Mammalian antioxidant protein complements alkylhydroperoxide reductase (ahpC) mutation in Escherichia coli

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INTRODUCTION

The MERS5 [now called the Aop1 (antioxidant protein 1) gene] was cloned as a transiently expressed gene of murine erythroleukaemia (MEL) cell differentiation and its antisense expression inhibited differentiation of MEL cells. We found that the Aop1 gene shows significant nucleotide sequence similarity to the gene coding for the C22 subunit of Salmonella typhimurium alkylhydroperoxide reductase, which is also found in other bacteria, suggesting it functions as an antioxidant protein. Expression of the Aop1 gene product in E. coli deficient in the C22-subunit gene rescued resistance of the bacteria to alkylhydroperoxide. The human and mouse Aop1 genes are highly conserved, and they mapped to the regions syntenic between mouse and human chromosomes. Sequence comparisons with recently cloned mammalian Aop1 homologues suggest that these genes consist of a family that is responsible for regulation of cellular proliferation, differentiation and antioxidant functions.

MATERIALS AND METHODS

Bacterial mutants and construction of the MERS5 gene mutants

The MERS5 cDNA, which encoded a 257-amino-acid protein showing no sequence identity with other known proteins, was cloned from RNA preferentially synthesized in murine erythroleukaemia (MEL) cells during the early period of MEL cell differentiation [1]. To understand the role of the MERS5 gene in the differentiation, we have transferred the MERS5 cDNA into MEL cells in both sense and antisense orientation. Only in the transformants with the antisense MERS5 cDNA, did their elevated expression inhibit differentiation, suggesting that the MERS5 gene product may promote early events in the differentiation of MEL cells [2]. However, its action mechanism has not been described. After we had published the results of that study, the sequence of a C22 subunit of bacterial alkylhydroperoxide reductase was reported [3,4] and we found that the bacterial gene shows 38% identity with the MERS5 gene. Thus we attempted to examine whether the MERS5 gene product has the same function as the bacterial gene product. In the present study we report that the MERS5 gene can complement bacterial mutation and that its product is a new type of mammalian antioxidant protein.

Abbreviations used: ahp, alkylhydroperoxide reductase locus; MEL, murine erythroleukaemia; IPTG, isopropyl β-D-(-)-thiogalactopyranoside; RFLP, restriction-fragment-length polymorphism; Bpag2, bulous pemphigoid antigen 2; Cstgmr, colony-stimulating-factor granulocyte macrophage receptor alpha chain; ORF, open reading frame; SOD, superoxide dismutase; Aop1, antioxidant protein 1 (Aop1 is the gene coding for it); PAG, human proliferation-associated gene; TSA, thiol-specific-antioxidant (TSA is its gene).

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Table 1  Comparison of the sequence of the MER5 product with those of the protein encoded by abpC (C22) and other proteins. 

The alignment was carried out using Genetyx version 6. The sequence similarity to the MER5 product shows the most matched alignment between MER5 and each gene product. Seven amino acid residues of cysteine-containing conserved regions (1 and 2) are shown.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Source of gene</th>
<th>Function</th>
<th>Sequence identity with the MER5 product (%)</th>
<th>Region 1</th>
<th>Region 2</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C22</td>
<td><em>S. typhimurium</em></td>
<td>Alkaline hydroperoxide reductase</td>
<td>38.0</td>
<td>TFVCPTF</td>
<td>GEVCPAK</td>
<td>[3,4]</td>
</tr>
<tr>
<td>Ahp4</td>
<td><em>B. subtilis</em></td>
<td>Unknown</td>
<td>56.3</td>
<td>TFVCPTF</td>
<td>GEVCPAG</td>
<td>[16]</td>
</tr>
<tr>
<td>26 kDa</td>
<td><em>H. pylori</em></td>
<td>Unknown</td>
<td>48.9</td>
<td>TFVCPTF</td>
<td>GEVCPAG</td>
<td>[16]</td>
</tr>
<tr>
<td>ORF3</td>
<td><em>C. pasteurianum</em></td>
<td>Rubredoxin</td>
<td>54.4</td>
<td>TFVCPTF</td>
<td>GGMCALD</td>
<td>[15]</td>
</tr>
<tr>
<td>TSA</td>
<td><em>S. cerivisiae</em></td>
<td>TSA</td>
<td>58.2</td>
<td>TFVCPTF</td>
<td>VTLPCN</td>
<td>[20]</td>
</tr>
<tr>
<td>29 kDa</td>
<td><em>E. histolytica</em></td>
<td>Unknown</td>
<td>47.2</td>
<td>TFVCPTF</td>
<td>GAVCPLN</td>
<td>[19]</td>
</tr>
<tr>
<td>Arch</td>
<td><em>M. thermoauto trophicum</em></td>
<td>Unknown</td>
<td>30.7</td>
<td>TFVCTEF</td>
<td>GVAAPAN</td>
<td>[14]</td>
</tr>
</tbody>
</table>

