Fusion Proteins with Anticoagulant and Fibrinolytic Properties: Functional Studies and Structural Considerations

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ABSTRACT

In an effort to combine the benefits of fibrinolytics, such as staphylokinase, with those of thrombin inhibitors for the prevention of vessel reocclusion after vascular injury, we have produced several chimeric proteins with plasminogen-activating and thrombin-inhibiting properties. Fusion proteins were constructed consisting of the modules staphylokinase (Sak), the factor Xa cleavage site, and various dipetalin (Dip) domains (H$_6$-Sak-Dip-I+II, H$_6$-Sak-Dip-I, and H$_6$-Sak-Dip-II). Sak stimulates fibrinolysis via activation of plasminogen, whereas dipetalin is a two-domain, Kazal-type inhibitor of thrombin. NMR spectroscopy of the fusion proteins revealed that the molecular structures of the modules are retained in the fusion protein and that no significant interactions occur between the modules in terms of their functionally relevant regions. In enzymatic thrombin inhibition tests and blood coagulation assays (thrombin, prothrombin, and activated partial thromboplastin times), no significant differences in anticoagulant capacity were observed between the fusion protein H$_6$-Sak-Dip-I+II and isolated Dip-I+II, even at nanomolar concentrations. Similar results (i.e., the inhibition of thrombin-induced platelet aggregation and the inhibition of thrombin-induced vascular relaxation) were obtained when the cellular thrombin effects were studied. The fusion protein containing Dip-I has less but still significant thrombin inhibitory effects compared with those of H$_6$-Sak-Dip-I+II. In contrast, the H$_6$-Sak-Dip-II protein failed to inhibit thrombin in each of the assays used. The plasminogen-activating and fibrinolytic activities of the fusion proteins are similar to those of wild-type Sak. The individual dipetalin domains do not activate plasminogen. In conclusion, the fusion protein H$_6$-Sak-Dip-I+II is a bifunctional molecule able to activate fibrinolysis via plasminogen activation and inhibit blood coagulation via direct inhibition of thrombin.

Plasminogen activators convert the zymogen plasminogen into plasmin, which initiates the lysis of blood clots. Staphylokinase (Sak), a plasminogen activator originally isolated from Staphylococcus aureus, exhibits a high thrombolytic efficacy (Collen and Van de Werf, 1993). Recent reports on staphylokinase-related research (Thomson, 1999; Okada et al., 2000; Wong and White, 2000; Goldhaber, 2001; Moons et al., 2001; Sazonova et al., 2001) demonstrate the growing interest in the development of Sak-based plasminogen activators. In addition, Sak is presently undergoing clinical trials (Armstrong et al., 2000; Heymans et al., 2000; Laroche et al., 2000) as a potential drug for thrombolytic treatment. Despite successful lytic therapy of thromboembolic disorders, such as acute myocardial infarction, reocclusion of the damaged vessels frequently reduces the therapeutic effect. Because there is a high procoagulatory potential with increased thrombin liberation at the injured site of the vessel,
Materials and Methods

