Characterization of a Monoclonal Antibody B1 That Recognizes Phosphorylated Ser-158 in the Activation Peptide Region of Human Coagulation Factor IX*

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Blood coagulation factor IX (FIX) undergoes various post-translational modifications such as γ-carboxylation and glycosylation. Non-phosphorylated recombinant FIX has been reported to rapidly disappear from plasma, indicating that phosphorylation of FIX plays an important role in the physiological activity of this coagulation factor. In this study, we characterized the human FIX activation peptide (AP) using a monoclonal antibody that recognizes phosphorylated Ser-158 in the AP region. Murine monoclonal antibody B1 against human FIX recognized FIX with an apparent Kd value of 5 nM in the presence of Ca2⁺ (EC50 = 0.58 mM). B1 bound to the isolated AP of FIX and retained the Ca2⁺ dependence of binding to the isolated AP. The deglycosylation of FIX did not affect the binding of B1 to AP, while B1 failed to bind to recombinant AP expressed in Escherichia coli. MALDI-TOF mass spectrometry showed that the m/z of plasma-derived deglycosylated AP is 82.54 Da greater than that of recombinant AP. The binding ability of B1 to AP was lost by the dephosphorylation of plasma-derived AP. B1 bound to synthetic peptide AP(5–19), including phosphoserine-13, but not to the non-phosphorylated AP(5–19) in the presence of Ca2⁺. These data provide direct evidence that Ser-13 of the plasma-derived FIX AP region (Ser-158 of FIX) is phosphorylated and that B1 recognizes the epitope, which includes Ca2⁺-bound phosphoserine-158. B1 should be useful in the quality control of biologically active recombinant FIX containing phosphoryserine-158.

Blood coagulation factor IX (FIX)γ is present in an inactive precursor form that consists of a γ-carboxyglutamic acid (Gla)-containing domain, two epidermal growth factor (EGF)-like domains, an activation peptide (AP) region, and a serine protease domain (1) (Fig. 1). FIX is activated physiologically by factor XIa or factor VIIa-tissue factor complex to generate FIXa via FIXα, an active intermediate, whereas RVV-X, a protease from Russell’s viper venom, can activate FIX to FIXαβ via FIXαε, an active intermediate (2). In both cases, AP is removed by peptide bond cleavage at two sites of FIX to generate the final form of activated FIX, FIXαβ, and an AP. Increased plasma FIX level is one of the risk factors of thrombosis (3), and along with the concentration of AP in plasma is a useful index for the activation of the blood coagulation system. Human coagulation FIX undergoes various post-translational modifications including γ-carboxylation of twelve Glu residues in the Gla domain, attachment of N-linked and O-linked oligosaccharides at the EGF domain and AP region, and β-hydroxylation of an Asp residue in the first EGF domain (4). Other post-translational modifications, such as sulfation of a Tyr residue and phosphorylation of Ser residue(s), also have been suggested (5) but precise data on these have not been reported to date. These post-translational modifications take place in the liver and may play important roles for specific activity, secretion, recovery, half-life in the circulation, and immunogenicity of FIX (6).

Ca2⁺ ions have been reported to bind to the Gla domain, the N-terminal EGF-like domain, and the serine protease domain of FIX, resulting in conformational changes important for the biological activity of FIX (7). Many Ca2⁺-dependent monoclonal antibodies against FIX have been reported and most of them recognize the Gla domain (8). Several of the previously described monoclonal antibodies recognize the AP region of FIX in a Ca2⁺-independent manner (9).

