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ORIGINAL ARTICLE

Overestimation of N-glycoPEGylated factor IX activity in a one-stage factor IX clotting assay owing to silica-mediated premature conversion to activated factor IX

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Essentials

- Nonacog beta pegol (N9-GP) activity is overestimated in clot method using silica-based reagents.
- Mimicking contact activation phase with silica reveals N9-GP activation before recalcification.
- Localization of N9-GP to silica facilitates activation by factor XIa and plasma kallikrein.
- Silica-based reagents to be used with caution when monitoring N9-GP therapy using clot method.

Summary. *Background:* Clinical laboratories routinely quantify factor IX (FIX) activity by measurement of the activated partial thromboplastin time (APTT) in a one-stage (OS) clotting assay. This assay can be performed with any of a plethora of differently composed APTT reagents, giving variable recovery when applied to nonacog beta pegol (N9-GP), an N-glycoPEGylated recombinant FIX. *Objective:* To identify the cause of observed overestimations of N9-GP activity in an OS FIX clotting assay when most APTT reagents containing silica are used as the contact activator, and to elucidate the underlying mechanism. *Methods:* Experiments mimicking the contact activation and clotting phases of the OS assay, combined with the use of plasmas with various deficiencies, were employed to shed light on the unique behavior of N9-GP. Confirmatory activations of N9-GP with purified enzymes and physical adsorption to silica particles were studied, and the influence of free polyethylene glycol (PEG) on these processes was investigated. *Results:* N9-GP, but not native FIX, added to FIX-deficient plasma was prematurely converted to activated FIX (FIXa) during the contact activation phase of the clotting assay. Activated FXI (FXIa) and plasma kallikrein (PK) were responsible for the activation of N9-GP, an event that appeared to require the presence of a silica-containing APTT reagent. PEG-dependent adsorption of N9-GP to silica particles could be demonstrated. *Conclusions:* The PEG moiety mediates colocalization of N9-GP with its activators FXIa and PK on silica surfaces, thereby facilitating premature conversion of N9-GP to FIXa during the contact activation phase, and leading to overestimation of the FIX activity in the OS clotting assay.

Keywords: blood coagulation tests; coagulation factor IX; hemophilia B; nonacog beta pegol; silicon dioxide.

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Introduction

Hemophilia is a congenital bleeding disorder requiring lifelong management, and should optimally be treated in a prophylactic manner to prevent the occurrence of bleeding episodes. In recent years, we have witnessed the development of new molecules for more convenient treatment of hemophilia, including hemophilia B, by the provision of higher circulating factor activity levels and prolonged duration of action, i.e. less frequent administration, making more patients amenable to prophylaxis [1]. The achieved circulatory half-life has been astonishing, especially for modified forms of factor IX (FIX). Successful construction of longer-acting FIX variants has been accomplished by fusion, either to an immunoglobulin Fc portion [2,3] or albumin [4,5], or by N-glycan modifications, either by the use of glycan-directed addition of a polyethylene glycol (PEG) moiety [6,7], or by creating additional glycosylation sites by protein engineering [8]. Challenges associated with assays to be used for potency assignment and monitoring of these new products for hemophilia treatment have been recognized, and are currently receiving considerable attention [9,10]. Although

the nature of these hurdles presumably varies between different engineered FIX molecules, they will definitely constitute an issue in the time to come.

Nonacog beta pegol (N9-GP) is a derivatized FIX molecule that is endowed with an extended pharmacokinetic and pharmacodynamic profile by the attachment of a 40-kDa PEG moiety to an N-linked glycan in the activation peptide [6], with a proven five-fold prolonged half-life in the circulation in patients as compared with regular FIX products [7]. Because the asparagine carrying the PEGylated glycan is removed upon activation, N9-GP is converted to native activated FIX (FIXa). N9-GP activity can be accurately assessed by measuring the activated partial thromboplastin time (APTT) by the use of a one-stage (OS) FIX clotting assay with select APTT reagents (e.g. SynthAFax), or by using chromogenic activity assays [11–14]. However, at the same time, the use of many, but not all, silica-based APTT reagents in the OS clotting assay is associated with a considerable, i.e. three-fold to five-fold, overestimation of N9-GP activity when it is assayed against a plasma-derived FIX standard [11–13]. Awareness of this phenomenon is important from a clinical perspective, because erroneously high recovery of N9-GP activity in connection with treatment monitoring would potentially constitute a safety risk, with ensuing suboptimal dosing of the drug leaving patients unprotected against subsequent bleeds.

