Plasma Hyaluronan-binding Protein Is a Serine Protease*

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CTCF is an essential factor for optimal transcription from the amyloid β-protein precursor promoter. A proteolytic activity detected in bovine, rabbit, horse, and human serum cleaves CTCF at three major sites, resulting in a modified mobility shift pattern of the fragments that retain DNA binding ability. The protease was purified to electrophoretic homogeneity, partially sequenced, and identified as the plasma hyaluronan-binding protein. The proteolytic activity was selectively abolished by various serine protease inhibitors, including the Kunitz-type protease inhibitor domain of amyloid β-protein precursor. Reduction with β-mercaptoethanol showed that the 70-kDa protein consists of two polypeptides with apparent molecular masses of 44 and 30 kDa. The serine protease domain was localized to the 30-kDa polypeptide as determined by [3H]diisopropylfluorophosphate binding.

The promoter of the amyloid β-protein precursor (APP) gene is a necessary element in the regulation of APP transcription and it has been shown to confer cell type-specific expression in transgenic mice (1, 2). The proximal APP promoter is devoid of CCAAT and TATA boxes but contains a prominent initiator (3). The integrity of the initiator element is essential for both start site selection and optimal transcriptional activity (3). In addition, transcription from the AP-1 promoter is critically dependent on the presence of an intact nuclear factor binding site designated APβ (3, 4). The core recognition sequence for this binding site is located between positions −82 and −93 and its elimination reduces transcriptional activity by ~70–90% (3, 4). The nuclear factor that activates transcription from APβ was identified as CTCF (5), a nuclear regulatory protein comprising 727 amino acids (6). It contains a centrally located DNA binding domain with 11 zinc finger motifs that are flanked by 267 amino acids on the N-terminal side and 151 amino acids on the C-terminal side. This protein was first identified as a factor that binds to the chicken c-myeg promoter (7) and to the silencer element of the chicken lysozyme gene (8, 9). A functional role for CTCF in both positive and negative transcriptional regulation has been documented (5, 6, 10, 11).

While purifying CTCF, we observed that binding to the

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The abbreviations used are: APP, amyloid β-protein precursor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; FCS, fetal calf serum; PHBP, plasma hyaluronan-binding protein; PAGE, polyacrylamide gel electrophoresis; DEAE, diethylaminoethyl; DFP, diisopropylfluorophosphate; KPI, Kunitz-type protease inhibitor. The APβ sequence became increasingly unstable as the level of purity increased. However, this instability could be overcome by supplementing the incubation mixture with the zwitterionic detergent CHAPS and large amounts of nonspecific protein (5). Routinely, fetal calf serum (FCS) was used as a source of such protein. While optimizing the conditions for the binding reaction, we noticed that if crude nuclear extract was preincubated with an excess of FCS prior to the binding reaction, substantial changes in the electrophoretic mobility shift pattern occurred, resulting in binding complexes with higher electrophoretic mobilities. We have here isolated the factor responsible for this altered mobility shift and identified it as a protease activity associated with the plasma hyaluronan-binding protein PHBP (12). The cDNA for this protein was previously cloned, and sequence analysis indicated the presence of a serine protease consensus domain. However, in the original preparations, proteolytic activity of PHBP was not demonstrated (12).

Materials and Methods

Nuclear Extracts, Mobility Shift Electrophoresis, and Protease Assays—Nuclear extracts were prepared from HeLa cells grown in suspension to a density of 5–8 ¥ 10^6 cells/ml (3, 13). The final protein concentration in extracts was 10–15 mg/ml in buffer D containing 25 mM Hepes, pH 7.6, 100 mM KCl, 2 mM MgCl_2, 0.2 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol. Extract preparations were aliquoted and stored at ~80 °C.

Double-stranded oligonucleotide APβ-WT (5) containing the CTCF recognition sequence was 5’-end-labeled with [γ-32P]ATP (14). 10 ng of labeled oligonucleotide (50,000–500,000 cpm) were incubated for 30 min at 25 °C with 10–20 μg of protein from nuclear extract in buffer D supplemented with 2 μg of poly(dI-dC), 5 μg of yeast tRNA, 2.5% CHAPS, and 1 mM of FCS in a total reaction volume of 30 μl. The incubation mixture was electrophoresed in 1% agarose or 6% polyacrylamide gels with 0.5 ¥ Tris-borate-EDTA (14) at 180 V constant voltage for 45 min. Gels were dried and autoradiographed for 2–4 h at ~80 °C.

