

**Residue  $\gamma$ 153Cys is essential for the formation of the complexes  $A\alpha\gamma$  and  $B\beta\gamma$ , assembly intermediates for the  $A\alpha B\beta\gamma$  complex and intact fibrinogen**

Fumiko Terasawa, Kiyotaka Fujita, Nobuo Okumura

Department of Biomedical Laboratory Sciences, School of Health Sciences, Shinshu University, Matsumoto Japan

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Correspondence to: Fumiko Terasawa, PhD

Department of Biomedical Laboratory Sciences, School of Health Sciences, Shinshu University, 3-1-1 Asahi, Matsumoto 390-8621, Japan

Phone:(81) 263 37 2387; Fax: (81) 263 37 2370;

E-mail: [fterasa@gipac.shinshu-u.ac.jp](mailto:fterasa@gipac.shinshu-u.ac.jp)

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 $A\alpha/\gamma$ m complex;  $B\beta/\gamma$ m complex

## **Abstract**

*Background:* Fibrinogen Matsumoto IV was found in a hypofibrinogenemia caused by a heterozygous missense mutation, i.e., the substitution of the fibrinogen  $\gamma$ -chain residue Cys153 by Arg.

*Methods:* To examine the precise basis for the fibrinogen deficiency, mixtures of any two vectors, the fibrinogen A $\alpha$ -, B $\beta$ -,  $\gamma$ - (153Cys) or  $\gamma$ m- (153Ala) chain were transfected into Chinese hamster ovary cells (CHO-A $\alpha$ / $\gamma$ , -A $\alpha$ / $\gamma$ m, -B $\beta$ / $\gamma$ , -B $\beta$ / $\gamma$ m). Expression and constitution of each of two chains and their complexes in the individual CHO cell lines were identified by SDS-PAGE and immunoblot analysis using polypeptide specific antibodies and two-dimensional electrophoresis (2DE).

*Results:* In the CHO-A $\alpha$ / $\gamma$  and -B $\beta$ / $\gamma$ , the A $\alpha$ / $\gamma$ - or B $\beta$ / $\gamma$ -complex was formed, whereas in the CHO-A $\alpha$ / $\gamma$ m and -B $\beta$ / $\gamma$ m, no A $\alpha$ / $\gamma$ m- or B $\beta$ / $\gamma$ m-complex was observed. These results demonstrate that  $\gamma$ 153Ala can not assemble with the A $\alpha$ - and B $\beta$ -chains, leading to impaired fibrinogen assembly and secretion.

*Conclusion:*  $\gamma$ 153Cys is an essential residue for the fibrinogen assembly which is dependent on A $\alpha$ / $\gamma$ - and B $\beta$ / $\gamma$ -complex formation.

## **Introduction**

Fibrinogen is a 340 kDa plasma glycoprotein consisting of a dimeric molecule of 3 polypeptide chains A $\alpha$ , B $\beta$ , and  $\gamma$ , linked by an extensive network of 29 intrachain and interchain disulfide bonds [1]. Fibrinogen is synthesized in hepatocytes at plasma concentration of 1.8 - 3.5 g/L. The chains are encoded by three genes, A $\alpha$ , B $\beta$ , and  $\gamma$  respectively, located in a cluster spanning some 50 kb on the long arm of chromosome 4 at 4q28-31 [2]. Each gene is separately transcribed and translated, and the nascent chains translocated into the endoplasmic reticulum (ER), where assembly occurs in a stepwise fashion [3]. Congenital hypo- and afibrinogenemia are associated with low and non-detectable levels of antigenic fibrinogen, respectively, and have been demonstrated in a variety of 54 cases mutations in all three genes, A $\alpha$ -chain; 27, B $\beta$ -chain; 14,  $\gamma$ -chain; 13 cases [4], including missense, nonsense and frameshift mutations; splice-site abnormalities, and larger deletions [5, 6]. It has not been demonstrated clearly, however, what the molecular mechanisms might be by which one amino acid substitution caused by a missense mutation can lead to impaired assembly or secretion of the fibrinogen.

