Elafin and Its Precursor Trappin-2 Still Inhibit Neutrophil Serine Proteinases when They Are Covalently Bound to Extracellular Matrix Proteins by Tissue Transglutaminase†

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Received July 20, 2005; Revised Manuscript Received September 27, 2005

ABSTRACT: Elafin and its precursor trappin-2 (also called pre-elafin) are potent protein inhibitors of neutrophil serine proteases such as leukocyte elastase and proteinase 3. Trappin-2 has unique conserved sequence motifs rich in Gln and Lys residues. These motifs are substrates for transglutaminases that may enable trappin-2 to be cross-linked to extracellular matrix proteins, thus anchoring the inhibitor at its site of action. We have used Western blotting and ELISA-based assays to demonstrate that both elafin and trappin-2 can be conjugated to various extracellular matrix proteins in vitro by a type 2 transglutaminase. Cross-linked elafin and trappin-2 still inhibited their target proteases. Surface plasmon resonance studies allowed the determination of the kinetic constants governing the interaction of fibronectin-bound elafin and trappin-2 with neutrophil elastase and proteinase 3. Both inhibitors were potent inhibitors when cross-linked to fibronectin by transglutamination, with equilibrium dissociation constants K_i for their interaction with target proteases of 0.3 nM (elastase−elafin), 20 nM (proteinase 3−elafin), 0.3 nM (elastase−trappin-2), and 12 nM (proteinase 3−trappin-2). The conjugated inhibitors reacted more slowly with their target enzymes than did the soluble inhibitors, perhaps due to their immobilization, with association rate constants of $2-7 \times 10^5$ M^{-1} s^{-1} for elastase and $1-4 \times 10^4$ M^{-1} s^{-1} for proteinase 3. We believe this is the first demonstration that transglutaminase-mediated cross-linking of serine protease inhibitors to proteins preserves their inhibitory capacities.

The chelonianin family of protease inhibitors (Family I17, Clan IP in the MEROPS database; http://merops.sanger.ac.uk/) includes the secretory leukocyte protease inhibitor (SLPI),[1] elafin, and trappin-2 (or pre-elafin), from which elafin is proteolytically released (*1*). These inhibitors are found in several tissues, including epithelia (*2*, *3*), lung (*4*), and macrophages (*5*), where they are involved in the physiological control of neutrophil serine proteinases. SLPI inhibits leukocyte elastase and cathepsin G (*6*), while elafin and trappin-2 target leukocyte elastase and proteinase 3 (*7*). Human trappin-2 is an unglycosylated 95-amino acid protein with an N-terminal domain (38 residues) or cementoin domain (*8*) containing several repeated motifs rich in Gln and Lys residues that serve as transglutaminase substrates, and a C-terminal inhibitory domain or whey acidic protein (WAP) domain containing four disulfide bonds (*9*) corresponding to elafin (57 residues). This region is structurally similar to SLPI. The N-terminal sequence of elafin also contains a motif containing Gln and Lys residues, similar to those of the cementoin domain. Elafin, also known as SKALP (skin-derived anti-leukoprotease), was first purified independently as an elastase inhibitor from the skin of patients with psoriasis by two groups in the early 1990s (*10*, *11*). It was subsequently isolated from human lung sputum by Sallenave et al. and called elastase-specific inhibitor (ESI) (*12*). Cloning the elafin cDNA revealed that the molecule was larger than expected due to the presence of an N-terminal noninhibitory domain (*13*−*15*). The whole molecule was then termed trappin-2; "trappin" is an acronym for transglutaminase substrate and wap domain containing protein (*16*).

The identification of sequence motifs that are substrates for transglutaminase in trappin-2 led to the initial hypothesis that this protease inhibitor could be covalently anchored to its site of action (*8*, *16*). Several members of the trappin family have now been identified in various species, and comparison of the sequences of their N-terminal domains indicates that they contain motifs having the general consensus sequence GQDPVK (*17*−*19*). Human trappin-2 has five such repeated motifs that are more adequately described by GQDXVK (Figure 1), four in the cementoin moiety and one at the very beginning of the elafin domain.

The fact that the sequences of these repeated motifs are similar to that of the seminal vesicle protein SVP-1, a trans-

† N.G. holds a doctoral fellowship from MENRT (Ministère de l'Education Nationale, de la Recherche et de la Technologie). This work was supported by the French cystic fibrosis association "Vaincre la Mucoviscidose".

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[1] Abbreviations: HNE, human neutrophil elastase; PR3, human neutrophil proteinase 3; ECM, extracellular matrix; SPR, surface plasmon resonance; RU, resonance units; SLPI, secretory leukocyte proteinase inhibitor; TGase, transglutaminase.

10.1021/bi051418i CCC: $30.25
Published on Web 11/05/2005

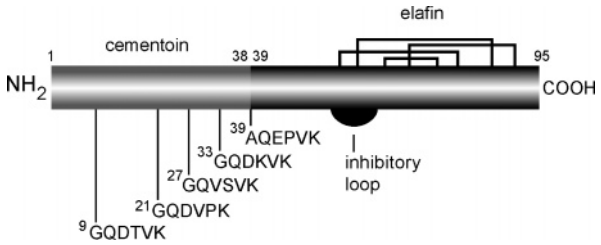

FIGURE 1: Diagram showing the structural organization of human trappin-2. The repeated sequence motifs conforming to the GQDX-VK consensus sequence that are transglutaminase substrates are shown. Four of them are located in the noninhibitory cementoin domain, and one is the beginning of the elafin inhibitory domain. The Gln residues (Q) of this motif may act as an acyl donor, while the Lys residues (K) may serve as an acceptor in the transglutaminase-mediated formation of an isopeptide bond between trappin-2 and other proteins. Also shown are the four disulfide bonds of the elafin domain (plain lines) and the inhibitory loop of the protease inhibitor domain.

