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A unique tetrameric structure of deer plasma haptoglobin – an evolutionary advantage in the Hp 2-2 phenotype with homogeneous structure

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Database
The sequence corresponding to deer Hp is available in the DDBJ/EMBL/GenBank database under the accession number EF601928

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Similar to blood types, human plasma haptoglobin (Hp) is classified into three phenotypes: Hp 1-1, 2-1 and 2-2. They are genetically inherited from two alleles ***Hp 1*** and ***Hp 2*** (represented in bold), but only the Hp 1-1 phenotype is found in almost all animal species. The Hp 2-2 protein consists of complicated large polymers cross-linked by α2-β subunits or $(\alpha 2\text{-}\beta)_n$ (where $n \geq 3$, up to 12 or more), and is associated with the risk of the development of diabetic, cardiovascular and inflammatory diseases. In the present study, we found that deer plasma Hp mimics human ***Hp 2***, containing a tandem repeat over the α-chain based on our cloned cDNA sequence. Interestingly, the isolated deer Hp is homogeneous and tetrameric, i.e. $(\alpha\text{-}\beta)_4$, although the locations of −SH groups (responsible for the formation of polymers) are exactly identical to that of human. Denaturation of deer Hp using 6 M urea under reducing conditions (143 mM β-mercaptoethanol), followed by renaturation, sustained the formation of $(\alpha\text{-}\beta)_4$, suggesting that the Hp tetramers are not randomly assembled. Interestingly, an α-chain monoclonal antibody (W1), known to recognize both human and deer α-chains, only binds to intact human Hp polymers, but not to deer Hp tetramers. This implies that the epitope of the deer α-chain is no longer exposed on the surface when Hp tetramers are formed. We propose that steric hindrance plays a major role in determining the polymeric formation in human and deer polymers. Phylogenetic and immunochemical analyses revealed that the ***Hp 2*** allele of deer might have arisen at least 25 million years ago. A mechanism involved in forming Hp tetramers is proposed and discussed, and the possibility is raised that the evolved tetrameric structure of deer Hp might confer a physiological advantage.

Haptoglobin (Hp) is an acute-phase protein (responsive to infection and inflammation) that is present in the plasma of all mammals [1–4]. A recent study has found that Hp also exists in lower vertebrates (bony fish) but not in frog and chicken [5]. The most frequently reported biological functions of the protein are to capture released hemoglobin during excessive hemolysis [6] and to scavenge free radicals during oxidative

Abbreviations
Hp, haptoglobin; β-ME, β-mercaptoethanol.

stress [7]. The captured hemoglobin is internalized by a macrophage/monocyte receptor, CD163, via endocytosis. Interestingly, the CD163 receptor only recognizes Hp and hemoglobin in complex, which indicates exposure of a receptor-binding neo-epitope [6]. Thus, CD163 is identified as a hemoglobin scavenger receptor. Recently, we have shown that Hp is an extremely potent antioxidant that directly protects low-density lipoprotein (LDL) from Cu^{2+}-induced oxidation. The potency is markedly superior to that of probucol, one of the most potent antioxidants used in antioxidant therapy [8–10]. Transfection of Hp cDNA into Chinese hamster ovary (CHO) cells protects them against oxidative stress [9].

Human Hp is one of the largest proteins in the plasma, and is originally synthesized as a single αβ polypeptide. Following post-translational cleavage by a protease, α- and β-chains are formed and then linked by disulfide bridges producing mature Hp [11]. The gene is characterized by two common alleles, ***Hp 1*** and ***Hp 2b***, corresponding to α1-β and α2-β polypeptide chains, respectively, resulting in three main phenotypes: Hp 1-1, 2-1 and 2-2. All the phenotypes share the same β-chain containing 245 amino acid residues. As shown in Fig. 1A, the α1-chain containing 83 amino acid residues possesses two available −SH groups; that at the C-terminus always cross-links with a β-chain to form a basic α-β unit, and that at the N-terminus links with another $(\alpha\text{-}\beta)_1$, resulting in an Hp dimer $(\alpha1\text{-}\beta)_2$, i.e. a Hp 1-1 molecule. In contrast, the α2-chain, containing a tandem repeat of residues 12–70 of α1 with 142 amino acid residues, is 'trivalent' providing an additional available −SH group (Cys15) that is able to interact with another α-β unit. As such, α2-chains can bind to either α1-β or α2-β units to form large polymers $[(\alpha1\text{-}\beta)_2\text{-}(\alpha2\text{-}\beta)_n$ in Hp2-1 and $(\alpha2\text{-}\beta)_n$ in Hp2-2] as shown in Fig. 1B.

Because of its weaker binding affinity to hemoglobin and retarded mobility (or penetration) between the cells, the polymeric structure of Hp 2-2 is dramatically more prevalent in some groups of patients with certain diseases, such as diabetes and inflammation-related diseases [7,12–14]. The human ***Hp 2*** allele has been proposed to have originated from ***Hp 1*** about two million years ago and then gradually displaced ***Hp 1*** as a consequence of nonhomologous crossing-over between the structural alleles (***Hp 1***) during meiosis [15–17], and is the first example of partial gene duplication of human plasma proteins [15,18,19]. Thus, only humans possess additional Hp 2-1 and 2-2 phenotypes.

In the present study, deer Hp protein was initially shown to be a homogeneous polymer using an electrophoretic hemoglobin typing gel. Following isolation

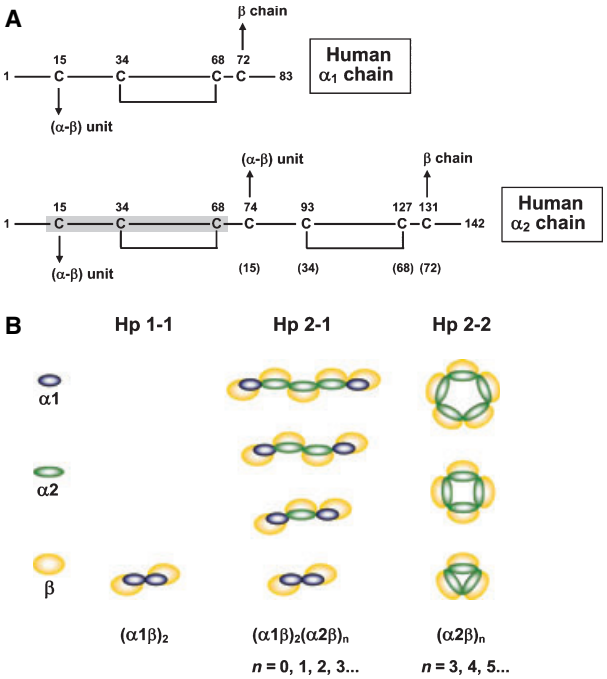

Fig. 1. Schematic drawing of the human Hp α-chain and the molecular arrangement of Hp phenotypes. (A) The human Hp α1-chain includes two avaiable −SH groups. That at the C-terminus always links to a β-chain to form a basic α1-β unit, and that at the N-terminus links either an α1-β unit or $(\alpha2\text{-}\beta)_n$ units. The sequence of α2 is identical to that of α1 except for a partial repeat insertion of residues 12–70. However, the extra Cys74 means that Hp 2-1 and 2-2 form complicated polymers. (B) Hp 1-1 forms the simplest homodimer $(\alpha1\text{-}\beta)_2$, whereas Hp 2-1 is polymeric in linear form, forming a homodimer $(\alpha1\text{-}\beta)_2$, trimer $(\alpha\text{-}\beta)_3$ and other polymers. Here, α represents α1- or α2-chains. Hp 2-2 forms cyclic structures: a trimer $(\alpha2\text{-}\beta)_3$ and other cyclic polymers.