The present study was aimed at identifying and describing the cause of the APTT reagent-dependent, elevated recovery of N9-GP activity obtained with the OS FIX clotting assay. To accomplish this, FIXa formation was quantified during the two temporally distinct phases of the OS FIX clotting assay, the contact activation and calcium-initiated clotting phases. In addition, activated FXI (FXIa) formation was followed during the contact phase. After we had pinpointed the apparently responsible event, pertinent specific tools, such as plasmas deficient in different individual coagulation zymogens, isolated enzymes, competition experiments with free PEG, and silica adsorption assessments, were employed to further dissect the mechanism underlying the overestimation of N9-GP activity that was operative during the contact phase of the clotting assay.

Materials and methods

Materials

N9-GP and non-PEGylated recombinant FIX (N9), which was used as control protein in the electrophoresis-monitored activation and silica adsorption studies, were produced by Novo Nordisk A/S (Måløv, Denmark), using described methodology [6]. The 4th international plasma-derived FIX standard (World Health Organization, 4th IS FIX, ref. 07/182) was obtained from the National Institute for Biological Standards and Control (NIBSC, Potters Bar, UK). BeneFIX was produced by Pfizer (New York, NY, USA), and optionally glycoPEGylated on terminal galactoses after desialylation, as described for N9-GP [6]. The following APTT reagents were used: HemosIL APTT-SP (liquid), HemosIL SynthAFax and HemosIL SynthASil from Instrumentation Laboratory (Bedford, MA, USA), TriniCLOT aPTT HS from Tcoag (Bray, Ireland), STA-PTT Automate from Diagnostica Stago (Asnières sur Seine, France), and Pathromtin SL from Siemens (Marburg, Germany). Plasmas depleted of either FIX, FVII, FXI, FXII or plasma prekallikrein were obtained from Precision BioLogic (Dartmouth, Canada). FXIa, activated FXII (FXIIa), plasma kallikrein (PK) and corn trypsin inhibitor were obtained from Enzyme Research Laboratories (South Bend, IN, USA), FIXa was obtained from Haematologic Technologies (Essex Junction, VT, USA), and aprotinin was obtained from Sigma-Aldrich (Munich, Germany). Rox Factor XIa and Rox FIX-A kits for measurements of FXIa and FIXa activity, respectively, were obtained from Rossix AB (Mölndal, Sweden). PEG hydroxylamine with an average molecular mass of 40 kDa (PEG40, Sunbright GL2-400CA) was obtained from NOF America (White Plains, NY, USA). The fibrin polymerization inhibitor I-2882 was obtained from Chiralix (Nijmegen, The Netherlands), and the PK substrate S-2302 was obtained from Chromogenix (Milan, Italy).

FIX sample preparation for activation studies

N9-GP, BeneFIX and 4th IS FIX were brought to room temperature before reconstitution. N9-GP was reconstituted with 10 mM histidine (pH 6.8), BeneFIX was reconstituted according to the manufacturer's instructions, and 4th IS FIX was reconstituted with 1 mL of water. After dissolution, the proteins were left at room temperature for 5 min, and then divided into aliquots and frozen at − 70 °C. Each FIX sample was thawed before use, diluted to 1 IU mL^{-1} in FIX-deficient plasma (or in plasma deficient for another coagulation zymogen), and then diluted to 0.05 IU mL^{-1} in 50 mM imidazole (pH 7.3), containing 0.1 M NaCl and 1% (w/v) bovine serum albumin, unless otherwise stated. The potency (unitage) of the N9-GP batch was assigned against 4th IS FIX with the OS FIX clot assay performed with SynthAFax.

Subsampling under conditions mimicking the contact activation phase and measurement of generated FXIa

A 50-μL FIX sample (0.05 IU mL^{-1}; kept on ice) was mixed with 50 μL of FIX-deficient plasma (kept on ice), and incubated at 37 °C for 2 min. The reaction was started by adding 50 μL of APTT reagent (APTT-SP or

SynthAFax kept at 37 °C). Samples of 10 μL were withdrawn at different time points, and quenched by mixing with 6 mL of ice-cold 50 mM MES (pH 5.7), containing 50 mM NaCl and 0.2% (w/v) bovine serum albumin. No FXIa activity was lost when it was stored in the MES buffer for up to 3 h. The zero-time sample was obtained by addition of MES buffer instead of APTT reagent followed by immediate dilution.

A slightly modified Rox Factor XIa method (the modifications being immediately successive addition of reagents 1 and 2 followed by 3 min of incubation) was used for FXIa quantification. A 50-μL quenched sample, or FXIa calibrator prepared in MES buffer, kept on ice was mixed with 25 μL of cold Tris buffer (pH 8.3), 50 μL of cold Rox Factor XIa kit reagent 1 (containing FIX, FVIII, and $CaCl_2$) and 50 μL of kit reagent 2 (containing FX, thrombin, phospholipids, and $CaCl_2$; kept at 37 °C), and incubated for 3 min at 37 °C. Activated FX (FXa) generation was terminated, and the FXa formed was quantified by the addition of FXa substrate in EDTA-containing solution. After 5 min at 37 °C, citric acid was added to quench FXa-catalyzed substrate hydrolysis, and the absorbance was read at 405 nm. The concentrations of FXIa in the samples were derived from a standard curve of calibrator potency assigned in U mL^{-1} versus NIBSC reference reagent for FXIa (human, 11/236). The same method was used to investigate the formation of FXIa in prekallikrein-deficient plasma with APTT-SP as the APTT reagent.

