Cell cycle-dependent expression of HERG1 and HERG1B isoforms in tumor cells

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 5, Issue of January 31, pp. 2947–2955, 2003
Printed in U.S.A.

Cell Cycle-dependent Expression of HERG1 and HERG1B Isoforms in Tumor Cells*

Received for publication, October 22, 2002, and in revised form, November 12, 2002
Published, JBC Papers in Press, November 12, 2002, DOI 10.1074/jbc.M210789200

Olivia Crociani‡, Leonardo Guasti‡, Manuela Balzi§, Andrea Becchetti¶, Enzo Wanke¶, Massimo Olivotto‡, Randy S. Wymore‖, and Annarosa Arcangeli‡**

From the ‡Department of Experimental Pathology and Oncology, University of Firenze, Viale G. B. Morgagni 50, 50134 Firenze, Italy, the ‖Department of Biological Science, University of Tulsa, Tulsa, Oklahoma 74104-3189, the §Department of Clinical Physiopathology, University of Firenze, Viale Pieraccini 6, 50134 Firenze, and the ¶Department of Biotechnology and Biosciences, University of Milano Bicocca, Piazza della Scienza 2, 20126 Milano, Italy

The role of K^+ channel activity during cell cycle progression has become a research topic of considerable interest. Blocking of K^+ channels inhibits the proliferation of many cell types, although the mechanism of this inhibition is unclear. There is speculation that K^+ channels differentially regulate the electrical potential of the plasma membrane (V_m) during proliferation. We have demonstrated that in tumor cells the value of V_m is clamped to rather depolarized values by K^+ channels belonging to the HERG family. We report here that tumor cell lines preferentially express the *herg1* gene and a truncated, *N*-deleted form that corresponds to *herg1b*. This alternative transcript is also expressed in human primary acute myeloid leukemias. Both HERG1 and HERG1B proteins are expressed on the plasma membrane of tumor cells and can form heterotetramers. The expression of HERG protein isoforms is strongly cell cycle-dependent, accounting for variations in HERG currents along the mitotic cycle. Moreover, the blocking of HERG channels dramatically impairs cell growth of HERG-bearing tumor cells. These results suggest that modulated expression of different K^+ channels is the molecular basis of a novel mechanism regulating neoplastic cell proliferation.

Potassium channels are the most diverse class of plasma membrane ion channels, and this hetereogeneity is reflected by the large variety of specific roles they exert in different cell types. Besides the regulation of excitability in nerve and muscle cells, and the linkage between plasma membrane and metabolic activity, there is now evidence that K^+ channels are involved in the regulation of cell proliferation (1). The cellular mechanisms linking K^+ channel activity and cell proliferation remain unclear, although a possibility is that activation of K^+ channels might be required for the passage of cells through a specific phase of the mitotic cycle (1, 2). K^+ channel blockage has been shown to be antiproliferative for numerous non-excitable as well as excitable cells (3–9); however, the link between K^+ channel activity and cell cycle progression remains elusive. One hypothesis is that K^+ channels might regulate cell volume, as well as the concentration of intracellular solutes critical for cell metabolism; alternatively, K^+ channel activity might serve to maintain permissive membrane potentials at critical cell cycle checkpoints (1). Furthermore, terminally differentiated G_0 cells display a hyperpolarized value of their membrane potential (V_m), whereas cycling and in particular tumor cells are quite depolarized (10).

We have shown previously (11) that the depolarized state of many tumor cell lines can be explained by the lack of classical inward rectifier K^+ channel-type inward rectifier K^+ currents accompanied by the expression of peculiar voltage-dependent K^+ channels, belonging to the HERG[1] family (12, 13). The *herg* (*human eag-related*) gene belongs to an evolutionarily conserved multigenic family of voltage-activated K^+ channels, the *eag* (*ether a-gò-gò*) family (15). *herg* genes and HERG currents (I_{HERG}) are preferentially expressed in neoplastic cell lines of different histogenesis, as well as in primary human endometrial cancers (11, 14). The functional properties of HERG channels are complex, and their contribution to the repolarization of the cardiac action potential well understood (16). For our purposes, however, it is sufficient to recall that the HERG activation and inactivation curves are such that their crossover produces maximal channel open probability between −30 and −50 mV in resting conditions (12, 13), thus contributing substantially to the resting potential of tumor cells (12, 13). In some neurons, the HERG role appears to be the regulation of the action potential firing frequency (17). Recent studies (18) indicate that in various normal tissues other than heart and brain, I_{HERG} and the *erg* gene are expressed only at very early stages of embryo development and are subsequently replaced by inward rectifier K^+ channel currents.

The molecular basis of I_{HERG} is being uncovered. HERG channels are tetramers, with each subunit consisting of six transmembrane domains, and both N and C termini are located intracellularly. The HERG proteins compose the α subunit of the channel, whereas a β subunit associating with HERG is

* This work was supported by grants from the Associazione Italiana Contro le Leucemie (Firenze) (to A. A.), Associazione Italiana Contro le Leucemie Comitato 30 ore (to A. A.), from the Ministero dell'Università e Ricerca Scientifica e Tecnologica (MURST, Cofin '99 and Cofin '01) (to A. A.), from the Associazione Italiana per la Ricerca sul Cancro (to M. O.), and from Ente Cassa di Risparmio di Firenze (to M. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ512214.

** To whom correspondence should be addressed: Dept. of Experimental Pathology and Oncology, Viale Morgagni 50, 50134 Firenze, Italy. Tel.: 39-055-4282326; Fax: 39-055-4282333; E-mail: annarosa.arcangeli@unifi.it.

[1] The abbreviations used are: HERG, *herg*-encoded protein; *herg*, human *eag*-related gene; I_{HERG}, HERG current; IRK, inward rectifier K^+ channel; RPA, RNase Protection Assay; RT-PCR, reverse transcription PCR; *hcyc*, human cyclophilin gene; PBS, phosphate-buffered saline; HU, hydroxyurea; RA, retinoic acid; PI, propidium iodide; RACE, rapid amplification of cDNA ends; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PAS, periodic acid-Schiff.

represented, at least in parts of the heart, by the MIRP1 protein (19). Three different ERG proteins have been cloned in mammals: ERG1, ERG2, and ERG3 (HERG1, HERG2, and HERG3 in humans), with the latter being specific to the nervous tissues (20). The recently characterized genomic structure of the *herg* gene encoding the HERG1 protein (*herg1* gene) consists of 15 exons, spanning about 19 kb on chromosome 7 (21, 22). Most of the exons code for the N and C termini, which therefore appear to be putative sites for alternative splicing. The HERG1 C terminus contains the cyclic nucleotide binding domain (15), and an alternatively spliced product of this region (named $HERG_{USO}$) has been identified in the heart (23), which cannot be expressed on the plasma membrane by itself but could modify the biophysical properties of I_{HERG}. Conversely, the N terminus is made up of two domains, the "eag" domain, comprising the first 135 amino acids of the HERG1 sequence, and the "proximal" domain, which extends from position 135 to about position 366. The former domain, a eukaryotic PAS domain, is involved in the regulation of channel gating (24–26), particularly with regard to deactivation rates, whereas the latter is apparently involved in regulating channel activation. An alternative transcript of the *herg1* gene, displaying a short N terminus, has been identified in mouse and human hearts, *merg1b* and *herg1b*, respectively (27, 28). Compared with *merg1*, *merg1b* has a different first exon (designated *1b*) located between exons 5 and 6 of the *Merg1* genomic sequence. Because the region upstream from exon *1b* may contain an alternate transcription initiation site, it is possible that *merg1b* represents an alternate transcript more than a splicing variant (27, 28). However, recent evidence (29) seems to exclude the expression of this transcript at the protein level in the hearts of various species.

We thus investigated the molecular structure of *herg* genes and HERG proteins in tumor cell lines. In particular, because I_{HERG} biophysical features (rapid deactivation kinetics and strong dependence of the activation gate on depolarized values of the V_m) as well as *herg* biomolecular characteristics (presence of multiple RNA bands ranging from 4.4 to 1.9 kDa as revealed in Northern blot experiments) in tumor cells are quite different from those displayed by the channel in the heart and in *herg1*-transfected cells (11, 30), the expression of different *herg* genes, as well as of alternate transcripts in tumor cells, was investigated.

We report here that tumor cell lines, as well as primary human tumors, preferentially express the *herg1* gene, along with *herg1b*. Both the full-length HERG1 and HERG1B proteins are coexpressed and can form heterotetramers on the plasma membrane of tumor cells. The expression of the two HERG protein isoforms turned out to be strongly cell cycle-dependent, suggesting a possible explanation for the variations in I_{HERG} along the mitotic cycle previously demonstrated in neuroblastoma cells (12). Moreover, the block of HERG channels dramatically impaired cell growth of HERG-bearing neuroblastoma cells.

On the whole, these results contribute to an understanding of the molecular basis of a novel mechanism regulating neoplastic cell proliferation, *i.e.* HERG K^+ channels.

