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# Detection of CD3ε polymorphism in cynomolgus monkeys by a method based on RFLP

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Abstract: We previously reported that peripheral lymphocytes from about 12% of cynomolgus monkeys lacked reactivity with anti-rhesus monkey CD3 monoclonal antibody (FN18). The nucleotide sequence analysis of the genes encoding CD3 component proteins revealed that a single amino acid substitutions found in the CD3ε chain determined the phenotype. In this study, we attempted to develop a method based on the restriction fragment length polymorphism (RFLP) and apply it for determination of the genotypes of individual monkeys. Comparison of the phenotype determined by fluorescence-activated cell sorter analysis with the genotype determined by RFLP analysis revealed that the FN18 -positive trait was dominant over the FN18-negative trait. It was also revealed that allele frequency was significantly different among macaques depending on the geographical region where their ancestors were derived from.

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## Introduction

Cynomolgus monkeys (*Macaca fascicularis*) are important experimental animals for biomedical research and understanding immunobiology of these animals is essential for interpretation of experimental data. The FN18 monoclonal antibody (mAb), which was raised against CD3 molecules of rhesus monkey (*Macaca mulatta*), is also able to be used for identification of T cells of cynomolgus monkey; however, it was shown that T cells from some cynomolgus monkeys and rhesus monkeys did not react with FN18 mAb [1–3, 7, 8].

The nucleotide sequence analysis of cDNAs coding for CD3 components showed that CD3ε chain from FN18 non-reactive cynomolgus monkeys had two common amino acid substitutions at positions 67 and 72 [8]. We have further shown that the amino acid at position 67 played a key role in determining the FN18 responsiveness by *in vitro* experiments using several mutated CD3ε genes [7].

In this study, we attempted to establish a method for genotyping individual monkeys based on the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method and used the method to determine the allele frequencies among cynomolgus monkeys derived from different geographical regions.

## Materials and Methods

### Animals

All the cynomolgus monkeys studied here were raised and reared in the Tsukuba Primate Center for Medical Science, NIID. Both genders were involved and the ages of the monkeys were between 2 and 16 years. This study was conducted in accordance with the Guide for Animal Experiments Performed at the National Institute of Infectious Disease.

### RFLP analysis

PolyA mRNA extracted from peripheral blood mononuclear cells (PBMCs) of FN18-reactive

cynomolgus monkeys using the Quickprep micro mRNA purification kit (Amersham, Uppsala, Sweden) was converted into cDNA using high fidelity RNA PCR kit (Takara, Shiga, Japan). Genomic DNA was extracted form PBMCs using the GeneTLE solution (Takara). PCR of CD3ε chain was performed in 20 μl reaction mixture containing ε-sense primer (5′-CTC CAT CTC TGG AAC CAC AGT A-3′) and ε-anti-sense primer (5′-CAG GTA GAG ATG ATG GCT CG-3′), 0.6–0.8 μg of genomic DNA or cDNA and 0.6 U of *ExTaq* polymerase (Takara). The size of PCR products was expected to be 207 bp. The reaction mixtures were heated at 95°C for 5 minutes, and then 40 cycles of amplification consisting of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s was carried out followed by an additional extension at 72°C for 7 minutes. About 0.3–0.5 μg of amplified DNA were digested with 2.5 U of *Mbo*II at 37°C for 2 hour followed by agarose gel electrophoresis using 4% gel. As similar results were obtained using both cDNA and genomic DNA as templates for PCR, most part of this study was conducted using the genomic DNA as template.

### Fluorescence-activated cell sorter (FACS) analysis

The PBMCs were isolated from fresh blood by standard Ficoll-Hypaque gradient centrifugation method. PBMCs were washed with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 containing 100 U/ml, 0.1 mg/ml streptomycin, and 10% foetal calf serum at a concentration of $10^6$ cells/ml. PBMCs ($10^5$) were incubated at 4°C for 1 hour with FN18 mAb (Biosource, Camlio, CA, USA), and washed twice with PBS containing 1% bovine serum albumin. After fixation with 1% paraformaldehyde at 4°C for 30 minutes, FACS analysis (FACS Caliber; Becton Dickinson, Cockeysville, MD, USA) was performed.

## Results

### Differentiation of genotype by the PCR–RFLP

As the nucleotide at position 200 of the FN18+ and FN18– cDNA clone was A and G, respectively, and the flanking sequence, GAAGA, gave rise to a recognition site by restriction enzyme *Mbo*II (Fig. 1), it was likely that the nucleotide difference could readily be differentiated by *Mbo*II digestion. As expected, the PCR amplicons derived from cloned FN18+ DNA was digested by *Mbo*II yielding two smaller bands, but that from FN18– clone was resistant to the digestion with *Mbo*II (Fig. 2A). We therefore attempted to apply this technique to determine the genotype of individual monkeys. There should be three distinct electrophoreic patterns of *Mbo*II-digested fragments. The PCR amplicons from homozygotes bearing A/A at position 200 should be resistant to *Mbo*II digestion, whereas those bearing G/G must be cut into two fragments. The PCR fragments from heterozygotes, however, would give rise to three fragments of 207, 113 and 94 bp, if properly digested. As incomplete digestion may be misleading, the PCR amplicons were subjected to complete digestion. In order to accomplish complete digestion, we used sufficient amount (2.5 U) of the enzyme to digest 0.3–0.5 μg of a short DNA fragment containing only one cutting site. Furthermore, the PCR amplicons from presumed heterozygotes were subjected to the nucleotide sequencing to assure that appearance of three fragments were due indeed to the heterozygocity. The results showed that both A and G were present at position 200 (data not shown), indicating that the presence of a 207-bp fragment after *Mbo*II digestion was because of the absence of *Mbo*II recognition site but not of incomplete digestion. These results indicate that RFLP analysis with *Mbo*II could be used as a powerful tool to determine the genotype of macaques.

### Inheritance of the polymorphism

To analyse how this polymorphism is inherited, a family consisted of three parents (one sire and two dams) and four offspring were selected and subjected to the PCR–RFLP analysis. They were bred and raised in the Tsukuba Primate Center for Medical Science, NIID. By FACS analysis it was shown that the sire (3028) was FN18 negative while the two dams (3032 and 1159) were positive

```
                                  67          72
          61   H N G  K N K E  D S G  D R L  F L P E  F S E   80
FN18 +/+ 181   CACAATGGTA AAAACAAAGA AGATTCTGGG GATCGGCTGT TTCTGCCGGA ATTTTCAGAA  240
FN18 -/- 181   ---------- ---------G ---------- ----AA---- ---------- ----------  240
          61   - - - - - - G - - - - Q - - - - - - - - -   80
```

*Fig. 1.* Nucleotide sequence of CD3ε. The nucleotide and the deduced amino acid sequences around polymorphic region were shown. Dots indicated identical nucleotide or amino acids. Recognition and cleavage site of *Mbo*II are indicated by underline and arrowhead, respectively.

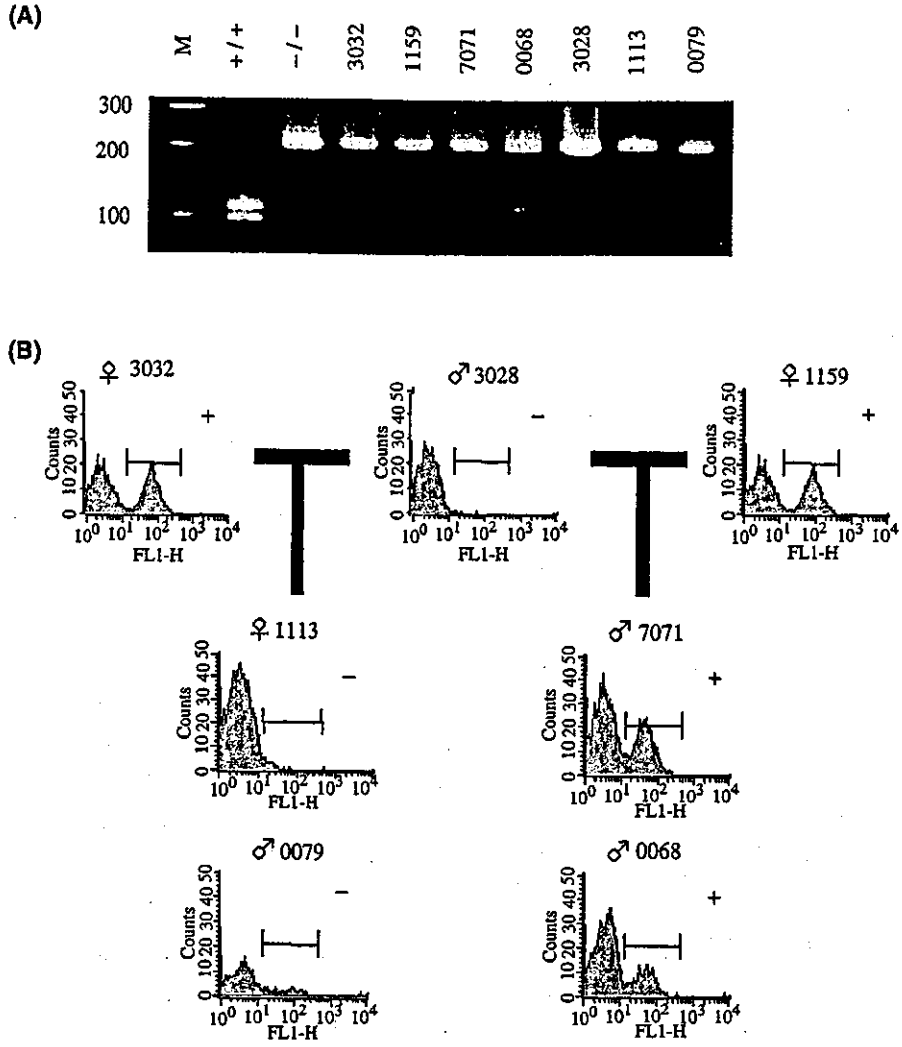


*Fig. 2.* The family pedigree demonstrating the inheritance of CD3ε genotypes and phenotypes. (A). The PCR products (207 bp) amplified from the genomic DNA of PBMCs were digested with *Mbo*II. The PCR products from cloned FN18 +/+ or −/− were also included as a positive or negative control. (B) The phenotypes regarding the reactivity with FN18 mAb were determined by FACS analysis. 1-H (*x*-axis): the fluorescence intensity of FN18 mAb.

