 1.2 M ammonium formate and 0.8 M HCl. GTP and GDP spots were visualised and marked under UV light. The plate was autoradiographed and the regions corresponding to GTP and GDP spots were recovered by scraping and [^{32}P] radioactivity was determined by liquid scintillation spectrometry. Finally, the ratio of GTP- to (GTP + GDP)-associated radioactivity was calculated.

2.9. Detection of the activated ERK

Cells cultured for 3 days in the presence or not of ribavirin (100 μM) were harvested, washed with PBS and lysed in boiling 63 mM Tris–HCl (pH 6.3) buffer containing 10% glycerol, 4% SDS, 1 mM orthovanadate and proteinase inhibitors. After centrifugation at 15,000 × *g* for 5 min, supernatant proteins (60 μg) were analysed by 10% acrylamide SDS-PAGE and electrotransferred onto nitrocellulose sheet. Immunoblots were probed with anti-active ERK followed by peroxidase-conjugated anti-rabbit antibody. Detection was performed using the ECL immunodetection system.

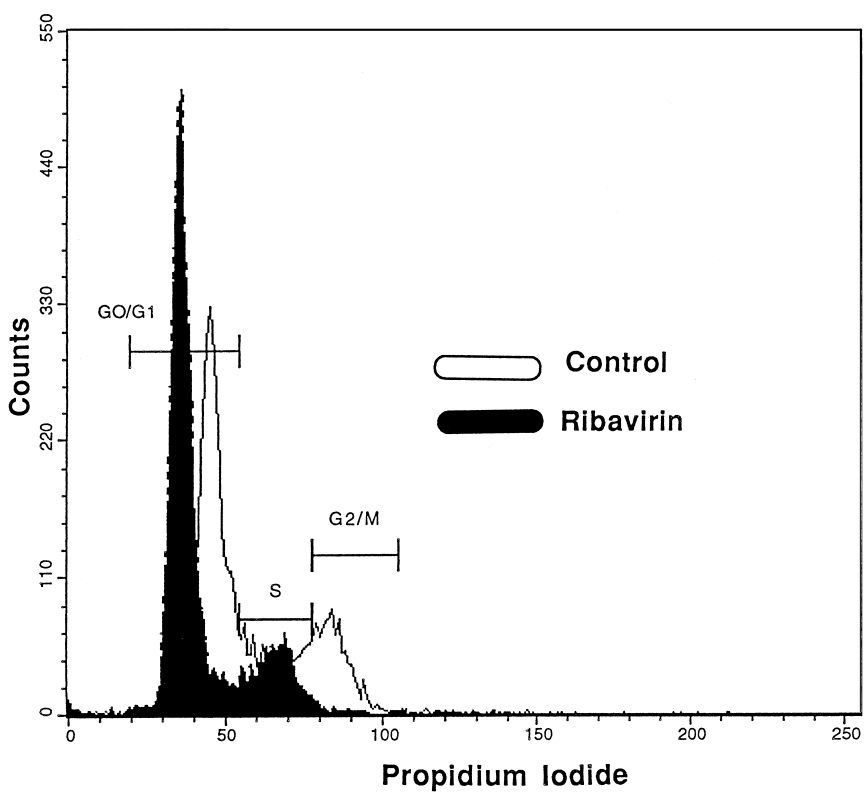


Fig. 2. Cytofluorometry analysis of the cell cycle of IGR39 cells. Cells were treated or not with 100 μM ribavirin for 3 days and stained with propidium iodide. Flow cytometric analysis was performed as described in Section 2.

Table 1
Effect of culture conditions on the cell cycle

Condition	Percentage of cells in the different phases of the cell cycle		
	G_0/G_1	S	G_2/M
Control	56.0	24.3	19.1
Ribavirin (100 μM, 3 days)	78.9	18.1	2.4
Without serum (3 days)	79.4	7.7	9.8
Vinblastine (50 nM, 18 h)	8.5	12.1	66.5

IGR39 cells were cultured for 3 days in the presence or absence of ribavirin (100 μM), without serum or in the presence of vinblastine for the last 18 h of culture.
Flow cytometric measurement of the percentage of cells present in the different phases G_0/G_1, S, and G_2/M of the cell-cycle was carried out. Calculation was performed using the mathematical method of Fried (see Section 2).

2.10. Quantification of protein

Protein concentration was determined using the BCA protein assay reagent as described by Brown et al. (1989) (with BSA as a standard).

3. Results

Previously, we have shown that ribavirin, when added for 3 days to an IGR39 cell culture, induced a strong decrease in intracellular GTP pools (Fouchier et al., 1996). The residual GTP concentration was about 30% of the normal level as measured using high pressure liquid chromatography (Fouchier et al., 1996). In contrast, no modification of ATP metabolism was detected. In the present work, we investigated the consequence of the reduction of the GTP level on the growth of IGR39 cells. Indeed, a number of cellular mechanisms related or not to cell division requires GTP synthesis or degradation.

