Clin Exp Immunol 1997; **110**:226–232

IgA nephropathy-specific expression of the IgA Fc receptors (CD89) on blood phagocytic cells

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(Accepted for publication 26 July 1997)

SUMMARY

We analysed the biochemical features of receptors for the Fc-region of IgA (FcαR, CD89) on blood monocytes and granulocytes of patients with IgA nephropathy (IgAN). FcαR on monocytes of IgAN were found to have a higher Mr (60–80 kD) than those of control monocytes (50–75 kD) and granulocytes (55–75 kD) in both IgAN and controls as shown by immunoprecipitation analysis. Removal of N-linked carbohydrates from FcαR on monocytes of IgAN revealed a 32–36 kD protein core, the Mr of which was still higher than that of controls (28–32 kD). When FcαR transcripts were analysed by reverse-transcription-PCR, only one prominent band was visualized in PCR products from IgAN monocytes. Since the results thus far show that IgAN monocytes express FcαR protein and mRNA differently from granulocytes and control monocytes, PCR products were then cloned and sequenced. The predominant band in PCR products from IgAN monocytes was identical to that of the FcαR a.1 transcript, and an additional 10 transcripts containing five novel transcripts were obtained from granulocytes and control monocytes. In three transcripts, we found an insertion sequence between the S2 and EC1 domains, suggesting the existence of a new exon. These results suggest a predominant usage of FcαR a.1 among various transcripts of FcαR in IgAN monocytes.

Keywords Fcα receptor IgA receptor IgA nephropathy

INTRODUCTION

IgA nephropathy (IgAN) is the most common type of glomerulonephritis and is characterized by mesangial IgA deposits [1]. Increased levels of the serum IgA and IgA-containing immune complexes have been detected in IgAN [2–4], one cause possibly being the delayed clearance of polymeric IgA [5–7]. Although the pathways for serum IgA catabolism are still unclear, two molecules have been thought to be involved—asialogycoprotein receptors (ASGP-R) on hepatocytes [8], and the specific receptor for the Fc-region of IgA (FcαR, CD89) on blood phagocytes [9,10]. Although a glycosylation defect of the hinge region of IgA1 molecules has been suggested [11], the ASGP-R system normally functions in IgAN [7]. On the other hand, increased FcαR expression on blood phagocytes has been observed in IgAN [12–14]. However, delayed FcαR-mediated endocytosis of IgA has been reported in IgAN monocytes [14]. Thus, it is speculated that the biochemical characteristics of FcαR are altered in IgAN.

FcαR have been shown to mediate IgA-dependent phagocytosis, antigen-dependent cellular cytotoxicity (ADCC), superoxide production, degranulation and infammatory mediator release [15]. The molecule is defined as heavily glycosylated protein with mol. wt ranging from 55 to 100 kD [9,16]. This wide range is partly due to various patterns of glycosylation [17], and alterations of glycosylation patterns have been reported in monocytes from alcoholic cirrhosis [18] and in eosinophils [16]. Another cause of the heterogeneity of the molecule is alternative RNA splicing. The FcαR gene contains five exons (S1, S2, EC1, EC2 and TM/C) and the deletions of each exon via alternative RNA splicing produce multiple isoforms [19]. In fact, blood monocytes express three transcripts [20] and granulocytes also express six transcripts [21]. On the other hand, alveolar macrophages preferentially express a specific isoform, namely FcαR a.2 [20]. Thus, FcαR differ from each other according to the cell types and the disease state due to differences in glycosylation patterns and alternative RNA splicing.

In this study, we analysed FcαR protein and mRNA expression on blood monocytes and granulocytes of IgAN. The whole molecule of FcαR on IgAN monocytes has a higher mol. wt and their protein core also has a higher mol. wt than control monocytes and granulocytes. This corresponds to the expression pattern of mRNA on IgAN monocytes that express FcαR a.1 predominantly. On the other

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hand, control monocytes and granulocytes express various FcαR transcripts via alternative RNA splicing. We also demonstrate the existence of a new exon between the S2 and EC1 domains.