(Fig. 2B). Similarly two offspring (1113 and 0079) were negative whereas the others (7071 and 0068) were positive. As the FACS profile of 0079 was rather ambiguous, we stained PBMC of this monkey with an mAb directed to monomorphic epitope of CD3ε (SP34). It was shown that SP34 positive cells did not react with FN18 confirming that 0079 was FN18 negative. The PCR–RFLP analysis using cDNA as templates showed that three monkeys (3028, 1113 and 0079) were homozygous (−/−) while the other monkeys (3032, 1159, 7071 and 0068) were heterozygous (+/−) (Fig. 2A). This finding indicates that these genes were co-dominantly expressed on RNA level. As there is no antibody available that would react with the protein expressed from the FN18 −/− genotype, the FN18-positive phenotype appeared to be inherited according to the Mendelian rules, and dominant over FN18.

### Allele frequency of monkeys from different geographical areas

We then applied the RFLP technique to determine the allele frequency of this particular single nucleotide polymorphism among cynomolgus monkeys whose ancestors were introduced from three different countries – Malaysia, Indonesia and Philippines. As shown in Table 1, the frequency of three genotypes, FN18 +/+, FN18 +/− and FN18 −/− in total, was 0.576, 0.339 and 0.085, respectively. It was noted, however, that the frequency of FN18 −/− was significantly higher (0.208) in the monkeys derived from Philippines

Table 1. The frequency of CD3ε genotype in cynomolgus monkeys

| | Country | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Malaysia | | Indonesia | | Philippine | | Total | |
| Genotype | Frequency | n | Frequency | n | Frequency | n | Frequency | n |
| FN18 +/+ | 0.808 | 38 | 0.675 | 52 | 0.226 | 12 | 0.576 | 102 |
| FN18 +/– | 0.149 | 7 | 0.299 | 23 | 0.566 | 30 | 0.339 | 60 |
| FN18 –/– | 0.043 | 2 | 0.026 | 2 | 0.208 | 11 | 0.085 | 15 |
| Total | 1.000 | 47 | 1.000 | 77 | 1.000 | 53 | 1.000 | 177 |

than in those from Malaysia (0.043) and Indonesia (0.026).

## Discussion

In this study, we established a simple method for the detection of CD3 polymorphism, and applied the method to analyse the mode of inheritance of the CD3ε polymorphism. We also determined allele frequency among monkeys originated from different countries. We found that the frequency of FN18 –/– genotype was higher in the Philippine population. This might be caused by bottleneck effect as mating was carried out among monkeys of the same origin. Another possibility was that FN18-negative gene had diffused widely into Philippine population as FN18-negative phenotype might be advantageous in reproduction or adaptation, in particular environment of Philippines.

There are several reports suggesting that the polymorphism found in human CD3 might be related to type I diabetes [4–6], but controversial results are also reported. It would be interesting to see whether there are any differences in biological properties between macaques of Philippine and other places. It also seems important to look at whether there are linkages between this polymorphism and certain diseases in cynomolgus monkeys.

In conclusion, we established a simple method to identify the polymorphism of CD3ε by genotyping using RFLP. The RFLP analysis of a large number of monkeys demonstrated that the frequency of the genotype of the CD3ε differed among cynomolgus monkeys of different origin of country.

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ORIGINAL PAPER

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# Detection of 14 alleles derived from the MHC class I *A* locus in cynomolgus monkeys

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**Abstract** A basic understanding of the major histocompatibility complex (MHC) class I, which, together with T-cell receptors, is a key player in antigen recognition by cytotoxic T lymphocytes, is necessary to study the cellular immune response to intracellular pathogens. The MHC has hardly been reported in cynomolgus monkeys (*Macaca facicularis*), although cynomolgus monkeys have been frequently used as the surrogate animal model. We attempted to determine the nucleotide sequences of the MHC class I *A* locus of cynomolgus monkeys (*Mafa-A*) and eventually 34 independent sequences of *Mafa-A* were obtained from 29 cynomolgus monkeys. These 34 sequences were classified into 14 *Mafa-A* alleles according to the results of phylogenetic analyses using the neighbor-joining method. One to three *Mafa-A* alleles were obtained from a single animal. We also tried to establish a multiplex PCR-SSP method for convenient typing of *Mafa-A* alleles. cDNA from a family of cynomolgus monkeys, which is composed of four sirs and four dams, were examined by multiplex PCR-SSP. The result of multiplex PCR-SSP showed that an individual cynomolgus monkey had two or three *Mafa-A* alleles, suggesting that the *A* locus of cynomolgus monkeys might be duplicated.

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**Keywords** Cynomolgus · Major histocompatibility complex · *Macaca facicularis* · Allele · PCR-SSP

## Introduction

The major histocompatibility complex (MHC) class I consists of heavy chain, $\beta_2$-microglobulin ($\beta_2$m), and antigen peptide (Hennecke et al. 2001). Human cells are known to express three highly polymorphic MHC heavy chains (*HLA-A*, *-B*, and *-C*) and three conserved MHC heavy chains (*HLA-E*, *-F*, and *-G*). *HLA-A*, *-B*, and *-C* present antigen peptides to cytotoxic T lymphocytes (CTL) and the CTL are then activated (Flynn et al. 1992; Hou et al. 1992; York and Rock 1996). These classical molecules, especially *HLA-C*, also provide both stimulatory and inhibitory signals to natural killer (NK) cells through killer cell immunoglobulin-like receptors (KIR) (Valiante et al. 1997).

The gene encoding the class I heavy chain is composed of eight exons. Exon 1 encodes the signal peptide, exons 2–4 specify the extracellular domains $\alpha_1$–$\alpha_3$, exon 5 codes for the transmembrane domain, and exons 6–8 code for the cytoplasmic domain. The $\alpha_1$ and $\alpha_2$ domains are the most polymorphic, while the $\alpha_3$ domain contributes to the $\beta_2$m association (Hebert et al. 2001) and interaction with the CD8 molecule (Salter et al. 1990).

The rhesus MHC has been extensively studied among non-human primates because rhesus monkeys are most frequently used as the surrogate animal model (Allen et al. 2001; Horton et al. 2001; Mothe et al. 2002) for HIV infection in human. Rhesus MHC (*Mamu*) class I *A* (Boyson et al. 1996b; Miller et al.1991; Urvater et al. 2000a; Voss and Letvin 1996; Watanabe et al. 1994), *B* (Boyson et al. 1996b; Voss and Letvin 1996; Yasutomi et al. 1995), *E* (Boyson et al. 1995), *F* (Otting and Bontrop 1993), *G* (Boyson et al. 1996a), *AG* (Slukvin et al. 1999), and *I* (Urvater et al. 2000b) have already been reported. Rhesus monkeys were shown to carry at least one *A* and two *B* loci, because three *Mamu-A* and five *Mamu-B* alleles have been identified in a single animal (Boyson et

al. 1996b). *HLA-C* homologues have been identified in the common chimpanzee, bonobo, gorilla, and orangutan (Adams et al. 1999, 2000; Cooper et al. 1998; de Groot et al. 2000; Lawlor et al. 1990, 1991), while no evidence of an *HLA-C* homologue was observed in old and new world monkeys (Adams and Parham 2001). Although SIV infection in cynomolgus monkeys is also used as the animal model for human HIV infection (McClure et al. 1990; Putkonen et al. 1992), there are few reports about cynomolgus MHC (*Mafa*) except for class II loci (Gaur and Nepom 1996; Kriener et al. 2000; Otting et al. 1992), class I *E* (Alvarez et al. 1997; Boyson et al. 1995), and *I* loci (Urvater et al. 2000b).

In this study, we have determined the nucleotide sequences of the genes coding for the cynomolgus MHC class I *A* molecules and found 14 *Mafa-A* alleles. In addition, we established a convenient method to detect the *Mafa-A* alleles.

## Materials and methods

### Animals

All the cynomolgus monkeys were raised and reared in the Tsukuba Primate Center for Medical Science, National Institute of Infectious Diseases. Both genders were involved and the cynomolgus monkeys were between 2 and 23 years old. This study was conducted in accordance with the Guide for Animal Experiments Performed at the National Institute of Infectious Diseases.