We established human FIX-specific monoclonal antibody-producing hybridoma cell lines and screened Ca2⁺-dependent monoclonal antibodies to determine the FIX binding site on snake venom-derived anti-coagulant protein, factor IX/factor X-binding protein (IX/X-bp) (10, 11). Most of the characterized Ca2⁺-dependent antibodies recognized the Gla domain of FIX and inhibited binding of IX/X-bp to FIX; they bound to FIXαβ as well as FIX. However, one of the Ca2⁺-dependent monoclonal antibodies, designated B1, was observed to bind to intact FIX, FIXα, and FIXαε, but not to FIXαβ lacking the AP region. Further characterization showed that B1 is the first monoclonal antibody that recognizes the AP region of FIX in the presence of Ca2⁺ ions. We report here that the epitope of FIX recognized by B1 contains a phosphoryserine residue at residue 13 of AP (Ser-158 of FIX), providing the first direct evidence of the existence of phosphoryserine in human FIX. We discuss the role of phosphorylation in human FIX.

EXPERIMENTAL PROCEDURES

Materials—Sheep γ S-200 HR and standard proteins for estimations of molecular mass were obtained from Amersham Biosciences LKB Biotechnology (Uppsala, Sweden). Formyl-Cellulofine and endo-α-N-acetyl-galactosaminidase and neuraminidase were purchased from Seikagaku Kogyo (Tokyo, Japan). N-glycanase F was purchased from Merck Biosciences (Darmstadt, Germany). Peroxidase-conjugated goat antibodies against mouse IgG were purchased from ICN Pharmaceuticals (Costa Mesa, CA). TOPO TA cloning kit for sequencing and pET directional TOPO expression kit were purchased from...
Invitrogen (Carlsbad, CA). All other reagents were of analytical reagent grade or better.

**Proteins**—Habu IX-bp was isolated from the venom of *Trimeresurus flavoviridis* by a previously described method (10). IX-bp-Cellulose was prepared by coupling IX-bp to formyl-Cellulose as previously described (11). Human FIX was purified from human plasma by affinity chromatography using IX-bp-Cellulose. RVV-X was isolated from the venom of *Daboia russelli* according to the method of Kisel et al. (12).

**Isolation of Human FIX AP**—Human FIX was activated by incubating RVV-X (1/40, mol/mol) in Tris-buffered saline, pH 8.0, containing 10 mM Ca2+ ions at 37 °C for 4 h. The AP fraction was separated from FIXαβ, FIX, and FIXα (by gel filtration on a Sephacryl S-200 HR column. Plasma-derived AP (pAP) was further purified using IX-bp-Cellulose column chromatography and reverse phase HPLC with a Finepak SIL C8-5 column (Japan Spectroscopic Co., Ltd., Tokyo, Japan).

**Expression of Recombinant AP**—Human FIX cDNA (nucleotides 139–753 corresponding to the region of mature FIX from the N-terminal residue to Phe-205) was isolated from a human liver cDNA library by PCR using two oligonucleotide primers, primers 1 (5′-TATAATCTACGTAAATGGAAAGAAG-3′) and 2 (5′-GAATGCATCAACTTGTTTACACA-3′), and was cloned into the pCR4-TOPO vector to prepare a plasmid, pCR-(139–753). cDNA corresponding to the region from Gly-133 to Phe-205 and containing the AP region (from Ala-146 to Arg-180) was synthesized by PCR using pCR-(139–753) as template, and primers 2 and 3 (5′-ACCAGGAAAGAGTTCGTGTTCACA-3′; the underlined sequence indicates a 4-nucleotide addition) as PCR primers. The predicted 219-bp PCR product was cloned into the pET 102/D-TOPO expression vector, which expresses a recombinant fusion protein containing thioredoxin at the N-terminal end and a His tag at the C-terminal end. The resulting expression construct, pET-(535–753), was transformed into E. coli (DE-3) competent cells and expressed as a soluble recombinant fusion protein in E. coli. The fusion protein was isolated by Ni-nitrilotriacetic acid column chromatography. The recombinant AP was purified with reverse-phase HPLC using the Finepak SIL C8 column (Japan Spectroscopic Co., Ltd., Tokyo) after digestion of the fusion protein with lysylendopeptidase and/or trypsin.

**Monoclonal Antibodies**—A BALB/c mouse was immunized with the pure human FIX. A panel of monoclonal antibodies against human FIX was prepared by the standard hybridoma technique. ELISA was used to select clones producing antibodies reactive to the antigen. The subtype of each antibody was determined using a typing kit (Bio-Rad).

