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Hominoid Evolution as Judged by Fibrinopeptide Structures*

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Summary. The fibrinopeptides A and B from gorilla, organgutan and siamang have been characterized, thereby completing a study of all six extant hominoids. The gorilla peptides were identical with the corresponding fibrinopeptides previously reported for human and chimpanzee. The orangutan peptide A was also identical with the human-chimpanzee-gorilla type A, but its fibrinopeptide B had two amino acid differences. The siamang A peptide differed from the others in one of its sixteen residues, but its peptide B was identical with the orangutan B. A cladogram based on the fibrinopeptide sequences of all six hominoids indicates that five amino acid replacements and one deletion can account for the evolution of present day sequences. It was also possible to deduce the amino acid sequence of the fibrinopeptides of the common ancestor of Old World monkeys and hominoids.

Key-Words: Hominoids — Fibrinopeptides.

The molecular relationship of man and his closest living relatives has been a topic of continuing interest and controversy ever since the publication of Darwin's classic work on the subject (1871) in which he considered not only anatomical and embryological evidence for relating man and the apes, but also developed pathological, cellular, and chemical arguments, concluding:

"...the correspondence in general structure, in the minute structure of the tissues, in chemical composition and in constitution, between man and the higher animals, especially the anthropomorphous apes, is extremely close."

The essential correctness of Darwin's molecular surmise was borne out by the heraldic work of Nuttall (1904) on the immunochemistry of serum

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Abbreviations Used. PITC, phenyl*iso*thiocyanate; DNS, dimethylaminonaphthalene sulfonyl-; PCA, pyrrolidone carboxylic acid; ASP, aspartic acid; ASN, asparagine; THR, threonine; SER, serine; GLU, glutamic acid; GLN, glutamine; GLY, glycine; ALA, alanine; VAL, valine, ILE isoleucine; LEU, leucine; PHE, phenylalanine.

proteins, a study which clearly showed that on a molecular level man's closest relatives were chimpanzees. In spite of these auspicious beginnings, however, twentieth century primate classification schemes, whether based on human vanity or mysticism, have continued to award man a separate grouping distinct from the Great Apes (Simpson, 1945; Fiedler, 1956; Walker, 1968).

A resurgence of interest in the molecular aspects of primate classification was sparked by the immunologic studies of Goodman (1961; 1962; 1963), on the basis of which he proposed a cladistic relationship which insistently placed man in the same family as the gorilla and chimpanzee, this family in turn belonging to a superfamily which included orangutans and gibbons (Goodman, 1963). Shortly thereafter a plethora of immunochemical data was reported (Hafleigh and Williams, 1966; Sarich and Wilson, 1966; Sarich and Wilson, 1967) which added to and were completely consistent with the taxonomic grouping suggested by Goodman. These observations were reinforced, if indeed they needed be, by detailed chromosome comparisons (Klinger *et al.*, 1963) and preliminary amino acid sequence data from hemoglobins and cytochromes c (for original references, see Dayhoff, 1969). In all areas the data were clear; the molecular relationship between man and the African apes is closer than the relationship between the latter group and other (Asian) apes, and it is likely that no molecular evolutionist today would quarrel with Darwin's position:

"It is therefore probable that Africa was inhabited by extinct apes closely allied to the gorilla and chimpanzee, and as these two species are now man's nearest allies, it is somewhat more probable that our early progenitors lived on the African continent than elsewhere."

What molecular evolutionists *do* quarrel about—among themselves and with others—is *when* man-ape progenitors made their appearance (Wilson and Sarich, 1970; Leakey, 1970; Goodman *et al.*, 1971).

In this article we report the amino acid sequences of the fibrinopeptides A and B from gorilla, organgutan and siamang. Taken in combination with recent reports from this laboratory on the fibrinopeptides of the chimpanzee (Doolittle and Mross, 1970) and the gibbon (Mross *et al.*, 1970) and the previously published structures of human fibrinopeptides (Blombäck *et al.*, 1966), these studies allow a comparison of all six major hominoid groups and the detailed tracing of individual amino acid replacements. The data are completely supportive of the immunochemical evidence cited above and previously published amino acid sequence data. Their unique value lies in their graphic completeness. Whereas it is still impossible to know what the underlying basis of an antigenic change might be in an immunologic system, amino acid sequence data reflect quantized events corresponding to single base substitutions in the genetic material. Furthermore, the peptides examined in this study rank among the most rapidly changing protein

regions known, and as such afford a sufficient number of changes within a small sector of the genome so that reliable confidence levels are often achieved without recourse to sophisticated computer analysis. Similarly, it is a relatively simple matter to reconstruct ancestral sequences for such mutable regions, and we have been able to deduce the structures of fibrinopeptides of the common ancestor of hominoids and Old World monkeys.