### RT-PCR and nucleotide sequencing

Peripheral blood mononuclear cells (PBMC) were isolated from the fresh blood of 29 cynomolgus monkeys by a standard Ficoll-Hypaque gradient method. PBMC were washed twice with PBS and suspended in 5 ml of RPMI-1640 (Sigma, St. Louis, Mo.) containing 100 U/ml penicillin (Meiji Seika Kaisha, Tokyo, Japan), 10% FCS (GIBCO-BRL, Grand Island, N.Y.), and 5 μg/ml concanavalin A (ConA; Pharmacia, Cleveland, Ohio) at a concentration of $10^5$ cells/ml. PBMC were cultured at 37 °C for 3–4 days. Messenger RNA extracted from the cultured PBMC (2–

**Table 1** Primers used for the amplification and sequencing of MHC class I cDNAs from cynomolgus monkeys

| Primer | Sequence | Concentration (pmol/sample) | Annealing temperature (°C) |
|---|---|---|---|
| Primers used for RT-PCR | | | 60 |
| Mafa-A-s | 5′-GCAGGATCCGAATCTCCCCAGACGCGCA-3′ | 10 | |
| Mafa-A-a | 5′-GCTCTAGACCTCACAAGGCAGCTGTC-3′ | 10 | |
| Mafa-A13-s | 5′-CGAACCCTCCTCCTGG-3′ | 10 | |
| Mafa-A1013-a | 5′-CTGAGAGTAGCTCCCTCCTTTTCTAT-3′ | 10 | |
| Primers used for multiplex PCR | | | |
| Primer set 1 | | | 72 |
| IA01-s | 5′-GCAGCGGGATGGAGAGGAA-3′ | 20 | |
| IA02-s | 5′-GCTGTGGTTGTGCCTTCTGGAAAA-3′ | 10 | |
| IA03-s | 5′-ACGCTGCAGCGCGCA-3′ | 2 | |
| IA04-s | 5′-GCGGCGGATGTGGCGGAGAG-3′ | 2 | |
| IA05-s | 5′-CTGCGACCTGGGGCCG-3′ | 2 | |
| IA-a | 5′-CCTGGGCACTGTCACTGCTT-3′ | 20 | |
| Primer set 2 | | | 72 |
| IA06-s | 5′-GGGCCTGTGCGTGGAGTCCCTG-3′ | 10 | |
| IA07-s | 5′-CACACTGACCTGGCAGCGT-3′ | 10 | |
| IA08-s | 5′-CTGCGACCTGGGGCCA-3′ | 10 | |
| IA09-s | 5′-CTACAACCAGAGCGAGGCCA-3′ | 10 | |
| IA10-s | 5′-GCAGCCCCGCTTCATCT-3′ | 10 | |
| IA-a | 5′-CCTGGGCACTGTCACTGCTT-3′ | 20 | |
| Primer set 3 | | | 70 |
| IA11-s | 5′-ACACATGTGACCCATCACCCT-3′ | 5 | |
| IA12-s | 5′-GCCGGAGTATTGGGACCA-3′ | 20 | |
| IA13-s | 5′-GGCCTGCAGGAGATGGAAA-3′ | 20 | |
| IA14-s | 5′-CGGACCTGGGGGCTCAA-3′ | 15 | |
| IA-a | 5′-CCTGGGCACTGTCACTGCTT-3′ | 20 | |
| Primers used for sequencing | | | |
| T7 primer | 5′-TAATACGACTCACTATAGGG-3′ | 3 | 55 |
| SP6 primer | 5′-ATTTAGGTGACACTATAG-3′ | 3 | 55 |
| Ia698 | 5′-TAGAAGCCCAGGGCCCAGGC-3′ | 3 | 55 |
| Is437 | 5′-ATTACATCGCCCTGAACGAG-3′ | 3 | 55 |

$10\times10^6$ cells) using a Quick Prep Micro mRNA Purification kit (Pharmacia Biotech, Uppsala, Sweden) were converted into cDNA using a High Fidelity RNA PCR kit (Takara, Shiga, Japan). The amplification of *A* locus was carried out by using specific primer sets, either Mafa-As/Mafa-Aa or Mafa-A13-s/Mafa-A1013-a (Table 1). Forty cycles of amplification were carried out at 94 °C for 30 s, at 60 °C for 30 s, and at 72 °C for 30 s, followed by an additional extension at 72 °C for 7 min using GeneAmp PCR System 9700 (Applied Biosystem, Norwalk, Conn.). The PCR products were cloned into pCR4-Blunt-TOPO plasmid using Zero Blunt TOPO PCR Cloning kit (Invitrogen, Carlsbad, Calif.). The clones were sequenced with sequencing primers T7, SP6, Ia698, and Is437 (Table 1) by an ABI model 310 DNA Sequencer (Applied Biosystem, Foster City, Calif.).

### The multiplex PCR-SSP

The multiplex PCR-SSP was carried out using cDNA from cynomolgus monkeys as the template. The primers used are listed in Table 1. Primer set 1 was a mixture of IA-01s, IA-02s, IA-03s, IA-04s, IA-05s, and IA-a, primer set 2 IA-06s, IA-07s, IA-08s, IA-09s, IA-10s, and IA-a, and primer set 3 IA-11s, IA-12s, IA-13s, IA-

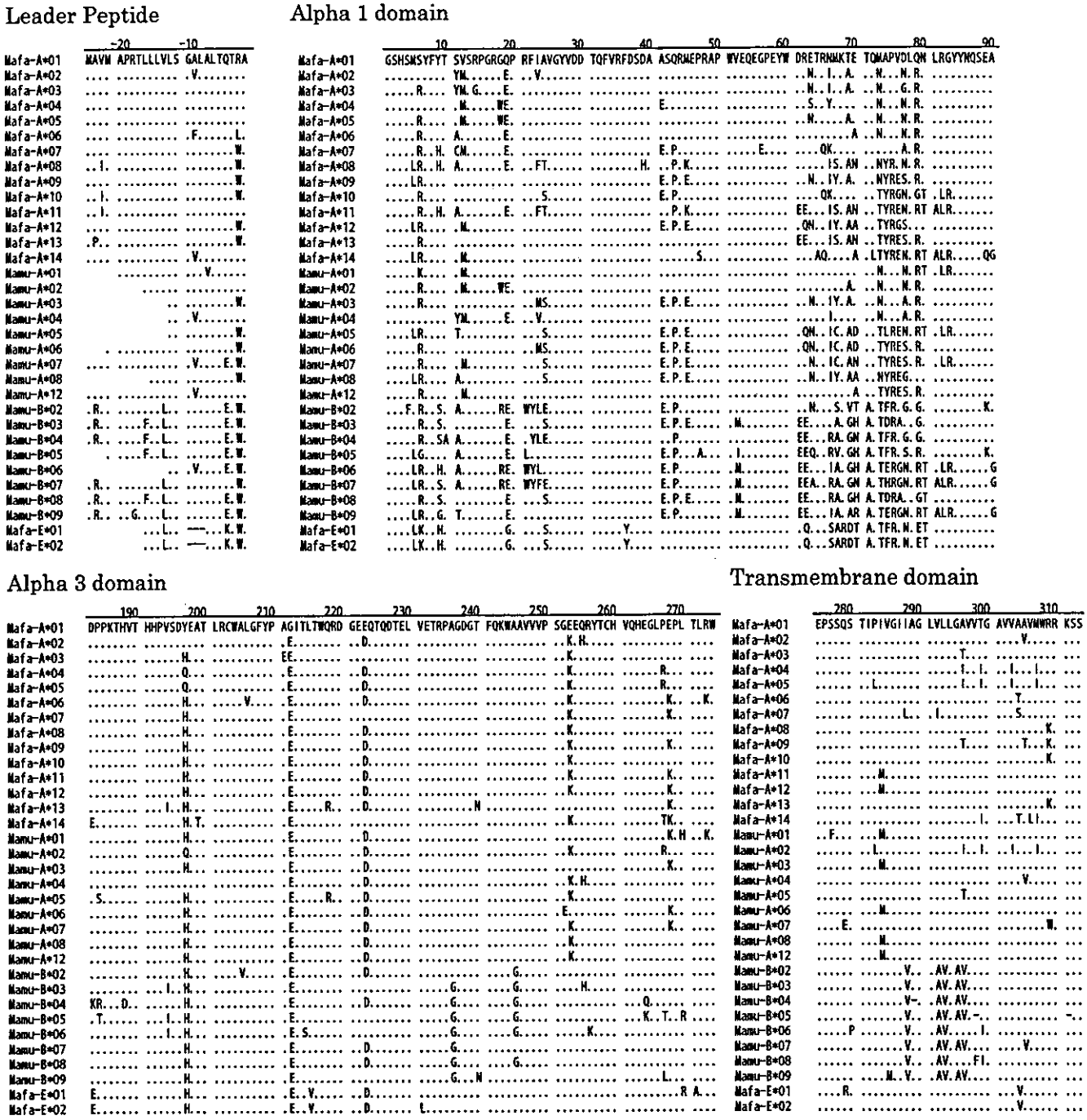


**Fig. 1** Alignment of predicted amino acid sequences of *Mafa-A* with previously reported *Mamu-A*, *Mamu-B*, and *Mafa-E* sequences. Identity to predicted amino acid sequence of *Mafa-A*01* is indicated by *dots*, whereas amino acid replacements are depicted by the conventional *one-letter code*. The deletions of amino acid are indicated by *hyphens*

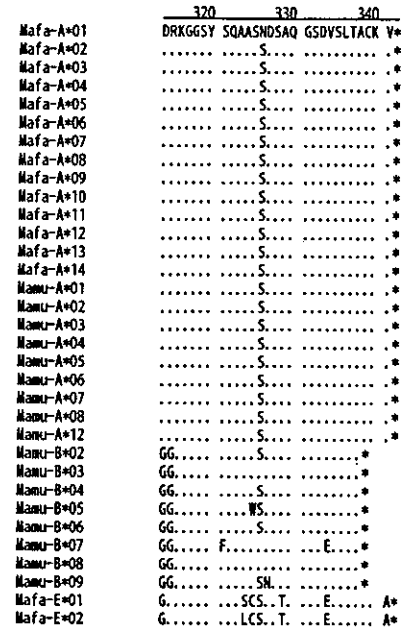


**Fig. 1** (continued)

14s, and IA-a. The concentrations of these primers were optimized after preliminary experiments (Table 1). The reactions were heated at 95 °C for 5 min, and then 25 cycles of amplification consisting of denaturation at 95 °C for 30 s, annealing either at 72 °C (for primer set 1 and set 2) or 70 °C (for primer set 3) for 30 s, and extension at 72 °C for 30 s. The amplicons were separated by 1.3% agarose gel electrophoresis and excised bands were purified using Freeze 'N Squeeze Spin Columns (Bio-Rad, Hercules, Calif.). The nucleotide sequences were determined by an ABI model 310 DNA sequencer (Applied Biosystem).

