Conserved and variable regions in protein Arp, the IgA receptor of *Streptococcus pyogenes*

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(Received 29 March 1993; revised 10 May 1993; accepted 24 May 1993)

The streptococcal M protein family, a number of cell surface molecules that interact with the human immune system, can be divided into two major classes, A and C, characterized by different types of repeats in the central part of the molecule. Class A and class C molecules are known to have a variable N-terminal region and a more conserved C-terminal region, but little is known about the mechanisms that give rise to this structural variation. In this report, we show that two variants of protein Arp, an IgA receptor in class C of the M protein family, have virtually identical signal sequences and C-terminal halves, but unrelated N-terminal sequences. Comparison of the sequences of the two genes and their flanking regions also demonstrates the presence of well-defined variable and conserved regions. Our results strongly suggest that the N-terminal sequence variation between the two variants of protein Arp was generated through an intergenic recombination event, rather than through intragenic recombination or accumulation of mutations.

Introduction

Antigenic variation is an important mechanism by which micro-organisms alter the structure of their surface, thereby escaping the immune system of the infected host (Birkbeck & Penn, 1986; Borst, 1991). Analysis of several different systems has demonstrated that such structural variation takes two different forms: antigenic *drift* that occurs by the gradual accumulation of mutations, and antigenic *shift* that occurs by an abrupt gene rearrangement (Borst, 1991). The latter mechanism often involves recombination, either with another sequence in the same genome or with exogenous homologous DNA that is taken up by transformation (Borst, 1991; Meyer, 1990).

An important human pathogen that exhibits antigenic variation is *Streptococcus pyogenes*, the group A Streptococcus. This bacterium expresses an antiphagocytic cell surface molecule, the M protein, that is a target for the immune response and occurs in numerous antigenic variants expressed by different strains (Lancefield, 1962; Fischetti, 1989; Scott, 1990). Sequence analysis of several

different M proteins has demonstrated that they are structurally related and are therefore encoded by a family of genes. In particular, all the known M proteins have homologous C-terminal parts, including the centrally located C repeat region (Fischetti, 1989; Scott, 1990). The N-terminal parts of the different M proteins vary in sequence, which explains the antigenic variation (Fischetti, 1989; Miller *et al.*, 1988; Scott, 1990), but the mechanism by which this variation is generated is not clear.

Sequencing of the genes for several Ig-binding cell surface proteins of *S. pyogenes* has shown that these molecules are also members of the M protein family (Frithz *et al.*, 1989; Heath & Cleary, 1989; Gomi *et al.*, 1990; Bessen & Fischetti, 1992; O'Toole *et al.*, 1992). Some of these Ig-binding proteins have C repeats, like the classical M proteins, while others have a different type of repeat, the A repeat, in the central part of the sequence. The M protein family can therefore be divided into two major classes, A and C, characterized by A repeats and C repeats, respectively (O'Toole *et al.*, 1992). Both class A and class C molecules exhibit N-terminal sequence variation and both classes of molecules comprise proteins that interact with the immune system of the host (O'Toole *et al.*, 1992). However, it is not yet known whether any of the Ig-binding proteins in class A and class C have antiphagocytic function, like the classical M proteins in class C.

One Ig-binding protein that has been extensively

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The nucleotide sequence data reported in this paper have been submitted to GenBank/EMBL and have been assigned the accession number Z22751.

studied is protein Arp, an IgA receptor expressed by some strains of *S. pyogenes* (Frithz *et al.*, 1989). This protein has C repeats, like the antiphagocytic M proteins, and exhibits N-terminal sequence variation, as demonstrated by N-terminal amino acid sequencing of Arp4 and Arp60, two variants of protein Arp expressed by group A streptococcal strains of different serotype (Frithz *et al.*, 1989; Lindahl & Åkerström, 1989). This similarity to the classical M proteins suggested that studies of protein Arp might shed new light on the mechanism of antigenic variation in the M protein family and prompted us to characterize the two variants of protein Arp in greater detail. The sequence of Arp60 has now been determined and compared with the previously reported sequence of Arp4 (Frithz *et al.*, 1989). The results show that the distinction between a variable N-terminal region and a conserved C-terminal region is more clear-cut in protein Arp than in any of the other class C proteins analysed so far, and they strongly suggest that the N-terminal sequence variation between the Arp60 and Arp4 proteins reflects an intergenic recombination event.