Although this article does not purport to answer the question of when man and the African apes last shared a common ancestor, we feel it will at the very least provide the protagonists in this controversy with some highly digitized data, on the basis of which they may or may not wish to re-evaluate their respective positions.

Materials and Methods

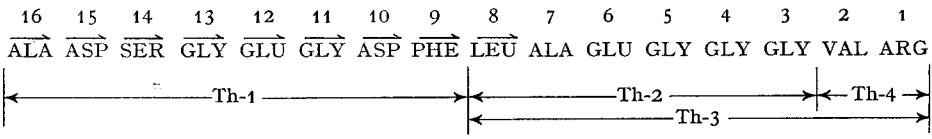
Blood plasmas from an orangutan and a gorilla were obtained from the Yerkes Primate Center, Atlanta, Georgia, U.S.A. Siamang plasma was supplied by the Laboratory for Experimental Medicine and Surgery in Primates, New York University Medical Center. Fibrinogen was prepared in each case by a modified Cohn ethanol fractionation scheme (Doolittle *et al.*, 1967). The fibrinopeptides were isolated in bulk fashion by chromatography on Dowex 50-X2 (Blombäck and Vestermark, 1958) and fibrinopeptide A separated from fibrinopeptide B by paper electrophoresis at pH 2. Amino acid analyses after total acid hydrolysis were conducted on a Spinco Amino Acid Analyzer. Chymotryptic digestions were performed at 37° for five hours in 0.1 M ammonium bicarbonate. Thermolysin digestions were limited to 40 min at 37°, in a 0.01 M Tris buffer, pH 8.1, containing 0.3 mM $CaCl_2$. Terminal pyrrolidone carboxylic acid (PCA) groups were removed from the fibrinopeptides B by incubation with pyrrolidone carboxylyl peptidase (Doolittle and Armentrout, 1968) for one hour at 30°. Isolation of peptides and fragments after digestion with any of these enzymes was accomplished by paper electrophoresis and subsequent elution from the paper. Sequential degradations were performed using the combination phenyl*iso*-thiocyanate-dansyl (PITC-DNS) procedure described by Gray (1967), using Taiwan polyamide paper for identification of DNS-amino acids (Woods and Wang, 1967). The positioning of glutamine and asparagine amides was based on the electrophoretic mobilities of peptides and peptide fragments at pH 4.1 and comparison with human fibrinopeptide and fibrinopeptide fragment controls.

Results

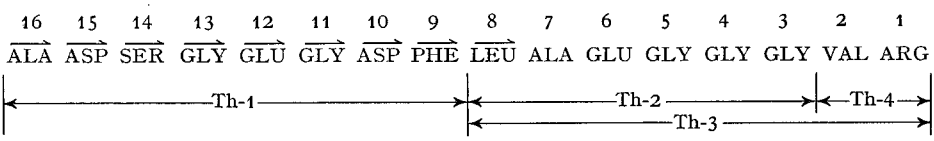
Gorilla Peptides. The gorilla fibrinopeptides were indistinguishable from human controls, including the fact that the A peptide is fractionally phosphorylated at serine-14. The patterns of enzymatic cleavage with thermolysin and the electrophoretic behavior and amino acid compositions of peptide fragments were also identical to those obtained with human fibrinopeptides. The bulk of the fibrinopeptide A sequence was confirmed by performing nine stepwise degradations with the PITC-DNS procedure. Although the acid hydrolysis involved in this method converts asparagine to aspartic acid and glutamine to glutamic acid, the mobilities of peptide fragments at pH 4.1 indicated that no sidechain amides were involved.

