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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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 (Domínguez, P. (1995) FEBS Lett. 376, 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).1 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 differences are unclear.

1 The abbreviations used are: SCGB, secretoglobin; UGB, uteroglobin; HGB, heteroglobin; HGB.A, HGB.B, HGB.B1, HGB.B2, subunits of the HGB heterodimer; ORF, open reading frame; PNGase F, peptide N-glycosidase F; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)] ethyglycine; trim, tryptic in matrix digest.

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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 antiserum 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 rabbits. Animals were injected subcutaneously in multiple sites with a preparation containing 500 µg of the conjugate emulsified with incomplete adjuvant. The parotid gland 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 with an anti-FHG22P3 (anti-FHG22-(76–95)) antisera that specifically recognized HGB.A, the product of the FHG22 gene (see "Results").

**Preparation of Antipeptide Antisera**—The peptides CAVKAVKVEVKKC (FHG22-(55–66)), and KEMMGKIAEVQYGKGTEN (FHG22-(76–95)), corresponding to amino, central, and carboxyboxyl sections of the FHG22 ORF (3), were synthesized with an AMS 422 automated multiple solid phase peptide synthesizer (Abimed, Langenfeld, Germany) using standard Fmoc (N-[9-fluorenylethoxycarbonyl) chemistry (44). The peptides FHG22-(24–45) and FHG22-(55–66) contain an extra Cys with respect to the deduced sequence in the carboxyl and amino terminus, respectively, for coupling purposes. The three peptides were coupled to keyhole limpet hemocyanin (Pierce) using Cys linking the antibody Sulfo-succinimidyl 4-N-maleimidomethyl-cyclohexane-1-carboxylic acid. These conjugates were used to elicit an antibody against polypeptide HGB.A (see "Results"). The antibody was used at a protein peak eluting between 0.3 and 0.36 M NaCl and analyzed by non-reducing and reducing SDS-PAGE, those showing contaminating proteins were discarded. Fractions of interest were pooled, dialyzed against 200 volumes of 10 mM 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 of the two isoforms of heteroglobin.

**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. The parotid gland 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 with an anti-FHG22P3 (anti-FHG22-(76–95)) antisera that specifically recognized HGB.A, the product of the FHG22 gene (see "Results").

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protein 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 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 Procise 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 hardierian gland cDNA library (37) as template. The common sense primer was a 20-mer oligonucleotide mix (5'-GAGYAGYCNATHGCNAARAC-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'-TCAGCTATGGCATCAACGTT-3') or T7 (5'-AGGCGC- CAGTGAATTTGTAAT-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 μg 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 hardierian 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, thymus, adrenal gland, hardierian gland, parotid gland, and submaxillary gland and also from ovary, uterus, prostate, seminal vesicle, and testes. Concentration, purity, and integrity of the RNA samples were assessed by A₂₆₀/₂₈₀ measurement and agarose electrophoresis. Total RNA (20–30 μg/lane) was separated using 1% agarose–formaldehyde gels and transferred to Duralose UV™ membranes (Stratagene) as described (41). Ethidium bromide fluorescence of the rRNA 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 samples. To determine the mRNA levels of the subunits HGB.A or (Stratagene) as described (41). Ethidium bromide fluorescence of the rRNA 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 samples.

RESULTS

HGB.A Is the Mature Polypeptide Encoded by the FHG22 mRNA—Northern analysis of the FHG22 mRNA (6.0 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 antiseraum AF22P3 (raised against the carboxyl-terminal peptide FHG22-(76–85) was found to detect a unique polypeptidic band with an apparent size of 5.6 kDa (Fig. 1, B and C). The band is found in hardierian, parotid, and submaxillary homogenates 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 hardierian 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 AF22P3 antiserum for the analysis of FHG22 mRNA negative tissues such as male and female thymus and is also undetected in other biological fluids such as female serum or vaginal discharge (data not shown).

For obvious reasons explained below, the 5.6-kDa polypeptide will be called HGB.A from now on. To demonstrate that it is indeed encoded by the FHG22 mRNA, the amino-terminal sequence of HGB.A was determined as follows. Two female parotid homogenate samples (100 μg of protein) were separated by reducing SDS-PAGE, and the gel was divided; one sample was stained with Coomassie, and the other was transferred to a nitrocellulose membrane and used for immunode-
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**Fig. 2. Immunodetection of oligomeric HGB and monomeric HGB.** 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 incubated in the absence (lane 1) or presence of 1.25 μg/ml of peptide FHG22-(76–95) (lane 2) or FHG22-(55–66) (lane 3).

**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

- Parotid
- Submaxillary
- Harderian
- Control

### Neur. O-gly.

- PNGase F

### Purification and Characterization of HGB

- Parotid gland (from females) was used as a source for purification of

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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, 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 glycoproteinic 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 molecules, 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 any 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
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Male parotid homogenate was used as a source for purification of HGB. Advance of the process was monitored by immunodetection with male parotid homogenate was used as a source for purification of HGB. The probe was successfully obtained using a mix of 384 oligonucleotide sequences (20-mers) that included all the possible codons for the sequence DDAAIKT except the 3rd position (see next paragraph).

