Modulation of Plasminogen Activation and Type IV Collagenase Activity by a Synthetic Peptide Derived from the Laminin A Chain†

Sharon Stack,‡ Robert D. Gray,§ and Salvatore V. Pizzo*,‡

Department of Pathology, Duke University Medical Center, Durham, North Carolina 27710, and Department of Biochemistry, University of Louisville Health Science Center, Louisville, Kentucky 40292

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ABSTRACT: Laminin is a large multidomain glycoprotein with diverse biological activities which include stimulation of neurite outgrowth, enhancement of tumor metastasis, and promotion of cell growth, adhesion, and differentiation. A 19 amino acid synthetic peptide derived from the E8 fragment of the laminin A chain (Cys-Ser-Arg-Ala-Arg-Lys-Gln-Ala-Ala-Ser-Ile-Lys-Val-Ala-Val-Ser-Ala-Asp-Arg-NH_2) was identified which promotes metastasis and stimulates collagenase IV activity in the culture medium of B16 melanoma cells (Kanemoto et al., 1990). We report that this peptide, here designated $LamA^{2091-2108}$, is also a potent stimulator of tissue plasminogen activator (t-PA)-catalyzed plasminogen activation, resulting in a 22-fold increase in the k_{cat}/K_m of the activation reaction. The activity of purified type I and type IV collagenase was inhibited by $LamA^{2091-2108}$ with IC_{50} values of 3 and 43 μM, respectively. These data support an alternative mechanism for the appearance of collagenase activity in the culture media of melanoma cells, namely, that the peptide stimulates plasminogen activation, subsequently generating collagenase activity.

Laminin, the major noncollagenous component of the basement membrane, is a large multidomain glycoprotein composed of three disulfide-linked polypeptide chains designated A, B1, and B2. Laminin contains binding sites for a variety of extracellular matrix (ECM) components including heparan sulfate proteoglycan (Woodley et al., 1983), type IV collagen (Terranova et al., 1980), and nidogen (Dziadek et al., 1985) and can self-assemble into polymers (Yurchenco et al., 1985). Various biological activities have been attributed to laminin including promotion of cell growth, adhesion, and differentiation (Charonis et al., 1988; Sephel et al., 1989), stimulation of neurite outgrowth (Liesi et al., 1989), and enhancement of tumor metastasis (Kleinman et al., 1989; Iwamoto et al., 1987). These activities have been localized to specific regions of the laminin molecule based largely on experiments involving proteolytic fragmentation and domain-specific antibodies. By use of this approach, the site responsible for heparin binding and promotion of cell adhesion has been localized to a 20 amino acid sequence of the B1 chain designated F9 (Charonis et al., 1988). The pentapeptide Tyr-Ile-Gly-Ser-Arg (YIGSR), also located on the B1 chain, has been shown to promote cell adhesion and migration (Kleinman et al., 1989; Graf et al., 1987) and block colonization of lungs by B16-F10 melanoma cells (Kleinman et al., 1989; Iwomoto et al., 1987). Another pentapeptide sequence, Ile-Lys-Val-Ala-Val (IKVAV), from the A chain of laminin has been identified which stimulates neurite outgrowth, cell adhesion, and cell migration (Tashiro et al., 1989). This pentapeptide is contained within an 18 amino acid fragment comprising residues 2091–2108 of the laminin A chain (Sasaki et al., 1988) with an additional amino-terminal Cys residue (designated PA22-2) which has been demonstrated to stimulate collagenase IV activity in the culture medium of B16-F10 melanoma cells and increase the incidence of lung colonization by B16-F10 cells in mice (Kanemoto et al., 1990).

We previously demonstrated that intact laminin stimulates the tissue plasminogen activator (t-PA)-catalyzed activation of plasminogen (Pg) to plasmin (Pm) (Stack et al., 1990). Plasmin has been implicated in a variety of physiological and pathological processes unrelated to fibrinolysis including extracellular matrix remodeling (Stickland et al., 1976; Highsmith 1981; Ossowski & Vassali, 1978) and procollagenase activation (Reich et al., 1988; He et al., 1989). Factors which modulate Pg conversion to Pm can indirectly influence procollagenase activation, resulting in apparent changes in collagenase activity and providing an alternative explanation for the increased collagenase activity previously observed in the culture medium of melanoma cells. In the present study, we examined the effect of the laminin A chain synthetic peptide PA22-2 (which we designate $LamA^{2091-2108}$) on t-PA-catalyzed Pg activation. To ascertain whether $LamA^{2091-2108}$ directly stimulates collagenase activity rather than procollagenase activation, we determined the effect of the peptide on the activity of purified interstitial (type I) and basement membrane (type IV) collagenase. We report that the peptide $LamA^{2091-2108}$ is a potent, specific stimulator of t-PA-catalyzed Pg activation whereas the activity of purified collagenases was inhibited by the peptide.