Protein Expression and Purification. The H6-Sak-Dip-I+II, H6-Sak-Dip-I, and H6-Sak-Dip-II fusion proteins were produced recombinantly by transforming Escherichia coli TG1 cells with the expression plasmids pMEXH6-Sak42D-(QF)-FXa-Dip-I+II, pMEXH6-Sak42D-(QL)-FXa-Dip-I, and pMEXH6-Sak42D-(QF)-FXa-Dip-II, respectively. Cell cultivation, stimulation of expression, cell harvesting, and the preparation of cell extracts were performed as described by Schlott et al. (1994). Fusion protein purification was achieved by a uniform, multistep chromatographic procedure: two repetitions of metal-chelating chromatography using NiII-chelating Sepharose (Amersham Biosciences, Freiburg, Germany) and Ni-nitrotriacyclic acid agarose (QIAGEN GmbH, Hilden, Germany) were followed by gel filtration (Superdex 75 HR 10/60 column; Amersham Biosciences) and anion exchange chromatography (MonoQ HR 5/5 column; Amersham Biosciences). The cleavage of the fusion proteins by FXa (New England Biolabs, Schwalbach, Germany) was performed as described by Schlott et al. (1997). Separation of the individual dipetalin domains from H6-Sak was performed by metal-chelating Ni-nitrotriacyclic acid chromatography (QIAGEN). Final Dip-domain purification from the column flow-through was achieved by gel filtration. After being eluted from the metal-chelate column, the H6-Sak protein was purified by cation exchange chromatography (MonoS HR 5/5 column; Amersham Biosciences). The purity and identity of the isolated proteins was confirmed by SDS-PAGE (Fig. 1), matrix-assisted laser desorption ionization-mass spectrometry, and N-terminal sequencing (data not shown). For preparation of the 15N-labeled fusion protein, the culture medium was supplemented with 15NH4Cl (Cambridge Isotopes Laboratories, Inc., Andover, MA) as described by Ohlenschlager et al. (1997, 1998). The protein isolation procedure was the same as detailed above. After adding 35 μL of D2O (Cambridge Isotopes Laboratories, Inc.), the sample had a volume of 600 μL in a sealable NMR tube, which yielded a final concentration of 1.0 mM U-[15N]-H6-Sak-Dip-I. All chemicals (i.e., buffer substances and components for SDS-PAGE) were of the highest quality commercially available.

NMR Spectroscopy. Two-dimensional [1H-15N]-HSQC experiments (Bodenhausen and Ruben, 1980) were recorded at 15°C on an INOVA 750-MHz four-channel NMR spectrometer (Varian, Inc., Palo Alto, CA). The NMR spectra were processed with the VNMR software (Varian, Inc.) and analyzed using the program XEASY (Barthels et al., 1995).

Clotting Assays. The thrombin time (TT), prothrombin time (PT), and activated partial thromboplastin time (aPTT) were measured using a Thrombotrack 8 coagulometer (Immuno, Heidelberg, Germany). Commercially available test kits (Immuno, Vienna, Austria) were used. All measurements were carried out in duplicate according to the guidelines of the manufacturer.

Activation of Plasminogen and Fibrinolytic Activity. Activation of plasminogen (final concentration, 1.5 μM) induced by the proteins tested (final concentration, 5 nM) was assayed at 37°C in 0.1 M phosphate buffer, pH 7.4, containing 0.1% (v/v) Tween 80. Aliquots (5 μL) were withdrawn at different time intervals (0–30 min) and diluted 50-fold in a 1 mM solution of the chromogenic plasmin substrate S-2251. The plasmin generated was determined at different time intervals by measuring the change in absorbance at 405 nm over a period of 1 min.

Kinetic analysis of plasminogen activation was performed as described by Collen et al. (1993b). Initial activation rates were obtained from plots of concentration versus activation time of plasmin generated. The kinetic constants Kd, kcat, and catalytic efficiency kcat/KM were calculated using the Lineweaver-Burk plot. Measurement of fibrinolytic activity using fibrin plates was performed according to the method of Astrup and Müllertz (1952).

Fig. 1. 13.75% SDS-PAGE of the purified fusion proteins and isolated dipetalin domains. Lanes 1 and 10, 10-kDa protein calibration mixture (Invitrogen, Carlsbad, CA) (the lowest band refers to 10 kDa); lane 2, H6-Sak-Dip-I; lane 3, Dip-I; lane 4, H6-Sak-Dip-II; lane 5, Dip-II; lane 6, H6-Sak-Dip-I+II; lane 7, Dip-I+II; lane 8, H6-Sak; and lane 9, Sak42D.
Plasma Clot Lysis Time. To 100 µl of human citrated plasma, 100 µl of the proteins tested (H₆-Sak-Dip-I+II and H₆-Sak; final concentration, 100 nM) were added. The determination of the clot lysis time was performed as two-step process with a Thrombotrack 4 coagulometer, which allows measurement of both clot formation and fibrinolysis. After the addition of 50 µl of thrombin (final concentration, 80–200 nM), clots were induced within <10 s, subsequently, and the lysis time was measured in the same experimental system over a period of 15 min.

