Characterization and Cloning of Two Isoforms of Heteroglobin, a Novel Heterodimeric Glycoprotein of the Secretoglobin-Uteroglobin Family Showing Tissue-specific and Sex Differential Expression*

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Javier Alvarez‡, Jorge Viñas‡‡, José M. Martín Alonso‡, Juan Pablo Albar‡, Keith Ashman‡, and Pedro Domínguez‡**

From the ‡Departamento de Bioquímica y Biología Molecular, Edificio Santiago Gascón, Universidad de Oviedo, 33006 Oviedo, Spain, ¶Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, Campus de Cantoblanco, Universidad Autónoma, 28049 Madrid, Spain, and Samuel Lunenfeld Institute, Toronto MSG IX5, Canada

Heteroglobin (HGB) is a 39-kDa heterodimeric protein detected under non-reducing conditions in harderian, parotid, and submaxillary glands and saliva of the Syrian hamster with antiseraum raised against the carboxy end deduced from the female harderian gland cDNA FHG22 (Dominguez, P. (1985) FEBS Lett. 176, 257–261). After reduction, only one 5.6-kDa polypeptide, named HGB.A, was immunodetected and identified by sequencing as the mature FHG22 product. Tissue-specific expression of HGB.A and HGB mimics that of FHG22 mRNA, with sex differences in submaxillary and harderian glands. Purification of HGB revealed it consists of HGB.A disulfide bonded to HGB.B, a 33.5-kDa N-glycosylated subunit that yields a 9-kDa core polypeptide after deglycosylation. Two highly homologous (96.2%) cDNA clones (HGB.B1 and HGB.B2) encoding 94 amino acid-long isoforms were identified by screening a female harderian gland library with an HGB.B probe. The corresponding mature polypeptides are 78 amino acids long with 12 differences, but 3 putative N-glycosylation sites are maintained. The expression of HGB.B mRNAs is parallel to that of HGB and HGB.A, but no HGB.B2 mRNA was detected in submaxillary glands. Homology studies indicate that HGB.A and HGB.B1/HGB.B2 belong to different subfamilies of the secretoglobin-uteroglobin family and form heterodimers as previously described.

The existence of a uteroglobin/Clara cell 10-kDa family of proteins including UGB/CC10 orthologs and paralogs (such as subunits of rat prostatein, cat Fel d 1 and mouse androgen-binding protein, and cDNAs like hamster FHG22) was previously suggested (1–4). New related proteins and cDNAs were described (5–9), and the family was formally established during a meeting in which a nomenclature committee (10) coined the generic name of secretoglobins (SCGBs). This family includes a diverse group of small, α-helical, secreted polypeptides (10–12) only described in mammals and reported to form dimeric structures bound by interchain disulfide bridges involving two or three conserved Cys residues (4, 8, 12). Five or six subfamilies have been defined by homology rather than by functional features (10, 13), in agreement with reported specific dimeric associations between members of subfamilies (13). Some SCGBs have also been shown to form heterotetrameric associations in which two disulfide-bound heterodimers are non-covalently bound (8, 14–16). A tissue-specific expression pattern linked to exocrine epithelia has been found for all the members (2–10) whose levels can also be regulated by hormones (4, 17–20), including sex steroids also found to be ligands for some SCGB oligomers (2, 12, 14, 15, 17, 21). The only homodimer and best-studied protein of the family is UGB (4, 17, 22, 29), for which several ligands have been described, including progesterone/steroids (24, 25), other hydrophobic ligands (12, 26–28), retinoids (29), and calcium (27, 30). Contrarily to calcium, it has been shown that lipophilic compounds bind to an internal cavity formed between the two polypeptides of the UGB homodimer (17, 27, 28), and the existence of such a hydrophobic pocket in heterodimeric SCGBs has also been proposed (10, 12). Several groups report UGB binding to cellular and matrix proteins and to a possible membrane receptor (31–33). Besides reports of different cellular and physiological actions (4, 17, 21), some of which arise from knock-out projects (34–36), the physiological role(s) of UGB and, in general, SCGBs is unclear.

We had previously prepared two sex-differential cDNA libraries and isolated male- and female-specific clones (3, 37) from Syrian hamster harderian glands. These are secretory organs from the orbital cavity related to the pineal gland and the gonads (38, 39), which in hamster show reversible sexual dimorphism regulated by hormones and other factors (40–42). The female harderian gland cDNA clone FHG22 was characterized in our laboratory and related to the UGB family as mentioned (3). The FHG22 mRNA was found to be expressed according to a tissue- and sex-specific pattern (3) only in three hamster exocrine glands; the highest levels are observed in parotid glands from either sex, and FHG 22 mRNA is present in female but not in male harderian glands and presents higher expression levels in female than in male submaxillary glands. These sexual differ-
ences led us to develop studies on hormone regulation; estradiol stimulates FHG22 mRNA expression in hardier gland both in vivo and in vitro, whereas no effect is observed using other sex steroids (20).