and identification of the protein, the α-chain was found to be similar to the human α2-chain based on its apparent molecular mass. We then cloned the cDNA of deer Hp, showing that the putative amino acid sequence mimics that of human Hp 2-2 (81.7% and 67.9% sequence homology in the β- and α-chains, respectively), and that the α-chain of deer Hp also possesses a unique tandem repeat. Interestingly, deer Hp α-chain comprises seven −SH groups, that are oriented exactly the same as in human Hp 2-2, but the molecular arrangement of deer Hp is strictly tetrameric, i.e. $(\alpha\text{-}\beta)_4$. It is thus totally different from human Hp 2-2, which has $(\alpha\text{-}\beta)_n$ polymers, where $n \geq 3$. Using an α-chain mAb as a probe and denaturing/renaturing experiments, we further demonstrated that steric hindrance of the Hp α-chain plays a major role in determining the polymeric formation of human $(\alpha\text{-}\beta)_n$ and the deer $(\alpha\text{-}\beta)_4$ tetramer. Amino acid sequence alignment demonstrated that the evolved amino acid

sequences of the ruminant β-chain are the most divergent among all mammals. By phylogenetic tree analysis, we identified the α-chain of dolphin and whale (a branch before the deer) as belonging to the α1 type. This suggests that the deer tandem repeat sequence arose between 25 and 45 million years ago, which is much earlier than the two million years proposed for humans. It is possible that the evolved tetrameric structure of deer Hp might confer a physiological advantage. We further proposed that a steric hindrance mechanism is involved in forming Hp tetramers.

Results

Identification of Hp phenotype

It has been claimed that the Hp of ruminants (cattle, sheep and goat) cannot enter polyacrylamide gels due to the large polymeric nature of the protein [20,21]. We tested whether this was also the case for the Hp of deer (another ruminant). Using a hemoglobin typing gel, we unexpectedly found deer plasma Hp to be a simple homogeneous molecule that is small enough to enter a 7% electrophoretic gel. An example of its phenotype and the electrophoretic properties of deer Hp, compared to human Hp 1-1, 2-1 and 2-2, is shown in Fig. 2. This shows that deer Hp mimics one of the polymeric forms of human Hp 2-1 or 2-2: either a linear or cyclic tetramer.

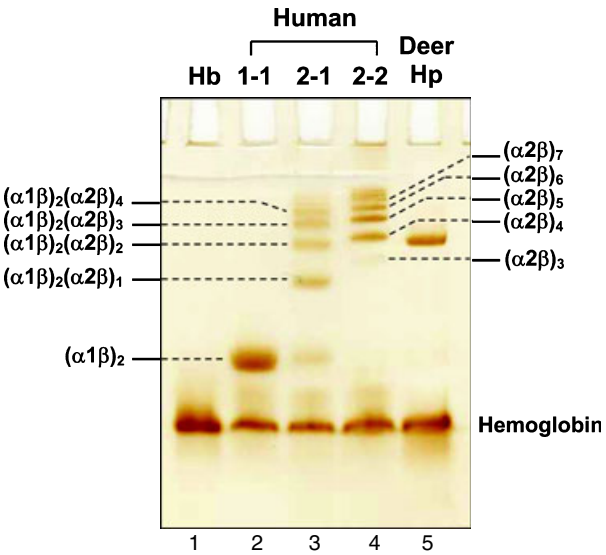

Fig. 2. Hemoglobin-binding patterns of deer and human plasma Hp on 7% native PAGE. Lane 1, hemoglobin only. Lanes 2, 3 and 4, human plasma of Hp 1-1, 2-1 and 2-2 phenotypes with hemoglobin, respectively. Lane 5, deer plasma with hemoglobin.

Isolation of deer Hp

The molecular size of the Hp α-chain has been conventionally used for identifying the phenotype of a given Hp protein. To further characterize the molecular form of deer plasma Hp, we attempted to isolate the protein using a Sepharose-based immunoaffinity column [22,23]. A mouse mAb prepared against the human α-chain (W1) was utilized for coupling to the Sepharose because this mAb was able to react with both human and deer α-chains on a western blot (described below). First, plasma samples enriched with Hp were pooled and applied to the affinity column. This procedure, however, failed to isolated deer Hp from the plasma due to the lack of binding of deer proteins to the column. Next, we used combined ammonium-sulfate fractionation and size-exclusion chromatography procedures [24] for the isolation. A size-exclusion chromatographic profile for the fractions containing Hp is shown in Fig. 3A (second peak). The homogeneity of isolated Hp was approximately 90%, as determined by SDS–PAGE (Fig. 3B). The presence of α-chains was

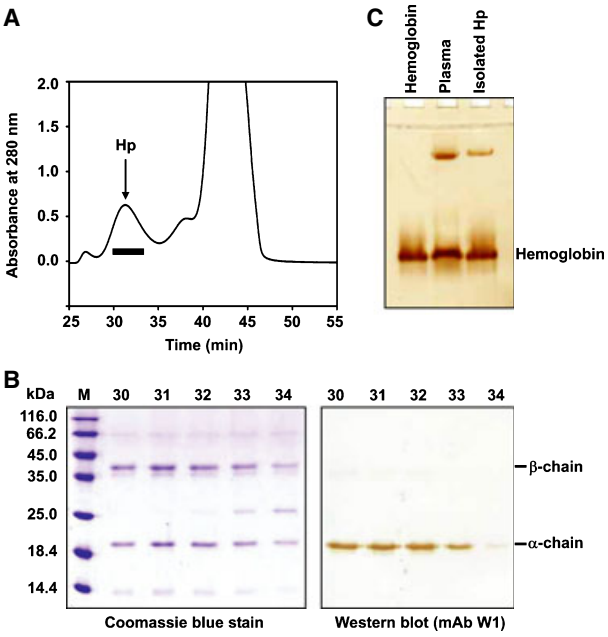

Fig. 3. Isolation of deer Hp using a size-exclusion Superose-12 column on an HPLC system. (A) A dialyzed supernatant of the 50% saturated ammonium sulfate fraction from plasma was applied to Superose-12 column (1 × 30 cm) at a flow rate of 0.3 mL·min^{-1}, using NaCl/Pi as the mobile phase. The bar represents the pooled fractions corresponding to Hp. (B) SDS–PAGE and western blot analyses of eluted Hp fractions. (C) Hemoglobin-binding properties of isolated Hp and plasma containing native Hp on 7% native PAGE. Lane M, molecular markers in kDa (Invitrogen).

confirmed by western blot using W1 mAb (Fig. 3B; right panel).