We have previously reported a hypofibrinogenemia caused by a heterozygous missense mutation, the substitution of the  $\gamma$ -chain residue Cys153 by Arg (Fibrinogen Matsumoto IV) [7]. To examine the molecular basis for this fibrinogen deficiency, the

variant fibrinogens of  $\gamma 153\text{Arg}$  and  $\gamma 153\text{Ala}$  were expressed in Chinese hamster ovary (CHO) cells. Immunoblot and metabolic pulse-chase analyses indicated that CHO cells transfected with the  $\gamma 153\text{Arg}$ - or  $\gamma 153\text{Ala}$ -coding vector synthesized the variant chains, but did not assemble the variant fibrinogen and did not secrete it into culture medium [7].

In a model of the processes involved in the assembly and biosynthesis of fibrinogen,  $\text{A}\alpha/\gamma$  and  $\text{B}\beta/\gamma$  dimers linked by a disulfide bonds are proposed to be intermediates which, upon addition of a third chain, form the  $\text{A}\alpha/\text{B}\beta/\gamma$  half-molecule and then mature fibrinogen  $(\text{A}\alpha/\text{B}\beta/\gamma)_2$  [8, 9]. Fibrinogen Matsumoto IV strongly suggests that the formation of the  $\text{A}\alpha/\gamma$ - and  $\text{B}\beta/\gamma$ -dimer is disturbed. Even though the  $\text{A}\alpha$ -,  $\text{B}\beta$ -,  $\gamma$ -chain are expressed together in CHO cells, it is a challenge to identify the components of the complexes, so we co-expressed each of two pair chains in the CHO cell lines, CHO- $\text{A}\alpha/\gamma$ , - $\text{A}\alpha/\gamma\text{m}$ , - $\text{B}\beta/\gamma$ , - $\text{B}\beta/\gamma\text{m}$ . In this report, we provide a molecular mechanism for inducing the impaired assembly of the intact  $\gamma 153\text{Ala}$  fibrinogen.

## Materials and Methods

### *Recombinant protein expression*

For synthesis of the  $\gamma 153\text{Ala}$  variant, the fibrinogen  $\gamma$ -chain expression vector, pMLP $\gamma$ , was altered by oligonucleotide-directed mutagenesis as previously reported ( $\gamma\text{m}$ -vector) [7].

CHO cells were cotransfected with mixtures of any two of the vectors, i.e., the fibrinogen  $\text{A}\alpha$ -,  $\text{B}\beta$ -,  $\gamma$ - or  $\gamma\text{m}$ -chains, along with a neomycine selection plasmid (pRSVneo), using the standard calcium-phosphate coprecipitation method. Individual cell lines were selected with a medium containing G418, and were examined for the expression of each polypeptide by means of immunoblot analysis under reducing conditions, and clones were established producing high as well as approximately equivalent levels of each of the two polypeptides, and were designated as CHO- $\text{A}\alpha/\text{B}\beta$ , CHO- $\text{A}\alpha/\gamma$ , CHO- $\text{A}\alpha/\gamma\text{m}$ , CHO- $\text{B}\beta/\gamma$ , and CHO- $\text{B}\beta/\gamma\text{m}$ , respectively. In addition, a CHO cell line secreting intact fibrinogen, CHO-NC, was used as a control.

### *Immunoblot analysis*

Cell lysates for immunoblot analysis were prepared as follows. After the individual cells were grown to confluence in 60-mm culture dishes, the media were removed and the cells were scraped into 100 $\mu\text{L}$  of non-reducing sample buffer for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). For the reducing conditions, 5% 2-mercaptoethanol (2ME) was added to the samples.

SDS-PAGE and immunoblot analyses for fibrinogen or individual polypeptides were performed according to the methods of Laemmli [10] and Huang et al. [11], respectively. For two-dimensional electrophoresis (2-DE), the first-run was performed on an 8% gel under non-reducing conditions and the second-run was performed on a 10% gel under reducing conditions. Briefly, individual lanes were cut from the gel of the first-run, incubated for 30 minutes in Tris-HCl buffer (pH6.8) containing 5% 2ME for sample reduction, and laid on secondary gels and underwent a run of electrophoresis. Immunoblots were developed with a rabbit anti-human fibrinogen antibody (DAKO, Carpinteria, CA), or a rabbit anti-human fibrinogen A $\alpha$ - or B $\beta$ -chain antibody (both from Chemicon International Inc., Temecula, CA), or anti-human fibrinogen  $\gamma$ -chain mouse monoclonal antibody 2G10 (Accurate Chemical & Scientific Corp. NY) [12]. Cross-reacting species were visualized with horseradish peroxidase-conjugated goat antirabbit IgG or antimouse IgG antibody (Medical and Biological Laboratories, Nagoya, Japan) and enhanced chemiluminescence (ECL) detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were exposed on Hyperfilm-ECL (Amersham Pharmacia Biotech).

