# Plasma Carboxypeptidases as Regulators of the Plasminogen System

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## Abstract

**Carboxy-terminal lysine residues on the surface of cells and fibrin bind plasminogen and control its activation. Since plasma contains basic carboxypeptidases, which remove carboxy-terminal lysines from protein substrates, we investigated if these enzymes are involved in the regulation of plasminogen binding sites. Plasma reduced plasminogen binding to cells, and this effect could be ascribed to the activity of the plasma carboxypeptidases. Purified carboxypeptidase N, which is constitutively active, and plasma carboxypeptidase B, which circulates as a zymogen, were both capable of significantly reducing plasminogen binding to cells. Dose titration experiments verified that plasma concentrations of either carboxypeptidase were sufficient to maximally affect plasminogen binding to cells. Furthermore, plasma carboxypeptidase B, but not carboxypeptidase N, reduced the rate of whole blood clot lysis induced by tissue-type plasminogen activator. These findings establish that plasma carboxypeptidases can modulate plasminogen binding to cells and control the rate of fibrinolysis. These functions delineate a novel role for the plasma carboxypeptidases in the regulation of the plasminogen system. (*J. Clin. Invest.* 1995. 96:2534–2538.) Key words: fibrinolysis • plasminogen activation • thrombolysis • plasminogen receptors**

## Introduction

Surfaces, either fibrin or cell surfaces, play a pivotal role in the regulation of the plasminogen system (for reviews see references 1 and 2). Surface-bound plasminogen is a better substrate for plasminogen activators, and bound plasmin is protected from rapid inactivation by $\alpha_2$-antiplasmin (3, 4). Interaction of plasmin(ogen) with surfaces is largely dependent upon the recognition of carboxy-terminal lysyl residues by the lysine binding sites associated with the kringle structures of plasminogen (5). Such lysines may exist as the naturally occurring carboxy termini of proteins or may arise as a result of limited proteolysis by enzymes including plasmin (6–11). The latter mechanism provides a positive feedback loop in which plasmin cleavage of cell surface proteins or fibrin up-regulates binding sites for plasminogen which favor further plasmin formation. Carboxy-terminal lysines are sensitive to exo-proteinases, such as pancreatic carboxypeptidase (Cp)[1] B, an enzyme which removes basic carboxy-terminal amino acids (lysines and arginines) from proteins. Indeed, Cp B has been used to implicate carboxy-terminal lysines in plasminogen binding to cells (5) and to partially plasmin-degraded fibrin (9, 11, 12).

Enzymes with a cleavage specificity similar to Cp B are present in blood. Cp N accounts for the constitutive Cp activity of plasma and is involved in the metabolism of numerous circulating peptides (for review see reference 13). Plasma Cp B (pCPB), which is structurally very similar to tissue Cp B, circulates as a proenzyme and is activated by plasmin and thrombin (14). Cp U is a basic Cp-activity that is detected in serum following coagulation (15). It has been suggested that pCPB contributes to Cp U because of functional similarities (16).

The existence of Cp activity in blood raises the possibility that these enzymes may influence plasminogen binding to surfaces. Using plasminogen binding to cell lines of leukocyte origin as a model system, we show that both Cp N and pCPB are potent modulators of plasminogen binding to these cells. Additionally, lysis of whole blood clots is significantly inhibited by pCPB. Taken together, these observations provide the basis to suggest a novel role for the basic Cp of plasma in the regulation of the plasminogen system.

## Methods

*Enzyme assays.* Basic Cp-activity was measured with furoylacroleyl-alanyl-lysine (lysine substrate) or furoylacroleyl-alanyl-arginine (arginine substrate) (both from Bachem, Philadelphia, PA) as substrates (17, 18). Test samples, diluted to 200 $\mu$l with 0.9% NaCl, were added to 300 $\mu$l of 0.5 mM substrate in 100 mM Hepes, 150 mM NaCl, pH 7.2, and the decrease in absorbance at 336 nm was measured. Plasmin activity was determined with the chromogenic substrate S2251 (Chromogenix, Molndal, Sweden) in 100 mM Hepes, 150 mM NaCl, pH 7.2. Both assays were performed at 22°C. Enzyme activity was expressed as substrate conversion: 1 U = 1 $\mu$mol/min under the conditions of the assays.

