Endogenous phosphorylation of the lipoprotein-associated coagulation inhibitor at serine-2

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Lipoprotein-associated coagulation inhibitor (LACI) inhibits activated Factor X (Xa) directly and, in an Xa-dependent fashion, inhibits Factor VIIa–tissue factor (TF), presumably by forming a quaternary Xa–LACI–VIIa–TF complex. LACI isolated from the conditioned media of HepG2 cells grown in the presence of [³²P]orthophosphate was observed to be covalently phosphorylated. Dephosphorylation of ³²P-LACI with phosphatase resulted in an almost complete removal of the radiolabel. Phosphoamino acid analysis of the purified ³²P-LACI established that the phosphorylation occurred on (a) serine residue(s). At its *N*-terminus, LACI contains a cluster of acidic residues *C*-terminal to the serine-2 residue. Such a site is characteristic of the sites phosphorylated by casein kinase II (CKII) in protein substrates. Edman degradation of endogenously labelled ³²P-LACI revealed that the serine-2 residue was a major site of phosphorylation. Phosphorylation of purified LACI by bovine CKII was observed to occur *in vitro*; amino acid sequence analysis demonstrated that CKII phosphorylated LACI at the serine-2 residue. Recombinant LACI expressed from mouse C127 fibroblasts transfected using a bovine-papilloma-virus expression vector was also found to be endogenously phosphorylated. By using site-directed mutagenesis, an altered form of LACI was produced in which the serine-2 residue had been changed to alanine. This altered LACI, although expressed in similar quantity to the wild-type LACI, was not detectably phosphorylated. Using the altered LACI in functional studies demonstrated that a serine residue at position 2, and thus the phosphorylation of this site, was not essential for LACI's inhibition of Xa and VIIa–TF activities.

INTRODUCTION

Upon vascular injury, blood coagulation is initiated when Factor VIIa, a plasma proteinase, is exposed to, and combines with, its essential cofactor tissue factor (TF). The VIIa–TF complex proteolytically activates its substrate Factors IX and X [1–5], which, through a cascade of events, leads eventually to the generation of a fibrin clot.

Lipoprotein-associated coagulation inhibitor (LACI) is associated with plasma lipoproteins and is a potent inhibitor of the VIIa–TF complex [6–9]. LACI has been purified by our laboratory from a human hepatoma cell line (HepG2) [10] and shown to bind to, and inhibit, activated Factor X (Xa) directly and to inhibit VIIa–TF activity in an Xa-dependent fashion, presumably by forming an Xa–LACI–VIIa–TF quaternary complex [11]. Further investigation demonstrated that, although LACI bound to and inhibited Xa which lacked its γ -carboxyglutamic acid (Gla)-containing domain, VIIa–TF activity was not inhibited by LACI in the presence of Gla-domainless Xa [11].

The primary amino acid sequence of LACI, deduced from cDNA sequences [12,13], shows that LACI contains three tandem Kunitz-type proteinase-inhibitory domains located between a cluster of negatively charged amino acid residues at its *N*-terminus and a cluster of positively charged residues near its *C*-terminus. Recent studies, employing site-directed mutagenesis to produce altered forms of LACI, have demonstrated LACI's first Kunitz domain (as it occurs from the *N*-terminus) probably binds to VIIa's active site, whereas the second Kunitz domain binds to the active site of Xa [14]. Primary amino acid sequence analysis indicates three potential sites for *N*-linked glycosylation, and studies indicate that at least two of these sites are glycosylated

[15]. The fraction of plasma LACI associated with high-density lipoproteins (~40%) has recently been shown to be covalently attached through disulphide linkage(s) to apolipoprotein A-II [16]. No other post-translational covalent modifications of LACI have been reported.

In the present study we demonstrate that HepG2 cells express LACI which exists in at least a partially phosphorylated state, with the dominant site of phosphorylation being serine-2. Studies *in vitro* are consistent with casein kinase II (CKII) being the kinase responsible for this post-translational modification.