Proteolytic activity was monitored by incubating 2 μl of nuclear extract aliquots prior to the binding reaction either with whole serum or with purified serum protein fractions in 5–10 μl of buffer D at 25 °C for 1 h. When needed, concentrated purified material was prediluted 10–100-fold with buffer D.

Protease Purification from Human Serum—Human serum (male) derived from whole clotted blood was purchased from Sigma. Solid ammonium sulfate was added to 500 ml of serum to 25% saturation. After centrifugation at 10,000 ¥ g for 15 min, the supernatant was collected, and proteins were further precipitated by increasing the ammonium sulfate concentration to 50% saturation. The centrifugation was repeated, and the resulting pellet containing the bulk of the proteolytic activity was resuspended in 100 ml of buffer T (20 mM Tris-HCl, pH 7.5, 2 mM MgSO_4, 0.1 mM EDTA, and 200 mM KCl). The solution was dialyzed overnight against 2 liters of buffer T with one change of buffer.

The dialyzed material was loaded on a 10-ml DEAE Sepharose Fast Flow (Amersham Pharmacia Biotech) column presaturated with buffer T. The column was subsequently washed with three 20-ml portions of buffer T containing 300, 350, and 400 mM KCl. The bulk of the proteolytic activity was eluted with 30 ml of buffer T containing 700 mM KCl. Proteins were precipitated in ammonium sulfate at 65% saturation. After centrifugation, the pellet was resuspended in 5 ml of buffer
D and dialyzed against 500 ml of the same buffer. The material was then loaded on a 1-ml HiTrap heparin-agarose column (Amersham Pharmacia Biotech) equilibrated with buffer D. The column was washed with 10 ml of buffer D, and the proteins were eluted with 18 ml of a linear KCl gradient (100–700 mM). Fractions of 1 ml were collected, and the proteolytic activity was monitored as described above.

Separation and Identification of CTCF Proteolytic Fragments—One milliliter of HeLa cell nuclear extract was incubated with 3 μl of heparin-agarose-purified protease for 1 h at 25 °C. The reaction was stopped by the addition of 15 μg of a peptide containing the KPI domain of APP (15). The KCl concentration in the reaction mixture was adjusted to 200 mM, and the reaction products were loaded on a 1-ml HiTrap SP Sepharose column equilibrated with buffer D containing 200 mM KCl. The column was washed with 5 ml of buffer D containing 200 mM KCl and 4 ml of the same buffer containing 300 mM KCl. Proteins were eluted with 20 ml of a 300–700 mM linear KCl concentration gradient. Fractions of 1 ml were collected and analyzed by mobility shift electrophoresis.

An Amersham Pharmacia Biotech Superose 6HR 10/30 gel filtration column was equilibrated with buffer D containing 500 mM KCl, 2.5% CHAPS, and calibrated with a set of globular proteins (Amersham Pharmacia Biotech HMW Calibration kit). SP Sepharose chromatography fractions 19–23 containing mobility shift activity were combined, supplemented with 2.5% CHAPS, concentrated on a Centricon-10 device (Amicon), and loaded on the gel filtration column. The gel filtration was performed at a 0.4 ml/min flow rate. Fractions of 0.5 ml were collected. The mobility shift from electrophoresis both under reducing and nonreducing conditions. The gel was stained with Coomassie Brilliant Blue R-250, impregnated with ECL procedure (Amersham Pharmacia Biotech), which was carried out according to manufacturer’s instructions.