In this paper, we report the use of an antipeptide antisera that specifically recognizes HGB.A, the product of the FHG22 mRNA, in monomeric or oligomeric form. HGB.A is the small subunit of a disulfide-bound heterodimer named heteroglobin (HGB), also formed by the large N-glycosylated subunit HGB.B. Sequencing of HGB.B enabled us to isolate two cDNAs corresponding to highly homologous isoforms (HGB.B1 and HGB.B2). HGB.A and HGB.B belong to different subfamilies of the SCGB family, and their mRNAs show a sex- and tissue-specific expression identical to that of HGB, but HGB.B2 is surprisingly absent in submaxillary glands.

**EXPERIMENTAL PROCEDURES**

**Animals**—Male and female Syrian hamsters (*Mesocricetus auratus*) were obtained from Charles River (Kingston, NY) and maintained under controlled temperature (20 ± 2 °C) and photoperiodic conditions (14:10 h, light/dark cycle) with food and water ad libitum. The animals were sexually mature (about 4 months old) when used for experimentation. For preparation of tissue homogenates or RNA extraction, the animals were killed by suffocation with carbon dioxide, and the tissues were rapidly dissected and washed with ice-cold phosphate-buffered saline. For the preparation of antipeptide antisera, outbred New Zealand male rabbits were maintained in controlled conditions for 40 days before the immunization protocol was started and bled when needed.

**Preparation of Hamster Saliva Samples and Tissue Homogenates**—Hamster saliva samples were obtained by inserting sterile cotton ear buds inside the animals’ mouths and allowing them to chew for 2 min. The cotton plugs were removed, inserted into a bottom-cracked 0.5-ml tube placed inside a 1.5-ml tube, and centrifuged at 15,000 × g for 5 min. Clarified saliva was collected, and the plugs were washed with one volume of 1 ml. Two intramuscular boosts of 200 μl of complete adjuvant (Difco) in a total with an equal volume of Freund’s complete adjuvant were used as room temperature for 30 min. Samples were finally heated at 100 °C for 10 min, electrophoresed in 10% or 15% analytical SDS-polyacrylamide gels, and either stained with Coomassie Brilliant Blue R-250 or transferred to nitrocellulose. The apparent molecular weight of protein bands was measured by interpolation from a linear semilogarithmic plot of Mr versus distance of migration using the MultiMark™ multi-colored mixture of protein standards (Novex, San Diego, CA). For Western blot analysis, proteins were electropheretically transferred from the gels to Duralose UV™ (Stratagene) essentially according to Towbin et al. (46), and membranes were stained with 0.01% Ponceau S in 0.1% acetic acid to verify protein transference. Blots were blocked in phosphate-buffered saline plus 0.1% Tween 20 containing 5% fat-free milk powder for 1 h at room temperature and were incubated with the antipeptide antiserum (1:2000) for 1 h. Immune reactive bands were visualized by an enhanced chemiluminescence reagent system (Amersham Biosciences, Inc.). After being found to be the only one able to detect polypeptide HGB.A (see “Results”), antiserum AF22P3 (anti-FHG22-(76–95)) was used for standard immunodetection.

**Enzymic Deglycosylation**—In the study of unpurified HGB deglycosylation, female hamster parotid, submaxillary, and hardier gland homogenates or saliva were digested with endoglycosidases to remove saccharides from the polypeptide chains, and the protein was then immunodetected with AF22P3. Briefly, the O-linked sugars were removed from proteins present in gland homogenates or saliva (40–80 μg of total protein/sample in adequate amounts to give similar band intensities) by incubating each of the samples in a total volume of 50 μl with 3 milliunits of neuraminidase plus 2 milliunits of O-glycosidase in 0.1× sodium phosphate buffer, pH 6.0, for 18 h at 37 °C. Similarly, N-deglycosylation of the proteins was performed by incubating samples with 0.6 units of PNGase F and 10× sodium phosphate buffer, pH 6.0, in reaction volumes of 50 μl for 18 h at 37 °C. Control reactions were incubated under the same conditions but in the absence of enzymes. Samples were then analyzed by non-reducing SDS-PAGE followed by AF22P3 immunodetection.

Complete removal of (N-linked) sugars from a purified parotid gland HGB preparation was achieved by incubating 10 μg of protein with PNGase F as described before but including 1 μl β-mercaptoethanol in the reaction mixture. The sample was then analyzed by SDS-PAGE and visualized by staining with Coomassie Blue as explained.