Materials and Methods

Materials. Synthetic peptides IKVAV-NH_2 and SRARK-NH_2 were purchased from the University of Louisville Peptide Synthesis Facility. All other peptides were from Multiple Peptide Systems, San Diego, CA. The composition and sequence of all peptides were verified by using an Applied Biosystems 420A derivatizer/analyzer amino acid analysis system and an Applied Biosystems 477A pulse liquid phase sequencer with "on-line" 120A PTH-amino acid analysis. Peptides with biological activity were further purified by reverse-phase HPLC. The synthetic substrates D-Val-Leu-

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* To whom correspondence should be addressed at the Department of Pathology, Box 3712, Duke University Medical Center, Durham, NC 27710.

‡ Duke University Medical Center.

§ University of Louisville Health Science Center.

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Lys-*p*-nitroanilide (VLK-pNA) and D-Ile-Pro-Arg-*p*-nitroanilide (IPR-pNA) and human fibrinogen were purchased from Helena Laboratories, Beaumont, TX. Fibrinogen was rendered plasminogen-free by chromatography on L-lysine–Sepharose (Deutsch & Mertz, 1970), and CNBr fragments of fibrinogen were generated according to the method of Blomback et al. (1968). Urokinase (u-PA) was purchased from Calbiochem, San Diego, CA, and coupled to CNBr-activated Sepharose according to the method of Cuatrecasas et al. (1968). Two-chain recombinant tissue plasminogen activator (t-PA) was supplied by Dr. Henry Berger at Wellcome Research Laboratories, Research Triangle Park, NC. Recombinant human fibroblast collagenase (type I) was kindly supplied by Dr. Jerry McGeehan at Glaxo Research Laboratories, Research Triangle Park, NC.

Proteins. Plasminogen (Pg) was purified from human plasma by affinity chromatography on L-Lys-Sepharose (Deutsch & Mertz, 1970) and separated into isoforms 1 and 2 by affinity chromatography on concanavalin A–Sepharose (Gonzalez-Gronow & Robbins, 1984). Affinity chromatography purified form 2 was utilized for all experiments. Plasmin (Pm) was generated by incubating 100 μg of Pg with 100 μL of urokinase-Sepharose in 20 mM Hepes, pH 7.4, for 1 h at 25 °C followed by centrifugation to remove the resin. Lys_{77}-Pg was prepared by limited proteolysis of Glu-Pg with Pm in a molar ratio of 10:1 for 30 min at 25 °C followed by chromatography on pancreatic trypsin inhibitor–Sepharose to remove Pm (Castellino & Powel, 1981). Protein concentrations were determined spectrophotometrically at 280 nm using an $A_{1\%/1cm}$ value of 16.8 and molecular weights of 92000, 83000, and 81000 for Glu-Pg, Lys_{77}-Pg, and Pm, respectively (Castellino, 1981).

Type IV collagenase/gelatinase was purified from the serum-free conditioned medium of porcine synovial membranes stimulated with phorbol 12-myristate 13-acetate by inhibitor-affinity chromatography (Stack & Gray, 1988). Briefly, pooled conditioned medium was concentrated by ultrafiltration, and the metalloproteinase zymogens were activated by incubation with 0.7 mM *p*-aminophenylmercuric acetate for 4 h at 35 °C. Type I collagenase and type IV collagenase were separated from other proteins by using an affinity matrix consisting of *N*-[1(*R,S*)-carboxy-*n*-butyl]-Leu-Phe-Ala coupled through the C-terminus to EAH-Sepharose 4B (Pharmacia). Chromatography on DEAE-Sephacel was used to separate type I collagenase from type IV collagenase.

