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Review

Evolutionary diversification of the mammalian defensins

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Abstract. Defensins are cysteine-rich cationic peptides that function in antimicrobial defense in both invertebrates and vertebrates. Three main groups of animal defensins are known: insect defensins; mammalian α-defensins and vertebrate β-defensins. It has been difficult to determine whether these molecules are homologous or have independently evolved similar features, but overall the evidence favors a distant relationship. The best evidence of this relationship is structural, particularly from their overall three-dimensional structure and from the spacing of half-cystine residues involved in intra-chain disulfide bonds. Some evidence favors a closer relationship between vertebrate β-defensins and insect defensins than between the two groups of vertebrate defensins. Examination of nucleotide substitutions between recently duplicated mammalian defensin genes shows that the rate of nonsynonymous (amino-acid-altering) substitution exceeds that of synonymous substitution in the region of the gene encoding the mature defensin. This highly unusual pattern of nucleotide substitution is evidence that natural selection has acted to diversify defensins at the amino acid level. The resulting rapid evolution explains why it is difficult to reconstruct the evolutionary history of these molecules.

Key words. α-Defensin; β-defensin; innate immunity; insect immunity; positive selection.

Introduction

Both vertebrate and invertebrate animals produce a variety of antibacterial peptides that function in innate immune defense [1–4]. The term 'defensin' is used for certain of these peptides in both insects and vertebrates, and known animal defensins can be placed in three very distinct groups: (i) insect defensins; (ii) mammalian α-defensins or 'classical' defensins, and (iii) vertebrate β-defensins [1–4]. No sequences for α-defensins are yet available from outside the mammals, while β-defensins have also been identified from birds, in which case they have been called 'gallinacins' [5]. All three types of defensins are characterized by being cationic and rich in half-cystine. There appear to be substantial differences among mammalian species with respect to the expression patterns of different defensins. α-Defensins were first detected in rabbit neutrophils and alveolar macrophages and in guinea pig neutrophils, and human neutrophils were found to express a number of distinct α-defensins [6]. In addition, certain α-defensins, sometimes termed 'cryptdins,' are expressed in Paneth cells of the mouse small intestine [6]. β-Defensins are expressed by leukocytes in both birds and mammals [4, 5] and in epithelial cells including those of human skin [7] and bovine tongue, trachea, and intestine [8–10].

The purpose of the present paper is to review the biology of the two gene families (α and β) encoding mammalian defensins from an evolutionary perspective. I address the following issues: (i) the relationships among three known families of animal defensins (i.e., insect defensins and vertebrate α- and β-defensins); (ii) the diversification of defensins within each of the two mammalian families, paying particular attention to the role of natural selection, and (iii) the coevolution of

charged residues in the propiece and the active peptide in the case of α-defensins. The antimicrobial peptides produced by plants and also called 'defensins' do not show any compelling evidence of homology with animal defensins and so will not be considered here. The symbols used for sequences used in analyses reported here, along with their Genbank accession numbers, are summarized in table 1.

Structures and relationships

The α- and β-defensins are both produced from precursors by proteolytic cleavage. The biologically active mature defensins share certain characteristics of sequence and structure, but there are also marked differences. Thus, it might be questioned whether the two gene families are in fact homologous; that is, whether they have descended from a single ancestral gene by gene duplication. In the case of α-defensins, the mature peptide is 29–35 amino acids in length, while β-defensins average somewhat longer (38–42 amino acids). In both cases, the primary structure of the mature peptide is characterized by numerous cationic residues, particularly arginine residues, and six half-cystine residues. These half-cystine residues are known to be involved in intra-chain disulfide bonds [3]. The resulting three-dimensional structures show a striking overall similarity, each consisting of a triple-stranded, antiparallel β-sheet [11].

However, five of the six half-cystine residues are the only amino residues that can be considered to be conserved between α- and β-defensins (fig. 1). There does not seem to be any reasonable alignment which would make the first half-cystine of α-defensins (which I refer to as C1′) correspond to the first half-cystine of β-defensins (which I refer to as C1). Furthermore, the pattern of disulfide bonding is different in α-defensins (half-cystines C1′-C6, C2-C4, and C3-C5) and β-defensins (half-cystines C1-C5, C2-C4, and C3-C6). Considering the insect defensins further complicates the picture. They appear to share all six homologous half-cystines residues with vertebrate β-defensins (fig. 1). Yet here again, the disulfide bonding pattern (C1-C4, C2-C5, and C3-C6) is distinct.

Thus, the structures of the three families of animal defensins are broadly similar but differ in important details. Further, aside from the half-cystine residues involved in disulfide bonding, there are no conserved residues found in all families. Thus, it might be argued that the three families are unrelated but have evolved a similar structure convergently. There are other possible cases of such convergent evolution of structure. For example, immunoglobulin domains and fibronectin type III domains both consist of three- or four-stranded β-sheets in the form of an 'all-β sandwich' [12, 13]. It is quite possible that these two structures evolved independently, and it is even possible that not all members of the 'immunoglobulin superfamily' are actually related but that the immunoglobulin fold itself has evolved independently two or more times.

