Molecular cloning, sequencing and expression studies of the human breast cancer cell glutaminase

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Phosphate-activated glutaminase (GA) is overexpressed in certain types of tumour but its exact role in tumour cell growth and proliferation is unknown. Here we describe the isolation of a full-length cDNA clone of human breast cancer ZR75 cells, by a combination of λ gt10 cDNA library screening and the rapid amplification of cDNA ends ('RACE') technique. The cDNA of human GA is 2408 nt with a 1806-base open reading frame encoding a 602-residue protein with a predicted molecular mass of 66309 Da. The deduced amino acid sequence contains a putative mitochondrial import presequence of 14 residues at the N-terminal end. Heterologous expression and purification in *Escherichia coli* yielded a product of the expected molecular size that was recognized by using antibodies against the recombinant human GA. Sequence analyses showed that human GA was

highly similar to the rat liver enzyme. Northern gel analysis revealed that the gene is present in human liver, brain and pancreas, in which a major transcript of 2.4 kb was demonstrated, but not in kidney, heart, skeletal muscle, lung or placenta. These results strongly suggest that the first human GA cloned, the GA from ZR-75 breast cancer cells, and presumably those from human liver and brain, are liver-type isoenzymes, in sharp contrast with the present view that considers the kidney type as the isoform expressed in all tissues with GA activity, with the exception of postnatal liver.

Key words: cDNA, glutamine, isoenzymes, mitochondria, tumour.

INTRODUCTION

Glutamine is the most abundant free amino acid in the human body and is essential for the growth of normal and neoplastic cells and for the culture of many cell types [1–3]. There is evidence that the clearance of circulating glutamine by bacterial glutaminase (GA) decreases tumour growth [4,5]. Clinically, decreasing glutamine levels by the administration of bacterial GA or by the use of glutamine antagonists is a common therapy for patients with acute lymphocytic leukaemia, although the mechanistic basis is unknown [6,7]. It has been reported that decreasing the glutamine concentration causes differentiation of the human myelomonocytic cell line U937 along the monocytic pathway [8]; in contrast, glutamine supplementation promotes a more malignant and less differentiated phenotype in human colon cancer cell lines [9].

Phosphate-activated GA (EC 3.5.1.2), the major enzyme of glutamine catabolism, has been implicated in the control of cell growth and the proliferation of tumour cells and rapidly growing normal cells [10–12]. In mice bearing the highly malignant Ehrlich ascites carcinoma, both tumour GA activity and mRNA levels ran in parallel, reaching maximum values during the exponential phase of tumour growth [13]. An enzymic imbalance in glutamine metabolism enzymes has been frequently detected in tumours such that glutamine synthetase specific activity is undetectable or almost negligible, whereas that of GA increases considerably [14,15]; tumour cells therefore have to trap the glutamine from the host tissues to sustain the active glutaminolysis needed to maintain its high proliferation rates [16,17]. Cytokines seem to be important in mediating this altered

glutamine metabolism in the host [18] as well as depressing an effective immune response against the tumour invasion [19].

There are two isoforms of mammalian GA, traditionally denoted kidney (K) and liver (L) types [20]. The former was cloned from rat kidney [21]; the main physiological role of the kidney isoenzyme is the control of the acid–base balance. The enzyme is induced in acidosis, in which a 6-fold induction of the mRNA levels occurred that was related to the increased demand of ammonia production. This K isoform is present in rat kidney, brain, small intestine, lymphocytes and fetal liver [20]. The cDNA of the rat liver enzyme has been isolated and sequenced [22]; it is expressed only in periportal hepatocytes of the postnatal liver, in which it couples ammonia production with urea synthesis [23]. Although the sequence comparison of both enzymes showed regions of considerable identity, there are also regions that lack significant similarity; Northern and Western analyses failed to recognize each other, which suggests that the enzymes belong to different genes [20]. The only tumour GA enzyme for which some molecular data are available, the Ehrlich ascites carcinoma enzyme, shows clear molecular differences from the rat liver and kidney enzymes [24], although it also has some kinetic and immunological properties similar to those of the K-type isoenzyme [25].

To understand at molecular level the role of this enzyme in the control of tumour cell growth and proliferation, we cloned, sequenced and characterized the first human and tumour GA, the enzyme from human breast cancer cells. Nucleotide and amino acid sequence similarity studies with the rat liver and kidney enzymes, as well as with partial putative cDNA human brain clones deposited in the GenBank database, are discussed;

Abbreviations used: GA, glutaminase; GSP, glutaminase-specific primer; GST, glutathione S-transferase; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RT–PCR, reverse-transcriptase-mediated PCR; UTR, untranslated region.

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surprisingly, the human breast tumour sequence shares a high degree of identity with both the human brain partial clones and the rat liver cDNA species, in sharp contrast with the currently accepted model that establishes the presence of the K isoenzyme in all non-hepatic tissues, with the L-type being expressed only in postnatal liver. However, our results with human GA differ considerably from that view and show a markedly different expression pattern in human tissues from that of rat enzymes.