**Enzyme-linked Immunosorbent Assay**—Microtiter plates were incubated with 50 µl of 1 µg/ml human FIX per well for 2 h at room temperature. All subsequent steps were performed at room temperature. After washing and blocking with 1% gelatin, 50 µl of test materials in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) and CaCl2 were incubated for 1 h. The plates were washed, 50 µl of anti-mouse IgG peroxidase conjugate was added per well, and plates were incubated for 1 h. After washing, enzyme substrate solution (1 mg/ml o-phenylenediamine and 0.06% H2O2 in 0.1 M sodium citrate, pH 5.5) was added, followed by the addition of 4 M H2SO4 to stop the reaction. Color development was measured at 492 nm.

**Electrophoresis—SDS-PAGE** was performed by the method of Laemml (13). The molecular mass markers used were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14 kDa).

**Western Blotting**—Samples such as intact FIX, FIX deglycosylated with N-glycosidase F, or desialylated and deglycosylated with neuraminidase and endo-α-N-acetyl galactosaminidase were subjected to electrophoresis on SDS-polyacrylamide (SDS-PAGE) gels. The proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA) using a blotting system from ATTO (Tokyo, Japan). In some analyses, a dot blotting system (Bio-Rad) was used to blot 0.3 pmol of FIX, 0.3 pmol of FIXαβ, and 100 pmol of pAP-2 onto a nitrocellulose membrane. After washing and blocking with 10% skim milk, the membrane was incubated with 10 mM B1 dissolved in 50 mM Tris-HCl, pH 8.0, containing 0.1 mM NaCl and 0.1% Tween 20 (TBS-T) with or without Ca2+ for 1 h. The membrane was washed in TBS-T with or without Ca2+, and then incubated with a solution of anti-mouse IgG peroxidase conjugate in TBS-T containing Ca2+ for 1 h. After washing, reactive species were detected with an ECL Western blotting detecting agent (Amersham Biosciences).

**Deglycosylation of Protein**—Intact FIX and pAP were treated at 37 °C for 3 h with N-glycosidase F (Roche Diagnostics GmbH, Mannheim, Germany), to remove N-linked oligosaccharide chains in 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM NaCl and 25 mM EDTA for intact FIX, or in 50 mM Tris-HCl buffer, pH 8.0, containing 0.1 mM NaCl for pAP. O-linked oligosaccharide chains were removed from intact FIX and pAP by treatment with neuraminidase and endo-α-N-acetylgalactosaminidase in 25 mM phosphate buffer, pH 6.0, at 37 °C for 1 h and 2 h, respectively. After digestion, deglycosylated FIX was used for SDS-PAGE and Western blotting, whereas pAP was applied to the column of Finepak SIL C8 (Japan Spectroscopic Co., Ltd., Tokyo, Japan) to separate peptide by reverse phase HPLC.

**Dephosphorylation of FIX and pAP**—Samples were dialyzed against 10 mM citrate buffer, pH 4.6, and dephosphorylated by potato acid phosphatase (Sigma) at 37 °C for 4 h. Dephosphorylated pAP was purified by reverse phase HPLC using Finepak SIL C8 column, and dissolved in 50 mM Tris-HCl buffer, pH 8.0, containing 0.1 mM NaCl. Dephosphorylated FIX was dialyzed against 50 mM Tris-HCl buffer, pH 8.0, containing 0.1 mM NaCl.

**Synthesis of Peptides**—Peptides were synthesized by Meiji Pharmaceutical University Core Facility (Tokyo, Japan) and the Peptide Institute Inc. (Osaka, Japan) using an ABI 431 Peptide synthesizer employing Fast Moc chemistry. Peptides were purified by reverse phase HPLC. To confirm purity and identity, the peptides were subjected to matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass
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FIGURE 2. Ca\(^{2+}\)-dependent binding of B1 to human FIX. The surface of microtiter plate wells was coated with human FIX at a concentration of 2 μg/ml and incubated with B1 in the presence of various concentrations of Ca\(^{2+}\). The binding of B1 was detected by ELISA and expressed as absorbance at 492 nm. Typical data from one of seven similar experiments are shown.

spectrometry and/or their amino acid sequences were analyzed. Insoluble peptides were dissolved in 1% Me\(_2\)SO.

