Tumorigenesis and Neoplastic Progression

RhoGTPases and p53 Are Involved in the Morphological Appearance and Interferon- α Response of Hairy Cells

Benjamin Chaigne-Delalande,* Lynda Deuve,* Edith Reuzeau,* Caroline Basoni,* David Lafarge,* Christine Varon,* Florence Tatin,* Guerric Anies,* Richard Garand,[†] Ijsbrand Kramer,* and Elisabeth Génot*

From Unité 441,* Institut National de la Recherche Médicale, University Victor Segalen Bordeaux, Bordeaux, and the European Institute of Chemistry and Biology, University of Bordeaux I, Talence; and the Laboratoire d'Hématologie,[†] Institut de Biologie, Centre Hospitalier Universitaire, Nantes, France

Hairy cell leukemia is an uncommon B-cell lymphoproliferative disease of unknown etiology in which tumor cells display characteristic microfilamentous membrane projections. Another striking feature of the disease is its exquisite sensitivity to interferon (IFN)- α . So far, none of the known IFN- α regulatory properties have explained IFN- α responsiveness nor have they taken into account the morphological characteristics of hairy cells. IFN- α profoundly alters cytoskeletal organization of hairy cells and causes reversion of the hairy appearance into a rounded morphology. Because cytoskeletal rearrangements are controlled by the Rho family of GTPases, we investigated the GTPase activation status in hairy cells and their regulation by IFN- α . Using immunolocalization techniques and biochemical assays, we demonstrate that hairy cells display high levels of active Cdc42 and Rac1 and that IFN- α down-regulates these activities. In sharp contrast, RhoA activity was low in hairy cells but was increased by IFN- α treatment. Finally, IFN- α -mediated morphological changes also implicated a p53-induced response. These observations shed light on the mechanism of action of IFN- α in hairy cell leukemia and are of potential relevance for the therapeutical applications of this cytokine. (*Am J Pathol* 2006, 168:562–573; DOI: 10.2353/ajpath.2006.050345)

Hairy cell leukemia (HCL) is a B-cell tumor affecting the preplasma stage of B-cell differentiation.¹ Although HCL is rare, this disease has been the subject of intense investigation because of its remarkable responsiveness to low-dose interferon (IFN)- α therapy. Most studies on HCL have focused on the profile of cell surface markers for classification purposes and to attempt to understand the unique sensitivity of hairy cells to IFN- α . However, alterations in the expression pattern of hairy cell surface molecules do not correlate with IFN- α sensitivity. In addition, none of the multiple known IFN- α regulatory properties can account for IFN- α responsiveness. Lastly, the proposed mechanisms of action of IFN- α in the disease do not take into account the phenotypic characteristics of hairy cells. These data suggest that IFN- α may target a specific defect in hairy cells and implies that the function and regulation of intracellular signaling molecules must be explored in these cells.

The immense progress in the understanding of cytoskeletal organization accomplished in the last decade has led us to consider the effects of IFN- α on hairy cell morphology. In various models, cytoskeletal dynamics have been shown to be driven by small G-protein members of the Rho family, a subclass of the Ras superfamily of which RhoA, Rac1, and Cdc42 are the best characterized members.² RhoGTPases cycle between an inactive GDP-bound and an active GTP-bound form.³ GTP-bound GTPases have the ability to interact with and thereby

Supported by grants from the Institut National de la Santé et de la Recherche Médicale; University of Bordeaux 1 and 2; GEFLUC; the Fondation pour la Leucémie; and fellowships from the Délégation Générale pour l'Armement (to B.C.); the Fondation pour la Recherche Médicale (to B.C.-D. and C.B.); and the French Ministry of Education, Research, and Technologies (to C.V. and G.C.).

Accepted for publication September 27, 2005.

Current address of L.D.: Stellenbosch University, Department of Zoology, Private Bag X1, Matieland 7602, South Africa.

Current address of D.L.: Laboratoire de Biogénèse Membranaire, CNRS, Université Bordeaux 2, Bordeaux, France.

Address reprint requests to Elisabeth Génot, Unité INSERM 441, European Institute of Chemistry and Biology, 2, rue Robert Escarpit, 33 600 Pessac, France. E-mail: e.genot@iecb.u-bordeaux.fr.

activate downstream targets, so called effectors.⁴ Guanine exchange factors (GEFs) catalyze the exchange of GDP for GTP and hence activate the GTPases whereas GTPase-activating proteins (GAPs) enhance the intrinsic GTPase activity, returning the GTPases under their basal GDP-bound state.³ Alternatively, specific alterations of the GTPases such as point mutations on key residues or covalent modifications by bacterial toxins, prevent nucleotide exchange or GTP hydrolysis and thereby lock the GTPase in one conformation or the other. Dominant-negative mutants act by sequestering GEFs, making them unavailable to endogenous GTPases when a stimulus is provided.

The actin cytoskeleton is composed of polymerized actin monomers (filamentous actin: F-actin) that can be rearranged into discrete configurations. Most studies have been performed in adherent cells, which undergo striking morphological changes and actin remodeling on RhoGTPase activation.⁵ Thus, RhoA regulates the formation of stress fibers, Rac1 regulates the formation of lamellipodia, a fine protrusive meshwork of actin filament at the leading edge of migrating cells, whereas Cdc42 regulates the formation of filopodia, finger-like protrusions, as well as cell polarity.⁵ Recent work revealed the role of RhoGTPases in hematopoietic cells.^{6,7} There is a great deal of interest in these proteins because in addition to their effects on the cytoskeleton, they control gene transcription and cell-cycle progression.^{8,9} RhoA, Cdc42, and Rac1 control the activity of the serum response element¹⁰ whereas elevated levels of monomeric actin decreases serum response factor activities.¹¹ Cell survival is also regulated by RhoGTPases. In this respect, Rac1 has recently received much attention when it was shown that constitutively active Rac1 could protect from cell death induced by various apoptosis inducers.^{12–14}

The tumor suppressor p53 is functionally connected to the RhoGTPase pathways.¹⁵ Moreover, p53 has recently been shown to be a regulator of cell morphology when it was shown that p53-null fibroblasts exhibit constitutive Cdc42-dependent membrane cytoplasmic extensions and that ectopic overexpression of p53 could antagonize their formation.¹⁶ IFN- α has recently been shown to suppress oncogene-induced transformation through p53,¹⁷ prompting us to examine the role of p53 in hairy cell morphology.

The coupling of cytoskeletal organization to cell-cycle regulation suggests that cytoskeletal integrity is determinant in the proper control of cell-cycle progression and survival.¹⁸ Because hairy cells present defects in the regulation of their cell cycle as well as alterations in their cytoskeleton, we hypothesized that RhoGTPases may play a role in the phenotypic aspect of hairy cells and could be involved in the response to IFN- α . We have explored the activity of RhoGTPases in hairy cells and examined the effect of IFN- α on this parameter. Because of the limited availability of fresh hairy cells and to their poor growth *in vitro*, such studies cannot be performed with primary hairy cells but can be achieved with suitable cell lines. In this study, two hairy cell lines derived from patients with HCL responsive to IFN- α were used, HCLL-7876¹⁹ and Eskol.²⁰ Two experiments were performed on

primary cells from one patient to confirm our observations. Our results demonstrate that RhoGTPases contribute to their cytoskeletal defects. In addition, IFN- α targets p53 and RhoGTPases to induce cellular responses that could be of prime importance in the therapeutic benefits of IFN- α in HCL.