MATERIALS AND METHODS

Cell Culture—The human neuroblastoma SH-SY5Y and LAN1 clone AE12 (kindly provided by Dr. G. Mugnai, University of Firenze, Italy) cell lines, the human rhabdomyosarcoma RD12 cell line (kindly provided by Dr. P. L. Lollini, University of Bologna, Italy), and HEK 293 cells (kindly provided by Dr. S. Heinemann, University of Jena, Germany) were cultured in DMEM containing 4.5 g/liter of glucose and 10% FCS (HyClone) and incubated at 37 °C in a humidified atmosphere with 5% CO_2. The human colon carcinoma H630 (kindly provided by Dr. E. Mini, University of Firenze, Italy), the human monoblastic leukemia FLG 29.1 (kindly provided by Dr. P. A. Bernabei, Hematology Unit, Firenze, Italy), and the human mammary adenocarcinoma SkBr3 and the human retinoblastoma Y-79 (kindly provided by Dr. A. Albini, IST, Genova, Italy) cell lines were all cultured in RPMI 1640 medium containing 5, 10, and 20% FCS, respectively, and incubated at 37 °C in a humidified atmosphere with 5% CO_2.

Cell Transfection—The HEK 293 cells, cultured on 100-mm Petri dishes, were transiently transfected with *herg1* cDNA cloned into *Hin*dIII/*Bam*HI sites of the pCDNA3.1 vector (Invitrogen) by the calcium phosphate method. Six hours before transfection the medium was replaced once. The precipitation solution was then added to the cell cultures. The precipitation solution was 400 μl of 2× BES-buffered saline (50 mM BES, 280 mM NaCl, 1.5 mM $Na_2HPO_4{\cdot}2H_2O$ (pH 6.96)) plus 400 μl of 0.25 M $CaCl_2$ and 36 μg of the cDNA construct. The medium was replaced 15 h later. Protein extraction, as well as control patch clamp analysis, was performed 48–72 h post-transfection. pCDNA3.1 without the insert was also transiently transfected as above in the same cell line, which was used as a negative control. These cells are referred to as MOCK.

RNase Protection Assay—The RNase Protection Assay (RPA) was performed essentially according to Dixon and McKinnon (31). Briefly, RNA was extracted from semiconfluent tumor cell lines (see above) by the guanidinium/isothiocyanate method (32). Commercially available human brain RNA (Clontech) as well as human heart RNA (Ambion) were used as controls for *herg1* and *herg3* expression and for *herg1b*, respectively. Thirty μg of total RNA was hybridized overnight at 48 °C with [^{32}P]UTP-labeled RNA probes. Digestion was then performed for 1 h at room temperature with RNase A (40 μg/ml) and T1 (2 μg/ml). Yeast tRNA (Invitrogen) was used as a negative control to test for the presence of probe self-protection bands. The samples were run on a 6.6% polyacrylamide gel and exposed for 1–8 days. The human *erg* probes used were the following: *herg1* (nucleotides 1401–1880, GenBank™ accession number NM 000238); *herg2* (nucleotides 2041–2245, GenBank™ accession number NM 030779); *herg3* (nucleotides 272–480, GenBank™ accession number AF 032897); the N-terminal *herg1* clone (nucleotides 184–589, GenBank™ accession number NM 000238) named $herg_{N135}$ was produced in our laboratory (14). The probe relative to *herg1b* was produced by RT-PCR (see below) from the FLG 29.1 cell line. Human cyclophilin (Ambion) was used as an internal loading control.

Reverse Transcription-PCR—Two μg of total RNA was retrotranscribed with Superscript reverse transcriptase (200 units) (Invitrogen) in the presence of random hexamers (2.5 μM). For *herg1b* amplification, the cDNA thus obtained was amplified using HotStarTaq polymerase (5 units) (Qiagen) and the following primers: herg1b-up 5′-CGATTCCAGCCGGGAAGGC-3′; herg1b-down 5′-TGATGTCCACGATGAGGTCC-3′ (product size, 363 bp), according to the sequence reported in Lees Miller *et al.* (28). It is worth noting that herg1b-up maps on exon 1b of the genomic sequence, whereas herg1b-down maps on exon 6 of the same sequence that is shared by both *herg1b* and *herg1* isoforms. Thirty five cycles of amplification were carried out after 15 min of enzyme activation at 95 °C as follows: 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min. The PCR product was cloned by means of the TA cloning system (Invitrogen) and then subcloned in pBluescript vector, sequenced, and used for RPA experiments as reported above. PCRs relative to human *eag* and *Kv 1.3* transcripts were performed according to Smith *et al.* (33), using the following primers: heag-up 5′-CGCATGAACTACCTGAAGACG-3′; heag-down 5′-TCTGTGGATGGGGCGATGTTC-3′ (product size, 479 bp); Kv 1.3-up 5′-TCGAGACGCAGCTGAAGAC-3′; Kv 1.3-down 5′-GGTACTCGAAGAGCAGCCAC-3′ (product size, ~350 bp).

Cloning of Herg1b from Tumor Cells—The *hergb* transcript was cloned from FLG 29.1 cells with two different approaches, RT-PCR and RACE-PCR. Briefly, poly(A) RNA was extracted by means of a poly(A) Pure kit (Ambion), and cDNA-retrotranscribed using either oligo(dT) primers (for RT-PCR cloning) or the 3′-adapter primer according to the 3′-RLM-RACE kit protocol (Ambion) (for RACE-PCR). PCR was performed with Takara polymerase (2.5 units) using the following primers: UP primer, 5′-AGGGAGCCAAGTCCTCCATGG-3′ (which maps into the sequence relative to the human exon 1b (dbEST Id: 8445856)); DOWN primer, 5′-GCGGCCGCACTGCCCGGGTCCGAG-3′ (which maps at the end of the *herg1* sequence with the addition of a *Not*I restriction site). The amplified band of ~2.5 kb was purified and cloned into the pCR2.1 vector (Invitrogen) using the TA cloning kit (Invitrogen). 3′-RACE PCR was performed according to the protocol provided in the Ambion kit, using 2.5 units of HotStarTaq polymerase (Qiagen), and using the same UP primer as reported above for first round. The second round of amplification was performed using *herg1b*-specific primers: UP

primer 5′-CAGGCAAAGCTTAGGGAGCCAAGTCCTCCATGG-3′ (which corresponds to the above reported up primer used for RT-PCR plus a *Hin*dIII restriction site); DOWN primer 5′-CAGCGCGCGGCC-GCCTGGGTGAGCCACGTGTC-3′ (which maps in the untranslated region of the *herg1* sequence (see above) and contains a *Not*I restriction site). In this case the amplified band was cloned into *Hin*dIII/*Not*I sites of pBluescript (Stratagene) vector. All the cloned bands were sent off for sequencing by PRIMM DNA Sequencing Service.

Protein Chemistry—For Western blot experiments both total cell lysate and membrane proteins were used as described previously (34). For membrane decoration two anti-HERG antibodies were used: an anti-ERG antibody raised against the C terminus (residues 1121–1137 of rat ERG1, Alomone Labs) and an anti-HERG antibody against the N terminus (residues 1–135 of human ERG1) developed in our laboratory (14). The latter serum was immunopurified on a column preadsorbed with the antigen and tested by means of enzyme-linked immunosorbent assay. *N*-Glycosidase F (Roche Molecular Biochemicals) was used following the manufacturer's instructions. For proteinase K (Roche Molecular Biochemicals) treatment, confluent cell cultures, seeded on 100-mm Petri dishes or on 25-cm^2 flasks, were washed with PBS and incubated with 3 ml of a solution containing 10 mM HEPES, 150 mM NaCl, and 2 mM $CaCl_2$ (pH 7.4) with or without 200 μg/ml proteinase K, at 37 °C for 30 min; enzyme activity was then stopped with 2 ml of ice-cold PBS containing 6 mM phenylmethylsulfonyl fluoride, 25 mM EDTA. After three washes in ice-cold PBS, membrane proteins were isolated and processed as above. For immunoprecipitation 3 mg of total protein lysate was cleared by incubation with protein A-Sepharose (50 μl of a 50% slurry) for 2 h at 4 °C. Anti-HERG antibody against the N terminus was added, and the samples were incubated on ice for 1 h. 40 μl of protein A-Sepharose was then added, and each sample was incubated overnight at 4 °C. The immunoprecipitates were washed in lysis buffer and ice-cold PBS prior to SDS-PAGE. Membranes were then immunoblotted with anti-ERG C terminus antibody. Super Signal (Pierce) was used for blot visualization.

Cell Synchronization and Cell Cycle Analysis—SH-SY5Y neuroblastoma cells were synchronized by hydroxyurea treatment according to Arcangeli *et al.* (12). Retinoic acid treatment was performed according to Arcangeli *et al.* (35). The distribution in the cell cycle phases was determined by flow cytometry; samples of cell suspension (10^6 cells/ml) were stained with propidium iodide (PI) as described by Vindeløv and Christensen (36). The samples were then analyzed using a FACScan flow cytometer (BD Biosciences) equipped with a 5-watt argon ion laser. The fluorescence of PI-stained nuclei was excited at 488 nm, and histograms of the number of cells *versus* linear integrated red fluorescence were recorded for 50,000 nuclei/sample. DNA histograms were analyzed using the MultiCycle DNA content and cell cycle analysis software (Phoenix Flow Systems, San Diego).