**Characterization of Two Isoforms of Heteroglobin**—The parotid glands from two female hamsters were extracted and treated as mentioned above to obtain the parotid homogenate from which the oligomer HGB was purified. The progress of purification was monitored by immunodetection of HGB.A with AF22P3 in the preparations and fractions obtained during the procedure. The parotid homogenate was first subjected to differential precipitation in ammonium sulfate at a temperature of 0 °C. The homogenate was brought up to 50% ammonium sulfate saturation, stirred for 1 h, and centrifuged at 15,000 × g for 20 min. The precipitate was discarded, and ammonium sulfate was added to the supernatant to reach 80% saturation, stirred, and centrifuged again as described. The precipitate was collected and resuspended in 3 volumes of 10× sodium phosphate, pH 7.5, and this preparation was called parotid 80–50 precipitate, with a protein concentration around 10 mg/ml. A precipitate sample (600 μl) was applied to an ion exchange chromatography DEAE-Sephacel (16/20) column coupled to a Gradient system (Amersham Biosciences, Inc.) equilibrated with 10 mM sodium phosphate, pH 7.5, at a flow rate of 18 ml/h, and the column was washed with 1 volume of equilibration buffer. Bound proteins were eluted with 150 ml of a linear gradient from 0 to 0.5 M NaCl in 1× sodium phosphate buffer. Each fraction (3 ml) containing HGB.A was monitored at a protein peak eluting between 0.3 and 0.36 M NaCl and analyzed by non-reducing and reducing SDS-PAGE; those showing contaminant proteins were discarded. Fractions of interest were pooled, dialyzed against 200 volumes of 10× ammonium bicarbonate, lyophilized, and finally resuspended in equilibration buffer to obtain a protein concentration around 5 mg/ml. Aliquots of this preparation were used for
characterization by SDS-PAGE and amino acid sequencing of the subunits.

Amino Acid Sequencing—Either 100 μg of parotid homogenate or 20 μg of purified HGB were separated by SDS-PAGE under reducing conditions, and the gels were stained with Coomassie Brilliant Blue as explained. Protein bands of interest were excised and in gel digestion with trypsin performed automatically in the Progest (Genomic Solutions) as explained (47). The tryptic peptides (trim) were separated by high performance liquid chromatography (48) and sequenced in a 474 Ponce peptide sequencer (Applied Biosystems).

PCR Cloning, Screening, and Isolation of cDNA Clones for HGB.B—To obtain a partial cDNA probe corresponding to the largest HGB subunit, two reactions of degenerate oligonucleotide-primed PCR (49, 50), were prepared using a female hamster harderian gland cDNA library (37) as template. The common sense primer was a 20-mer oligonucleotide mix (5′-GAGAYGAYCATHGCAARAC-3′) including the codon choices for the internal heptapeptide DDAIAKT (see Table I), and the two antisense primers corresponded to sequences of the promoters SP6 (5′-TCAGCTATGCACTAAAGCTT-3′) or TT (5′-ACGGCATCTATTGTAAA-3′) flanking the multiple cloning site of the phagemid pcDNAII (Invitrogen). The amplification reactions were set at a final volume of 50 μl containing 1 μl of template DNA, 10 pmol of each primer, 200 μM each dNTP, 50 mM KCl, 15 mM MgCl₂, and 1 unit of Taq DNA polymerase (Roche Molecular Biochemicals) and performed under the following conditions: an initial denaturation step at 94 °C for 5 min before the addition of the enzyme followed by 30 cycles at 95 °C for 1 min, 48 °C for 1 min, and 72 °C for 2 min and a final elongation step at 72 °C for 5 min. The reaction corresponding to the SP6 primer produced a 350-base pair fragment that was isolated, subcloned into the plasmid pGEM-T (Promega), sequenced, and used as a probe in the screening of 5 × 10⁶ colonies of the female hamster harderian gland cDNA library (37). Eight positive clones were isolated and completely sequenced from both ends, revealing the existence of two highly homologous cDNAs named thereafter HGB.B1 and HGB.B2.

RNA Preparation and Northern Analysis—RNA was extracted as previously described (3, 41) from the following male and female hamster tissues: spleen, brain, liver, small intestine, pancreas, lung, kidney, heart, parotid glands, submaxillary gland, harderian gland, prostate gland, seminal vesicle, and testes. Concentration, purity, and integrity of the RNA samples were assessed by Agarose-formaldehyde gels and transferred to Duralose UV™ membranes (Stratagene) as described (41). Ethidium bromide fluorescence of the RNA was used to check even loading and approximately quantify samples. To determine the mRNA levels of the subunits HGB.A or HGB.B in the different tissues, Northern blots were hybridized as described with [α-²³P]dCTP-labeled cDNA probes in the presence of 40% formamide at 42 °C using, respectively, the HGB.A-FHG22 cDNA (3) or a combination containing equimolar amounts of HGB.B1 and HGB.B2 cDNAs. Specific oligonucleotide probes with 20% mismatch to the opposite sequence were labeled with [α-²³P]dATP and polynucleotide kinase (Roche Molecular Biochemicals) and used to detect either HGB.B1 (5′-GTAATGCTGAACATCCAATAA-3′) or HGB.B2 (5′-TTTAGATCCGCACTAC-3′) mRNA by hybridization in the presence of 0.3 mM NaCl, 0.3 mM sodium citrate, 5× Denhardt’s solution, 50 mM phosphate buffer, pH 6.6, 0.1% SDS, and 0.1 mg/ml yeast RNA at 52 °C (4 °C below the Tm), as described by Sambrook et al. (51). The specificity and detection range of the oligonucleotides were determined using identical conditions with Southern blots having 0.01, 0.1, 1, and 10 ng of each HGB.B1 and HGB.B2 cDNAs run in adjacent lanes. Blots were washed thoroughly to eliminate the radioactivity when hybridized successively to more than one probe.