MALDI-TOF Mass Analysis—The mass of synthesized peptides, recombinant AP and N- and O-deglycosylated pAP, was measured by MALDI-TOF mass spectrometry using Voyager-DE (Applied Biosystems, Foster City, CA).

Equilibrium Dialysis—Equilibrium dialysis was performed at room temperature in a microdialyzer with 250-μl cells (Hoeffer Scientific Instruments, San Francisco, CA) at pH 8.0 (in 50 mM Tris-HCl, 0.1 M NaCl, pH 8.0) as described previously (10, 11). Aliquots (150 μl) of CaCl\(_2\) solution containing \(^{45}\)CaCl\(_2\) as a tracer (100,000 dpm/cell; PerkinElmer Life Sciences) were dialyzed against 150 ml of a 12.9 μM B1 protein solution for 20 h with constant rotation. Protein-bound Ca\(^{2+}\) ions were quantified by liquid scintillation counting.

Amino Acid Analysis and Sequence Analysis—Samples were hydrolyzed by treatment with a mixture of 5.7 M HCl and 1% phenol vapor at 110 °C for 24 h in tubes sealed under a vacuum. After evaporation, the hydrolyzates were analyzed on a Hitachi model L-8500 Amino Acid Analyzer by the method of Spackman et al. (14). Amino acid sequences of peptides were determined with Shimadzu Protein Sequencer (model PPSQ-21A, Shimadzu, Kyoto, Japan) or Applied Biosystems Protein Sequencer (Model 473A, Applied Biosystems).

RESULTS

Production of B1—Fusion products of one mouse spleen were seeded into 384 microplate wells, and all were positive in ELISA screening for antibodies to the immuno-fix FIX. The total of 119 wells were transferred to larger volume wells. Of these, antibodies from 46 wells were Ca\(^{2+}\)-dependent, 43 were Ca\(^{2+}\)-independent, and 30 were unclear. Antibodies from all but two of the Ca\(^{2+}\)-dependent fusion products reacted with FIXaβ, and B1 was cloned from one of these two products. The subtype of antibody B1 was determined to be IgG\(_1\). κ.

Binding Activity and Metal Ion Dependence of B1 on Binding to Human FIX—B1 bound to human FIX with an apparent K\(_d\) value of 5.0 ± 0.8 nM (n = 4) in the presence of 2 mM Ca\(^{2+}\) ions, but B1 did not bind to human FIXaβ or bovine FIX. Binding activity of B1 to human FIX was also supported in the presence of 2 mM Mg\(^{2+}\) ions with an apparent K\(_d\) value of 7.2 nM (data not shown). As shown in Fig. 2, the EC\(_{50}\) of Ca\(^{2+}\) on the binding between B1 and FIX was 0.58 ± 0.06 mM (n = 7). Equilibrium dialysis experiments using \(^{45}\)Ca\(^{2+}\) revealed that B1 had no direct Ca\(^{2+}\) ion binding ability (data not shown). The apparent K\(_d\) value of the binding between B1 and human FIX became slightly higher (~1.5-fold) in the presence of 100 mM choline chloride and 1 mM Ca\(^{2+}\) in place of 100 mM NaCl and 1 mM Ca\(^{2+}\) (data not shown). The affinity for B1 binding increased slightly in the presence of Na\(^{+}\), indicating that the conformation of the activation peptide of FIX is slightly sensitive to the binding of Na\(^{+}\) (Fig. 2).