Platelet Aggregation. Platelet aggregation was monitored using an aggregometer APACT 4 (Labor GmbH, Ahrensburg, Germany). Washed platelets were obtained from peripheral, citrate-anticoagulated blood of healthy volunteers according to Glusa et al. (1997). Measurement of ADP- and collagen-induced platelet aggregation was carried out with platelet-rich plasma. The platelet count was adjusted to 3 × 10⁹ platelets/ml. Proteins tested (final concentrations, 0.5–100 nM) were added to the platelet suspension 3 min before the addition of aggregation-inducing agents. Changes in light transmission of the platelet suspension were recorded continuously for a maximum of 10 min. The extent of aggregation was determined by measuring the maximum amplitude of an increase in light transmission. Percentage values were derived from the ratio of the extent of aggregation in samples with the test compounds compared with those without.

Vascular Relaxation Studies on Isolated Vessels. Porcine lungs were obtained from the local slaughterhouse. Small branches of pulmonary arteries were carefully prepared, cut into rings (2 to 3 mm long and 1.5 to 2 mm wide), fixed between two L-shaped platinum hooks in a 10-ml organ bath filled with modified Krebs-Henseleit solution at 37°C (Glua et al., 1996). Changes in tension were recorded isometrically. Resting tension was adjusted to 20 mN throughout each experiment. After an initial stabilization period of 60 min, the rings were stimulated at intervals of 45 min, once with KCl (45 mM) and threetimes with PGF₂α (3 µM), until the contractile response had become constant. The integrity of the endothelium was assessed functionally by measuring the relaxation after application of bradykinin to a final concentration of 10 nM. In endothelium-denuded vessels, this relaxation was absent. The relaxant response to thrombin was studied when the contraction had reached a plateau after the addition of PGF₂α. Dipetalins and fusion proteins were added to the organ bath 5 min before thrombin.

Data Analysis. Data are represented as mean ± S.E.M. for n separate experiments. Concentration-effect curves were fitted using the computer program Origin (OriginLab Corp., Northampton, MA). Comparison of means was made using Student’s t test modified according to the Bonferroni method. Differences were considered statistically significant at p < 0.05.

Results

NMR Spectroscopy of the Fusion Proteins. NMR spectroscopy was used to identify structural changes in the fused modules of the proteins H₆-Sak-Dip-I and H₆-Sak-Dip-I+II. The comparison of [¹H-¹⁵N]-HSQC data of Sak (Ohlenschlager et al., 1997) and Dip-I (Schlott et al., 2002) with the spectrum of the respective fusion protein identifies residues affected. For the sake of clarity, this approach is shown in Fig. 2a for H₆-Sak-Dip-I. The [¹H-¹⁵N]-HSQC spectrum of H₆-Sak-Dip-I consists of the sum of signal sets observed for the individual protein components. Only a few residues exhibited larger chemical shift variations in the ¹H and ¹⁵N dimensions. Figure 2b shows a mapping of these residues onto the staphylokinase solution structure (Ohlenschlager et al., 1998). With two exceptions (Lys⁹⁶ and Glu⁴⁶), all affected residues are located in the vicinity of the C terminus of the staphylokinase part of the fusion protein as expected because of the short linker element of six amino acids between staphylokinase and the dipetalin modules. A similar observation is made in the [¹H-¹⁵N]-HSQC spectrum of H₆-Sak-Dip-I+II, which can be described as the sum of the spectra of the three contributing domains. The [¹H-¹⁵N]-HSQC spectra also indicate that Sak and the dipetalin domains in the fusion protein display their native folds. In addition, only minor interactions between the plasminogen-activating and the thrombin-inhibiting modules take place, as shown by the low number of resonances shifted.

Plasminogen Activation and Fibrinolytic Activity. Using chromogenic substrate tests, it was shown that the fusion proteins (listed in Table 1) had a time course of plasminogen activation that was similar to that of the wild-type Sak and H₆-Sak (Fig. 3). Differences at the 5-min data point were not statistically significant. From 10 min onward, the plasmin generation rate stabilized at a steady-state level. In
inhibitory potency of Dip-I+II and H$_{6}$-Sak-Dip-I+II. In contrast, Dip-II, Dip-I, and the respective fusion proteins caused significantly weaker anticoagulant effects. The H$_{6}$-Sak did not affect the aPTT.