Sequence Comparisons—The nucleotide and amino acid sequences of HGB.B1, HGB.B2, and submaxillary homologates among themselves and also with the cDNA-deduced polypeptide sequences of available SCGB family members (11, 13) using the ClustalX application (52). A phylogenetic tree with subfamilies generated by homology was obtained, and the multiple alignments of the subfamilies including the HGB subunits are represented.

RESULTS

HGB.A Is the Mature Polypeptide Encoded by the FHG22 mRNA—Northern analysis of the FHG22 mRNA (0.6 kilobases) demonstrated a tissue-specific and sex differential expression in Syrian hamster (3). The mRNA expression pattern was properly reassessed here (Fig. 1A) and used as reference in the immunodetection of the expected polypeptide product. For this purpose, three antipeptide antisera corresponding to amino, central, and carboxyl parts of the mature polypeptide sequence deduced from the cDNA (3) were prepared by rabbit immunization and used for Western analysis of hamster samples after SDS-PAGE in reducing conditions. Under these conditions only the antisemur AF22P3 (raised against the carboxyl-terminal peptide FHG22 (76–95)) was found to detect a unique polypeptide band with an apparent size of 5.6 kDa (Fig. 1, B and C). The band is found in harderian, parotid, and submaxillary homologates and also in saliva; the highest levels appear in male and female parotid glands, whereas in submaxillary glands and in saliva, the levels are higher in females and absent in male harderian glands (Fig. 1B). This pattern of expression is the same as that of FHG22 mRNA such that a direct correlation between them is observed. The correlation is further supported by the fact that the band is not observed when using AP22P3 antiserum for the analysis of FHG22 mRNA negative tissues such as male and female thy-
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Fig. 2. Immunodetection of oligomeric HGB and monomeric HGB. A, Female hamster tissue homogenates (50 μg of protein per sample) were subjected to non-reducing or semi-reducing SDS-PAGE and used for immunodetection with AF22P3 antiserum. A, homogenates from female parotid, submaxillary, and harderian glands were subjected to long-run 10% SDS-PAGE in non-reducing conditions and used for immunodetection with AF22P3. B, three parotid homogenate samples were treated with 0.14 μl β-mercaptoethanol 30 min, subjected to 15% SDS-PAGE, and used for immunodetection with AF22P3 antiserum. 

Fig. 3. Partial deglycosylation of HGB from tissue homogenates and saliva. Female hamster tissue homogenates or saliva in adequate amounts to produce similar band intensities in Western analysis (40–80 μg of protein/lane) were incubated in the absence of enzymes (panel A), with 3 milliunits of neuraminidase (Neur.) plus 2 milliunits of O-glycosidase (O-gly.; panel B) or with 0.6 units of peptide N-glycosidase F (panel C) for 18 h at 37 °C. Samples were then subjected to 15% SDS-PAGE in non-reducing conditions followed by immunodetection using AF22P3 antiserum.

molecular weight standards. Using this method, HGB.A was only detected in fractions corresponding to a protein peak with an apparent Mr of 34,000 (data not shown), which demonstrates the lack of non-covalently bound heterotetramers and, hence, that the protein band detected by SDS-PAGE accounts for the complete oligomeric structure. However, some experimental observations indicated that the oligomer should be studied in the three expressing tissues. In fact, a more accurate determination of the band size in female homogenates after long-run SDS-PAGE in non-reducing conditions (Fig. 2A) permits visualization of the differences between the apparent sizes of the oligomer from submaxillary (Mr, 37,000) and those from parotid and harderian glands (Mr, 39,000), thereby suggesting that HGB.A can be bound to different counterparts. Because there is no defined physiological function, the name heteroglobin was given to these oligomeric proteins having the polypeptide HGB.A as subunit because of their heterodimeric structure, heterogeneity in size and biochemical composition, and heterotypic tissue expression, as shown in this work.

Deglycosylation of HGB—The observation that parotid HGB presents different sizes when measured by SDS-PAGE or chromatography could be due to a possible glycoproteic nature (55, 56). To detect the presence of saccharide chains in the molecule, female gland homogenates or saliva were digested either with neuraminidase plus O-glycosidase or with PNGase F and subjected to non-reducing SDS-PAGE followed by immunodetection with AF22P3 (Fig. 3). Removal of O-linked sugars does not affect HGB molecules, since the band pattern from tissues and saliva (Fig. 3B) is equivalent to that found in undigested control incubations (Fig. 3A). Rather, treatment with PNGase F alters migration of HGB molecules (Fig. 3C); in parotid glands, harderian glands, and saliva, the apparent size is reduced in 2 steps (from 39,000 to 33,000 and 25,000), suggesting that two N-linked oligosaccharide branches are being removed, whereas in submaxillary glands only one size reduction is clearly observed. Although this is positive proof of the presence of Asn-bound oligosaccharide chains in the HGB molecule, this procedure cannot be used for complete deglycosylation analysis due to the fact that full PNGase F action is only achieved in reducing conditions not compatible with detection using AF22P3. Because HGB.A cannot have a carbohydrate moiety because of its size, absence of consensus sequences for N-glycosylation, and the fact that no change in the band was observed after treatment with PNGase F (see Fig. 4), the oligosaccharide chains must be N-linked to the other subunit of the HGB molecule.