**RESULTS**

**Treatment of Nuclear Extract with Serum Changes the Mobility Shift Pattern of the APBβ Binding Complex**—When nuclear extract from HeLa cells was incubated with an 80-mer oligonucleotide containing the APBβ domain of the APP promoter, a characteristic mobility shift complex b was formed (Fig. 1, lane 2). This complex is the result of transcription factor CTCF binding to the APBβ recognition sequence (5). However, upon incubating the nuclear extract for 1 h at 25 °C with FCS before assembling the binding reaction, a substantial change in the electrophoretic mobility shift pattern occurred (Fig. 1, lanes 3–6). With increasing amounts of FCS in the preincubation reaction, the original mobility shift complex b was gradually eliminated as two new complexes, b1 and b2, with higher electrophoretic mobilities emerged. Moreover, at the highest concentration of FCS, complex b1 was also eliminated and only b2, the complex with the highest mobility, remained. A similar change in the mobility shift pattern was observed with adult bovine, horse, or rabbit serum (not shown), as well as with an 5–10-fold lower concentration of human serum (Fig. 1, lanes 7–10). They also occurred when crude nuclear extract was
replaced with purified CTCF. However, significantly lower amounts of serum were required to achieve comparable results (not shown). The most plausible explanation for this alteration in mobility shift behavior is a proteolytic cleavage of the CTCF protein by a serum enzyme.

**Fig. 2.** Purification of the putative proteolytic activity by chromatography on heparin-Sepharose. After initial fractionation by ammonium sulfate precipitation and anion exchange chromatography, the human serum fraction containing the hypothetical protease activity was loaded on a 1-ml Amersham Pharmacia Biotech HiTrap Heparin cartridge and eluted with a linear KCl gradient. Fractions of 1 ml were collected. A, A_{280} (thick line) and KCl concentration (thin line) profile of the elution gradient. B, agarose mobility shift electrophoresis with radiolabeled APB oligonucleotide and 2 μl of HeLa cell nuclear extract preincubated with 0.04 μl of elution fractions 9–20 (lanes 1–12). Free oligonucleotides (f) and binding complexes b, b1, and b2 are indicated by brackets. C, 4–20% gradient SDS-PAGE under nonreducing conditions of elution fractions 9–20 (lanes 1–12). Lanes were loaded with 10 μl from each fraction.

**Fig. 3.** Analysis of binding complexes separated by cation exchange chromatography and mobility shift electrophoresis. Nuclear extract was preincubated with heparin-agarose-purified protease. Resulting protein fragments were separated by cation exchange chromatography followed by mobility shift electrophoresis. A, polyacrylamide mobility shift electrophoresis with radiolabeled APB oligonucleotide and 2 μl of untreated HeLa cell nuclear extract (lane 1) or extract preincubated with 0.005 μl (lane 2), 0.01 μl (lane 3), 0.02 μl (lane 4), 0.04 μl (lane 5), 0.08 μl (lane 6), or 1.0 μl (lane 7) of heparin-agarose-purified protease. B, nuclear extract was preincubated with heparin-agarose-purified protease in preparative quantities. A 2-μl aliquot was analyzed by mobility shift electrophoresis (lane 1). The protease-treated extract was separated by SP-Sepharose cation exchange chromatography, and eluted material was collected in 1-ml fractions. Aliquots of 4 μl from elution fractions 18–24 were subjected to mobility shift electrophoresis (lanes 2–8). The positions of binding complexes (b, b0, b1, b10, and b2) are delineated by arrowheads, and free oligonucleotides (f) are shown by brackets.
serum, increasing amounts of partially purified active material in the preincubation reaction lead to a gradual increase in the amount of complex b2 at the expense of all other bands, which were progressively eliminated from the mobility shift pattern. At very high concentrations of material purified from human serum there was also a gradual decrease in complex b2 (Fig. 3A).

To demonstrate that the changes in the mobility shift patterns were indeed because of proteolytic digestion of the CTCF protein, we proceeded with separation of the treated nuclear extract and analysis of the resulting fractions by mobility shift electrophoresis and Western blotting. Initially, nuclear extract in preparative quantities was treated with heparin-agarose-purified active material. This resulted in a mobility shift pattern where the prominent complexes b, b1, and b2, as well as minor complexes b3 and b10, were represented (Fig. 3B, lane 1). The treated extract was loaded on a cation exchange column, and all proteins generating the mobility shift complexes eluted in fractions 19–24 (Fig. 3B, lanes 3–8). This purification step considerably reduced total protein concentration in the preparation, and the combined fractions 19–23 (Fig. 3B, lanes 3–7) were used as starting material (Fig. 4A, lane 1) for subsequent gel filtration.