Subsampling under conditions mimicking the contact activation phase and measurement of generated FIXa

A 50-μL FIX sample (0.05 IU mL^{-1}; kept on ice) was mixed with 50 μL of FIX-deficient plasma (kept on ice), and incubated at 37 °C for 2 min. Fifty microliters of APTT reagent (APTT-SP; kept at 37 °C) was then added. Samples were withdrawn at different time points, and mixed with an equal volume of stop solution (Tris buffer containing 7 mM EDTA, 20 μg mL^{-1} corn trypsin inhibitor, 12 600 kIU mL^{-1} aprotinin, and 0.5 mg mL^{-1} I-2882). The described procedure was not limited to use with FIX-deficient plasma, but was also used with FVII-deficient, FXI-deficient, FXII-deficient or plasma prekallikrein-deficient plasma (for FIX sample dilution and as a plasma source). This was feasible because the normal plasma level of FIX present in these plasmas, although 20-fold higher than that in a diluted N9-GP/FIX sample, resulted in a very low background generation of FIXa.

A modified Rox FIX-A method (the modifications being premixing of reagents 1 and 2 immediately prior to addition to the assay, followed by 2 min of incubation) was used for FIXa quantification. The quenched samples were diluted with an equal volume of FIXa diluent buffer, and kept at room temperature. Fifty microliters of sample or FIXa calibrator was mixed with 125 μL of a prewarmed (37 °C) mixture of Rox FIX-A reagents 1 (FVIII and FX) and 2 (thrombin, phospholipids. and $CaCl_2$), and this was followed by 2 min of incubation at 37 °C. Termination of FXa generation and FXa quantification were as described for FXIa measurement, except that FXa was allowed to act on the substrate for 4 min. The inhibitory effect of the stop solution on FX activation by FIXa, under the conditions used to determine FIXa in the presence of Ca^{2+}, phospholipids, and FVIII, was < 5%. The concentrations of FIXa in the samples were derived from a standard curve of calibrator potency assigned in IU mL^{-1} versus NIBSC reference reagent for FIXa (1st IS FIXa 97/562).

Subsampling under conditions mimicking the period after calcification (clotting phase) and measurement of generated FIXa

A 50-μL FIX sample (0.05 IU mL^{-1}; kept on ice) was mixed with 50 μL of FIX-deficient plasma (kept on ice), and incubated at 37 °C for 2 min. Fifty microliters of APTT reagent (APTT-SP or SynthAFax; kept at 37 °C) was then added, and this was followed by 5 min of incubation. Finally, 50 μL of 25 mM $CaCl_2$ (kept at 37 °C) was added to start the reaction, after which samples were withdrawn at different time points until clot formation, and mixed with an equal volume of stop solution, as described above for the determination of FIXa formed during the contact activation phase. The zero-time sample was taken immediately prior to $CaCl_2$ addition. The modified Rox FIX-A method described above was used for FIXa quantification.

Activation of N9-GP and FIX by purified enzymes

Fifty microliters of PK, FXIa or FXIIa, diluted to 400–0.18 nM in 50 mM imidazole (pH 7.3), containing 0.1 M NaCl and 1% (w/v) bovine serum albumin and kept on ice, was mixed with 50 μL of N9-GP or 4th IS FIX (diluted to 0.05 IU mL^{-1} in the same buffer and kept on ice), and left for 2 min at 37 °C. APTT-SP (50 μL, prewarmed to 37 °C) was added, and the mixture was incubated for 5 min at 37 °C; a 50-μL sample was then withdrawn, mixed with 50 μL of stop solution, and subjected to FIXa determination with the modified Rox FIX-A method as described above. N9-GP and 4th IS FIX were also incubated with a single concentration of PK or FXIa (final concentrations 30 nM and 1.5 nM, respectively) in the presence of APTT-SP, SynthAFax or imidazole buffer to investigate the APTT reagent dependence of FIXa formation. In addition, the incubation with APTT-SP was performed in both the absence and the presence of 2 μM PEG40.

The activation of N9-GP (and N9) by FXIa or PK was also monitored with SDS-PAGE. N9-GP/N9 was diluted to 1.5 μM, FXIa to 6 nM and PK to 90 nM in 50 mM

imidazole (pH 7.3), containing 0.1 M NaCl. Fifty microliters of N9-GP or N9 was mixed with 50 μL of FXIa or PK and 50 μL of APTT-SP, and incubated at ambient temperature. At each time point, a 16-μL sample was withdrawn, mixed with sample buffer containing SDS, and boiled. One microgram of FIXa was loaded as the reference. Electrophoretic analysis was performed by use of a 4–12% NuPAGE Novex Bis-Tris gel with MES running buffer (Invitrogen, Carlsbad, CA, USA), and the proteins were visualized by silver staining.