Purification and Characterization of HGB—Parotid gland (from females) was used as a source for purification of the
heterodimer due to the fact that it shows the highest levels of HGB.A (see Fig. 1 and 2) and convenient protein composition. A simple protocol followed by immunodetection of HGB.A after each step permits the purification of HGB from parotid homogenate in which it is a major protein, as shown by electrophoretic analysis (Fig. 4). Briefly, parotid homogenate (lane 1) was subjected to differential precipitation with ammonium sulfate; the precipitate (lane 2) was chromatographed through an ion-exchange column, and purified HGB was obtained from selected fractions (lane 3). This preparation was used to determine the size and subunit composition of the oligomer by SDS-PAGE. As expected, it migrates as a 39-kDa band in non-reducing conditions (lane 3), but after thiol-reducing treatment (lane 5), two bands are observed; the small one (5.6-kDa) was named subunit HGB.A as previously mentioned, and the large one (33.5 kDa) was named subunit HGB.B. Complete digestion of HGB with PNGase F (in reducing conditions) only affects one (33.5 kDa) was named subunit HGB.B, whose apparent size decreases from 54,000 to 28,000 to 19,000 (data not shown) and finally to 9,000 (lane 6). Thus, the carbohydrate part accounts for most of the HGB.B molecule, which is likely to have three N-linked oligosaccharide chains. Finally, the purity of the protein preparation was also analyzed by isoelectric focusing; oligomeric HGB migrates as a unique band with a very acidic pI around 2.8, in agreement with the expectable acidic nature of the carbohydrate moiety (data not shown).

Partial Sequencing of the Subunits HGB.A and HGB.B—

Purified parotid gland HGB was subjected to reducing SDS-PAGE, and the bands corresponding to the subunits HGB.A and HGB.B were processed for protein sequencing as described (47, 48). As expected, amino-terminal sequencing of HGB.A elicited an identical sequence to that found in the band from parotid homogenate (see Fig. 1C). The results of sequencing the amino terminus and tryptic peptides of the HGB.B band are shown in Table I, such that a 33 residue-long stretch including the former could be reconstituted. The lack of Met in the first position indicates that, as in HGB.A, the signal peptide has been removed, and the sequence corresponds to a mature polypeptide. Two consensus N-glycosylation sites are observed at positions 19 and 35, in which the Asn residues are probably true glycosylation sites as there is a gap in the sequencing signal (57). All the tryptic peptides can be located in the reconstructed amino-terminal sequence and identified after Lys residues in the two cDNA-deduced sequences shown in Fig. 5, including the artificial sequence of trim 17 which is composed of amino acids 91–94 followed by 77–82 (Table I). The positive identification of two peptides differing in one amino acid residue (trim 30 YTLPLYIR and trim 33 YTLPLYIR) corresponding to the sequences present in HGB.B2 and HGB.B1 (Fig. 5) demonstrates the presence of both polypeptides in parotid glands, which is also supported by the detection of residues from HGB.B2 (Pro-39) and HGB.B1 (Gln-48) in the sequence of trim 21. Finally, the cDNA combinations of the amino-terminal sequence of HGB.B were analyzed and utilized to obtain a DNA probe by mixed oligonucleotide–primed amplification of cDNA. The probe was successfully obtained using a mix of 384 oligonucleotide sequences (20-mers) that included all the possible codons for the sequence DDAIAKT except the 3′ position (see next paragraph).