Recognizing that the structural similarity of α- and β-defensins is not definitive proof that they are evolutionarily related, some authors have sought evidence on this question from other sources, for example, in linkage relationships. Liu et al. [14] reported that the human β-1 defensin gene is located within 100–150 kb of the α-defensin cluster on the short arm of chromosome 8 and proclaimed this linkage to be 'strong direct evidence for a common evolutionary origin of the two defensin families.' Unfortunately, this conclusion is unwarranted. Linkage in current-day mammalian genomes does not in itself provide any evidence of an evolutionary relationship. Indeed, there are many known cases of linkage between genes which are totally unrelated but whose products have similar expression patterns and function. One example is provided by the TAP transporter genes and LMP proteasome component genes linked to the major histocompatibility complex (MHC) genes of vertebrates, a linkage that evidently predates

Table 1. Sequences used in analyses reported in this paper.

Mammalian α-defensins
Mouse (*Mus musculus*) Cor (X15617), Def1 (U02994-5), Def2 (U02996), Def3 (U03000-1), Def5 (U03002-3), Def6 (U03002-3), 4C-2 (U12564), CRS4C-2 (U12564), CRS4C (S77610), 4C-4 (U12566), 4C-5 (U12566), CRS4C-5 (S77621)
Rat (*Rattus norvegicus*) NP1 (U16686), NP3 (U16683), NP4 (U16684)
Guinea pig (*Cavia porcellus*) 1A (D14119), 1B (D14118)
Rabbit (*Oryctolagus cuniculus*) NP3a (M64599), NP4 (M64601), NP5 (M64602), MCP1 (M28883), MCP2 (M28072)
Human (*Homo sapiens*) NP3 (X13621), NP4 (U18745), D5 (M97925), D6 (U33317)

Vertebrate β-defensins
Mouse BD1 (AF003524-5)
Rat BD1 (AF093536)
Human BD1 (X92744), BD2 (Z71389)
Rhesus (*Macaca mulatta*) BD1 (AF014016)
Pig (*Sus scrofa*) BD1 (AF031666)
Sheep (*Ovis aries*) BD2 (U75251)
Bovine (*Bos taurus*) BD2 (U75251), BD3 (AF016396), BD4 (AF008307), BDC7 (AF016395), BD9 (AF016394), EAP (AF000362), LAP (S76279), TAP (M63023)
Chicken (*Gallus gallus*) GAL1 (AF033335)
Turkey (*Meleagris gallopavo*) HP1 (AF033337)

Insect defensins
Anopheles gambiae (AF063402)
Aedes aegypti (S82860)
Drosophila melanogaster (Z27247)
Protophormia terraenovae (X55546)
Sarcophaga peregrina (J04053)
Stomoxys calcitrans (AF013146)

