Characterization of the Human Serum Trypanosome Toxin, Haptoglobin-related Protein*

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Haptoglobin-related protein (HPR) is a serum protein that is >90% homologous to the acute-phase reactant haptoglobin (Hp). Haptoglobin binds and removes free hemoglobin (Hb) from the circulation. Hpr levels are elevated with tumor progression in the serum of some cancer patients, but the relevance of this observation is not understood. HPR is an integral component of both trypanosome lytic factors. Previous data indicate that HPR represents the toxic component of both trypanosome lytic factors. It has been proposed that after uptake by the parasite, Hb bound to HPR causes lysis in a peroxidase-dependent process. We report that the molecular architecture of HPR in normal human serum is different from that of Hp and that HPR does not bind Hb in normal human serum. Immunodepletion of all detectable Hb from TLF1 does not deplete TLF1 of HPR or trypolytic activity, suggesting that the mechanism of parasite lysis is Hb-independent.

Haptoglobin (Hp)1 is an acute-phase plasma glycoprotein present in normal human serum (NHS) at concentrations of between 0.2 and 2 mg/ml. The major known function of Hp is to bind and remove potentially toxic free hemoglobin (Hb) from the circulation. Haptoglobin binds Hb with 1:1 stoichiometry with an extremely high affinity (Kd > 10^-15 M) (1), and Hp-Hb complexes are rapidly removed from the circulation via receptors in the liver. Haptoglobin is a heterodimer, and due to structural variation in the α chain of Hp α-β dimers, there are three Hp types in humans, namely Hp1–1, Hp2–1, and Hp2–2 (2). Types 2–1 and 2–2 exist as a series of disulfide-linked polymers (2).

Haptoglobin-related protein (HPR) displays more than 90% identity to Hp and is found at a much lower concentration in serum (3). The HPR gene arose by duplication of the Hp gene and is located 2.2 kilobase pairs downstream of the Hp gene on chromosome 16. There are 28-amino acid changes in HPR, 16 of which occur in the β chain. In addition, intron 1 of Hp is only 1.3 kilobase pairs, whereas that of HPR is 9.5 kilobase pairs and contains a retrovirus-like element (4). There is an Alu sequence in the 5'-flanking region of HPR (3, 4). The HPR gene is also present in apes and Old World monkeys and is a product of a gene triplication event that occurred early in primate evolution (5).

In NHS, HPR appears to be associated exclusively with either a small HDL subpopulation or a high molecular weight protein complex (6), both of which possess lytic activity against the African cattle parasite Trypanosoma brucei brucei. The HDL trypanolytic factor is termed TLF1 and is composed of phospholipid, apoA1, apoAII, paraoxonase and HPR (7). The second factor, TLF2, is a protein complex containing apoA1 and HPR (6, 8). The natural immunity of humans to T. brucei brucei but not to the morphologically indistinguishable human pathogens T. brucei gambiense and T. brucei rhodesiensese that cause sleeping sickness is due to the selective killing of T. brucei brucei by TLF1 and/or TLF2. Consistent with an important role for HPR in trypanosome killing, sera from nonhuman primates that contain the HPR gene (see above) are trypanolytic (9, 10). An exception is Chimpanzee serum, which is not trypanolytic. However, the HPR gene sequence in Chimpanzees was found to contain a frameshift mutation, resulting in premature termination of translation (5). Data indicate that trypanosome lysis is peroxide-dependent (7). It has been proposed that like Hp, HPR binds Hb, and that TLF1-associated HPR-Hb complexes possess peroxidase activity at acid pH. After uptake and delivery to parasite lysosomes, the HPR-Hb complexes would kill trypanosomes by oxidative damage (7).

Other than its role in trypanosome lysis, there is no known function of HPR. A form of HPR apparently distinct from that found in NHS is found in the sera of cancer patients. Hpr levels appear to be useful as a clinical diagnostic marker, since they are elevated in progression and decreased during regression of various carcinomas (11–14). Here we characterize this minor serum protein and further define its role as the toxin in the trypanocidal factors.

