

Correspondence

To the editor:

Evidence against a protein in plasma that is a product of a factor XI mRNA splice variant missing exons 6 and 7

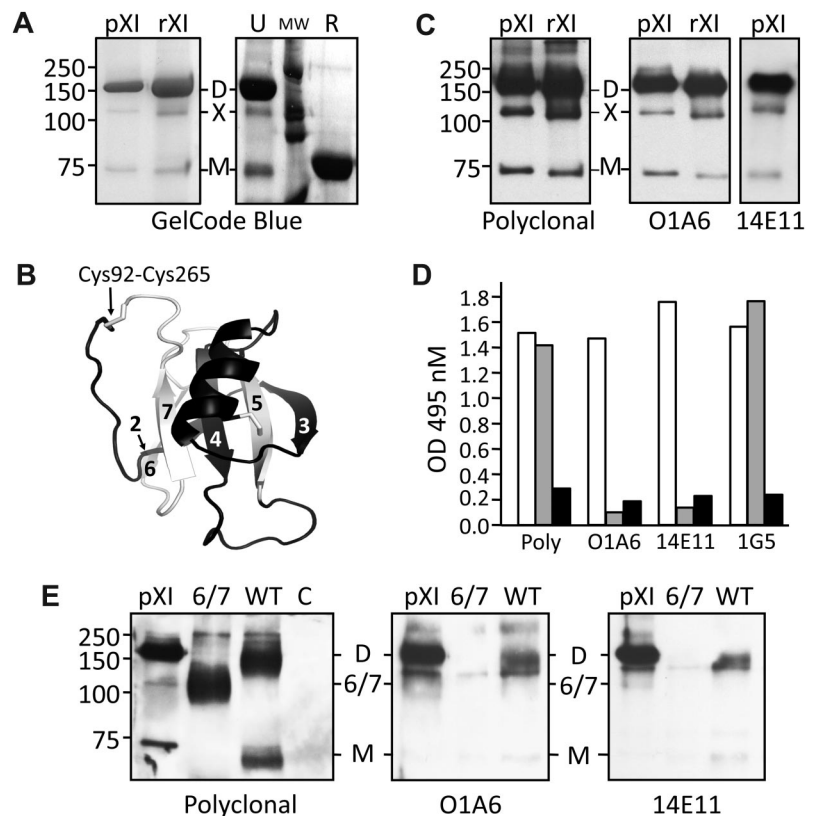
We read with interest the paper in *Blood* from Asselta et al¹ describing a form of human factor XI (fXI) encoded by an mRNA splice variant lacking exons 6 and 7 (fXI- Δ 6/7). We observe 3 bands when plasma fXI purified by IgG affinity chromatography is size fractionated by nonreducing gel electrophoresis (Figure 1A). The dominant 160 kDa form contains 2 identical subunits connected by a disulfide bond.²⁻⁴ The fainter band (70-80 kDa) migrating with reduced fXI represents single subunits. The third band, marked X in Figure 1A, is likely the approximately 105-kDa species observed in plasma immunoprecipitates by Asselta et al.¹ fXI subunits contain 4 apple domains (A1-A4, exons 3-10) and a protease domain (exons 11-15).^{2,3} A fXI- Δ 6/7 subunit would lack parts of A2 and A3 (amino acids 145-234). However, modeling (Figure 1B) indicates a complete apple domain could form from the N- and C-terminal portions of A2 and A3, respectively, encoded by exons 5 and 8.⁵ Asselta et al showed that fibroblasts transfected with fXI- Δ 6/7 cDNA synthesize a protein that may have this hybrid domain,¹ and postulated that fXI- Δ 6/7 and the 105-kDa form in plasma are identical based on electrophoretic mobility. Here we show that the 105-kDa plasma form is not likely to be fXI- Δ 6/7.

First, the 160- and 105-kDa species are both secreted by fibroblasts transfected with full-length factor XI cDNA (Fig 1A).^{6,7} Second, monoclonal antibodies that bind to the A3 (O1A6)^{8,9} or A2

(14E11)⁹ domains both recognize all 3 fXI forms on Western blots (Figure 1C). We transfected HEK293 fibroblasts with expression constructs for wild type fXI (WT-fXI) and fXI- Δ 6/7. In an enzyme-linked immunosorbent assay (ELISA), cell lysates for WT-fXI and fXI- Δ 6/7 both gave strong signals when polyclonal anti-human fXI IgG or a monoclonal IgG against the protease domain (1G5)^{6,7} is the capture antibody (Figure 1D). In contrast, only WT-fXI is captured by O1A6 or 14E11. Immunoprecipitates of cell lysates were studied by Western blotting. Again, polyclonal IgG recognized both WT-fXI and fXI- Δ 6/7 (Figure 1E left panel), whereas O1A6 and 14E11 recognized only WT-fXI (Figure 1E center and right panels). These data clearly show that the 105-kDa plasma protein is not identical to the protein from cells transfected with the fXI- Δ 6/7 cDNA.

The nature of the 105-kDa plasma species is not clear. It probably is a dimer, as it is not observed in reduced fXI (Figure 1A). Because both 160- and 105-kDa forms are secreted together by cells in serum-free media, they probably vary due to differences in posttranslational modifications. The apple domains of fXI form a tightly packed platform with extensive interdomain interfaces.^{5,10} Our initial evaluation indicates the hybrid domain in fXI- Δ 6/7 would not form interfaces properly, possibly resulting in an arrangement where the apple domains are like beads on a string.