Influence of Deglycosylation on the Binding of B1 to FIX—FIX was deglycosylated with N-glycosidase F or desialylated and deglycosylated with neuraminidase and endo-α-N-acetyl galactosaminidase. Binding of B1 to deglycosylated FIX was detected by Western blotting in the presence or absence of 3 mM Ca\(^{2+}\) (see supplemental Fig. S1). N-glycosidase F treatment increased the mobility of the FIX band on SDS-PAGE, indicating the loss of N-linked sugar residues. Whereas B1 bound to deglycosylated FIX in the presence of Ca\(^{2+}\), B1 failed to bind to deglycosylated FIX in the absence of Ca\(^{2+}\). The treatment of FIX with neuraminidase and endo-α-N-acetyl galactosaminidase slightly increased the mobility of the FIX band. As in the case of N-glycosidase F treatment, neuraminidase and endo-α-N-acetyl galactosaminidase treatments did not influence the binding ability and Ca\(^{2+}\) dependence of B1 binding to FIX.

Preparation and Characterization of pAP—Human FIX was activated with RVV-X and the released pAP was purified by reverse phase HPLC using Finepak SIL C\(_{2}\)S-5 column. Peptides were eluted with a linear gradient of 1–60% acetonitrile (CH\(_3\)CN) that contained 0.1% trifluoroacetic acid over the course of 90 min at a flow rate of 1 ml/min. Elution of peptide was monitored at 230 nm. Fractions corresponding to the peptide peaks indicated were collected.

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obtained in the sequence analysis of pAP-2 and recombinant AP (supplemental Fig. S2), indicating that Thr-14 of pAP-1 is partially modified.

Binding of B1 to AP—The binding activity of B1 to AP was investigated by ELISA in a competition assay. The binding of 6.7 nM B1 to solid-phase FIX was inhibited 50% by 20 nM FIX in solution (Fig. 4). Both pAP-1 and pAP-2 inhibited binding to the same extent at lower concentrations than intact FIX. The IC_{50} of pAP-2 was 4 nM. FIXaB did not influence the binding of B1 to FIX.

Metal Ion Dependence of the Binding of B1 to pAP—Metal ion dependence of the binding of B1 to pAP was tested by dot blotting (supplemental Fig. S3). FIX and isolated pAP (pAP-2) were spotted onto a nitrocellulose membrane and the binding of B1 in the presence of 2 mM Ca^{2+}, 2 mM Mg^{2+}, or 0.2 mM EDTA was tested. B1 bound to either FIX or pAP in the presence of Ca^{2+} ions or Mg^{2+} ions, but not in the absence of EDTA. 3B1, a Ca^{2+}-dependent monoclonal antibody against human FIX that recognizes the serine protease region bound to FIX even in the absence of metal ions but did not bind to pAP.

Effects of Synthetic Peptides on the Binding of B1 to FIX—Seven peptides (SKLTRAETVF (residues 141–145 of FIXaB light chain and residues 1–5 of AP), AETVPFDVDY (residues 1–10 of AP), PDVYVNSTEAETIL (residues 6–20 of AP), AETILDINITQTSQSF (residues 16–30 of AP), STQSFNDFTR (residues 26–35 of AP), AETVPFDVDYVNSTEAETIL (residues 1–20 of AP), PDVYVNSTEAETILDINITQ (residues 6–25 of AP), AETILDINITQTSQSFNDFTR (residues 16–35 of AP)) of the human FIX AP were synthesized, and their inhibitions of the binding of B1 to solid-phase FIX were measured to determine the epitope of AP detected by B1. These seven peptides did not show any significant inhibitory influences on the binding of B1 to solid-phase FIX even at a concentration greater than 100 μM, whereas 1 μM FIX almost completely abolished the binding of B1 to FIX (data not shown).