glutaminase substrate, suggested that this protease inhibitor is covalently anchored to its site of action, probably to extracellular matrix proteins (*8*, *14*, *16*). Molhuizen et al. (*14*) showed that transglutaminase-mediated cross-linking of trappin-2 can occur in the human epidermis both in vitro and in vivo. This group used biotinylated peptides derived from the transglutaminase substrate motifs to demonstrate that these peptides could be incorporated into skin proteins (*14*) or purified proteins (*16*) by the action of the endogenous transglutaminase(s) present in the scales from a psoriatic patient or by exogenous transglutaminases. In vitro studies showed that radiolabeled trappin-2 was an efficient TGase substrate for cross-linking to laminin or for the TGase-mediated incorporation of [^{14}C]methylamine (*14*). Western blotting analysis of molecular sizes of elafin and trappin-2 in the trachea mucous epithelium also indicated that this covalent cross-linking could occur in vivo. Immunodetection of these inhibitors at M_r values (~50 kDa) much higher than expected (~6−12 kDa) suggested that the cementoin domain was probably involved in covalent anchoring (*8*). Another study by Steiner and Marekov (*20*) identified, by N-terminal amino acid sequencing, several cross-linked proteins from human stratum corneum. These included trappin-2, and to a lesser extent elafin, conjugated to loricrin and accounted for ~6% of all proteins in the cornified cell envelope. This type of targeted immobilization of elafin or trappin-2 at their sites of action is most important for their use as aerosol-delivered inhibitors of neutrophil proteases in the treatment of inflammatory lung diseases. Although soluble elafin and trappin-2 are potent inhibitors of neutrophil elastase and proteinase 3, we do not know whether they remain inhibitory when immobilized on extracellular matrix proteins.

We have therefore measured the inhibitory capacity of elafin and trappin-2 covalently bound to extracellular proteins. We used several approaches to demonstrate that both elafin and trappin-2 may be cross-linked to various extracellular matrix proteins. They still behaved as potent protease inhibitors, despite having slightly reduced affinities for their target proteases.

MATERIALS AND METHODS

Materials. Recombinant elafin and recombinant trappin-2 were produced in *Pichia pastoris* as previously described (*7*). Human cathepsin G (EC 3.4.21.20) was obtained from MP Biomedicals (Vannes, France), and human neutrophil elastase (EC 3.4.21.37) and proteinase 3 (EC 3.4.21.76) were obtained from Athens Research and Technology (Athens, GA). Human lung tryptase (EC 3.4.21.59) was purchased from Calbiochem (VWR International, Strasbourg, France). Trypsin, N-Suc-AAPV-*p*-nitroanilide, human fibronectin, vitronectin, collagen IV, fibrinogen, bovine β-crystallin, bovine serum albumin, and guinea pig liver transglutaminase (EC 2.3.2.13) were obtained from Sigma-Aldrich (St. Quentin Fallavier, France). Mouse laminin V was from Chemicon (Euromedex, Souffelweyersheim, France). The intramolecularly quenched fluorogenic substrates Abz-APEEIMDRQ-EDDnp and Abz-VADCADQ-EDDnp were provided by L. Juliano (University of Sao Paulo, Sao Paulo, Brazil); 96-well plates were obtained from Nalge Nunc International (VWR International, Strasbourg, France). Goat polyclonal antibodies and mouse monoclonal antibodies directed against elafin were from HyCult biotechnology b.v. (Tebu Bio SA, Le Perray-en-Yvelines, France). Rabbit peroxidase-conjugated anti-goat antibodies were from DakoCytomation (Trappes, France). All other reagents were analytical grade.

Detection of Trappin-2 or Elafin Bound to Extracellular Matrix Proteins by Western Blotting. Recombinant trappin-2 or elafin (2 μg) was mixed with 4 μg of human plasma fibronectin and then incubated with 1.6×10^{-8} M guinea pig liver transglutaminase at 37 °C in 50 mM Tris-HCl buffer (pH 7.5), 2 mM $CaCl_2$, and 0.5 mM DTT (total volume of 15 μL) for 0−1 h. The reaction was stopped by adding sodium dodecyl sulfate (SDS) buffer without reducing agents. Samples were then separated by SDS−PAGE (10% gels) according to the method of Laemmli (*21*). Trappin-2 or elafin complexes were detected by Western blotting using a goat anti-elafin polyclonal antibody according to the method of Zani et al. (*22*).

Detection of Covalently Bound Trappin-2 or Elafin by an Enzyme-Linked Immunosorbent Assay (ELISA). Ninety-six-well ELISA microplates (Nunc) were coated (2 μg/well) with human fibronectin, vitronectin, fibrinogen, collagen IV, β-crystallin, mouse laminin V, or bovine serum albumin, diluted in 0.1 M sodium carbonate buffer (pH 9.5) by incubation overnight at 4 °C. The plates were washed with 25 mM potassium phosphate buffer (pH 7.4) and 0.15 M NaCl and free sites blocked by incubation in 50 mM Tris, 150 mM NaCl, and 2% Tween 20 for 1 h at 37 °C. Trappin-2 or elafin was cross-linked by transglutaminase with coated proteins as follows. Trappin-2 or elafin (4×10^{-7} M) was incubated at 37 °C in wells with guinea pig liver transglutaminase (1.25×10^{-7} M) in 50 mM Tris-HCl buffer (pH 7.5), 2 mM $CaCl_2$, and 0.5 mM DTT for various times up to 1 h. Reaction mixtures were removed by washing with the same buffer described above. Covalently linked trappin-2 or elafin was detected with goat anti-elafin polyclonal antibodies [diluted 1:2000 in potassium phosphate buffer (pH 7.4), 0.15 M NaCl, and 0.5% Tween 20] followed by peroxidase-conjugated anti-goat antibodies [1:2000 in potassium phosphate buffer (pH 7.4), 0.15 M NaCl, and 0.5% Tween 20]. Peroxidase activity was measured with *o*-phenylenediamine at 490 nm using a THERMOmax (Molecular Devices) microplate reader.