Methods

Preparation of DNA, restriction enzyme analysis and cloning. The λ clone used in this study carries a 15 kbp insert from *S. pyogenes* AW43, an M60 strain (Lindahl & Åkerström, 1989). This clone expresses the *arp60* gene, which encodes the IgA-binding protein designated Arp60. Phage stocks were prepared by confluent lysis on *Escherichia coli* LE392 as described by Arber *et al.* (1983). After polyethylene glycol precipitation and treatment with DNase and RNase, the phage particles were deproteinized using phenol and chloroform. The DNA was ethanol precipitated and purified by caesium chloride gradient centrifugation in the presence of ethidium bromide (Maniatis *et al.*, 1982). Digestion with restriction enzymes and ligations were performed under conditions recommended by the supplier (Boehringer Mannheim). Analysis of restriction enzyme fragments was performed using 0.8% agarose gels in Tris/acetate buffer (Maniatis *et al.*, 1982). Restriction enzyme fragments for subcloning in pUC18, pUC19, M13mp18 and M13mp19 (Yanisch-Perron *et al.*, 1985) were isolated from low gelling temperature agarose (BioRad) as described (Crouse *et al.*, 1983). Preparation of competent cells and transformation of *E. coli* JM83 (Yanisch-Perron *et al.*, 1985) and JM103 (Messing, 1983) were carried out as described by Morrison (1979). Plasmid DNA was isolated using an alkaline lysis procedure (Ish-Horowitz & Burke, 1981). The plasmid DNA was further purified by caesium chloride gradient centrifugation in the presence of ethidium bromide (Maniatis *et al.*, 1982). Southern blot analysis of restriction enzyme fragments was done using a standard protocol (Maniatis *et al.*, 1982) with Biotyne A (BNNG) membranes (PALL Corp.). DNA probes were prepared by nick-repair using [α - 32 P]dCTP and a kit from Amersham. Oligonucleotide probes were labelled in the 5' end with [γ - 32 P]ATP and polynucleotide kinase (Maniatis *et al.*, 1982).

DNA sequencing. Single stranded DNA from recombinant phages M13mp18 and mp19 was prepared as described by Messing (1983) and the nucleotide sequence of the inserts was determined by the dideoxy chain termination method (Sanger *et al.*, 1977, 1980) using [35 S]dATP (deoxyadenosine 5'-[α - 35 S]thiotriphosphate; Amersham) and Se-

quenase (Tabor & Richardson, 1987) (USB) following the protocol provided by the supplier. Part of the sequence was obtained from fragments cloned in pUC18 and pUC19 (Chen & Seeburg, 1985). Autoradiography was performed with Fuji X-ray film.

Colony immunoblot and protein analysis. The immunoblotting procedure using 125 I-labelled IgA for detection of colonies expressing IgA binding protein has been described (Frithz *et al.*, 1989). For isolation of protein Arp60, strain JM83 carrying the appropriate plasmid was grown in LB broth to early exponential phase and then subjected to osmotic shock (Anraku & Heppel, 1967; Russel & Model, 1982). The protein was purified from the osmotic shock lysate by affinity chromatography on IgA-Sepharose, as described (Frithz *et al.*, 1989).