human MER5 cDNA clone by the colony-hybridization method, *E. coli* containing cDNAs were seeded on a Luria-Bertani plate and the colonies formed were transferred on to nylon filters (Colon/Plaque Screen, du Pont). Filters containing 1 x 10^4 colonies/sheet were hybridized with a ^32P-labelled 798-bp *Pvu*II fragment containing almost-full-length MER5 cDNA [1]. Seven colonies were finally obtained from about 5 x 10^7 colonies of the library. All clones had the same length of cDNA (1.4 kb); thus one was selected and the cDNA fragment was converted into the Bluescript plasmid vector by a DNA Ligation Kit (Takara Shuzo, Kyoto, Japan). The sequences of both strands of the selected clone were determined using an M13 Sequencing Kit (Toyobo Co., Tokyo, Japan). Recording and analysis of the sequence were performed using the Genetyx and Genetyx-CD (Software Development Co., Tokyo, Japan).

Mouse chromosome mapping by the interspecific back-cross

The interspecific back-cross mapping panel has been typed for over 1500 loci that are well distributed among all the autosomes as well as the X chromosome [6]. C57BL/6J and *spretus* DNAs were digested with several restriction enzymes and analysed by Southern-blot hybridization for informative restriction-fragment-length polymorphisms (RFLPs) using a mouse cDNA *Aop1* probe. A 7.6 kb *M. spreitus EcoRI* RFLP was used to follow the segregation of the *Aop1* locus in back-cross mice. The mapping results indicated that *Aop1* is located in the distal region of the mouse chromosome 19 linked to bulbous pemphigoid antigen 2 (*Bpag2*) and colony-stimulating-factor granulocyte macrophage receptor alpha chain (*Csfgmrna*). Although 133 mice were analysed for every marker and are shown in the segregation analysis, up to 166 mice were typed for some pairs of markers. Each locus was analysed in pairwise combinations for recombination frequencies using the additional data. The ratio of the total number of mice exhibiting recombinant chromosomes to the total number of mice analysed for each pair of loci and the most likely gene order are: centromere-*Bpag2*-16/146-*Aop1*-3/166-*Csfgmrna*. The recombination frequencies [expressed as genetic distances in centimorgans (cM) ± S.E.M.] are: *Bpag2*-11.0 ± 3.0,*Aop1*-1.8 ± 1.0-*Csfgmrna*. For RFLP analysis, DNA isolation, restriction-enzyme digestion, agarose-gel electrophoresis, Southern-blot transfer and hybridization were performed essentially as described [7]. All blots were prepared with Zetabind nylon membrane (AMF-Cuno). The probe, a 1382 bp mouse cDNA clone, was labelled with [α-^32P]dCTP using a nick-translation labelling kit (Boehringer-Mannheim); washing was done to a final stringency of 0.8 x SSCP (96 mM NaCl/12 mM sodium citrate/12 mM Na_4HPO_4/4 mM NaH_2PO_4)/0.1% SDS at 65 °C. A major fragment of 8.8 kb was detected in the *EcoRI*-digested C57BL/6J DNA, and a major fragment of 7.6 kb was detected in *EcoRI*-digested *M. spreitus* DNA. The presence or absence of the 7.6 kb *M. spreitus-specific* EcoRI fragment was monitored in back-cross mice. A description of the probes and RFLPs for the loci linked to the *Aop1*, including *Bpag2* and *Csfgmrna*, has been reported previously [8,9]. Recombination distances were calculated as described [10] using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