## Results

Expression of each of the fibrinogen chains in cell lysates from transfected CHO cell lines was confirmed by reducing SDS-PAGE and immunoblot analysis with an antifibrinogen antibody. CHO-NC as a control exhibited expression of the A $\alpha$ -, B $\beta$ - and  $\gamma$ -chains, and other cell lines also clearly expressed two polypeptides among the A $\alpha$ -, B $\beta$ -,  $\gamma$ -, or  $\gamma$ m-chains (Fig1A). CHO cell lines expressing the B $\beta$ -chain also exhibited an extra band under the  $\gamma$ -chain (Fig1A), which might be a degradation product of the B $\beta$ -chain because the bands reacted with the antiB $\beta$ -chain antibody (data not shown). Although it is well known that the apparent mass of the complexes as estimated by SDS-PAGE under non-reducing conditions is usually larger than that calculated from the amino acid sequence [3], non-reducing SDS-PAGE and immunoblot analysis for established cell lines indicated the presence of some of the intermediates necessary for intact fibrinogen assembly (in addition to the non-assembly polypeptide-chain), except for CHO-A $\alpha$ / $\gamma$ m and CHO-B $\beta$ / $\gamma$ m. Briefly, CHO-NC exhibited kDa bands of 340 (fibrinogen) and 280, 166, 148, 118, and 93, CHO-A $\alpha$ /B $\beta$ ; the 166 and 148 kDa bands, CHO-A $\alpha$ / $\gamma$ ; the 166 kDa band, and CHO-B $\beta$ / $\gamma$ ; 141 kDa band (Fig.1B). On the other hand, CHO-A $\alpha$ / $\gamma$ m and CHO-B $\beta$ / $\gamma$ m exhibited no apparent complexes (Fig.1B).

To identify the polypeptide constitution of the intermediates in fibrinogen assembly

non-reducing SDS-PAGE and immunoblot analysis were performed using each of three polypeptide specific antibodies (Fig.1C-E). With the antiA $\alpha$ -chain antibody, in CHO-A $\alpha$ / $\gamma$ , the 166 kDa band and A $\alpha$ -chain were observed, but in CHO-A $\alpha$ / $\gamma$ m, only the A $\alpha$ -chain were observed (Fig.1C). With the antiB $\beta$ -chain antibody, in CHO-B $\beta$ / $\gamma$  cells the 141 kDa band and B $\beta$ -chain were observed, but in CHO-NC, -A/B and -B $\beta$ / $\gamma$ m cells, two faint bands around 141 kDa and the B $\beta$ -chain were observed (Fig.1D). The two faint bands around 141 kDa remained indeterminate by analysis using each of three polypeptide specific antibodies and 2-DE. These observations indicate that no A $\alpha$ /B $\beta$  complex was to be found in not only the CHO-A $\alpha$ /B $\beta$  but also the CHO-NC cell line (Figs.1C and D). Unfortunately, some of the undetermined, non-specific bands were observed when using the anti $\gamma$ -chain monoclonal antibody (Fig.1E).

