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Amino Acid Sequence of Human Histidine-Rich Glycoprotein Derived from the Nucleotide Sequence of Its cDNA†

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ABSTRACT: A λgt11 library containing cDNA inserts prepared from human liver mRNA has been screened with an affinity-purified antibody to human histidine-rich glycoprotein (HRG) and then with a restriction fragment isolated from the 5′ end of the largest cDNA insert obtained by antibody screening. A number of positive clones were identified and shown to code for HRG by DNA sequence analysis. A total of 2067 nucleotides were determined by sequencing 3 overlapping cDNA clones, which included 121 nucleotides of 5′-noncoding sequence, 54 nucleotides coding for a leader sequence of 18 amino acids, 1521 nucleotides coding for the mature protein of 507 amino acids, a stop codon of TAA, and 352 nucleotides of 3′-noncoding sequence followed by a poly(A) tail of 16 nucleotides. The length of the noncoding sequence of the 3′ end differed in several clones, but each contained a polyadenylylation or processing sequence of AATAAA followed by a poly(A) tail. More than half of the amino acid sequence of HRG consisted of five different types of internal repeats. Within the last 3 internal repeats (type V), there were 12 tandem repetitions of a 5 amino acid segment with a consensus sequence of Gly-His-His-Pro-His. This repeated portion, referred to as a "histidine-rich region", contained 53% histidine and showed a high degree of similarity to a histidine-rich region of high molecular weight kininogen.

Histidine-rich glycoprotein (HRG)[1] has been isolated and characterized from human serum (Heimburger et al., 1972; Morgan, 1978), plasma (Lijnen et al., 1980; Koide et al., 1982, 1985), and platelets (Leung et al., 1983) as well as from rabbit serum (Morgan, 1981). Although the physiological function of HRG has not yet been established, a number of biological properties have been reported including interaction with heparin (Heimburger et al., 1972; Koide et al., 1982; Lijnen et

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[1] Abbreviations: HRG, histidine-rich glycoprotein; HMW kininogen, high molecular weight kininogen; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton(s).

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al., 1983a; Kindness et al., 1984; Niwa et al., 1985), with the lysine binding site of plasmin(ogen) (Lijnen et al., 1980; Ichinose et al., 1984), and with thrombospondin (Leung et al., 1984). HRG will also cause an inhibition of autorosette formation between erythrocytes and lymphocytes (Rylatt et al., 1981; Lijnen et al., 1983b) and will bind to some divalent metals, heme and dyes (Morgan, 1978, 1981).

Human HRG consists of a single polypeptide chain with an apparent molecular weight of 75 000, including 14% carbohydrate. Its N-terminal sequence is Val-Ser-Pro-Thr-Asp (Koide et al., 1982). One of the striking characteristics of HRG is its unusual high content of histidine and proline, each of which exceeds 12% of the total amino acid content of the protein (Heimburger et al., 1972; Lijnen et al., 1980; Koide et al., 1985). Preliminary studies on the primary structure of HRG have shown that the N-terminal sequence is homologous to antithrombin III (Koide et al., 1982). The histidine-rich region of HRG is located in the C-terminal portion of the polypeptide chain (Koide et al., 1985). Detailed information of the primary structure of HRG is essential to understand the mechanism of the diverse functions of this protein and to clarify its physiological function in vivo. In this paper, the isolation and characterization of cDNAs coding for HRG are reported which establish the complete primary structure of the protein.

Materials and Methods

Materials. HRG was purified to homogeneity from human plasma as described previously (Koide et al., 1985). The purified protein migrated as a single band of apparent M_r 75 000 on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Antibody to human HRG was prepared as described previously (Koide, 1979) and purified by affinity chromatography using an HRG-coupled agarose column.

Screening of the λgt11 cDNA Library. A λgt11 cDNA library containing cDNA inserts prepared from human liver mRNA was kindly provided by Dr. Savio L. C. Woo. Approximately 2×10^6 phage were screened for HRG by the antibody-screening method of Young and Davis (1983a,b). The library was then rescreened by employing a restriction fragment of 291 nucleotides isolated from the 5′ end of the largest cDNA insert. The affinity-purified antibody was labeled with ^{125}I to a specific activity of 1.5×10^6 cpm/μg and was used to screen nitrocellulose filters containing phage plated at a density of 1.5×10^5 plaques per 150-mm plate. The cDNA probe was labeled with ^{32}P by nick translation to a specific activity of 1.7×10^8 cpm/μg. Positive clones were isolated, and each was plaque-purified.