Characterization of the Deglycosylated pAP and the Recombinant AP—To investigate the influences of sugar chains attached to pAP on the binding of B1 to pAP, N-linked sugar chains of pAP were removed by the treatment of pAP with N-glycosidase F. O-linked sugar chains attached to Thr-14 and Thr-24 of pAP were removed by the treatment of pAP with neuraminidase and endo-α-N-acetylgalactosaminidase. Both N-linked and O-linked oligosaccharides were removed by the treatment with N-glycosidase F, neuraminidase, and endo-α-N-acetylgalactosaminidase. These deglycosylated pAPs were purified by reverse phase HPLC. Recombinant AP without any modification was also prepared using E. coli expression system for comparison with pAP. The amino acid sequences of deglycosylated pAP (Fig. 5A) and recombinant AP (rAP) (Fig. 5B) were analyzed. The yield of PTH-derivative amino acids, including Ser-13, of rAP decreased at a constant ratio as the cycle advanced (Fig. 5B). Amino acid sequence analysis of deglycosylated pAP revealed that N-linked sugar chains attached to Asn-12 and Asn-22 and O-linked sugar chains attached to Thr-14 and Thr-24 of pAP were completely removed (Fig. 5A). Deglycosylation of N-linked sugar chains by N-glycosidase F yielded the change of Asn residues to Asp residues at positions 12 and 22 of pAP (Fig. 5A). Comparison of Ser-13 yield shows a yield in deglycosylated pAP of almost 10% of that expected based on the data for rAP (Fig. 5, A and B). Tyrosine sulfation at Tyr-155, Tyr-10 of AP, has previously been reviewed (5, 17). However, amino acid sequence analyses of pAP in our study revealed that the yield of the PTH-derivative of Tyr-10 of pAP and that of rAP were similar (Fig. 5, A and B). The results in our study indicate that the Tyr-10 residue of AP in plasma-derived FIX is essentially not sulfated.

Effects of Deglycosylated pAP and Recombinant AP on the Binding of B1 to FIX—Influences of the N- and O-deglycosylated pAP and the rAP on the binding of B1 to solid-phase FIX were examined by ELISA in the presence of 2 mM Ca^{2+} ions. B1 binding to FIX was inhibited 50% in the presence of 32.5 ± 7.0 nM intact pAP (Fig. 6A and Table 1). The concentrations for 50% inhibition of N-deglycosylated pAP, O-deglycosylated pAP, and N- and O-deglycosylated pAP were 22.5 ± 4.2 nM, 34.3 ± 5.5 nM, and 14.5 ± 2.6 nM, respectively. Recombinant AP did not inhibit the binding of B1 to FIX at concentrations less than 50 μM. These data indicate that B1 binds to pAP independently of the presence of sugar and suggest that pAP has some prosthetic group other than sugar.

MALDI-TOF Mass Analysis of N- and O-Deglycosylated pAP and Recombinant AP—The mass (m/z) of N- and O-deglycosylated pAP was 4051.9, and that of recombinant AP was 3969.36 (supplemental Fig. S4), indicating the mass of deglycosylated pAP to be 82.54 Da greater than that of rAP. Upon deglycosylation of N-linked sugar with N-glycosidase F, two Asn residues changed to Asp residues, with a corresponding increase in the mass of 2.0 Da. The remaining 80.54 Da account for the mass of PO_{3}H_{2}. These MALDI-TOF mass analyses suggest that a phosphate group attaches to pAP.

Influences of Dephosphorylation of Factor IX and pAP on the Binding with B1—The influences of dephosphorylation of FIX and pAP on binding with B1 were investigated. pAP was treated with potato acid phosphatase, and the amino acid sequence was analyzed. B1 did not bind to solid-phase dephosphorylated human FIX, even at a concentration of 100 nM (supplemental Fig. S5). The yield of PTH-derivative of Ser-13 increased by the dephosphorylation (supplemental Fig. S6). Dephosphorylated pAP also lost the ability to bind to B1 (Fig. 6B). pAP inhibited the binding of B1 to solid-phase human FIX but the dephosphorylated pAP did not.