Therefore an effort was made to examine the complex formation using 2-DE (Fig.2A-F). Briefly, the complexes were first separated by SDS-PAGE under non-reducing conditions and then run on SDS-PAGE again in the presence of 2-ME. In the CHO-NC cells, intact fibrinogen, A $\alpha$ /B $\beta$ / $\gamma$  (280 kDa)- and A $\alpha$ / $\gamma$  (166kDa)- complexes, two types of A $\alpha$ -oligomers (148 and 93 kDa), a B $\beta$ -oligomer (118 kDa), and single A $\alpha$ -, B $\beta$ -, and  $\gamma$ -chains were observed on the 2-DE gel (Fig.2A). For the CHO-A $\alpha$ /B $\beta$  cells both the 166kDa band was composed only of the A $\alpha$ -chain (Fig.2B). For the CHO-A $\alpha$ / $\gamma$  cells, the 166 kDa band was separated from the A $\alpha$ - and  $\gamma$ -chains and the CHO-B $\beta$ / $\gamma$  cells, the 141 kDa band was separated from the B $\beta$ - and  $\gamma$ -chains, respectively (Figs.2C and 2D). In contrast, for the CHO-A $\alpha$ / $\gamma$ m the faint 170 kDa band was an A $\alpha$ -oligomer and for the CHO-B $\beta$ / $\gamma$ m no complex was observed (Figs. 2E and 2F). These results suggested that  $\gamma$ m-chain is not able to assemble with the A $\alpha$ - or B $\beta$ -chains.

## Discussion

To examine the precise basis for the fibrinogen deficiency, mixtures of any two vectors, the fibrinogen A $\alpha$ -, B $\beta$ -,  $\gamma$ - (153Cys) or  $\gamma$ m- (153Ala) chain were transfected into CHO cells. The expression and constitution of each of the two chains and their complexes in the individual CHO cell lines were identified by immunoblot analysis using polypeptide specific antibodies and 2-DE.

In this report, evidence is provided which clearly demonstrates that the A $\alpha$ / $\gamma$  and B $\beta$ / $\gamma$  complexes, which are the assembly intermediates of fibrinogen, are formed in CHO-A $\alpha$ / $\gamma$  and CHO-B $\beta$ / $\gamma$  cells, respectively whereas  $\gamma$ 153Ala did not form these complexes with the A $\alpha$ - or B $\beta$ -chain. These results reveal that the substitution of the  $\gamma$ -chain residue Cys153 by Ala completely impairs the assembly of fibrinogen in CHO

cells as well as the subsequent secretion into medium, as has already reported [7].

Two groups have examined and reported the process of fibrinogen assembly from three polypeptide chains in BHK or COS-1 cells [8, 13]. They demonstrated that the  $\gamma$ -chain is a key polypeptide for fibrinogen assembly. Briefly, in a first step,  $A\alpha/\gamma$ - and  $B\beta/\gamma$ -complexes are formed, and in the second step, these two are assembled into the  $A\alpha/B\beta/\gamma$  complex and finally into intact fibrinogen,  $(A\alpha/B\beta/\gamma)_2$ . Interestingly, however, the  $A\alpha/B\beta$ -complex was not detected. Furthermore, Zhang JZ et al. [14] directly demonstrated that disruption of the disulfide loop of the  $\gamma$ -chain,  $\gamma 153\text{Cys}-\gamma 182\text{Cys}$ , resulted in the formation of fewer  $A\alpha/\gamma$ - and  $B\beta/\gamma$ -complexes and abolished fibrinogen assembly in COS-1 cells. Thus, the loss of Cys at residue 153 necessarily disrupts the disulfide bond between  $\gamma 153\text{C}$  and  $\gamma 182\text{C}$ , and the loss of the disulfide bond very likely induces profound changes in the overall structure of the C-terminal domain of the  $\gamma$ -chain. Our data demonstrate that the 153Ala mutation did not participate in assembly, and did not form intermolecular disulfide linked products with either the  $A\alpha$ - or  $B\beta$ -chains.