### GenBank accession numbers

The *Mafa-A* sequences described in this manuscript have been deposited at GenBank and were assigned accession numbers AB154760–AB154773. The GenBank accession numbers for all sequences used in this studies are as follows: *Gogo-B*0401,* AF157407; *Gogo-B*0501,* AF157408; *Gogo-B*0502,* AF157409; *Gogo-C*0103,* AF157410; *Gogo-C*0204,* AF157411; *HLA-A*0201,* AY365426; *HLA-A*0301,* L77702; *HLA-A*2402,* L47206; *HLA-A*2602,* M98453; *HLA-A*2901,* U83415; *HLA-A*8001,* L18898; *HLA-B*0801,* D83956; *HLA-B*1301,* D50290; *HLA-B*1802,* D25275; *HLA-B*2702,* L38504; *HLA-B*4201,* L76225; *HLA-B*57,* M32318; *HLA-B*7301,* U04787; *Mamu-A*01,* MMU50836; *Mamu-A*02,* MMU50837; *Mamu-A*03,* MMU41379; *Mamu-A*04,* MMU41380; *Mamu-A*05,* MMU41831; *Mamu-A*06,* MMU41834; *Mamu-A*07,* MMU41832; *Mamu-A*08,* AF243179; *Mamu-A*12,* AF157398; *Mamu-B*02,* MMU41833; *Mamu-B*03,* MMU41825; *Mamu-B*04,* MMU41826; *Mamu-B*05,* MMU41827; *Mamu-B*06,* MMU41828; *Mamu-B*07,* MMU41829; *Mamu-B*08,* MMU41830; *Mamu-B*09,* MMU41835; *Patr-A*0301,* AF500288; *Patr-A*0501,* AF500289; *Patr-A*0601,* AF500290; *Patr-A*11,* L47291; *Patr-A*14,* L47292; *Patr-B*01,* L47293; *Patr-B*16,* L47296; *Patr-B*17,* L47348; *Patr-C*03,* L47294; *Patr-C*04,* L47347; *Patr-C*05,* L47298; *Patr-C*06,* L47299; *Popy-A*0302,* AY034115; *Popy-A*0401,* AY034116; *Popy-A*0402,* AY034117; *Popy-A*0501,* AY034114; *Popy-B*0701,* AF118895; *Popy-B*0601,* AF118894; *Popy-B*0801,* AF118896; *Popy-B*0302,* AF118891; *Popy-B*0401,* AF118892; *Popy-B*0501,* AF118893; *Popy-C*0201,* AF118898; *Popy-C*0203,* AF470378; *Popy-C*0301,* AF470379; and *Popy-C*01, 012* AF470376.

## Results

### Detection of 14 MHC class I *A* locus alleles in cynomolgus monkeys

As there was no report on genes coding for class I MHC molecules in cynomolgus monkeys, we attempted to determine the nucleotide sequences of the alleles of the *A* locus in cynomolgus monkeys (*Mafa-A*). The amplicons obtained using *A*-locus-specific primers were cloned into pCR4-Blunt-TOPO plasmid and 8–48 independent clones were sequenced for each animal. When more than two clones from each animal showed the identical nucleotide sequences, the sequence was regarded as a consensus sequence representing a particular allele. Since PCR amplification and cloning procedures as well as sequencing are prone to misincorporation of nucleotides, amino acid sequences deduced from the nucleotide sequences were assessed by phylogenetic analysis using the neighbor-joining method (Saitou and Nei 1987) (data not shown). When the nucleotide sequence variation of the clone was negligible ($d$<0.025) compared with the consensus sequence, this particular clone was not considered to represent an independent allele. On the other hand, clones showing sequence variation of $d \geq 0.025$ were considered to represent independent alleles.

The deduced amino acid sequences of *Mafa-A* alleles are shown in Fig. 1, together with those published for *Mamu-A* and *-B*. All *Mafa-A* alleles encoded proteins of 365 amino acids. The putative glycosylation site was located at residue 86. In addition, conserved cysteine residues occurred at positions 101 and 164 in $\alpha_2$ and at positions 203 and 259 in $\alpha_3$. Other areas of similarity with *Mamu* included a region of variability at residues 77–83 near the C terminus of the $\alpha_1$ helix, analogous to the site of the Bw4/Bw6 motif in human.

To ascertain that these alleles were indeed derived from cynomolgus MHC class I *A* locus, they were compiled with other primate MHC class I loci (Fig. 2) and a phylogenetic tree was constructed using the amino acid sequences of $\alpha_1$ and $\alpha_2$ domains, since both domains were the most polymorphic regions of the molecule. The result showed that the sequences from cynomolgus monkeys aligned within the same cluster as those from other non-human primates, indicating that these cDNA clones were derived from distinct alleles of the MHC class I *A* locus of cynomolgus monkeys, *Mafa-A*.

A family consisting of eight animals was subjected to genetic analysis for better understanding of inheritance of *Mafa-A* alleles (Fig. 3, Table 2). By nucleotide sequence analysis, four *Mafa-A* alleles (*Mafa-A*03, -A*07, -A*11,* and *-A*14*) were found in this family. *Mafa-A*03* was detected in monkeys 2010 and 3005, whereas *Mafa-A*07* was detected in monkeys 8225 and 2010. *Mafa-A*11* was carried by monkeys 9234, 5117, 4045, 5076, and 1102,

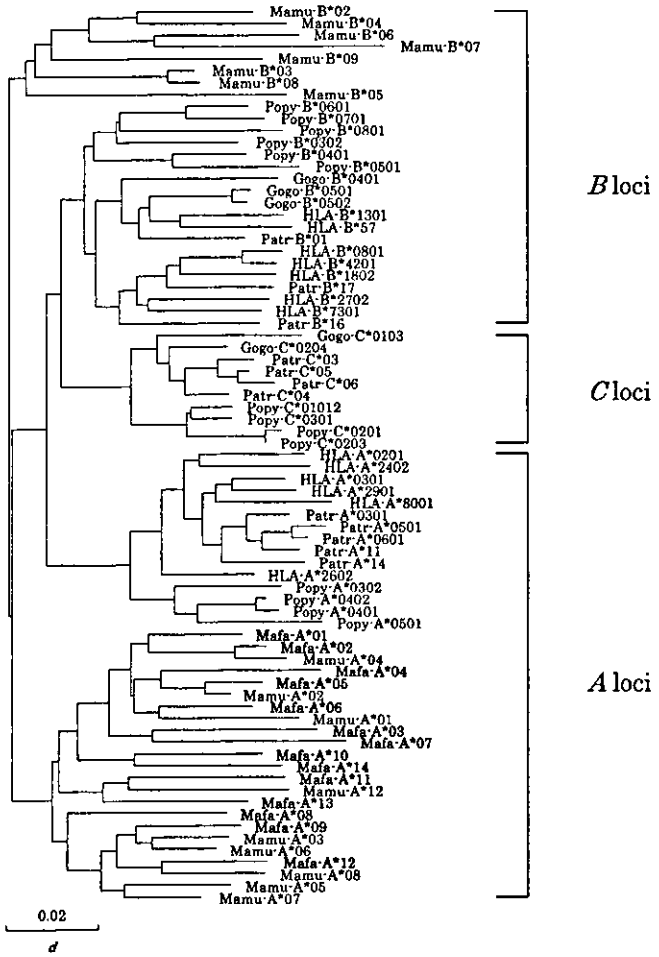


**Fig. 2** Phylogenetic analysis of primate class I MHC molecules. The phylogenetic tree of $\alpha_1$–$\alpha_2$ was constructed using nucleotide sequences with the neighbor-joining method by DNASIS pro (Hitachi software, Yokohama, Japan)

while *Mafa-A*14* was shared by monkeys 8225, 5117, 4045, 5076, and 3005. By nucleotide sequence analysis, only one allele (*Mafa-A*11*) was detected in 1102.

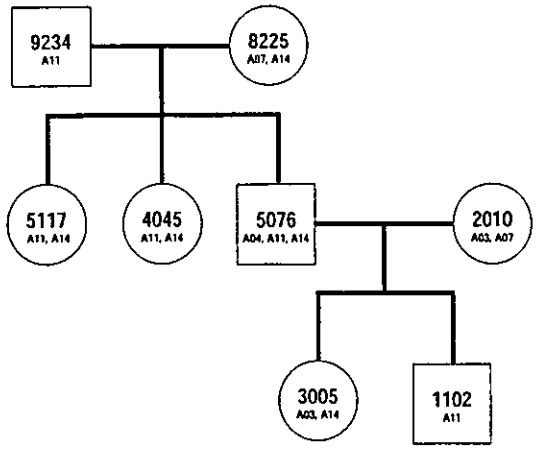


**Fig. 3** The family pedigree demonstrating the inheritance of alleles of the MHC class I *A* locus in cynomolgus monkeys. Male or female is denoted by a *square* or *circle*, respectively. The animal number and allele assigned to the animal are shown