Inhibitory Properties of Elafin or Trappin-2 Cross-Linked to Fibronectin. Trappin-2 or elafin (4×10^{-7} M) was cross-

linked to fibronectin in 96-well microplates as described above. Human neutrophil elastase (10^{-9} M) or proteinase 3 (10^{-10} M) was incubated in wells for 15 min at 37 °C in 50 mM Hepes buffer (pH 7.4), 0.75 M NaCl, and 0.05% IGEPAL CA-630. Elastase activity was measured with the fluorogenic substrate Abz-APEEIMDRQ-EDDnp (*23*) (final concentration of 20 μM), while the activity of neutrophil proteinase 3 was measured with 20 μM Abz-VADCADQ-EDDnp (*23*). The enzyme activity using both fluorogenic substrates was monitored at 320 nm (excitation wavelength) and 420 nm (emission wavelength) with a SPECTRAmax Gemini microplate reader (Molecular Devices). To allow fluorescence measurements, trappin-2 or elafin was cross-linked to fibronectin by transglutaminase as described above in Fluoronunc Maxisorp plates.

Surface Plasmon Resonance Analysis of the Interaction of Neutrophil Proteinases with Elafin or Trappin-2 Previously Bound to Fibronectin. Interactions between neutrophil proteinases and fibronectin-bound inhibitors (trappin-2 or elafin) were assessed by surface plasmon resonance (SPR) using a BIAcore 1000 instrument (BIAcore International, Uppsala, Sweden). All experiments were conducted at 25 °C.

Immobilization of Human Plasma Fibronectin. Human plasma fibronectin was covalently immobilized on a carboxymethylated dextran-coated sensor chip (CM5) using the amine coupling kit supplied by Biacore International. It was performed with 10 mM Hepes (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20 as a running buffer at a continuous flow rate of 5 μL/min. Dextran was activated as indicated by the manufacturer, and human plasma fibronectin (100 μg/mL) in 10 mM sodium acetate buffer (pH 4.0) was then injected (35 μL). Injection was stopped when the biosensor surface was coupled to 16 000 resonance units (RU). The surface was then deactivated with 1 M ethanolamine (pH 8.5, 50 μL) and washed for 5 min. The sensor chip surface was regenerated by injecting 10 mM HCl-glycine (pH 2.0, 5 μL) for 1 min.

Transglutamination. Transglutamination was performed at a flow rate of 1 μL/min using 50 mM Tris-HCl buffer (pH 7.5), 2 mM $CaCl_2$, and 0.5 mM DTT as a running and diluting buffer. Trappin-2 (100 μg/mL) or elafin (150 μg/mL) was mixed with guinea pig liver transglutaminase (50 nM). The mixture was injected (50 μL) three to five times until the sensor chip surface had bound 1200 RU (elafin) or 1600 RU (trappin-2). The chip was washed for 5 min, and mouse monoclonal anti-elafin antibodies (TRAB2F antibody, Tebu Bio SA, Le Perray en Yvelines, France) (50 μg/mL) were then injected (30 μL) for detection of elafin or trappin-2 bound to fibronectin.

Kinetic Measurements. All experiments were performed at a flow rate of 10 μL/min using 50 mM Hepes buffer (pH 7.4), 0.75 M NaCl, and 0.05% IGEPAL CA-630 as a running and diluting buffer. BIAcore 1000 sensorgrams [resonance units (RU) vs time] were recorded for 8 min using concentrations of human neutrophil elastase or proteinase 3 from 10^{-10} to 10^{-6} M. The biosensor surface was then washed for 10 min and regenerated by injecting 10 mM HCl-glycine (pH 2.0, 10 μL) for 1 min.

Calculation of Kinetic Constants. Collected data were analyzed with BIAeval software (version 3.1). The resulting binding curves were used to calculate the following kinetic constants: association rate constant (k_{ass}) and dissociation rate constant (k_{diss}). The data were fitted to a single-site interaction model [1:1 (Langmuir) binding, A + B $\leftrightarrow$ AB]. Assuming pseudo-first-order interaction kinetics, the rate of complex formation during sample injection is given by the equation $d[AB]/dt = k_{ass}[A][B] - k_{diss}[AB]$, which may be expressed as $dR/dt = k_{ass}CR_{max} - (k_{ass}C + k_{diss})R$, where dR/dt is the rate of change of the SPR signal, C is the concentration of analyte, R_{max} is the maximum analyte binding capacity in resonance units, and R is the recorded SPR signal in resonance units at time t. k_{diss} values were determined from the data collected during the dissociation phase ($dR/dt = -k_{diss}R$), while k_{ass} values were derived from the above rate equation for complex formation. The equilibrium dissociation constant was calculated from the kinetic rate constants ($K_i = k_{diss}/k_{ass}$). Details of the rate equations are described in the BIAeval version 3.1 software manual.