DNA Sequence Analysis. Phage DNA was prepared by the plate–lysate method (Maniatis et al., 1982), followed by banding on a cesium chloride step gradient essentially as described by Degen et al. (1983). The cDNA insert in the purified phage DNA was isolated by digestion with *Eco*RI and subcloned into the plasmid pUC9 (Vieira & Messing, 1982). Appropriate restriction fragments from the insert were subcloned into M13mp18 or M13mp19 for sequencing by the dideoxy chain termination method developed by Sanger et al. (1977). Sequencing reactions were carried out with deoxyadenosine 5′-(α-[^{35}S]thiotriphosphate) ([^{35}S]dATPαS) and run on buffer gradient gels containing 0.5–2.5× TBE buffer (0.089 M Tris-HCl/0.089 M boric acid/2 mM EDTA, pH 8.3) (Biggin et al., 1983). Over 90% of each strand of the cDNA insert was sequenced 2 or more times. Exonuclease *Bal*31 was employed to generate overlapping sequences at the 3′ end of λHHRG1 by using the systematic deletion method described by Poncz et al. (1982).

FIGURE 1: Partial restriction map and sequencing strategy for the cDNA inserts in clones λHHRG1, λHHRG2, and λHHRG3. The extent of sequencing is shown by the length of each arrow, and the direction of the arrow indicates the strand that was sequenced. The solid bars indicate the regions coding for the mature protein in each cDNA insert, and the open portion indicates the region coding for the leader sequence. Noncoding regions are shown by the lines at the end(s) of each cDNA insert.

M13mp18, M13mp19, and [^{35}S]dATPαS were purchased from Amersham. Restriction enzymes, nuclease *Bal*31, T4 DNA ligase, bacterial alkaline phosphatase, *Escherichia coli* DNA polymerase I (Klenow fragment), and other enzymes were from Bethesda Research Laboratories and/or New England Biolabs. Deoxynucleotide triphosphates and dideoxynucleotide triphosphates were from P-L Biochemicals. Na^{125}I was from New England Nuclear. DNA sequences were stored and analyzed by the computer programs of Staden (1977, 1978).

Results

Isolation and Characterization of cDNAs Coding for HRG. A human liver cDNA library cloned into a λgt11 phage expression vector was screened for cDNAs coding for HRG by the method of Young and Davis (1983a,b). In this method, ^{125}I-labeled affinity-purified antibody was used to detect phage directing the synthesis of a fusion protein between β-galactosidase and HRG. Twelve positive clones were isolated by screening 2×10^6 phage, and each was plaque-purified. The cDNA inserts were isolated from 10 clones by *Eco*RI digestion, and the 5′ and 3′ ends of each insert were sequenced. Out of 10 inserts that were characterized, 7 contained a poly(A) tail at the 3′ end. The clones which lacked a poly(A) tail were apparently derived from a single clone by amplification of the cDNA library since they were identical in length and had the same terminal sequences.

A total of 36 more positive clones were then isolated from the λgt11 library by screening 1.5×10^6 phage with a ^{32}P-labeled cDNA fragment. The cDNA from one phage with the largest insert (λHHRG3) obtained from the second screening and cDNA inserts obtained from λHHRG1 and λHHRG2 from the first screening were each subcloned into pUC9, and their nucleotide sequence was determined by using the strategy shown in Figure 1. A total of 2067 nucleotides for the three overlapping cDNA inserts is shown in Figure 2 along with the predicted amino acid sequence. This sequence contains 121 nucleotides from the 5′-noncoding end, 54 nucleotides coding for a leader sequence of 18 amino acids, 1521 nucleotides coding for 507 amino acids present in the mature protein, a stop codon of TAA, and 352 nucleotides of 3′-noncoding sequence, followed by a poly(A) tail of 16 nucleotides.