As shown in Fig. 5c, a significant prolongation of the PT was observed only in the presence of Dip-I+II and H$_{6}$-Sak-Dip-I+II. However, concentrations of 300 nM were required to inhibit coagulation.

**Plasma Clot Lysis Time.** In presence of H$_{6}$-Sak (100 nM), a clot was formed 4 to 7 s after the addition of 80 to 200 nM thrombin (Fig. 6a). In this system, clot lysis was completed after 608 ± 8 s (Fig. 6b). Addition of H$_{6}$-Sak-Dip-I+II (100 nM) to the plasma required higher thrombin concentrations (>80 nM) for inducing coagulation. At 90 nM thrombin, a clot was present after 25.1 ± 4.4 s, whereas at 100 nM, clot formation time was below 10 s (Fig. 6a). The subsequent clot lysis time was not significantly different from that measured in the presence of H$_{6}$-Sak or wild-type Sak (Fig. 6b). In control experiments, the thrombin-induced clot was stable against lysis over the period of 15 min, which rules out the contribution of endogenous plasminogen activators.

**Inhibition of Thrombin-Induced Platelet Aggregation.** Dip-I+II and H$_{6}$-Sak-Dip-I+II strongly inhibited thrombin-induced (1 nM) platelet aggregation (Fig. 7). A significant inhibitory effect was obtained at a concentration as low as 1 nM, whereas 20-fold higher concentrations were required to obtain similar inhibition of aggregation by Dip-I and H$_{6}$-Sak-Dip-I (Fig. 7). Dip-II and H$_{6}$-Sak-Dip-II produced no significant inhibitory effects on thrombin-induced platelet aggregation at comparably high concentrations (data not shown). In addition, the fusion proteins did not affect ADP- and collagen-induced aggregation.

**Influence on Vascular Relaxation.** In porcine pulmonary arteries with intact endothelium, thrombin (1 nM) caused a reversible relaxation of PGF$_{2\alpha}$-precontracted vessels of 84 ± 5% (n = 20). As shown in Fig. 8, Dip-I+II and H$_{6}$-Sak-Dip-I+II proved to be potent inhibitors of thrombin-induced, endothelium-dependent relaxation. Dip-I and H$_{6}$-Sak-Dip-I showed similar results, but 10 times higher concentrations were necessary to obtain significant inhibitory effects. In contrast to these findings, no inhibition of relaxation was obtained with Dip-II, H$_{6}$-Sak-Dip-II, and H$_{6}$-Sak. Thus, the present experiments confirm that the dipetalin domains and

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**Table 1**

Predicted molecular masses derived from the primary structure, masses determined by mass spectroscopy, and the $K_i$ values of the respective proteins for the interaction with thrombin.

<table>
<thead>
<tr>
<th>Structure</th>
<th>MALDI-MS Da</th>
<th>MALDI-MS $M^+$/pM</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_{6}$-Sak-Dip-I</td>
<td>23,167</td>
<td>23,264</td>
<td>353</td>
</tr>
<tr>
<td>Dip-I</td>
<td>6,083</td>
<td>6,077</td>
<td>75</td>
</tr>
<tr>
<td>H$_{6}$-Sak-Dip-II</td>
<td>23,717</td>
<td>23,750</td>
<td>&gt;353</td>
</tr>
<tr>
<td>Dip-II</td>
<td>6,598</td>
<td>6,576</td>
<td>&gt;353</td>
</tr>
<tr>
<td>H$_{6}$-Sak-Dip-I+II</td>
<td>29,096</td>
<td>29,158</td>
<td>0.78</td>
</tr>
<tr>
<td>Dip-I+II</td>
<td>12,011</td>
<td>11,995</td>
<td>&gt;353</td>
</tr>
<tr>
<td>H$_{6}$-Sak</td>
<td>17,102</td>
<td>17,175</td>
<td>&gt;353</td>
</tr>
<tr>
<td>Sak42D</td>
<td>15,583</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not determined; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry.