Effect of free PEG on FIXa generation in plasma

The effect of PEG40 on N9-GP activation in plasma was studied by mixing 25 μL of PEG40 (12 μM in imidazole buffer), 25 μL of N9-GP (0.1 IU mL^{-1} in imidazole buffer; kept on ice), and 50 μL of FIX-deficient plasma (kept on ice). The mixture was incubated at 37 °C for 2 min, and this was followed by the addition of 50 μL of APTT-SP (prewarmed to 37 °C). Samples were then withdrawn at different time points into an equal volume of stop solution, and analyzed with the modified Rox FIX-A method (see above).

Measurement of PK generation

N9-GP and 4th IS FIX were diluted as described under 'FIX sample preparation', but in this particular experiment they were diluted to 1 IU mL^{-1} in either FIX-deficient or FXI-deficient plasma, and then to 0.05 IU mL^{-1} in imidazole buffer. A 50-μL FIX sample (on ice) was incubated with 50 μL of FIX-deficient or FXI-deficient plasma for 2 min at 37 °C, after which APTT-SP (50 μL, 37 °C) was added. At time points up to 5 min, 20-μL samples were withdrawn and mixed with 220 μL of Tris buffer containing 20 μg mL^{-1} corn trypsin inhibitor. Twenty microliters of this mixture was further diluted with 80 μL of Tris-buffered corn trypsin inhibitor solution, and 100 μL of S-2302 (4 mM in Tris buffer) was subsequently added. Substrate hydrolysis was allowed to proceed for 17 min at 37 °C, and terminated with 50 μL of 2% (v/v) citric acid; the absorbance was then read at 405 nm. The PK concentrations in the samples were derived from a standard curve.

Assessment of N9-GP adsorption to silica and visualization with SDS-PAGE

Fifty microliters of a 50 μg mL^{-1} solution of N9-GP or recombinant FIX (N9) in 50 mM imidazole (pH 7.3), containing 0.1 M NaCl, was mixed with either 50 μL of APTT-SP preincubated with 2 μM PEG40 or 50 μL of APTT-SP to which only buffer had been added. After 10 min, the mixtures were centrifuged at 10 000 × *g* for 5 min to precipitate the silica particles. A sample (12 μL) for SDS-PAGE was taken from the supernatant. The silica pellet was resuspended in 100 μL of imidazole buffer, and centrifuged again. The washed pellet was suspended in 12 μL of imidazole buffer plus 4 μL of sample buffer, and subjected to SDS-PAGE analysis. A sample (6 μL) of the N9-GP/N9 solution was also taken before the addition of APTT-SP. Adsorption experiments with the other APTT reagents were performed identically. The samples were analyzed on a 4–12% NuPAGE Novex Bis-Tris gel (Invitrogen), and the proteins were visualized by Coomassie staining.

Assessment of silica-mediated N9-GP depletion from solution with the one-stage FIX clotting assay

Fifty microliters of a 50 μg mL^{-1} solution of N9-GP or N9 in 50 mM imidazole (pH 7.3), containing 0.1 M NaCl and 1% (w/v) bovine serum albumin, was mixed with either 50 μL of undiluted APTT-SP or 50 μL of a two-fold, four-fold, eight-fold or 16-fold dilution of APTT-SP in 50 mM imidazole (pH 7.3), containing 0.1 M NaCl, or 50 μL of buffer. After 10 min, the mixtures were centrifuged at 10 000 × *g* for 5 min to precipitate the silica particles and associated protein, and 50 μL of the supernatant was removed and frozen. The supernatant samples were diluted 10-fold in FIX-deficient plasma prior to analysis. The amount of N9-GP or N9 was determined in an OS FIX clotting assay (FIX:C) run on an ACL 9000 instrument (Instrumentation Laboratory), according to the manufacturer's instructions, with FIX-deficient plasma and SynthAFax as the APTT reagent.