*Carboxypeptidases.* Cp N was purified from fresh citrated human plasma (19, 20). Isolated Cp N displayed appropriate properties compared to described preparations (21), including a similar purification factor (5780) and specific activity (27 U/mg, lysine substrate), a four-

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*Received for publication 14 May 1995 and accepted in revised form 26 July 1995.*

J. Clin. Invest.

0021-9738/95/11/2534/05 $2.00
Volume 96, November 1995, 2534–2538

1. *Abbreviations used in this paper:* 6-AHA, 6-aminohexanoic acid; Cp, carboxypeptidase; GEMSA, guanidinoethylmercaptosuccinic acid; PCI, potato-carboxypeptidase inhibitor; pCPB, plasma carboxypeptidase B; t-PA, tissue-type plasminogen activator.

fold stimulation of peptidase activity by cobalt, and a threefold preference for lysine over arginine substrates. SDS-PAGE verified the expected composition of a 50- and a 90-kD subunit. "Activated" Cp N, obtained by purifying Cp N in the absence of protease inhibitors, showed partial degradation of the noncatalytic 90-kD subunit and increased specific activity (+50%), consistent with published data (19). The activity (lysine substrate) of both Cp N preparations on lysine substrate was > 95% inhibited by 0.1 mM GEMSA, an inhibitor of basic Cp (22). This concentration of GEMSA had only minimal inhibitory effect on the cleavage of the lysine substrate by pCPB, indicating that the contamination with pCPB-activity was < 5%.

An enriched precursor pCPB (pro-pCPB) fraction was isolated by affinity chromatography of citrated plasma, containing 1 mM PMSF, 5 mM benzamidine and 5 mM 6-AHA, on plasminogen coupled to CNBr-activated Sepharose (Pharmacia LKB, Piscataway, NJ) at 3 mg/ml. Bound pCPB was eluted with 50 mM 6-AHA. Absence of $\alpha_2$-antiplasmin was shown by the inability of such preparations to inhibit plasmin activity (t-PA [1 $\mu$g/ml] was used to activate plasminogen [1 $\mu$M], measured with S-2251). To generate the active pCPB, 200 $\mu$g/ml of the preparations were incubated with 10 $\mu$g/ml of plasmin for 1 h at room temperature immediately prior to use in experiments (14), followed by plasmin inhibition with 100 kallikrein inhibiting U/ml aprotinin. The activity of pCPB toward the arginine-substrate was inhibited by 1 mM GEMSA (85%) and 0.05 mg/ml potato carboxypeptidase inhibitor (100%). As the latter inhibitor did not affect Cp N, Cp N was absent in these preparations. For selected experiments, a more highly purified pCPB preparation, isolated from human plasma with a monoclonal antibody column followed by ion exchange chromatography (Mono Q HR 5/5, Pharmacia), was used. The material was homogenous as judged by SDS-PAGE (23).

*Cp-treatment of cells and plasminogen binding analyses.* Monocytoid U937 and lymphoid Molt-4 cells were cultured in RPMI, supplemented with 2 mM glutamine and 5% heat inactivated fetal calf serum. Cell viability was > 95% as assessed by trypan blue exclusion. Cp N, pCPB or Cp B was incubated in HBSS containing 0.1% BSA in the presence or absence of Cp-inhibitors for 30 min at 37°C and added to $2 \times 10^6$ cells in 100 $\mu$l. After 1 h at 37°C, the cells were washed by centrifugation, and $^{125}$I-plasminogen binding to the cells was assessed (3). When unfractioned plasma was used as the source of Cp, the procedure was slightly modified. Briefly, cells were treated with 5 mM diisopropylflourophosphate and 1 mM PMSF for 30 min to prevent activation of plasma plasminogen by cell-associated activators. This treatment was effective in neutralizing cell-surface associated plasminogen activating activity (data not shown). Citrated plasma was buffered with 50 mM Hepes, pH 7.2, preincubated with or without Cp-inhibitors for 30 min at 37°C, and 100 $\mu$l were added to $2 \times 10^6$ cells for 1 h at 37°C. Then, plasma plasminogen bound to the cells was eluted by addition of an equal volume of 200 mM 6-AHA in HBSS for 10 min at room temperature. In experiments in which $^{125}$I-plasminogen was added to plasma, this procedure was shown to elute > 99% of the radioactivity specifically bound to the cells. The cells were washed three times with HBSS before plasminogen binding was assessed (3). Neither of the above treatments caused a loss in cell recovery or viability.

