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

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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 (Vₘ) during proliferation. We have demonstrated that in tumor cells the value of Vₘ 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 herg1β. 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⁺ channel isoforms 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 heterogeneity 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 regulate cell volume, 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 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-go `-go`) family (15). herg genes and HERG currents (IHERG) 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). In 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 −50 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, IHERG and the eag gene are expressed only at very early stages of embryogenesis and are subsequently replaced by inward rectifier K⁺ channel currents.

The molecular basis of IHERG 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

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† The abbreviations used are: HERG, herg-encoded protein; herg, human eag-related gene; IHERG, HERG current; IRK, inward rectifier K⁺ channel; RPA, RNase Protection Assay; RT-PCR, reverse transcription-PCR; hcyt, 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 HERGneo) has been identified in the heart (23), which cannot be expressed on the plasma membrane by itself but could modify the biophysical properties of IHERG. 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 Ib) 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 $I_{\text{HERG}}$ biophysical features (rapid deactivation kinetics and strong dependence of the activation gate on depolarized values of the $V_m$) as well as herg1 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 heterotramers 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 $I_{\text{HERG}}$ 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. Mignani, University of Firenze, Italy) cell lines, the human rhabdomyosarcoma RD12 cell line (kindly provided by Dr. P. L. Lollini, University of Bologna, Italy), and HES293 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% CO₂. 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% CO₂.

Cell Transfection—The HEK 293 cells, cultured on 100-mm Petri dishes, were transiently transfected with herg1 CDNA cloned into HindIII/BamHI 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 Na₂HPO₄, 2H₂O (pH 8.90) containing 0.25 μg of complete medium and was replaced 15 h after. 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.

RNA 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. RNA was hybridized overnight at 48 °C with [32P]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-protecting bands. The samples were run on a 6% polyacrylamide gel and exposed for 1–8 days. The human erg1 probes used were the following: erg1 (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 herg1N135, was produced in our laboratory (14). The probe related 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 HotStarTag polymerase (5 units) (Qiagen) and the following primers: herg1b-up 5′-CGATTC-CAGCCGGGAGGCG-3′; herg1b-down 5′-TGATGCACGAGCTCAG-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 this transcript. A 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. PCR relative to human eog and Kv 1.3 transcripts were performed according to Smith et al. (33), using the following primers: heag-5′-CGCAT-GAACACTCTGGAAGACG-3′; heag-down 5′-CTGTGGTGGTGGGCCAGGCT-3′ (product size, 479 bp); Kv 1.3-up 5′-TGGAGCGGCCGCTGAA-GAC-3′; Kv 1.3-down 5′-GGTACTCGAAGAGCAGCCAC-3′ (product size, 550 bp).

Cloning of Herg1b from Tumor Cells—The herg 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 using Takara polymerase (2.5 units) using the following primers: UP primer, 5′-AGGGAGCCAACTGCTCATG-3′ (which maps to the sequence relative to the human exon 1b (dbEST Id: 8445866)); DOWN primer, 5′-GCCGCCCAGCTGGCGCGGTCAG-3′ (which maps at the end of the herg1 sequence with the addition of a NotI restriction site). The amplified band of ~2.5 kb was purified and cloned using the TOPO cloning system (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'-CAGGCAAGCTAGGAGCCAGCAATGCTGAGG-3' (which corresponds to the above reported up primer used for RT-PCR plus a HindIII restriction site); DOWN primer 5'-CAGCGCGCGCC-GCCTGCTAGGACCGAATGTC-3' (which maps in the untranslated region of the herg1 sequence (see above) and contains a Not1 restriction site). In this case the amplified band was cloned into HindIII/Not1 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-cm² flasks, were washed with PBS and incubated with 3 ml of a solution containing 10 mM HEPES, 150 mM NaCl, and 2 mM CaCl₂ (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 (10⁶ cells/ml) were stained with propidium iodide (PI) as described by Vindelov 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 in each phase of the cell cycle were calculated using Modfit software and 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 × 10⁴ and 1.2 × 10⁵ 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. Yeast tRNA as a negative control to test for probe self-protection bands. Human cyclophilin (hcyc) (Ambion) was used as an internal control and 1-day exposure. The protected bands corresponding to the above-mentioned genes were indicated by an arrow.

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).

The results of these experiments are shown in Fig. 1. As shown in Fig. 1A, all of the tumor cell lines tested express the herg1 gene, although at different intensities (see also the densitometric analysis reported in Fig. 3a). 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. 1B), 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 erk2 gene in the retina, at least in rat (20). As for herg3 expression (Fig. 1C), 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. 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 herg2, 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 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 be expressed at a lower level, especially in SH-SY5Y and FLG 29.1 cells and in the heart. Note that this lower-level expression reported recently (41) that the herg1 probe encompassing the first 135 amino acids of the N-terminal domain (herg1N135) as described under “Materials and Methods.”

The RNA samples were probed with the herg1 probe encompassing the first 135 amino acids of the N-terminal domain (herg1N135) 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. 1A: b, densitometric analysis of the herg1N135 expression reported in this figure. Signals of the herg1 and herg1N135 protected bands were normalized using the corresponding values of cyclophilin.

A probe was constructed (N135 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 N135 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. 4A, herg1b mRNA is indeed expressed in both the cell lines tested. The possibility of the simultaneous expression of herg1 and herg1b in tumor cell 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. 4B) 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. 4C, all of the
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 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. 5A), 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. 5B, 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 of ~100 kDa shifted to lower molecular weights. Furthermore, when cells were treated with proteinase K (Fig. 5B, 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. 5B, 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. 5C, 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 HERG1-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.

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 G1/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/G2 boundary after 8 h (Fig. 7A). Western blot experiments performed on the same cell preparation (Fig. 7B) 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 G1/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 G1 was achieved (Fig. 8A) (12). Under these conditions, a strong up-regulation of HERG proteins can be detected in Western blot experiments (Fig. 8B) 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-
tained by testing the effects of a specific IHERG 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 IHERG. Similar results were obtained with another HERG blocker, namely Way 123,398 (see insets in Fig. 9), in a different set of experiments. IHERG 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

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2 G. Hofmann, personal communication.
The phenotype of the transformed cell. In neuroblastoma and or oral features of both embryonic, highly immature cells and enmented that neoplastic cells display biochemical and behav-
turned off in fully differentiated cell types? This is an impor-
expression in tumor cells represent 1) the re-expression of an embryonic gene (see our results on quail
origins is less clear as neither
expressed in epithelial tissues in adults (20) or embryos (43).

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 IHERG biophysical properties (rapid deactivation kinetics and strong dependence of the activation gate on depolarizedVm values) as well as biomolecular features (presence of multiple RNA bands revealed by North-
eblot) 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 behav-
ioral 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 isforms. 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 isform 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 herg1bxn with the herg3 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 IHERG currents, but its puta-
tive 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 correspond-
ing 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 biophys-
ical 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 attributa-
able 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 G1 phase. These results give a molecular dimension to the previously demonstrated variations of HERG activation curves, as well as of Vm 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 G1/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

\[ A. \text{Arcangeli, A. Beechetti, L. Guasti, and O. Crociani, unpublished data.} \]
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 HERG 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, and colon-cancer tumors. Various strategies (specific drugs or specific antisense oligonucleotides) could be designed to block specifically the altered HERG currents in tumors without affecting the $I_{K_r}$ currents found in cardiac myocytes or other non-transformed cell types.

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