Materials and Methods

Cells and Culture Conditions

The HCL cell lines HCLL-7876 was obtained from Dr. Christel Uittenbogaart (University of California at Los Angeles, Los Angeles, CA) and Eskol from Dr. Milton Taylor (Indiana University, Bloomington, IN). Their characterization has been reported in the context of other studies.^{19,20} Fresh hairy cells were collected after written informed consent was given, in accordance with the rules and tenets of the revised Helsinki protocol, and cells were isolated as previously described.^{21,22} All cells were maintained in RPMI 1640 supplemented with glutamine, antibiotics (Gibco BRL, Grand Island, NY), and 10% fetal calf serum (GlobePharm, Surrey, UK).

Plasmids and Cell Transfection

p53 and various GTPase mutants were expressed as GFP fusion proteins encoded by eukaryotic expression vectors provided by Drs. P. Roux (CRBM/CNRS UPR1086, Montpellier, France) (pEGFP-p53 and pEGFP-p53H175R) and P. Fort (CRBM/CNRS UPR1086, Montpellier, France) (pEGFP-GTPase mutants). All plasmids were purified by equilibrium centrifugation in CsCl-ethidium bromide gradients using standard procedures. Cells were transfected by electroporation (Bio-Rad Laboratories, Hercules, CA). Briefly, 10 million cells/0.5 ml were pulsed in complete medium at 960 μ F and 310 V with 30 μ g of plasmid and cultured for 24 hours to allow plasmid expression. For *Clostridium botulinum* C3 exoenzyme intoxication, hairy cells were treated with 50 μ g/ml Tat-C3 for 18 hours.²³ Plasmids encoding GST-Rho-binding domain (RBD)-rhotekin and GST-Cdc42/Rac-interactive binding domain (CRIB)-PAK have been described elsewhere.²⁴

Reagents and Antibodies

Human recombinant IFN- α 2b (IntronA, Schering Plough) was reconstituted in phosphate-buffered saline and used at a final concentration of 1000 UI/ml for HCLL-7876 and 6000 UI/ml for Eskol.^{20,21} Mowiol 4-88 was from Calbiochem. Etoposide was from American Pharmaceutical Partner Inc. (USA) and used at a concentration of 20 μ mol/L unless otherwise indicated. Glutathione-Sepharose beads and various chemicals were from Sigma (St. Louis, MO). Tat-C3 was kindly supplied by Dr. J. Bertoglio (U461-INSERM, Chatenay-Malabry, France). Rhodamine-labeled phalloidin and fluorescein isothiocyanate-labeled secondary antibodies were purchased from Molecular Probes, Eugene, OR. Anti-RhoA and anti-p53

were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-Rac1 from Upstate Biotechnology (Lake Placid, NY), anti-CD20 from Serotec, anti-Cdc42 and anti-phospho-p53 antibodies from Transduction Laboratories (Lexington, KY). Peroxidase-labeled antibodies and chemiluminescence reagents were from Amersham International (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Immunofluorescence Microscopy

Studies were performed on suspended cells that were fixed with 3% paraformaldehyde prepared in cytoskeletal buffer (10 mmol/L morpholine ethanesulfonic acid, 150 mmol/L NaCl, 5 mmol/L EGTA, 5 mmol/L MgCl₂, and 5 mmol/L glucose, pH 6.1) for 10 minutes at room temperature, sedimented on poly-L-lysine-coated glass coverslips, and processed for immunofluorescence as previously described.²⁴ The coverslips were mounted on microscope slides with Mowiol 4-88 mounting medium. The hairy phenotype was considered suppressed only when F-actin-stained cells displayed no residual protrusion. Analysis of cytoskeletal alterations was assessed in three independent experiments in which at least 100 cells were counted. Confocal images were obtained by means of a confocal laser microscope system Nikon EclipseE800 (Amsterdam, The Netherlands), equipped with a $\times 60$ objective and driven by EZ2000 software. Each image was acquired sequentially using the appropriate filter sets and processed for publication using Adobe (San Jose, CA) Photoshop CS Software.

Separation of Membrane and Cytosol Fractions

The peculiar hairy cell membranes have led us to adapt a conventional fractionation protocol to optimize the preparation of membrane fractions. Cells were treated with IFN- α for 18 hours or left untreated, washed, and resuspended in hypotonic buffer containing 10 mmol/L Tris-HCl, pH 7.4, 1.5 mmol/L MgCl₂, 5 mmol/L KCl, supplemented with 1 mmol/L dithiothreitol, 0.2 mmol/L orthovanadate, 1 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride, 1 μ g/ml aprotinin, and 1 μ g/ml leupeptin. The suspension was homogenized with 30 strokes in a Dounce homogenizer and gently centrifuged to remove unbroken cells and nuclei. The remaining supernatant was centrifuged at $14,000 \times g$ for 30 minutes at 4°C, and the supernatant was collected as the cytosol fraction whereas pellets were resuspended in hypotonic buffer and collected as the membrane fraction. Protein concentrations were measured and equal quantities were loaded on gels and analyzed by Western blot using standard protocols. The amount of proteins detected by Western blotting was determined by scanning the autoradiogram followed by processing of the data with the NIH image software.

Rho, Rac, and Cdc42 Activity Assays

The RhoA, Rac1, and Cdc42 activity assays are based on the Rap1 activity assays²⁵ and were performed essentially as described in published protocols.^{26,27}

Effect of Etoposide on Hairy Cell Viability

Cell viability was determined using trypan blue exclusion assay. A total of 25×10^4 cells were seeded in 48-well plates and exposed to etoposide at the concentration specified or vehicle concentration alone. After 18 hours, cells were stained with trypan blue, and those excluding or including the dye were scored as alive or dead cells, respectively. Each point was performed in triplicate and 100 cells were scored for each condition.

Statistics

Data are expressed as the mean $\pm$ SD of at least three independent triplicate experiments. Significance was determined using the Student's *t*-test.

Results

F-Actin Phenotype of Hairy Cells and Alterations by IFN- α

To explore the overall cytoskeletal organization of hairy cells and the ability of IFN- α to alter this phenotype, polymerized actin was stained with rhodamine-labeled phalloidin and observed by confocal microscopy. Cells from HCLL-7876 and Eskol cell lines showed the same surface features as those isolated from malignant B cells from patients with HCL (primary cells, PR) (Figure 1). Most cultured hairy cells displayed an irregular profile, with a mixed phenotype made of both extended polymerized actin filaments organized in long protrusions and broader lamellipodium-like structures made up of a dense and compact mass of F-actin. Some cells exhibited thin and curved microspikes made up of long bundles of F-actin on the entire surface of the cell (Figure 1A). Other cells displayed shorter, finger-like protrusions highly reminiscent of filopodia (data not shown). In some instances ($\sim 20\%$ of the cells), cultured hairy cells displayed extreme polarization of their surface projections (Figure 1B). These structures correspond to the cytoplasmic projections and broad-based ruffles described previously by means of scanning electron microscopy.²⁸ This apparent heterogeneity is likely to reflect cytoskeletal dynamics.²⁹ Treatment of hairy cells with IFN- α smoothed cell surface outlines. A complete disappearance of these cytoplasmic hair-like projections was observed in $\sim 80\%$ of HCLL-7876 or Eskol cells after 4 days of treatment, in accordance with previous findings.²⁹ However, blunting of surface projections was already evident in $\sim 40\%$ of HCLL-7876 or Eskol cells after 18 hours of treatment or in primary hairy cells from a patient (Figure 1C), when other major effects of IFN- α are already

Figure 1. Cytoskeleton of hairy cells and alterations by IFN- α . HCLL-7876 and Eskol cells were either left untreated or incubated in the presence of IFN- α for 18 hours. Cells were harvested, fixed, and sedimented on poly-L-lysine-coated coverslips to be analyzed for F-actin with rhodamine-phalloidin staining. For both cell lines HCLL-7876 and Eskol and primary cells (PR), most untreated cells exhibited thin and curved microspikes made up of long bundles of F-actin on the entire surface of the cell (A) whereas other cells displayed extreme polarization of their surface projections (B). C: Treatment of hairy cells with IFN- α smoothed cell surface outlines. Images are three-dimensional reconstructions of stacks of confocal images. Scale bar, 4 μ m.

detectable.^{30,31} These morphological alterations obviously reflect IFN- α -induced changes in the cytoskeletal proteins.