*Lysis of whole blood clots.* For clot lysis studies, the procedure of Sabovic et al. (24) was adapted. Briefly, blood drawn from healthy volunteers was mixed quickly with tracer amounts, $2 \times 10^6$ cpm/ml, of $^{125}$I-fibrinogen, isolated and radiolabeled as previously described (25). Aliquots (500 $\mu$l) were clotted for 3 h at 37°C in glass tubes, and the resulting clots were washed three times for 1 min with HBSS. Clot associated radioactivity (80–90% of input) was determined in a $\gamma$-counter and assigned a value of 100%. The clots were then placed in HBSS containing 0.1% BSA and 1 $\mu$M plasminogen or buffered citrated plasma. Lysis was initiated by addition of t-PA. Lysis was allowed to proceed at 37°C with gentle agitation. Duplicate samples were removed at various time points and counted for released radioactivity to determine the extent of lysis. For selected experiments, plasma was depleted of pro-pCPB by passage over a monoclonal antibody column as described (23). As a control, plasma also was passed over an isotype-matched antibody column. Cp N activity in these plasma samples was unaffected.

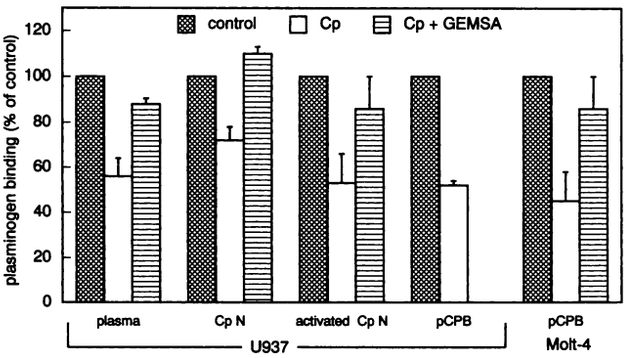


*Figure 1.* Effect of plasma and isolated plasma Cp on plasminogen binding to cells. U937 or Molt-4 cells were incubated in buffered citrated plasma or with the isolated plasma Cp in the presence or absence of 0.1 mM GEMSA (Cp N) or 1 mM GEMSA (pCPB) for 1 h at 37°C. After washing, plasminogen binding was assessed using 100 nM $^{125}$I-plasminogen. A buffer control was performed in each experiment and was assigned a value of 100%, and plasminogen binding to Cp-treated cells was expressed as percent of this value. The results are the means±SD of 3–6 experiments. Effect of plasma ($n$ = 3, $P$ = 0.01), Cp N ($n$ = 3, $P$ = 0.015), "activated" Cp N ($n$ = 6, $P$ = 0.0003), partially purified pCPB ($n$ = 3, $P$ = 0.0004), all on U937 cells; and effect of pCPB on Molt-4 cells ($n$ = 3, $P$ = 0.036) are shown.

*Reagents.* Diisopropylflourophosphate-treated carboxypeptidase B (Cp B) was purchased from Sigma Chemical Co., St. Louis, MO; t-PA was from Genentech Inc.; aprotinin was from Miles Inc., Kankakee, IL; plasmin (lysine and 6-AHA-free), GEMSA and potato Cp-inhibitor (PCI) were from Calbiochem, La Jolla, CA.