Fast protein liquid chromatography gel filtration was performed using a Amersham Pharmacia Biotech Superose 6 column. Mobility shift analysis of the loading material (Fig. 4A, lane 1) revealed the presence of all binding complexes that were observed prior to the cation exchange chromatography (Fig. 3B, lane 1). Meanwhile, Western blot analysis of the cation exchange-purified material with antibodies against either the N- (Fig. 4B) or C-terminal (Fig. 4C) end of CTCF recognized a protein with an apparent molecular mass of 140 kDa (band p) corresponding to native CTCF (Fig. 4, B and C, lanes 1 and 2). Antibodies against the N-terminal part of the protein also reacted with a 120-kDa band [p0], as well as with a much less pronounced band at 130 kDa [p01] (Fig. 4B, lane 2). In contrast, antibodies against the C-terminal end of CTCF recognized a new 70-kDa (p1) immunoreactive band (Fig. 4C, lane 2). The emergence of these lower molecular weight bands that are differentially recognized by antibodies either against the C- or the N-terminal end proves that CTCF indeed undergoes proteolytic cleavage during incubation with the purified fraction of serum.

Gel filtration of the fragments allowed an estimation of the number and relative positions of the cleavage sites. Both antibodies recognized the peak of intact CTCF that eluted in fractions 13 and 14 (Fig. 4, B and C, lanes 4 and 5). This represented an apparent molecular mass of 400 kDa as determined by calibration with globular protein standards. The corresponding binding complex b was observed in the same fractions by mobility shift electrophoresis (Fig. 4A, lanes 2 and 3). Fragment p0 eluted in fractions 15 and 16 (Fig. 4B, lanes 6 and 7), generating the corresponding binding complex b0 (Fig. 4A, lanes 4 and 5). Furthermore, fragment p1 eluted in fractions 17 and 18 (Fig. 4C, lanes 8 and 9) where the matching complex b1 was observed (Fig. 4A, lanes 6 and 7). The CTCF fragments corresponding to the complexes b10 and b2, which eluted in fractions 19–21, could not be recognized by either antibody (Fig. 4A, lanes 8–10; B and C, lanes 9–11). According to both SDS-PAGE and gel filtration, the CTCF fragment producing the faint band p01 migrated to a position between the full-length CTCF and fragment p0 (Fig. 4B, lanes 5 and 6). However, no binding complex has been identified that could be assigned to this fragment, presumably because its low prevalence does not allow detection. Alternatively, the hypothetical binding complex formed by p01 may not be separable from binding complexes b and b0 under the applied mobility shift electrophoresis conditions.

Assuming that CTCF binds to DNA as a monomer (10), the results of the gel filtration suggest that there are three prominent proteolytic cleavage sites on the CTCF molecule. One site
is located between the N terminus and the zinc finger DNA binding domain, and two are located between the zinc finger domain and the C terminus (Fig. 4D, arrows). Incomplete cleavage at these sites would produce numerous protein fragments. Among them, the fragments p0, p1, p10, and p10, which are schematically shown in Fig. 4D, would contain the zinc finger domain and produce the corresponding binding complexes. Fragments p0 and p0 would be recognized by the N-terminal but not the C-terminal antibody. Similarly, protein p1 would be only recognized by the C-terminal antibody. Finally, neither antibody would recognize the proteins p10 and p2 that produce binding complexes b10 and b2.

Depending on the conditions of the cleavage reaction, additional minor binding complexes could be observed on the mobility shift gel between the b1 and b2 bands. An arrowhead in Fig. 4A (fraction 18) indicates an example of such a weak binding complex, designated b2. Incidentally, an exceedingly weak band (p31) that reacted with the C-terminal antibody was detected migrating slightly ahead of fragment p1, in fractions 17 and 18 (Fig. 4C, arrowheads). This suggests the existence of an additional cleavage site on the CTCF molecule in close proximity to the major site that cleaves off the N terminus generating fragment p1 (Fig. 4D, arrowhead). Cleavage at that site would generate three additional hypothetical CTCF protein fragments containing the zinc finger DNA binding domain (Fig. 4D, fragments p31, p32, and p33, indicated by a bracket). Fragment p31 would thus retain an intact C-terminal sequence of CTCF and therefore react with antibodies against the C terminus. The same fragment could conceivably account for the appearance of the minor binding complex b3 observed in fraction 18 on the mobility shift gel. Because of the lower prevalence of CTCF protein cleavage at this site, it is possible for example that it only becomes accessible after cleavage at the N-terminal p1 site. However, we consider it to be a marginal cleavage site for the protease, and we therefore disregard it in the further discussion of the results.