MATERIALS AND METHODS

Subjects

Heparinized blood was obtained from 112 individuals (69 males and 43 females). Forty-eight individuals (34 males and 14 females) with IgAN proven by renal biopsy who varied in age from 8 to 17 years (mean 13·3 years) were selected for this study. None of the patients were on corticosteroid or any immunosuppressive therapies at the time of blood collection. The serum creatinine levels in all patients were below 1·5 mg/dl. Sixty-four other individuals (35 males and 29 females), ranging in age from 8 to 17 years (mean 13·5 years) and having mild inflammation of upper airway were used as controls. They had no histories or clinical features of renal diseases. Informed written consent was obtained from parents of all individuals.

Isolation of granulocytes and monocytes

Peripheral blood mononuclear cells (PBMC) and granulocytes were obtained via centrifugation over Mono-Poly resolving medium (Dainippon Seiyaku Co., Tokyo, Japan). Monocytes were enriched from PBMC by adherence to plastic tissue culture dishes for 1 h at 37°C and were recovered with pipetting. The purities of granulocytes from both IgAN patients and controls were >95%, whereas those of monocytes varied from 70 to 80%, as determined by morphological characteristics following Giemsa staining. The contaminating cells were lymphocytes.

Immunoprecipitation analysis

Immunoprecipitation analysis of FcαR protein was performed as described with some modifications [17]. In brief, granulocytes and monocytes (5×10^6 each) were surface-labelled with biotin-NHS (Amersham, Aylesbury, UK). After washing, cells were lysed in 1 ml of 0·5% NP-40 in PBS containing 0·02% azide, 1% aprotinin, 1 mM diisopropyl fluorophosphate, 5 mM iodoacetamide, and 1 mM PMSF. The lysates were precleaned four times with an excess of mouse monoclonal IgG (Pharmingen, San Diego, CA) coupled to protein G-Sepharose, and were incubated with mouse anti-FcαR MoAb (A59, Pharmingen, San Diego, CA) [17,18] or mouse monoclonal IgG coupled to protein G-Sepharose for 2 h at 4°C with constant rotation. After washing, bound materials were dissociated and subjected to *N*-glycanase (Genzyme, Boston, MA) treatment. The digested materials and the undigested ones were resolved by SDS–10% PAGE and were then electrically blotted onto PDVF membrane. After being blocked with 1% bovine serum albumin (BSA), the membrane was incubated with streptoavidin-conjugated horseradish peroxidase (Amersham) and the proteins were detected using the ECL system (Amersham).

RNA extraction and reverse transcription-PCR

Total RNA was extracted from 5×10^6 cells using ISOGEN (Nippon gene, Tokyo, Japan). The mRNA was reverse-transcribed with Moloney murine leukaemia virus reverse transcriptase (BRL Life Technologies, Gaithersburg, MD) using oligo (dT) primers (BRL). The FcαR transcripts were specifically amplified by PCR with specific primers encompassing the entire FcαR coding region (sense primer: 5′-ATGGACCCCAAACAGACC-3′; antisense primer: 5′-TCCAGGTGTTTACTTGCAGACAC-3′). Thirty-five cycles of denaturation (94°C for 45 s), annealing (62°C for 45 s), and elongation (75°C for 1·5 min) were performed in a thermocycler (Perkin-Elmer, Norwalk, CT). For standardization of cDNA samples from different donors, RT-PCR with β-actin primers (Clontech Labs Inc., Palo Alto, CA) was performed.

Cloning and DNA sequence analysis

The PCR-amplified DNA fragments were separated by electrophoresis on 2% agarose gel or 4% acrylamide gel, and were visualized by ethidium bromide staining. The DNA bands were excised from the gels and were purified by Prep-A-Gene DNA purification systems (BioRad, Hercules, CA). The purified DNA were cloned into the plasmid pMOSBlue T-vector (Amersham), and the nucleotide sequences were determined using a Thermo Sequence Fluorescence Labeled Primer Cycle Sequencing Kit