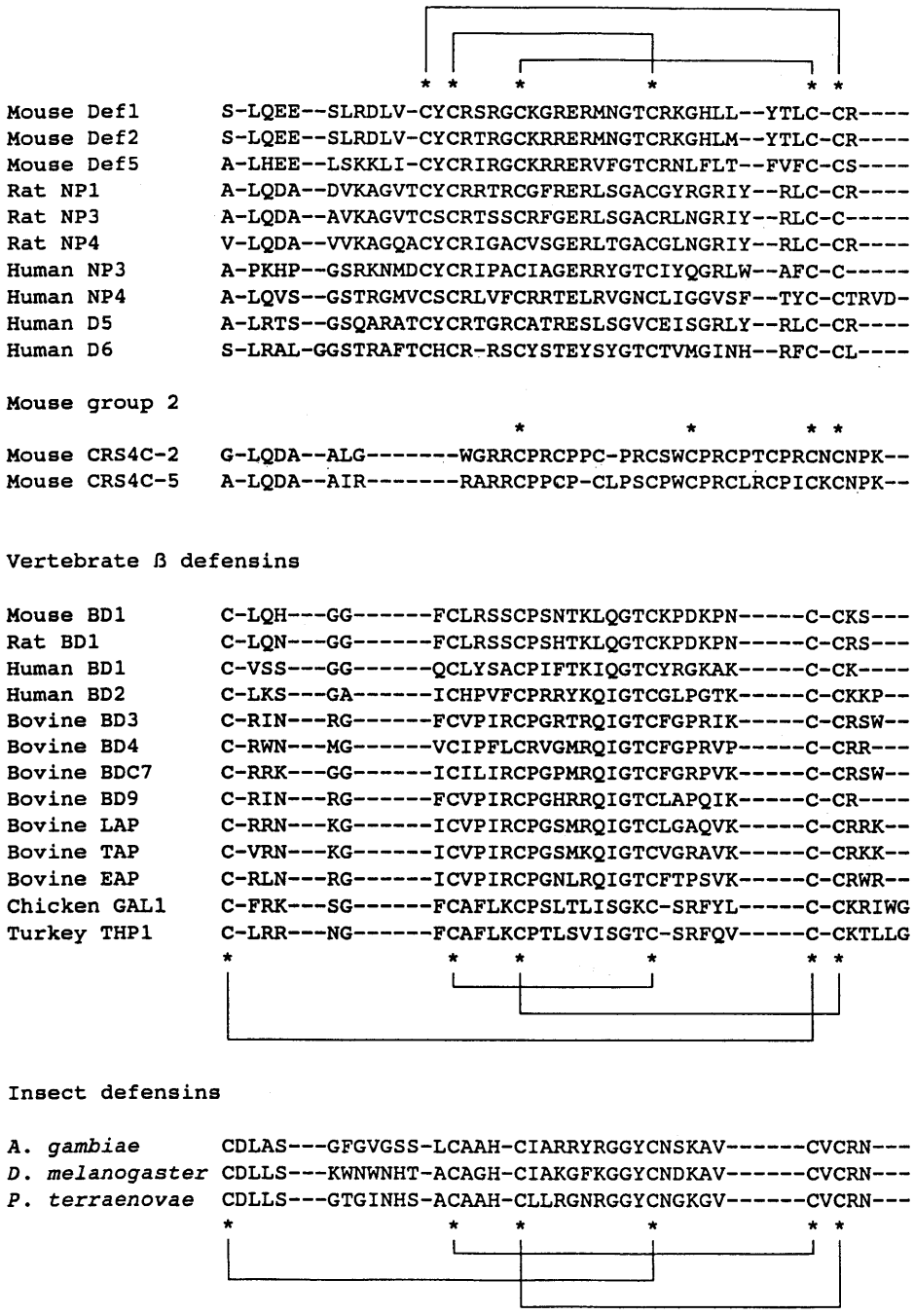

Figure 1. Hypothetical alignment of mammalian α-defensins, vertebrate β-defensins, and insect defensins. Intra-chain disulfide bonds are indicated by brackets.

the divergence of bony fishes and tetrapods [15]. TAP and LMP are evolutionarily unrelated to MHC genes but their products interact functionally with those of class I MHC genes. Given the similarity of tissue expression and function of mammalian α- and β-defensins, it remains a possibility that these genes originated separately but that their linkage in the genome has been selectively favored [16]. Of course, it is well known that there are many cases in which members of multigene families are linked in the genomes of vertebrates, but linkage in itself does not prove relationship.

Nonetheless, evidence from structural and sequence similarity appears to favor the hypothesis of an evolutionary relationship among the animal defensins, albeit a distant one. Perhaps the most suggestive evidence is seen in a region of the protein which I will call the 'defensin core.' This is the stretch from cysteines C3 to C4 (fig. 1). Although no other residues in the defensin core besides the half-cystine residues are conserved in all animal defensins, the number of residues in this stretch (11) is constant in all animal defensins, except certain mouse α-defensins, which have 10. These latter constitute the CRS4C group or 'mouse group 2,' as they are designated in figure 1; I will discuss their relationships further in the next section. The defensin core shows other conserved residues besides C3 and C4. In all defensins except mouse group 2, there is a glycine residue two positions before C4; and the residue between this glycine residue and C4 is usually threonine (fig. 1). Even outside the defensin core, there are a few additional signs of relationship among all defensins; for example, C6 is in most cases followed by a positively charged residue (arginine or lysine) (fig. 1).

As will be discussed below, there is strong evidence that both α- and β-defensins of mammals have evolved rapidly as a result of positive Darwinian selection favoring diversification at the amino acid level. Given such selection and the short length of the active defensin, it is not surprising that this region should have become so diversified over the course of evolution that evidence of homology has been nearly obliterated.

If animal defensins are indeed homologous, it is possible to ask if the two vertebrate families are more closely related to each other than either is to the insect defensins. In fact, this is a very difficult question to answer because there is no outgroup, that is, no more distantly related gene family that can be used to root the phylogeny of animal defensins. Figure 2 shows a phylogenetic tree of selected animal defensins based on the proportion of amino acid differences (p). (Only amino acid differences, not nucleotide differences, can be used for this analysis, because in the more distant comparisons, the nucleotide sequences are saturated with changes.) The tree is rooted in the midpoint of the longest internal branch. Given this rooting, vertebrate β-defensins cluster with insect defensins rather than with mammalian α-defensins (fig 2). This occurs because, on average, β-defensins are slightly more similar in amino acid sequence to insect defensins than they are to α-defensins.

β-Defensins and insect defensins share two other characteristics: both have a C1 cysteine, rather than the C1′ cysteine of α-defensins and both have a C3-C6 disulfide bond (Table 2). On the other hand, α-defensins and β-defensins share a C2-C4 disulfide bond (Table 2). The fact that there are more similarities between β-defensins and insect defensins than there are between either of these families and α-defensins (Table 2) suggests that β-defensins are more closely related to insect defensins than to α-defensins. This cannot yet be definitively concluded because, in the absence of an outgroup, it is impossible to establish the ancestral state of structural characters. For example, the fact that β-defensins and insect defensins share a C1 residue and a C3-C6 bond may not indicate a close relationship between these families if these traits were found in the common ancestor of all three families.