EXPERIMENTAL PROCEDURES

Sera—NHS from a single healthy donor of Hp haplotype 1–1 was stored at -70 °C in aliquots for up to 6 months. Sera from patients with paroxysmal nocturnal hemoglobinuria and Tangier disease were the kind gifts of Dr. W. Rosse (Duke University, NC) and Dr. Sachiya Ohtaki (Miyazaki Medical College, Fukuoka, Japan), respectively.

Parasites—Swiss Webster mice were inoculated intraperitoneally with T. brucei brucei TREU 667 stock (15), and the trypanosomes were harvested 2 days later from infected mouse blood and prepared for trypanolytic assay as described previously (16).

Purification of TLF1—Total lipoproteins were isolated from NHS by density gradient centrifugation, essentially as described (17). Solid KBr was added to serum to give a density of 1.25 g/ml, and after ultracentrifugation (Beckman NVTI 60 rotor, 16 h, 49,000 × g, 10 °C), the top 25% of the gradient containing lipoprotein was collected. The density of the lipoprotein fraction was then adjusted to 1.3 g/ml with KBr, and aliquots (4 ml) were layered under 0.9% NaCl (8 ml). After centrifugation for 3 h at 49,000 × g/10 °C (NVTI 60, Beckman), the HDL band was isolated. The crude TLF1 preparation was dialyzed against TBS at 4 °C and stored at -70 °C until use.

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The abbreviations used are: HPR, haptoglobin-related protein; NHS, normal human serum; TLF, trypanosome lytic factor; HDL, high density lipoprotein; PNH, paroxysmal nocturnal hemoglobinuria.

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with frequent buffer changes and then concentrated by ultrafiltration using Amicon (Beverly, MA) XM300 membranes. The TLF1 preparation (aliquots of 0.5 ml at about 50 mg protein/ml) was then fractionated by fast protein liquid chromatography (Pharmacia Biotech Inc.) on Superose 6 high resolution 10/30 and Superose 12 high resolution 10/30 columns connected in tandem (0.2 ml/min in TBS, pH 7.0). Fractions were analyzed by absorbance at 280 nm, and TLF1 fractions were identified by measurement of trypanosome lytic activity in each fraction (8, 18). Fractions with peak trypanolytic activity were pooled and concentrated using Centricron 100 devices (Amicon), and aliquots were stored at −70°C.

Anti-Hb Immunodepletion of TLF1—Anti-Hb monoclonal (Genzyme, Cambridge, MA) or polyclonal (Sigma) antibody was incubated with protein G-Sepharose (Pharmacia) for 2 h at 4°C. Ten μg of anti-Hb antibody was conjugated/μl of protein G-Sepharose. The conjugated beads were washed by centrifugation (phosphate-buffered saline, 0.1% bovine serum albumin) and resuspended to 50% (phosphate-buffered saline, 0.1% bovine serum albumin). After incubation for 1 h at 4°C, the beads were used to deplete TLF1 preparations of Hb. Fifty μl of TLF1 was treated with 4 μl of anti-Hb-Sepharose (or protein G-Sepharose as control) for 2 h at 4°C, and the immunoprecipitate was isolated by centrifugation. Immunoprecipitates were washed 5 × with phosphate-buffered saline containing 0.5 mM NaCl and 1% Nonidet P-40, followed by three washes with phosphate-buffered saline. Pellets and supernatants were analyzed as described below. Hb-HPr complexes were used in control immunoprecipitation experiments and were prepared as described (8).

SDS-Polyacrylamide Gel Electrophoresis and Immunoblot Analysis—All samples were separated in SDS-polyacrylamide gel electrophoresis 4–15% acrylamide gradient gels (Bio-Rad) under reducing or nonreducing conditions by standard procedures (19). Separated proteins were transferred to a nitrocellulose membrane, and prestained broad range markers (Bio-Rad) were used as an indicator of complete transfer. Membranes with transferred proteins were developed by either chemiluminescence (Amersham Life Science, Inc.) (as described by the manufacturer) or by standard alkaline phosphatase detection. For Hp detection, membranes were probed with rabbit anti-human Hp (Sigma) at either 1,000 × dilution (for alkaline phosphatase detection) or at 10,000 × dilution (for chemiluminescent detection). For Hb detection, membranes were probed with goat anti-human Hb (Chemicon, Temecula, CA) at 2,500 × dilution, and bound antibody was revealed by means of protein A-HPR antibody at 2,500 × dilution (Cappel, Durham, NC) and ECL reagents (Amersham).