In the case of the gorilla fibrinopeptide B, the terminal pyrrolidone carboxylic acid was enzymatically removed before stepwise degradations

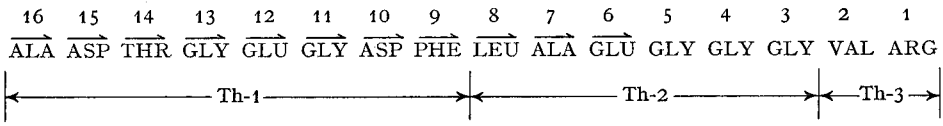
Gorilla A



Orangutan A

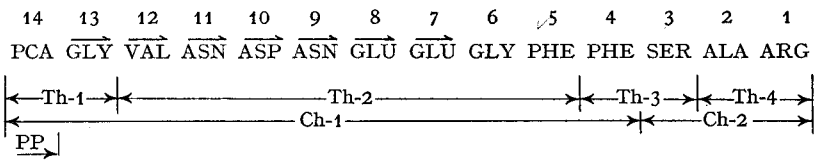


Siamang A

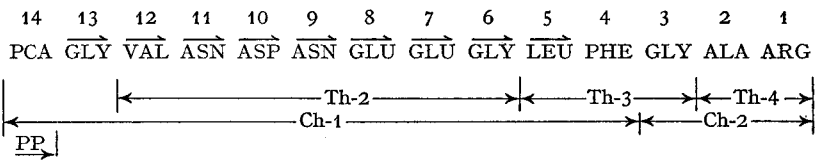


Scheme 1. Summary of data used to deduce the amino acid sequences of fibrinopeptides A. *Th* thermolysin. Half-arrows indicate identification by the PITC-DNS method

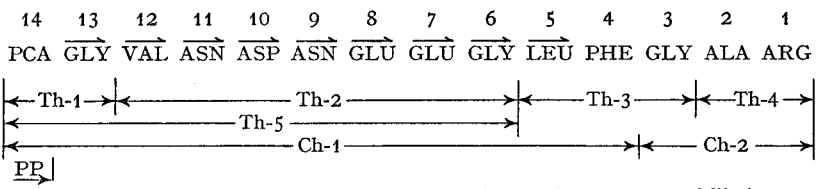
Gorilla B



Orangutan B



Siamang B



Scheme 2. Summary of data used to deduce the amino acid sequences of fibrinopeptides B. *Th* thermolysin; *Ch* chymotrypsin; *PP* pyrrolidone carboxylyl peptidase. Half-arrows indicate identification by PITC-DNS method

were performed. The positioning of the asparagines at positions B-11 and B-9 is arbitrary and is based on the fact that the fragments after each of the various enzymatic treatments all had the same electrophoretic mobilities as human peptide controls. The results are summarized in Schemes 1 and 2.

Orangutan Peptides. The orangutan fibrinopeptide A behaved identically with human A controls (and the gorilla A) in every way, including amino acid composition, enzymatic digestion features, and stepwise degradations. The B peptide, however, had an amino acid composition which indicated two amino acid replacements, a leucine and a glycine instead of a phenylalanine and a serine found in the human and gorilla B peptides. The positions of the replacements were readily localized by enzymatic fragmentation, the leucyl amino-specificity of thermolysin being especially helpful (Scheme 2). In comparison with the gorilla-human type B peptide, the orangutan B has leucine instead of phenylalanine at position B-5 and a glycine in place of a serine at B-3. The remainder of the sequence was confirmed by enzymatic removal of the terminal PCA followed by a series of PITC-DNS degradations (Scheme 2).

Siamang Peptides. The amino acid composition of the siamang fibrinopeptide A indicated that a single amino acid difference existed between this peptide and the human-gorilla-orangutan A peptides, there being a threonine residue instead of the solitary serine known to exist in the human fibrinopeptide A at position 14. Furthermore, in contrast to the other A peptides, there was no evidence of any phosphorylated siamang A peptide. As in the other peptides, electrophoretic mobilities at pH 4.1 indicated an absence of sidechain amides. A complete characterization of the thermolysin fragments coupled with a series of 11 stepwise degradations elucidated the entire sequence (Scheme 1).

The siamang B peptide behaved identically with the orangutan fibrinopeptide B in every way; the positions of the two amino acid differences compared with human and gorilla B peptides were similarly located at positions B-5 and B-3. The complete sequence was verified by performing nine stepwise degradations after the removal of the terminal PCA residue (Scheme 2).

Discussion

The amino acid sequences of the fibrinopeptides A and B from all six existing hominoid groups are listed in Scheme 3, as well as a set of composite sequences for the three species of Old World monkey whose peptides have been characterized. A cladogram depicting the stepwise changes leading to these sequences is presented in Scheme 4. Altogether, five different amino acid replacements have occurred along the various branches leading to the present-day hominoid structures; all of these are of the single base sub-

stitution variety (Marshall *et al.*, 1967). In addition, one deletion, reflecting the loss of three contiguous bases, has occurred on the path leading to the gibbon.