3.1. Analysis of cell growth and flow cytometry

As shown in Fig. 1, cell growth was arrested within h following the beginning of ribavirin treatment. This effect was inhibited by addition of guanosine, which restores GTP synthesis through the de novo pathway for guanine nucleotides (Johnson and Mukku, 1979). Hypoxanthine, which does not enter this pathway, was ineffective in restoring both the GTP level and cell growth. These obser-

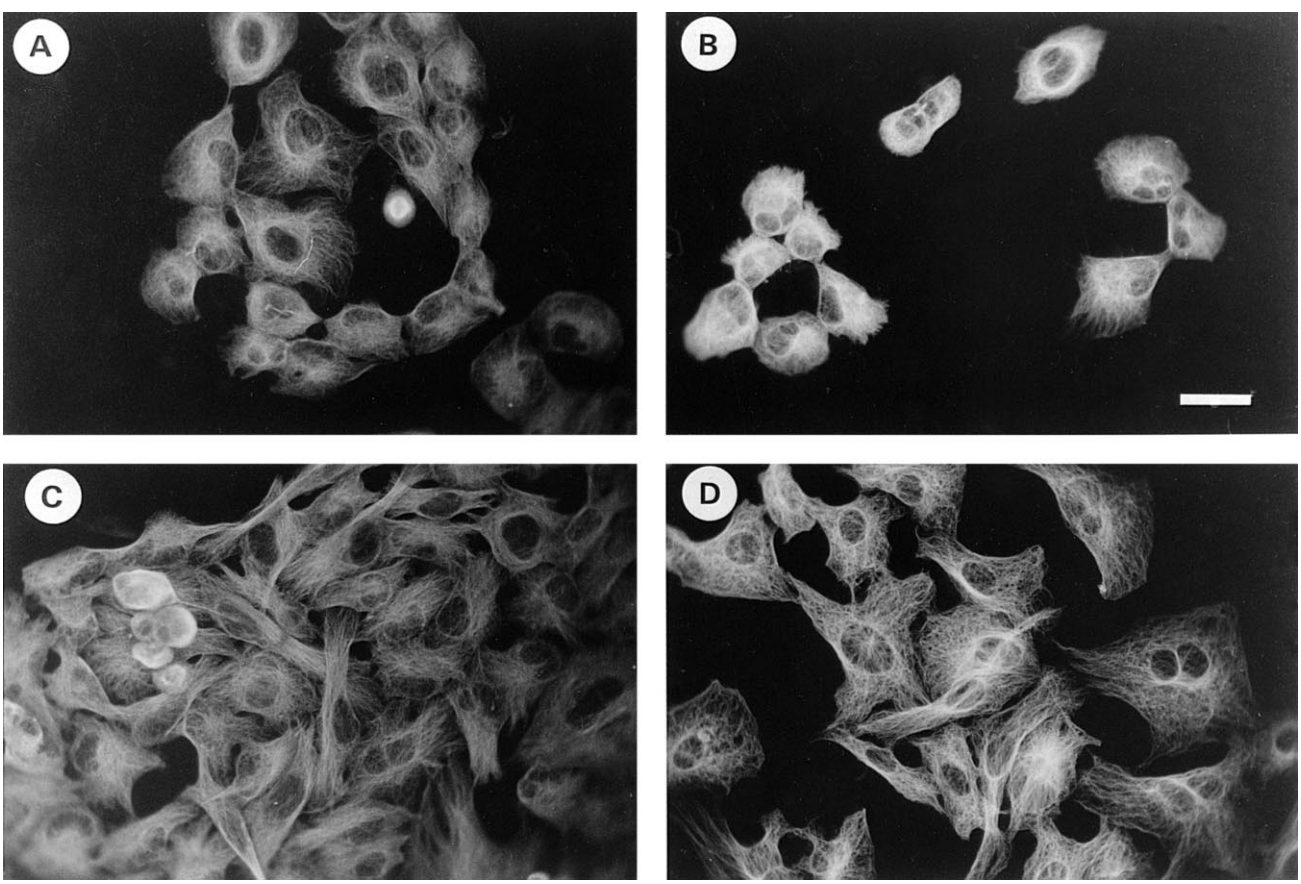


Fig. 3. Pattern of the microtubule network of ribavirin-treated IGR39 cells. Cells were cultured for 3 days with (B, D) or without 100 μM ribavirin (A, C), on Lab-tek chamber slides. For cold shock, cultures were placed at 4°C for 2 h and thereafter incubated at 37°C for 2 days in the same medium (C, D). (A, C) were left at 37°C during the whole experiment. Finally, cultures were fixed and immunofluorescence detection was performed using an antitubulin antibody as described in Section 2.

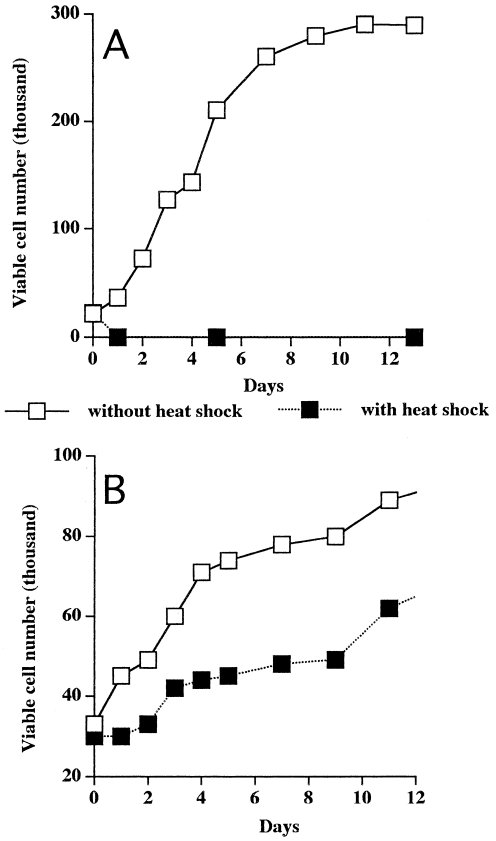


Fig. 4. Ribavirin-induced resistance of IGR39 cells to heat shock. Cells were cultured in the presence (B) or in the absence of ribavirin (A) at 37°C for 3 days. Some of the culture flasks were submitted (day 0) to heat shock at 45°C for 3 h (■). Then, new ribavirin-free medium was added and cells were returned to 37°C and culture was continued up to day 13. The number of cells in four wells was determined each day. Non-heat shocked cells (□).

vations showed that the ribavirin-induced growth inhibition was actually the result of the decrease in the intracellular GTP level through the blockade of GTP synthesis.