Assay Procedures—Quantitation of Hp, HPR, and Hb was performed using a Western blotting procedure as described previously (6). For assaying serum Hp and HPR concentrations, various dilutions of serum corresponding to 2–0.02 μl of serum were separated by SDS-polyacrylamide gel electrophoresis before transfer to nitrocellulose membranes (see above). Each gel run for quantitation purposes contained Hp and/or Hb standards of between 100 and 2.5 ng. Immunoblots were scanned, and immunostained bands were quantitated using NIH image 5.7 software. The distinction between Hp and HPR based on molecular weight and has been previously described (6). Haptoglobin used for standards was purified as described (18).

Parasite Lysis Assays—Assays were performed as described previously (18). Briefly, 2 × 10⁶ trypanosomes were incubated with the sample under test in a total volume of 200 μl in high glucose Dulbecco’s modified essential medium (Life Technologies, Inc.) containing 0.2% bovine serum albumin. Lysis was assessed by means of the fluorescent probe Calcein-AM (Molecular Probes, Eugene, OR) after a 15-min incubation at 37°C (18).

RESULTS

Structure of HPR—Haptoglobin is a disulfide-linked heterodimer, and Western blot analysis of reduced purified Hp1–1 using an anti-Hp polyclonal antibody revealed the expected 15-kDa α and 40-kDa β chains of the protein (Fig. 1, lane 2). The corresponding subunits for HPR are 12 and 35 kDa, respectively (8, 20) and are shown in Fig. 1, lane 1. Purified TLF1 was the source of HPR. Native Hp1–1 exists as a disulfide-linked tetramer (α2β2) (21), and as expected, an anti-Hp immunoblot of nonreduced Hp1–1 revealed a single 110-kDa protein band (Fig. 1, lane 4). In contrast, immunoblot analysis of nonreduced HPR revealed a major band of about 45 kDa and additional much fainter bands of about 90, 120, and 135 kDa, probably representing HPR multimers (Fig. 1, lane 3). Native Hp2–2 and Hp2–2 exist as disulfide-linked oligomeric complexes of between 200–700 kDa (21). Thus, the molecular architecture of native HPR is different to that of all Hp allotypes in that it appears to exist predominantly as a single α-β dimer.

Haptoglobin-related Protein Is Found in Blood of Patients with Intravascular Hemolysis—Hp binds free plasma Hb with high affinity, and Hp-Hb complexes are rapidly cleared from the circulation via hepatocyte receptors. We determined Hp and HPR concentrations in NHS and in sera from patients with paroxysmal nocturnal hemoglobinuria (PNH) or Tangier disease. These conditions lead to extensive intravascular hemolysis, resulting in excess free Hb in the circulation and subsequent Hp depletion. As expected, the pathological sera contained very low levels of Hp; between 500–5,000-fold less than in NHS (Table I). In contrast, the concentration of Hpr in both NHS and pathological sera was similar. Therefore, unlike Hp, HPR serum concentration does not decrease in the presence of excess circulating plasma Hb. The simplest interpretation of these data is that either HPR does not bind Hb or that the hepatocyte ligand within HPR-Hb complexes is absent or obscured.

Table I also shows that the concentration of HPR in NHS is about 50-fold lower than that of Hp (Table I). This is much higher than the previously predicted 1,000-fold difference that was based on a comparison of mRNA levels of Hp and HPR (3).

Hpr Does Not Contain Bound Hb—In NHS, most plasma HPR is associated with the TLF1 complex, a small HDL sub-
immunoprecipitate of control Hp and alkaline phosphatase-conjugated antibody. Western blot procedure (6). Nanogram quantities measured in 25-μl samples shown in lanes 3 and 4, respectively. Lane 7 represents anti-Hb immunoprecipitate of control Hp-Hb complexes. Immunoreactive bands were visualized by means of anti-Hp polyclonal antibody and a secondary alkaline phosphatase-conjugated antibody.