The leader sequence of 18 amino acids in HRG contains a typical region rich in hydrophobic amino acids (Blobel et al., 1979). The leader sequence is removed by the cleavage of an Ala–Val peptide bond by signal peptidase. A number of stop codons precede the initiation methionine at position

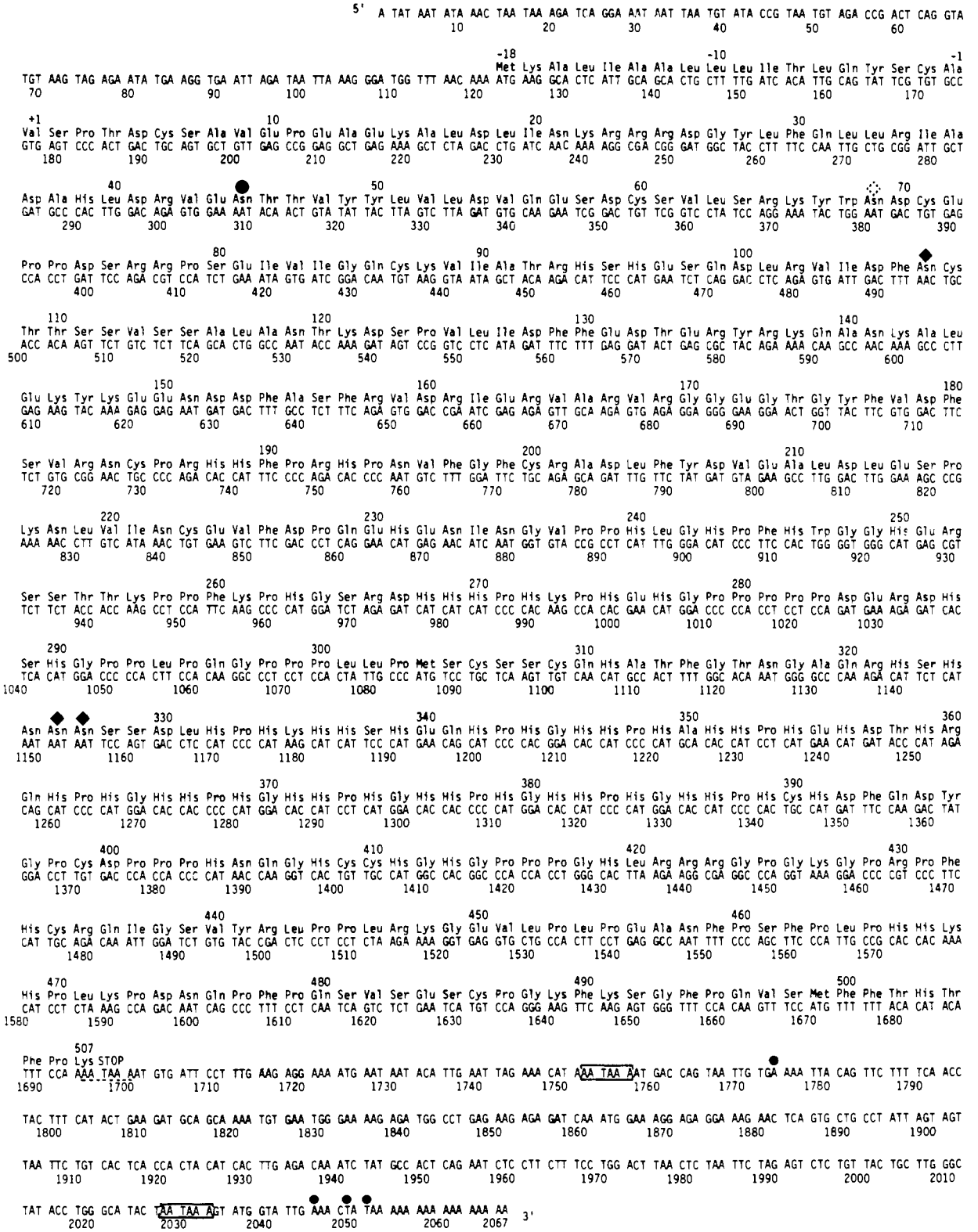

FIGURE 2: Nucleotide sequence of cDNA inserts in λHHRG1, -2, and -3 that code for human HRG. The predicted amino acid sequence contains the leader sequence (amino acids −18 to −1) and the mature protein (amino acids 1–507). (♦, ◇) Potential carbohydrate binding sites to asparagine residues; (●) sites of polyadenylylation in λHHRG clones (details in text). The polyadenylylation or processing sequences (AATAAA) are shown in boxes. A potential polyadenylylation or processing sequence is shown by a dashed underline.