Effect of Synthetic Peptides on Pg Activation. Coupled assays were used to evaluate the initial rate of Pg activation by t-PA or u-PA by monitoring the amidolytic activity of generated Pm (Wohl et al., 1980). Glu-Pg or Lys_{77}-Pg was incubated in 96-well microtiter plates at 37 °C in 20 mM Hepes, pH 7.4, in a total volume of 175 μL with the Pm substrate VLK-pNA (0.3 mM final concentration). Pg activation was initiated by the addition of 0.11 nM (4 IU/mL) t-PA or 0.8 nM (2 IU/mL) u-PA, and the Pm hydrolysis of VLK-pNA was monitored by measuring the change in absorbance at a wavelength of 405 nm at timed intervals using an Anthos Labtech Instruments Model 2001 plate reader. Initial velocities (v_i) were calculated from the slope (b) of plots of A_{405} vs t^2 by using the equation $v_i = b(1 + K_m/S_0)/Ek_e$ (Wohl et al., 1980) where K_m is the apparent Michaelis constant for VLK-pNA hydrolysis by Pm (0.3 mM), k_e is the empirically determined catalytic rate constant for Pm hydrolysis [3.2×10^4 M min^{-1} (mol of Pm)$^{-1}$], and E is the molar extinction coefficient of pNA at 405 nm (8800 M^{-1} cm^{-1}; Erlanger et al., 1965).

Table I: Effect of $LamA^{2091-2108}$ on Fibrinolytic Enzymes[a]

enzyme	substrate	activity (mol of substrate hydrolyzed/min) (−) $LamA^{2091-2108}$	activity (mol of substrate hydrolyzed/min) (+) $LamA^{2091-2108}$
t-PA	Glu-Pg	2.2×10^{-13}	27.0×10^{-13}
t-PA	Lys_{77}-Pg	5.1×10^{-13}	2.4×10^{-13}
t-PA	IPR-pNA	4.7×10^{-12}	1.4×10^{-12}
u-PA	Glu-Pg	7.3×10^{-11}	7.5×10^{-11}
plasmin	VLK-pNA	5.9×10^{-13}	5.0×10^{-13}

[a] Enzymes were incubated with peptide (500 μg/mL) for 10 min at 37 °C followed by addition of the indicated substrate as described under Materials and Methods.

The effect of the peptide $LamA^{2091-2108}$ and control peptides on Pg activation was determined by preincubating Pg with the peptide (0–500 μg/mL) at 37 °C for 10 min in 155 μL of 20 mM Hepes containing 0.3 mM VLK-pNA. After incubation, plasminogen activator was added and the reaction monitored as described above.

Effect of Peptide $LamA^{2091-2108}$ on Amidolytic Activity of Plasminogen Activators. The amidolytic activities of t-PA and u-PA in the presence of the peptide $LamA^{2091-2108}$ were determined by incubating t-PA (0.11 nM) or u-PA (0.8 nM) with peptide (0–500 μg/mL) at 37 °C in 155 μL of 20 mM Hepes, pH 7.4. The reaction was initiated by addition of IPR-pNA (0.3 mM), and substrate hydrolysis was monitored at a wavelength of 405 nm as described above.

Effect of Peptide $LamA^{2091-2108}$ on Pm Amidolytic Activity. The effect of peptide $LamA^{2091-2108}$ on Pm amidolytic activity was determined by incubating Pm (11 nM) with peptide (0–500 μg/mL) at 37 °C in 155 μL of 20 mM Hepes, pH 7.4. The reaction was initiated by addition of VLK-pNA (0.3 mM), and substrate hydrolysis was monitored at a wavelength of 405 nm as described above.

Effect of Peptide $LamA^{2091-2108}$ on Collagenase Activity. Activity of collagenases was assayed by using the synthetic collagenase substrate Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 (Dnp-PLGLWAR-NH_2) (5 μM) in 0.05 M Tris-HCl, 5 mM $CaCl_2$, and 0.2 M NaCl, pH 7.7 at 37 °C (Stack & Gray, 1989). Fluorometric measurements were performed with an SLM-Aminco SPF-500C spectrofluorometer. The initial rate of substrate hydrolysis was determined by monitoring the increase in fluorescence emission at a wavelength of 346 nm using an excitation wavelength of 280 nm. The effect of $LamA^{2091-2108}$ on two structurally and mechanistically related collagenases was determined by incubating human fibroblast type I collagenase (30 nM) or porcine synovial type IV collagenase/gelatinase (1.0 nM) with the synthetic substrate followed by addition of increasing amounts of $LamA^{2091-2108}$ (0.3–100 μM). Kinetic data for collagenase activity in the presence of $LamA^{2091-2108}$ were fit by least-squares regression analysis to the equation $\log (A_0/A_i - 1) = \log K + n \log [I]$, where A_0 and A_i represent the activity determined in the absence and presence of $LamA^{2091-2108}$, respectively, and n is the number of binding sites per enzyme molecule (Ambrose et al., 1950). IC_{50} concentrations were estimated from the slope and intercept values.