The reversibility of cell growth after a 3-day ribavirin treatment was investigated by either ribavirin removal or guanosine addition. In both cases, we found that cell growth resumed, denoting that the drug-induced modifications were totally reversible. Thereafter, following cessation of growth inhibition, the global rate of cell division was not constant: periods of high and low rates of cell division alternated, suggesting that ribavirin treatment caused synchronisation of the cell cycle. This assumption was verified using flow cytometry. These experiments showed that, following a 3-day ribavirin treatment, the cell cycle was blocked in the G_0/G_1 phase (Fig. 2). Controls, using cells cultured in the presence of vinblastine or in the absence of serum, showed arrest typically in G_2/M and G_0/G_1, respectively (Table 1).

3.2. Microtubular network

As GTP is involved in the mechanism of tubulin polymerisation and depolymerisation, one could consider that a possible action of ribavirin is at the microtubule level. Indeed, some antimitotic drugs are well known to impede microtubule dynamics and to block cell division as a result. However, usually these drugs block the cell cycle at the G_2/M stage. Though no evident alteration of normal spreading was noticed in ribavirin-treated cells, partial depolymerisation of the peripheral network was frequently observed with, apparently, some perinuclear condensation, as evidenced by immunofluorescence staining of the microtubular network (Fig. 3A and B). Ribavirin did not modify the time course of microtubule assembly during the recovery that followed a cold shock (Fig. 3C and D).

3.3. Heat shock

A number of papers have reported a low sensitivity of the centrosome to heat shock of tumour cell lines (Barrau et al., 1978; Debec, 1991; Malawista et al., 1983; Marcaillou et al., 1993). Since the centrosome is an organelle involved in cell division, this low sensitivity may account for the relative refractoriness to heat shock of some tumour cell lines (Barrau et al., 1978; Dewhirst, 1990; Marcaillou et al., 1993;). We have speculated that ribavirin-induced growth inhibition may modify this refractoriness.

IGR39 cells were especially resistant to heat shock at 45°C for 30 min but they were killed when the time of culture at this high temperature was extended to 3 h (Fig. 4A). Under these conditions, unexpectedly, pretreatment with ribavirin conferred resistance to long-lasting heat shock (45°C, 3 h) in IGR39 cells (Fig. 4B). Around 62% of cells remained viable and returned to division activity in the days following heat shock and ribavirin removal (Table 2). When blocked, heat-shocked cells did not suffer lethal damage and began to grow and proliferate again when DNA synthesis was allowed to resume.

This surprising observation suggested to us that it was the delay in mitosis (i.e. the failure to enter into S phase), occurring in response to the ribavirin treatment, which protected the cells from the irreversible effects of the high temperature treatment. As ribavirin-treated cells were blocked in G_1, one could conjecture that cells are espe-

Table 2
Effect of heat shock on cell viability

Heat shock (45°C, 3 h)	% Viable cells	% Viable cells	% Apoptotic cells
	−	+	+
Control	98.1	0.0	45.2 ± 9.3 ($n = 4$)
Ribavirin (100 μM, 3 days)	97.9	62.7	4.7 ± 1.1 ($n = 4$)

IGR39 cells were cultured for 3 days in the presence or absence of ribavirin (100 μM) and incubated at 45°C for 3 h. Subsequently, cell viability was determined using Trypan blue exclusion or cells were treated in order to measure cell apoptosis by DNA fragmentation determination as described in Section 2.

cially sensitive to a high temperature during any of the stages of mitosis subsequent to G_1 (i.e. S, G_2 or M).

However, the withdrawal of serum from the cell culture medium did not prevent the cell death caused by long-lasting heat shock (data not shown). Flow cytometric analysis of serum-deprived cells displayed a cell cycle arrest in G_1, suggesting that other processes in addition to the failure of cells to reach S phase have to be considered in order to explain the protective action of ribavirin.

The expression of Hsp proteins (Hsp70 and Hsp90) was analysed in control and heat-shocked cells treated or not with ribavirin and labeled with [^{35}S] methionine. Detection was carried out using both immunodetection and autoradiography. As expected, heat shock strongly enhanced Hsp70 biosynthesis (Fig. 5C), while decreasing the general level of protein synthesis (data not shown). Under heat-shock conditions, when cells were incubated in the presence of ribavirin, a slight further increase of both radiolabeled and immunodetectable Hsp70 was observed (Fig. 5A and C). We conclude that ribavirin enhanced Hsp70 renewal in cells submitted to heat shock. Surprisingly, after heat shock both the total cell content and biosynthesis rate of Hsp90 were reduced in cells not treated with ribavirin (Fig. 5B and D), while the presence of ribavirin prevented the heat shock-induced decrease of immunodetectable and radiolabeled Hsp90 (Fig. 5B and D). In conclusion, we believe that the treatment with ribavirin allowed the preservation of both the cell content and biosynthesis of Hsp90.

The rate of apoptosis induced by the heat shock was investigated by measuring the level of DNA fragmentation and this was compared to cell survival evaluated by the Trypan-blue exclusion method (Table 2). Following heat shock (45°C, 3 h), the number of cells engaged in the apoptotic process was strongly enhanced as compared to that of non-heat shocked cells. The number of apoptotic cells found in ribavirin-treated cultures was only slightly increased in response to the heat shock. Consequently, we assumed that heat shocked cells were killed by an apoptosis-dependent process and that ribavirin impeded the rate of programmed cell death. Therefore, ribavirin did not act as a standard microtubule inhibitor because, usually, these compounds induce arrest in G_2/M and cause apoptosis. For example, during a 20-h treatment, cells exposed to taxol have been reported to undergo DNA fragmentation characteristic of apoptotic cell death (Bhalla et al., 1993; Donaldson et al., 1994; Liu et al., 1994).

