Evolution of mammalian apolipoprotein A-I and conservation of antigenicity: correlation with primary and secondary structure

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Abstract We have evaluated the immunoreactivity of 20 monoclonal antibodies (mAbs) directed against human apolipoprotein (apo)A-I with a panel of high density lipoproteins (HDL) from 13 mammalian species. The pattern of cross-reactivity showed that 20 mAbs had different specificity. While not all mAbs recognized apoA-I from all of the different species, the antigenicity of some sequences was well conserved. Thus, mAb A05 cross-reacted with all species except guinea pig and rat. In contrast, the mAb 4H1, which recognized residues 2–8, required a specific proline in position 3, as no immunoreactivity was found in the species missing this amino acid. Furthermore, the presence of a threonine residue in place of serine (in position 6) in the cynomolgus monkey was associated with a 20-fold loss of immunoreactivity in radioimmunometric assay with 4H1. As most of the epitopes were found in CNBr fragments 2 and 3, we sequenced these regions in four species (horse, goat, sheep, and cat) and analyzed the alignment of most known sequences to evaluate their consensus. Except for the rat and the chicken, considerable identity was observed. This permitted us to deduce the involvement of the residues in some antigenic epitopes. In the middle of apoA-I, a conservative mutation $Asp_{103} \rightarrow Glu$ was found sufficient to eliminate all reactivity of this epitope for A11 (residues 99–105 . . . 126–132) in five species (rabbit, cow, goat, sheep, and rat). The residues essential to the expression of two other epitopes overlapping with A11 were also characterized. Edmundson-wheel representation of 18-residue repeated sequences of the different apoA-I species (for the eight amphipatic helices of residues 46–63, 68–85, 101–118, 123–140, 143–160, 167–184, 189–206, and 222–239) showed that secondary structure of apoA-I was more conserved than the antigenic epitopes. The N-terminal region, residues 1 to about 98, is rich in both strictly preserved sequences and epitope expression in most of the species surveyed. This evolutionary conservation of the N-terminal domain suggests an important yet unknown function.—**Collet, X., Y. L. Marcel, N. Tremblay, C. Lazure, R. W. Milne, B. Perret, and P. K. Weech.** Evolution of mammalian apolipoprotein A-I and conservation of antigenicity: correlation with primary and secondary structure. *J. Lipid Res.* 1997. **38:** 634–644.

Supplementary key words apoA-I • monoclonal antibody

Apolipoprotein A-I (apoA-I), the principal protein constituent of high density lipoprotein (HDL) (1), is believed to be important in plasma cholesterol transport (2 and see review 3), and lecithin:cholesterol acyltransferase (LCAT) activation (4). Human apoA-I consists of a single 243 residue polypeptide (1) and its primary structure has also been determined in many species such as cynomolgus monkey, rat, rabbit, chicken, dog, pig, cow, and recently in the mouse (5–7 and see review 8). A high degree of homology was observed between the sequences from these different species (6–10). Panels of monoclonal antibodies (mAbs) have been raised against human apoA-I (11, 12), and we have recently described the mapping of the antigenic sites for 29 mAbs on human apoA-I (13). The mapping of the epitopes on the primary sequence of apoA-I is important because mAbs have proven to be useful probes of apoA-I conformation at the surface of HDL (14, 15). Given the variety of mAbs available, we wanted to elucidate which mAbs cross-react with different animal species. These antibodies are very useful reagents to study apoA-I structure and, in some cases, the species sequence differences may help to map the residues that contribute to antigenic sites. Therefore, we present here data on *1*) the cross-reactivity of 20 mAbs with apoA-I from 13 mammalian species, *2*) a list of conserved antigenic epitopes, *3*) new partial amino-acid sequences of apoA-I from some species studied and their alignment with the known apoA-I, *4*) the sequence con-

Abbreviations: apoA-I, apolipoprotein A-I; HDL, high density lipoprotein mAb, monoclonal antibody.

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sensus that emerged, and 5) a comparison of the α-helices in an Edmunson wheel representation.

MATERIALS AND METHODS

Isolation of lipoproteins

Blood from different species was obtained at the Ecole Nationale Vétérinaire (Toulouse, France) and collected on disodium EDTA (1 mg/ml). Plasma was separated by low speed centrifugation at 1000 *g* for 20 min at 4°C. Sodium azide (0.01%) and phenylmethylsulfonyl-fluoride (1 mM) were immediately added and the samples were stored at −80°C under nitrogen. The lipoproteins were prestained with Sudan Black and separated by discontinuous gradient ultracentrifugation (16, 17).

Monoclonal antibodies (mAbs) against apoA-I and competitive radioimmunoassays

All the mAbs used in the present study have been described (13) and were obtained from mice immunized with human apoA-I or HDL (11, 12). Solid phase radioimmunoassay of apoA-I without Tween 20 was carried out as described earlier (15).

Dot-blot

The interaction between monoclonal antibodies and the apoA-I from different species was studied by spotting onto nitrocellulose 2 μg of HDL protein in 2 μl. The nitrocellulose paper was saturated with 3% polyvinylpyrrolidone (44,000 mol wt; BDH Inc., Montreal, Quebec) in Tris-buffered saline (1 h, 37°C) and further processed as described previously (13). Autoradiography was performed on XAR-5 film (Eastman Kodak Co., Rochester, NY) with an intensifier screen (Cronex; DuPont Instruments, Wilmington, DE). Images were digitized and volume integration of immunoreactive dots was made using a Molecular Dynamics Computing Densitometer, with correction for the background adjacent to each blot.

Purification and CNBr treatment of apolipoprotein A-I

ApoHDL (20 μg) from different species were electrophoresed in a 15% acrylamide gel (18). The proteins were transferred onto nitrocellulose and apoA-I was identified by immunoreaction. To determine internal amino acid sequences, apoA-I was treated with cyanogen bromide (CNBr) as described previously (11). ApoA-I was eluted from the membranes with 500 μl of formic acid (98%), diluted with water to 70%, and 2.5 mg of CNBr (25 mg/ml) was added. The mixture was purged with nitrogen for 2 min and incubated overnight in the dark at room temperature. The polypeptide fragments were separated as described before and electrotransferred to PVDF membranes (Immobilon) (11).