Results

Kinetics of Pg Activation in the Presence of $LamA^{2091-2108}$. The synthetic peptide $LamA^{2091-2108}$ caused a dose-dependent increase in the velocity of t-PA-catalyzed Pg activation (Figure 1), resulting in a 15-fold increase in activation velocity at a peptide concentration of 100 μg/mL. In contrast, no stimulation of u-PA-catalyzed Pg activation was observed in the presence of up to 500 μg/mL $LamA^{2091-2108}$ (Table I). The

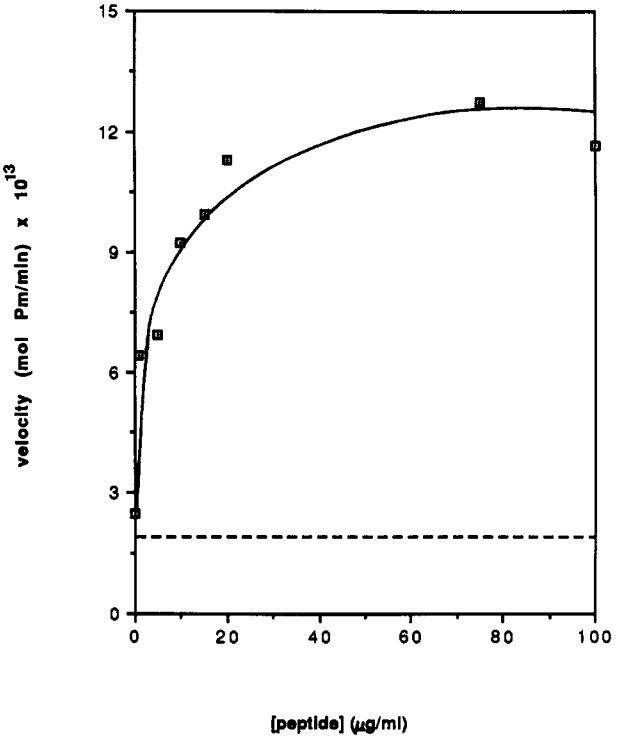

FIGURE 1: Concentration dependence of LamA$^{2091-2108}$ stimulation of t-PA-catalyzed Pg activation. Glu-Pg (0.3 μM) was preincubated with PA22-2 (0–100 μg/mL) in 20 mM Hepes, pH 7.4, in the presence of VLK-pNA (0.3 mM) for 10 min at 37 °C. After addition of t-PA (0.11 nM), the Pm hydrolysis of VLK-pNA was monitored at a wavelength of 405 nm. Reaction velocity in the absence of LamA$^{2091-2108}$ (---); reaction velocity in the presence of LamA$^{2091-2108}$ (—).

Table II: Effect of Synthetic Peptides on Plasminogen Activation by t-PA[a]

peptide	activity (mol of Pm/min) (×10^{12})
(A) 0	0.22
(B) CSRARKQAASIKVAVSADR-NH_2	3.43
(C) IKVAV-NH_2	0.19
(D) SRARK-NH_2	0.16
(E) SEASGMVTQL	0.06
(F) RDLKPAIVKVYDYYETDEFA	0.03
(G) PCPHPRDPDPASRTH	0.08
(H) YIGSR	0.09

[a] The designated peptide (100 μg/mL) was incubated with 0.3 μM Pg in the presence of 0.3 mM VLK-pNA for 10 min at 37 °C followed by addition of t-PA (0.11 nM).

amidolytic activities of t-PA and Pm were also unaffected by the addition of LamA$^{2091-2108}$ (Table I). A variety of synthetic peptides similar in size to LamA$^{2091-2108}$, but of unrelated amino acid sequence, were tested for the ability to stimulate Pg activation by t-PA. As shown in Table II (E–G), only LamA$^{2091-2108}$ had a stimulatory effect on Pg activation. An unrelated laminin-derived peptide, YIGSR, also had no effect on Pg activation (Table II, H). To determine whether the unpaired amino-terminal Cys residue added to the peptide fragment contributed to the stimulatory effect, the Cys was alkylated with iodoacetamide (Glazer et al., 1975). The stimulatory properties of the alkylated peptide were identical with those of the parent compound (data not shown). Two pentapeptide fragments of LamA$^{2091-2108}$ did not alter the rate of Pg activation (Table II, C, D), suggesting that the intact peptide is necessary for stimulation. In addition, treatment of the peptide with *Staphylococcus aureus* V8 proteinase, which introduces a single cleavage in the peptide (verified by