MATERIALS AND METHODS

Cell culture

Human breast cancer cell line ZR-75-1 [26] was purchased from American Type Culture Collection (A.T.C.C., Manassas, VA, U.S.A.). The cell line was grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum [26] at 37 °C under air/CO₂ (19:1).

cDNA probes and library screening

A human breast cancer cell ZR-75 library in λ gt10 was purchased from Clontech (Palo Alto, CA, U.S.A.). The library was screened with a putative human brain cDNA clone similar to GA supplied by the IMAGE consortium [clone ID no. 29788; THC (tentative human consensus) no. 70876] and purchased from A.T.C.C. The probe was labelled with the Klenow fragment of DNA polymerase and [α -³²P]dCTP by using the High Prime kit (Boehringer Mannheim, Mannheim, Germany). After 1.9×10^6 individual plaque-forming units had been screened, several positive signals were detected. After tertiary replating, only one positive clonal plaque was purified (λ GA-1) and the phage DNA was isolated from bacterial lysates by standard procedures [27]. The positive plaque was analysed for clonality and cDNA insert size by PCR with vector-specific primers that flank the *EcoRI* cloning site of the λ gt10 vector. One band of approx. 1.2 kb was amplified; the same insert size was obtained by digesting the isolated λ DNA with *EcoRI*.

Plasmid preparations and restriction analyses

The human brain cDNA clone used as a probe to screen the library was analysed with several restriction endonucleases. The 1.4 kb cDNA insert cloned into the *HindIII/NotI* cloning sites of the vector pLafmid BA was divided into three fragments: a *HindIII/PstI* 550 bp 5' fragment, a *PstI/PstI* 450 bp internal fragment and a *PstI/NotI* 400 bp 3' fragment. The three fragments were subcloned in the vector pBluescriptII SK (–) by standard methods [28]; the sequences of these cDNA species were determined in both orientations. DNA sequencing was performed with the Sanger dideoxy sequencing method [29] with Sequenase (U.S. Biochemical Corp.), [α -³²P]dCTP and manual sequencing, and also with the Thermo Sequenase dye-primer cycle kit (Amersham) on an ALF sequenator (Pharmacia, Uppsala, Sweden). The 1.2 kb λ GA-1 clone amplified by PCR was subcloned in the vector pCR-Script Amp SK(+) (Stratagene) in accordance with the manufacturer's instructions; the sequence of this cDNA was determined in both orientations as described above. Restriction endonucleases were from Boehringer Mannheim.

5' and 3' rapid amplification of cDNA ends (RACE)

The protocol outlined by Frohman [30] was essentially followed with slight modifications. Single-stranded cDNA was synthesized by reverse transcription of poly(A)⁺ RNA from ZR-75 cells by using SuperScript II Rnase H[–] reverse transcriptase (Gibco-

BRL) and the anti-sense primer 5'-CCGTGGGTCTAACTTC-CGAGCAC-3' (RT primer); this sequence was deduced from the positive λ GA-1. The cDNA species were 5' tailed with a poly(A)⁺ tail by using terminal deoxynucleotidyl transferase (Boehringer) and dATP; the primer excess was removed by ultrafiltration with Microcon-100 filters (Amicon). The cDNA species were subjected to PCR for 30 cycles with Advantage cDNA Polymerase Mix (Clontech). In these reactions the sense primer was a poly(T) anchor primer (Q_T) 5'-CCAGTGAGCAGAGTGACGAGGACCTCGAGCTCAAGCTTTTTTTTTTTTTTTTTT-3' and the anti-sense primer was a GA-specific primer (GSP-3, 5'-AGCT-GACTTGGCTGGCAGGCCAC-3') that lies 5' of the RT primer and whose sequence was also derived from λ GA-1. A second PCR was done using nested primers: 5'-GAGGACTC-GAGCTCAAGC-3' (primer Q₁, contained in Q_T) and the anti-sense primer 5'-CCAGAGAAGTCATACATGCCGAC-3' (GSP-4), which was approx. 40 bp upstream of GSP-3. To generate '3'-end' partial cDNA clones overlapping with the '5'-end' cDNA clones, 3'-RACE was performed and the poly(A)⁺ mRNA was reverse-transcribed with the Q_T anchor primer. Amplification was then performed with a primer, 5'-CCAGTG-AGCAGAGTGACG-3' (Q₀), containing part of this sequence and the GSP 5'-GGAGGTCACCTGTGAATCAGGCAG-3' (GSP-1) as the sense primer. A second set of amplification cycles was performed with the nested primers Q₁ and GSP-2 (5'-TTGCCCCATCACAGGCGAGAGTG-3'); the sequences of both sense primers GSP-1 and GSP-2 were deduced from the 5'-RACE fragment in such a way that 93 nt were overlapping between the end of the 5'-RACE cDNA and the beginning of the 3'-RACE cDNA. Confirmation of the identity of the RACE PCR products was determined by Southern blot analyses with the 29788 cDNA clone labelled with [α -³²P]dCTP (4500 Ci/mmol; ICN Biomedical). Custom-made single-stranded oligonucleotide primers were obtained from Pharmacia Biotech (Barcelona, Spain).

Subcloning and sequencing of the RACE products

Reaction products were analysed by agarose gel electrophoresis and selected products were excised from the gel and purified with the GeneClean kit (Bio 101). The 1.1 kb 5'-RACE product was ligated into the pCR-Script Amp SK(+) plasmid (Stratagene) and the 1.3 kb 3'-RACE product was ligated into the pGEM-T Easy vector (Promega), as recommended by the suppliers. The recombinant plasmids were transformed into competent *Escherichia coli* DH5 α cells. Individual clones were grown in Luria-Bertani broth containing 0.1 mg/ml ampicillin and their plasmids were isolated by using the Wizard Miniprep kit (Promega). Insert DNA species were sequenced on both strands with the forward and reverse M13 primers on an automated DNA Sequencer (ABI model 310) with the manufacturer's own protocol and reagents, and also with the Thermo Sequenase dye-primer cycle kit (Amersham) on an ALF sequenator (Pharmacia). Nucleotide and deduced amino acid sequences were compiled and similarities to GenBank, SwissProt, Protein Identification Resource and Protein Data Bank entries were determined by using BLAST [31] and the FASTA program of the University of Wisconsin Genetics Computer Group (Madison, WI, U.S.A.).