We have directly examined the role of the  $\gamma$ -chain by monitoring the synthesis of fibrinogen with increasing deletions from the C-terminus of the  $\gamma$ -chain. We found that residues near the C-terminus of the  $\gamma$ -chain were essential for fibrinogen assembly, and more specifically, that  $\gamma\text{Ile}387$  was critical [15]. Crystal structures of this  $\gamma$ -chain domain show that  $\gamma\text{Ile}387$  lies within a region of  $\beta$ -strand composed of the residues  $\gamma 381-388$ , which inserts in an anti-parallel fashion between the strands formed by residues  $\gamma 190-197$  and  $\gamma 244-251$  [16, 17]. We propose that the loss of  $\gamma\text{Ile}387$  markedly changes the 5-stranded anti-parallel  $\beta$ -strand sheet and thus the overall tertiary structure of  $\gamma$ -chain, preventing assembly  $\alpha\gamma$  and  $\beta\gamma$  dimers. The recombinant 153Ala  $\gamma$ -chain is speculated to have a marked disruption of tertiary structure of the C-terminal domain because  $\gamma 153\text{Cys}-\gamma 182\text{Cys}$  is adjacent to  $\beta$ -strand of  $\gamma 190-197$ . Our data indicate that this disruption also leads to assembly of the variant chain into fibrinogen, consistent with the conclusion that the overall structure of this region has a unique role for the assembly of normal fibrinogen [15].

Recently, hypo- or afibrinogenemia, which are caused by an amino acid substitution in the C-terminal domain of the  $\gamma$ -chain, have been reported in 4 cases, i.e., substitutions of  $\gamma 284\text{Gly}$  by Arg [18],  $\gamma 345\text{Asp}$  by Asp [19],  $\gamma 371\text{Thr}$  by Ile [20],  $\gamma 375\text{Arg}$  by Trp [21]. Although all 4 cases are not determined in terms of the assembly of variant fibrinogen by *ex vivo* protein expression analysis, two cases, namely  $\gamma 284\text{Arg}$  [18] and  $\gamma 375\text{Trp}$  [21], are associated with hepatic ER storage disease. We speculate from our observation that in these cases the variant  $\gamma$ -chains were not assembled into variant

fibrinogen. Interestingly, using transient transfection assays, Duga et al [22], Vu et al [23], and Spina et al [24] demonstrated that B $\beta$ -chains with substitutions in the C-terminal region of the B $\beta$ -chain, i.e., B $\beta$ 353Leu by Arg, B $\beta$ 400Gly by Asp, B $\beta$ 414Gly by Ser, and B $\beta$ 437Trp by Gly, assembled into normal 6-chain fibrinogens, but were not secreted into media. These data suggest that the tertiary structure of B $\beta$ -module may be critical to fibrinogen secretion but not assembly. These studies suggest that the tertiary structure of the  $\gamma$ -chain C-terminal domain is critical for fibrinogen assembly and the subsequent requisite secretion from hepatocytes.

Finally, the results presented here are completely in accord with the assembly process proposed in the above, and further reveal that the structure of the  $\gamma$ -chain C-terminal domain and/or  $\gamma$ 153Cys *per se* is essential for fibrinogen assembly, in particular the formation of the A $\alpha$ / $\gamma$ - and B $\beta$ / $\gamma$ -complexes. This abnormality is demonstrated to be one of the molecular mechanisms leading to congenital hypo- and afibrinogenemia.

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### Figure legends

Figure 1. Immunoblot analyses of the expressed fibrinogen chains. Fibrinogen chains in the cell lysates of transfected individual CHO cell lines were resolved on a 10% or 8% polyacrylamide gel under reducing (A) or non-reducing conditions (B-F) and transferred to nitrocellulose membranes. Blots were incubated with either a rabbit anti-human fibrinogen antibody (A, B), a rabbit anti-human A $\alpha$ -chain (C) or B $\beta$ -chain antibody (D), or the mouse anti-human  $\gamma$ -chain monoclonal antibodies, 4A5 (E) or 2G10 (F). Cross-reacting bands were developed by a horseradish peroxidase-conjugated goat anti rabbit IgG or anti mouse IgG antibody and enhanced chemiluminescence detection reagents. Lines indicate the molecular weights estimated for the A $\alpha$ - (67 kDa), B $\beta$ - (56 kDa),  $\gamma$ -chains (47.5 kDa) and intact fibrinogen (340 kDa). A: A $\alpha$ -chain, B: B $\beta$ -chain,  $\gamma$ :  $\gamma$ -chain, Fg: purified fibrinogen. Abbreviations of vector transfected CHO cell lines: NC, A $\alpha$ -, B $\beta$ - and  $\gamma$ -chain; A/B, A $\alpha$ - and B $\beta$ -chain; A/ $\gamma$ , A $\alpha$ - and  $\gamma$ -chain; A/ $\gamma$ m, A $\alpha$ - and  $\gamma$ m-chain; B/ $\gamma$ , B $\beta$ - and  $\gamma$ -chain; B/ $\gamma$ m, B $\beta$ - and  $\gamma$ m-chain.