Hemoglobin binding of isolated Hp

In the next experiment, we tested the hemoglobin-binding ability of isolated deer Hp. Fig. 3C shows that the isolated Hp was able to form an Hp–hemoglobin complex under 7% native PAGE. Furthermore, it demonstrates that the deer protein consists of one major molecular form that is identical to its native form in the plasma based on electrophoretic mobility. It appears that the Hp isolated under our experimental conditions was not significantly altered with regard to its molecular and biochemical properties.

Molecular mass estimation of deer and human Hp 2-2 using SDS–PAGE and western blot

Western blot analysis using the α chain-specific mAb W1 indicated that the mAb recognizes both human and deer α chains (Fig. 4A). It also reveals that the deer α-chain belongs to the α2 group, with a molecular mass of approximately 18 kDa on both SDS–PAGE and western blot. We therefore tentatively classified the deer Hp as phenotype 2-2. In isolated deer Hp, there was a trace amount of hemoglobin (approximately 14 kDa), with a molecular mass comparable to that of the human Hp α1-chain. The estimated molecular mass of the deer β-chain was about 36 kDa, slightly lower than that of human. The isolated deer Hp was further characterized using 4% SDS–PAGE under non-reducing conditions. Consistent with our hemoglobin binding assay, Fig. 4B (left panel) demonstrates that isolated deer Hp consists of only one specific tetrameric form, i.e. $(\alpha\text{-}\beta)_4$, with a molecular mass about 216 kDa, which is close to that of the human Hp 2-2 tetramer (230 kDa) based on the gel profile.

Unique immunoreactivity of deer Hp defined by mAb W1

We then attempted to ensure that the polymeric forms of human and deer protein were an Hp by western blot analysis using W1 mAb. Figs 3B and 4A clearly showed that this antibody was capable of binding both human and deer α-chains in its reduced form. Interestingly, Fig. 4B (right panel) shows that this mAb recognized all the human Hp 2-2 polymers, but not intact deer Hp 2-2. However, after adding a reducing reagent (β-mercaptoethanol; β-ME) directly to intact deer Hp, the immunoreactivity was recovered on a dot-blot assay (Fig. 4C). It appears that the antigenic epitope of deer α-chain is masked in the tetrameric form. This also explains why the W1 mAb-coupled affinity

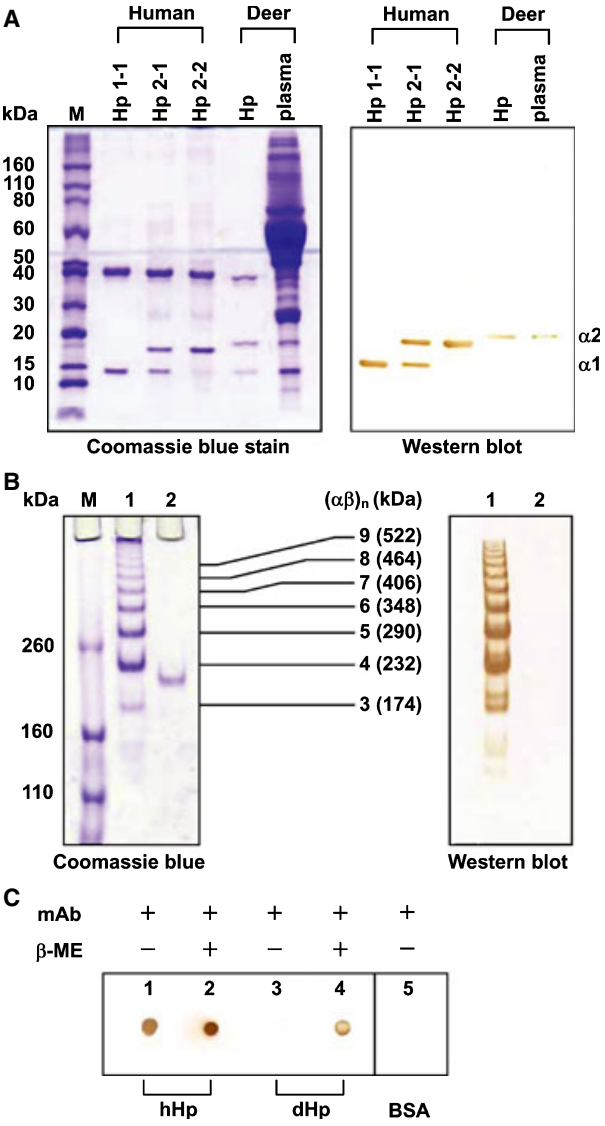

Fig. 4. SDS–PAGE, western blot and molecular mass analyses of isolated deer and human Hp. (A) The isolated proteins were run on 10–15% PAGE under reducing conditions. The western blot was performed using a human α-chain-specific mAb (W1) that cross-reacts with the deer α-chain. Lane M, molecular markers in kDa (Invitrogen). (B) Left panel: western blot analysis of the polymeric structure of isolated human and deer Hp under 4% non-reducing SDS–PAGE using α-chain-specific mAb W1. Lane M, molecular markers in kDa (Invitrogen). Lane 1, isolated human Hp 2-2. Lane 2, isolated deer Hp. Right panel: On the western blot, mAb W1 only recognizes human polymeric Hp, but not deer tetrameric Hp. (C) Dot-blot analysis of isolated human Hp (hHp) and deer Hp (dHp) using α-chain-specific mAb W1 in the presence or absence of the reducing reagent β-ME (143 mM). BSA was used as a negative control.

column failed to bind deer plasma Hp in the purification procedure described above.

Cloning of deer Hp cDNA

Evidently, the molecular form of deer 'Hp 2-2' totally differs from that of human Hp 2-2, with the latter found as typical polymers or the form $(\alpha\text{-}\beta)_n$, where n = 3–12 (Fig. 4B). It remains ambiguous as to whether deer Hp should be designated as a typical Hp 2-2. The most significant feature of the molecular structure of human Hp 2-2 is that it includes a tandem repeat in the α2-chain. To determine whether this is also true in deer Hp, we cloned the deer Hp cDNA. The complete linear nucleotide sequence corresponding to the α-β chain as determined by our laboratory has been submitted to GenBank (accession number EF601928). Based on the cDNA sequence, the deer α- and β-chains comprise 136 and 245 amino acid residues, respectively, which is similar to that of human, with 142 (α2) and 245 (β) residues (Fig. 5A,B). A tandem repeat of the deer α-chain was observed (discussed below).