## The establishment of the multiplex PCR-SSP method

We tried to develop a convenient method for MHC typing among relatively large numbers of animals. We have modified an existing PCR-SSP for detection of multiple allele simultaneously. The concentrations of primers and the annealing temperatures, which appeared critical, were described in Materials and methods in detail. To know whether this method was actually able to detect the *Mafa-A* alleles, plasmids harboring cDNA inserts for respective *Mafa-A* alleles were subjected to multiplex PCR-SSP. As shown in Fig. 4, distinct bands were amplified using appropriate primer sets, although the presence of non-specific bands was also noticed. The cDNAs obtained from members of the family mentioned above were analyzed by multiplex PCR-SSP. *Mafa-A*03* was found in monkeys 2010 and 3005. *Mafa-A*04* was detected in monkeys 5117, 4045, 5076, and 1102, while *Mafa-A*07* was detected in three monkeys: 8225, 2010 and 1102. *Mafa-A*08* was detected only in monkey 8225, while *Mafa-A*11* was shared by monkeys 5117, 4045, 5076, and 1102. *Mafa-A*14* was carried by monkeys 8225, 5117, 4045, 5076, and 3005. The results are summarized in

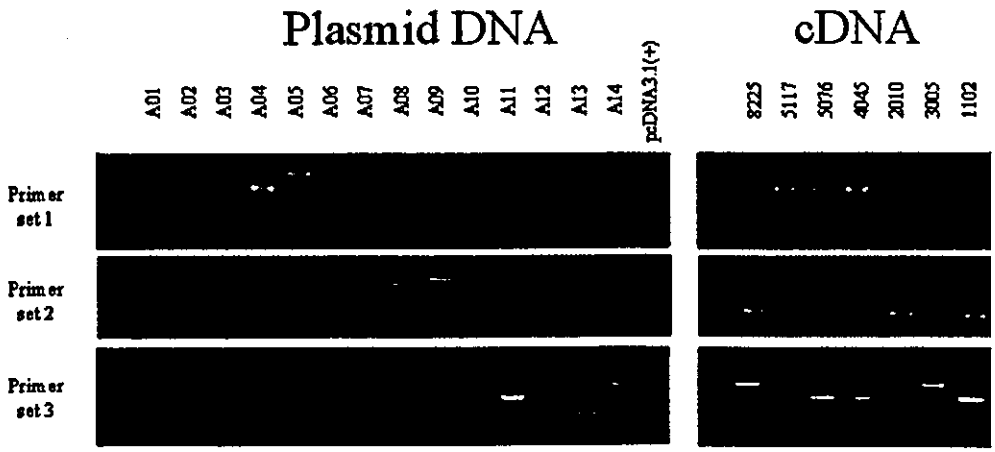


**Fig. 4** Multiplex PCR-SSP analyses of *Mafa-A* alleles. Identification of *Mafa-A*01* through *A*14* was conducted using cloned genes inserted into pcDNA3.1(−) vector (*left panel*). Copy DNAs obtained from seven cynomolgus monkeys were similarly subjected to multiplex PCR-SSP (*right panel*)

**Table 2** Summary of MHC class I cDNAs isolated from cynomolgus monkeys with RT-PCR and sequencing

| Animal | Allele | Number of copies | Primer pair/s |
|---|---|---|---|
| 9234 | *A11* | 8 | Mafa-A-s/Mafa-A-a |
| 8225 | *A07* | 5 | Mafa-A-s/Mafa-A-a |
| | *A14* | 31 | Mafa-A-s/Mafa-A-a |
| 5117 | *A11* | 20 | Mafa-A-s/Mafa-A-a |
| | *A14* | 11 | Mafa-A-s/Mafa-A-a |
| 4045 | *A11* | 35 | Mafa-A-s/Mafa-A-a |
| | *A14* | 7 | Mafa-A-s/Mafa-A-a |
| 5076 | *A04* | 6 | Mafa-A13-s/Mafa-A1013-a |
| | *A11* | 30 | Mafa-A-s/Mafa-A-a, Mafa-A13-s/Mafa-A1013-a |
| | *A14* | 10 | Mafa-A-s/Mafa-A-a, Mafa-A13-s/Mafa-A1013-a |
| 2010 | *A03* | 37 | Mafa-A-s/Mafa-A-a, Mafa-A13-s/Mafa-A1013-a |
| | *A07* | 9 | Mafa-A13-s/Mafa-A1013-a |
| 3005 | *A03* | 39 | Mafa-A-s/Mafa-A-a, Mafa-A13-s/Mafa-A1013-a |
| | *A14* | 15 | Mafa-A-s/Mafa-A-a, Mafa-A13-s/Mafa-A1013-a |
| 1102 | *A11* | 33 | Mafa-A-s/Mafa-A-a |

**Table 3** Summary of typing alleles with sequencing and multiplex PCR-SSP analysis on cynomolgus monkeys (*ND* not done)

| Animal | Sequencing | Multiplex PCR-SSP |
|---|---|---|
| 9234 | *A11* | ND |
| 8225 | *A07, A14* | *A07, A08, A14* |
| 5117 | *A11, A14* | *A04, A11, A14* |
| 4045 | *A11, A14* | *A04, A11, A14* |
| 5076 | *A04, A11, A14* | *A04, A11, A14* |
| 2010 | *A03, A07* | *A03, A07* |
| 3005 | *A03, A14* | *A03*, A14 |
| 1102 | *A11* | *A04, A07, A11* |

Table 3. Since the presence of the *Mafa-A*08* allele was only demonstrated by the multiplex PCR-SSP, the PCR amplicon was subjected to partial nucleotide sequence analysis. The result revealed that the DNA fragment identified as *Mafa-A*08* allele by multiplex PCR-SSP contained, in addition to that of an *Mafa-A* allele per se, the sequence derived from an allele that had not been found before. The results of similar analyses with large numbers of animals were consistent with the view that one to four *Mafa-A* alleles were present in an animal (data not shown). These results strongly suggested that the *A* locus in cynomolgus monkeys had been duplicated.

## Discussion

The polymorphic nature of MHC genes is particularly important in antigen recognition because it provides a way of presenting huge numbers of antigens by comparably limited numbers of MHC molecules. Numerous reports on the association of MHC alleles with susceptibility to HIV-1 infection in humans have been published; the prevalence of *HLA-B*14*, *-B*27*, and *-B*57* was shown to be significantly high among slow progressors, while a possible association of *HLA-B*35* and *-B*44* with shorter survival time was also demonstrated in HIV-1 infected patients (Gierowska et al.1999; Hendel et al. 1999; Migueles et al. 2000; Nelson et al. 1997). In SIV-infected rhesus monkeys, *Mamu-A*01* and *-A*1303* have been shown to be associated with not only longer survival time but also lower set-point viral load (Muhl et al.2002). However, as far as we know, there is no literature describing the associations of MHC polymorphism with progression of the disease in SIV-infected cynomolgus monkeys. Moreover, even the nucleotide sequence analyses have not been performed with regard to the class I MHC genes in cynomolgus monkeys. We therefore attempted to determine the nucleotide sequences of the MHC *A* locus genes. *Mafa-A* was amplified using *A*-locus-specific primer pairs. The results showed that at least 14 independent alleles of the class I *A* locus were found in cynomolgus monkeys.

We also have succeeded in developing a multiplex PCR-SSP method which enables us to readily detect several *Mafa-A* alleles simultaneously. When cDNA derived from cynomolgus monkey 8225 was analyzed by this method, however, the presence of a *Mafa-A* allele not detected by the usual RT-PCR was demonstrated. It seemed likely, therefore, that the primer pair designed to amplify *Mafa-A*08* was not specific enough to discriminate the *Mafa-A*08* allele from one with a very similar nucleotide sequence. Similarly, the primer pair designed to detect *Mafa-A*04* amplified a DNA fragment from not only the authentic *Mafa-A*04* allele but also an allele quite similar to *Mafa-A*04*. It seemed, therefore, premature to use only PCR-SSP for detection of either *Mafa-A*04* or *Mafa-A*08*; however, the method could be applied for detection of other alleles. Since we could not rule out completely the possibility that the primers designed to detect other alleles would cross-react with alleles yet to be found, further accumulation of the nucleotide sequences of *Mafa-A* alleles is required.

It was reported that rhesus MHC class I haplotype consisted of at least one *Mamu-A* and at least two *Mamu-B*

(Boyson et al. 1996b). No evidence for the presence of a *HLA-C* homologue was observed, suggesting that the evolution of the *C* locus in gorillas, chimpanzees, and humans is a fairly recent occurrence (Adams et al. 1999, 2000; Boyson et al. 1996b; Cooper et al. 1998; de Groot et al. 2000, Lawlor et al. 1990, 1991). This study showed that an individual cynomolgus monkey had two or three *Mafa-A* alleles, suggesting that the *A* locus of cynomolgus monkeys might be duplicated.