It is noteworthy that the cladogram is virtually indistinguishable from one constructed by Sarich and Wilson (1967) on the basis of immunological criteria. It is also worthy of comment that the fibrinopeptides A and B, as mutable as these structures are known to be in other mammals, are identical in human, chimpanzee and gorilla.

There are two conflicting interpretations of these and previous data of the same vein. Either man and the African apes have diverged much more recently than the paleontologists have led us to believe, perhaps as little as five million years ago (Wilson and Sarich, 1970), or the number of amino acid replacements per unit of historical time has been less than might be expected on the basis of data from other animals. Goodman (1961), for example, has long espoused a theory whereby hemochordial placental mammals like the higher primates should have a retarded rate of molecular evolution because of a higher rejection rate resulting from isoimmunization of foetuses by trespassing maternal circulations. In the "when" controversy, then, he sides with the paleontologists who cite 14 million year old fossils which have the features of early man (Leakey, 1970; Goodman *et al.*, 1971).

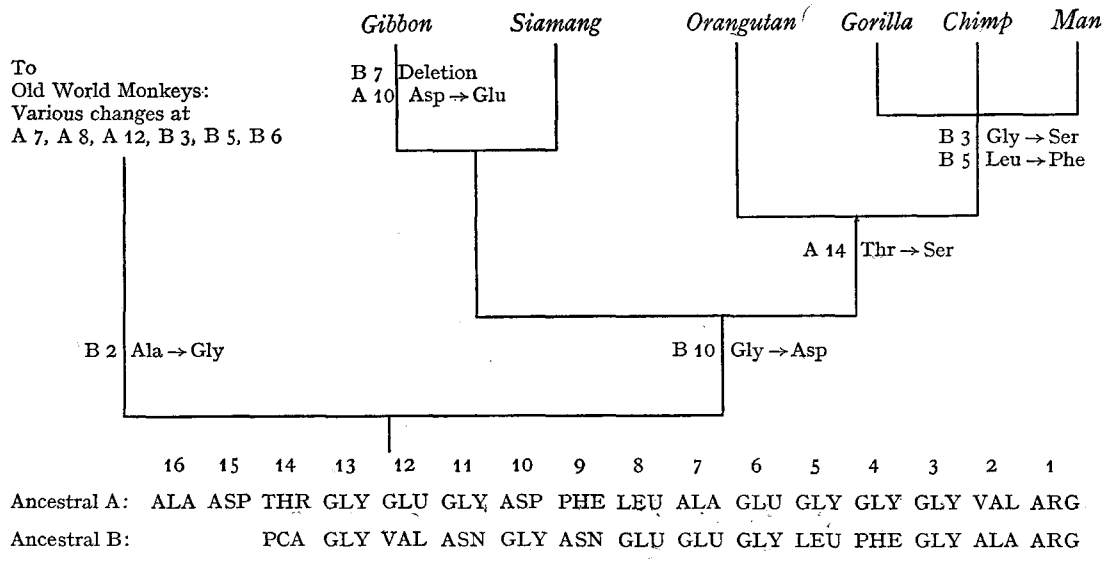
In many ways molecular evolutionists are handicapped in these arguments since their observations are restricted to extant organisms. On the other hand, the paleontologist, even more than the immunologist, has to rely on subjective interpretations of mysterious events. How many base substitutions, for example, does it take to go from six premolars in each jaw to four? Despite this shortcoming, it is likely that eventually enough fossils will be unearthed to permit a complete reconstruction of an anatomically continuous system. In combination with modern stratigraphy and improved radiodating technology these developments should settle the question.

In the meantime, we would suggest that the dispute is not as profound as the disputants contend. For example, which is more important when considering evolutionary relationships between groups, the absolute time in millions of years, or the numbers of biological generations which have occurred along their respective branches? If you are a paleo-ecologist interested in the interaction of co-existing creatures, obviously the former is more important. If you are a molecular biologist concerned with information transfer, however, then the number of opportunities for transmitting germinal changes may be more interesting. In fact, Laird *et al.* (1969) have reported DNA hybridization data which strongly indicate that molecular diversity between species is proportional to the number of generations between them rather than the number of years.