Cell Proliferation Assay—The human neuroblastoma cell lines SH-SY5Y and LAN1, cultured as above, were seeded in 96-well plates (Corning Glass) at a cell density of 1.8×10^4 and 1.2×10^4 cells per well, respectively, and then starved for 16 h in DMEM containing 1% FCS. After this time, DMEM containing 2.5% FCS, with or without HERG channel blocker (E4031 200 or 50 μM, and WAY 123,398, 50 μM, final concentrations), was added, and this was considered to be the time 0 of the experiment. At different times of incubation, cells were assayed using the colorimetric Cell Proliferation Reagent WST-1 (Roche Molecular Biochemicals), whose tetrazolium salt is cleaved by mitochondrial enzymes so that the amount of dye developed (read at 450 nm, reference at 630 nm) directly correlates to the number of metabolically active cells in the culture. Absorbance of culture medium plus wst-1 in the absence of cells was the blank position for the enzyme-linked immunosorbent assay reader (ELx-800, Biotek Instruments).

Data Acquisition and Analysis—RPA and Western blot images were acquired by an HP4C scanner, and the relative bands were analyzed by Scion Image software.

RESULTS

Experiments performed were aimed at determining the molecular basis of HERG currents in tumor cells. The first point to be explored was whether cancer cells expressed different *herg* genes, namely *herg1*, *herg2*, or *herg3*. For this purpose, RPA experiments were performed using appropriately cloned *herg1*, *-2*, and *-3* probes on tumor cell lines of different histogenesis: human neuroblastoma (SH-SY5Y), human rhabdomyosarcoma (RD12), human colon carcinoma (H630), human mammary carcinoma (SkBr3), and human monoblastic leukemia (FLG 29.1).

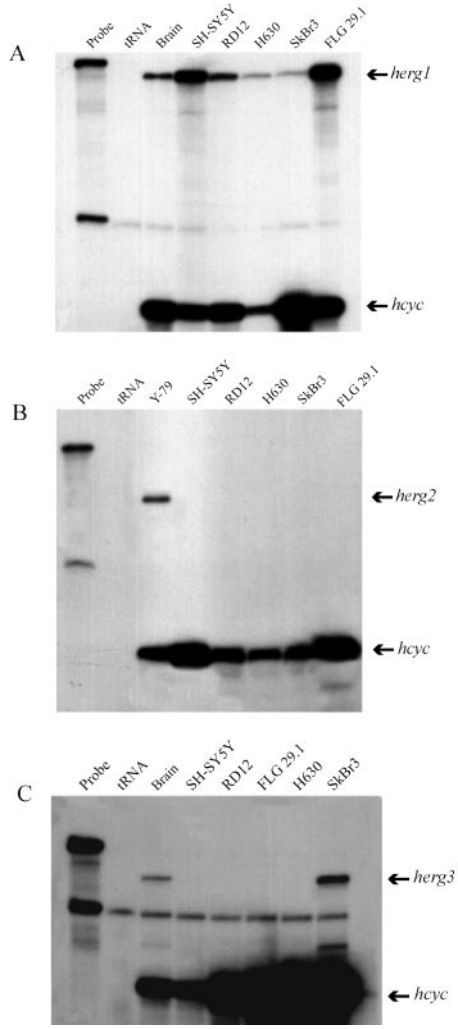

FIG. 1. ***herg-1*, *-2*, and *-3* expression in various human tumor cell lines.** RNA extracted from SH-SY5Y human neuroblastoma cells, RD12 human rhabdomyosarcoma cells, H630 human colon carcinoma cells, SKBr3 human mammary carcinoma cells, and FLG 29.1 human monoblastic leukemia cells was probed with the *herg1*, *-2*, and *-3* probes as described under "Materials and Methods." Human brain RNA was used as a control for *herg1* and *herg3*, whereas RNA from human retinoblastoma Y-79 cells was used as a control for *herg2* expression. Human cyclophilin (*hcyc*) (Ambion) was used as an internal control and yeast tRNA as a negative control to test for probe self-protection bands. *A*, *herg1* (1-day exposure); *B*, *herg2* (5-day exposure); *C*, *herg3* (1-day exposure). The protected bands corresponding to the above-mentioned genes are indicated by an *arrow*.

The results of these experiments are shown in Fig. 1. As shown in Fig. 1*A*, all of the tumor cell lines tested express the *herg1* gene, although at different intensities (see also the densitometric analysis reported in Fig. 3*a*). In particular, both SH-SY5Y and FLG 29.1 cells appear to overexpress the *herg1* gene, as suggested previously (11). On the other hand, no human tumor cell line expresses the *herg2* gene (Fig. 1*B*), except for the human retinoblastoma cell line Y-79. This expression, which represents the positive control in our experiments, is in keeping with the well known expression of *erg2* gene in the retina, at least in rat (20). As for *herg3* expression (Fig. 1*C*), only SkBr3 cells express the gene at good levels, as compared with human brain. Therefore, these cells express both *herg3* and *herg1*, with the latter expressed at relatively low intensity.

Because Kv channel encoding genes other than *herg*, like *eag* or *Kv 1.3*, have reportedly been linked to cell proliferation in different models (37, 38), we tested whether the above-mentioned genes were overexpressed in the tumor cell lines under

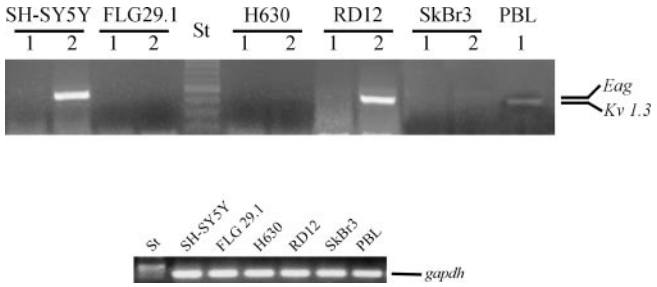

FIG. 2. **Expression of *eag* and *Kv 1.3* transcripts in tumor cell lines.** RNA was extracted from SH-SY5Y, RD12, H630, FLG 29.1, and SKBr3 tumor cell lines, as well as from normal peripheral blood lymphocytes (*PBL*). cDNA was retrotranscribed and amplified using specific primers for the *eag* and *Kv 1.3* K^+ channel genes as reported under "Materials and Methods." For each cell line used, *lane 1* represents amplification of the *Kv 1.3* gene, and *lane 2* represents amplification of the *eag* gene. Lymphocyte cDNA was amplified for the *Kv 1.3* gene and was used as the positive control. *Lower panel*, RT-PCR of the control gene *gapdh* in the various tumor cell lines as above.

study. Fig. 2 shows the expression of *eag* and *Kv 1.3* genes in SH-SY5Y, FLG 29.1, H630, RD12, and SkBr3 cells, as detected by RT-PCR. It is evident that, despite the good quality of all the cDNAs tested (see *gapdh* expression in the *lower panel* of Fig. 2), only SH-SY5Y, as reported previously (39), and RD12 cells, as expected (40), express the *eag* gene. *Kv 1.3* is not expressed in any of the tumor cell lines examined, although it is present in human peripheral resting lymphocytes as reported previously (33).

On the whole, data presented in Figs. 1 and 2 demonstrate that cancer cell lines preferentially express the *herg1* gene. Neither *herg2* nor *-3* nor the other Kv encoding genes that have been proposed to play a role in the control of cell proliferation (*i.e. eag* and *Kv 1.3*) are expressed at the RNA level, irrespective of the histological origin of the cancer cell lines tested. This result rules out the possibility that the *herg* RNA profile (11) as well as the HERG biophysical features specific of the different tumor cell lines that we have tested are due to coexpression of different proportions of the products of the three *herg* genes. The possibility of deletions as well as alternative splicing products of the *herg1* gene in tumor cells was then investigated. Because tumor I_{HERG} was demonstrated previously (30) to display fast deactivation kinetics, a feature associated with a deletion in the N-terminal domain, we first looked for the existence of *herg1* deletions and/or splicing modifications at this level.

A probe was constructed ($N_{(135)}$ *herg1* terminus) for RPA experiments, comprising the first 135 amino acids of the HERG1 sequence, *i.e.* the *eag* domain. The results of this experiment are reported in Fig. 3. The *eag* domain is present in the *herg1* transcript of all the tumor cell lines tested; however, when comparing the densitometric analysis of the results obtained with the *herg1* probe, encompassing a conserved region of the gene (Fig. 1), with the densitometric analysis of the experiments performed with the $N_{(135)}$ *herg1* terminus probe (see Fig. 3, *a* and *b*), it is evident that the *eag* domain is expressed at a lower level, especially in SH-SY5Y and FLG 29.1 cells. A possible explanation of these data is that tumor cells express both a full-length *herg1* mRNA, and a truncated form of the latter, lacking part or the entire N terminus.