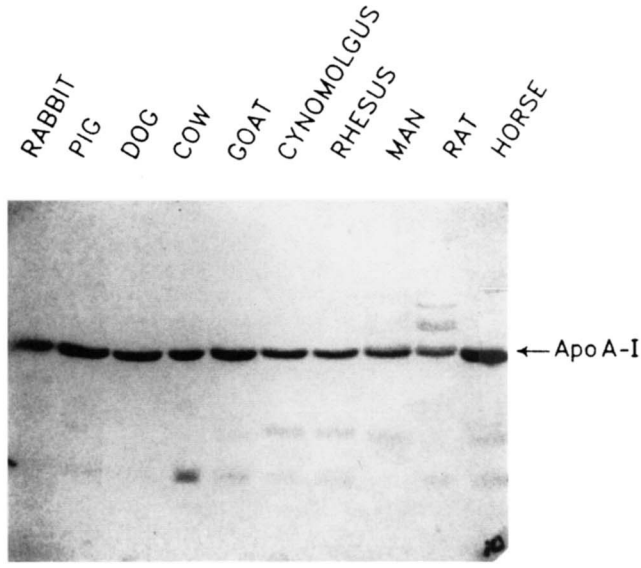


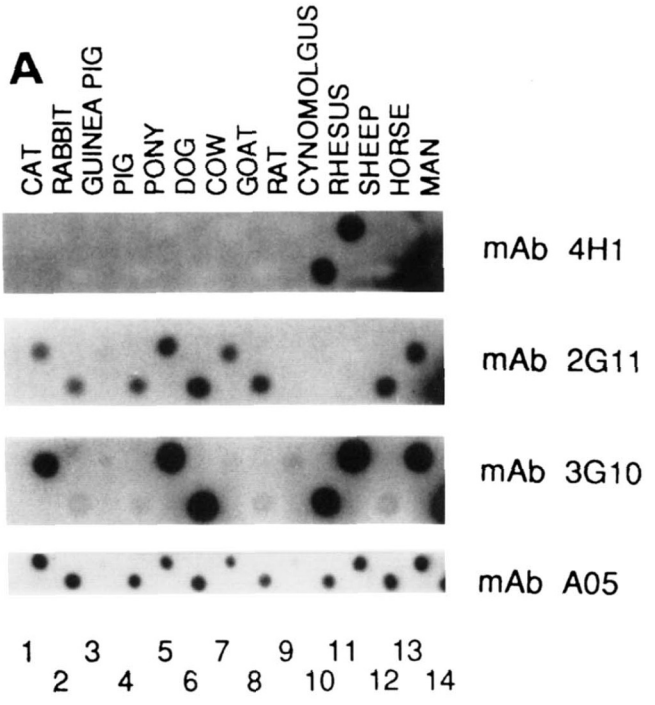
Fig. 1. SDS-PAGE of animal HDL protein (10 μg) electrotransfered onto nitrocellulose and stained with Ponceau red.

Analytical techniques

Complete protein sequences of human (APA1_HUMAN), cynomolgus monkey (APA1_MACFA), hamadryas baboon (APA1_PAPHA), rat (APA1_RAT), rabbit (APA1_RABIT), chicken (APA1_CHICK), dog (APA1_CANFA), bovine (APA1_BOVIN), pig (APA1_PIG), and mouse (APA1_MOUSE) apoA-I were from the SWISS protein sequence Data Bank (version 25). New amino acid sequences presented here were determined using an Applied Biosystems 470A sequencer with on-line PTH amino acid identification. Proteins were measured according to the method of Lowry et al. (19).

RESULTS

The lipoproteins from 13 animal species have been separated by discontinuous gradient centrifugation and the HDL were isolated. After SDS-PAGE separation, animal HDL proteins were electrotransferred onto nitrocellulose and stained with Ponceau Red. A major band of molecular weight of about 28,000 Da was visible in each sample (**Fig. 1**). Twenty three mAbs were tested for their reactivity with various apoA-I and HDL by dot-blot and Western blot. All the mAbs reacted with hu-