Results

FXI contact activation and FIXa generation during the two phases of the OS FIX clotting assay

Initially, we investigated whether the aims of the two steps in the OS FIX clotting assay were achieved, namely to generate FXIa during the contact activation phase, and then to activate FIX after recalcification during the subsequent clotting phase. Two APTT reagents were used in these model studies: APTT-SP as a representative of those reagents that overestimate N9-GP activity, and SynthAFax which gives the expected recovery. We found very similar contact activation phase generation of FXIa, reaching a level of ~ 2300 mU mL^{-1} after 5 min, with APTT-SP (Fig. 1A) and SynthAFax (data not shown), and the FXI activation rate was not influenced by the form of FIX present (recombinant glycoPEGylated or native plasma-derived). In contrast, the FIXa generation profiles obtained during the clotting phase in the presence of APTT-SP showed one striking difference between PEGylated and native forms of FIX. With N9-GP, a considerable amount of FIXa was present already at the beginning of the clotting phase, but the rate of conversion to FIXa during the actual clotting phase was

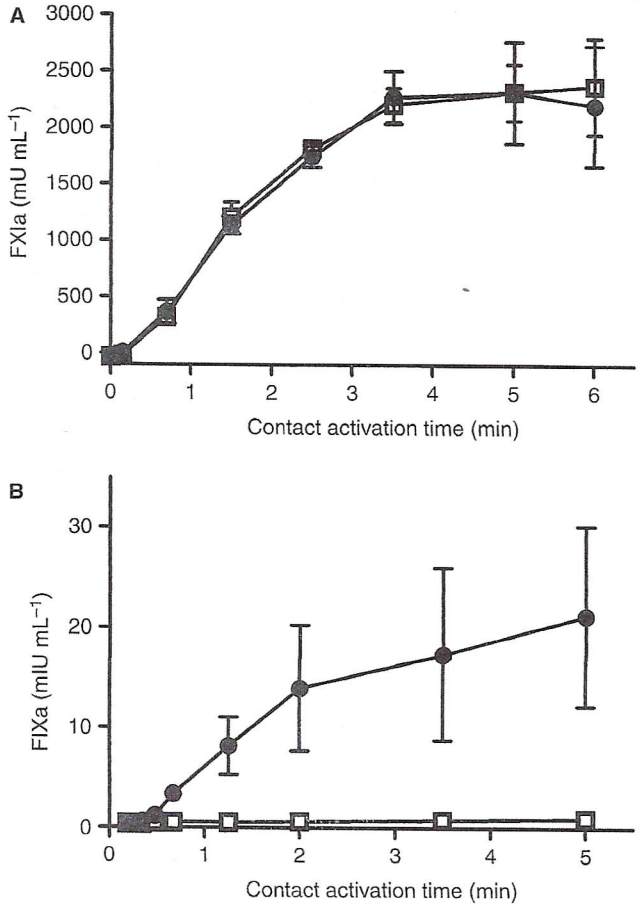

Fig. 1. Generation of activated factor XI (FXIa) and activated factor IX (FIXa) during the contact activation phase of the OS FIX clotting assay. (A) FXIa generation in the presence of APTT-SP and either nonacog beta pegol (N9-GP) (filled circles) or plasma-derived FIX (4th IS FIX, open squares). Indistinguishable FXIa generation was obtained with SynthAFax (data not shown). (B) FIXa formation from N9-GP (filled circles) and plasma-derived FIX (4th IS FIX, open squares) in the presence of APTT-SP. Neither N9-GP nor FIX was activated to FIXa when SynthAFax was used (data not shown).

indistinguishable for N9-GP and FIX (data not shown). This strongly suggested premature activation of N9-GP in the presence of APTT-SP during the preceding contact activation phase, and measurements of FIXa generation under conditions mimicking this phase did indeed reveal selective conversion of N9-GP to FIXa occurring before recalcification (Fig. 1B). The amount of FIXa that was generated from N9-GP and accumulated during the 5-min contact phase with APTT-SP roughly corresponded to that generated (from N9-GP or FIX) over the first 50 s after calcium addition (investigation of later time points was not feasible, owing to plasma clot formation). Thus, FIXa prematurely formed from N9-GP could definitely cause shortened clotting times in the OS FIX clotting assay, owing to rapid onset of FXa generation after Ca^{2+} addition. In the above experiments, glycoPEGylated BeneFIX behaved indistinguishably from N9-GP, and BeneFIX behaved like plasma-derived FIX (data not shown). This served to demonstrate that our results hold true for two different sources of the wild-type FIX scaffold.

Identification of the contact phase activators of N9-GP in the presence of APTT-SP

An initial experiment showed that activation of N9-GP during the contact activation phase could be monitored without an overwhelming background arising from the normal plasma level of FIX. This allowed the use of plasmas with various deficiencies to pinpoint the contact phase activator(s) of N9-GP in the presence of APTT-SP. The rates of conversion of N9-GP to FIXa were very similar in FIX-deficient and FVII-deficient plasma (data not shown), indicating that FVIIa was not involved in the activation of N9-GP. In contrast, the activation of N9-GP was reduced by ~ 50% in FXI-deficient plasma, and totally abolished in FXII-deficient and plasma prekallikrein-deficient plasmas (Fig. 2A). This pointed towards a role in N9-GP activation for FXIa, the only enzyme upstream of FIX activation in the intrinsic pathway of blood coagulation that was missing in FXI-deficient plasma, and also strongly indicated the existence of at least one additional activator of N9-GP that was responsible for the residual activation in FXI-deficient plasma. This activator(s) was presumably FXIIa and/or PK, as judged by the complete lack of N9-GP activation in the FXII-deficient and prekallikrein-deficient plasmas, as these plasmas are devoid of these two enzymes (as well as FXIa).