The possibility that such *N*-truncated RNA could belong to the already identified *herg1* alternative transcript named *herg1b* was then tested. First, the expression of *herg1b* was studied in two different tumor cell lines (SH-SY5Y and FLG 29.1 cells) by RT-PCR. As shown in Fig. 4*A*, *herg1b* mRNA is indeed expressed in both the cell lines tested. The possibility of the simultaneous expression of *herg1* and *herg1b* in tumor cell

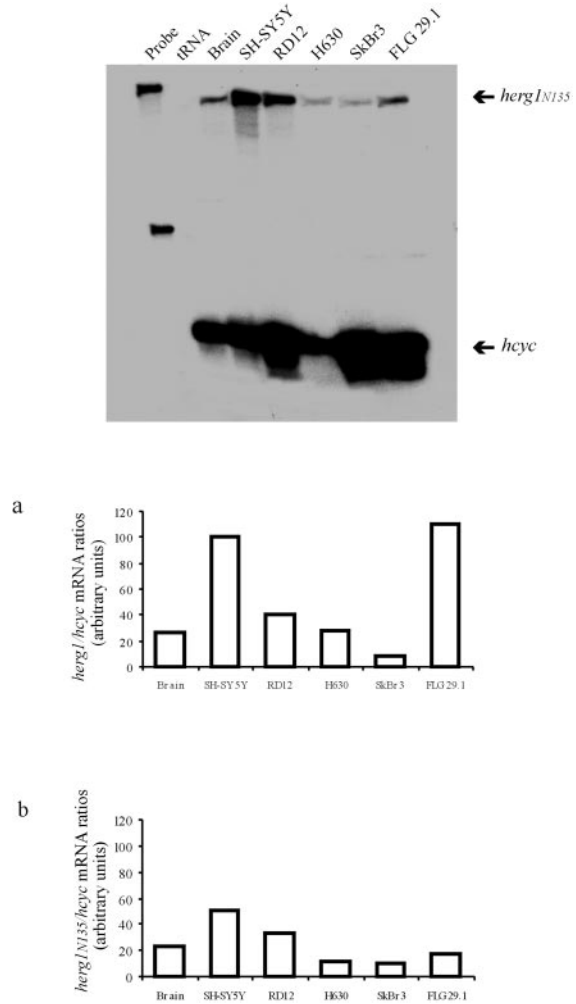

FIG. 3. **Expression of the *herg1* N-terminal domain in tumor cell lines.** The RNA samples were probed with the *herg1* probe encompassing the first 135 amino acids of the N-terminal domain ($herg1_{N135}$) as described under "Materials and Methods." Human cyclophilin (*hcyc*) (Ambion) was used as an internal control and yeast tRNA as a negative control to test for the presence of the probe self-protection bands. Exposure was 1 day. *herg1*- and *hcyc*-protected bands are indicated by an *arrow*. *a*, *densitometric analysis of the herg1* expression, relative to Fig. 1*A*; *b*, densitometric analysis of the $herg1_{N135}$ expression reported in this figure. Signals of the *herg1* and $herg1_{N135}$ protected bands were normalized using the corresponding values of cyclophilin.

lines was then investigated by constructing probes for RPA experiments comprising first the entire *herg1b* exon and part of exon 6, which is shared by *herg1* and *herg1b* genes (27, 28). If both *herg1* and *herg1b* are expressed in tumor cells, two RPA bands would be expected with molecular weights 258 and 363 bp, respectively. This result indeed occurred (see Fig. 4*B*) both in SH-SY5Y and FLG 29.1 cells and in the heart. Note that this is not common to all tissues expressing *herg1*, as only a lower RPA band was detectable in brain RNA, corresponding to the *herg1* gene. Moreover, observing the two bands present in FLG 29.1 cells, it is evident that the upper band (attributable to *herg1b*) has a higher intensity compared with the lower band corresponding to *herg1*. As tumor cell lines are deregulated in terms of their RNA expression, we analyzed whether *herg1b* mRNA could be detected in primary human tumors. We demonstrated recently (41) that the *herg1* gene is expressed in human myeloid leukemias; hence, we chose these cells as samples because they are not contaminated by other cell types, such as stromal or smooth muscle cells, that could express the *herg1b* transcript (27, 28). As shown in Fig. 4*C*, all of the

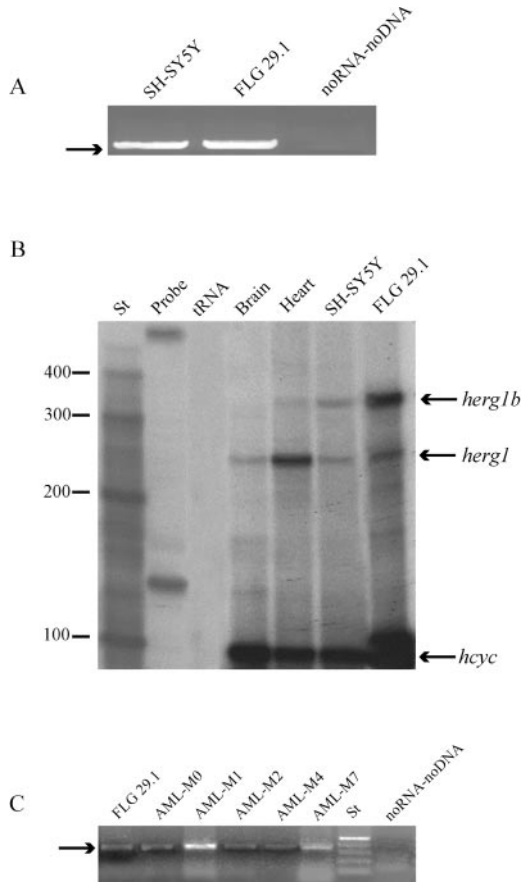

FIG. 4. ***herg1b* expression in tumor cell lines.** *A*, RT-PCR. RNA extracted from FLG 29.1 and SH-SY5Y (see *upper labels*) was retrotranscribed and amplified using primers specific for *herg1b* (see "Materials and Methods"). The lane labeled *noRNA-noDNA* represents the negative control. The two bands indicated by the *arrow* were sequenced and showed a 93% identity with the mouse *erg1b* (GenBank™ accession number AF034762). *B*, RPA experiments. RNA extracted from brain, heart, SH-SY5Y, and FLG 29.1 cell lines was hybridized with the *herg1b* probe cloned from FLG 29.1 (see "Materials and Methods"). *St*, ^{32}P-UTP-labeled molecular weight marker (Ambion). The results were obtained after 8 days of exposure and show a specific *herg1b*-protected band in all the samples tested except for the brain. Human cyclophilin (Ambion) was used as an internal control and yeast tRNA as a negative control to test for the presence of the probe self-protection bands. *C*, RT-PCR on primary acute myeloid leukemias. RNA was extracted from various primary acute myeloid leukemias of different FAB phenotypes (M0 to M7, see *upper labels*) and processed as described in the legend to *A*. RNA from FLG 29.1 human monoblastic cell line was used as positive control. Lane labeled as *noRNA-noDNA* represents the negative control.

primary myeloid leukemias we tested expressed the *herg1b* exon, ruling out the possibility that such expression is exclusively an artifact related to the altered gene expression occurring in established tumor cell lines.

The nature of the transcript containing the *herg1b* exon in tumor cells was then investigated by cloning the entire transcript from tumor cells. Clones obtained by RT-PCR and 3′-RACE PCR (see "Materials and Methods") were sequenced demonstrating that tumors cells do express the entire *herg1b* alternative transcript (GenBank™ accession number AJ512214). The *herg1b* transcript cloned from tumor cells was identical to that identified in human heart (27, 28), except for two polymorphisms (in position 689 and 953 of the submitted sequence), and identical to that reported for the *herg1* sequence cloned from neuroblastoma cells (11). It is worth noting here that, as stated in the Introduction and reported under "Materials and Methods," the sequence of the *herg1b* exon was confirmed on the genomic sequence of chromosome 7, suggesting the possibility that *herg1b* represents an alternate transcript more than a splice variant.

Furthermore, because data gathered from our RPA experiments showed that, in FLG 29.1 cells, *herg1b* represented the greatest amount of the total HERG mRNA, the next step was to determine whether the encoded protein HERG1B was expressed on the plasma membrane. Western blot experiments were, therefore, performed on SH-SY5Y neuroblastoma and FLG 29.1 leukemia cells, using anti-HERG antibodies, specific for both the C and N termini (see "Materials and Methods").