5' Primer extension analysis and elucidation of the full-length cDNA sequence of GA

The GA cDNA fragment obtained by 5'-RACE was used to design primers for use in primer extension analysis to determine whether we already had the 5' end of the GA cDNA. Three

primers were deduced from the first 180 nt of the 5'-RACE cDNA: anti-sense primers GSP-5, GSP-6 and GSP-7, lying approx. 150, 130 and 70 bp downstream respectively from the 5' end of the 5'-RACE fragment. The oligonucleotides were ^{32}P end-labelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and T4 polynucleotide kinase. Poly(A)⁺ mRNA of ZR-75 human breast cancer cells (approx. 0.5–1 μg) was mixed with the radioactive oligonucleotides and reverse transcribed with avian myeloblastosis virus reverse transcriptase, with the use of the protocol provided by the manufacturer (Primer Extension Analysis kit; Promega). A kanamycin-positive control RNA was run in parallel and the primer extension products were analysed on denaturing polyacrylamide analytical gels containing 8% (w/v) polyacrylamide, 7 M urea and 1 $\times$ TBE (Tris/borate/EDTA) buffer. To sequence the primer extension products obtained with GSP-5 and GSP-7, reverse-transcriptase-mediated PCR (RT-PCR) was performed as described above for the 5'-RACE experiments; in brief, for the GSP-5 products, the poly(A)⁺ mRNA was reverse transcribed with GSP-5 and PCR amplification was done with primers Q₀ and GSP-7. For the GSP-7 products, reverse transcription was performed with the GA RT primer, first PCR amplification with primers Q₀ and GSP-6, and a second round (nested) PCR with primers Q₁ and GSP-7. Reaction products were analysed, purified, cloned in pGEM-T Easy vector and sequenced as described above.

To construct the full-length cDNA sequence of tumour GA, the 5'-RACE product cloned in the pCR-Script vector was amplified with a sense primer corresponding to nt 9–27 of the sequence (GSP-8) and the anti-sense primer GSP-4; this yielded a product without the poly(T) tail at the 5' end. A first fusion was made between this product and the 5' end product RT-67 (obtained by RT-PCR with the RT primer and sequential PCR amplifications with primers GSP-6 and GSP-7); for that purpose, the 300 bp RT-67-specific GA fragment was polished with Klenow enzyme, leaving this product ending with primer GSP-7, starting with primer Q₁ and overlapping 88 nt with the 5'-RACE product, which was also Klenow-polished. After heat denaturation of the Klenow enzyme, the mixture was amplified with Advantage DNA polymerase and primers Q₁ (sense) and GSP-4 (anti-sense) for 30 cycles and the 1.2 kb fusion product was gel-purified. A second fusion was necessary to obtain the full-length clone by addition of the 3' end; approx. 100 ng each of the 1.2 kb fusion product and the 3'-RACE cDNA fragments (overlapping 93 nt) were blunt-ended by Klenow treatment to remove the 3'-A added by the *Taq* DNA polymerase. After heat treatment to denature the Klenow enzyme, the mixture was amplified with Advantage DNA polymerase and primer Q₁ for 30 cycles, as described above. The 2.4 kb fusion product, hGA-ZR75 full-length clone, was gel-purified and the complete hGA cDNA sequence was cloned in the pGEM-T Easy vector. Both strands from three independent clones were sequenced.

Northern and Southern blot analyses

Poly(A)⁺ mRNA was isolated from ZR-75 cells with the Micro FastTrack Poly(A)⁺ mRNA isolation kit (Invitrogen) by following the manufacturer's instructions. Total RNA was isolated from ZR-75 cells with a modified Chomczynski method [32] (TriReagent; Sigma). Total RNA (10–20 μg) was denatured in 50% (v/v) formamide/2.2 M formaldehyde and resolved by agarose/formaldehyde gel electrophoresis. RNA integrity was detected by staining with ethidium bromide. After destaining, the gel was transferred to positively charged nylon membrane Hybond-N⁺ (Amersham Ibérica, Spain) and hybridized with

radioactive cDNA probes labelled with $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ and the High Prime System. For hybridization, radiolabelled GA cDNA probes were added at 10⁶ c.p.m./ml. Pre-hybridization (3 h) and hybridization (10–12 h) were performed at 42 °C in 50% (v/v) formamide/5 $\times$ SSPE [SSPE is 0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA]/5 $\times$ Denhardt's solution/0.1% SDS/100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA. The membranes were washed twice in 2 $\times$ SSC (SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS at 42 °C followed by two washes in 0.5 $\times$ SSC/0.1% SDS and then 0.1 $\times$ SSC/0.1% SDS, both at 60 °C for 30 min. The mRNA corresponding to hGA was revealed with a PhosphorImager (Fuji Biosystems). The membrane was then exposed to Kodak X-OMAT AR films at –80 °C for 4–5 days. Autoradiograms were standardized with a glyceraldehyde-3-phosphate dehydrogenase cDNA probe [13] rehybridized in the same filter and analysed by transmittance densitometry. A human multiple-tissue Northern blot of poly(A)⁺ mRNA (Clontech) was hybridized with a gel-purified cDNA fragment of hGA-ZR75 labelled with ^{32}P by random priming. Prehybridization was done in 10 ml of ExpressHyb[®] Hybridization solution (Clontech) at 68 °C for 30 min. Hybridization was performed for 1 h in the same solution containing 10⁶ c.p.m./ml. The final wash was at 60 °C with 0.1 $\times$ SSC/0.1% SDS for 30 min.