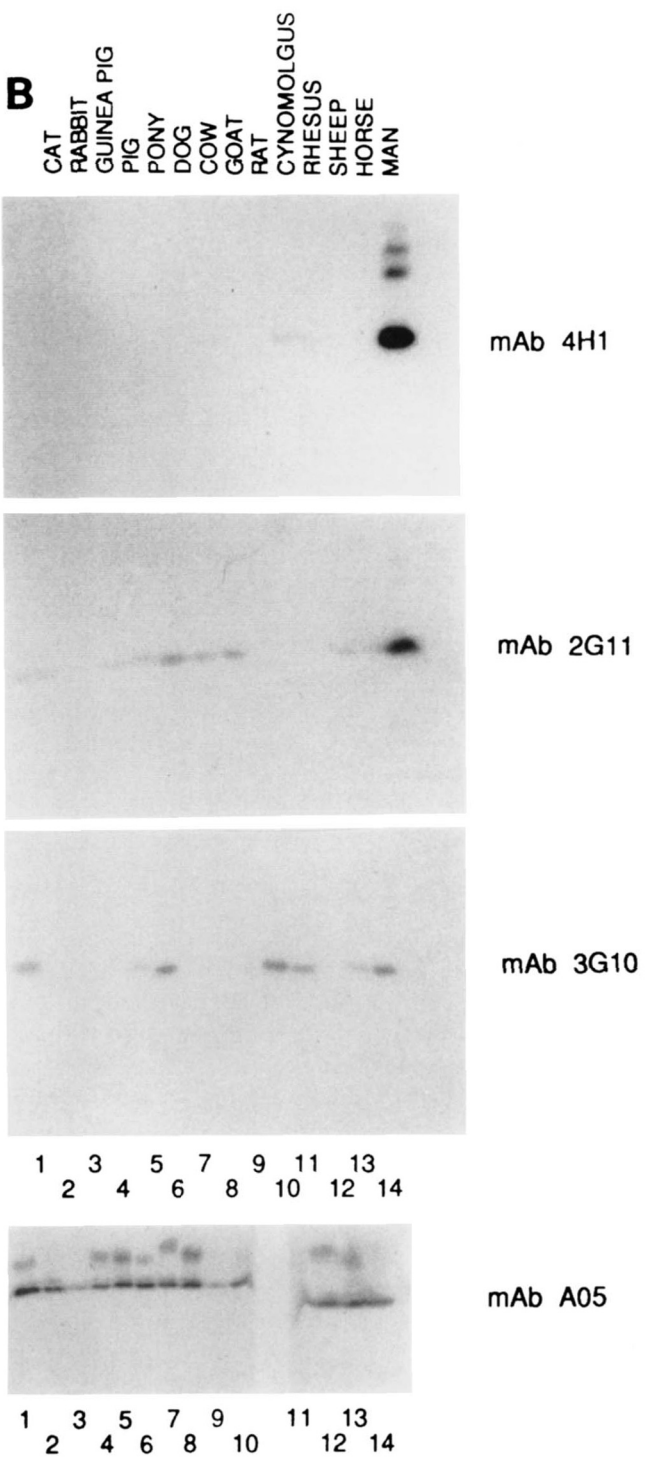


Fig. 2. A: Immunoreaction of four representative mAbs (4H1, 2G11, 3G10, and A05) with dot-blots of HDL (2 μg protein from various species) on nitrocellulose. B: Western blotting after SDS-PAGE of HDL (20 μg) with the same mAbs.

man apoA-I. The mAbs generally showed less reaction with HDL from the animal species than with human HDL in dot blots (**Fig. 2A**), and the same ranking order of reactivity was seen for animal apoA-I on Western blots after SDS-PAGE (Fig. 2B). While some species gave a strong reaction similar to human HDL, other species gave either weaker reactions or no detectable reaction over the background. The ranking order of reactivity of animal HDL was characteristically different for most of the mAbs. Out of the 20 different antibodies and the 13 different species, we found complete different patterns of reactivity (**Fig. 3** and **Table 1**). Thus a panel of different mammalian HDL allows rapid screening to identify differences among antibodies with unique specificities. Antibodies 4H1, 6B8, A10, and 4A12 reacted only with human, rhesus, and cynomolgus apoA-I. The mAb 4H1 recognizes a single linear epitope in the amino terminal region of human apoA-I spanning residues 2–8 (13) (Fig. 3 and Table 1). The antibody 4H1 requires a specific amino acid (Pro) in position 3 or 4, that is absent in the majority of species other than human (through deletion or mutation). Serine in position 6 appears important, as a substitution ($Ser_6 \rightarrow Thr$) led to a low immunoreactivity with apoA-I from the cynomolgus and rhesus monkeys. This was verified by competition radioimmunoassay, in which apoA-I from cynomolgus and rhesus HDL was about 20-fold less immunoreactive with 4H1 than with that of human

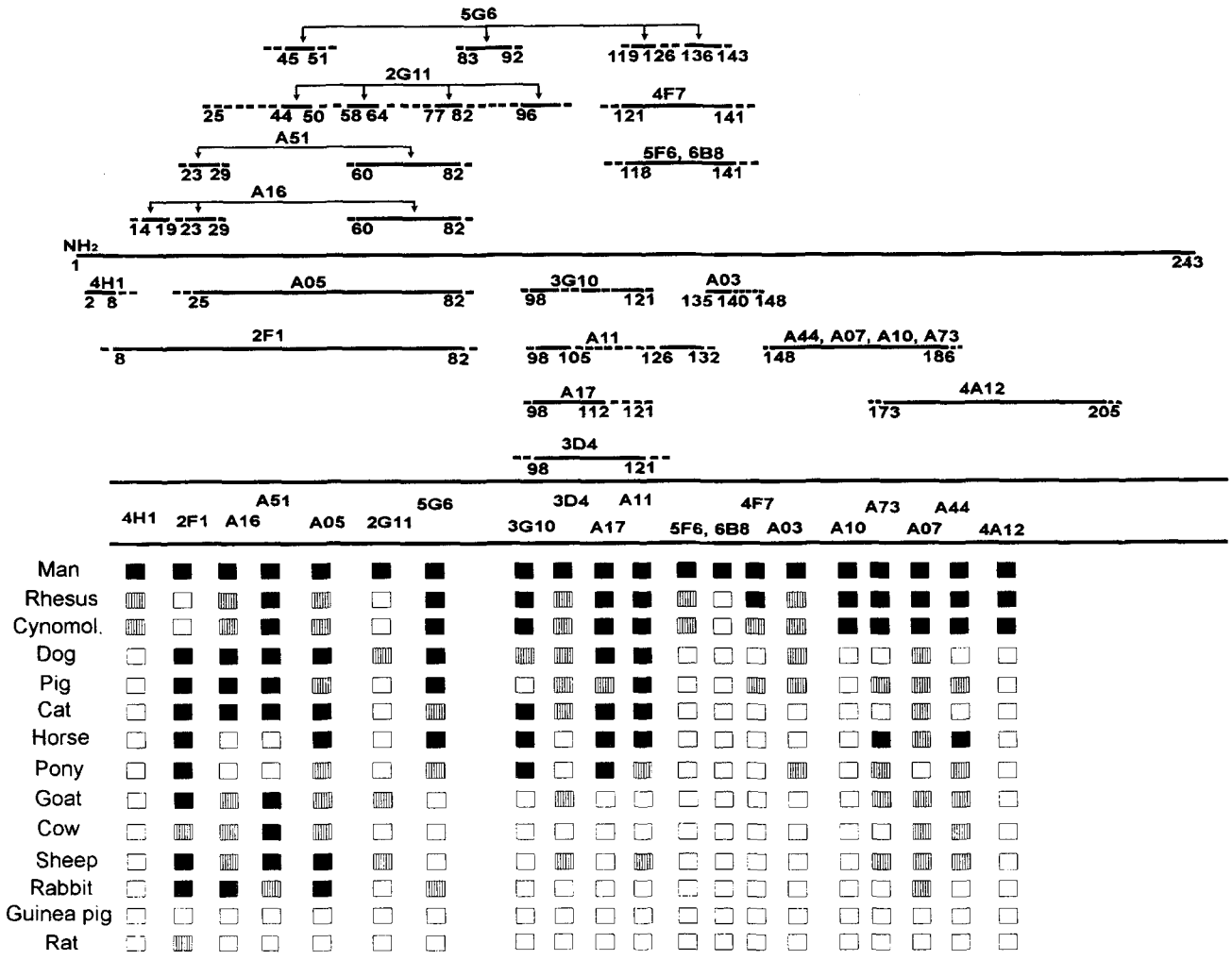


Fig. 3. Epitope map of apolipoprotein A-I and comparison of the immunoreactivity of 20 monoclonal antibodies with the apoA-I of different animal species. The data are consistent with both dot-blots of HDL and SDS-PAGE Western blots of HDL. Filled squares, immunoreaction with intensity comparable to the human sample (range 50–100% in Table 1); hatched squares, immunoreactivity with intensity less than the human sample (range 10–49% in Table 1); and open squares, no significant immunoreaction (0–9% in Table 1).