−18. This indicates that there is not a second methionine start site upstream from position −18.

The N-terminal sequence for the mature protein starts with valine at position +1. This sequence is in excellent agreement with the sequence of Val-Ser-Pro-Thr-Asp-Cys-Ser-Ala-Val-Glu-Pro-Glu-Ala-Glu-Lys-Ala-Leu-Asp-Leu-Ile-Asn-Lys determined from the intact protein (Koide et al., 1982) and a cyanogen bromide fragment (CN-50 kDa) of HRG (Koide et al., 1985). The C-terminal sequence starting with phenylalanine at position 500 is also in good agreement with the sequence of Phe-Phe-Thr-His-Thr-(Phe,Pro,Lys) determined from a small cyanogen bromide fragment of HRG (Koide et al., 1985). Two additional fragments have been isolated and characterized (CN-30 kDa and CN-14 kDa) and shown to

contain amino acid sequences of Ser-Cys-Ser-Ser-Cys-Gln-His-Ala-Thr-Phe-Gly and Pro-Pro-Pro-His-Asn-Gln-Gly-His-Cys-Cys-His-Gly-His-Gly-Pro-Pro-Pro-Gly-His-Leu, respectively (Koide et al., 1985). These sequences correspond to those starting with Ser-305 and Pro-401.

Polyadenylylation in the three cDNA clones coding for HRG occurred at different locations relative to the polyadenylation or processing sequence of AATAAA (Proudfoot & Brownlee, 1976). The cDNA insert in λHHRG2 was polyadenylylated two nucleotides upstream from the site used in λHHRG1, while the cDNA insert in λHHRG3 was six nucleotides upstream from that in λHHRG1. A number of other shorter clones that also coded for HRG were studied and shown to be polyadenylylated at one of the same positions as those described above. One clone, however, was polyadenylylated at nucleotide number 1774. This clone contains an AATAAA sequence 17 nucleotides upstream from the poly(A) tail. An additional potential polyadenylylation or processing sequence of AATAAA was identified, which includes the last two nucleotides of the coding region and the stop codon of TAA (Figure 2). This sequence, however, was not found to direct the polyadenylylation of any of the clones isolated thus far.

Discussion

In the present studies, a total of 2067 nucleotides have been sequenced from the cDNA inserts obtained from clones λHHRG1, -2, and -3. The derived amino acid sequence establishes the complete primary structure of HRG, consisting of 507 amino acid residues. This corresponds well with the estimate of 517 total amino acids from amino acid analysis (Koide et al., 1985). The amino acid composition of HRG as derived from the nucleotide sequence of the cDNA was as follows: Asp_{31}, Asn_{20}, Thr_{16}, Ser_{35}, Glu_{28}, Gln_{17}, Pro_{65}, Gly_{35}, Ala_{18}, $^1/_2$-Cys_{16}, Val_{26}, Met_2, Ile_{11}, Leu_{29}, Tyr_{10}, Phe_{26}, Trp_2, Lys_{21}, His_{66}, and Arg_{33}. The molecular weight calculated for the protein free of carbohydrate was 57 646.