High-molecular-mass genomic DNA was isolated from exponentially growing ZR-75 cells by a standard procedure (TriReagent). DNA (15 μg) was digested with selected restriction enzymes, size-fractionated on a 0.7% agarose gel and transferred to Hybond-N⁺ nylon membrane. The membranes were prehybridized at 68 °C for 30 min in QuickHyb solution (Stratagene) and hybridized with ^{32}P -labelled hGA cDNA (5'-RACE 1.1 kb fragment) in the same solution at 68 °C for 1 h. Washes were performed in 2 $\times$ SSC/0.1% SDS at room temperature for 15 min and twice with 0.1 $\times$ SSC/0.1% SDS at 42 °C for 15 min. The filters were exposed to Kodak X-OMAT AR films at –80 °C in the presence of intensifying screens for 5–7 days.

Expression of the hGA cDNA in *E. coli*

EcoRI and *XhoI* endonuclease restriction sites flanking the coding region of the hGA cDNA were constructed by PCR with the 2408 bp hGA cDNA in pGEM-T as template and Advantage cDNA polymerase. The reaction product was sequentially digested with *EcoRI* and *XhoI*, gel-purified and ligated in-frame in the *EcoRI/XhoI* site of expression vector pGEX-4t-1 (Pharmacia). The amino acid sequence of the 602 residues of the predicted open reading frame (ORF) region of hGA protein was expressed in *E. coli* as a glutathione S-transferase (GST) fusion protein (GST-hGA). The orientation and sequence of the hGA cDNA in the pGEX-4t-1 plasmid were confirmed by DNA sequencing with pGEX sequencing primers. For protein expression, this construct and empty pGEX-4t-1 vector were transformed into the *E. coli* BL21(DE3) cells. Bacterial cells were grown at 37 °C in accordance with the basic induction protocol of the manufacturer. The GST-hGA fusion protein was purified by using glutathione-Sepharose beads, as recommended by the supplier. The GST protein was separated from hGA by proteolysis with thrombin (1 i.u. for each 15 μg of recombinant fusion protein) for 18 h at 22 °C. Both the recombinant hGA and the GST-hGA fusion protein were analysed by SDS/PAGE and Western blotting as described elsewhere [24], employing anti-hGA antibodies as primary antibodies as well as antibodies against the GA from Ehrlich ascites tumour cells purified in accordance with a previously published protocol [24].

Generation of anti-hGA polyclonal antibodies

The amino acid sequence of residues 347–602 of the predicted C-terminal region of hGA protein was expressed in *E. coli* as GST fusion proteins (GST–CtGA). For the construction of plasmids encoding GST–CtGA, a *EcoRI*–*XhoI* fragment encompassing nt 1074–1909 was ligated to the *EcoRI*–*XhoI*-digested pGEX-6p-1 vector (Pharmacia). For protein expression, these constructs and empty pGEX-6p-1 vector were transformed into the *E. coli* BL21(DE3) cells. The GST–CtGA fusion protein was purified by using glutathione–Sepharose beads, as recommended by the supplier. The purified recombinant protein was used for hyperimmunization of New Zealand White rabbits and polyclonal antibodies were generated as described elsewhere [24].

Enzyme assays

The culture medium of ZR-75 cells was aspirated and cells were washed twice with 5 ml of 0.1 M Tris/HCl (pH 7.4)/0.2 M mannitol at 37 °C. Cells were then trypsin-treated and removed from Petri dishes with 10 ml of the same buffer. After centrifugation at 1700 *g* for 5 min at 4 °C, cells were resuspended in the Tris/mannitol buffer and counted with a Coulter Counter. Finally, cells were pelleted, resuspended in a small volume of Tris/mannitol at the desired cell concentration and frozen at –20 °C until analysis. GA activity in tumour cells, bacterial extracts and recombinant proteins was measured as described elsewhere [13].

RESULTS AND DISCUSSION

Correlation between growth curve and GA activity in ZR-75 cells

The oestrogen-positive ZR-75 human breast cancer cells were first analysed to measure the GA activity during tumour growth. As shown in Figure 1, the maximal GA activity in culture was high, in agreement with the general view that most tumours have the capacity to metabolize glutamine at high rates. There was also a dependence on the proliferation state: maximal activities were found at the beginning of the exponential growth phase, with a remarkable decrease at the stationary phase of growth, when confluence was achieved. The abundance of GA mRNA levels was measured in parallel (Figure 2). There was also a 5-fold increase in the relative levels of GA mRNA at the beginning of the exponential growth phase (day 2) in comparison with those shown when proliferation had already ceased (day 5 of culture). These results agree with those for Ehrlich ascitic tumour cells growing in the peritoneal cavity of Swiss mice [13] and indicate a long-term regulation of GA in tumours by differential gene expression, allowing maximum expression of the enzyme when the tumour cells are actively dividing, coincident with their maximum nitrogen requirement.