Results and Discussion

Nucleotide sequence of the *arp60* gene

The structural gene for protein Arp60 was previously shown to be present in a 15 kbp insert of streptococcal DNA in a λ EMBL3 clone (Lindahl & Åkerström, 1989). Digestion of this insert with both *Sal*I and *Hind*III generated a number of fragments, which were ligated to the plasmid vectors pUC18 and pUC19. After transformation into *E. coli* JM83, bacteria were tested for expression of IgA-binding activity using a colony immunoblot method. One pUC19 clone (pARP104) expressed IgA-binding and was shown to contain an insert composed of a *Sal*I-*Hind*III fragment of 0.9 kbp and a 1.8 kbp *Hind*III fragment (Fig. 1).

Hybridization studies indicated that at least part of the *arp60* gene was present on the 1.8 kbp *Hind*III fragment, since this fragment hybridized to a *Bgl*II fragment comprising most of the region encoding the previously sequenced *arp4* gene (Frithz *et al.*, 1989). Sequence analysis of the *Hind*III fragment indeed showed the presence of an open reading frame with extensive sequence identity to the C-terminal part of protein Arp4. However, the *Hind*III fragment did not contain sequences corresponding to a signal sequence and a promoter. This result suggested that the IgA-binding expressed by plasmid pARP104 was due to the expression of an incomplete *arp60* gene lacking a segment in the 5' region, including the streptococcal promoter.

A Southern blot analysis of the λ clone used for this work showed that the 5' region of the *arp60* gene was present on a 1.4 kbp *Pst*I fragment. This *Pst*I fragment was isolated and introduced into pUC18 to generate pARP103 (Fig. 1), which expresses IgA-binding. The sequence of the 1.4 kbp *Pst*I fragment was determined on both strands (Fig. 1) and was shown to contain a considerable overlap with the sequence in pARP104, as expected (Fig. 2). Combination of the two sequences demonstrated the presence of an open reading frame of 1206 nucleotides, encoding a protein of 402 amino acids with a calculated M_r of 45522. The identity of this

protein with Arp60 was confirmed by comparison with the previously determined N-terminal amino acid sequence of the processed form of the protein (Lindahl & Åkerström, 1989). The amino acid composition of Arp60 is similar to that of Arp4 and streptococcal M proteins (Fischetti, 1989; Frithz *et al.*, 1989). Possible promoter regions, indicated in Fig. 2, are identical to the promoter regions proposed for the *arp4* gene and are also similar to the ones proposed for several streptococcal *emm* genes (see Frithz *et al.*, 1989). Downstream (27 bp) of the stop codon there is an inverted repeat with the potential to form a stem-loop structure, which could function as a transcription terminator (Rosenberg & Court, 1979).

The sequence of the *arp60* gene described above was deduced from two different inserts of streptococcal DNA, none of which contains the complete *arp60* gene. To obtain expression of the whole gene in *E. coli*, a *Pst*I–*Bgl*II fragment from pARP103 and a *Bgl*II–*Hind*III fragment from pARP104 (Fig. 1) were ligated to pUC18 digested with *Pst*I and *Hind*III generating pARP102. The structure of pARP102 was verified by cleavage with the appropriate restriction enzymes. The *arp60* gene was expressed in *E. coli* JM83 carrying pARP102, and it was also expressed when the fragment of streptococcal DNA

Protein	Amino acid sequences of the C-repeats	Residue number in the processed protein
Arp60	LEAEKQKLAKKEKQISDASRQGLSRDLEASRAAKKELEAKHQK	121–162/C1
	--T-H---KE-----E---KV--DLAA	163–204/C2
	-T--H---KE-----E---KV--DLAE	205–246/C3
Arp4	-----D-----	112–146/C1
	---H---KE-----E---KV--DLAA	147–188/C2
	-T--H---KED-----E---KV--DLAE	189–230/C3

Fig. 5. Comparison of the C repeat sequences in proteins Arp4 and Arp60. The dashes represent identical amino acid residues. The data for protein Arp60 are from Fig. 2 and those for protein Arp4 are from Frithz *et al.* (1989).