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**Fig. 3.** Activation of plasminogen (final concentration, 1 µM) as a function of time by Sak42D (●), H$_{6}$-Sak (●), H$_{6}$-Sak-Dip-I+II (●), Dip-I+II (○), H$_{6}$-Sak-Dip-I (■), Dip-I (■), H$_{6}$-Sak-Dip-II (▲), and Dip-II (△) (final concentrations, 5 nM each). Data shown are mean ± S.E.M.; n = 5.

**Fig. 4.** Concentration-effect curves for fibrinolysis by H$_{6}$-Sak-Dip-I, H$_{6}$-Sak-Dip-II, H$_{6}$-Sak-Dip-I+II, H$_{6}$-Sak, and Sak on fibrin plates after 20 h. Data are given as mean ± S.E.M.; n = 8–9.
fusion proteins containing these domains block cellular effects of thrombin in a way that correlates with the inhibition of the proteolytic activity of thrombin.

Discussion

The synthesis of fusion proteins is a feasible way of designing multifunctional compounds of pharmacological interest. Therapy of thromboembolic disorders can be improved by the application of compounds that simultaneously dissolve a thrombus and inhibit the de novo formation of blood clots.
(Collen and Lijnen, 1995; van Zyl et al., 1997). These goals can be achieved by the combination of a thrombin inhibitor and a plasminogen activator. However, the results obtained to date (i.e., with hirudin N- or C-terminally fused to staphylokinase) have not been convincing.

The outcome of the present study of a new fusion protein that combines the advantageous effects of staphylokinase and the Kazal-type inhibitor dipetalin may indicate a benefit of this protein in therapeutic application. The construction of this Sak-Dip fusion protein was based on this rationale: staphylokinase is a globular protein and the smallest plasminogen activator currently known, with high efficacy in plasminogen activation and fibrinolysis (Collen et al., 1993a; Collen and Van de Werf, 1993); dipetalin is an efficient thrombin inhibitor composed of two molecular domains that are flexible due to an interdomain linker peptide. NMR data demonstrate that the folding of the individual domains of Dip is independent.

Our previous studies (Gase et al., 1996) have shown that Sak can be elongated by adding amino acids to the C terminus without any loss in its plasminogen-activating potency. Therefore, the dipetalin domains were C-terminally fused to Sak by a flexible linker element of six residues. This segment, together with the two flexible Dip domains, was designed to reduce steric hindrance between the individual components and, hence, also minimize restrictions on the bifunctional characteristics.

Some authors argue that the N-terminally fused Sak should be protected against proteolytic activities of plasmin (Szmraj et al., 2001). However, the products of proteolytic processing, mainly SakΔN10, arise during plasminogen activation in any case. Furthermore, we have shown that this N-terminal processing is an essential step in the activation process (Schlott et al., 1997, 1998). The N-terminal extension by a His tag to optimize the protein purification had no disadvantageous effect on the Sak function.

Finally, there was no evidence for an increase in affinity for fibrin when RGD sequences or kringle domains were inserted into a fusion protein (van Zyl et al., 2000; Szmraj et al., 2001). This approach would not seem to be productive, because Sak itself acts in a fibrin-specific manner (Collen et al., 1993b).

The rational design of an optimized bifunctional fusion protein was supplemented by structural studies using NMR spectroscopy. Comparison of the [1H,13C]-HSQC spectrum of the fusion protein with the spectra of Sak (Ohlenschlager et al., 1997) and Dip-I (Schlott et al., 2002) shows that the modules in the fusion protein exhibit the same globular fold. The three main areas of Sak identified as important for biological function (Silence et al., 1995) [i.e., residues 1–12 (N terminus), residues around Lys50, and residues in the α-helix from Glu65 to Asp69] are not affected, as demonstrated by the absence of pronounced chemical shift changes in the NMR spectrum of the fusion protein. A similar situation exists for Dip-I, where only a few residues show changes in resonance frequencies, indicating that the core-structural elements are not affected by domain interactions. It is known from heteronuclear relaxation data from a previous study (Schlott et al., 2002) that a higher conformational flexibility is observed at the N terminus and at the four C-terminal residues of the Dip-I module constituting the linker to the Dip-II domain. It also must be assumed that the Sak-Dip-I linker consisting of the six residues QLEEGGR does not introduce conformational restrictions. Thus, these flexible linkers allow an independent reorientation of the modules Sak, Dip-I, and Dip-II. Consequently, the NMR results are consistent with the retention of the plasminogen-activating and thrombin-inhibiting properties in the fusion protein.