Changes in RhoGTPase Activities Correlate with Changes in Hairy Cell Appearance

To explore the possibility that alteration in RhoGTPases activities may underlie the abnormal cytoskeleton configuration of hairy cells, experiments were designed to inhibit these activities individually. In most cell types, expression of dominant active form of Cdc42 (V12Cdc42 or L61Cdc42) induces the formation of cytoplasmic extensions and is associated with cell polarization,⁵ suggesting that Cdc42 could be involved in the formation of cellular protrusions in hairy cells. Endogenous Cdc42 activity can be inhibited by overexpressing the dominant-negative form of Cdc42, N17Cdc42, which traps Cdc42's guanine nucleotide exchange factor (GEF) and renders them unavailable for signaling functions. Another way to inhibit Cdc42-driven pathways is to block Cdc42 signaling to effectors. The formation of filopodia is dependent on the interaction of Cdc42 with the structural protein WASP^{27,32} (a protein defective in the Wiskott-Aldrich syndrome). Overexpressing only the CRIB domain of WASP prevents functional interaction of endogenous WASP with Cdc42 and thereby WASP-dependent filopodia formation.¹⁵

Once a transient transfection procedure was successfully established for HCLL-7876 cells (Eskol cells were found completely refractory to transfection), we pro-

ceeded to determine how hairy cells respond to inhibition of Cdc42. A GFP-tagged version of dominant-negative Cdc42 (GFP-N17Cdc42) was used to selectively examine cells that express the plasmid. Cytoskeletal alterations were visualized by rhodamine-phalloidin staining after cells had been sedimented on poly-L-lysine matrix-coated glass coverslips, 24 hours after transfection. After control transfection of a GFP-encoding vector, hairy cells were sometimes slightly more rounded than their control untransfected counterpart, with shorter microspikes and lamellipodia (see bottom of Figure 2C). We routinely observed 33% ($\pm 5\%$) transfected cells with 6% ($\pm 3\%$) smooth cells among the transfected cells. This low unspecific cell smoothing effect appeared in response to the electroporation method and not to the empty plasmid because electroporation in the absence of vector produced the same effect. To ease the analysis, this baseline ratio was arbitrarily counted as one. Inhibition of Cdc42 resulted in a decrease in both the size and density of microspikes in most of the transfected cells (Figure 2A). Quantitative analysis of N17-Cdc42-transfected cells showed 4.4 times more cells with a rounded morphology than the control (Figure 2B). The WASP-CRIB protein appeared as an even better inhibitor with six times more round cells among the transfected cells (Figure 2B). By contrast, transfection of a constitutively active mutant of Cdc42 (GFP-V12Cdc42 or GFP-L61Cdc42) accentuated the phenotype in all of the transfected cells. These cells exhibited longer and stronger spikes, together with a more intense actin staining (Figure 2C). Taken together, these results indicate that a Cdc42-WASP pathway (or a closely related GTPase, able to interact with WASP) is highly active in these cells and that inhibition of its activity corrects the aberrant cytoskeletal organization of the cells.

Because hairy cells also exhibit lamellipodia-like protrusions characteristic of cells harboring active Rac1 proteins, similar experiments were performed with Rac mutants. Expression of dominant-negative Rac1 (GFP-N17Rac1) significantly altered the hairy appearance with suppression of lamellipodia, decreased cortical actin staining, and reduced microspike length and density (data not shown), leading to a phenotype somewhat resembling Cdc42 inhibition. It is likely that inhibition of Rac1 interferes with the pathways involved in the aberrant cytoskeletal organization. Expression of dominant active Rac1 (V12Rac) did not induce any significant change in the overall cell morphology (Figure 2B), suggesting that Rac1 activity was already elevated in these cells.

A balance between Cdc42 and RhoA activities has been evidenced in several models²⁴ and therefore RhoA activity was also investigated. To inhibit Rho, a slightly different approach was used: cells were transfected with a plasmid encoding C3, a well-characterized bacterial toxin that specifically ADP-ribosylates Rho proteins, thereby inactivating them.³³ Several attempts to express C3 resulted in hairy cell death, suggesting that hairy cells could not survive inhibition of Rho proteins. An alternative milder treatment, consisting of the addition of recombinant fusion protein Tat-C3, was tested. Such treatment

Figure 2. Effects of RhoGTPase mutants on hairy cell morphology. HCLL-7876 cells were transiently transfected with a plasmid encoding either control GFP or GFP-tagged versions of RhoGTPase mutants. Twenty-four hours after transfection, cells were harvested, fixed, and analyzed for F-actin with rhodamine-phalloidin staining and examined for morphological alterations by confocal microscopy. Transfection efficiency was found very similar for all plasmids (data not shown). **A:** Expression of N17Cdc42 or V14RhoA inhibited cell protrusions (each image represents one confocal section of a cell). **B:** A quantitative analysis of the morphological alterations induced by RhoGTPase mutants is shown. The hairy phenotype was considered suppressed only when F-actin-stained cells displayed no residual protrusions. Loss of cellular protrusions was calculated on the basis of the control response obtained on transfection of the GFP encoding vector (*statistical significance of the loss of protrusions versus control, $P < 0.01$, **significant difference versus N17Cdc42, $P < 0.05$). Inhibition of Cdc42 or Rac, or expression of active RhoA, suppressed the hairy phenotype. **C:** In contrast, expression of dominant-active Cdc42 exaggerated the hairy phenotype by inducing the formation of longer and stronger actin spikes (three-dimensional reconstructions of stacks of confocal images). **D:** The same experiment performed with plasmids encoding Cdc42 effector-loop mutants revealed that signaling through WASP, and not through Rac1, accentuated the hairy phenotype. Results are normalized based on the control response (*significant difference versus control, $P < 0.01$). **E:** Cells transfected with either dominant-negative mutant or constitutively active Cdc42 became insensitive or refractory to IFN- α action. Scale bars, 4 μ m.

also led to hairy cell death (data not shown). In contrast, when dominant-active V14RhoA was expressed, a profound effect was observed with almost complete disappearance of actin spikes (Figure 2, A and B). Interestingly, this phenotype was indistinguishable from the one observed under Cdc42 inhibition (Figure 2A).