Effect of Tryptase and Trypsin on the Inhibitory Activity of Fibronectin-Bound Trappin-2. Trappin-2 (4×10^{-7} M) was cross-linked to fibronectin in 96-well microplates as described above. Fibronectin-bound trappin-2 was then incubated for 2 h at 37 °C with tryptase (from 10^{-10} to 10^{-7} M) in 25 mM sodium phosphate (pH 7.4), 0.1 mg/mL heparin, and 1 mM EDTA, or with trypsin (from 10^{-9} to 10^{-6} M) in 50 mM Tris-HCl (pH 8.1) and 0.15 M NaCl. The plates were washed, and leukocyte elastase was added to each well and incubated for 15 min at 37 °C to allow formation of protease−inhibitor complexes. Residual elastase activity was measured spectrophotometrically at 410 nm with 1 mM N-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide using a THERMOmax microplate reader.

RESULTS

Cross-Linking of Elafin and Trappin-2 to Extracellular Matrix Proteins by Transglutaminase. Transglutaminases catalyze protein cross-linking by forming intermolecular ϵ-(γ-glutamyl)lysine isopeptide bonds between a reactive glutamine and the ϵ-amino group of a reactive lysine residue. We performed cross-linking reactions and analyzed the results by Western blotting to determine whether guinea pig type 2 TGase could catalyze the formation of a covalent complex between trappin-2 and fibronectin. The immunoreactive band corresponding to native trappin-2 was steadily depleted when trappin-2 was incubated with TGase, as the two proteins became cross-linked to form high-molecular weight species. At least four low-mobility bands were detected using anti-trappin-2 polyclonal antibody (Figure 2A), suggesting that TGase catalyzed the covalent incorporation of trappin-2 into fibronectin. Elafin could also be cross-linked to fibronectin with transglutaminase (Figure 2B), but fewer bands were detected probably because there are fewer transglutaminase substrate sequences in elafin. Specificity was checked by adding excess EDTA, which totally inhibited the Ca^{2+}-dependent reaction protein cross-linking (data not shown).

To check potential self-cross-linking, recombinant trappin-2 was incubated with TGase alone. Faint bands were detected at approximately 26 and 35 kDa (Figure 2C), indicating that a small proportion of trappin-2 was covalently bound to itself by TGase. However, these bands were not observed in the presence of fibronectin, confirming that fibronectin is a good endogenous substrate of type 2

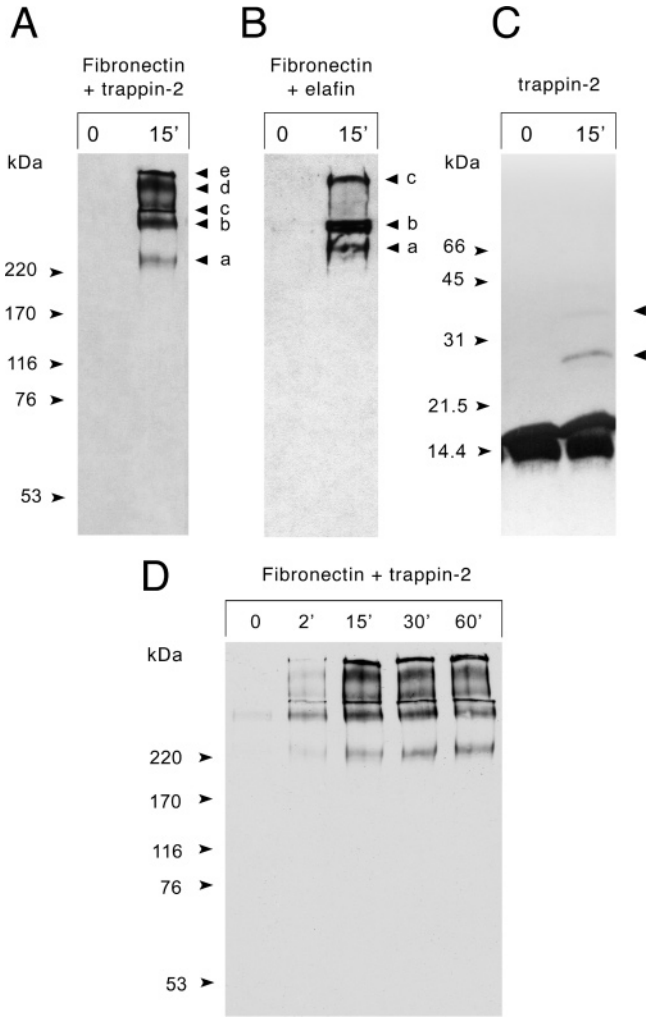

FIGURE 2: Transglutaminase-catalyzed cross-linking between elafin or trappin-2 and fibronectin analyzed by Western blotting. Recombinant trappin-2 (2 μg) or elafin (2 μg) was incubated with fibronectin (4 μg) and type 2 tissue transglutaminase in 15 μL of buffer [50 mM Tris-HCl (pH 7.5), 2 mM $CaCl_2$, and 0.5 mM DTT] at 37 °C for 15 min. Reaction mixtures were mixed with nonreducing sample buffer, heated, loaded onto 10% SDS−PAGE gels, and electrophoresed. The gels were subjected to transfer to nitrocellulose filters followed by immunostaining with anti-elafin antibodies. Arrows show the positions of products of the cross-linking reaction between fibronectin and inhibitors depicted as low-mobility bands ranging from a to e for trappin-2 (A) or from a to c for elafin (B). Panel C shows cross-linked products at ~26 and ~35 kDa (arrows) produced when trappin-2 was incubated with transglutaminase without fibronectin (16% SDS−PAGE gel). Panel D shows the appearance of cross-linked products of fibronectin and trappin-2 at times up to 60 min, analyzed by Western blotting. The left-hand lane in each panel contains molecular mass standards.

transglutaminases (*24*). Incubation with TGase from 2 to 60 min indicated that cross-linking was almost complete after 15 min (Figure 2D).