Table 1. Detection of each FcαR transcript in IgAN and controls

	Isoform	bp	IgAN	Control	*P*-value
Monocyte	X54150	873	48/48 (100·0%)	65/65 (100·0%)	NS
	D87855	837	22/48 (45·8%)	58/65 (92·3%)	<0·001
	D87853	807	16/48 (33·3%)	53/65 (81·5%)	<0·001
	D87857*	763	14/48 (29·2%)	48/65 (73·8%)	<0·001
	D87860*	627	22/48 (45·8%)	59/65 (90·8%)	<0·001
	D87854	585	39/48 (81·3%)	65/65 (100·0%)	<0·001
	D87856*	549	28/48 (58·3%)	57/65 (87·7%)	<0·001
Granulocyte	X54150	873	48/48 (100·0%)	65/65 (100·0%)	NS
	D87855	837	45/48 (93·8%)	60/65 (92·3%)	NS
	D87853	807	43/48 (89·6%)	55/65 (84·6%)	NS
	D87857*	763	40/48 (83·3%)	50/65 (76·9%)	NS
	D87860*	627	47/48 (97·9%)	60/65 (92·3%)	NS
	D87854	585	48/48 (100·0%)	65/65 (100·0%)	NS
	D87856*	549	46/48 (95·8%)	58/65 (89·2%)	NS

Data expressed as percentage of samples from each group with detectable mRNA transcripts on 4% polyacrylamide gel. χ^2 test was used to compare proportions between groups. *Novel sequences we have found.

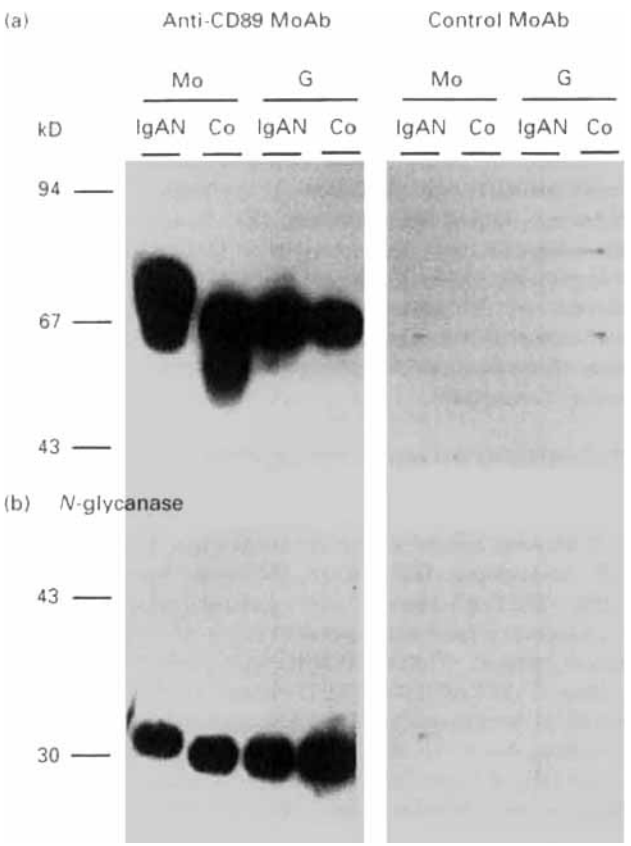


Fig. 1. Analysis of FcαR proteins on blood phagocytes. A representative experiment showing immunoprecipitation analysis of FcαR on blood monocytes (Mo) and granulocytes (G) from a control (Co) and IgAN patient (IgAN). Cells were surface-labelled with biotin-NHS and lysed in 0·5% NP-40 buffer. After removal of FcγR, as described in Materials and Methods, anti-FcαR MoAb-reactive and irrelevant MoAb-reactive molecules were incubated in the absence (a) or presence (b) of *N*-glycanase, and analysed by SDS–10% PAGE.

(Amersham) for DSQ-1 DNA sequencer (Shimadzu, Kyoto, Japan). The sequences of both strands were determined.

DNA analysis

The DNA sequences which were determined were analysed by computer software (GENETYX-Mac, Software Development Co, Tokyo, Japan) and databases of DDBJ (DNA Data Bank of Japan), GenBank (National Center for Biotechnology Information), and EMBL (European Molecular Biology Laboratory). Sequence homology searches were based on algorithms of FastA [22] and BLAST (Basic Local Alignment Search Tool) [23].

Statistical analysis

The χ^2 test was used to compare the appearance of each band on 4% acrylamide gel between each group. Statistical significance was set at a conventional 5% level.