Amino acid sequence alignment of deer and human Hp 2-2

The putative amino acid sequence alignment reveals that deer Hp is somewhat homologous to human Hp 2-2 (80% and 68% for β- and α-chains, respectively). The divergence and identity of the β-chain with that of other mammals are shown in Fig. 5C. The sequence for deer is relatively similar to that of cattle [25], another ruminant. We also created a brief phylogenetic tree for possible molecular evolution of the Hp β-chain using the CLUSTAL method in DNASTAR MEGALIGN software. The result shows that the evolved amino acid sequences of ruminant Hp β-chains are the most divergent among all mammals (Fig. 5D).

Analysis of –SH groups of the deer Hp α-chain and their implication for formation of the tetramer

As shown in Fig. 6 in the form of simplified ABC domains, the human α2-chain contains identical ABC

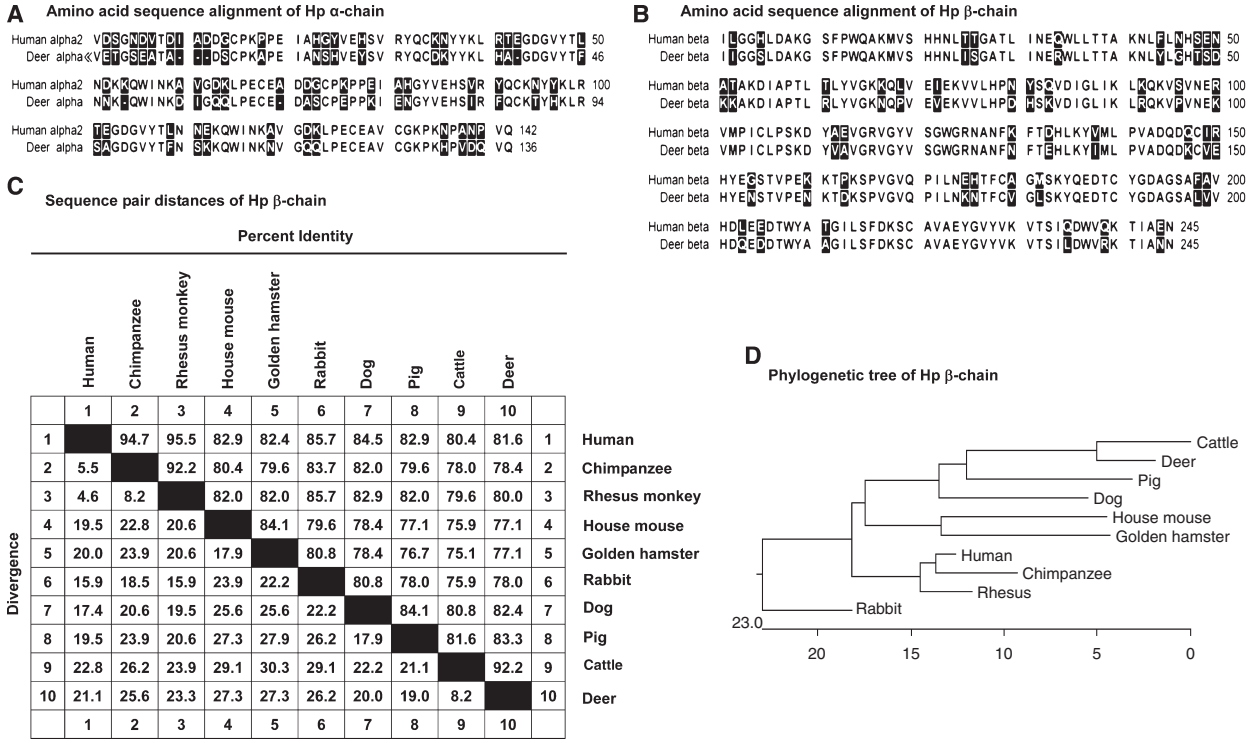

Fig. 5. Putative amino acid sequence analysis and divergence of mammal Hps. (A,B) Amino acid sequence alignment of the α- and β-chains of human and deer. Variable regions are shaded in black. The cDNA nucleotide sequence corresponding to deer Hp in this study has been deposited in GenBank under the accession number of EF601928. (C) Divergence of the amino acid sequences of Hp β-chains among ten mammals. (D) Phylogenetic tree constructed according to the amino acid sequences of Hp β-chains for ten mammals. The tree was plotted using the MEGALIGN program in the DNASTAR package. Branch lengths (%) are proportional to the level of sequence divergence, while units at the bottom indicate the number of substitution events.

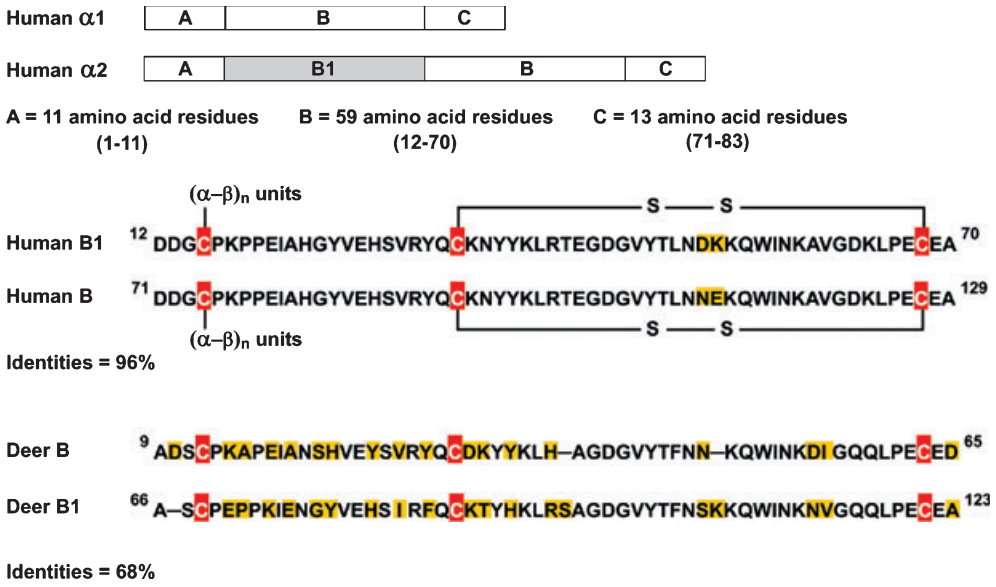

Fig. 6. Schematic drawing of tandem repeat region (B and B1) of deer and human α-chain. The most significant feature of human α2 is that it matches the ABC domains of α1 but with an additional insertion of a redundant sequence (B1 region). The repeat unit contains 59 amino acid residues between Asp12 and Ala70. The sequence homology in the repeat region of human is 96% (two amino acids mutated). Deer also have a redundant sequence (B and B1), but the sequence homology between the two repeat units is approximately 68%. The full length of the α-chain contains 142 and 136 residues in human and deer, respectively. The positions and number of Cys residues (total of seven) are completely identical between the two species (the one at the C-terminal region is not shown). Divergence of the amino acids within the species is marked in yellow.