3.4. Analysis of Ras–Raf-1 complex

An alternative way by which a decrease of cellular GTP content could regulate the cell cycle engine is at the level of the growth factor-induced transduction signals, in which G proteins are involved. In order to examine this point, we analysed using immunoprecipitation and immunoblotting the association of p21ras and Raf-1 kinase. Beforehand, control experiments were carried out: these showed that

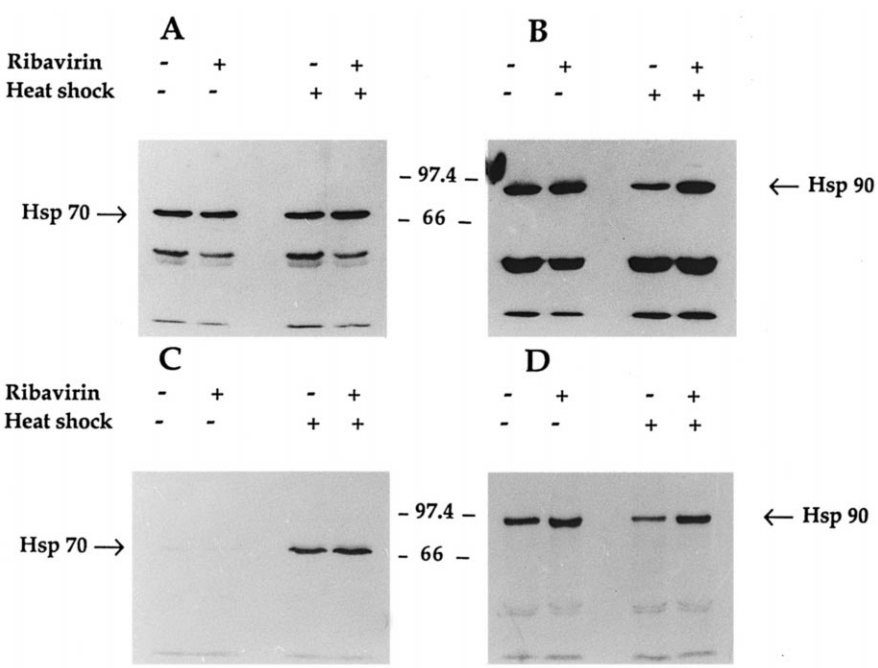


Fig. 5. Analysis of heat-shock proteins (Hsp) of heat-shocked IGR39 cells. Cells were cultured for 3 days in the presence or absence of ribavirin (100 μM) and then the cultures were incubated at 45°C for 30 min. The [^{35}S]-translabel was added for the last 15 min of thermic shock and the culture was returned to 37°C for 2 h. After cell lysis, extracts were immunoprecipitated with anti-Hsp70 (A, C) or anti-Hsp90 (B, D) antibody. Immunoprecipitates were analysed by immunoblotting. Blots were probed using either the anti-Hsp70 (A) or anti-Hsp90 antibody (B). Immunoreactivity detection (A, B) and autoradiography (C, D) were performed as described in Section 2.

the total cell content of p21ras and Raf proteins was not altered in ribavirin-treated cells (Fig. 6A and B). As shown in Fig. 6C, immunoprecipitation of Raf proteins was carried out using a specific antibody directed against this protein and p21ras was detected by immunoblotting in the same immunoprecipitate. However, when the immunoprecipitate was from ribavirin-treated cells, the coimmunoprecipitation of p21ras with Raf protein was strongly reduced, as revealed by immunoblotting (Fig. 6C). The identity of the band was confirmed using the peptide against which anti Raf-1 antibody is directed. When this peptide was added to the antibody before the immunoprecipitation, there was either a reduction or disappearance of the coimmunoprecipitate material that had a 21 kDa MW and was detected with the antiRas antibody (Fig. 6C).

The strong reduction of the association between Raf and Ras proteins in ribavirin treated cells was attributed to a change in p21ras affinity for Raf. This change could be induced by ribavirin treatment. Indeed, it has been established that p21ras must be in an activated state, linked to GTP, in order to efficiently bind the Raf kinase (Avruch et al., 1994; Moodie et al., 1993).

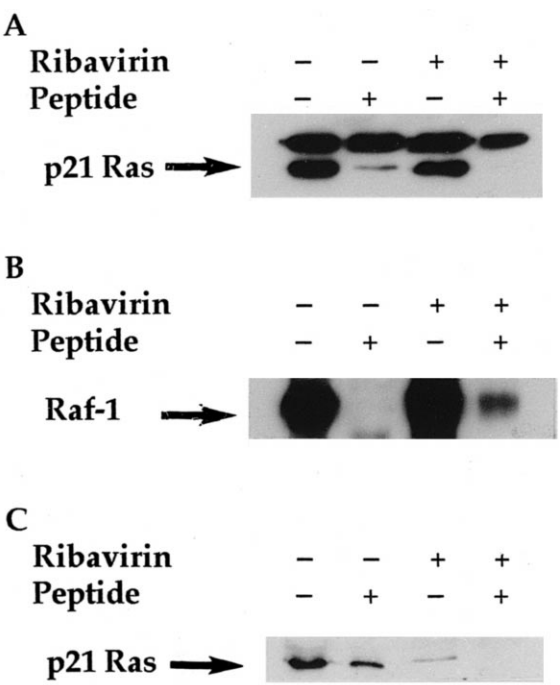
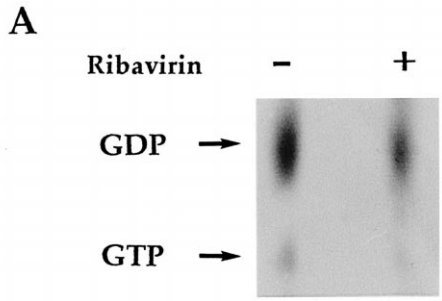


Fig. 6. Detection of Ras and Raf-1 proteins. IGR39 cells were cultured for 3 days in the presence or absence of ribavirin (100 μM). Then, the cells were lysed and immunoprecipitation using anti-Ras or anti-Raf-1 antibody was performed with or without a specific peptide (6 μg) blocking either anti-Raf- or anti-Ras-directed immunoprecipitation. Immunoprecipitates were analysed by immunoblot as described in Section 2: immunoprecipitation and probing with an anti-Ras antibody (A); immunoprecipitation and probing with an anti-Raf-1 antibody (B); immunoprecipitation with an antiRaf-1 antibody and probing with an anti-Ras antibody (C).