RESULTS

Immunoprecipitation analysis

First, we analysed the FcαR protein of granulocytes and monocytes by immunoprecipitation analysis using anti-FcαR MoAbs in 22 IgAN patients and 20 controls. Figure 1 shows a representative experiment. When cell surface proteins from granulocytes from both groups were immunoprecipitated with anti-FcαR MoAb, a broad 55–75 kD band was identified in all IgAN patients and controls. FcαR of monocytes from controls were identified as broader bands (50–75 kD) than granulocytes in 15 controls. The other five controls showed the same Mr as granulocytes. However, FcαR from monocytes from 16 IgAN patients showed narrower bands with higher Mr (60–80 kD, Fig. 1a) than those from control monocytes. The other six IgAN showed the same Mr as control monocytes (50–75 kD). To analyse the mol. wt of protein core, immunoprecipitates were digested by *N*-glycanase (Fig. 1b). Removal of *N*-linked carbohydrate moieties from FcαR molecules from IgAN monocytes also presented higher Mr (30–36 kD) than those from control monocytes (28–32 kD) in 16 patients. Other IgAGN patients showed the same Mr as controls. On the other hand, the protein core of granulocytes from all IgAN and controls showed the same Mr (28–32 kD) as those of control monocytes. These results indicate that IgAN monocytes express a different FcαR protein core from granulocytes and control monocytes and a different glycosylation pattern from that of control monocytes.

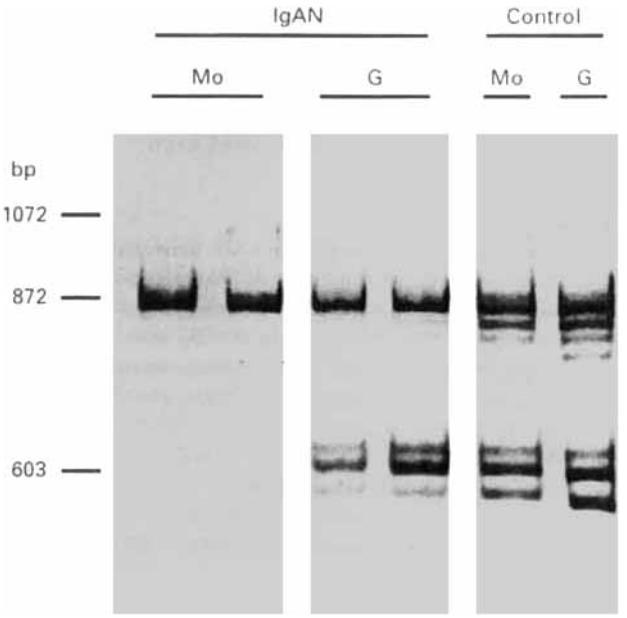
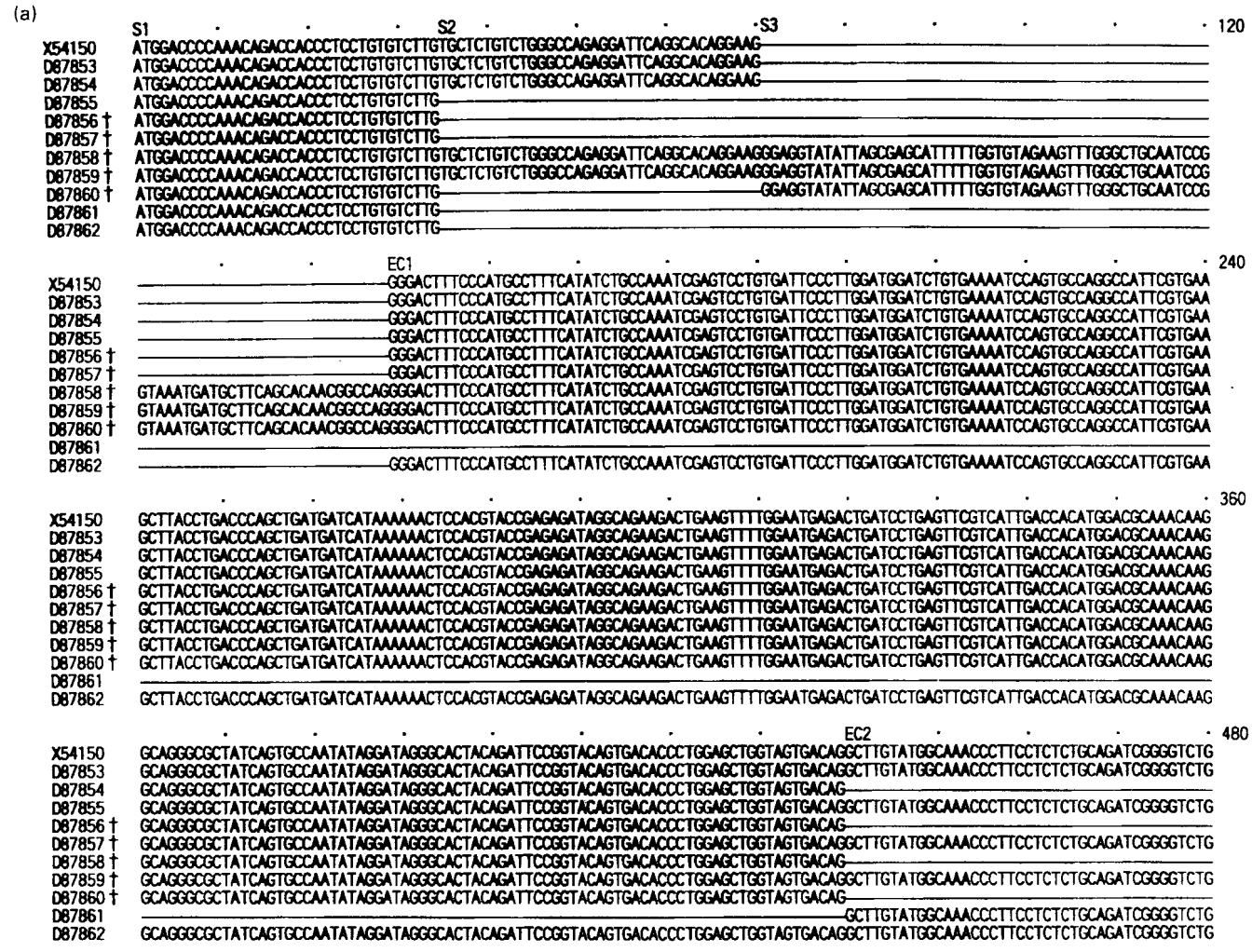