Human chromosome mapping by in situ hybridization

Fluorescence in situ hybridization was carried out with a biotinylated cDNA probe. A *PstI*-YbaI fragment (1318 bp), containing almost the entire coding region of the human *Aop1* gene, was labelled with biotin-16-dUTP by nick translation and used for in situ chromosome mapping by the FISH method described by Takeda et al. [11].

RESULTS AND DISCUSSION

The MER5 gene shows significant nucleotide sequence identity with bacterial genes

Recent 'homology' searches showed that the MER5 gene product has a significant amino acid sequence similarity to the C22 subunit of bacterial (*Salmonella typhimurium*) alkylhydroperoxide reductase [3,4] and products of other bacterial genes, as shown in Table 1. *Salmonella* alkylhydroperoxide reductase consists of two subunits, C22 and F52a; F52a shows strong sequence similarity to thioredoxin reductase [4]. This operon consisting of C22 and F52a genes is induced by H_2O_2 under the regulation of OxyR [12]. The sequence similarity between the *Salmonella* C22 subunit (amino acid residues 11-170) and the MER5 product (amino acid residues 73-234) was 38.0%, and
the region near the cysteine residue showed high similarity among eight different genes. The Bacillus subtilis ahpA gene product, which might have a similar function to the C22 protein of Salmonella, showed a stronger similarity (P. Zuber, personal communication). The open reading frame (ORF) of the nucleotide sequence linked to the superoxide dismutase (SOD) gene of the archaeabacterium Methanobacterium thermoautotrophicum shows considerable similarity to the MERS gene [14]. It is noteworthy that the archaeabacterium C22 homologue is linked to the SOD gene rather than thioredoxin reductase gene. ORF3 is linked to the gene for Clostridium pasteurianum rubredoxin, which is one of the iron–sulphur proteins present in a variety of anaerobic bacteria and considered as an electron carrier in the oxidation system of fatty acids and hydrocarbons [15]. Since ORF3 is linked to ORF1, which shows similarity to the F52a-component, the alkyl hydroperoxide reductase gene is linked to rubredoxin gene in C. pasteurianum. Helicobacter pylori 26 kDa protein was identified from a gene encoding a species-specific protein of the bacterium [16].

Complementation of alkylhydroperoxide reductase (ahpC) mutation in E. coli

Because of a striking conservation of structure of the MERS gene with the bacterial genes, we examined whether this gene can complement the deficiency of the C22-subunit gene in E. coli. By introducing the plasmid pGS01, which deletes entire ahp locus, the bacterium (ahp5/pGS01) with only the ahpC locus was generated [5]. Then, the MERS gene that had been linked to the lac promoter in sense and antisense orientation, was introduced into E. coli (ahp5/pGS01) (Figure 1a). The bacteria were grown in the presence of IPTG (1 mM) to induce the MERS gene product, and the expression of the MERS gene product was confirmed by the Western-blot analysis using the antiserum generated for an oligopeptide deduced from the MERS gene sequence [2] (Figure 1b). The resistance of the bacteria to alkylhydroperoxide was monitored by their viability (Table 2). A 10–50-fold increase in resistance to cumene hydroperoxide was obtained in the bacteria expressing the MERS gene product. The bacteria also acquired resistance to other hydroperoxides, such as H$_2$O$_2$ and t-butyl hydroperoxide. The resistance to H$_2$O$_2$ was especially high. These results indicate that the MERS gene product can complement the activity of the C22 subunit of bacterial alkylhydroperoxide reductase. Since the conserved regions of the MERS gene contain cysteine residues and the functional importance of two cysteine residues has been suggested [3], we mutated two cysteine residues (Figure 1a) and determined whether these mutations had lost their MERS function. The results clearly showed that Cys1 and Cys12 mutants completely lack activity, while the effect of the Cys2 mutation seems less than the other two mutations (Table 2). These studies suggest that the Cys$^{109}$ residue of MERS 5 is very important for the complementation of the C22 subunit of bacterial alkylhydroperoxide reductase in E. coli.