When experiments were performed with an anti-C terminus antibody (Fig. 5*A*), two main bands were detectable in *herg1*-transfected cells, weighing 135 and about 155 kDa, respectively, as expected (29, 42). On the other hand, in SH-SY5Y and FLG 29.1 cells, two main groups of bands could be seen: an upper group ranging from 135 to ~155 kDa, and a lower group ranging from 85 to ~100 kDa. It is worth noting that the upper group of bands is more evident in SH-SY5Y cells, whereas they are barely detectable in FLG 29.1 cells. The possibility that both the two groups represented HERG proteins expressed on the plasma membrane was then tested by performing experiments on cells treated with specific enzymes used to evaluate the glycosylation state (*N*-glycosidase F) as well as the plasma membrane expression (proteinase K) of HERG proteins. As shown in Fig. 5*B*, when membrane extracts from both SH-SY5Y and FLG 29.1 cells were treated with *N*-glycosidase F (*lanes 1*), both the bands of ~155 and those ~100 kDa shifted to lower molecular weights. Furthermore, when cells were treated with proteinase K (Fig. 5*B*, *lanes 3*) both the bands of ~155 and those of ~100 kDa disappeared, and only the bands of ~135 and ~85 kDa could be seen. The results were similar in both cell lines tested; the only difference was that all the bands of lower molecular weight were preferentially expressed in FLG 29.1 cells, whereas those of higher molecular weight were observed only after longer exposure of the autoradiographic film (see Fig. 5*B, inset*).

The results of these experiments suggest that both SH-SY5Y and FLG 29.1 cells express two HERG isoforms on their plasma membranes: one corresponding to the full-length protein (135 kDa), and its various glycosylated forms (the bands of ~155 kDa); and the second corresponding to HERG1B, which has a molecular mass of 85 kDa in the unglycosylated, immature form, and various glycosylated forms expressed on the plasma membrane (the bands of ~100 kDa). To verify this, experiments were performed using the antibody directed against the HERG N-terminal domain (see "Materials and Methods"). As shown in Fig. 5*C*, the reactivity of this antibody on HERG-transfected HEK 293 cells is comparable with that of the anti-C-terminal antibody (compare *C* with *A*), whereas in SH-SY5Y cells only the bands of higher molecular mass, ranging from 135 to 155 kDa, can be detected with this antibody. Similar results were obtained in FLG 29.1 cells with very long exposure (not shown).

On the whole, it appears plausible to conclude that tumor cell lines express two mature, highly glycosylated HERG proteins on their plasma membrane, a full-length HERG1 and the HERG1B isoform. In order to explore the possibility that these two proteins could form heterotetramers in cancer cells, as observed in transfected oocytes (27, 28), immunoprecipitation experiments were performed. Proteins from both *herg1*-transfected HEK 293 cells and SH-SY5Y cells were immunoprecipitated using the anti-N-terminal antibody that recognizes only the *herg1* product, and the subsequent Western blot was decorated with the anti-C-terminal antibody that is capable of recognizing both isoforms. As shown in Fig. 6, the immunoprecipitated proteins contain only HERG1 full-length in the trans-

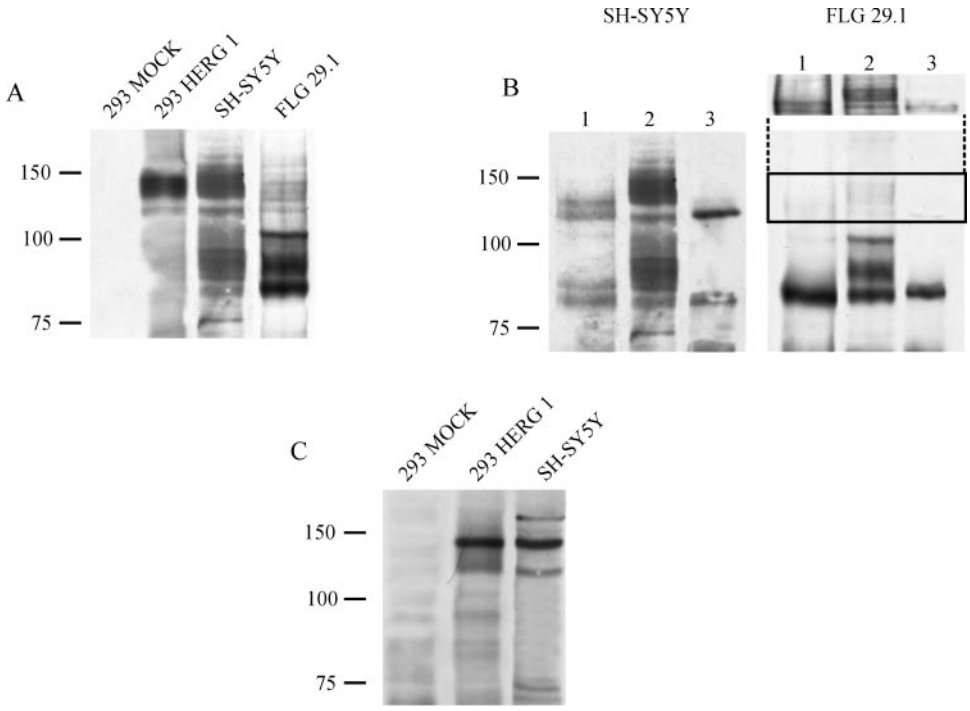

FIG. 5. **HERG protein expression in *herg1*-transfected cells and in tumor cell lines.** Membrane proteins were extracted, separated by SDS-PAGE, and immunoblotted with the anti-HERG C-terminal antibody (Alomone Labs) as reported under "Materials and Methods." *A*, HERG protein expression in HEK 293 MOCK-transfected cells (*293 MOCK*), in HEK 293 transfected with the *herg1* clone (*293 HERG1*), in SH-SY5Y cells, and in FLG 29.1 cells. The molecular weight of a protein standard (Bio-Rad) is reported on the *left.* Twenty μg of proteins were loaded in the case of HEK 293-transfected cells and 50 μg in the case of SH-SY5Y and FLG 29.1 cells. *B*, HERG protein expression in SH-SY5Y cells (*left panel*) and FLG 29.1 cells (*right panel*) treated with various enzymes as described under "Materials and Methods." *Lanes 1*, membrane extracts treated with *N*-glycosidase F; *lanes 2*, untreated control extracts; *lanes 3*, cells treated with proteinase K. The *inset* over the *right panel* relative to FLG 29.1 cells represents a longer exposure of the autoradiographic film corresponding to the full-length HERG protein bands (see "Results"). *C*, HERG protein expression in HEK 293 MOCK-transfected cells, in HEK 293 transfected with the *herg1* clone (293 HERG1), and in SH-SY5Y cells using an N-terminal antibody (see "Materials and Methods"). The molecular weight of a protein standard (Bio-Rad) is reported on the *left.*

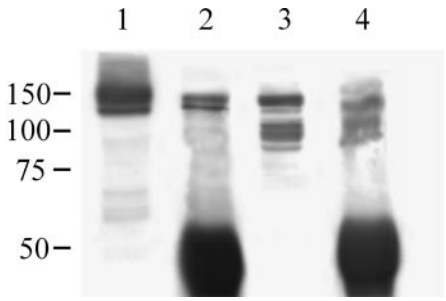

FIG. 6. **Immunoprecipitation of HERG proteins in *herg1*-transfected HEK 293 cells and SH-SY5Y cells.** Three mg of total cell extracts from *herg1*-transfected HEK 293 as well as SH-SY5Y (*lanes 2* and *4*, respectively) were immunoprecipitated using anti-HERG N terminus. The membrane was further decorated with anti-HERG C terminus. Twenty μg of HEK 293 proteins and 40 μg of SH-SY5Y proteins were also loaded (*lanes 1* and *3*, respectively). The molecular weight of a protein standard (Bio-Rad) is reported on the *left.*

fected HEK 293 cells (*lane 2*), whereas bands indicating the presence of both HERG1 and HERG1B proteins are detected in SH-SY5Y cells (*lane 4*).

It is, therefore, likely that tumor cell lines not only express both HERG1 and HERG1B proteins on their plasma membrane but that these proteins can form heterotetramers in these cells. Such heterotetramers can be composed of different amounts of each single protein, as suggested by the different appearance of Western blots on SH-SY5Y cells and FLG 29.1 cells.

We have demonstrated previously (12) that biophysical features, such as the activation voltage, of the HERG currents in neuroblastoma cells are cell cycle-dependent. The possibility now exists that such features can be accounted for by differential expression of HERG1B and full-length HERG1 proteins. Therefore, SH-SY5Y cells were synchronized by treatment with hydroxyurea (HU) and retinoic acid (RA), as reported previously (12). After a 15-h treatment with HU, neuroblastoma cells were blocked at the G_1/S boundary. Then, after HU withdrawal, cells enter almost synchronously into S phase, so that after 6 h a high percentage of cells is in the middle of the S phase and reaches the S/G_2 boundary after 8 h (Fig. 7*A*). Western blot experiments performed on the same cell preparation (Fig. 7*B*) showed that expression of the HERG1B mature form is significantly up-regulated in the middle of S phase as compared with unsynchronized cells, and to G_1/S HU-blocked cells (see also the densitometric analysis of the two HERG isoforms reported in *inset b*). Conversely, when SH-SY5Y cells were treated for 11 days with RA, a strong synchronization of the cells in G_1 was achieved (Fig. 8*A*) (12). Under these conditions, a strong up-regulation of HERG proteins can be detected in Western blot experiments (Fig. 8*B*) in agreement with results reported previously (35). This up-regulation apparently involves both of the isoforms, although a stronger increase in the intensity of the mature full-length HERG1 band can be observed (see the densitometric analysis reported in *inset b*).