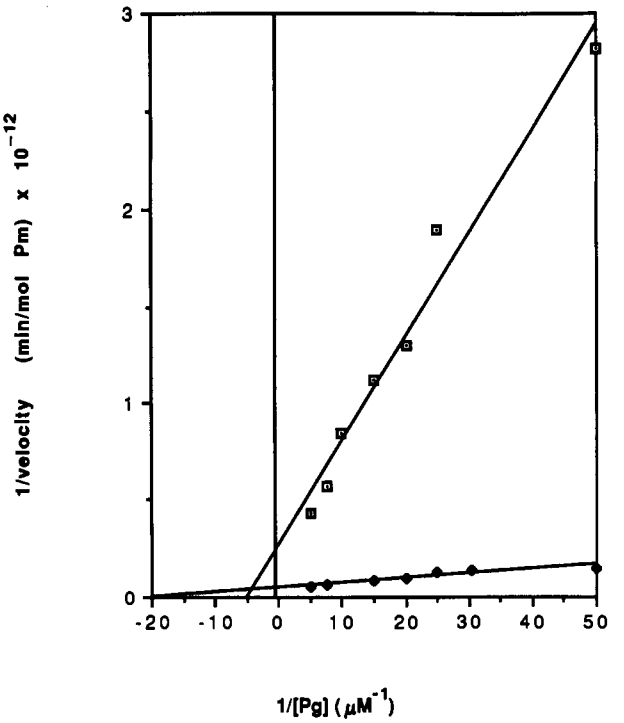

FIGURE 2: Lineweaver–Burk plot showing the kinetics of activation of Pg by t-PA in the presence of LamA$^{2091-2108}$. Glu-Pg was incubated for 10 min at 37 °C with LamA$^{2091-2108}$ (100 μg/mL) in 20 mM Hepes, pH 7.4, containing 0.3 mM VLK-pNA followed by addition of t-PA (0.11 nM). Initial velocities were determined from the slopes of plots of A_{405} vs time^2 as described under Materials and Methods. No LamA$^{2091-2108}$ (□); 100 μg/mL LamA$^{2091-2108}$ (♦).

HPLC, data not shown), removed the stimulatory effect, providing further evidence that the intact peptide is necessary for stimulation of Pg activation. The effect of LamA$^{2091-2108}$ (100 μg/mL) on the initial rate of Pg activation is shown in Figure 2. The k_{cat}/K_m of t-PA-catalyzed Pg activation calculated from these data increases from 3.2 μM^{-1} s^{-1} in the absence of peptide to 69.2 μM^{-1} s^{-1} in the presence of the peptide, resulting in an overall 22-fold increase in activation efficiency.

Fibrinogen fragments potentiate t-PA-dependent Pg activation, with maximum stimulation at a fragment concentration of approximately 20 μg/mL (Nieuwenhuizen et al., 1983). The effect of LamA$^{2091-2108}$ on fibrinogen stimulation of Pg activation is shown in Figure 3. Although fibrinogen fragments result in a greater overall stimulation of Pg activation, simultaneous addition of LamA$^{2091-2108}$ and fibrinogen fragments abolishes the additional stimulatory effect achieved with fibrinogen alone, indicating that LamA$_2^{091-2108}$ and fibrinogen may compete for similar binding sites on the Pg molecule.

Effect of LamA$^{2091-2108}$ on Collagenase Activity. The effect of LamA$^{2091-2108}$ on the activity of purified interstitial and type IV collagenase is shown in Figure 4. Addition of LamA$^{2091-2108}$ had an inhibitory effect on collagenase activity with an IC_{50} of 3 μM for type I collagenase and 43 μM for type IV collagenase. Since free Cys is a weak inhibitor of collagenase activity (IC_{50} of ~3 mM; Darlak et al., 1990), the amino-terminal Cys of LamA$^{2091-2108}$ was alkylated with *N*-ethylmaleimide (Glazer et al., 1975). The alkylated peptide retained slight inhibitory activity with an IC_{50} of approximately 100 μM for both collagenases (data not shown).