EXPERIMENTAL

Reagents

[³²P]Orthophosphoric acid (9000 Ci/mmol) and [γ -³²P]ATP (3000 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). The human hepatoma cell line (HepG2) and mouse C127 fibroblasts were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). The bovine-papilloma-virus expression vector pMON1123 and pSV2neo were provided by Monsanto Corp. (St. Louis, MO, U.S.A.). CKII, purified from bovine testes, was kindly provided by Dr. Edwin Krebs (University of Washington, Seattle, WA, U.S.A.). Alkaline phosphatase was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Acid phosphatase, t.l.c. plates (type 100 cellulose), and phosphoserine, phosphothreonine and phosphotyrosine standards were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Reagents for PAGE, including prestained protein molecular-

Abbreviations used: TF, tissue factor; LACI, lipoprotein-associated coagulation inhibitor; Gla, γ -carboxyglutamic acid; CKII, casein kinase II; VIIa etc., Factor VIIa etc.

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mass standards, were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). All other reagents were of reagent grade (or better) and came from either Fisher Scientific Co. (Pittsburg, PA, U.S.A.) or from Sigma.

LACI purification from ^{32}P -labelled-HepG2-cells' serum-free conditioned medium

Confluent cells were incubated with ^{32}P in serum-free, phosphate-free, Dulbecco's modified Eagle's medium. Cells received 5 mCi of ^{32}P in 10 ml of medium/75 cm² dish and were incubated at 37 °C for 3 days. LACI was purified from the cells' conditioned medium using a previously described three-step purification procedure which utilizes bovine Xa-affinity chromatography [17].

Phosphatase treatments

Purified LACI was dialysed into 20 mM-NH₄HCO₃, pH 8.0, and aliquots were dried with a SpeedVac Concentrator (Savant Instruments, Farmingdale, NY, U.S.A.). LACI was dissolved in 50 mM-sodium acetate, pH 5.0, for acid phosphatase treatment or 50 mM-Tris/HCl/100 mM-NaCl/5 mM-MgCl₂, pH 8.0, for alkaline phosphatase treatment. The appropriate phosphatase was added and the samples were incubated for 2 h at 30 °C. The samples were analysed by SDS/PAGE, followed by autoradiography. For functional studies, the LACI was repurified using bovine Xa-affinity chromatography to remove the phosphatase activity, dialysed and concentrated as described above.

Phosphorylation of LACI by CKII

To assay for the incorporation of [^{32}P]phosphate into LACI, purified LACI (1.6 μM) was incubated at 30 °C with CKII (1 nM) in 50 μl of 50 mM-Tris/HCl/150 mM-NaCl/10 mM-MgCl₂, pH 7.6, containing 5 μCi of [γ - ^{32}P]ATP (3000 Ci/mmol). To monitor the incorporation, 10 μl aliquots were removed at 15 min intervals, immediately spotted on to phosphocellulose paper (Whatman P81), washed in 150 mM-phosphoric acid and counted for radioactivity [18]. At 45 min, 5 μl aliquots were removed from the assay mixtures and added to 5 μl of SDS/PAGE 4 $\times$ sample buffer [60 mM-Tris/HCl/2 % (w/v) SDS/25 % (v/v) glycerol, pH 6.8] to stop the reactions. Samples were electrophoresed on 15 % (w/v) polyacrylamide gels and analysed by autoradiography. To ^{32}P -label the LACI for sequencing purposes, the above reaction was scaled up 7-fold and incubation was allowed to proceed for 2 h, after which the ^{32}P -LACI was separated from free label by using bovine Xa-affinity chromatography followed by further purification using gel-sieve chromatography [17].