B

Ribavirin	-	+
GTP/(GTP + GDP) n = 3	0.33 ± 0.008	0.176 ± 0.005

Fig. 7. Determination of p21ras bound GTP. IGR 39 cells were cultured for 3 days in the presence or absence of ribavirin (100 μM). Cells were [^{32}P] labeled, then lysed and immunoprecipitation was performed with the Ras Y13-259 antibody and with protein G-agarose as described in Section 2. The separation of Ras-associated nucleotides was carried out on PEI-cellulose plates. Autoradiography and radioactivity detection were as described in Section 2. A representative autoradiograph is presented (A) and the ratio of counts found in GTP versus GTP + GDP spots was calculated (B). The value represents the mean ± S.D. of three independent experiments.

3.5. Determination of p21ras bound GTP

The possible ribavirin-induced changes in the ratio between activated vs. basal states of p21ras was checked as follows: the GTP and GDP content of p21ras of control and Ribavirin-treated cells was measured in cells previously labeled using [^{32}P] orthophosphate. Immunoprecipitation of p21ras was performed using an antibody, which allows the nucleotide to remain bound to the Ras protein during the antigenic reaction. The complex p21ras/nucleotide was harvested and submitted to chromatography in order to separate p21-bound GTP and GDP. As expected, radioactivity incorporated in both nucleotides was lower in ribavirin-treated cells (Fig. 7A). In addition, the ratio of GTP- vs. GDP-associated radioactivity was lower in ribavirin-treated cells (Fig. 7B), suggesting a reduction of the level of the p21ras-activated state in these cells.

3.6. Detection of activated ERK

It is well known that activation of Raf protein kinase induces phosphorylation and activation of mitogen-activated protein (MAP) kinase kinase, which in turn phosphorylates and activates ERK. Using an antibody directed against the activated form of ERK, it was found that ERK was constitutively activated in IGR39 cells incubated without additional stimulus except foetal calf

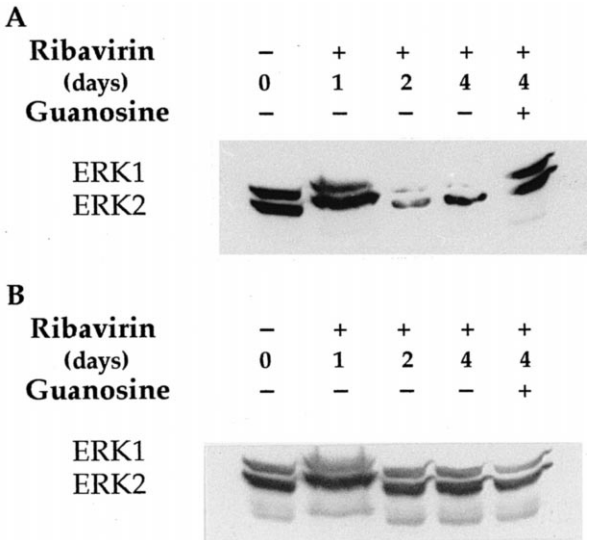


Fig. 8. Detection of activated ERK (A) and total ERK (B). IGR39 cells were cultured in the absence (day 0) or in the presence of 100 μM ribavirin (days 1, 2 and 4). Cells were lysed and the protein content was measured. Proteins were analysed by immunoblotting as described in Section 2 using an antibody directed against activated ERK. After stripping, the membrane was reprobed using an anti non-activated ERK antibody.

serum (Fig. 8). The extent of this activation was significantly lowered in cells treated with ribavirin for 2 or 4 days (Fig. 8). No reduction was detected in the case of a short treatment for 1 day. As expected, addition of guanosine to the medium abolished the effect of ribavirin on ERK activity. When probed with an anti non-active ERK antibody, the same blot displayed a similar ERK band intensity in each track, showing that no change in ERK expression was detectable during the time course of the experiment.

A faster migrating band was also detected, the apparent MW of which indicated that the band could correspond to p38 kinase isoforms. Note that the anti phospho-ERK-derived signal corresponding to this protein was weak and disappeared after ribavirin treatment for 2 or 4 days (Fig. 8).

4. Discussion

The present study shows that IGR39 cell proliferation was blocked in G_0/G_1 after a 1- to 3-day treatment with ribavirin. Guanosine was effective in preventing ribavirin action, thus providing evidence that the guanine nucleotides are critically involved in ribavirin-initiated cell cycle arrest. Hypoxanthine did not display such a salvage action, confirming that the ribavirin target was IMPDH, the rate-limiting enzyme of de novo GTP synthesis, in agreement with what is already reported concerning the overall impact of antipurine agents (Weber et al., 1994). The action of the drug on the cell cycle was totally reversible with no evident consequence on cell viability. Thus, strong depletion of GTP storage does not affect cell survival. In addition, upon ribavirin removal, cell growth resumed with a synchronisation of cell division.