## Results

*The effect of Cp on plasminogen binding to cells.* Cells were incubated in plasma or buffer for 1 h at 37°C. As shown in Fig. 1, the cells incubated in plasma consistently exhibited reduced plasminogen binding. In three experiments, the average reduction in plasma was 56% compared with the buffer control. This effect was not due to occupancy of cellular binding sites by plasma plasminogen as bound plasminogen was eluted with 100 mM 6-AHA before assessing $^{125}$I-plasminogen binding. More importantly, preincubation of plasma with 1 mM GEMSA, a Cp inhibitor, blocked the inhibitory effect of plasma (Fig. 1). This concentration of GEMSA had no direct effect on plasminogen binding to cells. Likewise, Cp N, at its plasma concentration (400 U/l, lysine substrate), decreased plasminogen binding to the U937 cells (Fig. 1). The extent of the decrease was ~ 30%. "Activated" Cp N, a proteolytic derivative of Cp N with enhanced peptidase activity, was somewhat more effective (47% inhibition, Fig. 1). The reduction in plasminogen binding induced by either Cp N preparation was fully blocked by 0.1 mM GEMSA. This effect of GEMSA not only implicates Cp activity in the inhibition of plasminogen binding but also indicates that contaminating pCPB, which is not efficiently inhibited by 0.1 mM GEMSA, did not contribute to the activity of the Cp N preparations. To verify that "activated" Cp N did not contain additional proteases that could degrade cell surface proteins, thereby reducing plasminogen binding, surface-labeled U937 cells (5) were treated with "activated" Cp N at its plasma

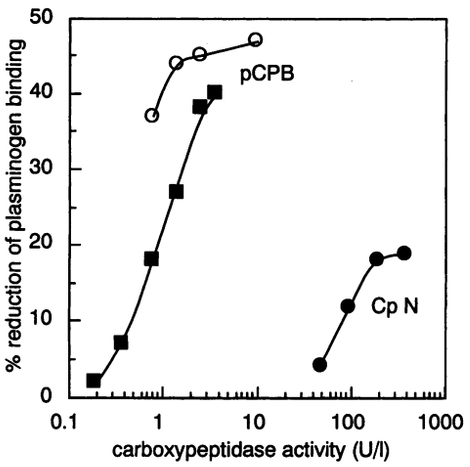


*Figure 2.* Dose-dependence of the Cp-effect on plasminogen binding to cells. U937 (*closed symbols*) or Molt-4 (*open symbols*) cells were incubated with increasing amounts of Cp as indicated for 1 h. After washing, plasminogen binding was assessed using 100 nM $^{125}$I-plasminogen. A buffer control was performed in each experiment and was assigned a value of 100%, and percent inhibition of plasminogen binding was plotted against the activity of the enzyme (arginine substrate for pCPB, lysine substrate for Cp N).

concentration (400 U/l, lysine substrate) for 1 h at 37°C. "Activated" Cp N did not cause release of cell-surface radioactivity, whereas a similar concentration of trypsin, 25 $\mu$g/ml, caused a 35% reduction of cell-associated radioactivity.

pCPB also caused a reduction in plasminogen binding to cells, 48% (Fig. 1). Additionally, the effect of pCPB on plasminogen binding to a second cell type, Molt-4 lymphoid cells, was assessed. A similar reduction in plasminogen binding, 56%, was observed. If pro-pCPB, without measurable Cp-activity, was added without prior plasmin activation, plasminogen binding was not significantly affected: $5.8\pm0.1 \times 10^5$ molecules plasminogen bound/cell, compared to $5.3\pm0.5 \times 10^5$ molecules/cell for cells incubated in buffer. Furthermore, the reduction of plasminogen binding was not observed in the presence of 1 mM GEMSA, consistent with an effect mediated by Cp activity. As a point of comparison, pancreatic Cp B (0.5 mg/ml) was used to treat U937 cells, resulting in similar reduction [48% ($n = 3$)] in specific plasminogen binding. Thus, plasminogen binding sites were sensitive to inactivation by each Cp. Moreover, the combination of "activated" Cp N (400 U/liter, lysine substrate) and Cp B (0.5 mg/ml) did not result in a further reduction of plasminogen binding (46%) compared with to "activated" Cp N alone (46%), suggesting that these Cp ultimately cleave the same carboxy-terminal lysine residues.