On the whole, results presented in Figs. 7 and 8 are consistent with the previously reported modulation of I_{HERG} activation curves during the cell cycle (12); in fact, prevalence of HERG1B expression shifts the activation voltage toward depolarized values (see the mouse *erg1b* encoded currents reported in Refs. 27 and 28), whereas the I_{HERG} of the full-length HERG has an activation voltage that is more hyperpolarized.

These results allow us to ask a fundamental question: what is the putative role of I_{HERG} in the regulation of cancer cell proliferation? A preliminary answer to this question was ob-

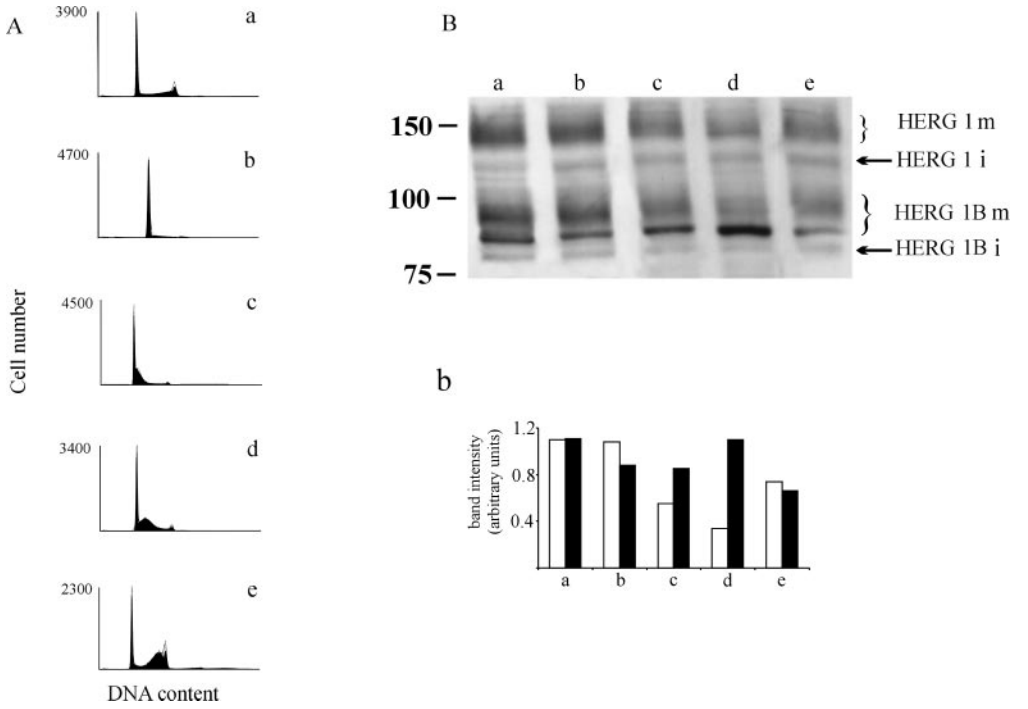

FIG. 7. **Cell cycle-dependent expression of full-length *versus* truncated HERG protein in SH-SY5Y cells.** SH-SY5Y cells were synchronized by HU treatment as reported under "Materials and Methods." At various times, cells were processed for both cytofluorimetric analysis and membrane extraction. Membrane extracts were then separated by SDS-PAGE and blotted; membranes were then decorated with the anti-HERG C-terminal antibody as reported above. Three separate experiments were performed, and a typical experiment is reported. *A*, cytofluorimetric analysis. *a*, control, unsynchronized cells (percentage of cells in G_1 = 54.4, S = 34.1, and G_2 = 11.5); *b*, cells treated with HU for 15 h (percentage of cells in G_1 = 80.5, S = 17.1, and G_2 = 2.4); *c*, cells after 3 h of HU withdrawal (percentage of cells in G_1 = 40.3, S = 57.1, and G_2 = 2.6); *d*, cells after 6 h of HU withdrawal (percentage of cells in G_1 = 39.8, S = 56.2, and G_2 = 3.9); *e*, cells after 8 h of withdrawal of HU (percentage of cells in G_1 = 29.7, S = 56.1, and G_2 = 14.2). *B*, Western blot of HERG proteins extracted from SH-SY5Y cells treated with HU as reported in *A*. *HERG1m*, HERG1 protein mature bands; *HERG1i*, HERG1 protein immature band; *HERG1Bm*, HERG1B protein mature bands; *HERG1Bi,* HERG1B protein immature band. *Inset b*, densitometric analysis of the bands corresponding to the mature form of HERG1 (*white bars*) as well as of HERG1B (*black bars*) isoform.

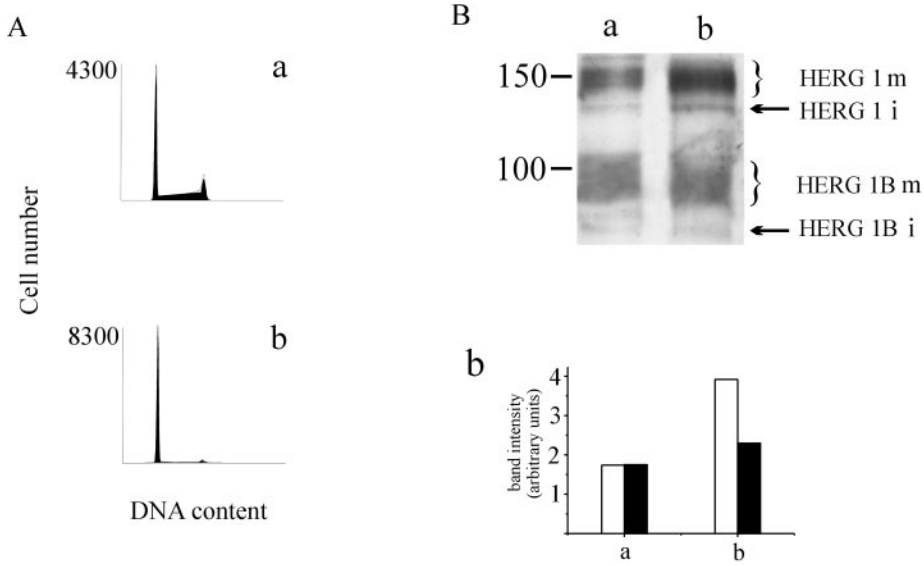

FIG. 8. **Expression of full-length *versus* truncated HERG protein in RA-treated SH-SY5Y.** SH-SY5Y cells were treated with RA for 11 days as reported under "Materials and Methods." The results are typical of four similar experiments. *A*, cytofluorimetric analysis. *a*, control, untreated cells (percentage of cells in G_1 = 45.9, S = 39.9, and G_2 = 14.2); *b*, RA-treated cells (percentage of cells in G_1 = 92.6, S = 5.3, and G_2 = 2.1). *B*, Western blot of HERG proteins extracted from SH-SY5Y cells treated with RA as reported in *A*. Labels on the *right* are the same as in Fig. 7. *Inset b*, densitometric analysis of the expression of the mature forms of HERG1 (*white bars*) as well as of HERG1B (*black bars*) isoforms.

tained by testing the effects of a specific I_{HERG} inhibitor (E4031) on proliferation of neuroblastoma cells, by utilizing the same approach used previously (41) in acute myeloid cells. The results of these experiments are reported in Fig. 9; it is evident that E4031 significantly impairs proliferation in SH-SY5Y cells (*left panel*), whereas it does not significantly affect cell growth in the LAN1 clone AE12 cells (*right panel*), which do not express I_{HERG}.[2] Similar results were obtained with another HERG blocker, namely Way 123,398 (see *insets* in Fig. 9), in a different set of experiments. I_{HERG} clearly affects proliferation of the SH-SY5Y cells; however, because the currents of both HERG1 and HERG1B proteins are blocked by these compounds, no conclusion about the differential role of the two HERG isoforms in cell growth can be inferred.

DISCUSSION

Results reported in this paper clearly show that various tumor cell lines preferentially express the *herg1* gene along

[2] G. Hofmann, personal communication.