Isolation and Characterization of cDNAs for HGB.B and HGB.A—A female hamster harderian gland cDNA library (37) was used as the template in degenerate oligonucleotide–primed PCR amplifications (49, 50), with the oligonucleotide mix defined by 5′-GAGGAYGCNATGCGCGAAC-3′ as sense primer and two antisense primers specific for the cloning vector. A 350-base-pair-long DNA fragment was successfully amplified, cloned, sequenced, and found to harbor an ORF starting with the sequence DDAIAKT and agreeing with the sequences described in Table I. It was then used as a probe to screen the female hamster gland cDNA library such that two highly homologous cDNAs could be identified (Fig. 5). Eight positive clones were isolated and sequenced during the process, seven of which are identical (HGB.B1), whereas the other (HGB.B2) was very similar (96.3% homology) and identical to the (partial) sequence of the cloned PCR product. A 477-nucleotide-long sequence containing a polyadenylation signal and followed by a poly(A) tail was obtained from the HGB.B1 clones, whereas the HGB.B2 sequence obtained was seven and eight nucleotides shorter at the 5′ and 3′ ends, respectively (Fig. 5). Parallel ORFs are observed in both cDNAs, starting at a Met with consensus sequence for translational initiation (58) and coding for 94-amino acid-long sequences showing 87.6% identity and 93.6% similarity (52). Comparison with the HGB.B amino terminus (Table I) revealed identical signal peptides with standard cleavage sites (53) between Cys-16 and Arg-17 in both sequences (Fig. 5). Remarkably, the untranslated and signal peptide nucleotide sequences are also identical, such that all the 17-base differences found are restricted to the 78 codons of the mature polypeptides, producing 12 amino acid substitutions (7 conservativeness) between mature HGB.B1 and HGB.B2 (Fig. 5). Despite these changes, three consensus sites for N-glycosylation are conserved at residues 19, 35, and 72, in agreement with the deglycosylation data from Figs. 3 and 4 and the gaps detected in the sequencing process. Also, both polypeptide sequences show three residues conserved in the SCGB family, Cys-23, Lys-64, and Cys-91, as well as Cys-66, conserved in all heterodimeric members of the family (11–13). According to their cDNA sequences, the calculated Mₐ of non-glycosylated HGB.B1 and HGB.B2 polypeptides are, respectively, 10,883 and 10,821 before and 9,048 and 8,986 after the action of the signal protease, in accordance with the apparent size of the deglycosylated HGB.B band (see Fig. 4).

Parallel Expression of HGB.B and HGB.A mRNAs—

To investigate whether the expression of both HGB subunits is transcriptionally coordinated, we determined the mRNA levels for HGB.B and HGB.A in a broad collection of RNAs from male and female hamster tissues by successively probing the same blots with equimolar HGB.B1/B2 or HGB.A cDNA probes as explained under “Experimental Procedures.” Homology between HGB.A and either HGB.B1 or HGB.B2 cDNA sequences (52) is around 47%, such that no cross-hybridization with mRNAs was expected in the conditions used. No expression for any HGB mRNA was found in male and female adrenal glands,
brain, heart, liver, kidney, pancreas, skeletal muscle, small intestine, spleen, and thymus or in ovary, seminal vesicle, and testes (data not shown). The result of hybridizing the HGB.B and HGB.A probes to RNA from some tissues of interest is shown in Fig. 6. Not surprisingly, the mRNAs showed almost indistinguishable sizes around 0.6 kilobases and very similar expression patterns: high levels in parotid glands from both sexes, lower levels with sexual differences (more in females) in submaxillary glands, and female-specific expression in harderian glands; no expression was observed in male harderian gland, prostate, uterus, or lung from any sex. Indeed, the patterns clearly concur with the protein distribution previously described (see Fig. 1 and 2), and it is interesting to highlight that no tissue with independent expression of HGB.A or HGB.B mRNAs was detected.

Differential Expression of HGB.B1 and HGB.B2 mRNAs

— Recurrent observation of such a specific expression pattern prompted us to determine the particular contribution of HGB.B1 and HGB.B2 mRNAs—Recurrent observation of such a specific expression pattern prompted us to determine the particular contribution of HGB.B1 and HGB.B2 mRNAs—Recurrent observation of such a specific expression pattern prompted us to determine the particular contribution of HGB.B1 and HGB.B2 mRNAs—Recurrent observation of such a specific expression pattern prompted us to determine the particular contribution of HGB.B1 and HGB.B2 mRNAs—Recurrent observation of such a specific expression pattern prompted us to determine the particular contribution of HGB.B1 and HGB.B2 mRNAs—Recurrent observation of such a specific expression pattern prompted us to determine the particular contribution of HGB.B1 and HGB.B2 mRNAs—Recurrent observation of such a specific expression pattern prompted us to determine the particular contribution of HGB.B1 and HGB.B2 mRNAs—Recurrent observation of such a specific expression pattern prompted us to determine the particular contribution of HGB.B1 and HGB.B2 mRNAs—Recurrent observation of such a specific expression pattern prompted us to determine the particular contribution of HGB.B1 and HGB.B2 mRNAs—Recurrent observation of such a specific expression pattern prompted us to determine the particular contribution of HGB.B1 and HGB.B2 mRNAs—Recurrent observation of such a specific expression pattern prompted us to determine the particular contribution of HGB.B1 and HGB.B2 mRNAs—Recurrent observation of such a specific expression pattern prompted us to determine the particular contribution of HGB.B1 and HGB.B2 mRNAs.