region in protein Arp4, and the corresponding nucleotide sequences are also virtually identical. Except for a 21 nucleotide deletion in the C1 region of the *arp4* gene, there are only eleven divergent nucleotide residues (of which eight are silent) in the 3'-terminal half of the two genes, which has a length of 723 nucleotides in *arp60*. At the protein level, the region of identity in the C-terminal half starts with the three C-repeats (amino acid residues 121–246). The C repeats in Arp60 and Arp4 are compared in Fig. 5, which shows that the C1, C2 and C3 repeats in protein Arp60 show a higher degree of homology to their counterparts in Arp4 than they do internally. This indicates that the C repeat region evolved in a common ancestor to *arp4* and *arp60*.

The striking conservation of the C-terminal region of protein Arp strongly suggests that the N-terminal sequence variation between Arp60 and Arp4 is the result of an intergenic recombination event. The origin of new variable regions and the nature of the postulated recombination event is not known, but it seems possible that recombination takes place after horizontal transfer of DNA between two streptococcal cells. In this context it should be noted that several other streptococcal cell surface proteins (the M49, H and emmL2 proteins) have the same overall structure as protein Arp (Haanes & Cleary, 1989; Gomi *et al.*, 1990; Bessen & Fischetti, 1992). The C-terminal parts of these proteins are homologous to the C-terminal parts of Arp4 and Arp60, but the N-terminal parts of the processed proteins do not show any homology to the Arp molecules. A similar pattern is seen when the sequences of the corresponding genes are compared. It therefore seems possible that the genes encoding these different proteins can recombine with the *arp* genes after transfer of DNA from one streptococcal cell to another.

The numbers of residues found in the C repeats of proteins Arp60 and Arp4 are multiples of seven (42 amino acid residues except for C1 in Arp4 which is 35 amino acid residues long). This finding supports the conclusion that the distribution of amino acid residues in protein Arp exhibits seven-residue periodicity, as in streptococcal M proteins (Fischetti, 1989; Frithz *et al.*, 1989).

Conserved sequences upstream and downstream of the *arp60* gene

Both the *arp4* and the *arp60* genes are flanked by open reading frames that have been sequenced or partially sequenced (Fig. 6). In the M4 strain, the *arp4* gene is located immediately downstream of the *mrp4* gene, which encodes a protein in class A of the M protein family (O'Toole *et al.*, 1992). Downstream of the *arp4* gene there is an open reading frame, *enn4*, from which one can deduce a protein in class C of the M protein family (Jeppson *et al.*, 1992). However, this sequence is probably not expressed at the protein level (Jeppson *et al.*, 1992). A similar arrangement is found in the M60 strain, where the *arp60* gene is closely linked to the *mrp60* gene (Stenberg *et al.*, 1992) and to the open reading frame *enn60*, which probably is silent (L.-O. Hedén, unpublished). It has been suggested that the silent *enn* genes can recombine with the homologous *arp* genes, thereby giving rise to structural variation (Haanes & Cleary, 1989; Jeppson *et al.*, 1992). However, recombination with the *enn* gene in the same chromosome cannot explain the N-terminal sequence variation between Arp4 and Arp60, since the N-terminal sequences of the putative Enn proteins are unrelated to the two Arp proteins. However, if an *arp* gene is horizontally transferred from another cell, it might be incorporated into the chromosome of the recipient by recombination in homologous regions, causing expression of a new variant of protein Arp in the recipient. To analyse the potential for such homologous recombination in the *arp* region, it was of interest to characterize the intergenic regions flanking the *arp* genes.