HRG contains about 14% carbohydrate (Heimburger et al., 1972). In the amino acid sequence reported here, four potential carbohydrate attachment sites having the sequence of Asn-X-Thr/Ser were found starting with Asn-45, Asn-107, Asn-326, and Asn-327. At the present time, it is not clear whether both of the adjacent asparagine residues (326 and 327) contain carbohydrate, but the presence of carbohydrate in one of these sites has been confirmed by the presence of glucosamine in a tryptic peptide isolated from a cyanogen bromide fragment (CN-30 kDa; T. Koide et al., unpublished results). A fifth potential carbohydrate attachment site is Asn-69 present in the sequence Asn-Asp-Cys. The sequence of Asn-X-Cys has been shown to contain carbohydrate in protein C (Stenflo & Fernlund, 1982). The addition of 14% carbohydrate to HRG would increase the molecular weight of the glycoprotein to about 67 000.

Most of the histidine and proline residues in HRG are found in the C-terminal region. Histidine residues are highly concentrated in a small region between residues 330 and 389 which forms the "histidine-rich" region in the protein. Proline residues, on the other hand, are more widely distributed, but many are concentrated in "proline-rich" regions prior to and following the histidine-rich region. These regions (residues 267–419) contain very few hydrophobic and basic amino acids.

A striking feature of the sequence organization of HRG is the internal repeats of 23 amino acid segments, as classified into 5 different types (types I–V, Figure 3). More than half of the amino acids in HRG are present in these five different types of internal repeats, most of which are successive and

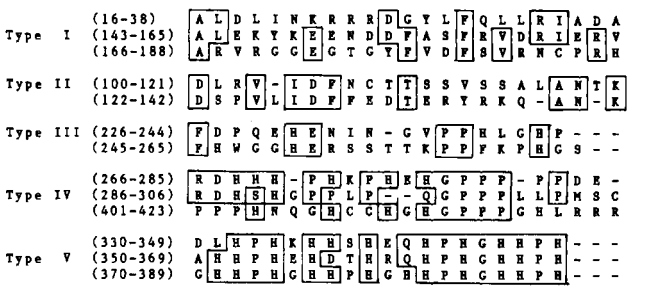

FIGURE 3: Five types of internal repeats present in HRG. Identical residues in each repeat are enclosed in boxes. Gaps were inserted for maximal homology in the amino acid sequences. The single-letter code for amino acids is as follows: Ala, A; Asn, N; Asp, D; Cys, C; Gln, Q; Glu, E; Gly, G; His, H; Ile, I; Leu, L; Lys, K; Met, M; Phe, F; Pro, P; Ser, S; Thr, T; Trp, W; Tyr, Y; Val, V.

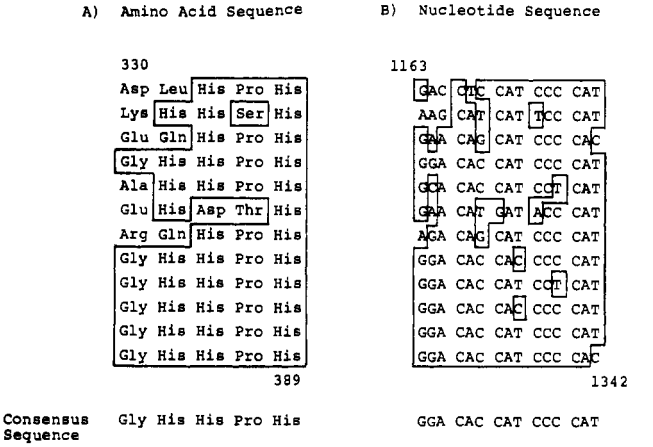

FIGURE 4: Tandem repeats in the cDNA and derived amino acid sequences of the histidine-rich region of HRG. Amino acid residues and nucleotides that are identical with the consensus sequence are enclosed in boxes.