### Table I

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Fig. 5. Nucleotide sequences and corresponding amino acid translations of HGB.B1 and HGB.B2. Nucleotide and amino acid (in **boldface**) numbers are counted by reference to the first position of initiating ATG codons as +1. **Lowercase** letters denote nucleotide differences, and **italic uppercase** letters denote amino acid differences. Underlined nucleotides show the polyadenylation signal in the 3'-untranslated region of the two cDNAs and two differential restriction sites mentioned under “Results.” Underlined amino acids indicate **N**-glycosylation motifs. Signal peptides are **boxed**, and relevant amino acids are **circled**. The HGB.B1 and HGB.B2 cDNA sequences have been entered into the EMBL/GenBank™/DDBJ data bases and found under GenBank™ accession numbers AJ252138 and AJ252139, respectively.
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Total RNA was extracted from male and female hamster tissues, and 30 µg of each preparation were electrophoresed in agarose-formaldehyde gels and transferred to nitrocellulose membranes. Membranes were used for detection of HGB.B mRNAs, washed thoroughly, and rehybridized for HGB.A mRNA detection. The HGB.B probe consisted of an equimolar mixture of HGB.B1 and HGB.B2 cDNAs, and the HGB.A probe was FHG22 cDNA (3). In addition to the tissues shown in the figure, no expression was detected in adrenal glands, brain, heart, kidney, liver, pancreas, skeletal muscle, small intestine, spleen, and thymus from any gender nor in ovary, seminal vesicle, and testes. The bottom panel shows ethidium bromide staining of ribosomal RNA corresponding to the indicated tissues.

When using the combined cDNA probe, the pattern already described for total HGB.B (see Fig. 6) was essentially repeated as expected. However, hybridizations of the RNA blot to each of the specific oligonucleotides demonstrate that HGB.B1 is expressed in parotid and submaxillary glands, whereas HGB.B2 is clearly present in parotid but not in submaxillary glands. To avoid misinterpretations, the ability of the specific probes to detect low amounts (10,000 target sequences in 0.01 ng of cDNA; not shown) of proper nucleic acid without showing cross-hybridization to higher amounts of the opposite is shown in the figure. Unfortunately, the very low hybridization to higher amounts of the opposite is shown in the Southern blot in Fig. 7. When using the combined cDNA probe, the pattern already described for total HGB.B (see Fig. 6) was essentially repeated as expected. However, hybridizations of the RNA blot to each of the specific oligonucleotides demonstrate that HGB.B1 is expressed in parotid and submaxillary glands, whereas HGB.B2 is clearly present in parotid but not in submaxillary glands. To avoid misinterpretations, the ability of the specific probes to detect low amounts (10,000 target sequences in 0.01 ng of cDNA; not shown) of proper nucleic acid without showing cross-hybridization to higher amounts of the opposite is shown in the control Southern analysis in Fig. 7. Unfortunately, the very low level of HGB.B mRNAs found in these female harderian glands impeded detection using these oligonucleotide probes; low HGB expression not related to estral cycle, age, or main environmental factors has occasionally been observed in female harderian glands.

In agreement with other authors (10, 13), because a definitive nomenclature has yet to be established, subfamilies including HGB.A and HGB.B have been named after them, and their multiple sequence alignments are shown in Fig. 8. HGB.A subfamily also includes lipophilins type A and B from man and rabbit (8, 9, 11), human lymphoglobin (13), and the C1 and C2 subunits of rat prostatein (59). HGB.B subfamily includes the two isoforms described in this paper, the two sequences reported for the prostatein C3 subunit (60, 61), human marmaglobin (5) and lipophillin C (or marmaglobin B or lacryogloblin) (6, 8, 62), and rabbit lipophilins type C (11). The three residues conserved in all the members of the SCGB family (Cys-23, Lys-64, and Cys-91; see Fig. 5 numbering) are shown in bold in Fig. 8. Cys-23 and Cys-91 are thought to be responsible for the formation of interchain disulfide bridges in all SCGBs, and Lys-64 is positioned in the calcium binding site (13, 30), whereas Cys-66 is only conserved in heterodimeric members and has been proposed to be involved in the formation of an additional disulfide bridge (8, 12). Although a common structure with four α-helices seems to be maintained in the whole family (12, 13), different residues are almost or absolutely conserved in each subfamily as illustrated (Fig. 8). Finally, it is worth mentioning that all HGB.B subfamily members show at least one N-glycosylation site, but only HGB.B1 and HGB.B2 have three.

DISCUSSION

This paper describes the identification and characterization of two isoforms of hamster HGB, a heterodimeric protein whose disulfide-bound subunits (HGB.A and HGB.B) belong to the expanding SCGB-UGB family (1–4, 10). The common small subunit HGB.A was first detected using antipeptide antiserum and later identified by sequencing as the mature polypeptide encoded by the tissue- and sex-specific hamster FHG22 mRNA (3). Indeed, immunodetection of HGB.A permitted purification of heterodimer from parotid gland, which in turn led to the cloning of two cDNAs coding for HGB.B1 and HGB.B2, the two

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isofoms of the N-glycosylated large subunit, and the study of their differential expression. With the lack of clues as to its function(s), the protein was named heteroglobin, thus reflecting heterogeneity because (i) it is a heterodimer, (ii) it is a glycoprotein, (iii) it has at least two isoforms, and (iv) subunits and isoforms show tissue and sex differences in expression. Additionally, the suffix “globin” has been chosen for the SCGB family, implying dimerization behavior to produce a conserved eight-helix bundle structure surrounding a hydrophobic pocket (10, 12).