To gain further insights into Cdc42 signaling in hairy cells, similar experiments were performed with a second generation of Cdc42 mutants. Cdc42 is able to induce diverse cellular responses via WASP, the PAK family, or ACK effectors through a region named effector-loop.³⁴ We used previously characterized Cdc42 effector-loop mutants as tools to dissect the molecular pathways in-

involved in cytoplasmic protrusion induction. In a constitutively active background (provided by the L61 mutation), an additional mutation in this sequence restricts the interaction with some but not all effectors. Accordingly, the L61Cdc42F37A mutant retains its ability to activate WASP but is deficient for Rac1 activation, whereas the L61Cdc42Y40C still signals to Rac1 but is unable to stimulate WASP.³⁴ Transient transfection of GFP-L61Cdc42 accentuated the hairy phenotype similar to that of GFP-V12Cdc42 (Figure 2D). Cells expressing the GFP-L61Cdc42F37A mutant displayed a morphology not significantly different from those expressing GFP-L61Cdc42 (data not shown), indicating that signaling to

Figure 3. Immunolocalization of endogenous RhoGTPases in hairy cells and relocalization on IFN- α treatment. HCLL-7876, Eskol, or primary cells were either left untreated or treated with IFN- α for 18 hours. Cells were harvested, fixed, and stained for F-actin (rhodamine-phalloidin) and RhoGTPases (primary antibodies recognizing the RhoGTPases were revealed with FITC-labeled secondary antibody) and observed by confocal microscopy. RhoGTPases (green) co-localizing with F-actin (red) at the cell cortex or within cellular protrusions appeared in yellow (merge). IFN- α induced the relocalization of Cdc42 (**A**), Rac1 (**B**), and RhoA (**C**). Each image is a confocal section. Scale bar, 4 μ m.

WASP was sufficient to exacerbate the hairy phenotype. In accordance with these data, expression of the GFP-L61Cdc42Y40C mutant impaired in WASP signaling did not mimic the effect of GFP-L61Cdc42 (Figure 2D). These results are congruent with the observation that transfection of a constitutively active form of Rac1 did not alter the hairy cell appearance.

Because both IFN- α treatment and overexpression of N17Cdc42 reduced the hairy-like phenotype, we next asked whether altering Cdc42 activity would change cytoskeletal responsiveness to IFN- α treatment in these cells. Cells were transfected as above and were subsequently either left untreated or treated with IFN- α for 18 hours. Data presented in Figure 2E revealed that plasma membrane smoothing was obtained in all cells overexpressing N17Cdc42, and IFN- α did not alter this effect. Cells transfected with active Cdc42 were refractory to IFN- α action. These data suggest that IFN- α receptor signaling acts either upstream or at the level of Cdc42 to regulate cytoskeletal organization.

IFN- α Alters GTPase Activities in Hairy Cells

Analysis of RhoGTPase Localization by Immunofluorescence Experiments

The results described above revealed that RhoGTPases could be involved in the defective cytoskeletal or-

ganization of hairy cells and that GTPases are potential targets of IFN- α treatment. Whether IFN- α affects the expression and activation states of RhoGTPases was next investigated. Inactive GTPases (ie, GDP-bound forms) reside in the cytoplasm, bound to the regulatory RhoGDI protein. On activation, RhoGDI is displaced and an aliphatic sequence is unmasked, allowing GTPases to anchor into membranes where they interact with effectors.³⁵ Consequently, active GTPases (GTP-bound) are found associated with membranes and this localization reflects their activation state. We therefore used immunofluorescence techniques to localize endogenous RhoGTPases in untreated hairy cells and explored the effects of IFN- α on their spatial distribution. Phalloidin staining revealed a thick ring of cortical polymerized actin underlining the contours of the plasma membrane and a loose network of F-actin inside the cell. Immunofluorescence studies performed with primary antibodies recognizing the RhoGTPases, followed by fluorescein isothiocyanate-labeled secondary antibody, were used to localize endogenous GTPases within the cells by confocal microscopy (Figure 3). Close association of GTPases with the plasma membrane was revealed by superposition of the two fluorochromes. In these experiments, Cdc42 was detected both at the plasma membrane (including that of villousities) and in the cytosol of both cell lines. When cells were treated with IFN- α , the reduction of membrane vil-

Figure 4. Distribution of RhoGTPases in cell fractionation experiments. HCLL-7876 and Eskol cells were either left untreated or treated with IFN- α for 18 hours. **A:** Proteins were fractionated into membrane and cytosolic fractions, and the CD20 protein was taken as a membrane marker. **B:** Equal amounts of membrane proteins were loaded on the gel and separated by electrophoresis followed by Western blotting using RhoGTPase-specific antibodies (one representative experiment). **C:** The amount of GTPases found in the membrane fraction was quantified in three experiments and is presented as percentage of the control response obtained in untreated cells. IFN- α induced a depletion of Cdc42 and Rac1 from the membrane fraction and the translocation of RhoA to the membrane fraction.

losities forced Cdc42 relocalization. However, most of Cdc42 remained at the cell periphery and did not translocate to the cytosol (Figure 3A). By contrast, Rac1 was easily detected at the plasma membrane of Eskol cells and was found to redistribute, at least in part, into the cytosol after IFN- α treatment (Figure 3B). Finally, RhoA was mainly cytosolic in untreated hairy cells. IFN- α treatment resulted in a clear relocalization of RhoA at the cortical actin ring, beneath the plasma membrane in both cell lines (Figure 3C). Similar subcellular localization of Cdc42, Rac, and RhoA were seen in fresh primary hairy cells (PR) (Figure 3, A–C).

Analysis of RhoGTPase Localization Using Cell Fractionation Experiments

Membrane-bound GTPases were also detected by cell fractionation experiments combined with Western blot analysis. Membrane and cytosolic fractions were prepared with cells that had been either left untreated or treated with IFN- α and the amount of each RhoGTPase present in both fractions was determined by Western blot using specific antibodies. When cells were exposed to IFN- α , significant changes were observed in GTPase distribution. Cdc42 and Rac1 were found depleted from

Figure 5. RhoGTPase activities analyzed by pull-down experiments. Hairy cells were either left untreated or incubated in the presence of IFN- α (18 hours). Cells were then lysed, and active GTPases were affinity precipitated with GST-RBD-rotoxin or GST-CRIB-PAK, eluted from the beads, and analyzed by Western blotting with the relevant antibodies. For each point, a fraction of the cell lysate harvested before precipitation was run to monitor GTPase content in each sample. **A:** IFN- α down-regulated Cdc42 and Rac1 activities and up-regulated that of RhoA in both cell lines as in primary cells. For each GTPase, quantification was made. The percentage of the GTPase under its GTP-bound form in the presence of IFN- α was divided by the one calculated in the absence of IFN- α . A graph showing these ratios is shown in **B**. IFN- α down-regulated Cdc42 and Rac1 activities and up-regulated that of RhoA.

plasma membranes whereas, in parallel, RhoA became more associated with these fractions (Figure 4).