The amino acids that composed the Mafa-A pocket were compared with those for the *Mamu-A* pocket. From a crystallographic analysis, a peptide of 8–11 amino acids was demonstrated to bind into the groove of the MHC molecule, which was made up of $\alpha_1$ and $\alpha_2$ domains. In the case of the most HLA molecules, the second amino acid of the peptide is thought to play a crucial role in binding to the HLA molecule in conjunction with the C-terminal amino acid of the peptide. In the case of *Mamu-A*01*, however, the most important anchor residue was

**Fig. 5** Amino acid residues of pockets B, C, D, and F probably determining the peptide-binding specificity of cynomolgus monkey MHC class I molecule. Amino acids identical with *Mamu-A*01* are indicated by *dashes*

B pocket residue No.

| MHC allele | 7 | 9 | 24 | 25 | 34 | 45 | 63 | 66 | 67 | 70 | 99 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Mamu-A*01 | Y | Y | A | V | V | M | E | N | M | E | V |
| Mamu-A*1303,NA4 | - | - | - | - | - | - | - | - | - | A | - |
| Mafa-A*01 | - | - | - | - | - | - | N | I | - | - | Y |
| Mafa-A*02 | - | - | - | - | - | - | N | I | - | - | Y |
| Mafa-A*03 | - | - | - | - | - | - | S | I | - | - | Y |
| Mafa-A*04 | - | - | - | - | - | - | N | Y | - | - | L |
| Mafa-A*05 | - | - | - | - | - | - | - | - | - | - | Y |
| Mafa-A*06 | - | - | - | - | - | - | - | - | - | A | - |
| Mafa-A*07 | - | H | - | - | - | - | - | K | - | - | Y |
| Mafa-A*08 | - | H | T | - | - | K | N | I | S | N | Y |
| Mafa-A*09 | - | - | - | - | - | E | - | I | Y | - | Y |
| Mafa-A*10 | - | - | S | - | - | - | - | K | - | - | Y |
| Mafa-A*11 | - | H | T | - | - | K | N | I | S | N | F |
| Mafa-A*12 | - | - | - | - | - | E | - | I | Y | A | Y |
| Mafa-A*13 | - | - | - | - | - | - | - | I | S | N | Y |
| Mafa-A*14 | - | - | - | - | - | - | - | - | - | A | Y |

C pocket residue No.

| MHC allele | 9 | 22 | 70 | 73 | 74 | 97 | 99 | 114 | 116 |
|---|---|---|---|---|---|---|---|---|---|
| Mamu-A*01 | Y | F | E | N | A | R | V | E | Y |
| Mamu-A*1303,NA4 | - | - | A | - | - | - | - | - | - |
| Mafa-A*01 | - | - | - | M | - | T | Y | - | F |
| Mafa-A*02 | - | - | - | - | - | M | Y | D | F |
| Mafa-A*03 | - | - | - | - | - | I | Y | S | F |
| Mafa-A*04 | - | - | - | - | - | - | L | D | H |
| Mafa-A*05 | - | - | - | - | - | K | Y | H | S |
| Mafa-A*06 | - | - | A | - | - | - | - | - | - |
| Mafa-A*07 | H | - | - | M | - | V | Y | S | - |
| Mafa-A*08 | H | - | N | - | Y | K | Y | - | F |
| Mafa-A*09 | - | - | - | - | Y | K | Y | - | S |
| Mafa-A*10 | - | - | - | T | Y | T | Y | - | - |
| Mafa-A*11 | H | - | N | T | Y | - | F | S | - |
| Mafa-A*12 | - | - | A | T | Y | - | Y | S | - |
| Mafa-A*13 | - | - | N | T | Y | - | Y | S | H |
| Mafa-A*14 | - | - | A | T | Y | T | Y | D | S |

D pocket residue No.

| MHC allele | 99 | 114 | 155 | 156 | 159 | 160 |
|---|---|---|---|---|---|---|
| Mamu-A*01 | V | E | S | M | Y | L |
| Mamu-A*1303,NA4 | - | - | R | - | - | - |
| Mafa-A*01 | Y | - | Q | - | - | - |
| Mafa-A*02 | Y | D | Q | H | - | - |
| Mafa-A*03 | Y | S | Q | W | - | - |
| Mafa-A*04 | L | D | - | - | - | - |
| Mafa-A*05 | Y | H | - | F | - | - |
| Mafa-A*06 | - | - | Q | I | - | - |
| Mafa-A*07 | Y | S | Q | H | - | - |
| Mafa-A*08 | Y | - | Q | H | - | - |
| Mafa-A*09 | Y | - | Q | - | - | - |
| Mafa-A*10 | Y | - | Q | W | - | - |
| Mafa-A*11 | F | S | - | F | - | V |
| Mafa-A*12 | Y | S | - | F | - | V |
| Mafa-A*13 | Y | S | Q | I | - | - |
| Mafa-A*14 | Y | D | Q | Q | - | - |

F pocket residue No.

| MHC allele | 77 | 80 | 81 | 116 |
|---|---|---|---|---|
| Mamu-A*01 | N | T | L | Y |
| Mamu-A*1303,NA4 | A | N | - | - |
| Mafa-A*01 | D | N | - | F |
| Mafa-A*02 | - | N | - | F |
| Mafa-A*03 | G | N | - | F |
| Mafa-A*04 | - | N | - | H |
| Mafa-A*05 | - | N | - | S |
| Mafa-A*06 | - | N | - | - |
| Mafa-A*07 | A | N | - | - |
| Mafa-A*08 | - | N | - | F |
| Mafa-A*09 | S | N | - | S |
| Mafa-A*10 | - | - | - | - |
| Mafa-A*11 | - | - | A | - |
| Mafa-A*12 | S | N | - | H |

identified at the third position of the antigen peptide (Allen et al. 1998; Dzuris et al. 2000; Sidney et al. 2000). On the other hand, anchor motif analysis of *Mamu-B*03* showed that the second as well as the C-terminal amino acids of the peptide was indispensable for binding to the MHC molecule (Dzuris et al. 2000). The second amino acid of the antigenic peptide interacts with the B pocket composed of the amino acids shown in Fig. 5. A close look at the amino acid sequences suggested that a peptide presented by *Mafa-A*06* might have a similar motif to the peptide presented by *Mamu-A*1303*, as amino acids involved in the formation of the B pocket were identical in both MHC molecules. Since it was shown that *Mamu-A*01* and -*A*1303* were associated with longer survival and lower set-point viral load (Nelson et al. 1997) in SIV-infected rhesus monkeys, it would be of interest to look at whether the presence of the *Mafa-A*06* allele in a cynomolgus monkey would affect the regulation of viral replication of SIV.

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# G-CSF Receptor-mediated STAT3 activation and granulocyte differentiation in 32D cells

**Research Article**

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## Summary

**Granulocyte colony-stimulating factor (G-CSF) receptor (GcR) mediates growth and differentiation signals in the granulocyte/monocyte lineage of hematopoietic cells. To investigate the differentiation signal via GcR, a conditional receptor activation system was constructed. Wild-type and mutant GcRs were controlled by fusion to a molecular switch derived from the hormone binding domain of the estrogen receptor (ER). GcR-associated signaling molecules were analyzed in 32D progenitor cells that possess a potential of granulocyte differentiation. While the wild-type GcR-ER fusion molecule induced a granulocyte differentiation in 32D cells, a substitution of phenylalanine for tyrosine 703 (Y703F) in GcR resulted in a differentiation block. The activation of the JAK1 and JAK2 kinases was indistinguishable between the cells expressing the wild-type fusion and the Y703F mutant, and phosphorylation of the STAT5 transcription factor was comparable, too. On the other hand, tyrosine phosphorylation of STAT3 was significantly decreased following activation of the Y703F mutant compared to the wild-type GcR fusion. The results suggested that tyrosine 703 was responsible, at least in part, for transmitting a differentiation signal via STAT3 in 32D. The fusion system with the estrogen binding domain provides a valuable tool to analyze mutant effector proteins in the natural cellular milieu while bypassing the endogenous counterparts.**

## I. Introduction

Recent advances in stem cell biology, together with gene transfer technology, have led to the prospect of a new generation of cell therapy. However, many obstacles must be overcome before this vision becomes a reality. One major hurdle is to control transplanted cells in the recipient's body, in particular, to expand the desired cell subsets so that they exhibit therapeutic benefit. We have developed a novel system for selective expansion of genetically modified cells to supplement current gene transfer vectors (Ito et al, 1997; Kume et al, 2002). In this system, the target cells are harnessed with a 'selective amplifier gene (SAG)' which encodes a fusion protein comprising the granulocyte colony-stimulating factor (G-CSF) receptor (GcR) and the hormone binding domain (HBD) of the estrogen receptor (ER). The ER-HBD works as a molecular switch so that the fusion protein generates a GcR-derived growth signal upon binding to estrogen (Mattioni et al, 1994). Besides the prototype SAG encoding a chimera of the full-length GcR and ER-HBD (GcRER), a series of derivative fusion receptors were constructed to attain altered ligand specificity and signal characteristics. The modifications include a deletion of the G-CSF binding site (ΔGcR) (Ito et al, 1997), replacement of the ER with a mutant specific for 4-hydroxytamoxifen (TmR) (Xu et al, 1999), and the substitution of phenylalanine for the most proximal tyrosine residue in the GcR cytoplasmic domain (Y703FGcR) (Matsuda et al, 1999a).

The Y703F mutant is of particular interest because this amino acid substitution apparently led to a differentiation block in myeloid progenitor 32D cells (Matsuda et al, 1999a). To explore the mechanisms of granulocyte differentiation in 32D cells, we examined

JAK-STAT pathways involved in GcR signaling, and identified reduced STAT3 phosphorylation associated with the Y703F mutation.

## II. Materials and methods

### A. Plasmids and cells

Bicistronic vector plasmids were constructed with the pMX retrovirus backbone and the encephalomyocarditis virus (EMCV)-derived internal ribosome entry site (IRES; nucleotides 259-833 of EMCV-R genome) (Duke et al, 1992; Onishi et al, 1996). pMX/ΔGcRER-IRES-CD8a encodes a fusion protein of ΔGcR and ER-HBD, and murine CD8a as a selectable marker (Fukunaga et al, 1991; Koike et al, 1987; Nakauchi et al, 1985). The Y703F mutation in the GcR part was introduced into this plasmid as previously described (pMX/ΔY703FGcRER-IRES-CD8a) (Matsuda et al, 1999a). The recombinant DNA experiments were carried out following the National Institutes of Health guidelines and approved by the Jichi Medical School Recombinant DNA Research Advisory Board.

The murine myeloid progenitor line 32D and its derivatives were maintained in RPMI-1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Bioserum, Victoria, Australia) and 0.5% conditioned medium of C3H10T1/2 cells transfected with a murine IL-3 expression plasmid pBMG-hph-IL-3 (Valtieri et al, 1987; Matsuda et al, 1999a; Xu et al, 1999).