TABLE 1. Immunoreactivity of apoA-I in different species relative to that in humans

	4H1	2F1	A16	A51	AO5	2G11	5G6	3G10	3D4	A17	A11	5F6	6B8	4F7	AO3	A10	A73	A07	A44	4A12
Human	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Rhesus	43	0	44	89	45	0	52	76	44	64	69	24	0	61	26	63	83	90	95	87
Cynomol.	35	0	47	92	41	0	58	61	40	77	74	26	0	46	36	78	72	78	81	70
Dog	0	105	59	60	60	14	63	49	24	99	71	10	0	2	22	3	2	27	0	0
Pig	0	87	52	88	44	8	50	0	38	11	53	4	0	11	15	1	26	23	21	0
Cat	0	78	77	94	68	6	34	75	30	81	78	0	0	1	4	0	4	36	0	0
Horse	0	72	0	0	50	8	71	68	9	68	71	1	0	5	0	0	50	14	61	0
Pony	0	81	0	0	45	9	42	68	6	63	43	0	0	4	12	0	47	8	45	0
Goat	0	93	36	58	38	14	1	0	12	7	7	0	0	5	5	2	22	29	29	0
Cow	0	43	21	55	29	9	0	0	5	6	5	0	0	4	7	2	0	21	16	0
Sheep	0	72	23	148	51	15	0	0	14	0	16	0	0	4	0	0	23	36	30	0
Rabbit	0	99	54	37	55	10	42	0	5	3	2	0	0	9	8	0	5	22	0	0
Guinea pig	0	0	0	0	1	0	0	0	0	7	4	0	0	0	3	0	3	0	0	0
Rat	0	49	0	0	1	0	0	4	9	5	8	0	0	1	0	1	0	0	0	0

Comparison of the immunoreactivity of 20 monoclonal antibodies with the apoA-I of different animal species. The data are consistent with both dot blots of HDL and SDS-PAGE Western blots of HDL. Images were digitized and surface integration of the immunoreactive dots was made using a Molecular Dynamics Computing Densitometer, with correction for the background adjacent to each blot.

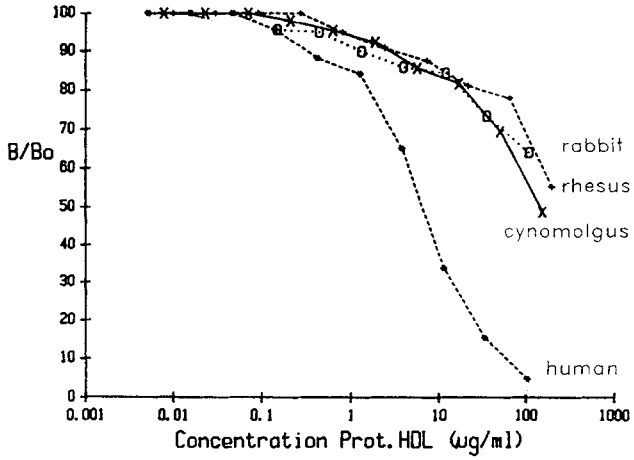


Fig. 4. Comparison of the immunoreactivity of human, cynomolgus, rhesus monkey, and rabbit HDL with monoclonal antibody 4H1 by competitive radioimmunoassay.

(**Fig. 4**). In the middle of the apoA-I sequence, a substitution $Asp_{103} \rightarrow Glu$ was found sufficient to eliminate any reactivity of this epitope for mAb A11 (residues 99–105 . . . 126–132) in six species (rabbit, cow, goat, sheep, rat, and guinea pig), whereas all species with Asp_{103} have highly reactive HDL. Antibody A17 which reacts with an epitope overlapping with A11 but which is more defined to the sequence 98–112, displays the same specificity, further supporting the role of Asp_{102}. Asp_{103} appears important also for antibody 3G10 which recognizes fragments 98–103 and 110–119 and is immunoreactive with the different species recognized by mAb A11 with the exception of the pig (Fig. 3). In addition to Asp_{103}, two other amino acids, Ala_{95} and Lys_{106}, could be involved in the immunoreactivity of mAb A11. Immunoreactive species with 3G10 express alanine or glutamine at position 95 and pig does not. However, pig also expresses Asn_{106} instead of lysine (**Fig. 5**).

In order to obtain some sequences of apoA-I fragments from goat, sheep, cat, and horse, which are not yet available, apoA-I were treated by CNBr. Different fragments were separated by electrophoresis. As previously described, apoA-I CNBr fragmentation was incomplete (not shown). Two gels were run in parallel, one was used for silver staining and the other was blotted with mAb A05. This antibody was shown to react with all species except for the rat and the guinea pig (see Table 1), and it is specific for human apoA-I CNBr fragment 2 (12). Amino acid sequences were determined after electrotransfer of the apoA-I CNBr fragments onto membranes. The apoA-I sequences from some species are presented in Fig. 5. We obtained for the goat and sheep, sequences at the NH_2 terminus (residues 1–13 and 1–21, respectively) and also for the goat, sheep, and cat, sequences starting from position 86 (86–115, 86–116, and 86–111, respectively), and corresponding to CNBr fragment 2. Concerning horse apoA-I, one fragment was sequenced between residues 148 and 178, in CNBr fragment 2.