Chromosome mapping of human and mouse Aop1 (MERS) genes

Because of a striking conservation of structure and function of the MERS gene with the bacterial antioxidant protein genes, we called the MERS gene product Aop1 (antioxidant protein 1) and examined gene conservation in the genomes of Man (Homo sapiens), chicken (Gallus gallus domestica), snapping turtle (Trionyx sinensis) and fruitfly (Drosophila melanogaster) by Southern-blot hybridization and demonstrated that these organisms contained regions of sequence identity (results not shown). Next, we compared its sequence similarity with the human gene by isolating it from a cDNA library of a human leukaemia cell line. The sequence similarity between the cloned human gene (256 amino acid residues) and the mouse gene (257 residues) was 86% (see Table 3). The chromosomal location of mouse Aop1 was determined by inter-specific back-cross analysis by using progeny derived from the mating of (C57BL/6J × M. spreitus) F1 mice with C57BL/6J mice [6–10]. The mapping
Table 2  Complementation of peroxidase resistance of the bacteria lacking the C22 subunit of alkylhydroperoxide reductase by the MERS gene products

<table>
<thead>
<tr>
<th>Peroxide</th>
<th>Expt.</th>
<th>Mutant...</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>(b)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>(c)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Table 3  Comparison of the amino acid sequences of the Aop1 gene family from mouse and Man

The alignment was carried out using Genetyx version 6. Sequence similarity to the MERS gene product and seven amino acid residues of the cysteine-containing conserved regions (1 and 2) are shown.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Sequence similarity to the MERS product (%)</th>
<th>Region 1</th>
<th>Region 2</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aop1 (human)</td>
<td>Unknown</td>
<td>86.0</td>
<td>TFVCPT</td>
<td>GEVCAP</td>
<td>[18]</td>
</tr>
<tr>
<td>MSP23 (mouse)</td>
<td>Stress protein</td>
<td>64.0</td>
<td>TFVCPT</td>
<td>GEVCAP</td>
<td>[18]</td>
</tr>
<tr>
<td>PAG (human)</td>
<td>Unknown</td>
<td>64.7</td>
<td>TFVCPT</td>
<td>GEVCAP</td>
<td>[17]</td>
</tr>
</tbody>
</table>

results indicated that Aop1 is located in the distal region of the mouse chromosome 19 linked to Bpag2 and Csfmgma as shown in Figure 2. The distal region of mouse chromosome 19 shares a region of sequence identity with human chromosome 10q and the pseudautosomal region of the X and Y chromosomes (summarized in Figure 2). Chromosomal localization of human Aop1 conducted by in situ hybridization [11] showed that the human Aop1 gene is located at 10q25–26, as shown in Figure 2. Thus both results are consistent with the location of the Aop1 gene in human and mouse chromosomes.