The logical procedure after cloning FHG22 was to identify the encoded polypeptic product; antiserum raised against the encoded polypeptide produced antibodies that (a) reacted with purified HGB, (b) reacted with the glycosylated large subunit, and (c) precipitated HGB when added to saliva, in which variations in HGB levels have been studied (20), but such a difference is not observed in saliva, in which variations in HGB levels have been studied in males and females but could not be related to hormonal or environmental factors (data not shown).

Despite the size difference observed in submaxillary glands, it is clear that HGB from any source is N-glycosylated at least twice, as shown in Fig. 3. Experiments performed with purified HGB demonstrate that all the sugar chains are bound to the large subunit HGB.A (M\(_r\) 33,500) as expected, whose core polypeptide showed an apparent M\(_r\) of 9,000 after complete PNGase F treatment (Fig. 4), in accordance with the sizes measured by SDS-PAGE (M\(_r\) 33,500) or by gel filtration chromatography (M\(_r\) 34,000), since it has been described that highly glycosylated proteins show an irregular behavior in SDS-PAGE gels (55, 56). Anomalous mobility could also account for the differences observed during deglycosylation of HGB (Fig. 3). However, the partial and average size differences observed during deglycosylation were higher than the expected size of most N-linked oligosaccharides (M\(_r\) 5,500–10,000 versus 3,000–4,000), perhaps due to alterations in mobility produced by charge shifts after sugar branches were removed from a small polypeptide (55, 56). Anomalous mobility could also account for the difference between apparent sizes of HGB from the parotid gland, measured by SDS-PAGE (M\(_r\) 39,000) or by gel filtration chromatography (M\(_r\) 34,000), since it has been described that highly glycosylated proteins show an irregular behavior in SDS-PAGE gels (55, 56). The fact that the protein is easily purified from a differential ammonium sulfate precipitate (Fig. 4) may also be due to the presence of sialic acic and sulfate residues that make some glycoproteins bind well to ion-exchange columns (63).

Besides the simultaneous expression of HGB.A, HGB.B, and HGB.B2 mRNAs in parotid glands (Figs. 6 and 7), proofs of the existence of HGB.A-HGB.B1 and HGB.A-HGB.B2 isoforms were obtained for the latter (20), but such a difference is not observed in saliva, in which variations in HGB levels have been studied in males and females but could not be related to hormonal or environmental factors (data not shown).
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two non-covalently associated heterodimers, A-B1 and A-B2, like C1-C3 and C2-C3 in protamine (14). This possibility might be ruled out because the size of the oligomer from parotid glands measured by gel filtration chromatography in native conditions (M, 34,000) is equivalent to the size of the heterodimer measured by SDS-PAGE (M, 39,000). Furthermore, such a tetrameric structure would imply similar tissue levels of HGB.B1 and HGB.B2, which is obviously not possible in submaxillary glands due to the lack of HGB.B2 mRNA (Fig. 7). Isolation of seven HGB.B1 and one HGB.B2 cDNA clones from the female harderian gland library supports the reverse transcription-PCR data showing that both mRNAs must be expressed but suggests that HGB.B1 is so to a higher extent, which is also in disagreement with the existence of an A-B1A-B2 oligomer in harderian glands.

The distribution of nucleotide differences between the HGB.B1 and HGB.B2 cDNA sequences indicates that they correspond to similar isoforms encoded by genes from different loci instead of alternatively spliced mRNAs or two expressed alleles. Thus, although the 5' and 3'-untranslated and the signal peptide sequences are identical (Fig. 5), it is remarkable to note that inside the 234 nucleotides encoding the mature polypeptide there are 17 differences affecting two exons (data not shown). The fact that the genes encoding HGB.B1 and HGB.B2 are differentially expressed at the transcriptional level (Fig 7) precludes the possibility that they might be alleles from a unique locus. Also, Southern analysis of hamster genomic DNA using different restriction endonucleases shows that an HGB.B cDNA probe hybridizes to two or more fragments in the genome DNA using different restriction endonucleases shows that an HGB.B cDNA probe hybridizes to two or more fragments.

that both HGB isoforms might play a different physiological role despite the fact that both can be supplied to the saliva through the parotid glands. Because no clear function has been established for heterodimeric SCGBs beyond lipid binding, we are not tempted to hypothesize any physiological role. The absence of clear orthologs among them (unlike UGB), mostly exocrine patterns of expression and promiscuity in heterodimeric associations, could be related to a species-specific role(s) and/or a widely maintained structural feature such as lipid affinity. The fact that HGB shows sexual differences in expression might support both possibilities, for example by binding pheromones.

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Characterization and Cloning of Two Isoforms of Heteroglobin, a Novel Heterodimeric Glycoprotein of the Secretoglobin-Uteroglobin Family Showing Tissue-specific and Sex Differential Expression

Javier Alvarez, Jorge Viñas, José M. Martínez Alonso, Juan Pablo Albar, Keith Ashman and Pedro Domínguez


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