The Proteolytic Activity Is Identified as PHBP—Initial attempts to further purify the protease under non-denaturing conditions were unsuccessful. Employing a variety of separation techniques, we observed the same major proteins were co-purified. Preliminary results indicated that the protease probably exists in serum as part of a high molecular weight multiprotein complex (data not shown). Therefore, we proceeded with the protease identification using preparative SDS-PAGE.

Proteins eluted from the heparin-agarose column were separated by SDS-PAGE under nonreducing conditions (Fig. 5A, lane 1). Four major protein bands (pp1-pp4) were extracted from the gel and further analyzed by SDS-PAGE. Under non-reducing conditions the extracted proteins produced homogenous bands (Fig. 5A, lanes 2–5). Treatment with β-mercaptoethanol differentially affected the electrophoretic behavior of proteins pp1-pp4 (Fig. 5A, lanes 7–10). In particular, protein pp2 migrated as a single 70-kDa band under nonreducing conditions (Fig. 5A, lane 3), whereas in the presence of β-mercaptoethanol it produced two bands with molecular masses of ~44 and 30 kDa (Fig. 5A, lane 8).

The extracted proteins were renatured and incubated with nuclear extract, followed by mobility shift electrophoresis (Fig. 5B, lanes 1–4). Proteolytic activity was detected only in the sample containing the pp2 protein (Fig. 5B, lane 2). As a control, the total heparin-agarose-purified protease fraction was denatured under reducing, as well as nonreducing, conditions and then renatured while omitting electrophoretic separation. Proteolytic activity could only be restored from the nonreduced sample (Fig. 5B, lanes 5 and 6). From these experiments we conclude that the proteolytic activity is associated with the 70-kDa protein pp2. This protein comprises two polypeptide chains with apparent molecular masses of 44 and 30 kDa, which are connected via disulphide bonds. Disruption of the bonds irreversibly abolishes activity.

To further characterize and identify the protease, the heparin-agarose-purified material was subjected to SDS-PAGE in the presence of β-mercaptoethanol. Proteins were subsequently transferred to polyvinylidene difluoride membrane and visualized by Amido Black staining. Both the 44- and 30-kDa proteins were excised, and the N-terminal sequences of the proteins were determined. The 44- and 30-kDa proteins contained the sequences SLLESLODTP and IYGGFKSTAGAKHP, respectively, and they displayed a perfect match with the sequence of

**Fig. 5. Identification of the protein responsible for the proteolytic activity.** Proteins eluted from the heparin-agarose column were separated by SDS-PAGE under nonreducing conditions, extracted from the gel, and renatured. A. SDS-PAGE of the extracted proteins without reducing agent (lanes 1–5) or with 5% β-mercaptoethanol (βME) (lanes 6–10). The starting material contained the four major proteins pp1–pp4 (lanes 1 and 6). These proteins were individually extracted from the nonreducing gel and separately analyzed under reducing and nonreducing conditions: pp1 (lanes 2 and 7), pp2 (lanes 3 and 8), pp3 (lanes 4 and 9), and pp4 (lanes 5 and 10). B. After extraction from the gel, proteins were renatured. The renatured proteins pp1–pp4 (lanes 1–4) were incubated with nuclear extract and analyzed by acrylamide mobility shift electrophoresis with radiolabeled APB oligonucleotide. In addition, the total heparin-agarose-purified material was denatured and renatured, without prior separation by SDS-PAGE, followed by mobility shift electrophoresis. Denaturation was carried out either under nonreducing (lane 5) or reducing (lane 6) conditions. Binding complexes b and b2 are indicated by arrowheads, free oligonucleotides (f) are indicated by a bracket. C, autoradiograph of the [3H]DFP-labeled protease. The heparin-agarose-purified protease was incubated with [3H]DFP and subjected to SDS-PAGE both under nonreducing (lane 1) and reducing (lane 2) conditions.
the human PHBP protein described by Choi-Miura et al. (12).