ApoA-I contains about the same number of basic residues, 28, and of acidic ones, 20. The NH_2 terminus of apoA-I contains many more basic residues (10 out of the first 51 amino acids) than acidic ones (3 residues). The protein contains 54 conserved hydrophobic residues (22%). Although the hydrophobic amino acids are distributed over the entire protein, there is, however, a greater concentration at the NH_2 terminus (18 of the first 60 residues). It is noteworthy that there are 3 conservative and consecutive hydrophobic amino acids VYV at positions 17–19. The two sequences most conserved in all species (18 and 13 amino acids) and where only homologous substitutions occur, are between residues 22–39 and 47–59. The sequences for which a strict conservation has been noted in eight different species are mostly found in the N-terminal half and include residues 17–19 (as noted above), residues 23–26, residues 32–34, residues (NLEKET) at positions 74–79, and residues 88–90. There are also a number of short amino acid sequences that are well conserved (64–68, 86–90, 96–99, 101–105, 115–119, 191–196, 203–209, and 216–224). This preponderance of well-conserved sequences in the N-terminal region is compatible with the well-conserved immunoreactivity of N-terminal epitopes in the different species. Indeed, out of seven epitopes characterized toward the N-terminus, only 4H1 and 2G11 react with only 3 and 4 species, respectively, while the others react with 9 or more species. Particularly noteworthy are antibodies 2F1 and A05 whose epitopes overlap between residues 8–82 and 25–82, and which react with 11 and 12 species out of the 14 tested. As indicated above, it is within this sequence that are found strictly conserved sequences (residues 17–19, 23–26, 32–34, and 74–79) and the sequence 46–51 which is also strictly conserved in all species but the mouse, where a conservative substitution, Asp48 to Glu, occurs.

We attempted to compare the conservation of different helical segments of apoA-I among various species as designed by Brasseur (20) using the Edmunson-wheel diagram (**Fig. 6**). The loop formed between helices 1 and 2 has the sequence-XGPXT- where X represents a hydrophobic amino acid (residue 64–68) and contains no charged amino acids (Fig. 5). Another sequence-UULXPXL- (where U represents an uncharged amino acid, H, Q, S, or G) in the loop between helices 7 and 8 (residues 216–222) displays no ionized residue. A very interesting conservation is observed in the helix 167–184 where there is a very well-conserved global charge upon two turns of the wheel (Fig. 6). In

position 172 there is a glutamic acid (red color) in dog, rat, and mice, and a glutamine in human, cynomolgus monkey, rabbit, cow, chicken, and pig, whereas in position 179 there is a glutamic acid in human, cynomolgus, rabbit, cow, chicken, and pig and a glutamine (dog), a threonine (rat) and an alanine (mice) (uncolored). However, the comparison of sequences using the Edmunson-wheel diagram did not help to further identify the epitopes and their conservation, possibly owing to the earlier report that most epitopes in this region of apoA-I appeared centered around the turns (13).

DISCUSSION

Epitopes may depend on protein conformation as well as amino acid sequence and for that reason, comparison of primary structure does not always allow us to uniquely assign each epitope. Furthermore, most characterized protein epitopes are composed of discontinuous regions of polypeptides (21–23), and this has been observed for many apoA-I epitopes, especially in the N-terminal half (13). Although the antibodies analyzed here did not include mAbs specifically selected to react with linear epitopes as described recently by Curtiss and Banka (24), we have identified, by comparison of sequences and epitope expression in different species, specific residues that are critical to the expression of several epitopes.

In the present study, we have compared apoA-I from different animal species that differ from human apoA-I only by a few amino acids. These differences have been used to map the residues that contribute to the immunogenicity of apoA-I. Pro_3 has been shown to be essential for the expression of the 4H1 epitope. It is noteworthy that this region of the sequence is hypervariable, as natural mutants have been described with proline deletion at position 3 or 4 of human apoA-I (9). This antibody may be useful to detect these natural mutations in human apoA-I. Several mouse mAbs react with overlapping epitopes spanning the residues 80–140 (Fig. 3). This region represents the main immunogenic region of apoA-I as suggested by Curtiss and Banka (24) using the Chou and Fasman algorithms (25) for prediction of antigenicity. We had also shown earlier that several epitopes are centered around the α-helix predicted between residues 99–121 (13), a region that we proposed to be a hinge domain (26). Three epitopes overlapping this sequence have been further delineated: Asp_{103} is essential to the expression of the epitopes for both A11 and A17, whereas the epitope for 3G10 requires not only Asp_{103} but also Ala_{95} and/or Asn_{106}. The antigenicity of this domain can be explained by its mobility and accessibility to proteolysis (27). Downstream of this sequence. Ehnholm and colleagues (28) identified a mAb that reacts with normal apoA-I but not with a genetic variant of apoA-I ($Glu_{136} \rightarrow Lys$). This point mutation induced a lack of immunoreactivity of the mAb with the epitope located between residues 113 and 148. Indeed residue 136 is close to a proline at position 139 which is thought to interrupt the amphipathic helix. This is in keeping with our earlier report (13) that many of the helix breaking regions of apoA-I constitute antigenic domains. Downstream of the putative hinge domain between residues 121 and 180, there is little in the way of consensus sequences or even consensus residues although most of the substitutions are conservative (Fig. 5). There is also little conservation of epitopes between species and no other epitope could be further delineated (Fig. 3).

We have observed that, in contrast in the N-terminal half of apoA-I, a number of epitopes are expressed consistently in most of the species studied (Fig. 3) and thus appear conserved through evolution. The strict conservation of a number of sequences distributed in this region probably explains the epitope conservation in the N-terminal region. With the exception of antibody 4H1, most epitopes at the extreme N-terminus have been shown to be discontinous (13). The presence of the discontinuous epitopes is also compatible with the observations and model of Nolte and Atkinson (29) who noted that the N-terminus domain (residues 1–57) contains the most ambiguously defined secondary structures. The model of the N-terminal region presented by these authors very interestingly suggests a complex tertiary structure based on amphipathic β-sheet where some of the strictly conserved sequences (residues 17–19 and 42–44) are found that are separated by random coil sequences also strictly conserved (residues 23–26 and 46–51).