Discussion

A variety of biological activities have been attributed to laminin including promotion of cell growth and adhesion,

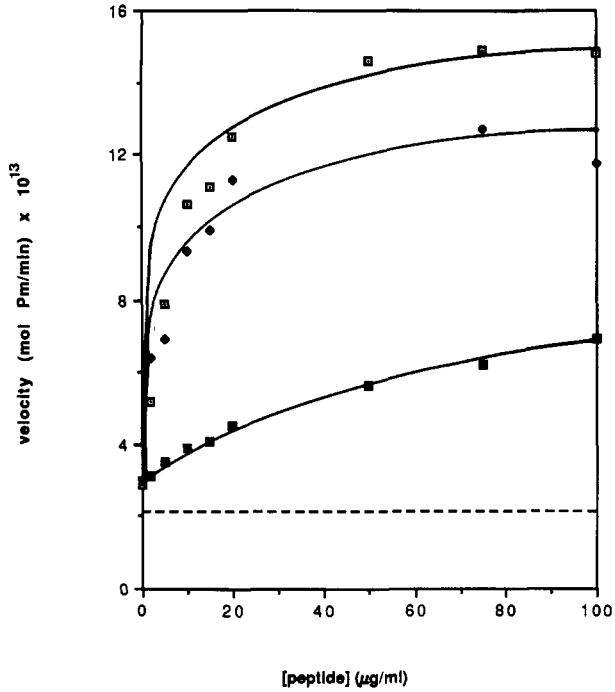

FIGURE 3: Stimulation of t-PA-catalyzed Pg activation by LamA$^{2091-2108}$ and fibrinogen fragments. Glu-Pg (0.3 μM) was preincubated with fibrinogen fragments (0–100 μg/mL) (□), LamA$^{2091-2108}$ (0–100 μg/mL) (♦), or fibrinogen fragments (0–100 μg/mL) with LamA$^{2091-2108}$ (100 μg/mL) (■) in 20 mM Hepes, pH 7.4, for 10 min at 37 °C in the presence of 0.3 mM VLK-pNA. t-PA (0.11 nM) was added to initiate the reaction, and the increase in absorbance was monitored at 405 nm. Reaction velocity in the absence of LamA$^{2091-2108}$ or fibrinogen fragments (---).

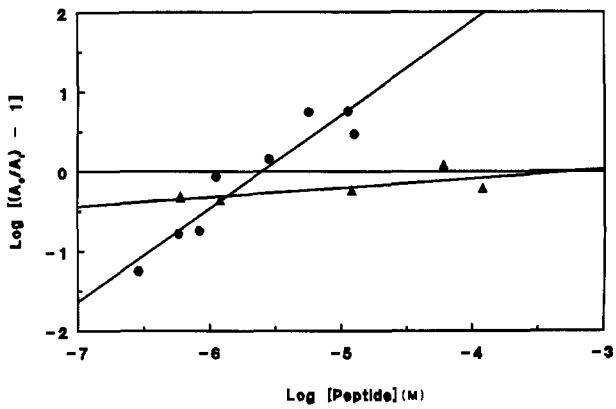

FIGURE 4: Inhibition of collagenase activity by LamA$^{2091-2108}$. The activities of human fibroblast collagenase type I (●) and porcine synovial collagenase type IV (▲) were determined by using the synthetic substrate Dnp-PLGLWAR-NH_2 (5 μM) in 0.05 M Tris-HCl, 5 mM $CaCl_2$, and 0.2 M NaCl, pH 7.7 at 37 °C. A_0 is the activity in the absence of peptide LamA$^{2091-2108}$, and A_i is the activity observed in the presence of peptide at concentration i.

tumor migration, and neural process formation (Martin & Timpl, 1987; Beck et al., 1990). These functions have been localized to specific regions of the laminin molecule. The peptide PA22-2 (LamA$^{2091-2108}$), a 19 amino acid synthetic peptide modeled from the E8 domain of the laminin A chain, has been shown to stimulate adhesion, migration, and metastasis of B16-F10 melanoma cells (Tashiro et al., 1989; Kanemoto et al., 1990) and to promote formation of processes on cerebellar neurons and PC12 cells (Tashiro et al., 1989). Additionally, it has been demonstrated that PA22-2 (LamA$^{2091-2108}$), as well as intact laminin, causes an increase in collagenase IV activity in the culture medium of B16-F10 and A2058 melanoma cells (Kanemoto et al., 1990; Turpeenniemi-Hujanen et al., 1986). In the present study, we have demonstrated that LamA$^{2091-2108}$ is also a potent stimulator of t-PA-catalyzed Pg activation while actually inhibiting collagenase activity.