Direct Analysis of RhoGTPase Activity by Selective Precipitation of GTP-Bound GTPases

To measure alterations in the ratio of GTP-bound versus GDP-bound GTPases, hairy cells were treated as before, cell lysates were made and subjected to the pull-down experiment.²⁵ GST fusion proteins containing GTPase-binding domains of effectors, CRIB of PAK for Rac and Cdc42²⁶ and rotoxin for RhoA,²⁷ were coupled to agarose beads to selectively precipitate GTP-bound GTPases from hairy cells. Western blot analysis of affinity-purified proteins revealed that prolonged IFN- α exposure induced a decrease in the GTP-Cdc42 content for both cell lines as well as for primary cells (Figure 5A). Similarly, under the same conditions, the amount of Rac1-GTP

Figure 6. Alteration of p53 status by IFN- α in hairy cells. HCLL-7876, Eskol, primary hairy, or BL41 cells were either left untreated or treated with IFN- α for various periods of time and cell lysates were prepared. Equal amounts of proteins (200 μ g per lane) were loaded on gels and separated by SDS-PAGE. **A:** p53 phosphorylation and expression were assessed by Western blot using relevant antibodies (shown is one representative experiment, HCLL-7876, Eskol, and PR blots were exposed for 10 minutes for autoradiography, BL41 was exposed for 1 minute). **B:** The amount of phosphorylated p53 protein measured 18 hours after transfection was quantified in three independent experiments and is presented as fold increase of the basal response obtained in untreated cells. IFN- α consistently induced a time-dependent increase in p53 phosphorylation on S15 in hairy cells but not in the Burkitt lymphoma cell line.

precipitated was also reduced. RhoA, again, displayed a distinct pattern, with a rise in the RhoA-GTP content in response to IFN- α treatment. Highly similar results were obtained with primary hairy cells treated *in vitro* with IFN- α (PR cells). The IFN- α -induced changes in the GTPase-GTP/total GTPase ratios were calculated for each GTPase (Figure 5B).

IFN- α Affects p53 Status in Hairy Cells

Taken together, the results presented above suggested to us that the IFN- α effect was mediated at the level of Cdc42, affecting Cdc42 activity and membrane localization. The tumor suppressor p53 is functionally connected to the RhoGTPase network and p53 is now known to counteract Cdc42-induced filopodia formation.¹⁶ Interestingly, hairy cells are often affected in their p53 status.^{36,37} In Eskol and HCLL-7876 cell lines, p53 expression was barely detectable when compared to a Burkitt B-cell line (Figure 6A) (hairy cell blots were exposed to

autoradiography for much longer periods and gave fainter bands). Because IFN- α has been shown to induce the expression and activation of p53 in tumor cells,¹⁷ we hypothesized that induction/activation of p53 could be involved in the disappearance of hairy cell protrusions. The low expression of p53 in the two cell lines was not increased by IFN- α , but a marked stimulation was detected on treatment with the robust p53-inducer etoposide (Figure 6A). Thus, p53 expression is efficiently stabilized by etoposide but only weakly by IFN- α . However, p53 phosphorylation at S15 increased in a time-dependent manner in response to IFN- α in hairy cell lines. In these experiments, a p53-deficient and IFN- α -sensitive tumor cell line, not derived from HCL but from a Burkitt lymphoma, BL41 cells, were used as a control to examine the specificity of the response. p53 phosphorylation was not increased in BL41 cells (Figure 6A). Primary hairy cells also responded to IFN- α by increasing S15 phosphorylation (Figure 6A), and p53 protein expression was below the threshold of detection by Western blot. When up-regulated on etoposide treatment, p53 accumulated under a form phosphorylated at S15 in HCLL-7876, Eskol, and BL41 cells. To determine whether p53 could be involved in the cytoskeletal response, cells were transfected with a p53-GFP fusion protein. Ectopic expression of p53 (GFP-p53; Figure 7A) led to a complete disappearance of the cytoplasmic protrusions in all transfected cells. When analyzed by Western blot, the ectopic p53 protein was found phosphorylated at S15 (Figure 7B). To confirm the effects of IFN- α treatment on p53, a GFP-tagged version of a naturally occurring dominant-negative p53 mutant (p53R175H)³⁸ was transfected in hairy cells and the morphological changes induced on IFN- α were investigated. The cell membrane-smoothing effect of IFN- α was greatly reduced in cells expressing the dominant-negative p53 mutant (Figure 7C). These results indicate that p53 is integrated in the overall IFN- α response leading to the disappearance of actin spikes. Finally, activation of p53 by etoposide ablated cell protrusions and killed hairy cells in a dose-dependent manner within 18 hours of treatment (Figure 7D).

Discussion

Hairy cells display an irregular profile, with numerous membrane extensions such as thin microspikes made up of long bundles of F-actin and broader lamellipodia-like structures. Cdc42 has been shown to control the formation of filopodia as well as other types of protrusions.³⁹ The various kinds of actin configuration observed in hairy cells are similar to those obtained when active forms of Cdc42 and Rac1 are overexpressed.

RhoGTPase Activities Contribute to Hairy Cell Morphology

Experiments performed to inhibit Cdc42 activity using transient transfection protocols revealed that constitutive signaling through Cdc42, or a closely related GTPase

Figure 7. p53 regulates hairy cell morphology. HCLL-7876 cells were transfected with an empty vector or with a plasmid encoding GFP, GFP-p53, or GFP-p53H175. Twenty-four hours after transfection, cells were harvested, fixed, stained for F-actin with rhodamine-phalloidin, and observed by confocal microscopy. **A:** Expression of GFP-p53 led to a complete disappearance of cell protrusions in all transfected cells whereas expression of p53H175-GFP did not affect the phenotype. **B:** Western blot analysis of these cells revealed basal phosphorylation of the fusion protein GFP-p53 on S15, which was further increased by IFN- α . HCLL-7876 cells were transfected with plasmids encoding either GFP or the dominant-negative mutant of p53, GFP-p53H175. Cells were allowed to express the plasmids for 24 hours and then treated or not with IFN- α for 18 hours. Cells were harvested, fixed, stained for F-actin with rhodamine-phalloidin, observed by confocal microscopy, and analyzed as described in Figure 2. Expression of dominant-negative p53 prevented morphological changes in response to IFN- α . **C:** Results are normalized based on the control response (*significant difference versus control, $P < 0.05$). To evaluate the effects of the p53 activator etoposide on cell viability, HCLL-7876 cells were treated with increasing concentrations of etoposide to stimulate p53 activity. After 18 hours, cell viability was determined, and results are presented as percentage of viable cells. **D:** Etoposide killed hairy cells in a dose-dependent manner within 18 hours of treatment. Scale bar, 4 μ m.

also able to interact with WASP, contributes to the unusual phenotype of hairy cells. Cytoplasmic processes were lost or dramatically decreased by inhibition of either Cdc42 or WASP. This latter protein, by recruiting the actin polymerization machinery (Arp2/3 complex), allows filopodia extension. Although filopodia can also be formed in the absence of WASP,^{40,41} our results obtained with the WASP-CRIB mutant indicate that cytoplasmic protrusions of hairy cells are WASP-dependent. Overexpression of a constitutively active form of Cdc42 exaggerated the phenotype by promoting the formation of longer spikes,

strengthening our conclusions. Finally, expression of an active Cdc42 mutant that selectively stimulates WASP (L61Cdc42F37A) confirmed this result. Although the organization of the actin filament system is cell-type-dependent, other GTPases related to Cdc42 such as TC10, RhoD, RhoG, and Wrch1 can also stimulate the formation of membrane protrusions and some of them are known to interact with WASP.^{2,42} Future studies will explore the role of these other RhoGTPase members in HCL.⁴³

Expression of V12Rac in hairy cells did not significantly affect F-actin organization, suggesting that Rac was already activated. These findings are consistent with the high levels of Cdc42 activity found in these cells as Rac1 is positioned downstream of Cdc42 in signaling cascades controlling cytoskeletal remodeling.⁵ Inhibiting Rac1 also results in significant smoothing of the hairy cell phenotype, suggesting that the defect leading to aberrant cytoskeleton organization is located upstream of Rac1. One plausible explanation for the inhibitory effect observed on dominant-negative Rac1 expression could reside in the ability of this protein to bind GEF(s) also active on Cdc42 family members. Several attempts to inhibit Rho activity resulted in hairy cell death, suggesting that hairy cells cannot survive inhibition of Rho. In contrast, expression of a constitutively active form of RhoA resulted in a normalization of the cellular morphology. Overexpression of V14RhoA had the same effect on cell morphology changes of hairy cells as N17Cdc42 or N17Rac1. Whether IFN- α -induced activation of RhoA is responsible for Cdc42 and Rac1 inhibition remains to be determined.