We determined the sequences of the intergenic regions upstream and downstream of the *arp60* gene (Fig. 1) and compared them with the corresponding sequences around the *arp4* gene, which are known from previous work (Jeppson *et al.*, 1992; O'Toole *et al.*, 1992). The results (Fig. 6) show that there are only five divergent residues and a deletion of one base in the region upstream of *arp60*, and that the intergenic region downstream is identical in the two systems. The variable parts of the *arp* genes are therefore flanked by long sequences that are

Fig. 6. Nucleotide sequences of the intergenic regions upstream and downstream of the *arp4* and *arp60* genes. The intergenic regions shown here include the postulated stop and initiation codons of the surrounding genes. The dashes represent identical nucleotide residues. The *mrp4* and *mrp60* genes encode proteins in class A of the M protein family (O'Toole *et al.*, 1992). The *mrp4* gene is known to be located immediately upstream of the *arp4* gene (O'Toole *et al.*, 1992), but it has not yet been conclusively shown that the *mrp60* gene is located immediately upstream of the *arp60* gene, as indicated by the dashed line. *enn4* and *enn60* are open reading frames from which one can deduce proteins in class C of the M protein family, but they are probably not expressed (Haanes & Cleary, 1989; Jeppson *et al.*, 1992; L.-O. Hedén, unpublished).

homologous in the two chromosomes. Upstream, this region of homology includes both the intergenic region and the sequence encoding the signal peptide. When the intergenic region upstream of the *arp* gene was compared with that downstream, the two regions were found to have only limited homology.

Concluding remarks

The sequence comparison of Arp60 and Arp4 reported here unequivocally shows that the processed form of protein Arp can be divided into a variable N-terminal region and a conserved C-terminal region. Comparison of the corresponding genes also shows that the *arp* gene has a well-defined variable region, which is surrounded by conserved regions. Upstream, the conserved region corresponds to the sequence between the *mrp* and *arp*

genes and to the sequence encoding the signal peptide. Downstream, the conserved region covers most of the *arp* gene and the entire sequence between the *arp* and *enn* genes (Fig. 6).

Jones *et al.* (1985) first postulated that the streptococcal M proteins have variable N-terminal and conserved C-terminal parts, and suggested that the structural variation in the N-terminal region is the result of a selective pressure that causes this part of the molecule to evolve faster in response to immunological selection by the host. This conclusion, which was made before extensive sequence data were available, was based on analysis of the reactivity of a set of monoclonal antibodies. Sequencing work subsequently led to the suggestion that the variable regions might be inserted by homologous recombination (Miller *et al.*, 1988; Haanes-Fritz *et al.*, 1988). Our results support the hypothesis that recombination between two different genes contributes

to the generation of structural variability in the M protein family, since it seems very likely that the N-terminal sequence variation between Arp60 and Arp4 is the result of a recombination event. Although it cannot be formally excluded that the N-terminal variation between Arp60 and Arp4 is the result of gradual accumulation of mutations, it would be difficult to reconcile such an explanation with the almost complete sequence conservation in the C-terminal part of the protein (and the corresponding part of the gene). The mechanism of the postulated recombination event is not understood, but it seems possible that new variable regions could be acquired from exogenous sources through transduction, transposition or transformation (Haanes-Fritz *et al.*, 1988; Laible *et al.*, 1991; Maynard Smith *et al.*, 1991).

Although the data discussed above suggest that intergenic recombination is an important mechanism for the generation of N-terminal sequence variation in the M protein family, it seems likely that accumulation of mutations also contributes to the structural variation (Fig. 4; see also Jones *et al.*, 1985). Furthermore, there is evidence that intragenic recombination between short repeated regions in the 5' part of the *emm6* gene can give rise to antigenically distinct variants of the M6 protein (Jones *et al.*, 1988; Fischetti, 1989). Taken together, these various data indicate that structural variation in the M protein family arises by several different mechanisms.

We are indebted to Miss E. Frithz for valuable contributions during the initial stages of this work.

This work was supported by grants from the Swedish Medical Research Council (projects 9096 and 9490), the Medical Faculty of the University of Lund, the Royal Physiographic Society in Lund, the Foundations of Bergvall, Cornell, Crafoord, Kock and Österlund, by Hightech Inc. and by King Gustaf V's 80-year foundation.

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