The natural selection that favors the conservation of functionally important proteins is a widely accepted idea in evolutionary theory. The antigenicity index of an important and invariant functional domain in protein is very low whereas the regions outside the functional domain vary and can be highly antigenic. As well, primary structures, and thus antigenic epitopes, that may have important metabolic functions are conserved in a variety of species. In keeping with this principle, Nelson et al. (30) noted that epitopes spatially located near the recognition site of apoB have a greater tendency to be conserved in a variety of animal species, a result confirmed by Young et al. (31). The carboxyterminal region with its two α-helices at residues 189–206 and 222–239 is a highly conserved part of the apoA-I molecule (Figs. 5 and 6), and only two antibodies have been reported to bind to this region (32). This low anti-

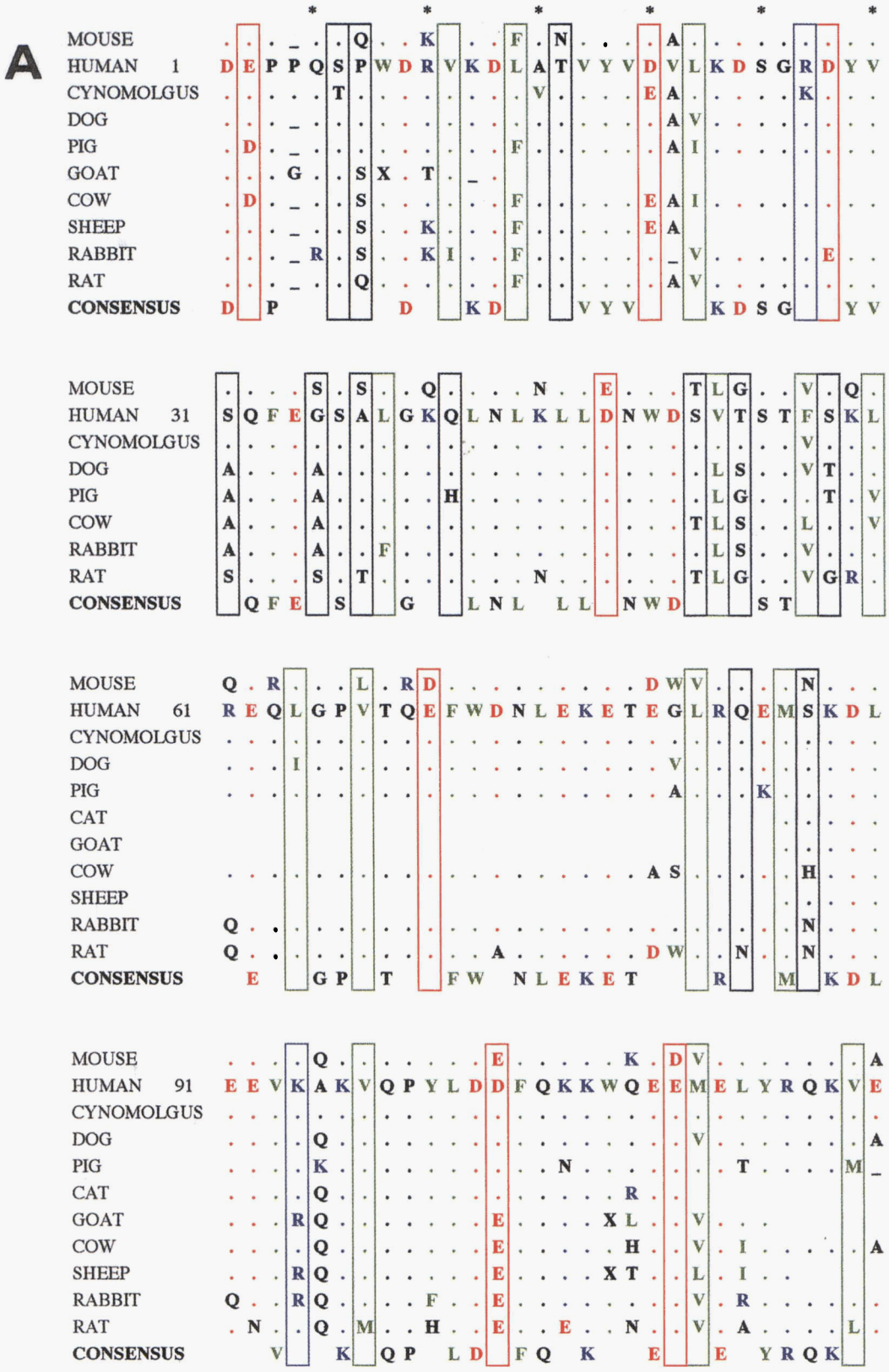


Fig. 5.