**Figure 2. Constitutional analyses for the intermediate complexes in CHO cell lines by two-dimensional SDS-PAGE.** The first dimension was run on 8% SDS-PAGE under non-reducing conditions and the second dimension was run on 10% SDS-PAGE under reducing conditions. After transfer to nitrocellulose membranes, blots were developed with a rabbit anti-human fibrinogen antibody and developed with horseradish peroxidase-conjugated goat anti rabbit IgG and enhanced chemiluminescence detection reagents. The reduced purified fibrinogens (Fg) are shown on the left side and the constitutional polypeptides at the bottom of each gel as A: A $\alpha$ -chain, B: B $\beta$ -chain, and  $\gamma$ :  $\gamma$ -chain. Abbreviations of vector transfected CHO cell lines: NC, A $\alpha$ -, B $\beta$ - and  $\gamma$ -chain (A); A/B, A $\alpha$ - and B $\beta$ -chain (B); A/ $\gamma$ , A $\alpha$ - and  $\gamma$ -chain (C); B/ $\gamma$ , B $\beta$ - and  $\gamma$ -chain (D); A/ $\gamma$ m, A $\alpha$ - and  $\gamma$ m-chain (E); B/ $\gamma$ m, B $\beta$ - and  $\gamma$ m-chain (F). (A): (a);280 kDa, (b);166 kDa, (c);148 kDa, (d);118 kDa, (e);93 kDa band in NC cells.