IFN- α Regulates Cdc42, Rac1, and RhoA Activities

Hairy cells incubated in the presence of IFN- α lose their characteristic villousities. Expression of mutants able to inhibit Cdc42 or expression of an active RhoA mutant mimics the effects of IFN- α in suppressing cytoplasmic processes. The striking resemblance of the resulting phenotypes suggested to us that activating RhoA could result in Cdc42 inhibition. To address this point, subcellular immunolocalization, membrane fractionation, and pull-down experiments allowed us to compare the relative levels of active endogenous GTPases in IFN- α -treated versus control cells. Direct measurements of RhoGTPase activities using the pull-down method showed that Cdc42 and Rac1 activities were reduced on prolonged (18 hours) exposure to IFN- α , whereas that of RhoA was clearly increased. The three approaches converged on the same conclusions for Rac1 and RhoA. Pull-down experiments demonstrated a reduction in the total pool of Cdc42 activity and depletion of Cdc42 from membrane fractions after IFN- α treatment. However, although Cdc42 did redistribute when cytoplasmic protrusions were reduced, Cdc42 immunostaining remained co-localized with cortical actin at the cell periphery. A plausible explanation for these contrasting findings is that a Cdc42 membrane-targeting signal exists in hairy cells and remains in IFN- α -treated cells as indicated by the co-local-

ization of cortical actin and Cdc42 seen using the immunofluorescence approach. Pull-down experiments showed that Cdc42 cannot be activated in the presence of IFN- α or that GTP-bound Cdc42 is instantly hydrolyzed by a putative increase in GAP activity. The fact that hairy cells transfected with the GAP-deficient Cdc42 mutant were found refractory to IFN- α action supports this conclusion.

RhoGTPases Are Likely to Contribute to the Tumorigenesis of HCL

Cdc42 is a Ras-related protein that has been implicated in the control of normal cell growth and, when improperly regulated, in cellular transformation and invasiveness.⁴⁴ The presence of a transforming gene in hairy cells was documented by established methods,⁴⁵ but the oncogene involved has not been identified yet. Increased expression of cyclin D1 occurs in HCL.⁴⁶ Cyclin D1 is under the control of Rac1 to control cell-cycle progression, and high levels of active Rac1 could thereby contribute to tumorigenesis by disturbing the normal cell cycle. Hairy cells also exhibit abnormal constitutive expression of an AP1 complex containing junD.⁴⁷ Regulation of AP1 is dependent on Cdc42/Rac1 signaling, in agreement with our findings. Finally, inhibitory mutants of Cdc42 and Rac were shown to reduce cell growth in another hairy cell line.⁴⁸

Cdc42 is unlikely to be oncogenic by itself because its activity could be down-regulated by IFN- α as measured by pull-down experiments. Dominant-negative mutants exert their inhibitory effects by titrating GEF, and the inhibition of cytoplasmic protrusions observed with both N17Cdc42 and N17Rac suggest that a common GEF might be involved in this process as already proposed above. The oncogenic potential involving RhoGTPase in tumors is often driven by alterations in GEF or GAP activities.^{43,49} Future studies will explore the expression and regulation of these proteins in hairy cells.

Interestingly, a previous report from Zhang and colleagues⁴⁸ compared RhoGTPase expression and status in leukemic cells from HCL, B-cell lymphocytic leukemia, and B-cell acute leukemia and concluded that RhoGTPases are uniquely activated in HCL. However, in this study RhoA was found constitutively activated when compared to its status in non-HCL leukemic cells. In the present study we compared RhoA activity in the presence and absence of IFN- α . We did not assess the basal activation status of RhoA but demonstrate that RhoA activity is further increased by IFN- α . In the same study, it was reported that only dominant-negative Rac could reduce cell protrusions. However, the approach used by Zhang and colleagues⁴⁸ differs from ours in that dominant-negative mutants were expressed in stable cell lines whereas we used transient transfection protocols. More importantly, the BNBH-1 hairy cell line used in their study has been derived from a Japanese hairy cell variant type of HCL, which differs in many respects from typical HCL seen in western countries and from which HCLL-7876 and Eskol cells have been derived. The predominant role

of Cdc42 in hairy cells could be another distinctive feature of typical HCL, not occurring in Japanese HCL disease.

Our data show that IFN- α increases RhoA activity. These findings are in agreement with those reported by Badr and colleagues²³ in mature normal B cells. This study reported a regulatory effect of IFN- α on RhoA in the context of ligand-induced chemokine receptor internalization in normal B cells.²³ The regulation of RhoA in B cells seems different from that described for fibroblasts and macrophages.^{23,50} In adherent cells, activation of RhoA has often been linked with tumorigenicity, but recent work suggests that RhoA can also induce cell-cycle arrest via its effector ROCK.⁵¹ In addition, prolonged inhibition of RhoA in lymphocytes *in vivo* has been shown to induce development of lymphoma,⁵² suggesting that elevating levels of RhoA may antagonize a positive signal for proliferation/tumorigenesis.

IFN- α Regulation of RhoGTPase Activities and Increased Activation of p53 May Have Anti-Tumoral Benefit

Tumor expansion often results from the accumulation of cancer cells that are unable to apoptose. In HCL patients in which IFN- α therapy is successful, hairy cells are rapidly cleared from the blood, suggesting the occurrence of tumor cell apoptosis.⁵³ Extensive *in vitro* studies have established that IFN- α is not a direct inducer of programmed cell death for hairy cells but restores their sensitivity to apoptosis-inducing agents.⁵³ Interestingly, cells expressing active Rac1 have been demonstrated to be resistant to apoptotic inducers of various origins.^{12,14} It is tempting to speculate that the high levels of active Rac1 found in hairy cells prevent effective killing by the immune system. We previously reported that hairy cells exhibit high levels of intracytoplasmic calcium $[Ca^{2+}]_i$,³¹ a phenomenon that could also protect hairy cells from programmed cell death. $[Ca^{2+}]_i$ was found reduced on IFN- α treatment.⁵⁴ Because Rac1 has recently been shown to increase $[Ca^{2+}]_i$,⁵⁵ the observed reduction in Rac1 activity could be involved in the normalization of $[Ca^{2+}]_i$. Tumor cells are prone to apoptose, but high $[Ca^{2+}]_i$ ³¹ or active Rac1 (this study) could protect hairy cells from programmed cell death. By decreasing Rac1 activity, IFN- α could down-regulate certain cellular processes to a threshold where cells become responsive to apoptosis inducers.