Phospho amino acid analysis

Purified ^{32}P -LACI was dialysed into 20 mM-NH₄HCO₃, dried, redissolved in 100 μl of 6 M-HCl and incubated under argon for 2 h at 110 °C. Samples were dried, resuspended in 20 μl of pH 1.9 buffer [formic acid (88 %)/acetic acid/water (25:78:897, by vol.)] and spotted on to cellulose t.l.c. plates along with phospho amino acid standards. Phospho amino acids were resolved by electrophoresis in pH 1.9 buffer for 3 h at 500 V. The plates were stained with ninhydrin and analysed by autoradiography [19].

Phospho-LACI sequencing and identification of phosphorylated residues

^{32}P -labelled LACI samples were sequenced by automated Edman degradation, using two methods. In one method, ^{32}P -LACI was sequenced using a Beckman 890C spinning-cup sequencer. The sample from each cycle, including a blank (begin) cycle, was counted directly for radioactivity, to determine ^{32}P release [20]. The second method employed the split-filter tech-

nique of Wang *et al.* [21]. In this method ^{32}P -LACI was applied to a glass-fibre sequencing filter coated with Polybrene. The filter was cut into four equal sections and gas-phase sequencing was performed. A section of the filter was removed after a blank (begin) cycle and after the first three sequencing cycles. Free ^{32}P and protein were extracted from the filter sections with 88 % formic acid, dried and resuspended in 20 μl of pH 3.5 buffer [pyridine/acetic acid/water (1:10:189, by vol.)]. These samples were spotted on to cellulose t.l.c. plates, which were then electrophoresed at 500 V for 1 h in pH 3.5 buffer. The plates were analysed by autoradiography [19].

Construction and transfection of the LACI(Ala-2) mutant

The construction and transfection of recombinant wild-type LACI [LACI(wt)] has been described [13]. The same LACI cDNA insert engineered into the bovine-papilloma-virus vector for expression in C127 fibroblasts was also ligated into M13mp18, and site-directed mutagenesis was performed [22] to convert the TCT codon encoding for serine at position 2 (residues are numbered from the *N*-terminus of mature LACI) into a GCT codon encoding for alanine. This mutant LACI cDNA was engineered into the bovine-papilloma-virus expression vector and used to transfect C127 fibroblasts as described in [13].

SDS/PAGE and Western blotting

SDS/PAGE was performed by the method of Laemmli [23] using a 15 % separating gel (4 % stacking gel). Western blotting was performed as previously described, using a rabbit polyclonal anti-LACI IgG fraction [24].

Functional assays of LACI

LACI(wt) and LACI(Ala-2) were assayed using the appropriate cloned cells' serum-free conditioned medium diluted in TBSA (100 mM-NaCl/50 mM-Tris/HCl/0.1 % BSA, pH 7.4). Alkaline-phosphatase-treated and CKII-phosphorylated LACI were repurified using Xa-affinity chromatography before assaying. The LACI concentration for each sample was determined in a particle-concentration immunofluorescence assay using two non-competitive anti-LACI monoclonal antibodies [25]. Xa-inhibitory activities were determined as previously described using 25 ng of bovine Xa/ml (final concn.) [13]. VIIa-TF inhibitory activities of the LACI's were measured by a ^3H -IX-activation-peptide release assay as previously described [13].

RESULTS

Phosphorylation of LACI by HepG2 cells

LACI purified from the serum-free conditioned medium of HepG2 cells incubated for 3 days in the presence of [^{32}P]orthophosphate was found to be radiolabelled as determined by SDS/PAGE, followed by autoradiography (Fig. 1a, lanes 1 and 3). The ^{32}P incorporated into LACI was almost completely removed by treatment with either acid phosphatase or alkaline phosphatase (Fig. 1a, lanes 2 and 4). Western-blot analysis demonstrated the phosphatase treatments were not accompanied by any detectable alterations in the quantity of LACI or in LACI's relative mobility in the gel system (Fig. 1b). Phospho amino acid analysis of purified ^{32}P -LACI labelled by HepG2 cells revealed phosphorylation occurred on serine residue(s), but not on threonine or tyrosine residues (Fig. 1c).