Isolation and analysis of cDNA clones

When we started this study, only the full-length GA cDNA of rat kidney [21], about half of the rat brain cDNA [33] and less than half of the rat liver cDNA [23] were known. On the basis of this information we tried to design degenerate oligonucleotides as heterologous probes to amplify the GA from human breast cancer ZR-75 cells by RT–PCR. However, when several amplified products were cloned and sequenced, they turned out to be non-specific amplifications. We then screened the λ gt-10 ZR-75 library with a partial putative human brain GA clone from the IMAGE consortium (clone 29788); the clone had approx. 400 bp sequenced with 71.5% identity with the rat kidney GA nucleotides. After screening 2×10^6 plaque-forming units, we isolated a

Figure 1 Growth curve (●) and GA activity (○) of ZR-75 human breast cancer cells

GA activity was measured as described in the Materials and methods section. The amount of enzyme which catalyses 1 μ mol of glutamate formation/min is defined as one unit. Values are means $\pm$ S.E.M. for three different experiments. Abbreviation: mU, m-units.

Figure 2 Northern blot analysis of total RNA from ZR-75 human breast cancer cells growing *in vitro* at different days of culture

32 P-labelled cDNA from the 5'-RACE clone of hGA was used as a probe. The signal for control mRNA [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] is shown below the GA autoradiogram; it was obtained by rehybridization of the same filter.

1.2 kb positive plaque (λ GA-1) that contained two GA sequences of 134 and 95 nt, having 91–92% identity with the rat liver GA (GenBank accession number J05499). These two GA-similar sequences were separated by a segment of 142 nt without any significant similarity to the database.

After identification of the λ GA-1 clone, we additionally screened 4×10^6 plaque-forming units without finding any new positive signal. The inability to clone larger GA cDNA species from the library by using the 29788 human brain cDNA clone as a probe was unexpected because the quality (recombinant titre and average size) of the library used was normal. However, as can be seen in Figure 2, the GA mRNA expression was highly dependent on the proliferation state of the tumour cells; when confluence was achieved there was a poor abundance of GA RNA. Therefore if the library was made with confluent cells it could explain the poor representation of GA mRNA. In addition, it is possible (1) that the GA transcripts contained structural features that led to the generation of truncated transcripts, or (2) that GA recombinants are unstable and their proportions in the library are decreased.

Scheme 1 Diagram of the cloning strategy for hGA

In this representation the double-stranded cDNA species generated are shown as rectangles flanked by the initial and end primers; when necessary, internal primers are also depicted to clarify the fusion of products. Starting with the poly(A)⁺ mRNA of ZR-75 cells, 5'-RACE and 3'-RACE were performed with the indicated primers to obtain GA fragments of 1.1 and 1.3 kb respectively. Primer extension analysis confirmed that we did not have the complete 5' end, so RT-PCR with poly(A)⁺ mRNA was performed with primers flanking the 5' termini of the 1.1 kb fragment; this yielded the RT-67 product, which extended 180 bp upstream of the 5'-RACE fragment. The 1.1 kb 5'-RACE product was amplified by PCR to delete the poly(T) tail and then fused to RT-67 (overlapping around GSP-7); the fusion product was amplified and a 1.2 kb hGA 5'-end insert was obtained. This product was finally fused to the 3'-RACE fragment (overlapping around GSP-2); after amplification by PCR with primer Q₁, the full-length hGA ZR-75 clone of 2.4 kb was obtained.

Cloning of the full-length cDNA

To overcome this problem, 5'- and 3'-RACE were used to obtain the remaining sequence of the hGA cDNA. The sequences obtained from λGA-1 were employed to design the primers; the RACE-generated GA fragments were then used to PCR amplify from the 5' end of the sequence through the complete ORF, generating the full-length hGA cDNA sequence. To facilitate the description of these studies, the structure of the different cDNA species generated and how were they assembled is shown diagrammatically in Scheme 1, along with the primers employed. The 5'- and 3'-RACE products have 1077 and 1242 nt respectively and overlap by 93 nt. They were ligated to yield a product of

2226 nt; although the size of this cDNA agrees with estimates of the size of the GA mRNA obtained by Northern gel analysis (Figure 2), we wished to ensure that the whole 5'-end sequence was present in this construct. For that purpose, primer extension analysis was done with several anti-sense primers flanking the 5' end: one of them, GSP-7, gave a single-strand cDNA product of approx. 240 bases (Figure 3), which was considerably larger than the product theoretically expected starting from GSP-7 and assuming that we had the full-length hGA cDNA (96 nt). This product (RT-67) was amplified by RT-PCR with the anti-sense RT primer and the sense GSP-6 and GSP-7 primers (Scheme 1), then cloned into the pGEM-T vector and sequenced. The RT-67 product contained the first 96 nt of the 2226 nt fragment starting

Figure 3 Primer extension analysis of ZR-75 cells RNA

Oligonucleotides GSP-5 and GSP-7 flanking the 5' termini of the 5'-RACE clone were ^{32}P end-labelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and T4 polynucleotide kinase. Poly(A)⁺ mRNA of ZR-75 human breast cancer cells (approx. 0.5–1 μg) was mixed with the radioactive oligonucleotides and reverse-transcribed with avian myeloblastosis virus reverse transcriptase; the products from primer extension reactions were separated by electrophoresis on an 8% (w/v) denaturing polyacrylamide gel. The size of the single-stranded cDNA products (in bases) was estimated approximately by comparison with ϕX174 *Hinf*I DNA markers shown at the right. Lane 1, GSP-5 with mRNA omitted (control); lane 2, GSP-5; lane 3, GSP-7 with mRNA omitted (control); lane 4, GSP-7; lane 5, DNA markers. The arrow indicates the specific GA product of approx. 240 bases obtained by reverse transcription with primer GSP-7.

from GSP-7, but also added a further 180 nt; this length was slightly higher than the size deduced from the single-stranded cDNA fragment in the primer extension analysis (Figure 3). No other specific GA products were obtained in the primer extension analysis employing primers GSP-5 (Figure 3) and GSP-6 (results not shown): for GSP-5, several products were amplified by RT-PCR, then cloned and sequenced; none was GA-specific (results not shown).