Flow cytometric analysis of ribavirin-blocked cells displayed a pattern different from that observed on cells treated with drugs acting as microtubule inhibitors, suggesting an alternative action for ribavirin. It is likely that it was the G_1/S transition, which was impeded in the presence of this drug. However, the pattern was also different from that of thymidine-blocked cells (experiment not shown), which suggests that the growth arrest was not due only to an unavailability of guanine metabolite to sustain basal DNA synthesis. In fact, the pattern was more comparable to that displayed by serum-deprived-cells, suggesting that an alteration in the regulation of growth factor-dependent mitosis had taken place.

The function of tubulin is regulated by GTP and tubulin dimers can polymerise only when the β-subunit carries GTP. However, GTP hydrolysis is not required for microtubule assembly. Microtubules assemble from tubulin in the presence of slowly hydrolysed analogs of GTP, such as guanylyl methylenediphosphonate (GMPCPP) (Drechel and Kirschner, 1994), and grow at normal rates, but exhibit no dynamic instability. Indeed, the hydrolysis reaction is crucial only for the dynamic instability of microtubules: only those that contain GDP-tubulin can undergo a transition from growing to shrinking phase. Perhaps, the stability of microtubules is modulated by proteins that regulate the GTPase activity of tubulin in the polymer. Globally, the dynamic instability of microtubules is dependent on the availability of cellular GTP (Drechel and Kirschner, 1994).

Antitubulin agents induce modifications of the microtubule network: depolymerisation of microtubules with colchicinoids and Vinca alkaloids (Rowinsky and Donehower, 1991), or stabilisation of microtubules with formation of bundles (in interphasic cells) or abnormal mitotic asters with taxoids (Kumar, 1981; Schiff and Horwitz, 1980). These antimitotic drugs induce G_2/M arrest, which is correlated with their cellular toxicity (Horwitz, 1992). Neither of these patterns was observed in the presence of ribavirin. The partial depolymerisation of the peripheral network, together with the lack of modification of the time course of microtubule assembly during the recovery which follows cold shock, led us to conclude that a reduction of cellular GTP content modifies only the stationary state of the microtubular network in the vicinity of the cytoplasmic membrane, but does not affect the growing phase, nor probably the formation of mitotic spindles.

The particular sensitivity of some tumour cell lines to heat shock has been repeatedly reported (Dewhirst, 1990). Centrosome integrity and activity are especially sensitive to various stresses, including heat shock (Debec, 1991; Marcaillou et al., 1993). We speculated that a change in the GTP content of living cells can affect the activity of the organising centre of microtubule nucleation and its

response to heat shock and we were interested in testing this hypothesis.

Surprisingly, 62% of ribavirin-treated IGR39 cells appeared to be in some way safeguarded from the deleterious effects of heat shock. It has been known for a long time that yeast cells starved of nutrients (Murray and Hunt, 1993) or mammalian cells deprived of growth factors (Murray and Hunt, 1993) arrest early in G_1 in a state called G_0. Arrested cells display a decreased rate of protein synthesis and have often increased resistance to temperature extremes and other environmental stresses (Murray and Hunt, 1993). Entry into this specialised resting state can be thought of as a form of cell differentiation designed to ensure survival under adverse conditions. It can be hypothesised that such a phenomenon occurred as a result of the treatment of IGR39 cells with ribavirin.

However, arrest in G_0/G_1 by itself is not sufficient to account for the protective effect. Indeed, serum withdrawal, which also induces arrest in G_0/G_1 did not protect these cells whereas Hsp70 renewal was up-regulated with either of the treatments. It is conceivable that this increase in Hsp synthesis and renewal ensures an adaptation, which contributed to protect cells against a subsequent stress. However, with ribavirin treatment, some other mechanisms appeared to be involved to support the resistance to long-term heat shock.

The mechanism of ribavirin-mediated enhancement of Hsp70 renewal is probably connected to protein synthesis inhibition, which has already been reported to occur during the treatment with ribavirin of both primary cultures of several cell types and established cell lines (Ilyin et al., 1998). The reduction of GTP levels affects certainly the small GTPase Ran, which is involved in the nucleocytoplasmic transport of RNA across the nuclear envelope (Cole and Hammell, 1998). A global diminution of GTP levels could down-regulate the Ran protein and thus reduce general protein synthesis owing to the decrease in mRNA availability in the cytoplasm (Dahlberg and Lund, 1998). Ran might also be involved in a proofreading function related to the assembly of import complexes (Melchior and Gerace, 1998). Recently, this small GTPase has been reported to be required for the control of the cell cycle (Dasso, 1995). However, it has been known for a long time that ribosome-associated accessory factors, such as the initiation factor (eIF5) or elongation factors, display GTPase activity, which is required for their function. As a consequence of either mutations affecting these factors or a low availability of GTP, translation could be aborted and some wrong translation products could be produced. This accumulation of un- or wrong-folded peptides could induce a stress response and consequently Hsp synthesis.

Others have previously reported a down-regulation of c-*ras* gene expression at the transcription level (Olah et al., 1990) and a decrease of Ras-GTP content of tiazofurin-treated K562 cells (Hata et al., 1993). The *ras* protooncogene product is a GTP-binding protein and is thought to transduce signals regulating cell proliferation and differentiation.

It is now well established that forms of activated Ras interact specifically with Raf-1 (Koide et al., 1993; Van Aelst et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993). In addition, MAP kinase activity is only associated with activated forms of Ras (Moodie et al., 1993). These observations have traced out the major steps in the Ras pathway for transmitting growth signals to the cell nucleus (Avruch et al., 1994).

Raf can be activated by Ras (Wood et al., 1992), and this process is mediated by direct interaction of these proteins (Wood et al., 1992; Zhang et al., 1993). The Raf protein kinase functions as a MAP kinase kinase kinase by phosphorylating and activating the MAP kinase kinase (Dent et al., 1992; Howe et al., 1992; Kyriakis et al., 1992), which in turn phosphorylates and activates MAP kinase (Wartmann and Davis, 1994). The stimulated MAP kinase then translocates into the nucleus, where many of the physiological targets of the MAP kinase signal transduction pathway are located (Ahn et al., 1992).