Detection of Covalently Bound Trappin-2 or Elafin by an Enzyme-Linked Immunosorbent Assay (ELISA). An ELISA-based experiment was performed with the wells first coated with ECM proteins (fibronectin, fibrinogen, vitronectin, laminin V, and collagen IV), β-crystallin, or bovine serum albumin (BSA) to better characterize the TGase-mediated incorporation of elafin or trappin-2 into various proteins. Unadsorbed proteins were removed by washing, and elafin or trappin-2 was added together with transglutaminase and the mixture allowed to react for 1 h. The wells were washed

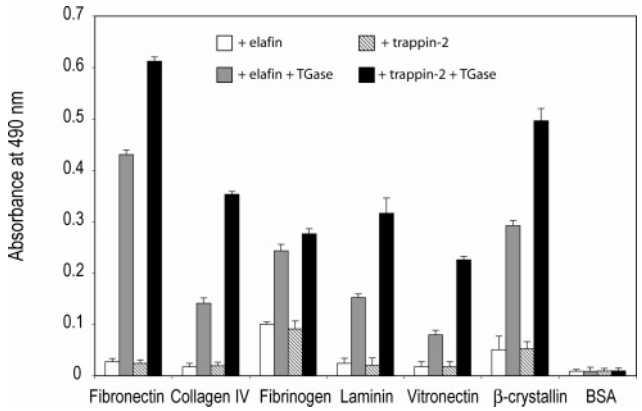

FIGURE 3: Transglutaminase-mediated binding of elafin and trappin-2 to immobilized proteins determined by an ELISA. Elafin or trappin-2 was incubated in wells coated with fibronectin, collagen IV, fibrinogen, laminin V, vitronectin, β-crystallin, or bovine serum albumin (BSA), with or without transglutaminase (125 nM), for 1 h at 37 °C. The wells were washed and covalently fibronectin-bound elafin, or trappin-2 was detected with goat polyclonal anti-elafin antibodies and rabbit peroxidase-conjugated anti-goat antibodies as the second antibody. Peroxidase activity was measured at 490 nm using *o*-phenylenediamine as a substrate. Error bars reflect the standard deviation of three independent determinations.

thoroughly, and the amount of covalently bound elafin or trappin-2 was measured with polyclonal anti-elafin antibodies, followed by peroxidase-conjugated anti-IgG.

Both elafin and trappin-2 were anchored to ECM proteins (Figure 3). Trappin-2 seemed to be more readily incorporated into all the ECM proteins that were tested than was elafin (except fibrinogen). As observed in Western blot experiments, fibronectin appeared to be the most favored substrate for TGase, although apparent differences could be due to different incorporation rates or different stoichiometries for binding. β-Crystallin, which has been shown previously to efficiently cross-link biotinylated peptides derived from the transglutaminase substrate sequences of trappin-2 (*16*), also incorporated both elafin and trappin-2 following transglutamination. Bovine serum albumin (BSA) was used as a negative control and did not appear to be a transglutaminase substrate (Figure 3).

Specificity was checked by omitting TGase (Figure 3) or by adding excess EDTA (not shown). No signal was detected under these conditions, indicating that elafin and trappin-2 did not interact reversibly with the proteins tested prior to covalent coupling catalyzed by TGase. The high background signal observed with fibrinogen and β-crystallin when no TGase was added may be due to the nonspecific binding of immunoglobulins in our ELISA.

Time-course ELISA studies (Figure 4) indicated that trappin-2 was more rapidly incorporated into fibronectin than was elafin. Trappin-2 was almost totally cross-linked after incubation for 15 min with the highest concentration of TGase (125 nM), while elafin was covalently bound more slowly. As expected, the cross-linking of both inhibitors depended on the TGase concentration used in the assay (Figure 4).

Inhibitory Properties of Elafin or Trappin-2 Cross-Linked to Fibronectin. Since fibronectin appeared to be a good TGase substrate, we examined the inhibitory capacity of fibronectin-bound inhibitors (Figure 5). We used an enzyme-based assay to measure the residual proteolytic activity of

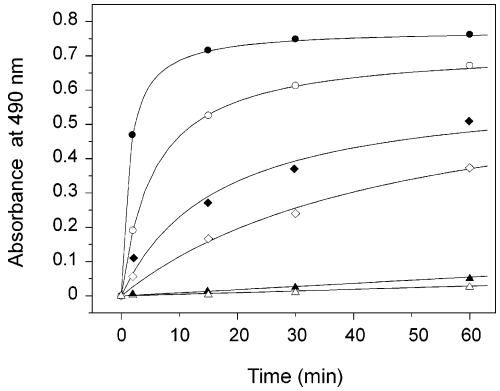

FIGURE 4: Analysis of binding of elafin and trappin-2 to immobilized fibronectin by an ELISA as a function of time. Elafin or trappin-2 was incubated with 12.5 nM TGase [elafin (◇) and trappin-2 (○)] or 125 nM TGase [elafin (◆) and trappin-2 (●)] at 37 °C in microtiter wells coated with fibronectin for different times. The wells were washed and cross-linked elafin, or trappin-2 was detected with anti-elafin polyclonal antibodies. Elafin (△) and trappin-2 (▲) were incubated with BSA using the highest TGase concentration (125 nM) as a negative control.