*Relative potency of the Cp in reducing plasminogen binding to cells.* Dose titration experiments were performed to determine the minimal concentration of isolated Cp required for a maximal reduction in plasminogen binding to U937 cells in 1 h (Fig. 2). With pancreatic Cp B, the saturating concentration was 500 mg/liter, corresponding to 100,000 U/l (arginine substrate) (not shown). Approximately 40 mg/liter or 400 U/liter (lysine substrate) of Cp N were required to give maximal effect. Based on activity, 3 U/liter (arginine substrate) were sufficient with pCPB, corresponding to less than 4 mg/liter. A similar saturation curve was obtained for pCPB treatment of Molt-4 cells (see Fig. 2). Since plasmin can both activate and inactivate pCPB (14), the pCPB preparation may be a mixture of latent, active and inactive pCPB. Thus, this value for pCPB could be considerably lower. To compare these concentrations with the concentrations of Cp-activities in plasma, constitutive and latent Cp was determined in blood samples from seven healthy donors (15). The activity determined in citrated plasma was attributed to Cp N. The Cp-activity in serum after 1 h of coagulation at 37°C was defined as the sum of Cp N and latent (Cp U) activity, to which pCPB is likely to be a major contributor (16), and we defined latent Cp-activity in blood samples as pCPB. pCPB activity was then calculated by subtracting plasma Cp-activity from serum activity for each blood donor tested. Based on these data, plasma concentrations (average 400 U/liter, lysine substrate) of Cp N resulted in maximal reduction of plasminogen binding to cells in our assay system. Much lower concentrations of pCPB (3 U/liter) were required for maximal effect on cellular plasminogen binding. Plasma concentrations of inducible Cp ranged from 15–50 U/liter. Thus, at < 20% of plasma concentration, an effect on plasminogen binding is still predicted. In summary, pCPB is the more potent Cp in reducing plasminogen binding to cells, with pCPB > Cp N ≫ Cp B. Cp B is unlikely to contribute to the plasma Cp-activity because of the high concentrations required and its absence from normal plasma (26).

*Effect of carboxypeptidases on whole blood clot lysis.* The carboxy-terminal lysines in partially degraded fibrin enhance plasminogen activation and accelerate fibrinolysis (12). Plasma Cp could remove these carboxy-terminal lysines and, thereby, blunt fibrin degradation (11, 12). To test this possibility, the lysis of whole blood clots was followed in the absence and presence of highly purified, pro-pCPB or Cp N. Blood clots, formed with tracer $^{125}$I-fibrinogen, were placed in buffer with t-PA and plasminogen. In this experimental setting, pro-pCPB is activated by the generated plasmin. These clots are stable, both in plasma or buffer, with < 5% lysis in 30 h. Over an observation period of 3 h, lysis was significantly inhibited by the addition of pro-pCPB (Fig. 3 *A*). Consistent with an indirect inhibitory effect via the clot, pCPB did not inhibit plasmin activity, measured with S2251 using the same concentrations of plasminogen and t-PA in the absence of a clot. The inhibition of clot lysis was overcome by addition of PCI, a specific inhibitor of pCPB, indicating that the inhibition of clot lysis was dependent on the catalytic activity of pCPB. Similar results were obtained in three separate experiments using blood from two different donors. The delay in the inhibitory effect of pro-pCPB may reflect the time required for its activation to pCPB. Plasma concentrations of Cp N, on the other hand, inhibited clot lysis only marginally under these conditions: after 1.5 h, the extent of lysis was 75%, both in the absence and presence of Cp N. We next explored if inhibition of pCPB by PCI influenced fibrinolysis in plasma. As shown in Fig. 3 *B*, addition of PCI to plasma resulted in a significant acceleration of clot lysis, consistent with Fig. 3 *A*. Spontaneous lysis (< 5%/30 h) was not affected by PCI, indicating that the inhibitor preparation does not contain fibrinolytic activity. Cp N inhibition (0.1 mM GEMSA), on the other hand, had no effect on clot lysis in plasma by t-PA (not shown). To more directly show that this stimulatory effect was, indeed, due to inhibition of pCPB, we depleted plasma of pCPB by passage over a column coupled with a monoclonal antibody against pCPB (23). As shown in Fig. 3 *C*, pCPB-depletion resulted in a accelerated clot lysis compared to plasma passed over a column coupled with an