Purified enzymes were employed to corroborate the identities of the N9-GP contact phase activators inferred from the plasma experiments conducted in the presence of APTT-SP. At the concentration (~ 1.5 nM) obtained during the contact activation phase of the OS FIX clotting assay, FXIa could activate N9-GP, whereas detectable FIX activation required considerably higher FXIa concentrations. PK, even at a concentration of > 100 nM, was a totally selective activator of N9-GP. Finally, FXIIa (up to 140 nM) was unable to activate any form of FIX (data not shown). These results clearly illustrated that FXIa and PK, but not FXIIa, were able to convert N9-GP to FIXa, and supported their involvement in the premature N9-GP activation.

The roles of silica and the conjugated PEG moiety in N9-GP activation

A more detailed characterization of the FXIa-catalyzed and PK-catalyzed conversion of N9-GP to FIXa revealed the absolute requirement for a particular type of reagent, such as APTT-SP, because no activation occurred if it was replaced with SynthAFax or buffer (Fig. 2B). The early observation that both activators preferred N9-GP over FIX as the substrate strongly indicated that there

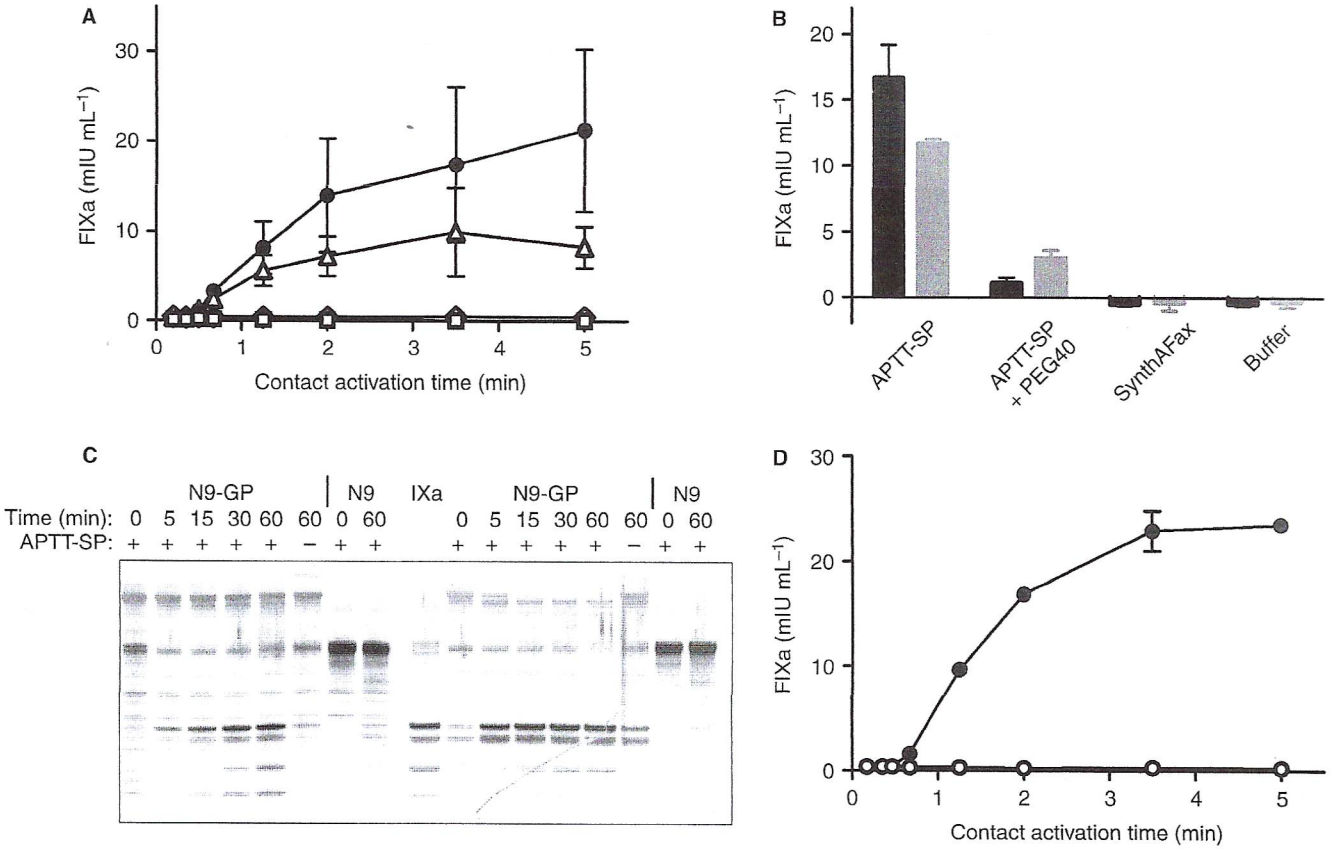

Fig. 2. Identification of nonacog beta pegol (N9-GP) activators and requirements for activation. (A) N9-GP activation in deficient plasmas under contact activation phase conditions in the presence of APTT-SP. The data are from activation experiments in FIX-deficient (filled circles), FXI-deficient (open triangles), FXII-deficient (open diamonds) and prekallikrein-deficient plasma (open squares). (B) Plasma kallikrein (PK)-catalyzed and activated FXI (FXIa)-catalyzed N9-GP activation requirement for APTT-SP and abrogation by free PEG40. PK (30 nM, black bars) and FXIa (1.5 nM, gray bars) were used at concentrations that adequately represented the amounts generated during the contact phase of the OS FIX clotting assay. The activated partial thromboplastin time (APTT) reagent used, if any, and the presence of PEG40 are indicated below the graph. (C) Accelerated conversion of N9-GP to activated FIX (FIXa) by PK and FXIa in the presence of APTT-SP. N9-GP or non-PEGylated recombinant FIX (N9) was incubated with 30 nM PK (left of the 'FIXa' reference) or 2 nM FXIa (right of the 'FIXa' reference) for the indicated times in the presence (+) or absence (−) of APTT-SP. Time-zero samples were withdrawn immediately after addition of APTT-SP. (D) Inhibition of N9-GP activation in plasma by free PEG40. The generation of FIXa from N9-GP spiked into FIX-deficient plasma to which APTT-SP had been added is shown in the absence (filled circles) and presence (open circles) of 2 μM free PEG40.