domains to α1 with insertion of a tandem repeat region (B1). The latter contains amino acid residues between Asp12 and Ala70 (a total of 59 residues). The sequence homology between the repeat regions of the human α2-chain is 96%, with only two amino acids mutated (replacement of Asn52 and Glu53 in the B region by Asp52 and Lys53 in the B1 region). This tandem repeat is responsible for the formation of Hp polymers due to the extra –SH group (Fig. 1A). Such repeats also exist within the deer α-chain (B1 and B repeat), where the B1 region is residues 9–65. Thus, at the molecular level, the deer α-chain belongs to the α2 group, and is identical to the human α2-chain in possessing a tandem repeat. Interestingly, the sequence homology between the two repeat units (B1 and B) of deer is only 68% (Fig. 6).

As shown schematically in Fig. 1A, the human α2-chain consists of seven –SH groups (Cys15, 34, 68, 74, 93, 127 and 131) in 142 residues. Among these, there are two disulfide linkages within the α-chain (Cys34 and 68 and Cys93 and 127), and the one at the C-terminal region (Cys131) cross-links with the β-chain (Cys105) to form a basic α-β unit. Under such an arrangement, Cys15 and Cys74 are available to link with other α-β units. As a result, human α2 forms $(\alpha\text{-}\beta)_n$ polymers (where $n \geq 3$) as shown in Fig. 4B. Interestingly, the number and location of –SH groups in the deer α2-chain are identical to those in human (Fig. 6), but the deer Hp only yields a tetrameric $(\alpha\text{-}\beta)_4$ form. As the identity between the tandem repeats of deer is only 68% (compared with 96% in human), we hypothesized that these amino acid differences determine the conformation between Cys15 and 74 and drive the construction of the $(\alpha\text{-}\beta)_4$ structure of deer Hp (see Discussion).

To test whether the deer Hp can also form multiple polymers *in vitro*, we denatured the protein using 6 M urea with addition of 143 mM β-ME. Under these conditions, the deer protein was completely dissociated, similar to the profile shown in Fig. 4A for SDS–PAGE analysis (data not shown). We then slowly renatured the deer Hp by stepwise dialysis in order to determine possible formation of other large polymers (greater than tetramer). Figure 7 shows that the renatured protein retained the tetramer form, and no other polymers larger than tetramers were observed on SDS–PAGE, although some trimers were produced. Under the same conditions, human Hp 2-2 was renatured to $(\alpha\text{-}\beta)_n$. The data suggest that formation of deer Hp tetramer is specific, not randomly assembled. This assembly seems to be dependent on the unique orientation of the –SH groups within the Hp. In addition, each renatured protein retained its hemoglobin-binding ability (Fig. 7). A hypothetical model explaining the formation of Hp tetramers is described below.

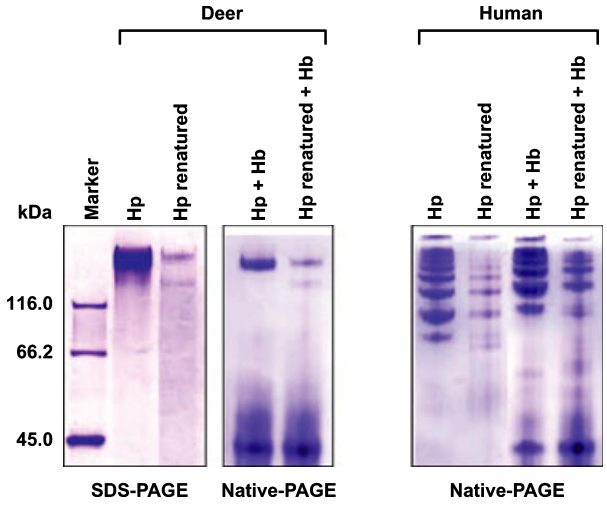

Fig. 7. SDS–PAGE and native PAGE analyses of renaturation of deer and human Hp polymers. Denaturation of deer Hp using 6 M urea under reducing conditions (143 mM β-ME) followed by renaturation resulted in the formation of $(\alpha\text{-}\beta)_4$ and some $(\alpha\text{-}\beta)_3$.

Discussion

Isolation of deer native Hp

We have recently developed several lines of human Hp mAb and routinely utilized these antibodies for the isolation of human Hp 1-1, 2-1 and 2-2 phenotypes [22,26]. As only W1 (specific to the α-chain) is able to cross-react with the deer α-chain on a western blot, we attempted to utilize this mAb for the affinity isolation of deer Hp in this study. Interestingly, the W1 mAb only recognizes the human Hp but not deer Hp in its intact form (Fig. 4B,C). We therefore used a previously described HPLC-based size-exclusion chromatography procedure [24] for the isolation of deer Hp. However, this procedure is only suitable for isolating the Hps with a homogeneous structure, and is not suitable for human Hp 2-2 or 2-1 [22]. One minor disadvantage of the method was the contamination of the isolated Hp by a trace amount of hemoglobin (Fig. 4A). This is observed mainly because Hp–hemoglobin complexes are formed prior to the purification; as such, hemolysis should be kept to a minimum in order to reduce the hemoglobin level while collecting the blood.

Presence of Hp in deer plasma

Not all deer possess a high level of plasma Hp. About 30% of the plasma samples that we screened (total $n = 15$) exhibited low Hp levels in the hemoglobin-binding assay (Fig. 2). Based on chromogeneity, the concentrations of deer plasma were approximately 1 $mg{\cdot}mL^{-1}$ of those used for purification when compared with human Hp 1-1 standard. In reindeer ($n = 6$), a mean plasma value of 0.6 $mg{\cdot}mL^{-1}$ has been reported [27].

Primary structure of the deer α-chain and its relationship to Hp polymers

There are several lines of evidence support the conclusion that the genotype of deer Hp is ***Hp 2***, with an Hp 2-2 phenotype. First, analysis of mercaptoethanol-reduced plasma indicates a molecular mass of 18 kDa for the α-chain, which is similar to that of human α2 based on a western blot (Fig. 4A). Second, the molecular mass of the α-chain from a purified sample was also similar to that of human α2 (Fig. 4A). Third, by putative amino acid sequence alignment, the deer α-chain contains a tandem repeat that is consistent with that found in human. Fourth, the total number of −SH groups and their location resulting from the tandem repeat are completely identical to that of human, although the sequence homology between the repeats was 68% in deer, compared to 96% in human (Fig. 6).

