# Exosites expedite blood coagulation

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**A careful balance between active-site and exosite contributions is critically important for the specificity of many proteases, but this balance is not yet defined for some of the serine proteases that serve as coagulation factors. Basavaraj and Krishnaswamy have closed an important gap in our knowledge of coagulation factor X activation by the intrinsic Xase complex by showing that exosite binding plays a critical role in this process, which they describe as a "dock and lock." This finding not only significantly enhances our understanding of this step in the coagulation cascade and highlights parallels with the prothrombinase complex, but will also provide a novel rationale for inhibitor development in the future.**

The ability of blood to clot locally depends on the assembly and function of the two essential complexes of blood coagulation, called the Xase and prothrombinase complexes. These related protein complexes assemble in a similar manner on cell surfaces; the Xase complex converts factor X (FX) to factor Xa (FXa), and prothrombinase converts prothrombin to thrombin (Fig. 1). Both are essential for normal blood clotting (hemostasis) and are involved in pathological clotting (thrombosis). Current treatments for thrombotic disorders—active-site inhibitors of thrombin—can cause bleeding as a side effect. It is therefore important to understand how these complexes assemble and function to provide new avenues for the development of novel anticoagulants.

One challenge in developing protease inhibitors is in creating a molecule that is sufficiently specific to act only on the target of interest. This is particularly a problem for serine proteases like the promiscuous trypsin, which is structurally similar to Xase and prothrombinase. Yet Xase and prothrombinase complexes are extremely specific for their respective substrates, raising the question of how they discriminate among all the possible substrates present in blood plasma. Several advances have been made in recent years to better understand these structure-function relationships (1, 2). Whereas a number of structures of coagulation factor serine protease domains are available that provide insights into the enzymatic mechanisms of zymogen activation, how this process works in the context of the full-length enzymes and larger molecular assemblies is less well-understood and experimentally more difficult to tackle. Moreover, for many coagulation factors, it is not clear how specificity and activity may be influenced by regions external to the active site. Whereas some examples of exosites have been uncovered (3), the best-characterized complex in this respect sits at the "end" of the cascade: the prothrombin complex releases thrombin to initiate blood clotting in an exosite-dependent manner (4), which is also naturally exploited for inhibition of this key protease (5).

The intrinsic Xase complex is a closer structural equivalent to the prothrombinase complex and is composed of factor IXa (FIXa) and factor VIIIa (FVIIIa) at the membrane (Fig. 1). The role of the intrinsic Xase complex is to activate FX, but it can also be activated by the extrinsic Xase, consisting of factor VIIa and tissue factor. The activation of FX thus sits at the crossroads of the intrinsic and extrinsic blood coagulation cascade and thus represents an important target for the development of anticoagulants (6). However, studying the mechanistic details of the activation of FX by the Xase complex has proven challenging due to the difficulty of accurately measuring rates for FIXa due to interference of cosolvents with complex stability and general low peptidolytic activity requiring maturation of the active site in the context of the Xase complex (7). Basavaraj and Krishnaswamy (8) have now circumvented this problem by using the fluorogenic peptidyl substrate PF-3688, which allows reliable kinetic parameters to be determined in the absence of alcohols.

Using a clever combination of this substrate and the pan-specific competitive inhibitor 4-aminobenzamidine (pAB), an arginine analog that binds to the S1 pocket of arginine-specific serine proteases, they reveal that cleavage by FIXa in isolation or in the context of the complex with FVIIIa and small unilamellar phospholipid vesicles (PCPS) displays competitive inhibition behavior, whereby pAB and PF-3688 binding is mutually exclusive. However, pAB binding did not affect FX binding to intrinsic Xase in a mutually exclusive way, whereby an increase in pAB concentration decreased $V_{max}$ without significantly increasing the $K_m$ for FX. Hence, they were able to conclude that the affinity of FX for Xase did not rely on the active site, so the initial contact involves exosites in the Xase complex that recognize FX prior to engaging the FX scissile bond with the Xase active site.

To further validate this mechanism, two FX mutants, active-site serine $X_{S195A}$ and the $X_{R15Q}$ mutant deficient in FIXa (and Xase) engagement, were employed. Both mutants acted as competitive inhibitors of FX activation, showing that interference with exosite binding must be the cause for this behavior as the mutants are unable to engage the active site of FIXa. To confirm that the $X_{R15Q}$ mutant is indeed unable to be cleaved by Xase, gel-shift assays using SDS-PAGE analysis were carried out after incubating Xase with either FX from plasma or the

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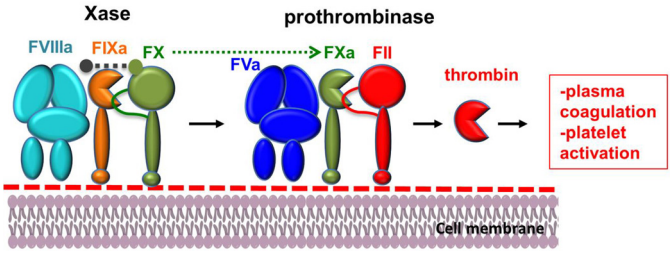


**Figure 1. Schematic diagram showing the assembly of the intrinsic Xase in the surface of the activated phospholipid cell membrane (*red dotted line*), consisting of cofactor FVIIIa (*cyan*) with FIXa (*orange*) required to cleave substrate FX (*green*).** The critical importance of exosites involved in this step as highlighted by Basavaraj and Krishnaswamy (8) is indicated by a *dashed line*. The resulting proteolysis generates FXa, which subsequently drives the prothrombinase complex formed in a similar way with cofactor FVa, resulting in the cleavage of prothrombin (*FII*) to generate thrombin, which then goes on to cleave a variety of substrates, driving plasma coagulation and platelet activation.