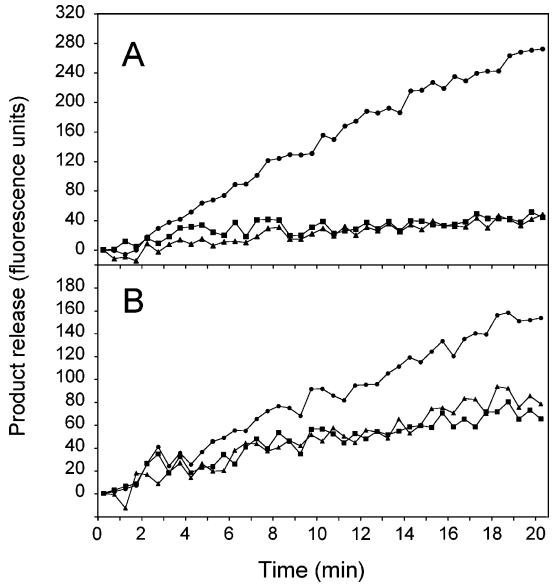

FIGURE 5: Inhibition of leukocyte elastase and proteinase 3 by elafin and trappin-2 bound to fibronectin. Typical inhibition experiments showing the hydrolysis rate of fluorogenic substrates for HNE (A) or PR3 (B). Elafin or trappin-2 was bound to fibronectin by TGase and unbound inhibitor was washed. HNE (final concentration of 10^{-9} M) or PR3 (final concentration of 10^{-10} M) was placed in the wells containing immobilized elafin or trappin-2 for 15 min at 37 °C, and the residual protease activity was then measured using fluorogenic substrates [Abz-APEEIMDRQ-EDDnp (20 μM) for HNE and Abz-VADCADQ-EDDnp (20 μM) for PR3]. The concentration of the fluorescent product released by HNE (A) or PR3 (B) was measured spectrofluorometrically over the course of 20 min in the presence of fibronectin-bound elafin (▲) or fibronectin-bound immobilized trappin-2 (■). Trappin-2 was incubated in fibronectin-coated wells without TGase (●) in control experiments. The rate of substrate hydrolysis by both enzymes was similar in fibronectin-coated wells containing no inhibitor (data not shown).

HNE and PR3 in the presence of conjugated elafin and/or trappin-2. Fibronectin was adsorbed onto high-adsorption ELISA plates and the bound fibronectin incubated with TGase and trappin-2 or elafin for 1 h. Then elastase or proteinase 3 was added and the mixture incubated for 15 min to form reversible complexes between immobilized inhibitors and their target proteases. The residual activity of each enzyme was then measured using highly sensitive fluorogenic substrates previously developed in our laboratory (*23*). The proteolytic activities of both HNE and PR3 were significantly inhibited by conjugated elafin and trappin-2 (Figure 5). The apparent percentage inhibition of HNE (~85%) was higher than that of PR3 (~55%) under the same coupling conditions for both inhibitors. This probably reflects differences in K_i values of the enzyme−inhibitor pairs; since the percent inhibition for a given enzyme: inhibitor ratio depends directly on the enzyme concentration to K_i ($[E]_0/K_i$) ratio (*25*) once equilibrium is reached, the higher the $[E]_0/K_i$, the higher the percent inhibition. This suggests that the K_i values for the interaction of PR3 with both immobilized inhibitors are higher (i.e., PR3 has lower affinity) than those expected for HNE. This was confirmed in our SPR study. Cathepsin G was not inhibited, in agreement with data for soluble elafin and trappin-2.

Kinetics for Binding of Leukocyte Elastase and Proteinase 3 onto Fibronectin−Elafin/Fibronectin−Trappin-2 Sensor Chips. We determined the binding kinetics for the interaction of fibronectin-bound inhibitors with neutrophil proteinases using surface plasmon resonance. Fibronectin was first immobilized on a carboxymethylated dextran matrix on a BIAcore CM5 sensor chip by random amine coupling. Elafin or trappin-2 was then conjugated to fibronectin by simultaneously injecting a mixture of inhibitor and TGase until the surface was loaded to approximately 1500 RU. Injecting anti-elafin monoclonal antibodies confirmed the efficiency of the TGase-mediated coupling reaction (not shown). HNE or PR3 was injected over immobilized elafin or trappin-2 followed by buffer to analyze the interaction of fibronectin-bound inhibitors with neutrophil proteinases. The reaction was assessed in real time by recording the changes in surface plasmon resonance. The sensorgrams were analyzed and fitted to a single-site (1:1) interaction model. The association (k_{ass}) and dissociation (k_{diss}) rate constants were determined (Table 1) for HNE concentrations of 0.1−30 nM and PR3 concentrations of 10−1000 nM (Figure 6). Equilibrium dissociation constants (K_i) were calculated from the rate constants and found to be 0.3 nM for the interaction of HNE with both immobilized inhibitors; the K_i value for the inhibition of PR3 with immobilized elafin was 20 nM, and that for trappin-2 was 12 nM (Table 1). The TGase-mediated immobilization of elafin and trappin-2 slightly altered the inhibitory potency of both molecules (in terms of affinity) as compared to those of soluble inhibitors (*7*). Nevertheless, they remained tight-binding inhibitors of their target enzymes. But we did observe a decrease in k_{ass} values and consequently an increase in K_i values when we used an analyte flow rate that was sufficiently high (10 μL/min) to prevent mass transport limitations (the transport of analyte molecules across the stationary layer). Thus, it can be hypothesized that the observed reduction in the k_{ass} value may be due to a greatly reduced frequency of collision between the two partners, which governs the rate of complex formation of a bimolecular system (*26*), due to inhibitor immobilization. Most remarkably, the resonance units did not completely recover after protease injection was stopped at high protease concentrations (Figure 6A, curve 7), indicating that fibronectin, which is highly sensitive to HNE degradation (*27*), was partially proteolyzed. This suggests