Isolation and Characterization of cDNAs for HGB.B1 and HGB.B2—A female hamster hardier gland cDNA library (37) was used as the template in degenerate oligonucleotide-primed PCR amplifications (49, 50), with the oligonucleotide mix defined by 5'-GAYGAYGCNATHGNNARAC-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 DDAAIKT and agreeing with the sequences described in Table I. It was then used as a probe to screen the female hardier 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 conservaties) 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 M, 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 mature polypeptides, producing 12 amino acid substitutions (7 conservaties) between mature HGB.B1 and HGB.B2 (Fig. 5).

**Non-reducing**

**Reducing**

![Fig. 4. Purification of HGB from hamster parotid glands. Female parotid homogenate was used as a source for purification of HGB.](http://www.jbc.org/)
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 to the HGB.B pool. New RNA samples from parotid, submaxillary, and harderal glands were added to the analysis. The results are presented in Table I, showing the amino acid sequence of the HGB.B subunit from parotid HGB.

#### Table I

**Amino acid sequencing of the HGB.B subunit from parotid HGB**

Purified HGB (20 μg) was treated with 1 M β-mercaptoethanol 30 min, and subjected to 15% SDS-PAGE, and the gel was stained with Coomassie Blue. The band containing the 34.5-kDa polypeptide (HGB.B) was excised from the gel and used for amino-terminal and tryptic peptide sequencing.

<table>
<thead>
<tr>
<th>Peptide Position in ORF</th>
<th>Sequence</th>
<th>Position in ORF</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT (N) SSGCNAL</td>
<td></td>
<td>17–26</td>
</tr>
<tr>
<td>TI (N) SSVPMEEYHQVK</td>
<td></td>
<td>33–49</td>
</tr>
<tr>
<td>TI (N) SSVPMEE</td>
<td></td>
<td>33–42</td>
</tr>
<tr>
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</tr>
<tr>
<td>ETFAYK</td>
<td></td>
<td>65–70</td>
</tr>
<tr>
<td>CSGYNVFVME</td>
<td></td>
<td>91–94/77–82</td>
</tr>
<tr>
<td>N-term</td>
<td></td>
<td>17–49</td>
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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 are found under GenBank™ accession numbers AJ252138 and AJ252139, respectively.
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were prepared, blotted, and successively hybridized to three
probes, one able to detect both mRNAs (equimolar combination
of HGB.B1 and HGB.B2 cDNAs) and two differentials able
to detect each mRNA (specific oligonucleotides with 20% mis-
match to each other’s mRNA); the results are shown 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 ex-
pressed 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-
detection to higher amounts of the opposite is 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 ovari,
seminal vesicle, and testes. The bottom panel shows ethidium bromide staining of
ribosomal RNA corresponding to the indicated tissues.

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). The HGB.B subfamily includes
the two isoforms described in this paper, the two sequences
reported for the prostatein C3 subunit (60, 61), human mam-
maglobin (5) and lipophilin C (or mammaglobin B or lacryglo-
bin) (6, 8, 62), and rabbit lipophilins type C (11). The three
residues conserved in all 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 responsi-
ble 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 abso-
lutely conserved in each subfamily as illustrated (Fig. 8). Fi-
ally, it is worth mentioning that all HGB.B subfamily mem-
bers 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
(5). 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
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 polypeptide product; antiserum raised against the carboxyl terminus was able to immunodetect a 5.6-kDa band (Fig. 1), demonstrating that it and also present in saliva (Fig. 1). The comparison of amino-acids showing the same tissue and sex distribution as FHG22 mRNA with the estradiol activation of HGB.A mRNA expression reported for the latter (20), but such a difference is not observed in saliva, in which variations in HGB levels have been studied due to the presence of sialic acids and sulfate esters that make anomalous mobility could also account for the differences observed during deglycosylation of HGB (Fig. 3) and Fig. 5). However, the partial and average size differences observed during deglycosylation of HGB (Fig. 3) and HGB.B2 (Fig. 5). 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.B (\(M_r 33,500\)) as expected, whose core polypeptide showed an apparent \(M_r 9,000\) after complete PNGase F treatment (Fig. 4), in accordance with the sizes calculated for mature HGB.B1 and HGB.B2. Amino acid sequencing of the HGB.B polypeptide showed an apparent size of most \(N\)-glycosylated proteins show an irregular behavior in SDS-PAGE due to the presence of sialic acids and sulfate esters that make anomalous mobility produced by charge shifts after sugar branches were removed from a small polypeptide (55, 56). Anomalous mobility could also account for the differences observed during deglycosylation of HGB (Fig. 3) and HGB.B 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 gel (55, 56). The fact that the protein is easily purified from a differential ammonium sulfate precipitate (Fig. 4), in accordance with the sizes calculated for mature HGB.B1 and HGB.B2. 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two non-covalently associated heterodimers, A-B1 and A-B2, like C1-C3 and C2-C3 in parietin (14). This possibility must be ruled out since the size of the oligomer from parotid glands measured by gel filtration chromatography in native conditions (M<sub>r</sub> 34,000) is equivalent to the size of the heterodimer measured by SDS-PAGE (M<sub>r</sub> 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 hardier 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-B1:A-B2 oligomer in hardier gland.

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 polypeptides 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 from a unique locus. 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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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