Identification of the site in LACI phosphorylated by HepG2-cells

Scanning LACI's primary amino acid sequence, predicted from cDNA sequence [12,13], revealed that a potential re-

Fig. 1. (a) Phosphorylation of LACI by HepG2 cells, (b) Western-blot analysis and (c) phospho amino acid analysis

(a) LACI was purified from the serum-free conditioned medium of HepG2 cells grown in the presence of [32 P]orthophosphate. LACI was incubated without (lane 1) or with (lane 2) 5 units of acid phosphatase or without (lane 3) or with (lane 4) 5 units of alkaline phosphatase. The samples were electrophoresed on an SDS/15%-(w/v)-polyacrylamide gel. The gel was dried and exposed to X-ray film. The resulting autoradiograph is shown. Apparent molecular masses of prestained protein standards are shown on the left. (b) An identical gel was transferred to nitrocellulose. A rabbit anti-LACI IgG was used to probe for LACI, and the blot was developed colorimetrically as described in the text. Apparent molecular masses of prestrained standards are shown on the left. (c) Endogenously labelled LACI was subjected to partial acid hydrolysis and analysis by thin-layer electrophoresis. An autoradiograph of the thin-layer plate is shown. The positions of phospho amino acid standards, revealed by ninhydrin staining, are indicated. P_i designates the mobility of inorganic phosphate.

Fig. 2. Identification of the dominant residue phosphorylated in LACI (HepG2)

(a) 32 P radioactivity released from a spinning-cup sequencer during sequential cycles of Edman degradation. Endogenously labelled 32 P-LACI(HepG2) was applied to a spinning cup for sequencing. The material released into the butyl chloride phase during a blank cycle (B) and each of the first six sequencing cycles (1–6) was counted for 32 P. (b) Thin-layer-electrophoretic analysis of 32 P extracted from a gas-phase sequencer filter after sequential cycles of Edman degradation. 32 P-LACI was applied to a glass-fibre filter containing Polybrene and subjected to gas-phase sequencing. A filter segment was removed after a blank (begin) cycle (lane 1) and after Edman degradation cycles 1 (lane 2), 2 (lane 3) and 3 (lane 4). Radioactivity was extracted from each segment with formic acid and analysed by thin-layer electrophoresis at pH 3.5. In lanes 3 and 4 the radioactivity migrating as P_i represents 52% and 49% of the total activity of each sample respectively, as determined by scraping the plate and measuring radioactivity.

cognition site for phosphorylation by CKII exists at the serine-2 residue at LACI's N-terminus (see below). To determine if this site was phosphorylated, endogenously labelled 32 P-LACI was subjected to sequencing by Edman degradation using a spinning-cup sequencer. Fig. 2(a) shows the 32 P radioactivity released after a blank (begin) cycle and six sequencing cycles. As expected for the serine-2 being phosphorylated, the peak release of 32 P occurred after the second sequencing cycle (third cycle overall, including the begin cycle). The endogenously labelled LACI was sequenced a second time using the split-filter technique of Wang *et al.* [21]. Radioactivity was extracted from the pieces of sequencing filter removed after the begin cycle and the first,

second and third cycles of Edman degradation, and free 32 P was separated from 32 P-LACI by thin-layer electrophoresis. As the autoradiograph in Fig. 2(b) shows, free phosphate appears after the second sequencing cycle. Of the total radioactivity, 52% and 49% in cycles 3 and 4 respectively migrated as P_i as determined by scraping the plate's radioactive areas and counting. These results demonstrate serine-2 is a major site of phosphorylation in LACI by HepG2 cells.