Relationships among α-defensins

The active mammalian α-defensin is cleaved from a primary translation product consisting of a signal peptide (19 amino acids), a propiece (37–51 amino acids), and the active or mature defensin (29–34 amino acids) [17]. Because the relationships among α-defensins were poorly resolved by a phylogenetic tree based on the active defensin alone (fig. 2), a phylogeny was constructed on the basis of the signal peptide and the propiece (fig. 3). The resulting phylogeny is essentially identical to a previously published phylogeny based on the entire primary translation product [18]. The tree shows that α-defensins form species-specific clusters (fig. 3). This implies that α-defensins have duplicated repeatedly after divergence of these species [18].

The most remarkable within-species diversity seen is that in the mouse, in which there are two distinct subfamilies of α-defensins (labeled '1' and '2' in fig. 3). Group 2 members are characterized by the absence of the first two of the consensus half-cystine residues that characterize typical α-defensins. Mouse CRS4C-2 and CRS4C-5 provide examples (fig. 1). The highly divergent structure of the mature peptide region in mouse group 2 α-defensins has led to the proposal that these molecules are not even homologous to defensins at all. Rather, it has been suggested that the group 2 defensin gene combines an exon 1 (encoding the signal peptide and propiece) of an α-defensin with an exon 2 of a totally unrelated gene [19]. Presumably this hybrid gene arose as a result of a recombinational event.

This hypothesis may be correct. One piece of evidence in favor of it is the existence of yet another mouse molecule called CRS1C, which shows some resemblance to group 2 defensins, particularly in its 5′ end, but almost none to α-defensins [19]. CRS1C is rich in half-cystines but not as cationic as a typical defensin. On the other hand, the mouse group 2 defensins do show some resemblance to α-defensins, especially in the defensin core (fig. 1). In the phylogeny of mature defensins, group 2 defensins group among the α-defensins (fig. 2). However, this phylogeny remains relatively poorly sup-

ported, since the number of sites analyzed is small. Given the positive selection acting on these genes and their resulting high rate of evolution, it is possible that group 2 defensins are simply divergent members of the