**TABLE II**

| Sample                        | Hb     | Hp    | HPR   |
|-------------------------------|--------|-------|-------|
| Mock-depleted TLF1 supernatant| 18.34  | 51.74 | 86.28 |
| Anti-Hb-depleted TLF1 supernatant | –      | 30.56 | 90.92 |
| Mock-depleted TLF1 precipitate | 23.25  | 21.22 | –     |
| Anti-Hb-depleted TLF1 precipitate | 33.58  | 41.94 | –     |
| Mock-depleted Hp · Hb supernatant | –      | 20.19 | –     |
| Anti-Hb-depleted Hp · Hb precipitate | 34.4   | 35.33 | –     |

Fig. 2. Western blot analysis under reducing conditions of TLF1 fractions immunoprecipitated with anti-Hb monoclonal antibody and revealed by antibodies to Hp. Lanes 1 and 2 represent supernatant from two TLF1 fractions after control incubation with protein G beads. One of the fractions is heavily contaminated with Hp (lane 2). Lanes 3 and 4 represent supernatants from samples of the same two fractions after immunoprecipitation with anti-Hb monoclonal antibodies. Lanes 5 and 6 represent anti-Hb immunoprecipitates from samples shown in lanes 3 and 4, respectively. Lane 7 represents anti-Hb immunoprecipitate of control Hp-Hb complexes. Immunoreactive bands were visualized by means of anti-Hp polyclonal antibody and a secondary alkaline phosphatase-conjugated antibody.

population that possesses trypanosome lytic activity (6, 7). Partially purified TLF1 was fractionated by gel filtration, and two lytic TLF1 fractions eluting at slightly different molecular weights were selected. One contained a high HPR and a low contaminating Hp concentration (Fig. 2, lane 1), and one contained low HPR and high Hp concentration (Fig. 2, lane 2). Each of these fractions was treated with anti-Hb monoclonal antibody bound to protein G beads, and the immunoprecipitate and supernatant were analyzed by anti-Hp Western blot. A quantitative determination of Hp, HPR, and Hp in the various anti-Hb immunodepleted fractions is shown in Table II. Fig. 2 and Table II shows that an anti-Hb antibody coimmunoprecipitated contaminating Hp from TLF1 preparations, but failed to coimmunoprecipitate HPR (Fig. 2, lanes 5 and 6, and Table II). Analysis of supernatants after anti-Hb immunoprecipitation shows that HPR remains in the supernatant (Fig. 2, lanes 3 and 4, and Table II). Not all Hp present is expected to contain bound Hb, so not all Hp was immunoprecipitated. Anti-Hb antibody was added in excess, as indicated by the quantity of control Hp-Hb complexes that were immunoprecipitated (Fig. 2, lane 7 and Table II). Further analysis of anti-Hb-treated fractions by anti-Hb Western blot revealed the removal of all detectable Hb from TLF1 (Fig. 3, Table II).

Immunodepletion of Hp from TLF1 Does Not Deplete Trypanolytic Activity—It has been proposed that Hb bound to HPR kills *T. brucei* in a peroxidase-dependent mechanism (7). However, the data presented above indicate that HPR does not contain bound Hb in NHS. Furthermore, Fig. 4 shows that immunoprecipitation of TLF1 with either monoclonal or polyclonal antibodies to Hb did not remove trypanolytic activity from a TLF1 preparation.

Table II shows that a fully lytic TLF1 preparation that has been immunodepleted of all detectable Hb (<3 ng) contains about 90 ng of HPR. Thus, even if very low levels of Hb are bound to HPR in TLF1, there is at a minimum a 30-fold excess of HPR over Hb (native molecular weights of HPR and Hp are similar). In a final experiment, excess exogenous Hb was added to TLF1, and its trypanolytic activity was measured. If Hb bound to HPR and represented the lytic moiety of TLF1, any...
exogenous Hb added to TLF1 would be expected to complex with the excess HPR and result in an increase in trypanolytic activity. The addition of Hb to TLF1 did not significantly affect TLF1 trypanolytic activity (Fig. 5). We conclude that Hb does not play a role in trypanolysis.