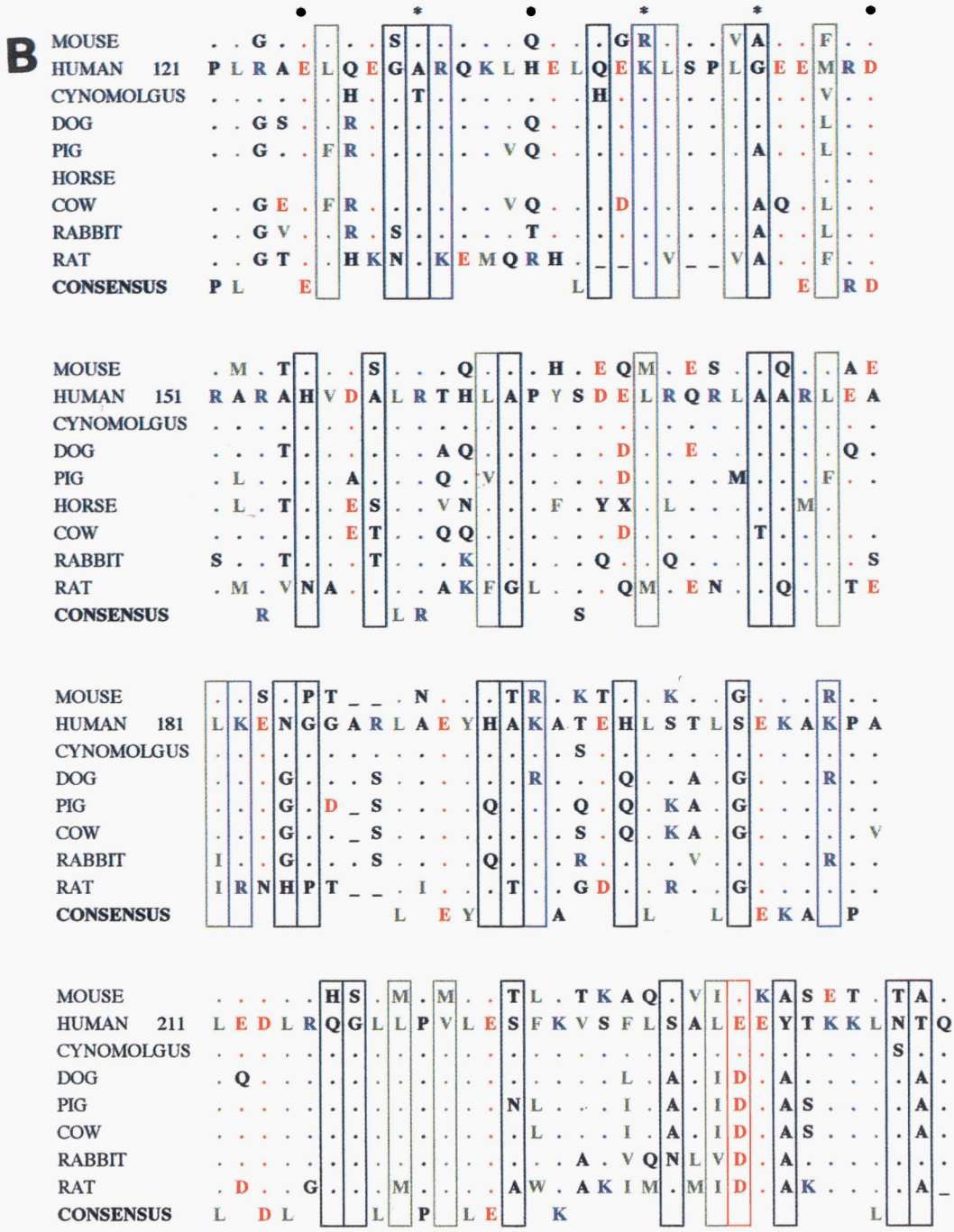


Fig. 5. Comparison of apoA-I amino acid sequences. Colors indicate aspartic acid or glutamic acid (red, D and E); arginine or lysine (blue, R and K); and hydrophobic residues methionine, valine, leucine, isoleucine, phenylalanine, tyrosine, or tryptophan (green, M, V, L, I, F, Y, and W). The remaining neutral amino acids at physiological pH, glycine, alanine, serine, threonine, asparagine, glutamine, histidine, and cysteine (G, A, S, T, N, Q, H and C) are uncolored. The column containing amino acids of the same color that possess the same character are boxed.

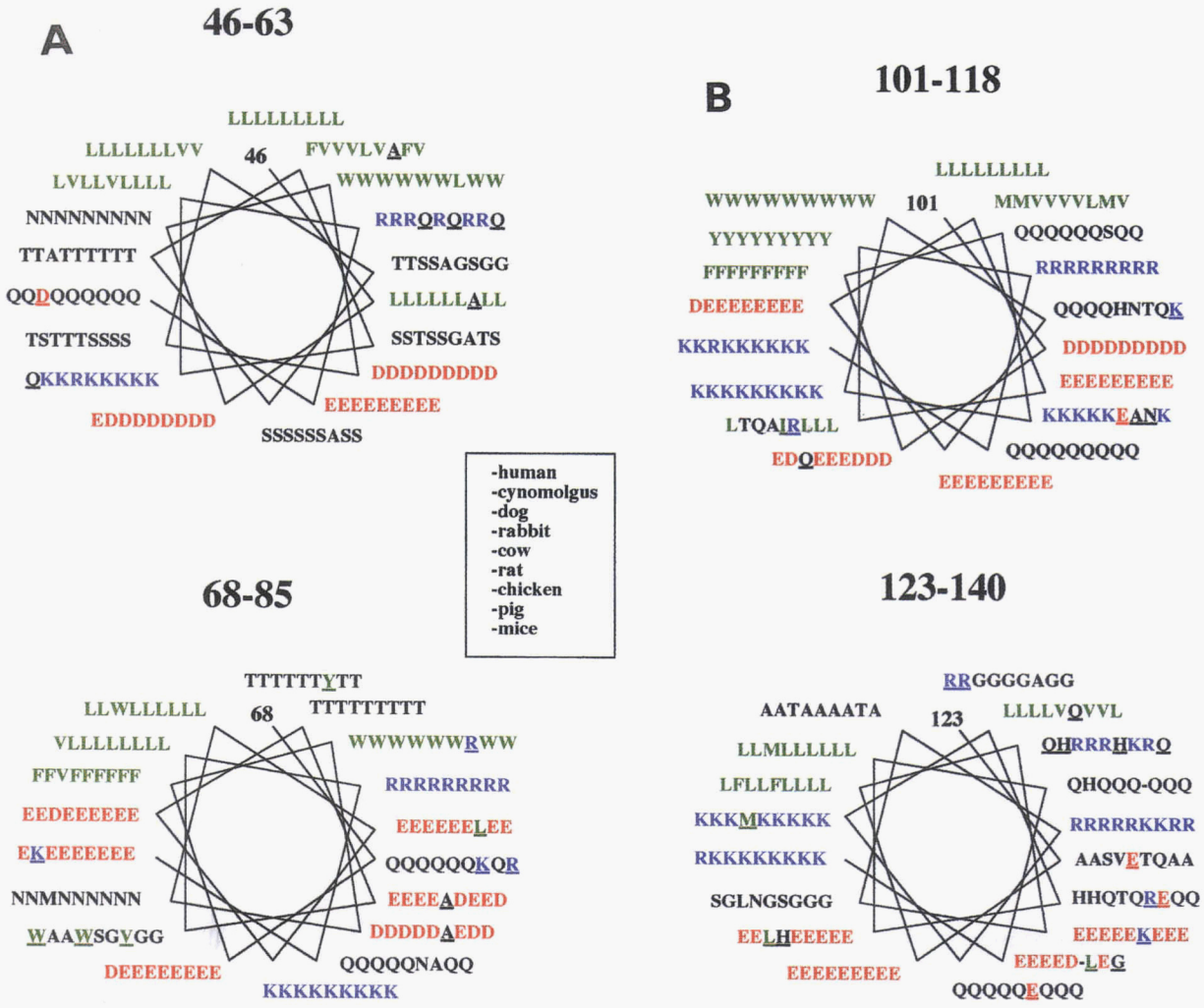


Fig. 6. Edmundson-wheel diagram of 18-residue repeated sequences of the different apoA-I species from human, cynomolgus, dog, rabbit, cow, rat, chicken, pig, and mice. Amino acids at each position are listed from inside to the exterior of the wheel, in the order in which these proteins are listed. Eight amphipathic α-helices are represented 46–63, 68–85, 101–118, 123–140, 143–160, 167–184, 189–206, and 222–239. The colors correspond to these of Fig. 5. The amino acids that possess a different character are underlined.