contiguous. Types I, IV, and V are triplications, and types II and III are duplications (Figure 3). These last repeats include 12 tandem repeats of 5 amino acids. The tandem repetition becomes striking when the amino acid sequence of the histidine-rich region (residues 330–389) is rearranged in blocks, as shown in Figure 4A. It is evident that a sequence of 5 amino acids is repeated 12 times in tandem in this region. Also, the last five repeated sequences are identical, representing exact repetitions of a consensus sequence of Gly-His-His-Pro-His. The nucleotide sequence in this region revealed a more strictly conserved repeat of 15 nucleotides (Figure 4B). One possibility is that this region evolved from a short stretch of 15 nucleotides (nucleotides 1328–1342) by successive gene duplication. This could occur in the following way: an ancestral gene coding for Gly-His-His-Pro-His was initially duplicated to generate a gene coding for 10 amino acids. Duplication of the latter sequence would generate a gene coding for a repeat of 20 amino acids composed of 4 tandem repeats of the original 5 amino acids (type V, Figure 3). Duplication of the repeat of 20 amino acids would yield the second and third type V repeats. It also seems possible that the tandem repeats of Gly-His-His-Pro-His are responsible for heme and metal binding to HRG, since two imidazole rings of histidine residues in His-Pro-His will form a configuration that is ideal for metal and heme binding. Furthermore, this probability is supported by experiments with rabbit HRG (Morgan, 1985). In these experiments, a peptide which contained most of the histidine and proline residues in HRG was isolated by limited plasmin digestion of HRG and shown to have the ability to bind heme and metal ions similar to intact HRG.

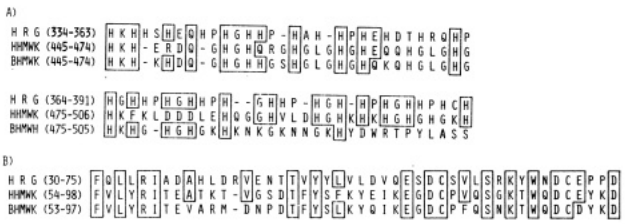

FIGURE 5: Comparison of the amino acid sequences of human HRG with human and bovine HMW kininogen in their histidine-rich regions (A) and amino-terminal regions (B). Amino acid residues identical with those of HRG are enclosed in boxes. Gaps were inserted for maximal homology. The numbering of the amino acid residues for human and bovine HMW kininogen was taken from Takagaki et al. (1985) and Kitamura et al. (1983), respectively. HHMWK, human HMW kininogen; BHMWK, bovine HMW kininogen.

Two other plasma proteins are well known to have a histidine-rich sequence in their polypeptide chains, including high molecular weight (HMW) kininogen (Han et al., 1975; Kitamura et al., 1983; Takagaki et al., 1985) and ceruloplasmin (Kingston et al., 1979). A comparison of the histidine-rich region in HRG with the histidine-rich regions in human and bovine HMW kininogen is shown in Figure 5A. These regions show a high degree of sequence homology (about 50%). Furthermore, many of the nonidentical residues differ by just one base in their respective codons. These data suggest that the histidine-rich regions of HRG and HMW kininogen are evolutionarily and functionally related. The histidine-rich regions of HRG and HMW kininogen are also similar to each other in that their hydrophobic amino acid content is very low. The hydrophobic amino acid content of the histidine-rich region of ceruloplasmin, however, is high. There also appears to be no sequence similarity to ceruloplasmin. The histidine-rich region of HMW kininogen is known to bind to kaolin in the kaolin-mediated initiation of blood coagulation (Sugo et al., 1980). On the basis of the high sequence homology of histidine-rich regions, HRG may have some function as a modulator of the contact activation of blood coagulation mediated by the histidine-rich region. A computer search also revealed a high degree of sequence homology of HRG with another part of HMW kininogen (Figure 5B). In this comparison, 20 residues out of 46 were identical (43% homology), where the homology between human and bovine HMW kininogen is 73%. This region in HMW kininogen is also present in low molecular weight kininogen (Ohkubo et al., 1984; Takagaki et al., 1985).

The primary structure of the histidine-rich protein of the malaria parasite has recently been reported (Ravetch et al., 1984). This protein contains 73% histidine and also has several different types of internal repeats. However, no sequence homology has been found between this protein and the histidine-rich region of HRG.