PHBP cDNA sequence data (12) suggested that the protein contains a putative serine protease domain. In such a case the active center of the protease might form a covalent bond with DFP. To provide additional evidence that the protease is PHBP, we performed [3H]DFP labeling of the protease. We incubated the heparin-agarose-purified material with tritium-labeled DFP and analyzed the reaction products by SDS-PAGE incubated with HeLa cell nuclear extract before assembling the binding reaction with radiolabeled APPβ oligonucleotide. Binding complexes (arrowheads) and the free oligonucleotide (bracelet) were separated by acrylamide mobility shift electrophoresis. B, SDS-PAGE of the elution fractions 14–23 from the cation exchange column described in A under nonreducing conditions (lanes 1–10). The 70-kDa PHBP protein band and a contaminating 140-kDa band in fractions 14–19 (lanes 1–6) are indicated. C, SDS-PAGE of the elution fractions 14–23 in the presence of β-mercaptoethanol (lanes 1–10). 44- and 30-kDa PHBP polypeptide bands are indicated. βME, β-mercaptoethanol.

In contrast, fractions 20 and 21 displayed much higher concentrations of the 70-kDa PHBP protein (Fig. 6B, lanes 7 and 8), whereas no other protein band could be detected in these fractions. Similarly, the corresponding concentration peak of the 44- and 30-kDa polypeptides eluted in the same fractions under reducing conditions (Fig. 6C, lanes 7 and 8). The protease purified to homogeneity is very unstable. Any prolonged handling of the nonfrozen material lead to degradation and reduction in activity (data not shown). The 30-kDa polypeptide chain is especially labile. Thus, two independent experiments, [3H]DFP labeling and chromatographic purification of the protease to homogeneity, confirmed our assignment of the proteolytic activity to the PHBP protein.

The PHBP Protease Is Specifically Inhibited by the KPI Domain of APP—Several common protease inhibitors were examined for their effect on the protease activity. Heparin-agarose-purified PHBP was premixed with commonly used maximal concentrations of either phenylmethylsulfonyl fluoride, aprotinin, pepstatin, or leupeptin and subsequently incubated with HeLa cell nuclear extract followed by mobility shift electrophoresis (Fig. 7, lanes 1–6). The protease was inhibited by high concentrations of the serine protease inhibitors phenylmethylsulfonyl fluoride, aprotinin, and leupeptin, whereas the aspartic protease inhibitor pepstatin did not significantly affect the PHBP protease activity (Fig. 7, lanes 3–6). Furthermore, a peptide containing the KPI domain of APP completely inhibited the protease at a concentration of 12 μg/ml whereas the inhibitory effect was still significant at 1.2 μg/ml concentration (Fig. 7, lanes 7–9). In contrast, a mutated version of the KPI domain peptide did not affect the PHBP protease activity even at a concentration of 800 μg/ml (Fig. 7, lane 10). This mutation in which the arginine at position 301 was substituted with isoleucine, leads to a shift in the inhibition specificity of the mutant peptide from trypsin-like to elastase-like proteases. 2

2 W. Van Nostrand, personal communication.
concentrations of 125
...domain of APP was added to the preincubation mixture in decreasing
...radiolabeled APB
...preincubated with protease inhibitors and subsequently incubated with
...KPI domain (12). In a further development, the same
...introduced an internal cleavage on the N-terminal side of iso-
...putative signal peptide from the N terminus of the protein and
...underwent posttranscriptional processing, which removed the
...(12). The authors concluded that the PHBP protein in serum
...cDNA encoding the PHBP protein and the deduced sequence of
...was designated as PHBP2 (17).