To construct the full-length hGA cDNA we devised a strategy outlined in Scheme 1: first, we fused the 5'-end fragment RT-67 with the 5'-RACE product. For that purpose, the 5'-RACE product was PCR amplified with the sense primer GSP-8 (GSP-4 as the anti-sense primer) just at the beginning of its sequence, to remove the poly(T) tail at the 5'-end while maintaining an overlapping region of 88 nt with RT-67 (Scheme 1). Afterwards, we ligated this 1.2 kb fusion product with the 3'-RACE cDNA fragment (93 overlapping nucleotides) and amplified the fused product by means of PCR with primer Q₁ alone: a 2.4 kb double-stranded cDNA fusion product was obtained and cloned into the pGEM-T vector.

Nucleotide and predicted amino acid sequence of hGA

The human full-length GA cDNA isolated from ZR-75 breast cancer cells is 2408 nt long (Figure 4); the size of this cDNA agrees with estimates of the size of the GA mRNA obtained by

Northern blot analysis. The complete hGA cDNA contains an ORF of 1806 nt that extends from the first translation consensus sequence [34] of an initiation codon at position 52 to the first termination codon at position 1858. The entire cDNA contains 51 and 551 nt of 5' and 3' untranslated sequences respectively. Translation of the cDNA results in a predicted protein of 602 residues with a molecular mass of 66309 Da (Figure 4). The first 14 residues, when plotted as a helical wheel, have the potential to form an amphipathic α -helix; furthermore, this fragment possesses seven basic residues, no acidic residues and a 93.88% probability of being a mitochondrial signal peptide, as predicted by the program MitoProtII, which was devised for the analysis of mitochondrial import sequences [35].

Secondary structure analysis of the mRNA of hGA with the MFOLD program of the GCG package showed inverted repeats and stem-loop structures likely to be formed in the 5' untranslated region (UTR) of the clone, namely between nt 13 and 51 of the sequence. This should help in understanding the difficulties found in obtaining the complete 5' end sequence by RT-PCR: in addition, the first 200 nt of the hGA are quite GC-rich, which thus hinders reverse transcription because reverse transcriptase tends to stop or pause in regions of high secondary structure in the template RNA. Because prematurely terminated first-strand cDNA species are tailed by terminal transferase just as effectively as full-length cDNA species in the RACE technique, cDNA populations composed largely of prematurely terminated first strands result primarily in the amplification and recovery of cDNA ends that are not full-length either [30]. To avoid the generation of heterogeneous extension products in the primer extension analysis experiment, we took care to use primers whose target sequences were located within 100–150 nt of the 5' terminus of the mRNA [28]. However, GAs have been in some way refractory to reverse transcription [22]. Furthermore, in a study with the rat kidney enzyme, specific reverse transcription was not achieved with GSPs or random hexamers, with the best priming method for reverse transcriptase being oligo(dT) [36].

Sequence similarity of hGA to other proteins

Sequence comparisons between the cDNA of hGA and the NCBI GenBank[®], EMBL, DDBJ and PDB sequence databases showed that it was highly similar (89% identity) to the rat liver GA cDNA. This result was unexpected, because the data so far available for GA in mammals indicate that the L isoform of GA is present only in adult liver; all other tissues, including fetal liver, with GA activity have the kidney (K-type) isoenzyme [20]. In contrast, the human GA showed only 68.5% identity with the rat kidney GA cDNA, a percentage similar to that found between rat liver and kidney GA cDNA species [22]. In addition, the cDNA sequence of hGA is highly related to several partial human cDNA clones of unknown function in the NCBI GenBank non-redundant expressed sequence tag ('EST') database. Similarities are as high as 80–100%; most of the clones matched the 3' UTR region and were isolated from human fetal liver-spleen, testis, fetal heart and brain. Furthermore, after sequencing the 29788 human brain partial cDNA clone employed in the screening of the library, we found almost 100% identity with the hGA cDNA isolated from ZR-75 cells, encompassing nt 1070–2408 of hGA; that is, the C-terminal half and all the 3' UTR region. Moreover, another partial cDNA human brain clone (clone 23817) has 100% identity with hGA in 832 nt comprising the last 300 nt of the ORF and the whole 3' UTR region. These results strongly suggest a great similarity between the human GAs from breast cancer cells and brain; again, this was unexpected because rat brain GA is also a K-type enzyme [33].

Figure 4 Nucleotide and deduced amino acid sequences of hGA

Deduced amino acids are shown under the coding sequence. The putative mitochondrial signal peptide is underlined. The primers used for PCR to obtain the full-length clone are shown in boxes and denoted by arrows. Amino acid 1 is the initiation methionine and the stop codon is indicated by the asterisk. A polyadenylation consensus sequence is doubly underlined.

With regard to the deduced amino acid sequence, hGA shares a considerable degree of identity (94 %, similarity 96 %) with the rat liver enzyme (Figure 5) but, curiously, the human tumour enzyme extends over 67 residues at the N-terminal end, including the putative signal peptide of 14 amino acids that the rat liver enzyme lacks. This putative signal sequence has the requirements

for a mitochondrial presequence: a propensity to form amphiphilic α -helix, an abundance of basic and hydrophobic residues and a lack of acidic amino acids. In addition, it is quite similar to the putative signal sequence of the rat kidney enzyme constituted by the first 16 residues [21] (Figure 5); both presequences were unambiguously identified as mitochondrial

Figure 5 Comparison of the predicted amino acid sequences of human breast cancer cell GA, rat liver GA and rat kidney GA

The alignment was performed with the PILEUP program and the result was displayed with the PRETTYBOX program (Genetics Computer Group, Madison, WI, U.S.A.). Identical amino acids are indicated in black boxes and conservative amino acid substitutions in grey boxes. For simplicity, the first 70 residues of the rat kidney GA sequence are omitted.