Table 1: Surface Plasmon Resonance Analysis of the Interaction of Trappin-2 and Elafin Covalently Bound to Fibronectin by Transglutaminase with Neutrophil Elastase and Proteinase 3[a]

	neutrophil elastase				proteinase 3			
	k_{ass} (M^{-1} s^{-1})	k_{diss} (s^{-1})	K_i (M)	relative K_i[c]	k_{ass} (M^{-1} s^{-1})	k_{diss} (s^{-1})	K_i (M)	relative K_i[c]
fibronectin-bound trappin-2	$(1.5 \pm 0.01) \times 10^5$	$(4.5 \pm 0.1) \times 10^{-5}$	$(0.3 \pm 0.01) \times 10^{-9}$	10	$(1.1 \pm 0.003) \times 10^4$	$(1.3 \pm 0.01) \times 10^{-4}$	$(11.8 \pm 0.10) \times 10^{-9}$	66
soluble trappin-2[b]	$(3.6 \pm 0.5) \times 10^6$	$(1.1 \pm 0.2) \times 10^{-4}$	$(0.3 \pm 0.01) \times 10^{-10}$		$(2.0 \pm 0.1) \times 10^6$	$(3.7 \pm 1.1) \times 10^{-4}$	$(0.18 \pm 0.06) \times 10^{-9}$	
fibronectin-bound elafin	$(6.7 \pm 0.1) \times 10^5$	$(2.0 \pm 0.08) \times 10^{-4}$	$(0.3 \pm 0.01) \times 10^{-9}$	4	$(4.0 \pm 0.06) \times 10^4$	$(8.0 \pm 0.2) \times 10^{-4}$	$(20 \pm 0.53) \times 10^{-9}$	170
soluble elafin[b]	$(3.7 \pm 0.1) \times 10^6$	$(3.2 \pm 0.1) \times 10^{-4}$	$(0.08 \pm 0.005) \times 10^{-9}$		$(3.3 \pm 0.03) \times 10^6$	$(4.0 \pm 0.3) \times 10^{-4}$	$(0.12 \pm 0.01) \times 10^{-9}$	

[a] Kinetic constants were determined from binding curves shown in Figure 6 using BIAeval (version 3.1). Standard deviations are given. [b] From ref *7*. [c] K_i(bound inhibitor)/K_i(soluble inhibitor).

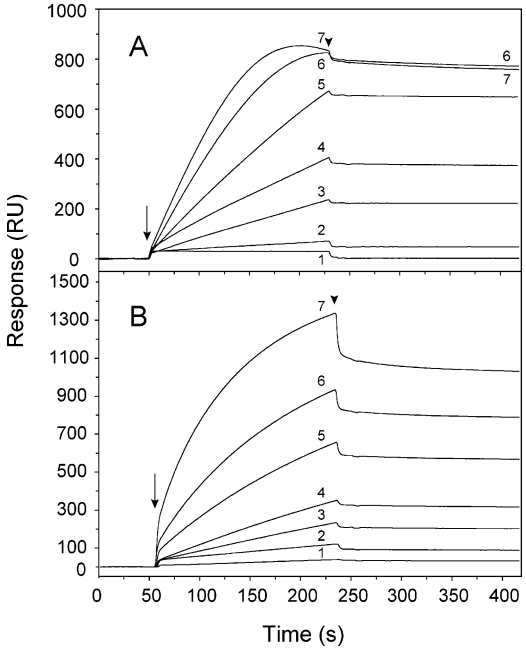

FIGURE 6: Analysis of binding of leukocyte elastase (HNE) and proteinase 3 (PR3) to immobilized fibronectin-bound trappin-2 by surface plasmon resonance. Fibronectin was immobilized on a CM-5 chip inserted in the BIAcore 1000 system. Then a mixture containing trappin-2 and transglutaminase was injected to produce transglutaminase-mediated cross-linking of the inhibitor to fibronectin. The chip was washed thoroughly, and various concentrations of HNE or PR3 were run over the immobilized trappin-2. Its association was monitored in real time while registering the resonance signal (response), as shown by the sensorgrams. The concentrations of enzyme in both panels were 0.1, 1, 5, 10, 15, 25, and 30 nM for curves 1−7 for HNE (A) and 10, 25, 50, 100, 250, and 500 nM and 1 μM for curves 1−7 for PR3 (B), respectively. The arrow indicates the injection of protease and the arrowhead the end of the injection. Kinetic constants were calculated from these curves using BIAeval (version 3.1). Curve 7 of panel A was not included in the calculations because the fibronectin was hydrolyzed by HNE, as indicated by the weaker response obtained during the complex dissociation phase as compared to curve 6.

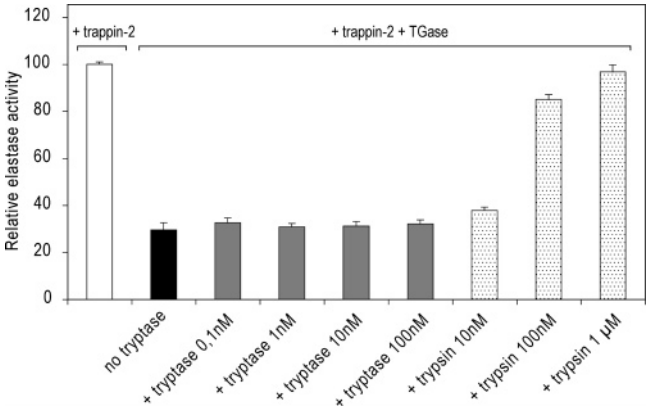

FIGURE 7: Effect of tryptase and trypsin on the inhibition of neutrophil elastase by fibronectin-bound trappin-2. Microtiter wells were coated with fibronectin, and TGase and trappin-2 were added together to cross-link trappin-2 to fibronectin. The plates were washed thoroughly. No tryptase (black bar), tryptase (0.1−100 nM) (gray bars), or trypsin (10 nM, 100 nM, and 1 μM) (dotted bars) was added and the mixture incubated for 2 h at 37 °C to release elafin (the inhibitory domain) from trappin-2. The plates were again washed. HNE was added to each well and the mixture incubated for 15 min. Its residual activity was then measured at 410 nm using the chromogenic substrate N-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide. Residual elastase activity is expressed as relative activity compared to the control experiment (white bar) without added TGase. Values are means ± the standard error of three wells.

that the total inhibitory capacity of the sensor chip was overwhelmed by the slight excess of protease, allowing HNE proteolytic activity. Fibronectin was rapidly degraded, as assessed by a large loss of resonance units (not shown), when no inhibitor was conjugated or when injecting cathepsin G, which is not inhibited by elafin or trappin-2.