Fig. 2. RT-PCR analysis of FcαR mRNA in blood phagocytes. Total RNA was isolated from monocytes (Mo) and granulocytes (G) and subjected to RT-PCR analysis with specific primer pairs for FcαR. PCR products were separated on 4% polyacrylamide gel. DNA markers are indicated on the left and the sizes of the PCR products that have been cloned and sequenced are indicated on the right. † Novel sequences we have found.

FcαR transcripts in blood phagocytic cells from IgAN

When PCR-amplified FcαR transcripts from granulocytes and control monocytes were separated on 4% polyacrylamide gel, at least seven DNA bands were seen. However, an 873 bp band was remarkable in those from IgAN monocytes. Figure 2 shows a representative experiment from two IgAN patients. This 873 bp band was identified in all the 48 patients, whereas the other bands were seen in only some of the patients (Table 1). RT-PCR with β-actin primers was performed for standardization of cDNA samples from different donors (data not shown). The 873 bp band was identical to the FcαR a.1 (ΔS3, Fig. 3) transcript (see below), which was the longest one in all the transcripts. These results were



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in agreement with those of the immunoprecipitation analysis of FcαR protein.

New members of FcαR

Each DNA band was purified from the gel, cloned into the pMOSBlue T-vector, and sequenced by a cycle sequencing technique. Eleven transcripts were detected, their sequences being shown in Fig. 3. Schematic representation of these transcripts and structure of CD89 genome are shown in Fig. 4. We found five novel alternatively spliced forms of FcαR, that is, ΔS2S3EC2, ΔS2S3EC2, ΔEC2, Δ66EC2, ΔS2EC2. The seven DNA bands on 4% acrylamide gels corresponded to ΔS3 (873 bp), ΔS2S3 (837 bp), ΔS366EC2 (807 bp), ΔS2S374EC2 (763 bp), ΔS2EC2 (627 bp), ΔS3EC2 (585 bp) and ΔS2S3EC2 (549 bp). ΔS3 was identical to previously described FcαR a.1 transcript [18]. Similarly, ΔS366EC2 and ΔS3EC2 were identical to FcαR a.2 and FcαR, a.3 respectively. Three transcripts (ΔS366EC2, Δ66EC2, and ΔS2S366EC2) had deletions of 66 bp in EC2 and one transcript (ΔS2S374EC2) lacked 74 bp in EC2. In three transcripts (ΔEC2, Δ66EC2, and ΔS2EC2), we found a 78 bp insertion sequence (S3) between S2 and EC1. 5′splicing donor sites and 3′splicing accepter sites conformed to GT-AG rule [24] in all transcripts except those containing S3, and 5′ splicing occurred after the first G in a glycine codon (G/gt) [19]. This rule also applies to intraexon splicing within EC2. Some transcripts (ΔS366EC2, ΔS3EC2, ΔS2S3, and ΔS2S366EC2) differed at nucleotide positions 402, 415 and 535 from the known FcαR splice variant [21].

DISCUSSION

We have found that IgAN monocytes preferentially express FcαR a.1 transcript, whereas granulocytes and control monocytes express multiple transcripts via alternative RNA splicing. This alteration of mRNA expression of FcαR on IgAN monocytes corresponds to the changes in mol. wt of the protein core of the molecule. That is to say, we have found that the protein core of FcαR from IgAN monocytes shows a higher Mr (28–32 kD) than granulocytes and control monocytes (32–36 kD) by the treatment of the molecule with *N*-glycanase. Further, we have found that all the FcαR molecules from IgAN monocytes have a higher Mr (60–80 kD) than those from granulocytes and control monocytes (55–75 kD). The differences in Mr of whole molecules between IgAN monocytes and controls were larger than in Mr of protein cores. So it is suggested that the glycosylation pattern of IgAN monocytes differs from those of control monocytes. This corresponds to the