Requirement of serine-2 for endogenous phosphorylation of LACI

To investigate further the phosphorylation of LACI, site-directed mutagenesis was used to prepare a LACI mutant in which the serine-2 residue was changed to alanine. As previously described for recombinant LACI(wt), the mutant DNA was also introduced into the bovine-papilloma-virus vector pMON1123 and co-transfected with pSV2neo into mouse C127 fibroblasts. A clone expressing the altered human LACI protein, designated LACI(Ala-2), was identified by Western-blot analysis (Fig. 3a). LACI(wt) and LACI(Ala-2) were purified from serum-free conditioned media from their respective cells which had been incubated for 3 days in the presence of [32 P]orthophosphate. The extent of incorporation of radioactivity into the respective LACIs is shown in the autoradiograph in Fig. 3. Although both cell lines expressed roughly similar amounts of recombinant LACIs, only the LACI(wt) was 32 P-labelled. Thus C127 fibroblasts are capable of phosphorylating LACI, and the serine-2 residue of LACI is necessary for this phosphorylation to occur.

Phosphorylation of LACI by CKII *in vitro*

The serine-2 in LACI precedes five consecutive negatively charged amino acid residues. Such a site is characteristic of the sites phosphorylated by CKII in protein substrates [16]. The ability of CKII to catalyse the phosphorylation of purified LACI(HepG2) *in vitro* was investigated. The results in Fig. 4(a) demonstrate that LACI can be phosphorylated *in vitro* by CKII (lane 1) and that LACI does not undergo autophosphorylation (lane 2). The radiolabelled band observed in the kinase assay without LACI (lane 3) is characteristic of CKII undergoing autophosphorylation [26]. Phosphoamino acid analysis demonstrated that phosphorylation of LACI had occurred predominantly on serine residue(s) and only a trace of radiolabelled phosphothreonine was detected. [Although

Fig. 3. Requirement of serine-2 for the phosphorylation of LACI

LACIs were purified by Xa-affinity chromatography from the serum-free conditioned medium of the indicated cells which were grown in the presence of [32 P]orthophosphate. Eluted materials were concentrated and electrophoresed on identical SDS/15% polyacrylamide gels. (a) Expression of recombinant LACI(wt) and LACI(Ala-2). A Western blot of LACI(HepG2) (lane 1), C127 cells (lane 2), LACI(wt) (lane 3) and LACI(Ala-2) (lane 4) is shown. A rabbit anti-LACI IgG was used to probe for LACI, and the blot was developed colorimetrically. Apparent molecular masses of prestained standards are shown on the left. (b) Phosphorylation of recombinant LACI(wt) and LACI(Ala-2). An autoradiograph of one gel is shown. Lane assignments for samples are as in (a).

Fig. 4. (a) Phosphorylation of LACI by CKII and (b) phospho amino acid analysis

LACI (1.6 μ M) was incubated with [γ - 32 P]ATP in the presence (lane 1) or absence (lane 2) of CKII (1 nM) or CKII alone (lane 3) was incubated with [γ - 32 P]ATP. Reaction products were electrophoresed on a SDS/15% polyacrylamide gel, and [32 P]phosphate incorporation was monitored by autoradiography. Apparent molecular masses of prestained protein standards are shown on the left. (b) CKII phosphorylated 32 P-LACI underwent partial acid hydrolysis and analyses by thin-layer electrophoresis as described in Fig. 2. An autoradiograph of the thin-layer plate is shown. The positions of phosphoamino acid standards, revealed by ninhydrin staining, are indicated. P_i designates the mobility of inorganic phosphate.

phosphothreonine and phosphotyrosine were not resolved in this system, ion-exchange h.p.l.c. demonstrated the low level of activity co-migrating electrophoretically with the phosphothreonine/phosphotyrosine position was phosphothreonine (T. J. Girard, D. McCourt & R. Thoma, unpublished work).] Sequence analysis demonstrated that more than 90% of the 32 P incorporated into LACI by CKII was located on the serine-2 residue (T. J. Girard & D. McCourt, unpublished work).

Fig. 5. Functional activities of LACI(Ala-2)

(a) Inhibition of Xa. Xa-inhibitory activities were determined as previously described [13] using 25 ng of bovine Xa/ml (final concn.). Samples are LACI(wt) (●) and LACI(Ala-2) (■) serum-free conditioned media diluted in TBASA. The LACI concentration for each serum-free conditioned medium was determined in a particle-concentration immunofluorescence assay [24] using two non-competitive anti-LACI monoclonal antibodies. (b) Inhibition of VIIa-TF activity. VIIa-TF inhibitory activities were measured by a 32 P-IX activation peptide release assay [13]. Samples are as in (a).