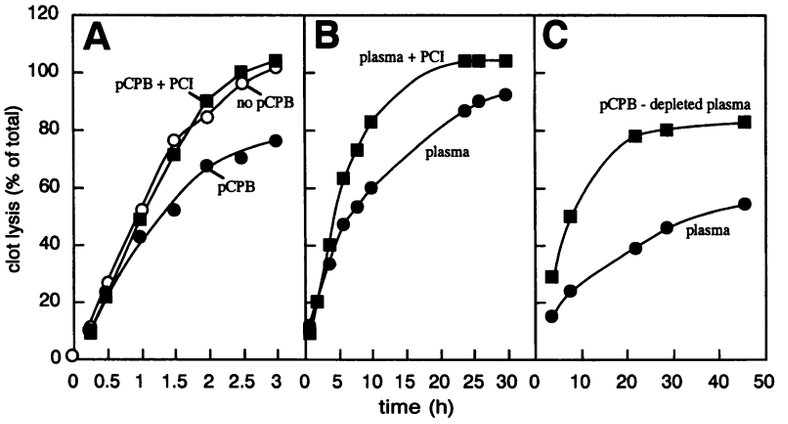


*Figure 3.* Effect of plasma Cp on clot lysis. Whole blood clots, labeled with $^{125}$I-fibrinogen, were incubated with 1 $\mu$M plasminogen in HBSS (*A*) or buffered (50 mM, Hepes pH 7.2) citrated plasma (*B* and *C*) and t-PA. Clot lysis was followed by measuring the release of radioactivity into the supernatant fraction at the indicated time points. (*A*) (t-PA at 1 $\mu$g/ml); (●) pro-pCPB highly purified (20 $\mu$g/ml, equivalent of 15 U/l); (■) pro-pCPB and 100 $\mu$g/ml PCI; and (○) control (t-PA and plasminogen alone). (*B*) (t-PA at 0.1 $\mu$g/ml); plasma without (●) or with (■) 20 $\mu$g/ml PCI; (*C*) (t-PA at 50 $\mu$g/ml); (■) pCPB antibody-depleted plasma; or (●) control antibody-treated plasma.

isotype-matched control antibody. To address the basis for the differential effect of pCPB and of Cp N on clot lysis, we used partially plasmin digested whole blood clots (11), treated them with plasma concentrations of either Cp N or pCPB, and then assessed binding of $^{125}$I-plasminogen to these clots. Nonspecific binding was determined in the presence of 200 mM 6-AHA. While treatment with pCPB resulted in extensive removal (> 90%) of specific binding sites, Cp N removed < 50% of the specific binding. Thus, as with plasminogen binding to cells, Cp N is less effective in removing carboxyterminal lysyl residues from fibrin surfaces.

## Discussion

In this study, we show that plasma Cp function as previously unrecognized regulators of the plasminogen system. Plasma Cp are capable of reducing plasminogen binding to cells and counteracting the plasmin-induced up-regulation of cellular plasminogen binding sites. Furthermore, the plasminogen binding Cp, pCPB, inhibits whole blood clot lysis, implicating this enzyme in the regulation of fibrinolysis. These data support the model depicted in Fig. 4. Cells and fibrin express a constitutive plasminogen binding capacity. Proteolysis of cell surfaces and fibrin by plasmin and other proteinases can generate new carboxy-terminal lysines (6–11). These residues support additional plasminogen binding, which, in turn, facilitates further plasminogen activation. Controlling this up-regulation of plasminogen binding are the plasma Cp. By removing the carboxy-terminal lysines, the Cp down-regulate plasminogen binding and activation on the surface of cells and fibrin, thereby dampening proteolysis. Thus, the Cp complete a proteolytic amplification loop, returning plasminogen binding to a basal state.

Both Cp N and pCPB suppressed plasminogen binding to cells. Compared on the basis of activity towards small peptide substrates, pCPB was more potent in reducing plasminogen binding to cells than Cp N and Cp B. pCPB also was effective in reducing the rate of fibrinolysis, whereas Cp N was not. pCPB is activated by both plasmin and thrombin and interacts directly with plasminogen (14). Accordingly, it is tempting to speculate that pCPB functions as a thrombin-activated stabilizer of blood clots by dampening fibrinolysis. In addition, during ongoing fibrinolysis, more pCPB would be activated by plasmin and serves as a "brake" on the amplification of proteolysis induced by plasmin-mediated generation of carboxy-terminal