The present functional studies provide evidence that the fusion proteins, like their individual modules, exhibit both anticoagulant and fibrinolytic effects. The functional experiments comprise global clotting and fibrinolysis tests as well as thrombin-induced platelet aggregation and vascular relaxation. The clotting assays confirm the strong anticoagulant activity of the domain Dip-I+II. Generally, in all tests, including the cellular experiments, the thrombin inhibitory potency of the domains Dip-I and Dip-II was less than that of Dip-I+II. It was previously shown in tests with chromogenic substrates that the combination of Dip-I and Dip-II did not exceed the efficacy of Dip-I alone. Therefore, Dip-II seems to be important for thrombin inhibition only when it is linked to the Dip-I domain via a flexible linker peptide, as indicated from the structural NMR studies (Schlott et al., 2002). Knowledge of the tertiary structure of Dip-II, currently under investigation, is required to clarify whether Dip-II binds to the thrombin anion-binding exosite 1.

There were no significant differences between the anticoagulant activities of the fusion proteins with Sak and the individual Dip domains. On the other hand, the Sak fusion proteins are active as plasminogen activators and fibrinolytic agents with the same potency as the wild-type Sak, whereas the protein domains without Sak are ineffective. Thus, the functional studies confirm the structural NMR results with the fusion proteins as discussed above. Among the different coagulation tests, the TT is the most sensitive method to test the anticoagulant effect of H6-Sak-Dip-I+II and Dip-I+II. Higher concentrations of the fusion proteins were required to prolong the aPTT or PT. For comparison, the anticoagulant effects of H6-Sak-Dip-I+II and Dip-I+II are in the same concentration range as that for hirudin, the well-known, most potent recombinant thrombin inhibitor (Markwardt, 1994c).

Moreover, it was of interest to demonstrate whether the fusion proteins also inhibited thrombin-induced cellular effects mediated by stimulation of protease-activated receptors. Thrombin is the most potent agonist of platelet activation, and at very low concentrations, it causes human platelet aggregation and relaxation with the same potency observed for the inhibition of aggregation. The same thrombin concentration (1 nM) was used for the inhibition of aggregation and relaxation, the concentration-response curves for the inhibition of both effects had a similar shape. The present results with H6-Sak-Dip-I+II and Dip-I+II correlate well with the results for the inhibition of these effects by hirudin and triabin, whereas hirudin had slightly greater effects on the inhibition of thrombin (Markwardt, 1991). Triabin is a thrombin anion-binding exosite 1 inhibitor of thrombin that inhibits the cellular effects, probably by interfering with the thrombin recognition site of protease-activated receptor 1, thereby preventing the cleavage of the N terminus of the receptor.

There were no significant differences between the abilities of the fusion proteins and wild-type Sak to activate plasminogen. These findings were corroborated by the fibrin plate assay. The lysis zones induced by all proteins tested were the same size as
those produced by wild-type Sak. Thus, the plasminogen-activating and fibrinolytic properties remain unchanged in the fusion proteins compared with those of wild-type Sak and H₆-Sak.

To demonstrate whether the fusion protein is capable of simultaneously discharging its two separate functions (i.e., the inhibition of thrombin and the activation of plasminogen), plasma clot lysis tests were performed. In this test system, H₆-Sak caused a complete lysis of the thrombin-induced clots without interference with the clotting time. In the presence of the fusion protein H₆-Sak-Dip-I-II, the clot formation was inhibited when the thrombin concentration exceeded the capacity of Dip-I-II to bind thrombin, but the subsequent clot lysis was in the same range as seen with H₆-Sak. Thus, the fusion protein retains its bifunctionality when thrombin is bound, thereby providing evidence for the formation of the ternary thrombin/II-plasminogen complex. This has to be expected when also considering the strong interaction of the dipetalin domain with thrombin as shown by a

Acknowledgments

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References