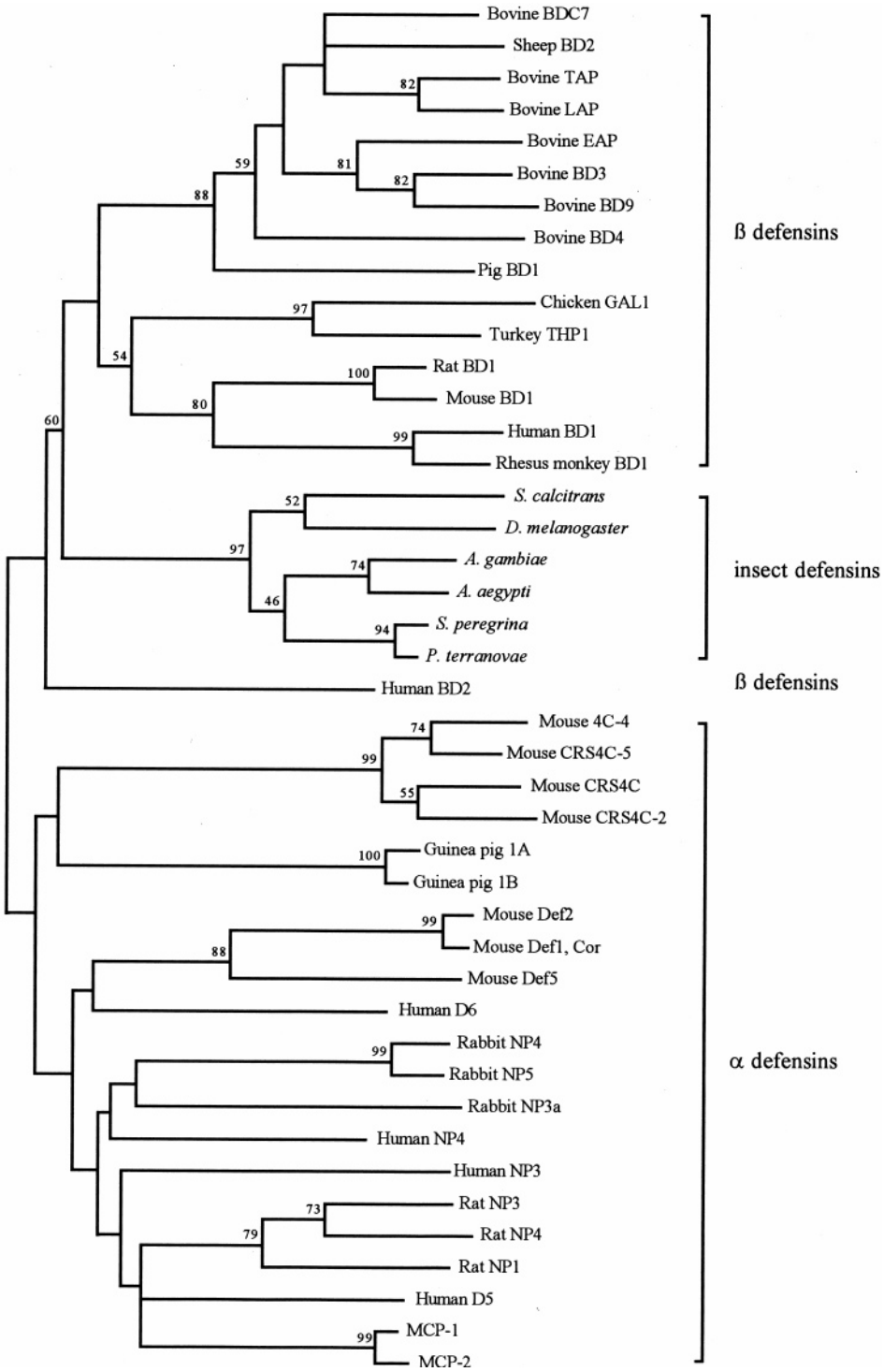

Figure 2. Phylogenetic tree of mature defensins, constructed by the neighbor-joining method [33] on the basis of the proportion of amino acid differences (p). The reliability of the branches in the tree was tested by bootstrapping, which involves repeated sampling from the data set with replacement [34]. Numbers on the branches indicate the percentage of 1000 bootstrap samples supporting the branch; only values $\geq 50\%$ are shown.

Table 2. Character sharing by the three major groups of animal defensins.

Character	α and β	α and insect	β and insect
C1 residue			+
C2-C4 disulfide bond	+		
C3-C6 disulfide bond			+
Average sequence similarity			+

α-defensin family. Their evolution from typical α-defensins can easily be explained by amino acid replacements and deletions. Because positive selection can greatly accelerate the rate of amino acid change, group 2 defensins may have diverged from other α-defensins quite recently; indeed, as the phylogeny of figure 3 indicates, after the divergence of mouse and rat, which took place some 40 million years ago.

Positive selection

Gene duplication is a key step in the process by which new proteins with new functions evolve [20], but the mechanism by which functional novelties arise remains uncertain. In a widely cited model, Ohno [21] proposed that gene duplication is followed by a period during which one gene copy is redundant and thus able to accumulate mutations at random; by chance such mutations may fit such a redundant gene for a new function. However, there are many problems with this hypothesis [22, 23]. One problem is that there is now evidence, from several gene families, that gene duplication is in some cases followed not by random accumulation of

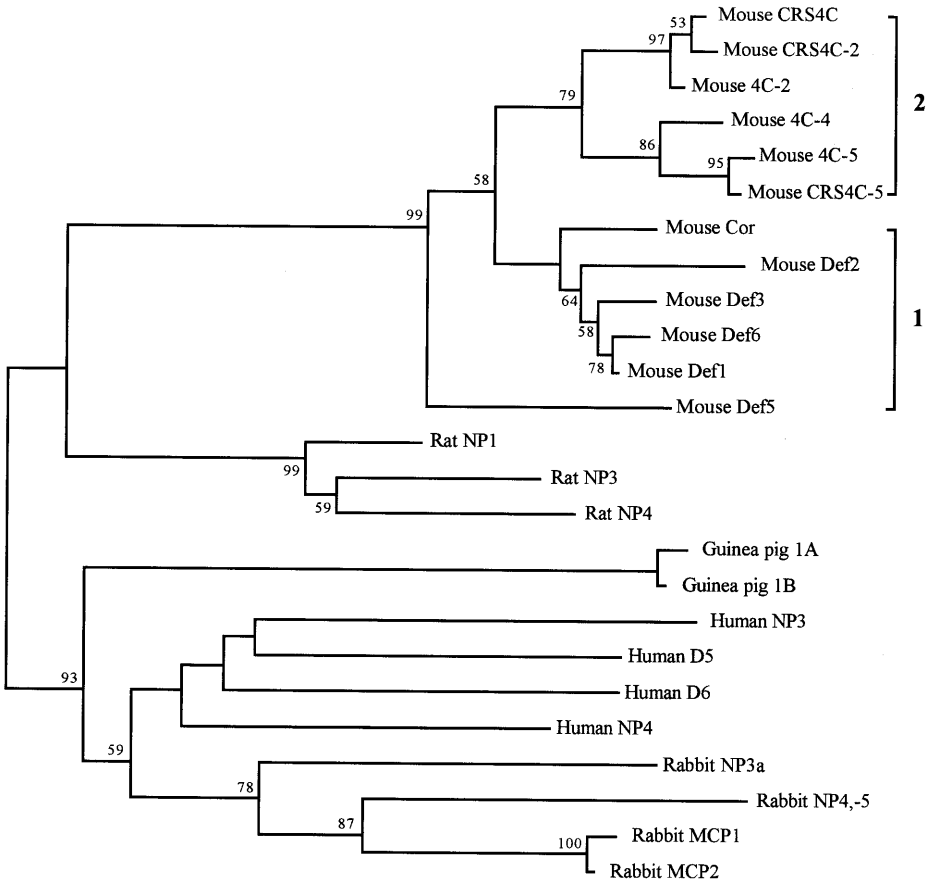

Figure 3. Phylogenetic tree of signal peptide and propiece of α-defensins, constructed by the neighbor-joining method [33] on the basis of the proportion of amino acid differences (p). The reliability of the branches in the tree was tested by bootstrapping, which involves repeated sampling from the data set with replacement [34]. Numbers on the branches indicate the percentage of 1000 bootstrap samples supporting the branch; only values $\geq 50\%$ are shown.