### B. Immunoprecipitation and western blotting

32D cells were deprived of serum and IL-3 for 3 hours at a density of 5 x $10^5$ cells/ml, and incubated in RPMI medium containing 1 mM $Na_3OV_4$ for an additional 1 hour at 1 x $10^7$ cells/ml. After starvation, cells were stimulated with either $10^{-7}$ M $E_2$ (Sigma, St. Louis, MO) or $10^{-9}$ M recombinant human G-CSF (provided by Chugai Pharmaceuticals, Tokyo, Japan) for given periods, then washed with ice-cold phosphate-buffered saline (PBS) containing 100 μM $Na_3OV_4$. Subsequently, cells were solubilized in lysis buffer (1% NP-40, 20 mM Tris-HCl [pH 7.4], 137 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin and 2 mM $Na_3OV_4$) on ice for 30 minutes, and centrifuged for 10 minutes. The soluble proteins were measured by Protein Assay (Bio-Rad, Hercules, CA).

For immunoprecipitation, the cell lysate containing 1 mg of protein was incubated with one of the following antibodies for 8 hours at 4°C: anti-JAK1 (Upstate Biotechnology, Lake Placid, NY), anti-JAK2 (Upstate Biotechnology), anti-STAT3 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-STAT5 (C-17; Santa Cruz Biotechnology). The immune complexes were absorbed by protein G-Sepharose beads (Sigma) for 2 hours at 4°C. The beads were washed with the lysis buffer and boiled in sample buffer (60 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol and 5% 2-mercaptoethanol) for 3 minutes. After centrifugation, the supernatants were subjected to SDS-7.5% polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride membranes (Immobilon-P; Millipore, Yonezawa, Japan). After blocking treatment with 5% bovine serum albumin (Fraction V; Roche Diagnostics, Mannheim, Germany), the membranes were incubated with an anti-phosphotyrosine antibody (4G10; Upstate Biotechnology) for 1 hour at room temperature. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Little Chalfont, UK). In some instances, membranes were stripped by incubation in denaturing buffer (62.5 mM Tris-HCl [pH 6.7], 2% SDS and 100 mM 2-mercaptoethanol) for 30 minutes at 50°C and reprobed with another antibody.

## III. Results

### A. Construction of conditionally activated G-CSF receptors

Structures of the chimeric receptors used in this study are shown in **Figure 1.** The fusion protein system is based on the fact that ER-HBD functions as an estrogen-specific molecular switch to control heterologous effector proteins, in our case, GcR (Mattioni et al, 1994). GcR belongs to the type I cytokine receptor superfamily, and its cytoplasmic domain comprises functionally distinct subdomains: the membrane-proximal region is sufficient for mitogenic signaling, and the membrane-distal portion is essential for granulocyte maturation (Dong et al, 1993; Fukunaga et al, 1993; Avalos, 1996; Koay and Sartorelli, 1999). All of the four conserved tyrosine residues in the cytoplasmic domain of GcR (at positions 703, 728, 743 and 763 in the murine GcR) are in the membrane-distal region and phosphorylated upon G-CSF stimulation. Among these, the tyrosine at position 703 (Y703) was most prominently phosphorylated and involved in granulocyte differentiation (Yoshikawa et al, 1995). However, previous studies on functional domains of GcR were carried out with ectopically expressed wild-type and mutant molecules in receptor-negative cells. It may be more informative if mutant receptors are analyzed in the natural intracellular environment where the endogenous molecule functions. From this viewpoint, the ER-HBD fusion system provides a valuable experimental tool. Estrogen specifically activates the introduced GcRER (and its derivatives) without influencing the endogenous GcR in the same cell, and the downstream events can be studied independently.

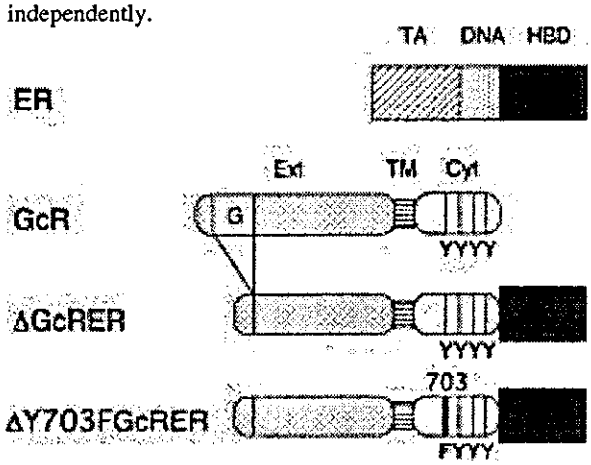


**Figure 1.** Structures of the chimeric receptors involved in this study. GcRER is a fusion of the full-length murine granulocyte colony-stimulating factor (G-CSF) receptor (GcR) and the hormone binding domain (HBD) of rat estrogen receptor (ER). ΔGcRER is a derivative of GcRER deleted of the G-CSF binding site (amino acids 5-195). ΔY703FGcRER carries a substitution of phenylalanine for a cytoplasmic tyrosine at position 703 (Y703F) in GcR. Ext, extracellular domain; G, G-CSF binding site; TM, transmembrane domain; Cyt, cytoplasmic domain; TA, transactivation domain; DNA, DNA binding domain; YYYY, conserved tyrosine residues in GcR cytoplasmic domain; FYYY, Y703F mutation in GcR.

In our previous report, the biological response to the ΔGcRER- and ΔY703FGcRER-mediated signal was evaluated in murine myeloid progenitor 32D cells (Δ designates a deletion of amino acids 5-195 required for G-CSF binding; Matsuda et al, 1999a). Parental 32D cells are dependent on interleukin-3 (IL-3) for continuous growth, and switching from IL-3 to G-CSF makes the cells differentiate into morphologically mature neutrophils (Valtieri et al, 1987). By retrovirus-mediated gene transfer, stable clones expressing ΔGcRER (32D/ΔGcRER) or ΔY703FGcRER (32D/ΔY703FGcRER) were established and stimulated by estrogen. While estrogen-treated 32D/ΔGcRER cells underwent granulocyte differentiation indistinguishable from that seen in G-CSF-treated cells, 32D/ΔY703FGcRER cells showed a distinct phenotype. Estrogen supported a long-term proliferation of 32D/ΔY703FGcRER with myeloblastic appearance, indicating that the Y703F mutation abrogated the differentiation signal (Matsuda et al, 1999a). This observation prompted us to characterize signaling molecules downstream of GcR in more detail.

Following ligand-induced homodimerization, GcR induces a wide array of intracellular signaling events (Avalos, 1996). Like many other cytokine receptors, GcR has no intrinsic kinase activity; instead, it recruits and activates other cytoplasmic kinases such as Janus kinases (JAKs), signal transducer and activation of transcription (STAT) proteins, Src family kinases and components of the mitogen-activated protein kinase pathway. The activation of JAKs is one of the earliest events in the GcR signaling cascade, followed by the tyrosine phosphorylation of STATs and GcR itself (Nicholson et al, 1994; Dong et al, 1995). Since the signal transduction for granulocyte differentiation has been ascribed to the JAK-STAT pathway, we focused on these molecules in ΔGcRER and ΔY703FGcRER cells.

## B. Estrogen-induced phosphorylation of JAK1 and JAK2 via fusion receptors

First, we examined the tyrosine phosphorylation of JAK1 and JAK2. As shown in **Figure 2**, these kinases were not tyrosine-phosphorylated in resting 32D/ΔGcRER and 32D/ΔY703FGcRER cells. Addition of G-CSF rapidly induced phosphorylation of JAK1 and JAK2; this event was induced by dimerization of the endogenous GcR, and maximal activation was observed within 10 minutes (data not shown). Similarly, $10^{-7}$ M 17β-estradiol ($E_2$) induced tyrosine phosphorylation of JAK1 and JAK2 in these cells (**Figure 2**). The estrogen-induced activation of JAK1 and JAK2 was mediated by chimeric receptors, at a slower rate than the activation mediated by the endogenous GcR; the maximal phosphorylation was observed 60 minutes after $E_2$ addition (time course not shown). The difference in kinetics of JAK1/JAK2 phosphorylation may be due to different mechanisms of receptor activation. While G-CSF directly crosslinks GcR at the extracellular domain, the activation of ER-HBD fusion receptors is a ligand-induced derepression that involves other proteins such as HSP90 (Mattioni et al, 1994). Nevertheless, the levels of JAK1/JAK2 phosphorylation were comparable whether the cells were stimulated with G-CSF or estrogen. As shown in **Figure 2**, the levels of estrogen-induced JAK1/JAK2 phosphorylation in 32D/ΔY703FGcRER cells were comparable to those seen in 32D/ΔGcRER cells. Reprobing of the blots with anti-JAK1 and anti-JAK2 antibodies showed that approximately equal amounts of the kinases were loaded on these lanes (not shown). Thus, we concluded that the Y703F mutation had little, if any, effect on the tyrosine phosphorylation of JAK1 and JAK2. Considering that JAK1 and JAK2 are constitutively associated with the membrane-proximal region of GcR which is sufficient to activate them (Nicholson et al, 1994; Dong et al, 1995; Avalos, 1996), it is conceivable that the kinases were not affected by the GcR mutation in the membrane-distal region.

## C. Comparable STAT5 phosphorylation following fusion receptor activation

Next, we investigated the activation of STAT proteins in 32D/ΔGcRER and 32D/ΔY703FGcRER cells. It was shown that G-CSF-induced signaling involves STAT1, STAT3 and STAT5 (Tian et al, 1994; de Koning et al, 1996; Tian et al, 1996; Shimozaki et al, 1997; Dong et al, 1998; Chakraborty et al, 1999; Ward et al, 1999). Since the membrane-distal cytoplasmic region of GcR was not required for STAT1 activation (de Koning et al., 1996), we addressed whether the phosphorylation of STAT5 and STAT3 is affected by the Y703F mutation. **Figure 3** shows the time course of STAT5 activation in 32D/ΔGcRER and 32D/ΔY703FGcRER cells (upper panel). STAT5 was not tyrosine-phosphorylated in unstimulated 32D cells, and addition of $10^{-9}$ M G-CSF induced a rapid phosphorylation of this molecule through crosslinking of the endogenous GcR. On the other hand, $10^{-7}$ M of $E_2$ induced a slower and less extensive phosphorylation of STAT5.