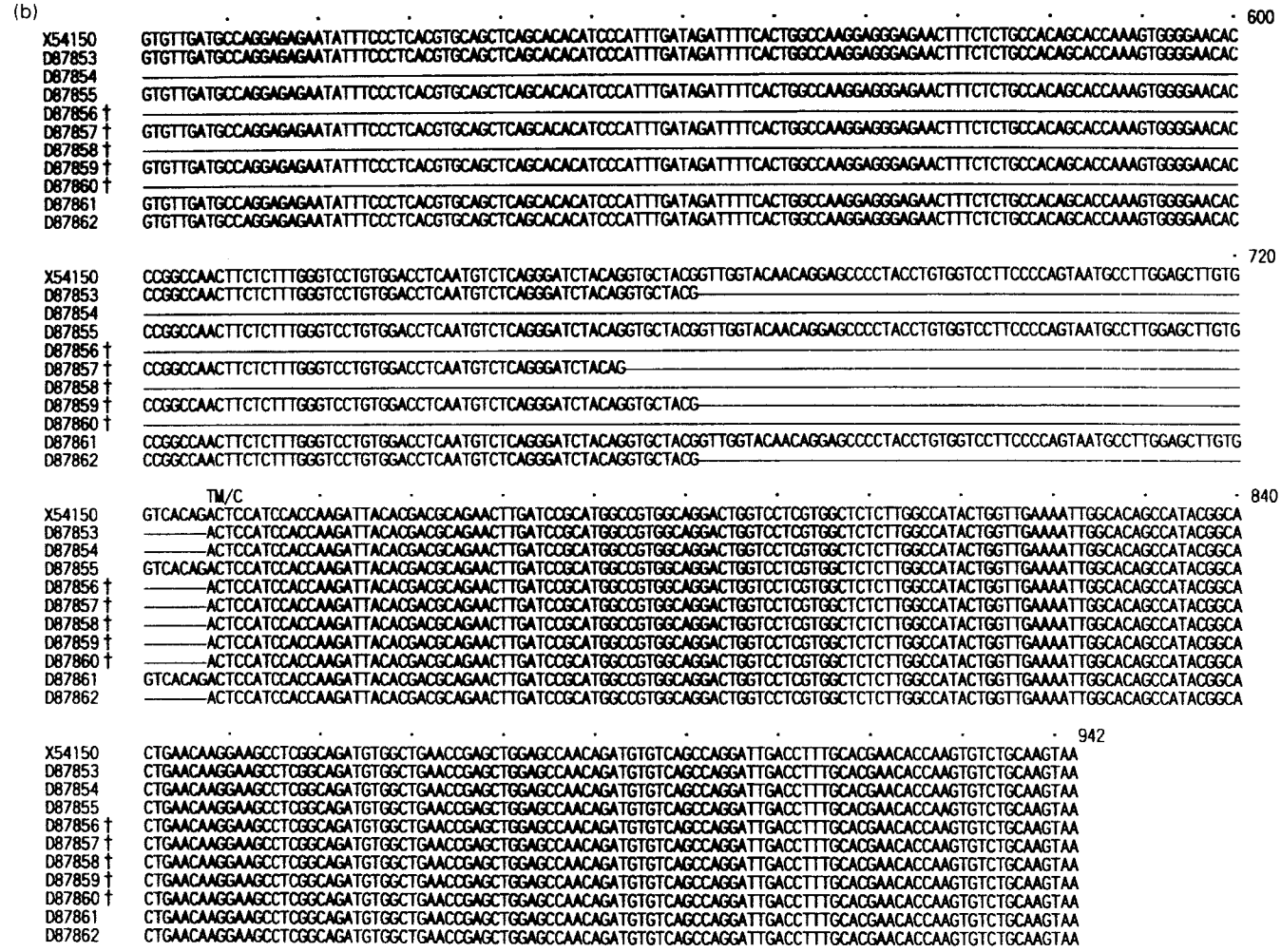

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Fig. 3. (a, b) Aligment of nucleotide sequences of 11 FcαR splicing variants. Numbers in the left hand margin are the DDBJ/GenBank/EMBL accession numbers. Nucleotide positions are specified in the right margin. S1, S2, S3, EC1, EC2, and TM/C indicates first nucleotide of each exon. † Novel sequences we have found.

predominant expression of FcαR a.1 in IgAN monocytes, since they have the most sequences of possible *N*-glycosylation sites. These results suggest that alteration in both protein core and glycosylation patterns are involved in the change in mol. wt of FcαR from IgAN monocytes.

Altered glycosylation pattern of FcαR has been reported in monocytes from patients with alcoholic cirrhosis, and IFN-γ is suggested to be responsible for such alterations in that disease [18]. Moreover, FcαR on eosinophils has been reported to have a higher mol. wt than that on neutrophils [16]. However, protein cores of FcαR on monocytes from alcoholic cirrhosis and eosinophils are the same Mr (about 32 kD) as normal monocytes and neutrophils. Thus, unlike IgAN, only the glycosylation pattern is up-regulated in these states. On the other hand, alveolar macrophages express FcαR with smaller Mr (28 kD) as a result of dominant expression of FcαR a.2 isoform [20]. It is not clear whether the isoform lacking each of these domains functions differently or not. However, it has recently been reported in FcγRI that endocytosis requires only EC domain, whereas phagocytosis is dependent on an interaction between TM domain and the γ-subunit [25]. Thus, deletions of each domain of FcαR may cause the changes in the functions and signalling pathways, such as endocytosis. Therefore the specific expression of FcαR a.1 on IgAN monocytes could be related to delayed FcαR-mediated endocytosis in monocytes from the disease.