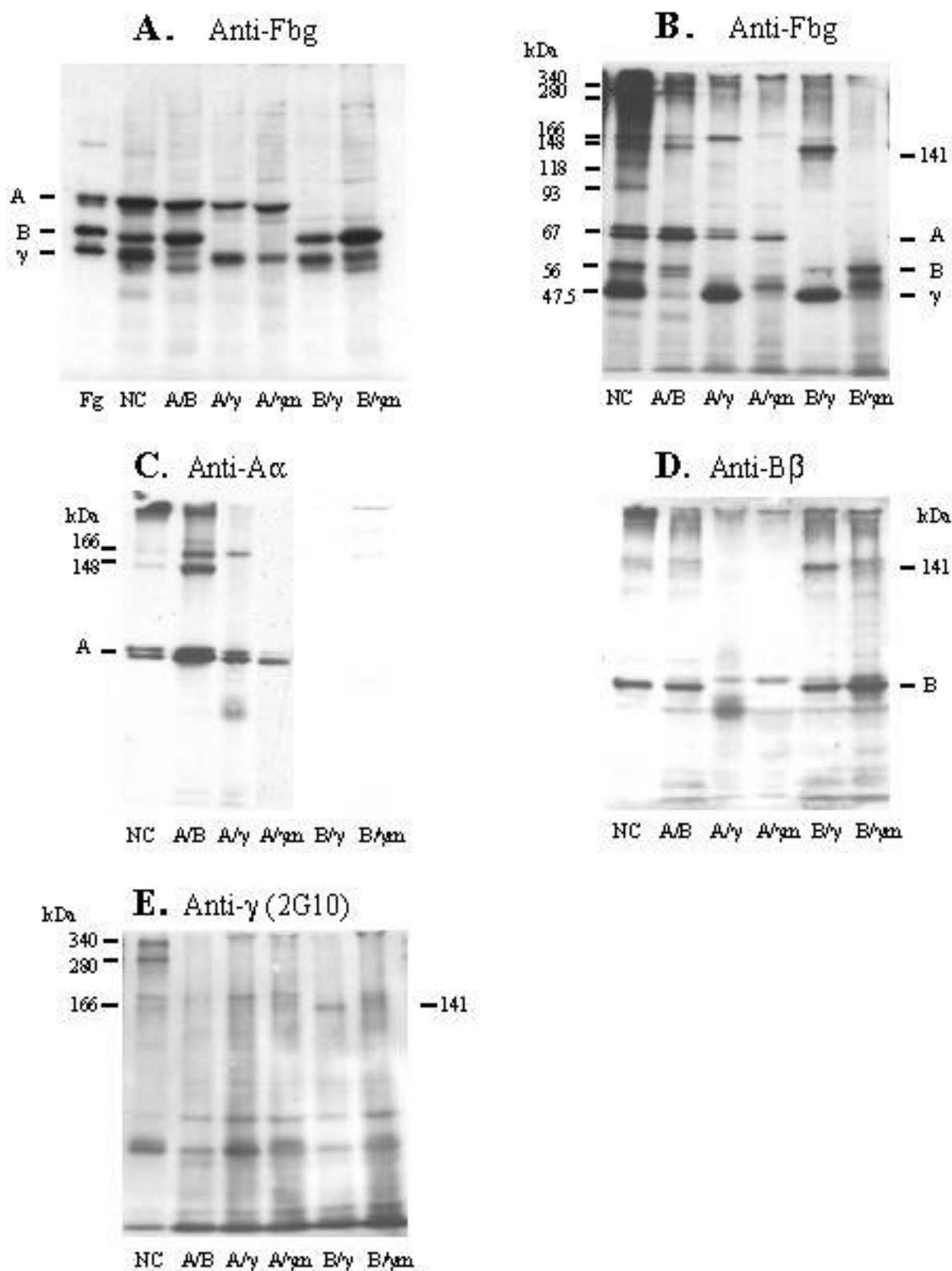


Fig. 1

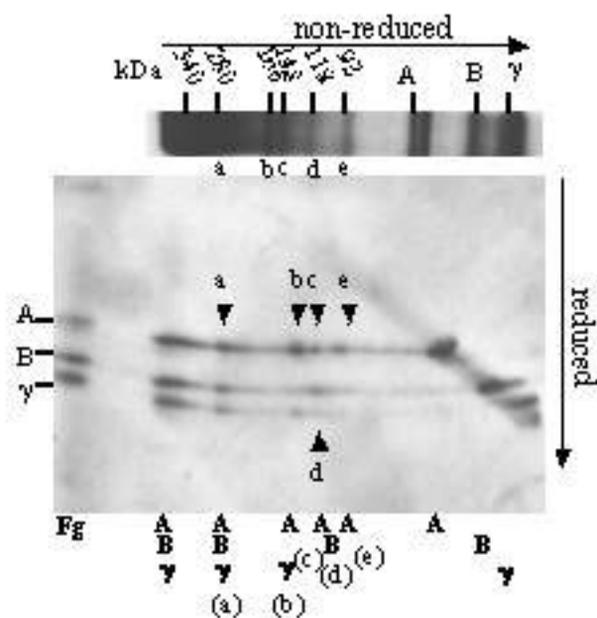
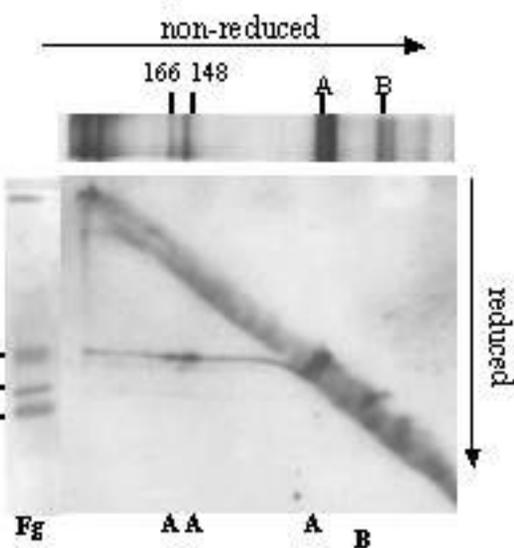
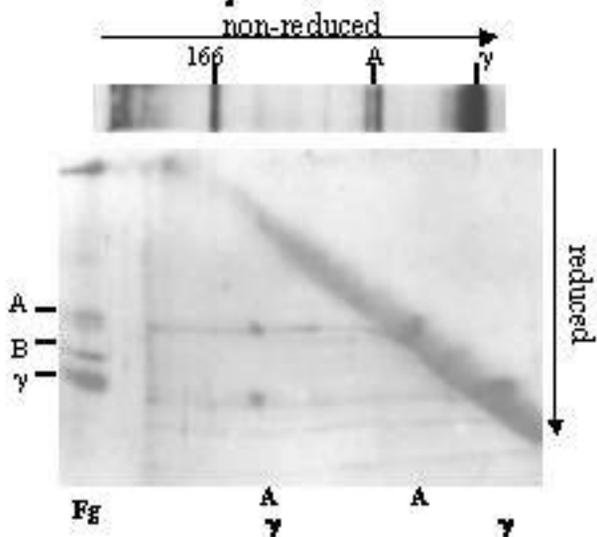
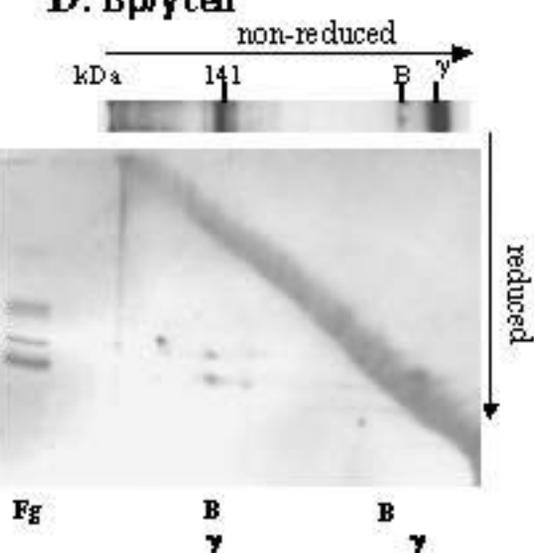
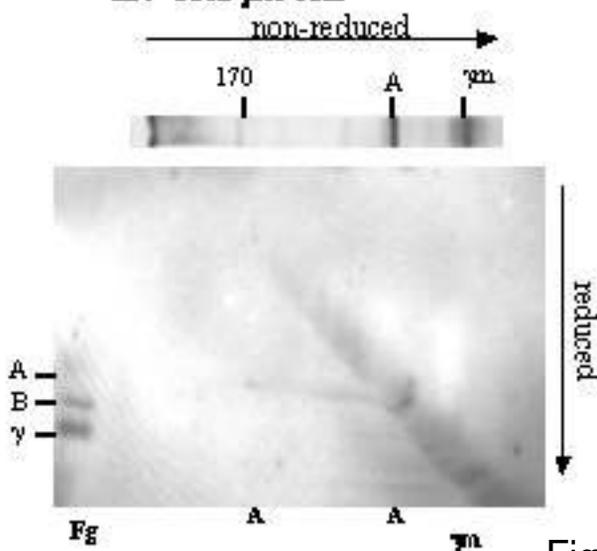