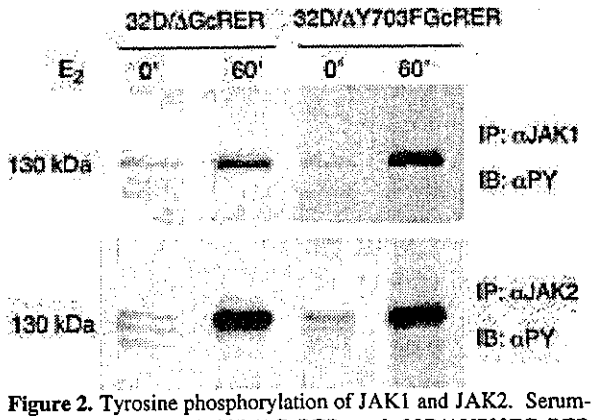


**Figure 2.** Tyrosine phosphorylation of JAK1 and JAK2. Serum- and cytokine-starved 32D/ΔGcRER and 32D/ΔY703FGcRER cells were harvested before (0') and after 60 minutes (60') of incubation with $10^{-7}$ M of estradiol ($E_2$). Lysates from 32D/ΔGcRER and 32D/ΔY703FGcRER cells were immunoprecipitated (IP) with either an anti-JAK1 (αJAK1; upper panel) or an anti-JAK2 (αJAK2; lower panel) antibody. Immunoblotting (IB) was carried out with an anti-phosphotyrosine antibody (αPY).

The estrogen-induced STAT5 activation was comparable in 32D/ΔGcRER and 32D/ΔY703FGcRER cells at 60 minutes after stimulation, and reprobing of the blot with an anti-STAT5 antibody showed that approximately equal amounts of STAT5 were loaded (**Figure 3**, lower panel). The delay in STAT5 phosphorylation may be associated with a slower JAK1/JAK2 activation through estrogen-induced dimerization of the chimeric receptors. The reason for the reduced STAT5 phosphorylation in the $E_2$-stimulated cells is currently unknown; we speculate that the linking of ER-HBD to the C-terminal of GcR might hinder STAT proteins from freely accessing the membrane-distal region of the receptor. In any case, STAT5 appeared to be phosphorylated to the same extent in 32D/ΔGcRER and 32D/ΔY703FGcRER cells. Others demonstrated that STAT5 was activated even when the membrane-distal region of GcR was deleted or the receptor tyrosine phosphorylation was abrogated (Shimozaki et al, 1997; Tian et al, 1996). Taken together with our observation that JAK1 and JAK2 were activated in both 32D/ΔGcRER and 32D/ΔY703FGcRER cells (**Figure 2**), we concluded that the Y703F mutation did not affect the tyrosine phosphorylation of STAT5.

### D. Decrease in STAT3 Activation by Y703F G-CSF Receptor Mutant

Finally, we addressed whether the Y703F mutation in GcR affects tyrosine phosphorylation of STAT3. After cytokine starvation, 32D/ΔGcRER and 32D/ΔY703FGcRER clones were incubated with $10^{-7}$ M of $E_2$ for 60 minutes. While estrogen induced a significant tyrosine phosphorylation of STAT3 in 32D/ΔGcRER, only a slight activation of STAT3 was detected in 32D/ΔY703FGcRER clones (**Figure 4**, upper panel, arrow). Reprobing of the membrane with an anti-STAT3 antibody revealed an even loading of STAT3 in these lanes (**Figure 4**, lower panel).

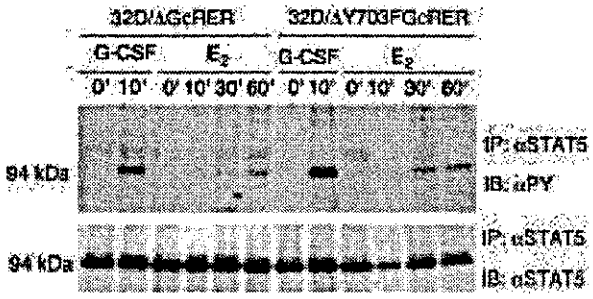


**Figure 3.** Tyrosine phosphorylation of STAT5. Starved 32D/ΔGcRER and 32D/ΔY703FGcRER cells were harvested before (0') and after 10, 30, and 60 minutes (10', 30', 60') of incubation with $10^{-9}$ M of G-CSF or $10^{-7}$ M of estradiol ($E_2$). Lysates were immunoprecipitated (IP) with an anti-STAT5 antibody (αSTAT5) and immunoblotted (IB) with an anti-phosphotyrosine antibody (αPY; upper panel). The blot was reprobed with the anti-STAT5 antibody to confirm the equal loading of STAT5 (lower panel).

Repeated experiments constantly demonstrated a decreased STAT3 phosphorylation in 32D/ΔY703FGcRER. Consistent with this observation, Tian et al showed that the G-CSF-induced STAT3 activation was greatly abrogated in UT-7epo cell transfectants by deleting a membrane-distal part including Y703 from GcR (Tian et al, 1996). We therefore concluded that Y703 in GcR was involved in STAT3 activation, and that the event is crucial to granulocyte differentiation in 32D cells.

## IV. Discussion

The phosphotyrosine residues in GcR create potential docking sites for the recruitment of signaling molecules such as STATs that contain a Src homology 2 (SH2) domain. STAT3 is recruited via the interaction of its SH2 domain with receptor tyrosine residues that are present in a tyrosine-X-X-glutamine (YXXQ) sequence (Stahl et al, 1995). Among four conserved tyrosine residues in the cytoplasmic region of GcR, only Y703 provides a YXXQ motif, accounting for the reduced STAT3 activation by the Y703F mutant. However, there was a residual level of STAT3 activation in ΔY703FGcRER and other GcR mutants devoid of this motif, which suggested the presence of another STAT3 binding site in GcR or some bridging molecule (Avalos, 1996; Chakraborty et al, 1999). We observed a few additional phosphorylated proteins coimmunoprecipitated with STAT3 including a 130 kDa species (**Figure 4**, upper panel, arrowheads). These proteins are yet to be identified; at least they did not react with an antibody against GcR in a subsequent reprobing (data not shown).

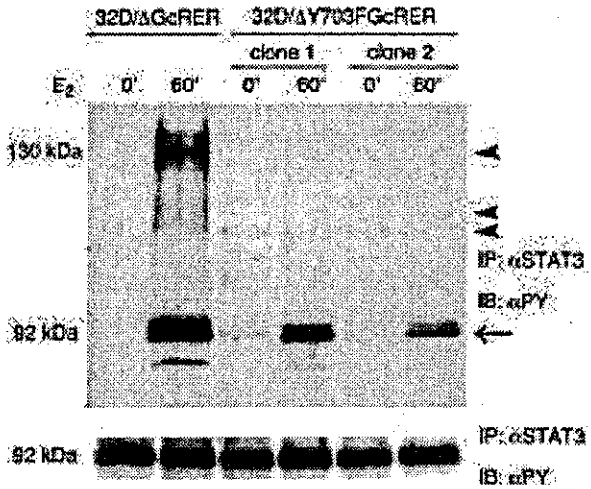


**Figure 4.** Tyrosine phosphorylation of STAT3. Starved 32D/ΔGcRER and 32D/ΔY703FGcRER (clone 1 and clone 2) cells were harvested before (0') and after 60 minutes (60') of incubation with $10^{-7}$ M of estradiol ($E_2$). Lysates were immunoprecipitated (IP) with an anti-STAT3 antibody (αSTAT3) and immunoblotted (IB) with an anti-phosphotyrosine antibody (αPY; upper panel). The blot was reprobed with the anti-STAT3 antibody to confirm the equal loading of STAT3 (lower panel). Besides STAT3 (92 kDa, arrow), several phosphoproteins including a 130 kDa species (arrowheads) were coimmunoprecipitated.

A consensus has been reached that tyrosine phosphorylation of GcR and activation of STAT3 is crucial to granulocyte differentiation, but there remains some controversy over the relative contribution of each tyrosine residue depending on the cells used (Tian et al, 1994, 1996; de Koning et al, 1996; Shimozaki et al, 1997; Chakraborty et al, 1999; Ward et al, 1999). Previous reports employed either GcR-negative cells to examine the function of the receptor and associated molecules, or overexpression of dominant-negative forms of GcR to elucidate the mechanisms for growth and differentiation. By using ER-HBD fusion proteins to bypass endogenous GcR, we herein provided additional data suggesting the major involvement of Y703 in STAT3 activation. It is of particular note that the cells retained the expression of wild-type GcR and downstream signaling molecules, thereby rapidly undergoing granulocyte differentiation in response to G-CSF, indistinguishable from the parent 32D cells (Matsuda et al, 1999a).

Contrary to its promoting function in myeloid cell differentiation, STAT3 was shown to play a central role in the maintenance of the pluripotent phenotype of embryonic stem cells (Matsuda et al, 1999b; Niwa et al, 1998). STAT3 appears to dictate widely divergent instructions such as differentiation and proliferation depending on the cell type. Thus, it is crucial to set up an appropriate venue to study the physiological molecular interaction involving a promiscuous molecule such as STAT3. The HBD fusion system provides a powerful tool to examine the behavior of mutated proteins controlled by specific ligands, in the exact milieu where the wild-type molecules coexist but remain unstimulated.

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