We have found 11 FcαR splicing variants including five novel sequences in monocytes and granulocytes. Three of those have 78 bp insertion sequence (S3) between S2 and EC1 domain, which has not been reported in known cDNA sequence of FcαR. We searched database DDBJ/GenBank/EMBL, and were unable to find any homologous sequences to S3. Since the sequence of the intron between S2 and EC1 exon in the genome of FcαR is unknown, it is suggested that S3 is a new exon within the intron. Insertion of the S3 dose not cause frame-shift and results in an insertion of 26 amino acid residues. Although several FcγR and FcεR exhibit common structural features [26,27], they have no sequences like S3. Therefore, this sequence is suggested to be unique for FcαR. All FcαR transcripts lack S2, S3, EC1, EC2 or a part of the 3′ end of EC2 domain, and some of these splicing variants are identical to known splicing variants that have been reported in blood

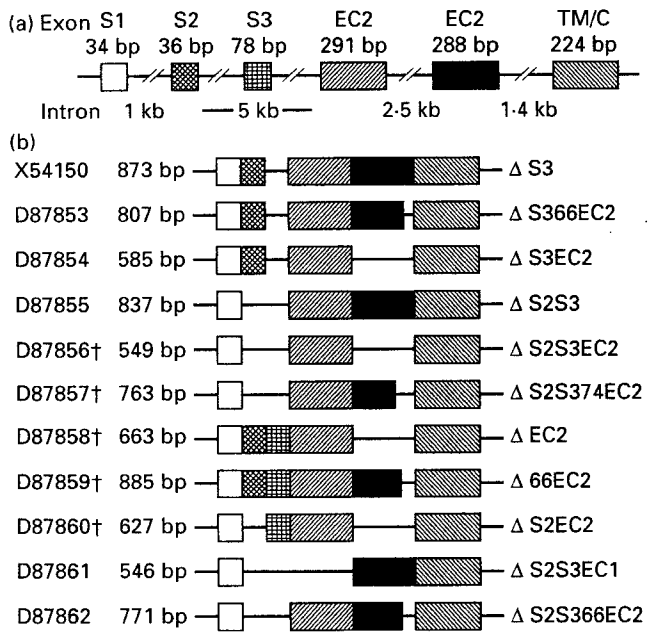

Fig. 4. (a) Structure of the FcαR gene. (b) Schematic representation of 11 FcαR transcripts. Numbers in the left hand margin are the DDBJ/GenBank/EMBL accession numbers. † Novel sequences we have found.

monocytes and granulocytes. That is to say, blood monocytes express three transcripts [20], and granulocytes express at least six transcripts [21]. We found three sequences that have two types of intraexonal splicing in the EC2 domain, that is, the deletion of 66 bp and of 78 bp from the 3′ end of EC2 (Fig. 4). Alternative RNA splicing within exons like these sequences has been reported in other molecules, such as membrane form of Ig [28]. Further, we have found seven transcripts lacking S2 domain (Fig. 4), at the site of peptidase cleavage [29,30]. The deletions of short signal sequence as these transcripts have been described in FcγR and FcεR [27,31]. These transcripts are thought to produce 'proreceptors' that are tethered to the membrane and not expressed on the surface of the cells until an alternate proteolytic event [32,33]. So it is suggested that these isoforms could not be detected by the immunoprecipitation analysis using anti-FcαR MoAbs.

The protein core of FcαR on granulocytes and control monocytes looks like a single molecule in immunoprecipitation analysis, in spite of the expression of multiple FcαR transcripts. One reason for this difference is the existence of the 'proreceptors' that are not expressed on the surface of the cells, and an additional reason is the deletions or changes of epitopes recognized by anti-FcαR MoAbs. The anti-FcαR antibody A59 that we used recognizes the protein core of FcαR [17], and the epitopes exist outside of the IgA-binding site. By *in-vitro*-translation analysis, FcαR a.1 and FcαR a.2 are recognized by this antibody, whereas FcαR a.3 is not. Since the FcαR a.3 lacks entire EC2 domain, it seems that the antibody recognizes epitopes located in the domain [20]. We found four splicing variants lacking EC2 domain, and it is possible that they are not detected by immunoprecipitation analysis using the antibody. Due to these reasons, some of the splicing variants that we found are not detected through the immunoprecipitation analysis, although the proteins translated from these cDNAs are expressed in the cells.

In conclusion, the higher mol. wt of FcαR on monocytes of IgAN is due to predominant usage of FcαR a.1 among various transcripts of FcαR. On the other hand, the low mol. wt of FcαR on granulocytes and control monocytes seen in the assay of immunoprecipitation is due to the multiple usage of the transcripts. The specific expression of FcαR a.1 on IgAN monocytes may be related to delayed FcαR-mediated endocytosis in monocytes from the disease.

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