Addition of LamA$^{2091-2108}$ stimulated t-PA-catalyzed Pg activation in a concentration-dependent manner with a maximum stimulation observed at a peptide concentration of 100 μg/mL (Figure 1). A variety of other peptides ranging in size from 5 to 20 amino acids had no stimulatory effect on Pg activation (Table II), indicating that the stimulation observed with LamA$^{2091-2108}$ is sequence-specific. Smaller fragments of LamA$^{2091-2108}$ (Table II, C, D) as well as V8 proteinase treated peptide failed to stimulate Pg activation, indicating that the intact peptide is necessary for stimulation.

We previously reported that isolated protein components of the ECM, including laminin, stimulate Pg activation by t-PA (Stack et al., 1990). The present data indicate an increase in activation efficiency using the peptide LamA$^{2091-2108}$ (k_{cat}/K_m = 69 μM^{-1} s^{-1}) over that observed with intact laminin (k_{cat}/K_m = 6 μM^{-1} sl^{-1}), suggesting that additional domains of the intact laminin molecule modulate its interaction with Pg and t-PA. This is supported by the observation that the pentapeptide YIGSR, derived from the laminin B1 chain, actually has a slight inhibitory effect on Pg activation (Table II, H).

The t-PA-catalyzed activation of proteolytically modified Lys_{77}-Pg was unaffected by up to 500 μg/mL LamA$^{2091-2108}$ (Table I). Glu-Pg has been demonstrated to undergo large conformational changes following ligand binding, changing from a relatively inactivatable "closed" form to a more readily activated "open" form (Castellino, 1981; Mangel et al., 1990). However, the conformation and activation kinetics of Lys_{77}-Pg are not dramatically altered by ligand binding (Castellino, 1981). Since LamA$^{2091-2108}$ had no effect on the activation rate of Lys_{77}-Pg, our kinetic data suggest that the peptide may stimulate activation of Glu-Pg by promoting a conformational change to the "open" form of the molecule. This is supported by the data of Figure 3 which indicate that LamA$^{2091-2108}$ competes with fibrinogen fragments for Pg binding. Fibrinogen fragments are known to interact with specific ligand binding sites on Pg, inducing a large conformational change in the molecule and stimulating the activation by t-PA. The amidolytic activities of t-PA and Pm were unaffected by addition of LamA$^{2091-2108}$ (Table I), providing additional evidence that LamA$^{2091-2108}$ stimulates Pg activation through interaction with the substrate. Previous data from our laboratory showed a 7-fold increase in t-PA amidolytic activity in the presence of intact laminin (Stack et al., 1990), suggesting that this direct stimulatory effect on t-PA activity is contained within a different segment of the laminin molecule.

The peptidolytic activities of both type I and type IV collagenase were inhibited in the presence of LamA$^{2091-2108}$ (Figure 4). Several investigators have reported that intact laminin as well as peptide PA22-2 (LamA$^{2091-2108}$ increased the observed type IV collagenase activity in the culture medium of A2058 and B16-F10 melanoma cells (Turpeennieni-Hujanen et al., 1986; Kanemoto et al., 1990). Type IV collagenase and related matrix metalloproteinases are secreted as zymogens which are activated extracellularly (Stricklin et al., 1977; Wilhelm et al., 1987; Collier et al., 1988). Although the exact physiological mechanism of procollagenase IV activation remains unclear, in vitro activation of both interstitial and type IV procollagenase is a plasmin-dependent process (Reich et al., 1988; He et al., 1989). This indicates that factors which stimulate Pg activation could result in increased levels

of procollagenase activation, leading to an observed increase in collagenase activity. Our data demonstrate that LamA$^{2091-2108}$ stimulates plasmin formation but does not directly enhance collagenase activity, suggesting an alternative mechanism for increased levels of collagenase activity in the culture medium of peptide-treated cells. Thus, we propose that the increase in collagenase activity previously reported could be a consequence of the stimulation of plasminogen activation.

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Registry No. t-PA, 105913-11-9; Pg, 9001-91-6; collagenase, 9001-12-1; LamA$^{2091-2108}$, 131435-36-4.

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