Binding of B1 with Synthetic Peptide Containing Phosphoserine—Two peptides containing phosphoserine at position 13 of AP were synthesized (Fig. 7A). Peptide AP-(7–17) and peptide AP-(5–19) correspond to the sequence from Asp-7 to Glu-17 and from Phe-5 to Ile-19, respectively. Both peptides AP-(7–17) and AP-(5–19) include phosphoserine. Peptide AP-(5–19) at concentrations from 100–360 μM (the mean value from 4 experiments, 255 μM) inhibited the binding of B1 (10 nM) to solid-phase FIX by 50%, whereas peptide AP-(7–17) did not, even at concentrations greater than 1 mM (Fig. 7B).
DISCUSSION

Phosphorylation of pAP—The mass (m/z) of deglycosylated pAP determined by MALDI-TOF mass spectrometry was 82.54 Da greater than that of recombinant AP (supplemental Fig. S4). FIX AP has two N-linked and two O-linked oligosaccharides (4, 18). Upon deglycosylation of N-linked sugar with N-glycosidase F, two Asn residues changed to Asp residues, with corresponding increase in mass of 2.0 Da. The remaining 80.54 Da corresponds to the mass of PO₃H₂. Dephosphorylation of pAP by acid phosphatase resulted in the recovery of yield of the PTH-derivative of Ser-13 (supplemental Fig. S6), indicating that Ser-13 of pAP was phosphorylated.

Human FIX lost the ability to bind to B1 upon dephosphorylation by potato acid phosphatase (supplemental Fig. S5), clearly indicating that the phosphoryl group(s) of human FIX contributes to the binding of B1 to FIX. Binding of B1 to solid-phase human FIX was inhibited by pAP from human FIX but was not inhibited by dephosphorylated pAP (Fig. 6B), indicating that the binding ability of B1 to pAP is dependent upon phosphoserine at position 13 (Ser-13) of pAP.

To further localize the epitope of FIX detected by B1, two peptides containing phosphoserine, peptide AP-(5–19) and peptide AP-(7–17), were synthesized. Whereas AP-(7–17) failed to inhibit the binding of B1 to solid-phase human FIX, AP-(5–19) inhibited the binding, as shown in Fig. 7B. These data indicate that the minimal epitope recognized by B1 cannot be formed by phosphoserine alone, but also requires hydrophobic amino acids located at the amino and carboxyl ends of peptide AP-(5–19). It is likely that the three-dimensional structure of this epitope is formed by hydrophobic interactions of these residues.

Kaufman (5) previously reviewed the full phosphorylation of plasma-derived human FIX at Ser-158, but experimental data had not been shown. The extent of serine phosphorylation in recombinant FIX synthesized by myotube and Chinese hamster ovary (CHO) cell was analyzed using an anti-phosphoserine antibody (17); only 7% of recombinant FIX was phosphorylated (6). White et al. (17) also suggested that plasma-derived FIX was almost fully phosphorylated but that recombinant products of FIX produced by CHO cells were not. Although both recombinant FIX was not phosphorylated, it had spe-
specific clotting activity in vitro assays, similar to that of plasma-derived FIX (6, 17). These data indicate that post-translational modifications in cells other than hepatic cells are not identical with those in the liver and that phosphorylation of Ser residue(s) of human FIX does not affect biological activity in vitro. However, CHO cell- and myotube-synthesized recombinant FIX had lower recovery in plasma than plasma-derived FIX after intravenous infusion to mice, indicating that the post-translational modifications such as phosphorylation play an important role in clearance by the liver of FIX (6, 17). Phosphorylation at Ser-158 of human FIX should contribute to the prevention of coagulation FIX clearance by the liver.

Sugar Chain Attached to Human FIX AP—Human FIX AP contains two N-linked sugar chains at Asn-12 and Asn-22 (4). In addition, attachment of O-linked sugar chains occurs at two sites, including Thr-14 and Thr-24 (18). In our studies, two molecular species, pAP-1 (26%) and pAP-2 (74%) are obtained from human plasma-derived FIX AP fraction (Fig. 3). Sequence analysis of the two pAPs revealed that Asn-12 and Asn-22 of the two pAPs are modified by N-glycosylation (supplemental Fig. S2). Thr-14 and Thr-24 are modified by O-glycosylation in pAP-1, and Thr-14 and Thr-24 were not modified in pAP-2 (supplemental Fig. S2). These data show that almost all human FIX molecules are glycosylated at Asn-12 and Asn-22, whereas Thr-14 and Thr-24 are partially glycosylated (Fig. 8).