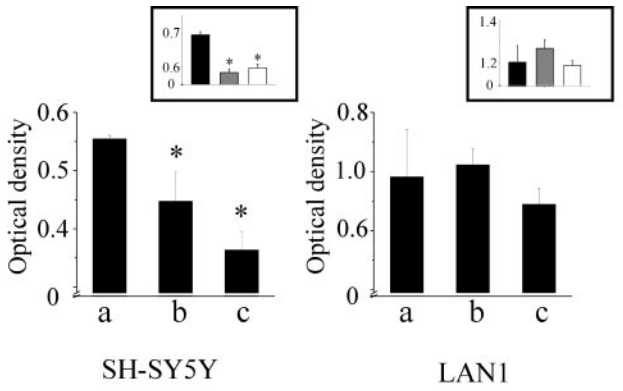

FIG. 9. **Effect of I_{HERG} block on cell proliferation of SY5Y and LAN1 (clone AE12) cells.** Cells were serum-starved overnight with 1% FCS and then cultured in 2.5% FCS in the absence (*a*) or in the presence of the HERG inhibitor E4031 (50 μM (*b*) and 200 μM (*c*), final concentrations), as reported under "Materials and Methods." Cell proliferation was assessed by means of the cell proliferation reagent WST1 assay after 48 h of incubation. *Insets,* comparative effect of 50 μM E4031 (*gray bars*), 50 μM Way 123,398 (*white bars*) as compared with the controls (*black bars*) on cell proliferation of SH-SY5Y (*left inset*) and LAN1 (clone AE12) (*right inset*) after 72 h of incubation. Values are expressed as optical density. Each experimental point represents the mean ± S.E. of four determinations carried out in two separate experiments. Student's *t* test for paired samples was carried out. *, significantly different at the $p < 0.05$ level as compared with the control.

with a truncated form of the HERG1 protein that corresponds to the alternative transcript of *herg1* first discovered in the heart, *herg1b*. The *herg1b* isoform is also expressed in primary human tumors. Moreover, the data reported here show that the corresponding proteins, HERG1 and HERG1B, are differentially expressed during cell cycle phases and that HERG currents are capable of modulating cell proliferation in tumor cells.

The expression of *herg1*, *-2*, and *-3* was studied in various human tumor cell lines in order to define better the molecular basis of HERG currents discovered in tumor cells. It was demonstrated previously (11, 30) that I_{HERG} biophysical properties (rapid deactivation kinetics and strong dependence of the activation gate on depolarized V_m values) as well as biomolecular features (presence of multiple RNA bands revealed by Northern blot) in tumor cells are quite different from those displayed in the heart and in *herg1*-transfected cells. As reported here, all the tumor cell lines tested expressed the *herg1* gene, whereas *herg2* expression is limited to human retinoblastoma cells and *herg3* to a human mammary carcinoma cell line. Whereas the expression of *herg2* in retinoblastoma cells is in keeping with the well documented expression of *erg2* in the retina of the rat (20) and quail (43), the presence of *herg3* in a cell line of epithelial origin is quite surprising, because *erg3* was first characterized as a nervous system-specific gene (20).

The following question thus arises: does the *herg* gene(s) expression in tumor cells represent 1) the re-expression of an embryonic gene or 2) the ectopic expression of a gene that is turned off in fully differentiated cell types? This is an important point for cancer researchers, as it has been well documented that neoplastic cells display biochemical and behavioral features of both embryonic, highly immature cells and novel, often metaplastic characteristics, completely altering the phenotype of the transformed cell. In neuroblastoma and rhabdomyosarcoma cells, *herg1* expression appears to be the re-expression of an embryonic gene (see our results on quail embryos (43)). The type of expression for tumors of epithelial origins is less clear as neither *herg1* nor *herg3* genes are expressed in epithelial tissues in adults (20) or embryos (43).

The data reported here also demonstrate that tumor cell lines and primary human tumors express an alternative transcript of *herg1*, which results in an N-terminal truncated form. It is worth noting that in human tumors many cancer-associated genes are alternatively spliced (44). Although the function of most of these variants is not well defined, some of them have antagonistic activities related to cell death mechanisms. In many types of cancer and cancer cell lines, the ratios of the splice variants are frequently shifted toward the anti-apoptotic splice isoforms. In this regard, the *herg1* gene may be among such genes that are alternatively spliced in neoplastic cells, raising interesting questions as to the different roles exerted by the full-length *versus* the spliced isoform in tumor cell establishment and maintenance.

These results also indicate that the truncated *herg1* is the alternative transcript *herg1b*. The whole transcript of *herg1b* was cloned by RT-PCR and 3′-RACE in FLG 29.1 leukemia cells. This is the first report of the entire transcript in humans obtained thus far (GenBank™ accession number AJ512214). During the 3′-RACE cloning procedure, we also cloned another *herg* variant that resulted in the fusion of the *herg1b* exon with the $herg_{USO}$ sequence. This transcript, lacking the 104 amino acid C-terminal domain necessary for channel recapitulation, is not expressed at the protein level on the plasma membrane and thus does not contribute to I_{HERG} currents,[3] but its putative role in tumor cells is now under investigation.

In contrast, the HERG1B protein is expressed on the plasma membrane and does form heterotetramers with the *herg1* gene product in tumor cells, as demonstrated clearly by immunoprecipitation experiments reported in this paper. The *herg1b* transcript was first identified in heart (27, 28), but the corresponding protein is not expressed in adult hearts (29). Although it was suggested (29) that the protein could be expressed during development, the exact role of HERG1B in cell physiology is still unknown. Therefore, the demonstration that this channel protein is expressed in tumors, while confirming that tumor cells often express embryonic and/or alternatively spliced genes, opens new and interesting perspectives on the role of *herg1* isoforms in tissues other than heart.

The expression of *herg1b* in neoplastic cells could explain both the peculiar pattern of mRNA expression and the biophysical features of HERG currents observed in tumor cells (11, 12, 30). HERG currents in such cells, especially in leukemia cell lines, display fast deactivation kinetics that may be attributable to expression of an N-terminal truncated HERG in these cells (30). As described previously (27, 28), the current encoded by *herg1b* displays most of the above biophysical features when expressed in oocytes.

Further experiments performed on synchronized cells show that whereas the truncated HERG1B form is up-regulated during the S phase, the full-length HERG1 protein increases its expression on the plasma membrane during the G_1 phase. These results give a molecular dimension to the previously demonstrated variations of HERG activation curves, as well as of V_m during cell cycle progression of neuroblastoma cells (12). This result is also in keeping with other reports demonstrating cell cycle modulation of K^+ channel expression (1), and in general with the reported link between K^+ channels and cell cycle progression (45, 46). In different models (1) it has been reported that an increase in K^+ channel expression and activity occurs at the G_1/S boundary and that such an increase is necessary for cells to traverse the cell cycle. In HERG-bearing tumor cells, such as SH-SY5Y, an increase in the HERG1B/HERG1 ratio on the plasma membrane occurs as cells proceed

[3] A. Arcangeli, A. Beechetti, L. Guasti, and O. Crociani, unpublished data.

through the S phase. This increase could account for the depolarization of V_m occurring during S phase progression of neuroblastoma cells, as reported previously (12), and the necessity of HERG channel activity for proliferation. This is demonstrated by impaired neuroblastoma and leukemia (41) cell proliferation in the presence of HERG-specific inhibitors. In other words, a tightly clamped V_m value is required for each cell cycle phase and can be obtained by alternatively switching the above-mentioned ratio; the V_m oscillations are apparently necessary for neuroblastoma cell cycling, so that cell proliferation stops either when the full-length HERG1 isoform is turned on and V_m hyperpolarizes (see RA-treated cells) or when the HERG isoforms-based clock is impaired by totally blocking HERG currents.

Finally, the truncated HERG1B isoform lacks the PAS domain, an oxygen-sensing domain of basic helix-loop-helix proteins, like HIF-1. The latter is a transcriptional activator that is up-regulated by hypoxia and is responsible for gene activation under hypoxic conditions (47). It is worth noting here that hypoxia is a main determinant of tumor progression and is currently regarded as a major hindrance to cancer therapy (48, 49). The ability of tumor cells to express two types of HERG proteins, one endowed with and the other lacking the PAS domain, could be an advantage for cancer growth and progression. In hypoxia, cells could thus sense the decreased oxygen tension by PAS, lowering the HERG1B/HERG1 ratio, thus leading to a shifting of the activation curve of HERG currents and to hyperpolarize V_m, limiting K^+ loss (50). This could permit the cell to survive in G_1 without entering into the apoptotic pathway, because K^+ efflux is recognized as one of the earliest events in cells undergoing apoptosis (51). When the oxygen supply is restored and/or growth factors are produced, HERG-bearing tumors could undergo a remodeling of their HERG channels on the plasma membrane, increasing the HERG1B/HERG1 ratio, thus clamping V_m to depolarized values compatible with sustained cell growth.

Data presented in this paper, and in particular the demonstration of protein expression of the *herg1b* transcript, provide a novel perspective for a therapeutic approach to control HERG-expressing human primary tumors like endometrial cancers (14), acute myeloid leukemias (41), astrocytomas,[4] and colo-rectal cancers.[5] Various strategies (specific drugs or specific antisense oligonucleotides) could be designed to block specifically the altered HERG currents in tumors without affecting the I_{kr} currents found in cardiac myocytes or other non-transformed cell types.

Acknowledgments—We thank Dr. E. Gherardi (MRC Centre, Cambridge, UK) and Dr. R. T. Wymore (University of Tulsa, Oklahoma) for reading the manuscript.