**DISCUSSION**

We have studied certain properties of HPR in NHS and report several new findings. The molecular architecture of native HPR is distinct from that of Hp. Hpr levels in Hp-depleted patient sera are comparable to levels found in NHS, indicating either that HPR does not bind Hb or that HPR-Hb complexes (unlike Hp-Hb complexes) are not cleared from the circulation. We have not measured the turnover of HPR, but it is unlikely that the maintenance of HPR levels in serum of PNH patients reflects compensatory synthesis after clearance of HPR-Hb complexes. In fact, HPR in NHS does not contain any detectable bound Hb. Therefore, HPR does not share the principal function of Hp, which is to bind and remove free Hb from the circulation. This is consistent with earlier studies reporting no detectable peroxidase activity in HPR identified on native polyacrylate gels (22). Finally, the present experiments show that the concentration of HPR in NHS is about 50-fold less than that of Hp, contrasting with the 1,000-fold difference previously predicted from mRNA quantitation (3).

The sequence of HPR is more than 90% identical with that of Hp, and the predicted Hb binding region of Hp is conserved. The inability of HPR to bind Hb may be due to a different pattern of disulfide bonding that results in structural differences. Although both Hp and HPR contain the same total number of cysteines, the cysteine at position 15 of the Hp α chain that stabilizes the tetramer through cross-linking the monomers is replaced by phenylalanine in HPR (4). This is consistent with our observation that HPR exists predominantly as an α-β dimer. Though HPR can polymerize into higher order complexes, these are minor components (based on the faint staining with anti-Hp). It is also possible that the cysteines in HPR are conformationally shielded from one another, thus preventing oligomer formation.

In NHS, total serum HPR appears to be distributed between two protein species termed TLF1 and TLF2, both of which have trypansomolytic lytic activity. Susceptibility of trypanosomes to these lytic factors determine their human infectivity and defines their host range. There is compelling evidence that HPR represents the toxic component of these factors (6, 7). The unusual hydrophobic N terminus of HPR in NHS (7) may be involved in its targeting to TLF1 (an HDL particle) and to TLF2 (a protein complex that contains the amphipathic apolipoprotein A1) (6). However, the current data does not support the hypothesis that HPR-mediated trypanolysis is due to peroxidase activity of bound Hb (see the introduction). Although Smith et al. (7) detected Hb in their TLF1 preparation (as do we in the TLF1 preparations used in this study), the current data indicate that Hb is a contaminant and not a component of TLF1.

So how does HPR mediate trypansomolytic lysis? The previous measurement of peroxidase activity in TLF1 may have been due to contaminating Hb, but the inhibition of parasite lysis by catalase indicates that endocytosed TLF1 causes parasite lysis via a peroxide-dependent mechanism (7). In addition to Hb, heme/hemin, Fe³⁺, and Cu²⁺ can participate in peroxidation of lipids and proteins and cause cell damage through reactive oxygen intermediates. Hp is known to bind Cu²⁺ (24) and heme (2), and it is possible that HPR may similarly bind one or more of these molecules, resulting in a parasite lysosome-inducible peroxidase activity. An alternative and intriguing possibility is that the HPR in TLF may induce a parasite-specific peroxidase activity. Further characterization of HPR, in particular identification of a putative peroxidase-confering moiety, may provide a better understanding of its precise role in trypanosome lysis. Such knowledge may be useful in creating drug delivery systems for African trypanosomes such as trypanolytic recombinant fusion proteins incorporating HPR and parasite receptor ligands.

Other than its role in trypanolysis, there is no known function of HPR. Increased levels of HPR have been reported in sera from patients with various cancers, and HPR levels have been reported to correlate with tumor progression (11–14, 25). However, the analysis of cancer-related HPR revealed that it contains an N-terminal extension not found in HPR present in NHS (23). This N-terminal extension appears to be due to an alternative processing of the HPR pre-mRNA, resulting in transcription of intron 1 and translation from the first AUG in intron 1 with the loss of exon 1. The relationship between the two forms of HPR is not known.

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