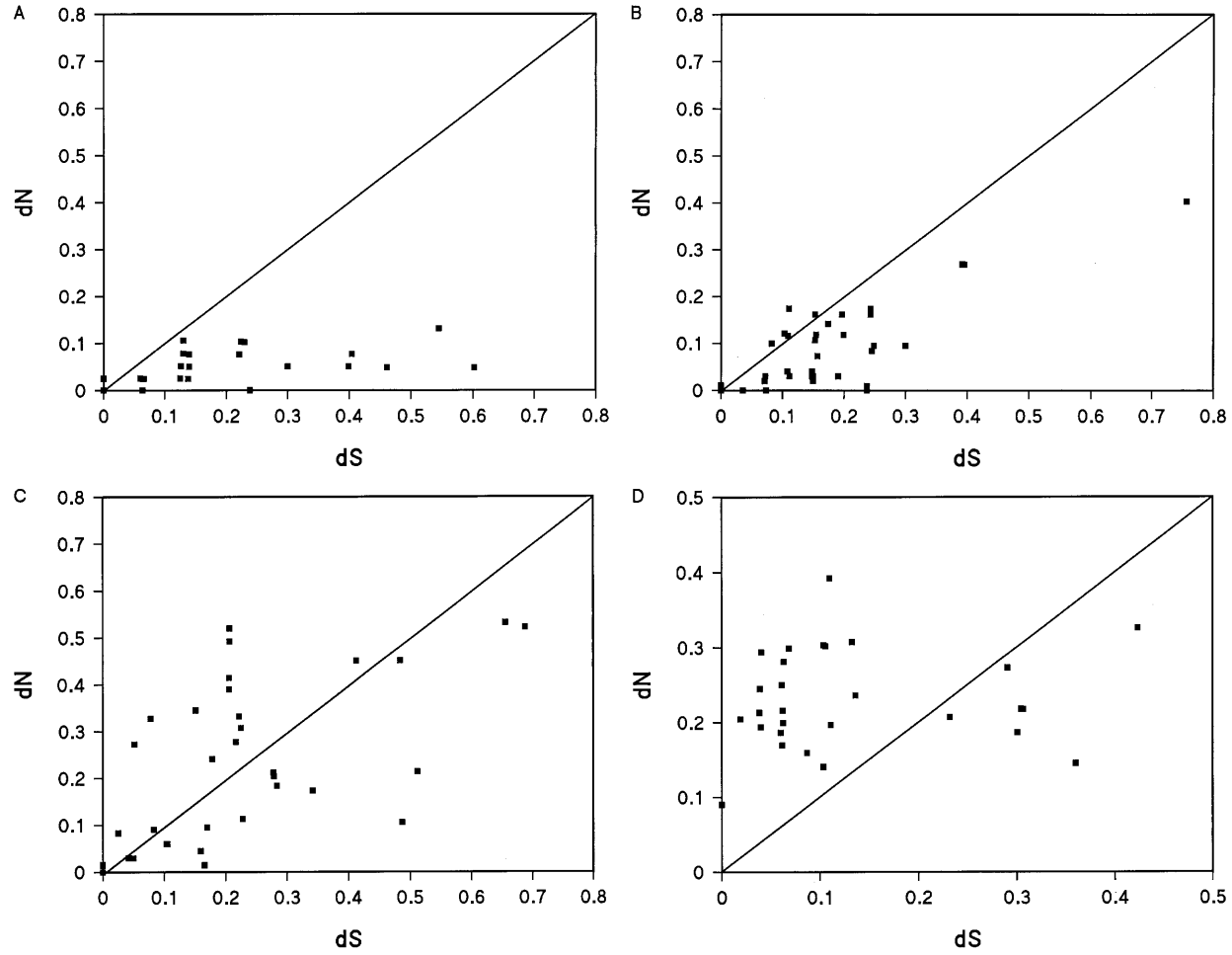

Figure 4. Number of nonsynonymous substitutions per nonsynonymous site (d_N) plotted against number of synonymous substitutions per synonymous site (d_S) [35] for pairwise comparisons among closely related rodent α-defensins: In each case, a 45-degree line is drawn; thus a point above the line indicates a comparison for which dN > dS. (*A*) Signal peptide, (*B*) propiece, (*C*) mature defensin. (*D*) A similar plot for mature β-defensins of bovine and sheep.

mutations but by positive Darwinian selection leading to functional specialization of the products of the daughter genes.