REFERENCES

1. Wonderlin, W. F., and Strobl, J. S. (1996) *J. Membr. Biol.* **154,** 91–107
2. Wang, S., Melkoumian, Z., Woodfork, K. A., Cather, C., Davidson, A. G., Wonderlin, W. F., and Strobl, J. S. (1998) *J. Cell. Physiol.* **176,** 456–464
3. Xu, B., Wilson, B. A., and Lu, L. (1996) *Am. J. Physiol.* **271,** C2037–C2044
4. Price, M., Lee, S. C., and Deutsch, C. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 10171–10175
5. Shirihai, O., Merchav, S., Attali, B., and Dagan, D. (1996) *Eur. J. Physiol.* **431,** 632–638
6. Nilius, B., and Wohlrab, W. (1992) *J. Physiol.* (*Lond.*) **445,** 537–548
7. Skryma, R. N., Prevarskaya, N. B., Dufy-Barbe, L., Odessa, M. F., Audin, J., and Dufy, B. (1997) *Prostate* **33,** 112–122
8. Yao, X., and Kwan, H. Y. (1999) *Life Sci.* **65,** 55–62
9. Vaur, S., Bresson-Bepoldin, L., Dufy, B., Tuffet, S., and Dufy-Barbe, L. (1998) *J. Cell. Physiol.* **177,** 402–410
10. Binggeli, R., and Weinstein, R. C. (1986) *J. Theor. Biol.* **123,** 377–401
11. Bianchi, L., Wible, B., Arcangeli, A., Taglialatela, M., Morra, F., Castaldo, P., Crociani, O., Rosati, B., Faravelli, L., Olivotto, M., and Wanke, E. (1998) *Cancer Res.* **58,** 815–822
12. Arcangeli, A., Bianchi, L., Becchetti, A., Faravelli, L., Coronnello, M., Mini, E., Olivotto, M., and Wanke, E. (1995) *J. Physiol.* (*Lond.*) **489,** 455–471
13. Faravelli, L., Arcangeli, A., Olivotto, M., and Wanke, E. (1996) *J. Physiol.* (*Lond.*) **496,** 13–23
14. Cherubini, A., Taddei, G. L., Crociani, O., Paglierani, M., Buccoliero, A. M., Fontana, L., Noci, I., Borri, P., Borrani, E., Giachi, M., Becchetti, A., Rosati, B., Wanke, E., Olivotto, M., and Arcangeli, A. (2000) *Br. J. Cancer* **83,** 1722–1729
15. Warmke, J. W., and Ganetzky, B. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 3438–3442
16. Sanguinetti, M. C., Jiang, C., Curran, M. E., and Keating, M. T. (1995) *Cell* **81,** 299–307
17. Chiesa, N., Rosati, B., Arcangeli, A., Olivotto, M., and Wanke, E. (1997) *J. Physiol.* (*Lond.*) **501,** 313–318
18. Arcangeli, A., Rosati, B., Cherubini, A., Crociani, O., Fontana, L., Ziller, C., Wanke, E., and Olivotto, M. (1997) *Eur. J. Neurosci.* **9,** 2596–2604
19. Abbott, G. W., Sesti, F., Splawski, I., Buck, M. E., Lehmann, M. H., Timothy, K. W., Keating, M. T., and Goldstein, S. A. (1999) *Cell* **97,** 175–187
20. Shi, W., Wymore, R. S., Wang, H. S., Pan, Z., Cohen, I. S., McKinnon, D., and Dixon, J. E. (1997) *J. Neurosci.* **17,** 9423–9432
21. Curran, M. K., Splawski, I., Timothy, K. W., Vincent, G. M., Green, E. D., and Keating, M. K. (1995) *Cell* **80,** 795–803
22. Itoh, T., Tanaka, T., Nagai, R., Kamiya, T., Sawayama, T., Nakayama, T., Tomoike, H., Sakurada, H., Yazaki, Y., and Nakamura, Y. (1998) *Hum. Genet.* **102,** 435–439
23. Kupershmidt, S., Snyders, D. J., Raes, A., and Roden, D. M. (1998) *J. Biol. Chem.* **273,** 27231–27235
24. Wang, J., Trudeau, M. C., Zappia, A. M., and Robertson, G. A. (1998) *J. Gen. Physiol.* **112,** 637–647
25. Cabral, J. H. M., Lee, A., Cohen, S. L., Chait, B. T., Li, M., and McKinnon, R. (1998) *Cell* **95,** 649–655
26. Viloria, C. G., Barros, F., Giraldez, T., Gomez-Varela, D., and De La Pena, P. (2000) *Biophys. J.* **79,** 231–246
27. London, B., Trudeau, M. C., Newton, K. P., Beyer, A. K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Satler, C. A., and Robertson, G. A. (1997) *Circ. Res.* **81,** 870–878
28. Lees-Miller, J. P., Kondo, C., Wang, L., and Duff, H. J. (1997) *Circ. Res.* **81,** 719–726
29. Pond, A. L., Scheve, B. K., Benedict, A. T., Petrecca, K., Van Wagoner, D. R., Shrier, A. S., and Nerbonne, J. M. (2000) *J. Biol. Chem.* **275,** 5997–6006
30. Schönnher, R., Rosati, B., Hehl, S., Rao, V. G., Arcangeli, A., Olivotto, M., Heinemann, S., and Wanke, E. (1999) *Eur. J. Neurosci.* **11,** 753–760
31. Dixon, J. E., and McKinnon, D. (1996) *Eur. J. Neurosci.* **8,** 183–191
32. Maniatis, T., Fritsh, E. F., and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., pp. 194–195, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
33. Smith, G. A. M., Tsui, H.-W., Newell, E. W., Jiang, X., Zhu, X.-P., Tsui, F, W. L., and Schlichter, L. C. (2002) *J. Biol. Chem.* **277,** 18528–18534
34. Hofmann, G., Bernabei, P. A., Crociani, O., Cherubini, A., Guasti, L., Pillozzi, S., Lastraioli, E., Polvani, S., Bartolozzi, B., Solazzo, V., Gragnani, L., Defilippi, P., Rosati, B., Wanke, E., Olivotto, M., and Arcangeli, A. (2001) *J. Biol. Chem.* **276,** 4923–4931
35. Arcangeli, A., Rosati, B., Crociani, O., Cherubini, A., Fontana, L., Passani, B., Wanke, E., and Olivotto, M. (1999) *J. Neurobiol.*, **40,** 214–225
36. Vindeløv, L. L., and Christensen, I. J. (1990) *Cytometry* **11,** 753–770
37. Pardo, L. A., Bruggemann, A., Camacho, J., and Stuhmer, W. (1998) *J. Cell Biol.* **143,** 767–775
38. Cahalan, M. D., Wulff, H., and Chandy, K. G. (2001) *J. Clin. Immunol.* **21,** 235–252
39. Meyer, R., and Heinemann, S. H. (1998) *J. Physiol.* (*Lond.*) **508,** 49–56
40. Occhiodoro, T., Bernheim, L., Liu, J. H., Bijlenga, P., Sinnreich, M., Bader, C. R., and Fischer-Lougheed, J. (1998) *FEBS Lett.* **434,** 177–182
41. Pillozzi, S., Brizzi, M. F., Balzi, M., Crociani, O., Cherubini, A., Guasti, L., Bartolozzi, B., Becchetti, A., Wanke, E., Bernabei, P. A., Olivotto, M., Pegoraro, L., and Arcangeli, A. (2002) *Leukemia* (*Baltimore*) **16,** 1791–1798
42. Zhou, Z., Gong, Q., Ye, B., Fan, Z., Makielski, J. C., Robertson, G. A., and January, C. T. (1998) *Biophys. J.* **74,** 230–241
43. Crociani, O., Cherubini, A., Piccini, E., Polvani, S., Costa, L., Fontana, L., Hofmann, G., Rosati, B., Wanke, E., Olivotto, M., and Arcangeli, A. (2000) *Mech. Dev.* **95,** 239–243
44. Mercatante, D., and Kole, R. (2000) *Pharmacol. Ther.* **85,** 237–243
45. Ouadid-Ahidouch, H., Le Bourhis, X., Roudbaraki, M., Toillon, R. A., Delcourt, P., and Prevarskaya, N. (2001) *Receptors Channels* **7,** 345–356
46. Mcfarlane, S. N., and Sontheimer, H. (2000) *Glia* **30,** 39–48
47. Semenza, G. L. (1998) *J. Lab. Clin. Med.* **131,** 207–214
48. Coleman, C. N. (1998) *J. Natl. Cancer Inst.* **80,** 310–317
49. Guillemin, K., and Krasnow, M. A. (1997) *Cell* **89,** 9–12
50. Fontana, L., D'Amico, M., Crociani, O., Biagiotti, T., Solazzo, M., Rosati, B., Arcangeli, A., Wanke, E., and Olivotto, M. (2001) *Biochem. Biophys. Res. Commun.* **286,** 857–862
51. Yu, S. P., Canzoniero, L. M. T., and Choi, D. W. (2001) *Curr. Opin. Cell Biol.* **13,** 405–411

[4] A. Masi, personal communication.
[5] E. Lastraioli, personal communication.