It remains unclear why the apparent molecular mass of the deer α-chain on PAGE is somewhat higher than that of human. We therefore attempted to determine whether it was due to additional carbohydrate moieties on the deer α-chain. However, using Pro-Q Emerald glycoprotein gel stains (Molecular Probes, Eugene, OR, USA), we did not identify any carbohydrates associated with the α-chain of either species (data not shown).

Hypothetical model for the formation of the deer Hp tetramer

The ability of the deer Hp to refold and reassemble into its tetrameric form *in vitro* indicates that the assembly of α- and β-chains into predetermined polymers is dependent on their biochemical nature (Fig. 7). As shown in Fig. 8A, we proposed a model to explain the formation of tetramers. This suggests that the two −SH groups of the deer α-chain are located on two flat surfaces at different angles to each other. Under these conditions, a homodimer cannot form due to the availability of another free −SH group of the α-β unit for cross-linking with another α-β unit. Figure 8B illustrates that there is no steric hindrance for tetramer formation, although there are two possible configurations for the tetramer. Some trimers may form, but there is some hindrance preventing the subunits from coming

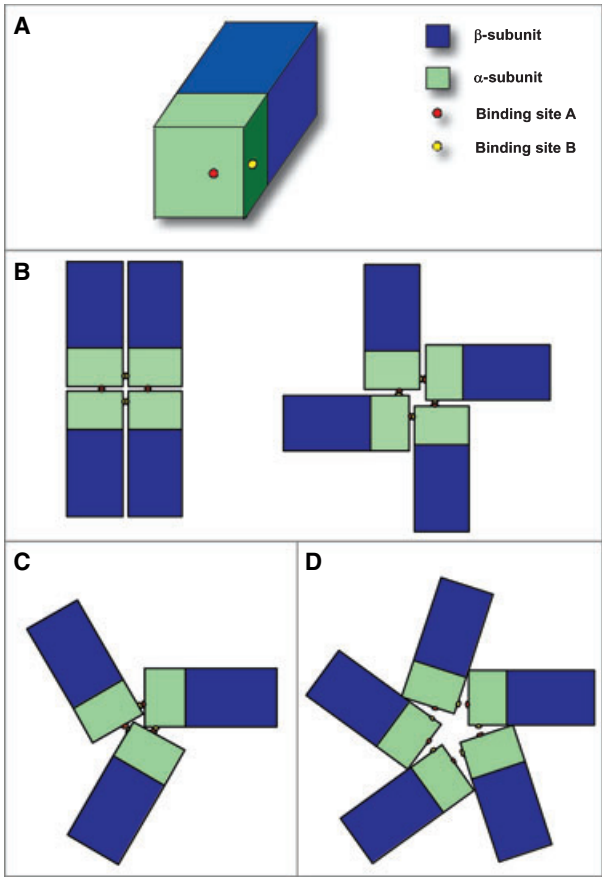

Fig. 8. A hypothetical model illustrating the steric hindrance involved in formation of a deer Hp tetramer. (A) A basic Hp subunit comprising one α- and one β-subunit. The –SH groups that connect the Hp subunits into polymers are assumed to be located with steric hindrance between the SH binding sites A and B. (B) The two different possible forms of tetramers. (C) A trimeric form of deer Hp is possible to assemble according to this model, but steric hindrance is seen which prevents the –SH groups from linking to some extent. (D) Formation of a pentamer or higher-order polymer is not possible.

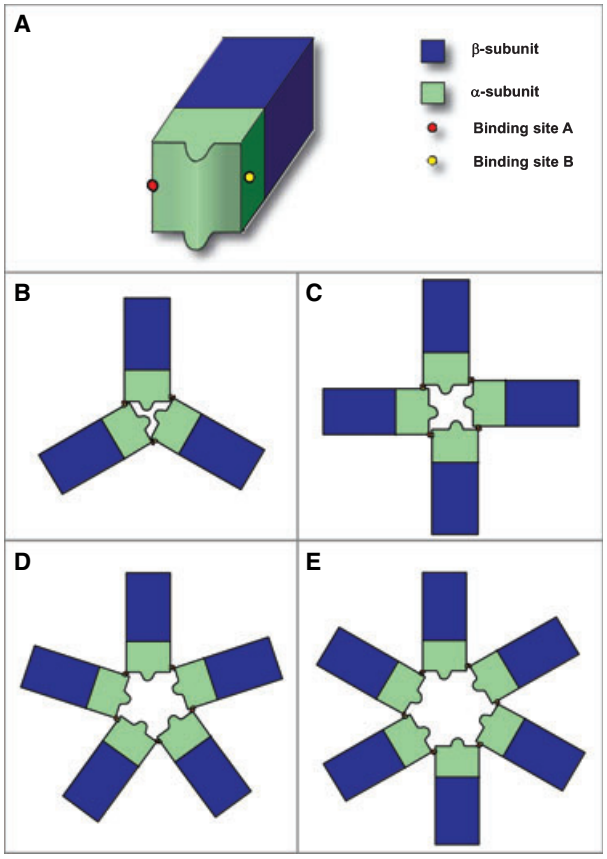

Fig. 9. Model of formation of human Hp 2-2 polymers. The positioning of the –SH groups involved in polymer formation differs from those in deer Hp. (A) A basic human Hp 2-2 subunit comprising one α- and one β-subunit. The –SH groups that connect the subunits into polymers are located at the edge of the surface. The hindrance between the –SH binding sites A and B prevents formation of a dimer. (B) A trimer is able to form to some extent with some steric hindrance. (C–E) Polymers of a higher order than tetramers can form without any steric hindrance.

close together in the cyclic center (Fig. 8C). Therefore, the formation of trimers takes place to a much lower extent than that of tetramers. No higher-order polymers are formed, because the distance between the –SH groups is too great to allow cross-linking for $(\alpha\text{-}\beta)_5$ pentamers or other larger polymers (Fig. 8D).

For a higher-order polymer ($n > 5$), the angle (θ) between the sides containing the –SH groups of two polymers would be $90–360/n$ degrees. If the distance between the –SH sites is approximately 90°, and the side of the Hp subunit contributes the base of the triangle, the distance is proportional to $\sin\theta$. As θ approaches 90° as n approaches infinity, the distance between the –SH sites also comes close to a maximum as n increases. In fact, few trimers are seen in our renaturing experiment (Fig. 7) and no polymers of an order of five or higher are observed.