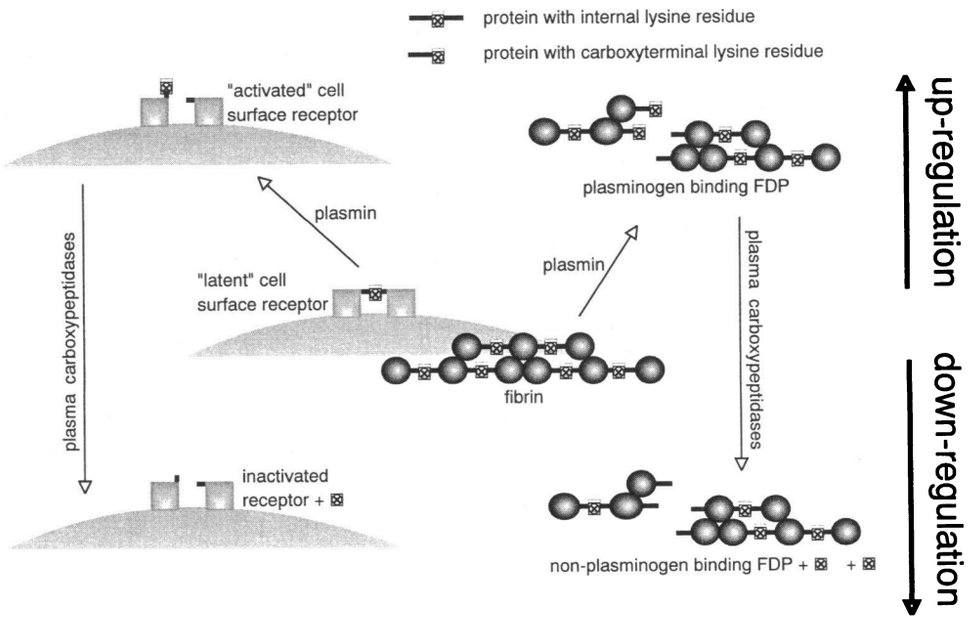


*Figure 4.* Model for the role of plasma proteinases in the modulation of plasminogen binding to cell and fibrin surfaces. Carboxy-terminal lysines on cells and fibrin, particularly partially degraded fibrin, can serve as plasminogen binding sites by interacting with the lysine binding sites of plasminogen. Proteinases, such as plasmin, can generate such carboxy-terminal lysines, thereby up-regulating plasminogen binding sites. Plasma carboxypeptidases, such as Cp N and pCPB, cleave carboxy-terminal lysines. Thus, the carboxypeptidases down-regulate plasminogen binding sites and dampen fibrinolysis and cell-associated proteolysis.

lysine residues on cell surfaces (6–8) and fibrin (10, 11). Although Cp N appears to be considerably less potent than pCPB, particularly in affecting fibrinolysis, it may still regulate the availability of carboxy-terminal lysine residues in the absence of coagulation and plasminogen activation. This constitutive removal of plasminogen binding sites by Cp N may explain why freshly isolated leukocytes express a two orders of magnitude lower number of plasminogen binding sites compared to cultured cells (27, 28). In addition, limited proteolysis of Cp N increases its activity toward peptide substrates and plasminogen binding sites, suggesting that plasmin or other plasma proteases involved in hemostasis may "activate" Cp N. A question yet to be resolved is whether all inducible Cp-activity (Cp U) is attributable to pCPB, as proposed in ref. (16). Alternatively, other inducible Cp may be present in plasma that, in concert with pCPB and Cp N, regulate the availability of plasminogen binding sites in the vasculature.

In clot lysis experiments, pCPB directly affected the kinetics of fibrinolysis. The concentration of pCPB used in the clot lysis experiments would maximally generate 15 U/l Cp-activity (based upon the arginine substrate with plasmin as the pro-pCPB activator), which falls within the range of inducible Cp-activity in blood. Considerable variation of latent Cp-activity, both in healthy individuals and in inflammatory disease (29) has been observed. Thus, individual differences in clot lysis kinetics may be linked to the potency of the Cp-system. This possibility further suggests that selective modulation of pCPB activation and activity might be an approach to enhance the efficacy of thrombolytic therapy. Cp N was ineffective in clot lysis experiments, which may be a result of its reduced ability to remove carboxy-terminal lysyl residues from the clot-surface and/or its inactivation by plasmin (20).

## Acknowledgments

This work was supported by National Institutes of Health grant HL-17964. A. Redlitz was the recipient of a Postdoctoral Fellowship of the American Heart Association, Northeast Ohio Affiliate.

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