The strong decrease of anti-Raf-1-mediated coimmunoprecipitation of Ras seen in ribavirin-treated cells suggests that the transduction of the growth signal was impeded in response to incubation in the presence of this drug. Moreover, we demonstrated that a low GTP concentration in IGR39 cells reduced the availability of activated, GTP-bound Ras and impeded MAP kinase-mediated signal transmission to the nucleus, and consequently the progression through the cell cycle, which involves MAP kinase activation. However, it is currently thought that inactivation of ERK promotes apoptosis (Alessi et al., 1995). Observations reported here suggest, on the contrary, that such an inactivation appears connected to protection against apoptosis. As discussed further, the Hsp-promoted, mitochondria-targeted Raf-1 localisation could induce Raf-1 to display an anti-apoptotic function, canceling out the pro-apoptotic function of membrane-located Raf-1.

It appears that the anti-phosphoERK antibody recognised weakly another band corresponding to the p38 kinase. This band also disappeared after a 2-day treatment with ribavirin. A pathway, which leads to activation of p38 via MKK4 is regulated by Rac/cdc42. Rac belongs to GTP-regulated small G-proteins. Consequently, this observation suggests that a decrease of GTP level could modify Rac activity and more downstream p38 kinase phosphorylation. It has been suggested that the balance between ERK and JNK/p38 pathways is important in determining whether a cell survives or undergoes apoptosis (Birkenkamp et al., 1999). Since the p38 inhibitor SB203580 (Lee et al., 1999) prevented cells from undergoing apoptosis, it is thought that enhanced JNK/p38 activity leads to apoptosis (Birkenkamp et al., 1999). So, these observations suggest a balance between on one hand an apoptosis signal induced by the down-regulation of MAP kinase activity and on the other two possible survival signals: one being

induced by mitochondrial-targeted Raf-1 and the other through the down-regulation of p38 kinase.

What is the possible relationship, if any, between suppression of Raf-1 activation and heat-shock resistance?

The cytoplasmic Raf protein kinase exists as a high molecular mass complex with Hsp90 and p50 (Wartmann and Davis, 1994): Hsp90 and p50 were coimmunoprecipitated with the membrane-bound Raf protein kinase, indicating that it is the Raf–Hsp90–p50 complex that associates with the membrane during activation of the Raf protein kinase (Wartmann and Davis, 1994). Raf-1 binds to Hsp90 via its COOH-terminal catalytic site and remains complexed to Hsp90 even when bound to Ras at the plasma membrane, and it has been proposed that Hsp90 is involved in Raf-1 transport to the cell membrane.

It is only the Ras-induced membrane-bound Raf complex that exhibits increased protein kinase activity (Ahn et al., 1992). As the Ras requirement for Raf activation is completely bypassed by targeting Raf to the plasma membrane through a Ras-independent mechanism (Avruch et al., 1994; Leevers et al., 1994; Stokoe et al., 1994), it was hypothesised that the Ras-induced mobilisation of the Raf–Hsp90–p50 complex at the membrane allows protein–protein interactions, which represent the mechanism of regulation of Raf protein kinase activity (Schulte et al., 1995).

The Ras-activated kinase cascade completes a sequence of biochemical reactions that links signals initiated by mitogenic receptors to the cytoskeletal and transcriptional targets that mediate cell division or differentiation (Avruch et al., 1994; Hall, 1994; Liu et al., 1995). However, much remains to be learned about this pathway and its relationship to the cytoskeleton. While the interaction of protein kinase C with the cytoskeleton, via other G proteins such as Rac, is established (Best et al., 1996), only some observations suggest a possible interaction between Raf and the cytoskeleton (Leevers et al., 1994; Stokoe et al., 1994). For example, the transport of Raf at the membrane (Wartmann and Davis, 1994) and its implication for the relocalisation of Hsp90, which is reported to associate with the microtubules, are consistent with such an interaction. Authors have concluded that, following its recruitment by Ras, Raf-1 tightly associates with cytoskeletal components because once the translocation to the plasma membrane was completed, Raf could no longer be solubilised with the detergent NP 40 (Hall, 1990; Hancock et al., 1989, 1990). The Hsp70 protein, which is known to be associated with tubulin subunits (Marchesi and Ngo, 1993), constitutes another possibility to link cytoskeleton, heat-shock effects and survival or apoptosis. This Hsp was reported to be involved in an anti-apoptotic message at the level of the mitochondrial membrane (see below).

Consequently, all these observations and our results taken together make it conceivable that Raf-1 transport to the membrane needs a polymerised network around and under the membrane, and that Raf-1 transport to the membrane is impeded by disruption of the peripheral microtubular network we observed in ribavirin-treated cells. Thus, this default at the level of the cytoskeleton constitutes possibly an additional mechanism leading to the reduction of the Ras/Raf interaction.

Results from the immunodetection and radiolabeling experiments showed an enhancement of the renewal of Hsp proteins in response to treatment with ribavirin. This relative destabilisation of Hsp90 indicates that a change in the interactions and/or the localisation of this protein had taken place. As Raf activation requires direct binding with Hsp90/70, the lack of Raf activation suggests some particular state of Hsp90/70–Raf interaction in ribavirin-treated cells. We hypothesise that a special conformation or localisation of this protein may have influenced the response to heat shock, inducing during the experiment an enhancement of cell protection against the irreversible effects of heat shock.