genicity may be related to the evolutionary preservation of this segment. Alternatively, the carboxyl terminal domain that is the main lipid binding domain (33) may also have undergone many silent mutations which, while preserving its functionality, i.e., the amphipathic characteristics of α- helices, did not preserve the immunoreactivity. In contrast, it is clear from the summary of cross-species expression of epitopes in Figs. 3 and 5, that there is a highly preserved set of overlapping epitopes in the N-terminal domain, essentially between residues 25 and 82. This may reflect the conservation of a domain with a complex tertiary structure which must serve an important but yet unknown function.

Analysis of evolutionary relationships among different apoA-I sequences shows that apoA-I is a highly conserved protein (7–9). The major ambiguity in any sequence alignment concerns the insertions that are required to improve the overall alignment. The insertions and/or deletions generally occur in the more variable loop regions of the structure. Estimates of substitution rates show that apoA-I evolved about 25% faster than an average gene in mammalian lineage as already calculated by Januzzi et al. (6). However, all portions of the coding regions evolve at roughly similar rates, suggesting that global conformation is conserved. This is corroborated by a conservation of the substituted amino acids of similar character (Fig. 5).

In conclusion, we have identified in this report the

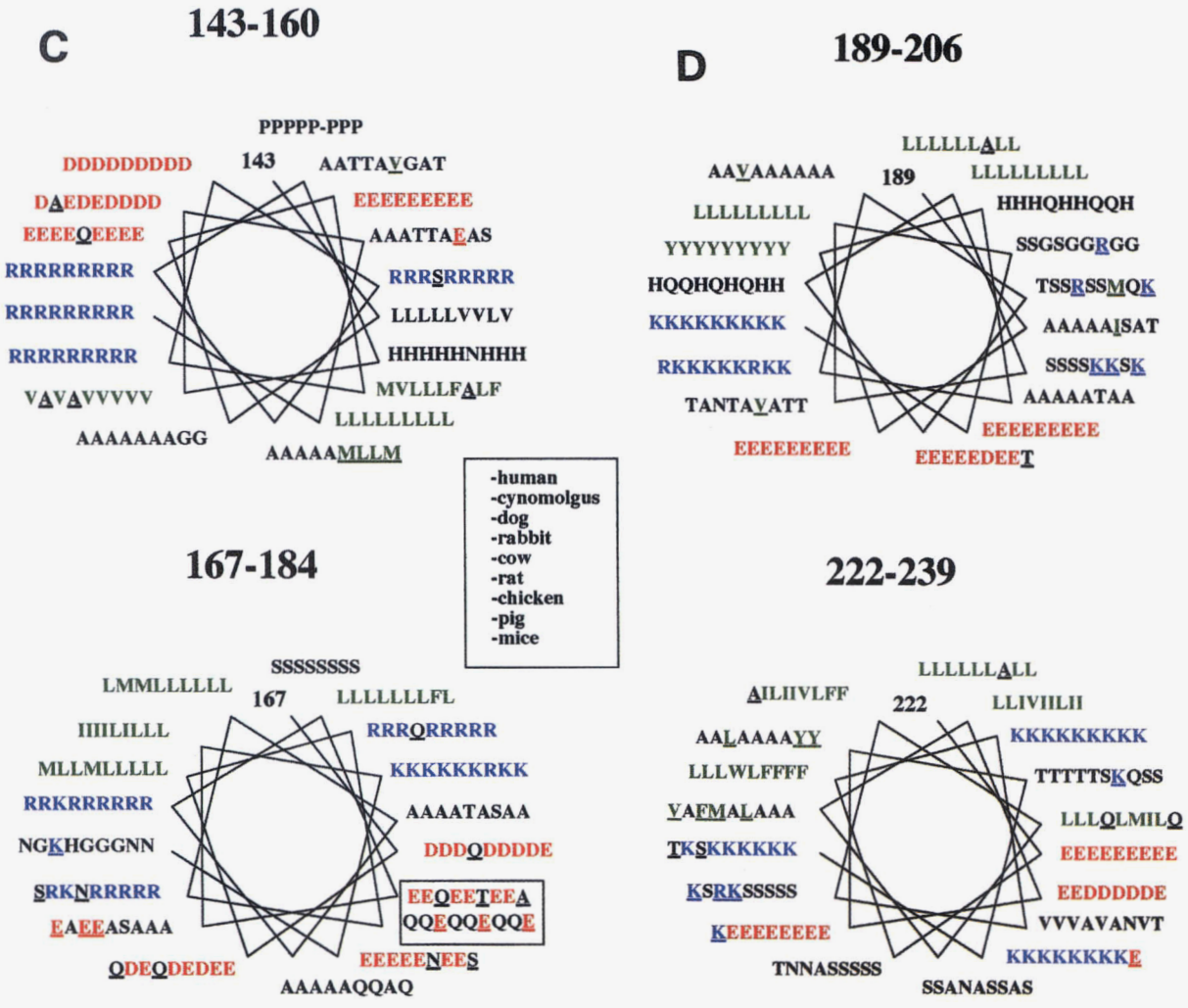


Fig. 6.

mAbs that cross-react with apoA-I of different animal species and defined further some of their epitopes. This enables us to select the mAbs that are appropriate for studies of apoA-I in animal species, to define more precisely some antigenic sites, and, finally, to identify conserved structures in apoA-I. This approach may help us to understand the evolution of apoA-I and other exchangeable apolipoproteins.

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