For human Hp 2-2, on the other hand, the formation of higher-order polymers is possible (Fig. 9). The assumed positions of the –SH groups differ from those in deer Hp. They are located at the edges of the same plane, so formation of an identical 'stacking' dimer or $(\alpha\text{-}\beta)_2$ is not possible due to steric hindrance between the two –SH groups (Fig. 9A). However, formation of some trimers by linking together via the two –SH groups at the edge is possible, but not to a great extent due to the limited space in the cyclic center (Fig. 9B). This explains why there are only trace amount of trimers in all the human Hp 2-2 samples (Fig. 2). The cyclic center provides sufficient room to facilitate

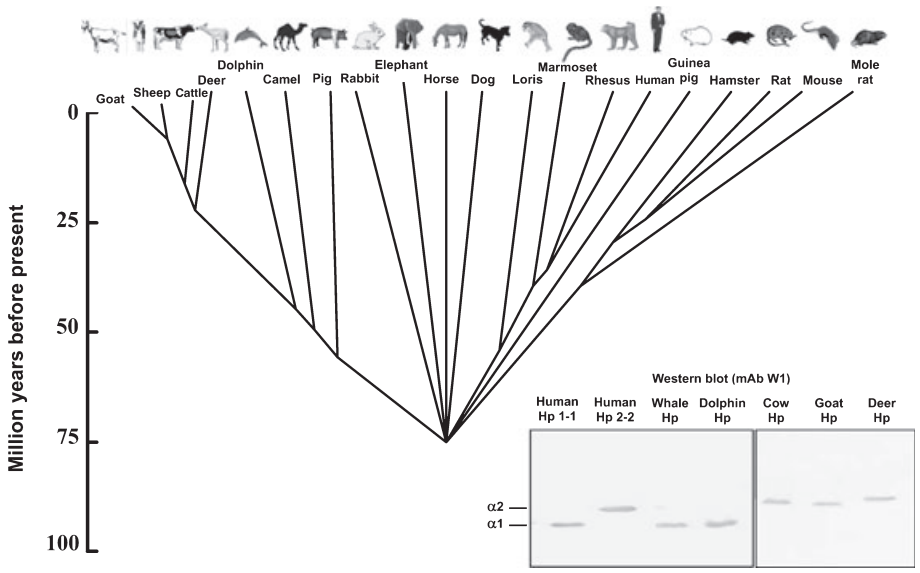

Fig. 10. Phylogenetic tree illustrating the molecular evolution of mammals, and phenotyping of human, whale, dolphin and ruminant α-chains. The tree is constructed by assuming that all eutherian orders radiated at about the same point in evolutionary time, approximately 75 million years ago. Alternative branching orders give essentially identical results. Within a eutherian order, branch points are assigned using evolutionary times based on fossil records [30]. Western blot analysis of Hp of six mammals (with a branching point before and after deer) was conducted using a 10–15% SDS–PAGE gradient gel under reducing conditions with an α-chain-specific mAb (W1) prepared against human Hp.

formation of polymers of an order greater than four α-β units. Such configuration also allows binding of the W1 mAb. In contrast, the cyclic center of deer Hp tetramers is totally blocked and is not accessible for mAb binding (Fig. 4B,C).

Evolution

In vertebrates, a recent study has suggested that the Hp gene appeared early in vertebrate evolution, between the emergence of urochordates and bony fish [5]. All mammalian species studied to date have been shown to possess Hp. Analysis of the electrophoretic patterns of Hp–hemoglobin complexes has suggested that most of these Hps are similar to human Hp 1-1 [28]. Only the protein found in ruminants (cattle, sheep and goat) resembled polymeric forms of human Hp 2-2 [20], but whether they also possess a tandem repeat remains unexplored [25].

It is thought that humans originally had a single Hp 1-1 phenotype [29]. Maeda *et al.* [15] proposed that the tandem repeat sequence of human α2 evolved two million years ago from a nonhomologous unequal crossover between two ***Hp 1*** alleles (***Hp 1S*** and ***Hp 1F***) during meiosis. A unique feature of the ***Hp 2*** allele is that it is present only in humans and is not found in any primates, including New and Old World monkeys, chimpanzees and gorillas [17]. We have recently found that cattle also possess ***Hp 2*** as the sole genotype [25]. It is likely that ruminants including deer, cattle, goat and sheep may all possess a sole ***Hp 2***-type allele. In the present study, we have shown that the inserted tandem repeat region in deer Hp appears to have extensively evolved, as 32% of the repeated region has undergone mutation, compared to that of only 4% (two amino acid residues) in human Hp (Fig. 6). Thus, we propose that the occurrence of the tandem repeat in deer was much earlier than in humans.

Figure 10 depicts a phylogenetic tree constructed by assuming that all eutherian orders (mammals) radiated at about the same point in evolutionary time (approximately 75 million years ago) [30]. The phylogenetic analysis indicates that crossing-over of deer α-chains occurred after divergence of the line leading to ruminants and pig, as pig possesses only the Hp 1-1 phenotype [24]. As dolphins and whales are the closest divergences before the ruminants, we further examined the size of the α-chain in whales and dolphins as well as other ruminants (cattle and goat) to determine the possible time of the tandem repeat evolution in deer Hp. Interestingly, the inserted panel of Fig. 10 shows that the α-chains of all the ruminants tested are the α2 type, except for dolphins ($n = 5$) and whales ($n = 5$).

These data suggests that the crossing-over resulting in the tandem repeat in ruminants occurred at least 25 million years ago or between 25 and 45 million years ago (Fig. 10), which is much earlier than the two million years proposed in humans [15]. The molecular evolution of the ruminants, which are the latest mammals in the phylogenetic tree (diverging after dolphins), is remarkably rapid, based on molecular evolution models for growth hormone and prolactin, when compared with other mammals [31,32]. This model appears to be consistent with the overall amino acid alterations (32%) within the tandem repeat of deer Hp α-chain. A similar alteration in cattle has also been reported recently [25].

Whether this alteration is adaptive during evolution remains to be addressed. For example, in cattle, there is an extensive family of at least eight prolactin-like genes that are expressed in the placenta [33,34]. These genes appear to be arranged as a cluster on the same chromosome. Phylogenetic analysis suggests that all of these genes are the consequence of one or more duplications of the prolactin gene; detailed analysis suggests that a rapid adaptive change has played a role in molecular evolution [35].

Evolutionary advantage of deer Hp protein being a tetramer

In addition to the superior binding affinity of Hp to hemoglobin, Hp is an anti-inflammatory molecule and a potent antioxidant [9]. In humans, the large complicated polymers of Hp 2-2 are a risk in the association of diabetic nephropathy [36,37]. One explanation is that the large polymer dramatically retards penetration of the molecule into the extracellular space [36]. We have shown in the present study that deer Hp 2-2 was not able to form complicated polymers, because the diversity in amino acid sequence between the tandem repeat of α-chain has produced steric hindrance (Fig. 8) that may be advantageous to deer.