Aop genes consist of a family that is responsible for proliferation, differentiation and antioxidant function

After cloning of human Aop1 gene, we identified the sequence similarity to other mammalian genes (Table 3). Human proliferation-associated gene (PAG), which codes for a 22 kDa protein, shows 64.7% identity with the Aop1 gene [17]. Therefore our human Aop1 gene is not identical with PAG. MSP23 was cloned as a 23 kDa stress-induced mouse peritoneal macrophage protein [18]. It is noteworthy that MSP23 shows more similarity to the PAG than the Aop1 product, and these two gene products are about 60 amino acids shorter than Aop1 and human Aop1 gene products. The sequence similarity suggests that the PAG product is a human homologue of MSP23. Aop1, PAG and the MSP23 gene may consist of a family. It would be interesting to know whether more family members exist and whether these genes co-localize as a cluster within the same chromosomal region.

In addition, we found significant sequence identity with genes of two pathogenic human parasites. Entamoeba histolytica 29 kDa surface antigen protein shows significant identity with the Aop1 [19]. E. histolytica is a pathogenic protozoan causing extensive mortality and morbidity worldwide through diarrhoeal disease and organ abscess formation. The surface antigen may play an integral role in the modulation of host–parasite interactions. H. pylori 26 kDa protein [16] also showed significant sequence identity. H. pylori, a prevalent human-specific pathogen, is a causative agent in chronic active gastritis, gastric and duodenal ulcers and gastric adenocarcinoma, one of the most common forms of cancer in humans. It would be worthwhile to examine whether these proteins have an antioxidant function and are involved in pathogenesis.

In Saccharomyces cerevisiae (baker’s yeast), a thiol-specific antioxidant (TSA) gene was isolated and a tsu mutant, generated by homologous recombination, was produced. It was shown that the TSA product is not essential for cell viability under aerobic conditions, but under anaerobic conditions, especially in the presence of Methyl Viologen or a peroxide such as t-butyl peroxide and H2O2, the tsu mutant showed slow growth, suggesting that TSA acts as an antioxidant [20]. The sequence similarity between the Aop1 genes and TSA suggests that these genes function in an oxidation–reduction system. It would be worthwhile to test whether the Aop1 genes can complement the
of proliferation and differentiation. In fact, we have shown previously that the *Aop1* gene product enhances differentiation [2]. The *Aop1* gene products may be a new type of protein involved in regulation of proliferation/differentiation and antioxidant functions through redox regulation in mammalian cells.

We thank Dr. G. Storz for sending us the bacterial strains and plasmids and for helpful suggestions, and to Dr. S. Shibahara for providing human leukemia cDNA library. This work was partly supported by Grant-in-Aid for Scientific Research from Ministry of Education, Science and Culture of Japan and by the National Cancer Institute, Department of Health and Human Services, under contract NO1-CO-74101 with A. B. L.

**REFERENCES**

13. Reference deleted.

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**Figure 2** Chromosome localization of human and mouse *Aop1*

(a) *Aop1* maps in the distal region of mouse chromosome 19. *Aop1* was placed on mouse chromosome 19 by interspecific back-cross analysis. The segregation patterns of *Aop1* and flanking genes in 133 back-cross animals that were typed for all loci are shown at the top of the Figure. For individual pairs of loci, more than 133 animals were typed. Each column represents the chromosome identified in the back-cross progeny that was inherited from the (C57BL/6J × *M. spreitus*) F1 parent. The black boxes represent the presence of a C57BL/6J allele and white boxes represent the absence of the *M. spreitus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 19 linkage map showing the location of *Aop1* in relation to linked genes is shown at the bottom of the Figure. Recombination distances between loci in centimorgans are shown to the left of the chromosome and the positions of loci in human chromosomes, where known, are shown to the right. References for human map positions of loci cited in the present study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by the William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD, U.S.A.). (b) In situ localization of human *Aop1* gene on chromosome 10q25-26. Among 550 cells, 60 signals (single and twin spots in the Figure) were found on 10q25-26 [statistical significance (P) < 0.01].

*tsa* mutant of yeast. Perhaps because the *Aop1* gene was isolated as differentiation-related gene [1,2], proliferation-associated gene (*PAG*) [17] and stress-induced proteins (MSP23 and TSA) [18,20], it is likely that redox regulation may be involved in the regulation...