The best source of evidence for positive selection at the molecular level is the comparison of rates of synonymous and nonsynonymous nucleotide substitution [24]. It is expected that, in the case of most protein-coding genes, the majority of nonsynonymous (i.e., amino-acid-altering) mutations will be deleterious to protein function and thus to the organism's fitness; these will then be quickly eliminated by natural selection (conservative or 'purifying' selection). By contrast, synonymous mutations, because they do not change the amino acid, will be selectively neutral or nearly so [25]. Thus, in most genes, the number of synonymous nucleotide substitutions per synonymous site (d_S) will be greater than the number of nonsynonymous nucleotide substitutions per nonsynonymous site (d_N). On the other hand, when diversification of proteins at the amino acid level is selectively favored, d_N will exceed d_S. This is a highly unusual pattern in sequence comparisons, but a number of cases have been described in recent years [23]. Of particular interest with regard to the mechanism of origin of new protein function, there are a number of cases in which d_N has been found to exceed d_S in comparisons between recently duplicated genes, suggesting that natural selection has acted to favor functional diversification after gene duplication [22, 23].

Mammalian α-defensins provide a striking example of such a pattern [18]. In the signal peptide and the propiece, d_S exceeds d_N in most comparisons (Fig. 4A, B) as is true of a typical gene. However, in the case of the

mature defensin, d_N often exceeds d_S, particularly when d_S is relatively low (fig. 4C). Since synonymous mutations are selectively neutral, the degree of nonsynonymous difference between two coding sequences should be a function of the time since their last common ancestor. Thus, the pattern of nucleotide substitution seen in α-defensins seems to be one in which a burst of nonsynonymous substitutions occurs shortly after gene duplication but thereafter, the rate of nonsynonymous substitution slows down. Such a pattern is consistent with natural selection favoring specialization of defensins after gene duplication. Interestingly, comparison of numerous β-defensins from Bovidae (bovine and sheep) shows a very similar pattern (fig. 4D). There are many different β-defensins known from bovine, which are expressed in a variety of tissues. Like α-defensins, these also seem to have diversified as a result of positive Darwinian selection.

The charge balance hypothesis

Observing that the propiece of α-defensins has an anionic character, Michaelson et al. [17] proposed that the propiece plays a role in neutralizing the cytotoxicity of the defensin until it is ready for an antimicrobial attack. As evidence for this view, these authors plotted net negative charge in the propiece against net positive charge in the mature defensin for seven mammalian α-defensins. The resulting relationship was linear, suggesting that over the course of evolution, amino acid substitutions have occurred in both of these regions in