In fact, a link between apoptosis/survival and Raf-1 activation could be the newly discovered Bcl-2-associated anti-death gene 1 (BAG-1) protein, which binds and inhibits Hsp70/Hsc70-mediated refolding of an unfolded protein substrate (Hohfeld and Jentsch, 1997) and which was first identified based on its ability to bind and enhance the antiapoptotic activity of Bcl-2 (Takayama et al., 1995). When overexpressed, this factor promotes cell survival (Clevenger et al., 1997; Takayama et al., 1995). In addition, BAG-1 can bind to the catalytic domain of Raf-1 and stimulate Raf-1 kinase activity through a Ras-independent mechanism (Wang et al., 1996). In fact, as Hsp90 is constitutively associated with Raf-1, this interaction may permit protein complexes containing BAG-1 and Hsp70 to alter indirectly the conformation of Raf-1, through effects of Hsp/Hsc70 on Hsp90 (Takayama et al., 1997), thus resulting in kinase activation (Takayama et al., 1997). As heat-shock proteins have been described as cooperating with Bcl-2 in the maintenance of cell survival, this rather complex network of interacting proteins suggests a link between cell signaling, cell death and stress response.

It appears that Raf-1 can form a trimolecular complex with Bcl-2 and BAG-1 (Wang et al., 1996). Given that Bcl-2 is located primarily on the membrane of mitochondria (Reed et al., 1996), the BAG-1/Bcl-2 complex might target Raf-1 to a different location and to a different substrate from those associated with the plasma membrane where Raf-1 participates in growth factor receptor signal transduction. One such substrate is the pro-apoptotic protein BAD, which when unphosphorylated associates with Bcl-2, thus preventing it from dimerising with Bax and preventing its function as blocker of cell death (Yang et al., 1995). The function of BAD is regulated in response to Raf-induced phosphorylation, when the Raf-1 kinase is activated locally in the vicinity of Bcl-2 (Wang and Reed, 1998; Takayama et al., 1995). Once phosphorylated, BAD no longer associates with Bcl-2, which is then released and binds to Bax. This dimerisation with Bax promotes the

anti-apoptotic function of Bcl-2. In summary, the binding of BAG-1 to Raf-1 raises the possibility that Raf-1 may become activated locally in the vinicity of Bcl-2 through a mechanism involving a protein–protein interaction, thus potentially targeting Raf-1 to unique substrates presumably involved in the regulation of apoptosis as opposed to the MAP kinase-signaling pathway in which Raf-1 has traditionally been involved (Wang and Reed, 1998).

Consequently, the various effects of ribavirin on the cytoskeleton, Hsp renewal and Ras–Raf activation might induce relocalisation of Raf-1 kinase in the mitochondrial membrane, thus promoting an anti-apoptotic message and cell survival via an interaction with BAG-1 and phosphorylation of the BAD protein.

We have shown in this paper that the strong reduction by 70% of the GTP content of IGR39 cells cultured for 3 days in the presence of ribavirin was responsible for the cell cycle arrest in the G_0/G_1 stage. Indeed, guanosine abolished completely the ability of ribavirin to inhibit cell cycle progression. This inhibition was found to be totally reversible: washing out the drug led to resumption of the cell cycle and synchronisation of cell growth. Consequently, the dose of ribavirin, which induced cell cycle arrest as a result of a strong reduction of GTP level was non-toxic and did not induce apoptosis of IGR39 cells.

Ribavirin-treated cells displayed a reduced level of GTP-associated p21ras, and this protein became unable to maintain the association with the protein kinase Raf-1. Consequently, the Ras/Raf pathway appeared altered, as shown by the decreased level of activated ERK. Taken together, these three pieces of experimental evidence strengthen the hypothesis of down-regulation of the Ras/Raf pathway as being in part responsible for the ribavirin-mediated cell cycle arrest.

Cells exposed to ribavirin displayed a partial disappearance of the peripheral microtubular network, whereas no obvious effect was noticed on tubulin repolymerisation. Moreover, culture in the presence of ribavirin led to an up-regulation of Hsp renewal and to a thermotolerance against a 3-h, 45°C thermic shock. Considering the recent literature, especially that on the function of the new discovered protein BAG-1, these later observations suggest a ribavirin-induced mechanism in which complexes including Raf kinase and Hsp proteins are relocalised from the cytoplasmic to mitochondrial membrane leading, via regulation of BAD, to both cell cycle arrest and apoptosis protection and survival.

In conclusion, our results led us to hypothesise that the ribavirin-induced depolymerisation of the peripheral cytoskeleton in IGR39 cells modifies the translocation of protein complexes involving Raf. Consequently, this perturbation, in addition to the low availability of GTP to convert p21ras into an activated state, which is certainly responsible for the weak MAP kinase activity, could be the basic mechanism underlying both heat-shock resistance and cell cycle arrest without induction of cell apoptosis.

The antiapoptotic effect of ribavirin we observed in this study is important to consider with the view to clinical trials with other tumour cells besides those of leukaemic origin. Indeed, as shown here with melanoma cells, ribavirin-induced cell cycle arrest is reversible. The synchronisation of cell division, which occurred when the cell cycle resumed, upon drug removal, could enhance the efficiency of a subsequent treatment with another drug acting at a different phase of the cell cycle. Possible targets interesting to examine can be those involved in the anti-apoptotic mechanism and specially those affecting either Raf kinase translocation or BAD phosphorylation. Recently, a synergistic action was reported when quercitin, a dietary flavonal, was combined or applied after ribavirin in OVCAR-5 cells (Li et al., 1999). These two drugs attack different enzyme targets and stop the cell cycle at different phases.

The clear assessment of the possible clinical implication of our observations requires further investigations in order to examine whether ribavirin induces similar effects on other cell lines and tumours. Paradoxically, owing to the reversibility of the ribavirin-induced cell cycle arrest, this drug may be of interest in the design of new anticancer bi-therapies.

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