As stated above, the genetic mechanisms underlying the pathogenesis of HCL are unknown, although the tumor suppressor p53 is frequently deleted or mutated compared to other hematological malignancies.³⁶ In normal cells, Rac1 and Cdc42 extinction activates endogenous p53, and apoptosis induction in response to inhibition of Rac1 and Cdc42 requires p53.¹⁶ In addition, p53 exerts a selective effect on Cdc42-mediated cell functions.¹⁵ In p53-null mice, either overexpression of wild-type p53 or activation of endogenous p53 counteracts Cdc42-induced filopodia formation. Mechanistic studies indicate that p53 prevents the initiating steps of filopodia

formation operating at the level of Cdc42 itself or immediately downstream of it.¹⁶ In our models of hairy cells, p53 expression was low, suggesting mono-allelic deletion and/or protein instability. In these cells, overexpression of wild-type p53 or activation of endogenous p53 reduces cell protrusions, indicating that a similar mechanism could be involved. The p53 activator etoposide completely ablated cell protrusions and killed hairy cells in a dose-dependent manner.

Although the consequences of IFN- α -induced cytoskeletal reorganization on hairy cell behavior remains an open question, alterations of RhoGTPase status in hairy cells are likely to correlate with their distinctive profile of integrin receptors, activation status, and invasive behavior of the bone marrow and spleen.^{26,56,57} The normalization of RhoGTPase activities and the increased activation of p53 may have therapeutic anti-tumoral benefits by reducing tumor expansion, invasiveness, and/or increasing apoptosis. Our data illustrate the link between p53 and the cytoskeleton in a pathological situation in which p53 regulates RhoGTPase-dependent cell effects that control actin cytoskeletal dynamics. This novel function of p53 may contribute to anti-tumor activity.

Acknowledgments

We thank Dr. J.P. Vial and Pr. J.F. Viallard (Haut-Levêque Hospital, Pessac, France) for phenotyping the cell lines and providing primary hairy cell samples; Dr. J. Bertoglio (INSERM-U461, Chatenay-Malabry, France) for providing the Tat-C3; Dr. J. Collard (The Netherlands Cancer Institute, Amsterdam, The Netherlands) for GST-fusion proteins; Dr. A. Hall and Dr. S. Etienne-Manneville (University College of London, London, UK) for dominant-negative WASP; Dr. F. Ichas (IECB/INSERM, Pessac, France) for the gift of etoposide; Dr. T. Taniguchi and Dr. A. Takaoka (University of Tokyo, Tokyo, Japan) for helpful discussions; Dr. P. Fort for GFP-GTPase fusion proteins encoding plasmids; and Dr. P. Roux (CRBM-CNRS-UPR-1086) for GFP-p53 fusion proteins encoding plasmids and critical reading of the manuscript.

References

- Bouroncle BA: Leukemic reticuloendotheliosis (hairy cell leukemia). *Blood* 1979, 53:412–436
- Aspenstrom P, Fransson A, Saras J: Rho GTPases have diverse effects on the organization of the actin filament system. *Biochem J* 2004, 377:327–337
- Van Aelst L, D'Souza-Schorey C: Rho GTPases and signaling networks. *Genes Dev* 1997, 11:2295–2322
- Aspenstrom P: Effectors for the Rho GTPases. *Curr Opin Cell Biol* 1999, 11:95–102
- Nobes CD, Hall A: Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 1995, 81:53–62
- Woodside DG, Wooten DK, Teague TK, Miyamoto YJ, Caudell EG, Udagawa T, Andruss BF, McIntyre BW: Control of T lymphocyte morphology by the GTPase Rho. *BMC Cell Biol* (serial online) 2003, 4:2
- Bird MM, Lopez-Lluch G, Ridley AJ, Segal AW: Effects of microinjected small GTPases on the actin cytoskeleton of human neutrophils. *J Anat* 2003, 203:379–389
- Blanchard JM: Small GTPases, adhesion, cell cycle control and proliferation. *Pathol Biol (Paris)* 2000, 48:318–327
- Olson MF, Paterson HF, Marshall CJ: Signals from Ras and Rho GTPases interact to regulate expression of p21Waf1/Cip1. *Nature* 1998, 394:295–299
- Hill CS, Wynne J, Treisman R: The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell* 1995, 81:1159–1170
- Sotiropoulos A, Gineitis D, Copeland J, Treisman R: Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. *Cell* 1999, 98:159–169
- Zhang B, Zhang Y, Shacter E: Rac1 inhibits apoptosis in human lymphoma cells by stimulating Bad phosphorylation on Ser-75. *Mol Cell Biol* 2004, 24:6205–6214
- Genot EM, Arriemerlou C, Ku G, Burgering BM, Weiss A, Kramer IM: The T-cell receptor regulates Akt (protein kinase B) via a pathway involving Rac1 and phosphatidylinositol 3-kinase. *Mol Cell Biol* 2000, 20:5469–5478
- Murga C, Zohar M, Teramoto H, Gutkind JS: Rac1 and RhoG promote cell survival by the activation of PI3K and Akt, independently of their ability to stimulate JNK and NF-kappaB. *Oncogene* 2002, 21:207–216
- Lassus P, Roux P, Zugasti O, Philips A, Fort P, Hibner U: Extinction of rac1 and Cdc42Hs signalling defines a novel p53-dependent apoptotic pathway. *Oncogene* 2000, 19:2377–2385
- Gadea G, Lapasset L, Gauthier-Rouviere C, Roux P: Regulation of Cdc42-mediated morphological effects: a novel function for p53. *EMBO J* 2002, 21:2373–2382
- Takaoka A, Hayakawa S, Yanai H, Stoiber D, Negishi H, Kikuchi H, Sasaki S, Imai K, Shibue T, Honda K, Taniguchi T: Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence. *Nature* 2003, 424:516–523
- Welsh CF, Roovers K, Villanueva J, Liu Y, Schwartz MA, Assoian RK: Timing of cyclin D1 expression within G1 phase is controlled by Rho. *Nat Cell Biol* 2001, 3:950–957
- Kanowitz-Klein S, Saxon A, Uittenbogaart CH: Constitutive production of B cell differentiation factor-like activity by human T and B cell lines. *Eur J Immunol* 1987, 17:593–598
- Harvey W, Srouf EF, Turner R, Maze R, Starrett B, Kanagala R, Pereira D, Merchant P, Taylor M, Jansen J: Characterization of a new cell line (ESKOL) resembling hairy-cell leukemia: a model for oncogene regulation and late B-cell differentiation. *Leuk Res* 1991, 15:733–744
- Genot EM, Meier KE, Licciardi KA, Ahn NG, Uittenbogaart CH, Wietzerbin J, Clark EA, Valentine MA: Phosphorylation of CD20 in cells from a hairy cell leukemia cell line. Evidence for involvement of calcium/calmodulin-dependent protein kinase II. *J Immunol* 1993, 151:71–82
- Genot E, Mathiot C, Kolb JP: Modulation of the response to B-cell growth factor (BCGF) of hairy cells from a patient under IFN-alpha therapy. *Blut* 1987, 55:65–67
- Badr G, Borhis G, Treton D, Richard Y: IFN[alpha] enhances human B-cell chemotaxis by modulating ligand-induced chemokine receptor signaling and internalization. *Int Immunol* 2005, 17:459–467
- Moreau V, Tatin F, Varon C, Genot E: Actin can reorganize into podosomes in aortic endothelial cells, a process controlled by Cdc42 and RhoA. *Mol Cell Biol* 2003, 23:6809–6822
- Franke B, Akkerman JW, Bos JL: Rapid Ca2+-mediated activation of Rap1 in human platelets. *EMBO J* 1997, 16:252–259
- Sander EE, Collard JG: Rho-like GTPases: their role in epithelial cell-cell adhesion and invasion. *Eur J Cancer* 1999, 35:1302–1308
- Castellano F, Montcourrier P, Guillemot JC, Gouin E, Machesky L, Cossart P, Chavrier P: Inducible recruitment of Cdc42 or WASP to a cell-surface receptor triggers actin polymerization and filopodium formation. *Curr Biol* 1999, 9:351–360
- Garniel H, Hiraoka A, Golomb HM: The effect of cultivation and interferon treatment on the surface morphology of hairy cell leukemia cells. *Cancer Invest* 1987, 5:389–399
- Miyoshi EK, Stewart PL, Kincade PW, Lee MB, Thompson AA, Wall R: Aberrant expression and localization of the cytoskeleton-binding pp52 (LSP1) protein in hairy cell leukemia. *Leuk Res* 2001, 25:57–67
- Genot E, Valentine MA, Degos L, Sigaux F, Kolb JP: Hyperphospho-