Figure 6 Organ distribution of hGA ZR-75 mRNA

A human multiple-tissue Northern blot containing approx. 2 µg of poly(A)⁺ mRNA per lane was hybridized with the 5' region of hGA cDNA from ZR-75 breast cancer cells (5'-RACE fragment). Lanes contained, from left to right, RNA from human heart (H), brain (B), placenta (Pl), lung (Lu), liver (L), skeletal muscle (M), kidney (K), and pancreas (P). RNA size marker bands are indicated at the left. A single band of approx. 2.4 kb is apparent in liver, brain and pancreas RNA samples.

targeting sequences by the MitoProtII program [35]; in contrast, no such putative signal peptide was predicted for the rat liver protein. A lower degree of identity (72 %) was found with the rat kidney protein (Figure 5), which is 72 residues longer at the N-terminal end. The pattern of differences reflects that most of them are situated in the last 50 residues of the C-terminal region, although there are non-conservative scattered substitutions through the whole sequence too. Finally, it is noteworthy that hGA also has similarity to two putative proteins of *Caenorhabditis elegans*: the deduced amino acid sequences of the *C. elegans* clones DH 11.1 (SwissProt number Q19013, 571 residues) and F30F8.2 (SwissProt number Q93650, 529 residues) have 64 % and 59 % similarity respectively to human GA. The lengths of the aligned sequences were 519 and 459 residues respectively; thus these sequences of the *C. elegans* genome would constitute a putative GA enzyme.

A large group of biosynthetic enzymes have a glutamine amidotransferase domain that possesses GA activity [37] and on the basis of sequence similarity these enzymes are separated into the trpG-type and purF-type glutamine amidotransferases [38]. For the trpG-type enzymes, an essential cysteine residue activated by a neighbouring histidine has been implicated in the chemical mechanism of the hydrolysis of glutamine [39]. On the basis of chemical modification studies with both the purified and native membrane-bound GA from Ehrlich ascites tumour cell, essential cysteine and histidine residues were also postulated in the catalytic mechanism of tumour GA [40]. However, the consensus amino acid sequences of the GA domain of the trpG-type glutamine amidotransferases [41] were not found in the deduced amino acid sequence of hGA, in agreement with the rat GA enzymes [20]. In contrast, hydropathy analysis [42] of hGA, as well as primary amino acid sequence analysis with a neural network system to predict transmembrane helices [43], did not reveal any transmembrane segment in the amino acid sequence. Thus the human tumour GA is not likely to be an integral

membrane protein. This is consistent with previous membrane topography studies of the Ehrlich ascites tumour cell GA, which discounted a transmembrane localization for the protein, although anchorage on the matrix side of the inner mitochondrial membrane with as yet unidentified components was also suggested [44].

Expression of hGA in human tissues

To reinforce the results suggested above by sequence comparisons, we performed a Northern blot analysis to probe polyadenylated mRNA from eight different human tissues (Figure 6). A single GA transcript of 2.4 kb was present in human liver, brain and pancreas, whereas no signal was observed in heart, placenta, lung, skeletal muscle or kidney. The most intense expression was seen in liver and brain, in agreement with the sequence comparison results. Thus the breast cancer cell GA had a pattern of expression in human tissues qualitatively different from that shown by its liver counterpart in rat, because the liver GA cDNA did not hybridize with RNA isolated from rat tissues other than liver, including brain, pancreas and fetal liver [20,22]. Accordingly, with the use of a rat brain GA cDNA as a probe, hybridization was achieved in kidney and brain mRNA samples but was not detectable in liver RNA [33]. To assess the relative abundance of the GA isoforms in human tissues, we also probed the human Northern blot with a 1.1 kb rat kidney cDNA [13,21]. The tissues that showed hybridization with this K-isoform probe were mainly kidney and brain, whereas weaker signals were also obtained with heart, lung, pancreas and placenta; no hybridization was detected in liver and skeletal muscle (results not shown). Interestingly, the size of the RNA transcript revealed with this kidney probe was approx. 4.4 kb in all the tissues, whereas the mRNA transcript detected in human liver, brain and pancreas with the ZR-75 probe was 2.4 kb. It can be concluded that from the human tissues analysed, only brain and pancreas expressed both types of transcript, K and L; although the L type was predominant in pancreas, similar levels of both types were seen in brain. In liver, only the L type was detected; kidney, heart, placenta and lung hybridized with the K type, whereas no transcript was seen in skeletal muscle.