was also a role for the conjugated PEG moiety in the activation process. The influence of free PEG40, in the presence of APTT-SP, was investigated to look for evidence of competition with the glycan-linked PEG moiety in N9-GP and attenuation of N9-GP activation. Activation, both by FXIa and by PK, was indeed clearly influenced by the presence of free PEG40, and was dramatically reduced at 2 μM (Fig. 2B). Using gel electrophoretic analysis, we confirmed that FXIa and PK were capable of activating N9-GP to yield FIXaβ composed of the familiar heavy and light chains in a process that was greatly accelerated by the presence of APTT-SP (Fig. 2C). In agreement with our other findings, conversion of N9-GP to FIXaβ was largely dependent on the PEGylation. It is worth mentioning that sensitivity limitations required the use of higher protein concentrations than in the activity-based assays, even when silver staining visualization was employed. Finally, we confirmed the inhibitory effect of free PEG40 on N9-GP activation under contact phase conditions in the environment that is most relevant for the OS FIX clotting assay, namely in FIX-deficient plasma with added APTT-SP, where 2 μM PEG40 completely prevented N9-GP activation (Fig. 2D).

To investigate whether the conjugated PEG moiety of N9-GP actually mediates adsorption, and thereby localization, to silica particles, N9-GP was mixed with APTT-SP, and this was followed by precipitation of the silica particles by centrifugation and quantification of the amount of protein pulled down. Significantly more extensive silica absorption of N9-GP than of FIX was evident, as judged by the more intense staining (Fig. 3A). The adsorption of N9-GP was reduced to the low (background) level seen with FIX if the APTT-SP reagent was preincubated with free PEG40. The adsorption of native FIX was low and largely unaffected by free PEG40. Taken together, the data corroborated silica association

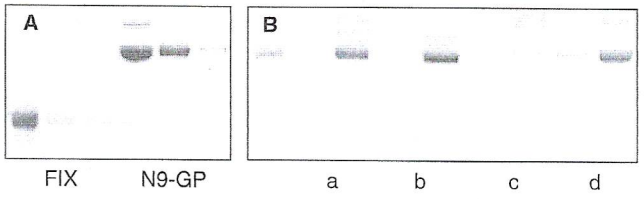

Fig. 3. Physical adsorption of nonacog beta pegol (N9-GP) to silica particles. (A) Polyethylene glycol (PEG)-mediated adsorption of N9-GP to silica particles in APTT-SP. The three lanes shown each for FIX and N9-GP represent the control protein solution before addition of APTT-SP (left), and protein adsorbed to silica in the absence (middle) and presence (right) of 2 μM free PEG40, respectively. The controls show that comparable amounts of protein were added. (B) Adsorption of N9-GP to silica particles in various APTT reagents. The outer left lane shows N9-GP prior to incubation with APTT reagent. Pairs of lanes show any residual N9-GP in the solution phase (first lane) and silica-adsorbed N9-GP (second lane) after incubation with APTT-SP (a), TriniCLOT aPTT HS (b), STA-PTT Automate (c), and Pathromtin SL (d).