- rylation of CD20 in hairy cells. Alteration by low molecular weight B cell growth factor and IFN- α . *J Immunol* 1991, 146:870–878
31. Genot E, Bismuth G, Degos L, Sigaux F, Wietzerbin J: Interferon- α downregulates the abnormal intracytoplasmic free calcium concentration of tumor cells in hairy cell leukemia. *Blood* 1992, 80:2060–2065
32. Leung T, Chen XQ, Tan I, Manser E, Lim L: Myotonic dystrophy kinase-related Cdc42-binding kinase acts as a Cdc42 effector in promoting cytoskeletal reorganization. *Mol Cell Biol* 1998, 18:130–140
33. Paterson HF, Self AJ, Garrett MD, Just I, Aktories K, Hall A: Microinjection of recombinant p21rho induces rapid changes in cell morphology. *J Cell Biol* 1990, 111:1001–1007
34. Lamarche N, Tapon N, Stowers L, Burbelo PD, Aspenstrom P, Bridges T, Chant J, Hall A: Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of p65PAK and the JNK/SAPK MAP kinase cascade. *Cell* 1996, 87:519–529
35. Del Pozo MA, Kiosses WB, Alderson NB, Meller N, Hahn KM, Schwartz MA: Integrins regulate GTP-Rac localized effector interactions through dissociation of Rho-GDI. *Nat Cell Biol* 2002, 4:232–239
36. Konig EA, Kusser WC, Day C, Porzolt F, Glickman BW, Messer G, Schmid M, de Chatel R, Marcsek ZL, Demeter J: p53 mutations in hairy cell leukemia. *Leukemia* 2000, 14:706–711
37. Vallianatou K, Brito-Babapulle V, Matutes E, Atkinson S, Catovsky D: p53 gene deletion and trisomy 12 in hairy cell leukemia and its variant. *Leuk Res* 1999, 23:1041–1045
38. Ory K, Legros Y, Auguin C, Soussi T: Analysis of the most representative tumour-derived p53 mutants reveals that changes in protein conformation are not correlated with loss of transactivation or inhibition of cell proliferation. *EMBO J* 1994, 13:3496–3504
39. Adams JC, Schwartz MA: Stimulation of fascin spikes by thrombospondin-1 is mediated by the GTPases Rac and Cdc42. *J Cell Biol* 2000, 150:807–822
40. Snapper SB, Takeshima F, Anton I, Liu CH, Thomas SM, Nguyen D, Dudley D, Fraser H, Purich D, Lopez-Illasaca M, Klein C, Davidson L, Bronson R, Mulligan RC, Southwick F, Geha R, Goldberg MB, Rosen FS, Hartwig JH, Alt FW: N-WASP deficiency reveals distinct pathways for cell surface projections and microbial actin-based motility. *Nat Cell Biol* 2001, 3:897–904
41. Pellegrin S, Mellor H: The Rho family GTPase Rif induces filopodia through mDia2. *Curr Biol* 2005, 15:129–133
42. Aronheim A, Broder YC, Cohen A, Fritsch A, Belisle B, Abo A: Chp, a homologue of the GTPase Cdc42Hs, activates the JNK pathway and is implicated in reorganizing the actin cytoskeleton. *Curr Biol* 1998, 8:1125–1128
43. Karnoub AE, Symons M, Campbell SL, Der CJ: Molecular basis for Rho GTPase signaling specificity. *Breast Cancer Res Treat* 2004, 84:61–71
44. Keely PJ, Westwick JK, Whitehead IP, Der CJ, Parise LV: Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. *Nature* 1997, 390:632–636
45. Lane MA, Sainten A, Cooper GM: Stage-specific transforming genes of human and mouse B- and T-lymphocyte neoplasms. *Cell* 1982, 28:873–880
46. Bosch F, Jares P, Campo E, Lopez-Guillermo A, Piris MA, Villamor N, Tassies D, Jaffe ES, Montserrat E, Rozman C, Cardesa A: PRAD-1/cyclin D1 gene overexpression in chronic lymphoproliferative disorders: a highly specific marker of mantle cell lymphoma. *Blood* 1994, 84:2726–2732
47. Nicolaou F, Teodoridis JM, Park H, Georgakis A, Farokhzad OC, Bottinger EP, Da Silva N, Rousselot P, Chomienne C, Ferenczi K, Arnaout MA, Shelley CS: CD11c gene expression in hairy cell leukemia is dependent upon activation of the proto-oncogenes ras and junD. *Blood* 2003, 101:4033–4041
48. Zhang X, Machii T, Matsumura I, Ezoe S, Kawasaki A, Tanaka H, Ueda S, Sugahara H, Shibayama H, Mizuki M, Kanakura Y: Constitutively activated Rho guanosine triphosphatases regulate the growth and morphology of hairy cell leukemia cells. *Int J Hematol* 2003, 77:263–273
49. Boettner B, Van Aelst L: The role of Rho GTPases in disease development. *Gene* 2002, 286:155–174
50. Saci A, Carpenter CL: RhoA GTPase regulates B cell receptor signaling. *Mol Cell* 2005, 17:205–214
51. Bhowmick NA, Ghiassi M, Aakre M, Brown K, Singh V, Moses HL: TGF- β -induced RhoA and p160ROCK activation is involved in the inhibition of Cdc25A with resultant cell-cycle arrest. *Proc Natl Acad Sci USA* 2003, 100:15548–15553
52. Cleverley S, Henning S, Cantrell D: Inhibition of Rho at different stages of thymocyte development gives different perspectives on Rho function. *Curr Biol* 1999, 9:657–660
53. Baker PK, Pettitt AR, Slupsky JR, Chen HJ, Glenn MA, Zuzel M, Cawley JC: Response of hairy cells to IFN- α involves induction of apoptosis through autocrine TNF- α and protection by adhesion. *Blood* 2002, 100:647–653
54. Yano S, Tokumitsu H, Soderling TR: Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. *Nature* 1998, 396:584–587
55. Price LS, Langeslag M, ten Klooster JP, Hordijk PL, Jalink K, Collard JG: Calcium signaling regulates translocation and activation of Rac. *J Biol Chem* 2003, 278:39413–39421
56. Sahai E, Marshall CJ: RHO-GTPases and cancer. *Nat Rev Cancer* 2002, 2:133–142
57. Burthem J, Cawley JC: Specific tissue invasion, localisation and matrix modification in hairy-cell leukemia. *Leuk Lymphoma* 1994, 14(Suppl 1):19–22