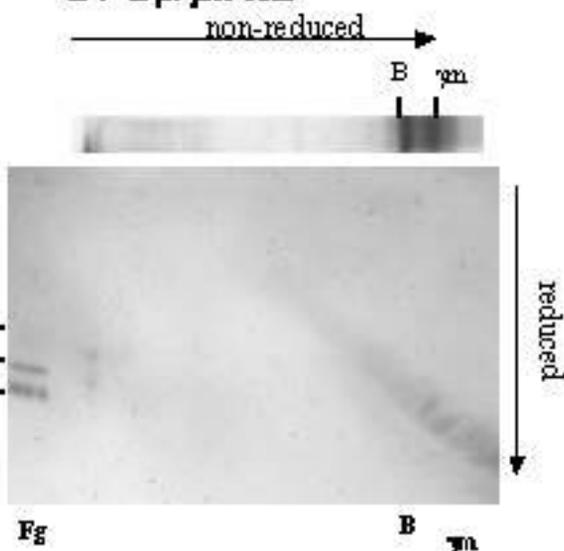
**A. NC cell****B. A $\alpha$ /B $\beta$  cell****C. A $\alpha$ / $\gamma$  cell****D. B $\beta$ / $\gamma$  cell****E. A $\alpha$ / $\gamma$ m cell****F. B $\beta$ / $\gamma$ m cell**

Fig. 2