$X_{R15Q}$ mutant. No bands indicating FXa formation were observed for the mutant.

Moreover, fluorescence anisotropy binding assays in the presence of PCPS were carried out to further verify active site–independent interactions between FX and intrinsic Xase. For these experiments, the active site of IXa was covalently blocked by Phe-Phe-Arg-chloromethyl-ketone modified by fluorescein 5-maleimide to generate factor IXai (FIXai), a long-established method (9) to study Xase interactions. First the authors confirmed that they could observe formation of Xase. When increasing amounts of thrombin were added, FVIII cleavage in a reaction containing inactivated FIXai increased anisotropy, indicative of a tight interaction between FIXai and FVIIIa on the membrane surface. Next, FIXai was used to construct an inactive Xase complex that, when titrated with $X_{S195A}$ or $X_{R15Q}$ mutants, also yielded an equivalent increase in anisotropy. Remarkably, from these experiments, it was possible to estimate a dissociation constant of ~15 nM, which demonstrates the existence of high-affinity binding exosites for FX in the Xase complex. Whereas previous research on FX activation had provided some clues that exosites might be involved, these data provided compelling new evidence that exosite binding is part of the catalytic pathway. The strategy of using competitive and noncompetitive, reversible and irreversible inhibitors in kinetic and binding assays affecting the interplay between these three critical coagulation factors in combination with uncleavable variants will be applicable for the investigation of other dynamic multienzyme assemblies that are difficult to investigate by standard means.

This research will open up exciting new possibilities for targeting this specific interaction in the future with exosite-specific inhibitors, which—given what has been shown for targeting such sites in the prothrombin complex—may open up a whole new chapter for anticoagulants. It is known that FIXa has at least two exosites, exosite I (calcium-binding site) and II (heparin-binding site), in the catalytic domain, and it is predicted that all four domains of FIXa wrap across FVIIIa, generating inter- and intraprotein binding sites in the Xase complex (10). It will now be paramount to identify the structural characteristics of the exosite and full Xase complex structures to define the residues involved at the interface, which may in turn provide a scaffold for the development of novel anticoagulants with a reduced bleeding side effect.

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*Abbreviations*—The abbreviations used are: PF-3688, H-d-leucyl-phenyl-glycyl-arginine-7-amino-4-methylcoumarin; pAB, 4-amino-benzamidine; PCPS, small unilamellar phospholipid vesicles; $X_{S195A}$, factor X with an Ala substitution at $Ser^{195}$; $X_{R15Q}$, factor X with a Gln substitution at $Arg^{15}$.

## References

1. Goettig, P., Brandstetter, H., and Magdolen, V. (2019) Surface loops of trypsin-like serine proteases as determinants of function. *Biochimie* **166,** 52–76 CrossRef Medline
2. Li, X., Sim, M. M. S., and Wood, J. P. (2020) Recent insights into the regulation of coagulation and thrombosis. *Arterioscler. Thromb. Vasc. Biol.* **40,** e119–e125 CrossRef Medline
3. Mohammed, B. M., Matafonov, A., Ivanov, I., Sun, M.-F., Cheng, Q., Dickeson, S. K., Li, C., Sun, D., Verhamme, I. M., Emsley, J., and Gailani, D. (2018) An update on factor XI structure and function. *Thromb. Res.* **161,** 94–105 CrossRef Medline
4. Krishnaswamy, S. (2013) The transition of prothrombin to thrombin. *J. Thromb. Haemost.* **11,** 265–276 CrossRef Medline
5. Huntington, J. A. (2014) Natural inhibitors of thrombin. *Thromb. Haemost.* **111,** 583–589 CrossRef Medline
6. Zögg, T., and Brandstetter, H. (2011) Complex assemblies of factors IX and X regulate the initiation, maintenance, and shutdown of blood coagulation. *Prog. Mol. Biol. Transl. Sci.* **99,** 51–103 CrossRef Medline
7. Kristensen, L. H., Olsen, O. H., Blouse, G. E., and Brandstetter, H. (2016) Releasing the brakes in coagulation Factor IXa by co-operative maturation of the substrate-binding site. *Biochem. J.* **473,** 2395–2411 CrossRef Medline
8. Basavaraj, M. G., and Krishnaswamy, S. (2020) Exosite binding drives substrate affinity for the activation of coagulation factor X by the intrinsic Xase complex. *J. Biol. Chem.* **295,** 15198–15207 CrossRef Medline
9. Lollar, P., Parker, E., Curtis, J. E., Helgerson, S. L., Hoyer, L. W., Scott, M. E., and Scandella, D. (1994) Inhibition of human factor VIIIa by anti-A2 subunit antibodies. *J. Clin. Invest.* **93,** 2497–2504 CrossRef Medline
10. Venkateswarlu, D. (2014) Structural insights into the interaction of blood coagulation co-factor VIIIa with factor IXa: a computational protein-protein docking and molecular dynamics refinement study. *Biochem. Biophys. Res. Commun.* **452,** 408–414 CrossRef Medline

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