As shown in Fig. 4, pAP-2 binding to B1 was slightly stronger than to pAP-1, suggesting that the O-linked sugar chains of pAP-1 hinder the interaction between AP and B1. As the removal of bulky N-linked sugar chains of pAP more effectively decreased IC50 values of the binding between B1 and FIX, the existence of steric hindrance by N-linked sugar was indicated (Table 1). However, the influence of deglycosylation was not as significant.

Ca2⁺ Dependence of the Binding between B1 and pAP—Most previously described Ca2⁺-dependent anti-FIX monoclonal antibodies recognize the Gla domain (8). Several monoclonal antibodies that recognize FIX AP independent of Ca2⁺ ions have been reported (9) but a Ca2⁺-dependent monoclonal antibody with binding activity to FIX AP is not known to date. To investigate whether isolated pAP maintains Ca2⁺ dependence in binding to B1, pAP-2 was blotted onto a nitrocellulose membrane, and the binding to B1 was measured in the presence and absence of Ca2⁺ ions. B1 recognized pAP-2 only in the presence of Ca2⁺ or Mg2⁺ ions but not in the presence of EDTA (supplemental Fig.
S3). B1 is the first Ca$^{2+}$-dependent monoclonal antibody that recognizes the AP region of human FIX.

The O-linked sugar chain attached to human FIX AP consists of GalNAc, Gal, and sialic acid (18). Because charged groups such as on sialic acid play an important role in cationic binding in various proteins, and sialic acids are known to have a relatively high affinity for Ca$^{2+}$ ions, we studied the possibility that the Ca$^{2+}$ dependence of the binding between B1 and pAP comes from the presence of sialic acids in pAP. However, B1 bound not only to intact FIX but also to deglycosylated FIX (supplemental Fig. S1). Furthermore, pAP-2, which lacks the O-linked sugar chain, bound to B1 with higher affinity than pAP-1, which contains the O-linked sugar chain (Fig. 4). These results indicate that B1 recognizes FIX independent of the presence of the sugar chain, including sialic acids, and that the Ca$^{2+}$ dependence is caused by mechanisms other than Ca$^{2+}$ binding to the sugar chains.

With respect to an anti-protein C monoclonal antibody, an anticoagulant serine protease precursor having a similar domain structure with FIX, a Ca$^{2+}$-dependent monoclonal antibody that recognizes AP was reported (19). This antibody, named HPC4, has a low affinity metal ion binding site as in the case of protein C (21). The conformation of the activation peptide of protein C was influenced by Na$^{+}$ binding (20). The serine protease domain of human FIX is predicted to contain a Na$^{+}$ binding site as in the case of protein C (21). As mentioned above, the affinity for B1 binding increased slightly in the presence of Na$^{+}$ compared with in the presence of choline chloride, indicating that the conformation of the AP of FIX is slightly sensitive to the binding of Na$^{+}$.

The above-mentioned results suggest that Ca$^{2+}$ binds to phosphoserine-13 of AP (phosphoserine-158 of FIX) and that B1 recognizes the epitope formed by Ca$^{2+}$-bound phosphoserine-13 and several amino acids including amino acids around the phosphoserine and hydrophobic amino acids such as Phe-5 and Ile-19.

In conclusion, we showed in this study that Ser-158 of human FIX is phosphorylated and that a monoclonal antibody B1 recognizes FIX AP containing the phosphoserine residue (Fig. 8). The functional role of the phosphoryl group of FIX AP on FIX activation as well as the three-dimensional structure of the AP of FIX has not yet been clarified. B1 will be a valuable tool in the investigation of the role of AP on blood coagulation and the three-dimensional structure of the FIX zymogen. Further, B1 promises to be useful in the quality control of biologically active recombinant FIX containing phosphoserine-158 that plays an important role in clearance by the liver of recombinant human FIX.

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