Figure 1. SDS-polyacrylamide gel electrophoresis, western immunoblots, and ELISA of human factor XI. (A left panel) GelCode blue-stained 10% polyacrylamide-sodium dodecyl sulfate gel of plasma (pXI) or recombinant (rXI) human fXI (2-4 μ g) purified using a monoclonal IgG against the A3 domain (pXI) or protease domain (rXI). (A right panel) Recombinant fXI (4 μ g) run unreduced (U) or reduced (R). MW indicates molecular weight markers. (B) Ribbon model of a hypothetical hybrid apple domain encoded by the fXI Δ 6/7 mRNA, based on the primary amino acid sequence encoded by exons 5 (black ribbon) and 8 (white ribbon) and the crystal structure of human fXI.⁵ The domain is bounded by a disulfide bond between Cys92 and Cys265. Strands 2-7 of the β -sheet are numbered. (C) Chemiluminescent Western blots of plasma (pXI) or recombinant (rXI) fXI using goat polyclonal anti-factor XI IgG (left panel), monoclonal IgG O1A6 (recognizes A3 domain; center panel), or monoclonal IgG 14E11 (recognizes A2 domain; right panel) as the primary antibody. (D) ELISA of cell lysates from HEK293 fibroblasts transiently transfected with expression constructs for WT-fXI (\square), fXI- Δ 6/7 (\blacksquare), or empty vector (pJVCMV^{6,7}; \blacksquare). The ELISA capture antibodies were a goat polyclonal anti-human fXI IgG (Poly), O1A6, 14E11, or a monoclonal antibody against the protease domain (1G5). The detection antibody was a goat polyclonal anti-human fXI IgG conjugated to horseradish peroxidase. (E) Chemiluminescent Western blots of immunoprecipitates of lysates of HEK293 cells transfected with WT-fXI (WT), fXI- Δ 6/7 (Δ 6/7), or empty vector (C). pXI is a plasma fXI control. Immunoprecipitation was done with IgG 1G5.^{6,7} Primary antibodies are indicated below each blot. For panels A, C, and E, positions of molecular mass standards (in kDa) are indicated on the left. D indicates fXI dimer; M, fXI monomer; X, putative fXI Δ 6/7 splice variant; and 6/7, fXI-6/7 splice variant.



The disruption of the platform would likely interfere with binding to high-molecular-weight kininogen,¹⁰ a protein that forms a complex with fXI in plasma. This, and the absence of fXI coagulant activity,¹ raise questions about the function of such a protein, if it was expressed.

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14E11; A.G. and E.I.T. generated and characterized IgG O1A6 and 14E11; and J.E. modeled fXI apple domains. All authors contributed to writing the final version of the letter.

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Response

Further thoughts on the “phantom” $\Delta 6/7$ FXI isoform

We are pleased that our work raised the interest of the scientific community. Indeed, the main aim of the paper was to characterize the pattern of alternative splicing of the factor XI (F11) gene and to study its role in the regulation of F11 expression by the coupling of nonproductive splicing and nonsense-mediated mRNA decay.

In their letter, Gailani and coworkers focused their efforts in providing evidence against the existence of a factor XI (FXI) protein isoform in plasma originating from the skipping of exons 6 and 7 and therefore lacking the C-terminal half of the A2 domain and the N-terminal half of the A3 domain (FXI- $\Delta 6/7$). The rationale of their strategy was to show that the FXI- $\Delta 6/7$ is not recognized by monoclonal antibodies against A2 and A3 domains, whereas these same antibodies readily react against an approximately 105-kDa band immunoprecipitable from both human plasma and media conditioned by cells expressing full-length recombinant FXI. However, Gailani and colleagues' position is based on the assumption that the 105-kDa band corresponds to the hypothetical $\Delta 6/7$ band we found in human plasma. This is unlikely to be true for the following reasons: (1) The 105-kDa band was easily detectable by Coomassie staining loading just 2 to 4 μg of FXI. Conversely, we could detect the putative $\Delta 6/7$ FXI isoform by Coomassie-stained gels only loading at least 10 μg of commercial FXI, or by Western blotting after FXI immunoprecipitation from 1.5 mL of human plasma. (2) At variance with Gailani et al, we cannot see a 105-kDa band (or any band of a similar molecular weight) when expressing

recombinant full-length FXI in eukaryotic cells. The same result was found in other papers in the literature.^{1,2} Moreover, also in the data presented by Gailani et al, this band is undetectable in lysates of FXI-expressing HEK293 cells (compare panels C and E of Figure 1 in Gailani et al). (3) When immunoprecipitating FXI from human plasma with a mouse monoclonal antibody, we detected a band (under nonreducing conditions) migrating below FXI that was shown, by mass-spectrometry analysis, to correspond to contaminating mouse IgGs (data not shown in Asselta et al³). (4) The matching between the 105-kDa species and the putative $\Delta 6/7$ FXI isoform is further hampered when considering that more than one band is detectable in nonreducing SDS-PAGE of plasma FXI purified by affinity chromatography with goat anti-human FXI polyclonal antibody (our unpublished data). Mass spectrometry of these additional excisable bands demonstrated that they correspond to contaminating proteins (ie, ceruloplasmin, complement factor H, haptoglobin, and serum albumin).

In conclusion, we agree that our data do not unequivocally demonstrate the existence of a circulating FXI isoform originating from the $\Delta 6/7$ FXI transcript (as thoughtfully stated in our original paper³). However, we feel that the results presented by Gailani and colleagues are still not conclusive. A future possibility could be to purify enough FXI from human plasma to be able to analyze fast-migrating FXI-specific bands by mass spectrometry after digestion with a protease (such as Glu-C) generating $\Delta 6/7$ -specific peptides.