In conclusion, we have shown that deer possess an ***Hp 2*** allele with a tandem repeat that could have occurred at least 25 or between 25 and 45 million years ago based on the phylogenetic analysis. The phenotypic and biochemical structure of their Hp is markedly homogeneous, with a tetrameric arrangement due to the orientation of the two available –SH groups, preventing the formation of the complicated Hp polymers found for human Hp 2-2. In terms of molecular evolution, this steric hindrance may have conferred an advantage on deer Hp that compensates for the undesired tandem repeat in the α-chain.

Experimental procedures

Animal plasma

Animal plasma of deer (*Cervus unicolor swinhoei*), goat (*Capra hircus*), cattle (*Bos taurus*), pig (*Sus scrofa domestica*), dolphin (*Steno bredanensis)* and whale (*Delphinapterus leucas*) were obtained from the Pingtung County Livestock Disease Control Center and the Veterinary Medicine Teaching Hospital, National Pingtung University of Science and Technology, Taiwan.

Phenotyping

Hp phenotyping was performed by native PAGE using hemoglobin-supplemented serum or plasma [22]. Briefly, 6 μL plasma were premixed with 3 μL of 40 $mg{\cdot}mL^{-1}$ hemoglobin for 15 min at room temperature. The reaction mixture was then equilibrated with 3 μL of a sample buffer containing 0.625 M Tris (pH 6.8), 25% glycerol and 0.05% bromophenol blue, followed by electrophoresis on a 7% native polyacrylamide gel (pH 8). Electrophoresis was performed at 20 mA for 2 h, after which time the Hp–hemoglobin complexes were visualized by shaking the gel in a freshly prepared peroxidase substrate (30 mL $NaCl/P_i$ containing 25 mg of 3,3′-diaminobenzidine in 0.5 mL dimethyl sulfoxide and 0.01% H_2O_2). The results were confirmed by western blot using an α-chain-specific mAb prior to phenotyping.

Preparation of mouse mAb and human Hp

Mouse mAb W1 specific to the human Hp α-chain was produced in our laboratory according to standard procedures [38]. Native human Hp was isolated from plasma using an immunoaffinity column followed by size-exclusion chromatography on an HPLC system using previously described procedures [22].

Purification of deer haptoglobin

Plasma samples enriched with Hp were prepared from deer blood containing 0.1% EDTA, followed by centrifugation at 1200 ***g*** for 15 min at 4 °C to remove the cells. Isolation was performed according to the method previously established for porcine Hp [24]. Saturated ammonium sulfate solution was added to the plasma to a final saturated concentration of 50%. After gentle stirring for 30 min at room temperature, the precipitate was discarded by centrifugation at 4000 ***g*** for 30 min at 4 °C. The supernatant was then dialyzed at 4 °C for 16 h against $NaCl/P_i$ containing 10 mM phosphate (pH 7.4) and 0.12 M NaCl with three changes. After dialysis, the sample was concentrated and filtered through a 0.45 μm nylon fibre prior to size-exclusion chromatography. An HPLC system (Waters, Milford, MA, USA), consisting of two pumps, an automatic sample

injector and a photodiode array detector, with a Superose-12 column (1 × 30 cm) (GE Healthcare, Uppsala, Sweden) pre-equilibrated with NaCl/P_i, was used for further purification. The column was run for 60–80 min at room temperature with a flow rate of 0.3 mL·min^{-1} using NaCl/P_i as the mobile phase. Fractions containing Hp were pooled and concentrated to a final volume of 1 mL using an Amicon centrifugal filter (Millipore, Billerica, MA, USA), and stored at −20 °C until use.

Gel electrophoresis

SDS–PAGE was performed according to the method described by Laemmli [39] with some modifications, using 5% polyacrylamide as the stacking gel [40]. In general, samples containing 143 mM β-ME were preheated at 100 °C for 10 min in a buffer containing 12 mM Tris–HCl (pH 6.8), 0.4% SDS, 5% glycerol and 0.02% bromophenol blue before loading to the gel. The samples were run on a step gradient of polyacrylamide gel (10 and 15%) for about 1.5 h at 100 V and stained using Coomassie brilliant blue. For determination of the molecular mass of Hp, the tested samples were prepared under the non-reducing conditions using the SDS gel. Alternatively, the SDS gel was prepared in a 0.04 M phosphate buffer (pH 7.0) containing 4% polyacrylamide, and the samples were run for about 6 h at 30V. The molecular mass standard for SDS–PAGE, containing three prestained proteins (260, 160 and 110 kDa), was purchased from Invitrogen (Carlsbad, CA, USA).

Immunoblot analysis

Western blot analysis was performed using a method similar to that described previously [40]. In brief, the electrotransferred and blocked nitrocellulose was incubated with anti-Hp mAb W1, followed by washes and incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (Chemicon, Temecula, CA). The membrane was developed using 3,3′-diaminobenzidine containing 0.01% H_2O_2. Dot blots were performed by applying the samples (reduced or non-reduced) onto a nitrocellulose membrane using anti-Hp mAb W1 as the primary antibody.

Cloning and sequencing analysis of deer Hp

The entire procedure was similar to that described previously [9,10]. Briefly, total RNA was extracted from deer whole blood using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The gene for deer Hp from total RNA was reverse-transcribed and PCR-amplified using proofreading DNA polymerase (Invitrogen), forward primer 5′-TTCCTGC AGTGGAAACCGGCAGTGAGGCCA-3′ and reverse primer 5′-CGGAAAACCATCGCTAACAACTAAGCTT GGG-3′. The PCR cycling profile was as follows: denaturation at 94 °C for 5 min, then 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 90 s, then final extension at 72 °C for 10 min. The PCR product was analyzed by electrophoresis through a 1% agarose gel, and purified using a gel extraction kit (BD Biosciences, Palo Alto, CA). The purified PCR product was cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA), and then the ligated plasmid was transformed into *Escherichia coli* JM109 (Qiagen). Finally, the sequence of deer Hp was confirmed by DNA sequencing.

Sequence alignment and phylogenetic analysis

The cDNA and amino acid sequence alignment, sequence pair distances and phylogenetic tree construction were performed using DNASTAR software (Lasergene, Madison, WI, USA).

Denaturation and renaturation of deer and human Hp 2-2

Purified deer Hp (0.1 mg·mL^{-1}) or human Hp 2-2 (2 mg·mL^{-1}) were mixed with NaCl/P_i containing 6 M urea and 143 mM β-ME and incubated at room temperature for 30 min. The reaction mixture was first dialyzed in 200 mL NaCl/P_i at 4 °C for 6 h, and this was repeated three times (total 24 h) to allow renaturation. The mixture was finally dialyzed against 2 L NaCl/P_i overnight. The concentrated Hp samples with or without reduction were incubated with hemoglobin for use on a typing gel as that for plasma phenotyping, and then stained by Coomassie brilliant blue.

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