The amino acid sequences of the proline-rich regions of HRG are compared with those of proline-rich peptides (Saitoh et al., 1983a,b) and proline-rich proteins (Azen et al., 1984; Ziemer et al., 1984) from parotid saliva in Figure 6. The homology of HRG and proline-rich peptides and proteins is as high as 49% (Figure 6, top) and 45% (Figure 6, bottom). The characteristic sequence of Gln-Gly-Pro-Pro-Pro- in the proline-rich regions of proline-rich peptides and proteins is also present in HRG as a Gln/His-Gly-Pro-Pro-Pro sequence. Circular dichroism studies on proline-rich peptides (Isemura et al., 1983; Shibata et al., 1984) and glycoprotein (Aubert et al., 1982) demonstrated the existence of poly(L-proline) form II conformation in their polypeptide structures due to sequences of continuous proline residues. Recently, similar

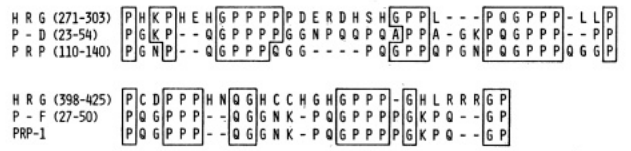

FIGURE 6: Comparison of the amino acid sequences of two proline-rich regions in HRG with those of proline-rich peptides and proteins from parotid saliva. Amino acid residues that are identical with those of the proline-rich regions in HRG are enclosed in boxes. Gaps were inserted for maximal homology. The sequences of proline-rich peptides (abbreviated as P-D and P-F) were taken from Saitoh et al. (1983a,b, respectively) and those of proline-rich proteins (abbreviated as PRP and PRP-1) from Ziemer et al. (1984) and Azen et al. (1984), respectively.

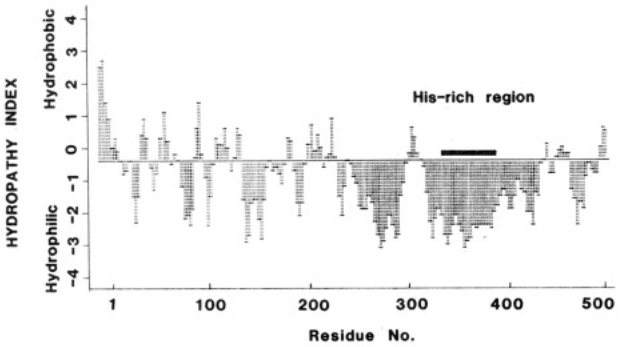

FIGURE 7: Hydropathy index for human HRG. Analyses were made by the method of Kyte and Doolittle (1982).

observations have also been reported with HRG, suggesting the presence of sequences of continuous proline residues (Morgan, 1985). In the present studies, four Pro-Pro, three Pro-Pro-Pro, and one Pro-Pro-Pro-Pro-Pro were identified in HRG. Furthermore, the amino acid sequences of two proline-rich regions in HRG are homologous to those of the proline-rich peptides and proteins. From these observations, it appears likely that the two proline-rich regions of human HRG may also have an elongated bending and twisting conformation similar to that suggested for the proline-rich protein (Ziemer et al., 1984) and rabbit HRG (Morgan, 1985).

Hydropathy analysis by the method of Kyte and Doolittle (1982) showed that HRG is hydrophilic throughout the molecule (Figure 7). The histidine-rich region (residues 330–389) is particularly hydrophilic, giving large negative values in its hydropathic index. The secondary structure of HRG predicted by the method of Garnier et al. (1978) consists of 8% helix, 14% β-sheet, 46% β-turn, and 32% random coil. The C-terminal half of the molecule (residues 215–507), however, would be predicted to contain no helical structure where the histidine-rich region consists of only β-turn and random coil.

In spite of the high content of histidine and glycine residues and the frequent occurrence of a Gly-His sequence, there were no Gly-His-Lys sequences in HRG. This tripeptide, which has been detected in plasma, has been shown to promote cell growth in rat hepatoma cells (Pickart et al., 1973).

Homologies of HRG with HMW kininogen and proline-rich peptides and proteins in the C-terminal portion, together with homology of HRG with antithrombin III in the N-terminal portion (Koide et al., 1982, 1986), suggest that multifunctional properties of HRG may be due to the organization of the polypeptide chain into multidomains.

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Registry No. DNA (human liver histidine-rich glycoprotein mRNA complementary), 100857-87-2; glycoprotein (human liver histidine-rich precursor protein moiety reduced), 100857-88-3; glycoprotein (human liver histidine-rich protein moiety reduced), 100857-89-4.

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