of N9-GP mediated by the PEG moiety, and demonstrated effective blocking by free PEG40. Because FIX was not found to be activated to a detectable extent during the contact activation phase in plasma in the presence of APTT-SP, a low level of adsorption to silica does not apparently induce conversion to FIXa by FXIa or PK. The inclusion of several silica-based APTT reagents revealed that adsorption of N9-GP to silica particles is a general phenomenon with reagents containing a sufficient amount of this contact activator, because SDS-PAGE analysis in all cases showed adsorption and concomitant solution-phase depletion of N9-GP (Fig. 3B). STA-PTT Automate depleted the solution of N9-GP as efficiently as, for instance, APTT-SP, but adsorbed N9-GP gave a band of relatively weak intensity, presumably because of release of protein in the washing step. The disappearance of N9-GP (but not FIX) from the solution after incubation with APTT-SP was corroborated by OS FIX clotting assay measurements on the supernatant, with SynthAFax as the APTT reagent. Less than 5% of N9-GP remained in solution, whereas an 8–10-fold dilution of APTT-SP sufficed to prevent detectable removal of N9-GP from solution (data not shown). In the OS assay, no adsorption to particles could be demonstrated with SynthASil, a silica-based reagent not causing overestimation of N9-GP activity. This potentially solely reflects an inherently low silica content, as judged by the absence of a visible pellet.

Discussion

Like any APTT reagent, APTT-SP should provide a surface for FXIIa and PK generation in the OS clotting assay and set the stage for contact activation of FXI to FXIa. However, because APTT-SP contains silica in a certain amount, and possibly of a particular quality, as the contact activator, this reagent also turned out to mediate premature conversion of N9-GP to FIXa in the absence of Ca^{2+}. This process is catalyzed by FXIa and PK, and in both cases two cleavages occur, resulting in removal of the PEGylated activation peptide and formation of the familiar FIXaβ. The premature occurrence of this event is untimely from the perspective of the OS FIX clotting assay, in which FIX conversion to FIXa should not occur until after recalcification, and it consequently leads to overestimation of N9-GP activity (in the case of APTT-SP, approximately five-fold).

As judged by gel electrophoretic quantification, a similar extent of N9-GP adsorption could be observed with several reagents containing a sufficient amount of silica particles. This was true for APTT-SP, TriniCLOT aPTT HS, STA-PTT Automate, and Pathromtin SL, but not for SynthASil, with its much lower silica content. Therefore, it is a reasonable assumption that overestimation of N9-GP activity is a common problem associated with most members (not SynthASil) of the family of silica-based APTT reagents, which is in agreement with previous data from OS FIX clotting assay measurements [11–13]. Moreover, because premature activation to FIXa was only observed with N9-GP and not with native FIX, a link between the conjugated PEG moiety and silica became evident. This was supported by the demonstration of selective, physical adsorption of N9-GP to silica particles, and by the ability of free PEG40 to prevent both the adsorption to silica and the activation of N9-GP to FIXa in plasma during the contact activation phase. However, preliminary data from our laboratory clearly show that the OS FIX clotting assay cannot be made suitable for N9-GP quantification with silica-based APTT reagents simply by adding free PEG. In conclusion, a number of observations suggest that an APTT reagent such as APTT-SP, containing silica as the contact activator, and a PEG moiety conjugated to FIX are the crucial prerequisites for N9-GP activation to take place during the contact activation phase. We propose an activation mechanism involving colocalization of N9-GP with FXIa and PK. These enzymes are generated on, and perhaps to some extent remain bound to, the silica surface, and their cleavage of N9-GP was accelerated in the presence of silica. This phenomenon would promote (premature) FIXa generation in the absence of calcium ions, and hence lead to erroneously high recovery of N9-GP activity in the OS FIX clotting assay.

We have unambiguously shown that overestimation of N9-GP activity in the OS FIX clotting assay, which appears to be a common issue with most silica-containing APTT reagents, arises from an assay artefact mediated by a combination of silica and the conjugated PEG moiety. It has no relationship with any other property, functional or physical, of the N9-GP molecule. Nevertheless, the erroneous overestimation of N9-GP activity could potentially lead to underdosage of the drug in future clinical practice, and certain silica-based APTT reagents should

therefore be avoided when the OS assay is used to monitor patients with hemophilia B receiving N9-GP therapy.

Addendum

P. Rosén and S. Rosén designed the research, performed experiments, analyzed data, and revised the manuscript. M. Ezban designed the research and revised the manuscript. E. Persson designed the research, performed experiments, analyzed data, and wrote the manuscript.

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Disclosure of Conflict of Interests

P. Rosén is a co-owner of, and S. Rosén is a consultant for, Rossix AB, the manufacturer of the chromogenic assay kits used in the study. M. Ezban and E. Persson are employees of Novo Nordisk A/S, the manufacturer of N9-GP.

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