Functional activities of the LACI(Ala-2) mutant

Serum-free conditioned media were used to assess the ability of the LACI(Ala-2) molecules to inhibit Xa and VIIa-TF activities. As the results in Fig. 5 show, both LACI(wt) and LACI(Ala-2) were equally potent in inhibiting Xa and VIIa-TF activities. Likewise, alkaline-phosphatase-treated LACI possessed inhibitory activities similar to those of untreated LACI (results not shown). These results demonstrate that a serine residue at position 2 in LACI, and thus the phosphorylation of this site, is not necessary for the inhibition of Xa or VIIa-TF activities.

DISCUSSION

In the present paper we demonstrate that LACI isolated from HepG2 serum-free conditioned medium is covalently phosphorylated. Sequence analysis clearly identified the serine-2 residue as a major site of phosphorylation, though other sites of phosphorylation could not be ruled out. The N-terminal sequence of mature LACI is DSEED... This sequence, a serine residue followed by a cluster of negatively charged aspartic acid and/or glutamic acid residues, is characteristic of the sites phosphorylated by CKII in protein substrates [16]. We have shown that, *in vitro*, CKII phosphorylates LACI on serine-2.

Recombinant LACI(wt) expressed by mouse C127 fibroblasts was observed to be phosphorylated; however, the altered recombinant LACI(Ala-2), in which the serine at residue 2 has been replaced with alanine, was not detectably phosphorylated. This is consistent with serine-2 being the only site phosphorylated in LACI or, alternatively, that a serine or a phosphoserine residue at position 2 is a prerequisite for phosphorylation to occur elsewhere in the LACI molecule. For example, CKII phosphorylation of some proteins potentiates their phosphorylation by glycogen synthase kinase 3/Fa [27–29].

The observation that purified LACI is endogenously phosphorylated and that this LACI can also be further phosphorylated by CKII *in vitro* demonstrates that the serine-2 residue in purified LACI(HepG2) is only partially phosphorylated when isolated. To what extent endogenous phosphorylation has occurred is not known.

CKII is widely distributed among eukaryotic organisms [30],

and phosphorylation of DNA topoisomerase II [31] and RNA polymerases I and II [32] by CKII have been reported to regulate activity. Assays with *in vitro* recombinant LACI(Ala-2) and alkaline-phosphatase-treated LACI indicate the phosphorylation state of LACI at the serine-2 residue is not required for LACI's inhibition of Xa or VIIa-TF activities. This is not unexpected, since we have previously shown that the first and second Kunitz domains of LACI are responsible for these activities [14].

Human plasma LACI is associated with lipoproteins (low-density > high-density > very-low-density lipoproteins) [6,8,15]. Analysis of LACI's primary amino acid sequence revealed no substantial hydrophobic region which might interact with lipoproteins, and the mechanism of LACI's lipoprotein association remains unknown. Yang *et al.* [33] have described a post-translational modification of bovine myelin basic protein where the hydrophobic moiety phosphatidylinositol is covalently attached to a phosphoserine residue in the protein. It is conceivable that a phosphate group on serine-2 in LACI serves as a site for a similar modification which could account for LACI's association with lipoproteins. The observation that most of the ³²P in endogenously labelled LACI(HepG2) was susceptible to phosphatase treatment (see Fig. 1a) indicates that this phosphate group was not substantially further modified in LACI(HepG2). However, preliminary data indicate that, unlike purified plasma LACI, purified LACI(HepG2) does not associate with lipoproteins (W. F. Novotny, unpublished work). The extent of modification of the serine-2 residue in plasma LACI, and whether this region of LACI plays a role in LACI's lipoprotein association, require investigation.

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