In the process of our studies on the binding of transcription factor CTCF (7) to the APBβ element of the APP promoter, we
...discovery that preincubation with serum resulted in proteolytic digest of CTCF. The proteolytic activity was purified and identified as PHBP. At the final stage of PHBP purification, SDS-PAGE revealed several protein bands in the fractions containing the protease activity. Proteolytic activity was recovered only under nonreducing conditions from the protein band with an apparent molecular mass of 70 kDa. In the presence of β-mercaptoethanol this protein yielded two bands with molecular masses of 44 and 30 kDa. The N-terminal sequence that we determined here for the 44-kDa protein perfectly matched the major N-terminal sequence of the 50-kDa PHBP fragment. There was also a perfect match between the N-terminal sequence that we determined for the 30-kDa protein and the minor N-terminal sequence of the 17-kDa PHBP polypeptide identified by Choi-Miura et al. (12). Moreover, the circumstance that we observed a 30-kDa fragment instead of a 17 kDa fragment as the C-terminal PHBP polypeptide suggests that in our preparation the serine protease domain of the protein remained intact. This conclusion was further confirmed by [3H]DFP labeling of the purified protease, which identified the 30-kDa fragment as the carrier of the active serine protease domain of PHBP. This difference in molecular mass of the C-terminal polypeptide generated by the two independent purification procedures can be explained by either alternative processing of the PHBP molecule or by nonspecific proteolytic degradation. Indeed, in our experience the PHBP protein purified to homogeneity is exceedingly unstable. A possible explanation for this instability is that PHBP is capable of self-inactivation by digesting its own protease domain. This might explain why Choi-Miura et al. (12) observed neither the 30-kDa fragment nor a proteolytic activity in their preparations of the PHBP protein.

Whereas PHBP is prominently present in serum, there is currently no evidence that it functions in the processing of nuclear transcription factor CTCF. However, PHBP can be useful in investigating some functional properties of CTCF. For example, It was established from the cDNA sequence that the actual molecular mass of CTCF is only 82 kDa. However, during SDS-PAGE, CTCF migrates slower than expected, resulting in an apparent molecular mass of ~140 kDa. The source of this abnormal rate of migration was traced to the N-terminal end of the protein, presumably resulting from the unusual shape of the molecule (18). Furthermore, sedimentation velocity experiments suggested that unbound CTCF exists in a monomeric form (10). However, based on calibration with established molecular weight standards, whole CTCF eluted from the gel filtration column as a protein with the surprisingly high apparent molecular mass of 400 kDa (Fig. 4). This value was also confirmed in independent experiments where CTCF was not treated with protease prior to separation by gel filtration (data not shown). Such an apparent deviation from the actual 82-kDa molecular mass suggested that additional evaluation of the monomeric state of CTCF was desirable.

Our assignment of the number and relative position of the proteolytic cleavage sites on the CTCF molecule was based on the assumption that CTCF binds to the APBβ element in the form of a monomeric protein (10). The gel filtration results could thus be explained by the presence of three major proteolytic cleavage sites on the CTCF molecule (Fig. 4D). According to this interpretation one of the sites would be located between the N terminus and the zinc finger DNA binding domain and the other two between the zinc finger domain and the C terminus. However, if the hypothetical possibility that CTCF binds to the APBβ element as a dimer is considered, a similar mobil-
ity shift pattern could be generated by an incomplete digestion of the dimer assuming that each subunit contains a single cleavage site. In addition, because complexes b0 and b01 are much less pronounced than complexes b, b1, and b2, they would have to be disregarded under this model as resulting from nonspecific cleavage. Complex b would then be attributed to the intact homodimer, complex b1 to the heterodimer with one subunit cleaved and another intact, and complex b2 to the homodimer comprising two cleaved subunits. The heterodimer forming complex b1 would then be found in the gel filtration matrix. They specifically proposed inter-

PHBP could participate in the proteolysis of the extracellular

In the original PHBP study (12) the authors hypothesized that

including the determination of the proteolytic target sequence.

be used as a substrate for some aspects of this investigation, though it has not been established whether CTCF is a natural

target for processing by PHBP within the cell, it could indeed

suggests that in some cases PHBP proteolysis might have a transient character in a specific location. For example, high levels of hyaluronic acid are accumulated locally during wound healing (19), which could attract PHBP to the affected area.

This localization, as well as the potentially transient character of the proteolysis, suggests a possible function for PHBP in the wound healing process.

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