Results on other tumour GAs at the molecular level are scarce and studies done at the immunological and kinetic levels showed results compatible with the existence in these tumour cells of the K-type enzyme [16,45], reinforcing the accepted current view of the K isoenzyme's being ubiquitous. The rat kidney GA possesses two mRNA transcripts of 6 and 3.4 kb whose levels are affected co-ordinately in response to changes in acid-base balance [46]. In Ehrlich ascites tumour cells, a rat kidney cDNA probe hybridized with a major transcript of approx. 7 kb: the profile of relative GA mRNA levels was roughly correlated with GA specific activities such that maximum expression occurred when the tumour cells were actively dividing during the exponential growth phase [13]. Furthermore, these results suggest that the enzyme from Ehrlich tumour cells is K-type, in agreement with other kinetic data and in spite of notable differences at the molecular level between the Ehrlich tumour cell and rat kidney proteins [24]. On the basis of immunological data, the rat hepatoma HTC cell GA was another tumour enzyme that seemed to belong to the K type [45]. The GA mRNA from ZR-75 human breast cancer cells also showed a long-term regulation depending on the cell proliferation state (Figure 2); however, the expression and sequence results reported here strongly support the contention that this human tumour enzyme is L-type and is expressed in other human tissues. Furthermore, Northern blots of ZR-75 cells probed with the rat kidney cDNA failed to detect any transcript,

Figure 7 Southern blot analysis of ZR-75 cell DNA

Samples containing 15 µg of ZR-75 breast cancer cell DNA were digested with indicated restriction enzymes, fractionated by agarose-gel electrophoresis and hybridized with the 5'-RACE fragment as a probe. *HindIII*-digested λ DNA was analysed in the first lane; numbers at the left are the relative mobilities of the fragments used as size markers.

at least under our standard conditions of high-stringency washes and 4–5 days of exposure.

Southern analysis

Human genomic DNA isolated from ZR-75 breast cancer cells was analysed by Southern blotting to assess the genomic organization of human GA. Total DNA was digested with different enzymes that did not cut within the hGA cDNA 2.4 kb sequence, then subjected to electrophoresis and blotted to a nylon membrane probed to a 1.1 kb 5'-RACE cDNA-coding fragment (Figure 7). DNA cut with *HindIII*, *EcoRI* and *XbaI* each yielded one fragment of 23, 6.4 and 6.6 kb respectively, whereas only *XhoI* yielded two bands (more than 10 kb and 3.7 kb respectively). Although the very simple hybridization pattern observed suggests that there was only one gene coding for GA in the human genome, the existence of other members closely related that did not cross-hybridize with our probe should also be considered. The nucleotide sequence for a mammalian GA gene has not been determined in any species; therefore the genomic organization, the related gene products and even the number of GA genes remain unknown [20], although initial progress in cloning and identifying the promoter for the rat enzymes has been made [20,22]. Southern analysis in LLC-PK₁-

Figure 8 SDS/PAGE and immunoblot analyses of recombinant hGA

The ORF of the hGA cDNA was cloned in-frame with the GST cDNA into the pGEX-4t bacterial expression vector, and the GST-hGA fusion protein was purified by glutathione–Sepharose affinity chromatography. Lane 1, molecular mass markers; from top to bottom these were: phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa). Lane 2, Coomassie staining of GST-hGA; lane 3, Coomassie staining of hGA after cleavage of the fusion protein with thrombin; lane 4, immunoblot analysis of recombinant hGA revealed with anti-(human GA) polyclonal antibodies; lane 5, immunoblot analysis of recombinant GST-hGA fusion protein revealed with anti-(human GA) antibodies.

F⁺ cells (pig renal proximal-tubule-like cell line) probed with the 3' UTR region of rat kidney GA cDNA indicated the presence of a single gene [47].

Heterologous expression of hGA as a fusion protein in *E. coli*

The ORF of the hGA cDNA was cloned downstream from a tac promoter in the pGEX-4t vector as a GST protein (GST-hGA). The construct was transformed into *E. coli* BL21. Positive clones were initially grown as 10 ml cultures and later induced in the late exponential phase by the addition of isopropyl β-D-thiogalactoside. Aliquots of cell extract were analysed by SDS/PAGE. Extracts from induced cultures showed the increased expression of a band with a molecular mass of approx. 92 kDa in comparison with that of extracts from the uninduced cultures (results not shown). The size of the protein fragment observed after SDS/PAGE is in accordance with that expected from a fusion protein of GST and hGA (Figure 8). The fusion protein was purified from the cell extract by using a glutathione–Sepharose column, then digested with thrombin. Analysis by SDS/PAGE (Figure 8) yielded a band with an apparent molecular mass of 66 kDa, the expected size of the precursor hGA (phGA), that was also recognized by anti-hGA antibodies raised against the affinity-purified recombinant C-terminal GA fusion protein (Figure 8). Furthermore, both proteins (GST-hGA and hGA) were recognized by polyclonal antibodies against the purified Ehrlich ascites tumour cell GA [24] (results not shown). Enzymic analyses for GA activity in bacterial extracts and in purified recombinant GST-hGA and hGA proteins were unsuccessful; at present it is therefore uncertain whether this mitochondrial protein can be properly folded in bacteria or whether an eukaryotic expression system will be required to obtain active GA enzyme.

Concluding remarks

This is the first report of the isolation of a cDNA for human GA. The completion of the molecular cloning of hGA provides a foundation for future studies on the regulation of expression of GA and the elucidation of its role in neoplastic transformation. In addition, the results described here provide the first molecular

evidence of the existence of an L-type GA in a tissue different from postnatal liver. The fact that hGA bears identity with the L-type GA, and its expression in other human tissues such as liver, brain and pancreas, suggests that, instead of a new tumour-specific isoform, tumour GA would differ from that of normal tissues in the mechanism governing its expression, which elicits an up-regulation of the enzyme during the exponential phase of tumour growth, becoming a positive regulator of proliferation. Interestingly, we have found recently that tumour cells transfected with anti-sense constructs of GA showed a significant decrease in the rate of cell proliferation that was correlated with a diminished GA expression (C. Lobo, M. A. Ruiz-Bellido, J. C. Aledo, J. Márquez, I. Núñez de Castro and F. J. Alonso, unpublished work).

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