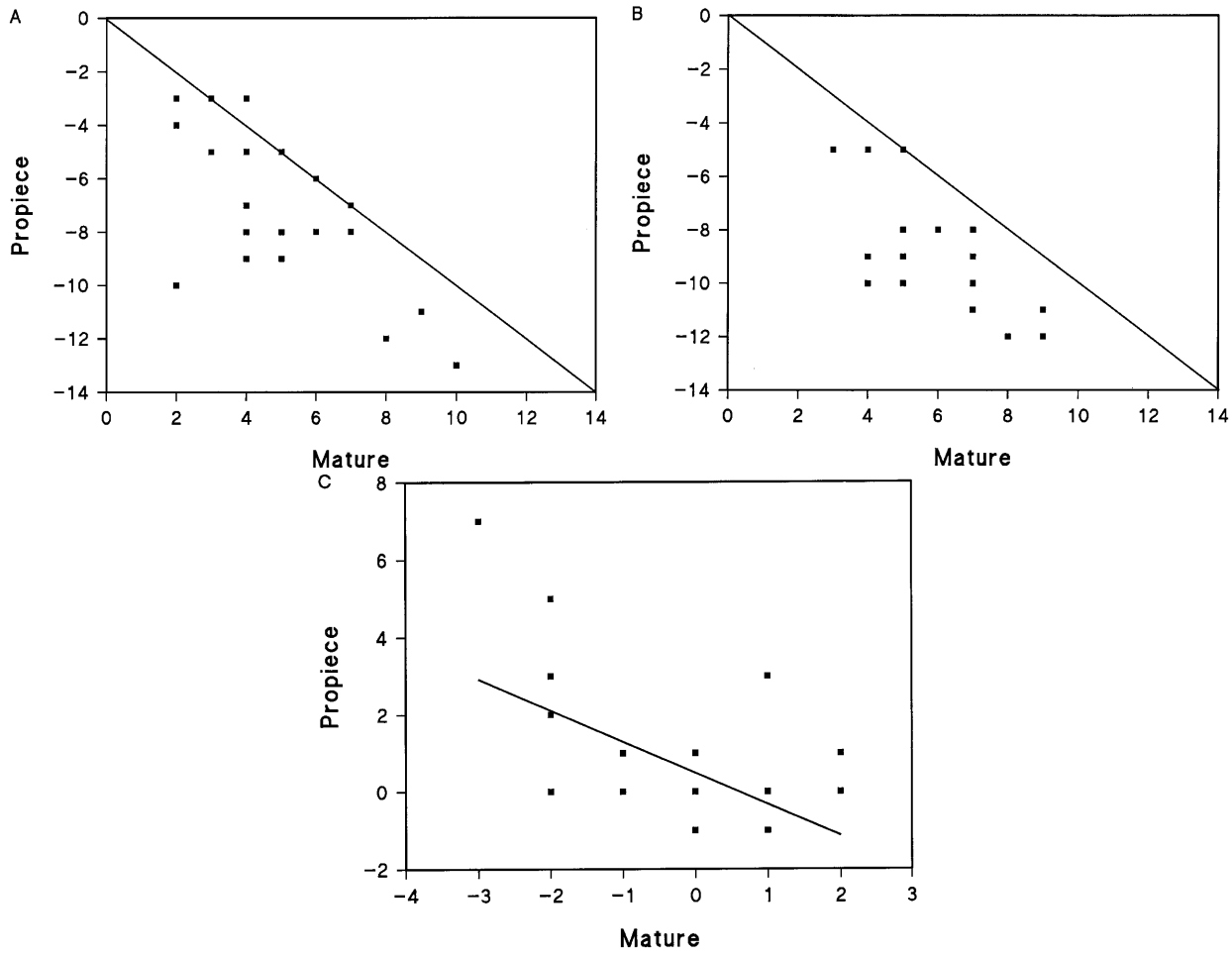

Figure 5. (*A*) Net charge in the propiece vs that in the mature defensin for 28 mammalian α-defensins ($r = -0.742$; $P < 0.001$). The line drawn is a 45° line. (*B*) Net charge in the propiece vs that in the mature defensin for 22 reconstructed ancestral mammalian α-defensins [18] ($r = -0.755$; $P < 0.001$). The line drawn is a 45° line. (*C*) Charge change in the propiece vs that in the mature defensin as reconstructed to have occurred in the evolution of mammalian α-defensins [18] ($r = -0.548$; $P < 0.001$). The line drawn is the linear regression line ($y = 0.486 - 0.808x$).

such a way as to balance charge [17]. Figure 5A shows a similar plot for 29 α-defensins (including mouse group 2), which provides further support for the hypothesis of Michaelson et al. [18].

Given the evidence of positive diversifying selection on defensins and the charge balance between propiece and mature defensin, an interesting question is raised: how have propiece and defensin coevolved so that this charge balance has been maintained even while the amino acid sequence of the mature defensin has undergone change? Hughes and Yeager [18] approached this question by reconstructing ancestral amino acid sequences using a maximum-likelihood method [26]. This method reconstructs ancestral sequences for a given substitution model and a given phylogenetic tree [26]. In this case a phylogeny of rodent α-defensin sequences similar to that of figure 3 was used [18].

The reconstructed ancestral sequences also showed charge balancing between propiece and mature defensin, reminiscent of extant sequences (fig. 5B).

Furthermore, when reconstructed changes in residue charge in the propiece were plotted against those in the mature defensin, there was a significant correlation (fig. 5C). As the defensin became more cationic in the course of evolution, the propiece became more anionic. Conversely, when the defensin became less cationic, the propiece became less anionic.

Conclusions

It is now commonly agreed that the specific immune system of vertebrates (including MHC, T cell receptors, and immunoglobulins) is unique to the vertebrates [27, 28]. Thus, this system is believed to have evolved in the vertebrate lineage. By contrast, it has frequently been asserted that the innate immune defense system of vertebrates represents an ancient system that has been conserved since the common ancestor of vertebrate and invertebrate animal phyla [29–31]. By and large, however, molecular data argue against extensive evolutionary continuity between invertebrate and vertebrate immunity [32]. In the case of most gene families that have members with immune system functions in both arthropods and vertebrates, these immune functions have evolved independently in the two lineages [32]. One clear exception are the lysozymes [32], which have a similar function in both vertebrates and arthropods. The defensins may be another exception if, in fact, as structural similarities suggest, vertebrate and insect defensins share a common origin.

Because the mature defensin is a short peptide and because the genes encoding these molecules have evidently frequently been subject to positive diversifying selection, it will be difficult, if not impossible, to reconstruct fully the early evolutionary history of this family. However, there has been quite recent diversification of both α- and β-defensins in a variety of mammalian species, and this recent evolution may be much more amenable to evolutionary study. Because of their recent origin and their important function in antimicrobial defense, these molecules may represent an ideal system for studying adaptive evolution at the molecular level.

It would be of particular interest to know why selection has favored evolution of a distinctive diversified α-defensin repertoire in each mammalian species studied to date. Likewise, why has a highly diversified repertoire of β-defensins evolved in bovine? The most obvious hypothesis to explain such diversity is that different defensins are needed to deal with different microbial pathogens. Unravelling the possible relationships between defensin diversity and pathogen diversity represents a challenge for future investigations in this field.

The innate immune system of vertebrates is sometimes considered to be no more than a remnant of an ancient defensive strategy and to be almost completely superseded by the sophisticated defenses involved in specific immunity. The defensins show that such a view is an oversimplification. Though presumably phylogenetically ancient, the defensins of mammals are still evolutionarily active, apparently continuing to respond to challenges faced by mammalian lineages in their radiation over the past 100 million years.

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