Fibrinopeptides A and B

	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
Human A	ALA–	ASP–	SER–	GLY–	GLU–	GLY–	ASP–	PHE–	LEU–	ALA–	GLU–	GLY–	GLY–	GLY–	VAL–	ARG
Chimpanzee A	ALA–	ASP–	SER–	GLY–	GLU	(GLY,	ASP)	PHE	(LEU,	ALA,	GLU,	GLY,	GLY,	GLY,	VAL)	ARG
Gorilla A	ALA–	ASP–	SER–	GLY–	GLU–	GLY–	ASP–	PHE–	LEU	(ALA,	GLU,	GLY,	GLY,	GLY)	VAL–	ARG
Orangutan A	ALA–	ASP–	SER–	GLY–	GLU–	GLY–	ASP–	PHE–	LEU	(ALA,	GLU,	GLY,	GLY,	GLY)	VAL–	ARG
Siamang A	ALA–	ASP–	THR–	GLY–	GLU–	GLY–	ASP–	PHE–	LEU–	ALA–	GLU–	GLY–	GLY–	GLY–	VAL–	ARG
Gibbon A	ALA–	ASP–	THR–	GLY–	GLU	(GLY,	GLU)	PHE	LEU	(ALA,	GLU,	GLY,	GLY,	GLY,	VAL)	ARG
Old World Monkey A (composite)	ALA	ASP	THR	GLY	GLU/ASP	GLY	ASP	PHE	LEU/ILE	ALA/THR	GLU	GLY	GLY	GLY	VAL	ARG
Human B			PCA	–GLY–	VAL–	ASN–	ASP–	ASN–	GLU–	GLU–	GLY–	PHE–	PHE–	SER–	ALA–	ARG
Chimpanzee B			PCA	(GLY,	VAL,	ASN,	ASP,	ASN,	GLU,	GLU,	GLY,	PHE)	PHE	(SER,	ALA)	ARG
Gorilla B			PCA	–GLY–	VAL–	ASN–	ASP–	ASN–	GLU–	GLU–	GLY–	PHE–	PHE–	SER–	ALA–	ARG
Orangutan B			PCA	–GLY–	VAL–	ASN–	ASP–	ASN–	GLU–	GLU–	GLY–	LEU–	PHE–	GLY–	ALA–	ARG
Siamang B			PCA	–GLY–	VAL–	ASN–	ASP–	ASN–	GLU–	GLU–	GLY–	LEU–	PHE–	GLY–	ALA–	ARG
Gibbon B			PCA	(GLY,	VAL,	ASX,	ASX,	ASX,	GLX,	——,	GLY,	LEU)	PHE	(GLY,	ALA)	ARG
Old World Monkey B (composite)			PCA	GLY	VAL	ASX	GLY	ASN	GLU	GLU	GLY/SER	LEU/PRO	PHE	GLY/SER	GLY	ARG

Scheme 3. Complete listing of hominoid amino acid sequences. The composite Old World monkey sequences represent the fibrinopeptides of rhesus macaque and green monkey (Blombäck *et al.*, 1965) and the mandrill (Doolittle *et al.*, 1969)



Scheme 4. Simple cladogram depicting evolution of hominoid and Old World monkey fibrinopeptides (rhesus macaque, green monkey, and mandrill). The directions of the changes at B-2 and B-6 are based on our unpublished sequence data for the fibrinopeptides of New World monkeys. All the changes indicated in the cladogram are of the single-base-substitution type

It seems clear from what we know of the reproductive biology of higher primates that a much longer generation time exists for these creatures than for most other mammalian groups. For instance, in the original discussion of fibrinopeptide structure and evolution, Doolittle and Blombäck (1964) used an average generation time of five years in analyzing data from artiodactyls. Most hominoids don't even reach puberty until they are 7–8 years old (Walker, 1968), and a reasonable estimate for a mean generation time might be 15 years. Is it fair, then, to note that humans, chimpanzees and gorillas all have identical fibrinopeptides, whereas differences occur in the fibrinopeptides of such closely related (and recently diverged) pairs as cat-lion, dog-fox, donkey-horse, or water buffalo-cape buffalo (Dayhoff, 1969)? Of course it is, as long as one keeps in mind exactly what one is comparing. Is a person any less related to his grandfather if his grandfather was born 80 years before him instead of 40? In other words, it seems to us, on the basis of the fibrinopeptide data presented, that man and African apes are very closely related, perhaps as closely as the other pairs of animals cited. On the other hand, the divergence between man and these apes may have taken place considerably earlier in absolute historical time than the divergences of pairs cited for carnivores, artiodactyls and perissodactyls, all of which have shorter mean generation times. The greater time involved, however, in no way compromises the proximity of their relationship. If this interpretation is correct, then the fibrinopeptide sequences and other data on hominoid evolution may be reconciled with the fossil record without resort to more circuitous explanations such as *in utero* isoimmune effects.

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