Effect of Tryptase and Trypsin on the Inhibitory Activity of Fibronectin-Bound Trappin-2. We have previously shown that tryptase releases elafin from soluble trappin-2 very efficiently and specifically (*1*). We therefore tested the effect of tryptase on fibronectin-bound trappin-2 to determine whether the susceptibility of trappin-2 to tryptase was altered. Incubation with various concentrations of tryptase did not alter the inhibitory capacity of bound trappin-2, suggesting that elafin is no longer released from trappin-2 (Figure 7). The highest tryptase concentration used was 100 nM, whereas 2.5 nM is enough to release elafin from soluble trappin-2 (*1*). Perhaps tryptase, whose active form is a tetramer, could not access its proteolytic sites in bound trappin-2 because of steric hindrance. However, trypsin did gradually reduce the inhibitory activity of immobilized trappin-2 at the lowest concentrations that were used, whereas HNE inhibition was almost destroyed by 1 μM trypsin (Figure 7). Hence, elafin can be released from immobilized trappin-2 by trypsin, as is the soluble inhibitor, although it is always possible that trypsin degrades fibronectin to release fibronectin fragments bearing bound trappin-2. Trappin-2 appears to lose its high sensitivity to the elafin-releasing

action of tryptase once trappin-2 is incorporated into fibronectin.

DISCUSSION

Both elafin and trappin-2 are good substrates for transglutaminase, which can cross-link these inhibitors to various ECM proteins. Fibronectin appeared to be the best ECM protein we have tested for covalently binding elafin or trappin-2 catalyzed by a transglutaminase. Elafin can also be cross-linked by transglutamination, although it is incorporated more slowly than trappin-2. Nara et al. (*8*) used the same type 2 TGase (guinea pig liver TGase) as us and found that elafin alone was not cross-linked with itself, unlike trappin-2, suggesting that elafin is not a TGase substrate. However, they used an elafin moiety fused to maltose binding protein, whose properties could be different from those of native elafin. The extent of cross-linking of trappin-2 or elafin by TGase in vivo is difficult to analyze, since cross-linking renders the molecule insoluble so that it cannot be extracted from tissues without using reagents that also cleave peptide bonds. Several studies, including immunohistochemical ones, have found elafin cross-linking in vivo (*20*, *28*−*30*); however, the published data often do not clearly indicate whether elafin or its precursor trappin-2 is involved. Steinert and Marekov (*20*) digested the cornified cell envelope of human foreskin epidermis with proteinase K to identify several distinct proteins that are cross-linked protein components of the cell envelope structure. Elafin was estimated to represent ~6% of all proteins (*20*). Direct N-terminal sequencing of released soluble peptide fragments identified the reactive glutamine acceptor and lysine donor of elafin or trappin-2 participating in the covalent attachment of this inhibitor to epidermal proteins. This approach revealed that Gln40, Gln95, Lys36, Lys38, and Lys44 (trappin-2 sequence numbering) were involved in the formation of isopeptide bonds mainly with loricrin, and to a lesser extent with keratin 1 and desmoplakin (*20*). The observation that some peptide sequences started at the sequence corresponding to that of native elafin (AQEPV) suggests that elafin alone could be cross-linked by transglutamination, in agreement with the results presented here. This may occur via Gln2 or Lys6 of the Ala1-Gln2-Glu3-Pro4-Val5-Lys6 motif (elafin numbering). However, proteolytic cleavage may also take place at the junction between cementoin and elafin once trappin-2 is cross-linked via residues of its elafin moiety. Trappin-2 is also incorporated via two Lys residues (Lys36 and Lys38) of the fourth transglutaminase substrate motif of the cementoin domain. Why the motifs starting at Gly9, Gly21, and Gly27 (Figure 1) are not involved in elafin or trappin-2 cross-linking is not clear, but it probably reflects the fact that the two motifs starting at Gly33 and Ala39 are more exposed on the protein surface and/or more accessible to the TGase active site. Steiner and Marekov (*20*) also found that the C-terminal Gln57 (elafin numbering) of elafin or trappin-2 is involved in covalent cross-linking, suggesting that this terminal glutamine is also a TGase substrate, although it is not in a GQDXVK motif. Thus, the rules governing the recognition of residues in trappin-2 suitable for transglutamination are not yet clear. Further studies will be necessary to identify residues involved in the TGase-mediated cross-linking of trappin-2 to fibronectin or other proteins.

The relevance and importance of the cross-linking of elafin or trappin-2 as a protease inhibitor in situations other than the participation of elafin or trappin-2 in forming the cornified envelope of the epidermis and in stabilizing the extracellular matrix (*31*) are not clear yet. This study shows that trappin-2 and elafin conjugated to fibronectin by a TGase still inhibit their protease targets, neutrophil elastase and proteinase 3. As a direct consequence of immobilization, rates of association of bound inhibitors with their target proteases are somewhat lower than those of the soluble inhibitors, which reduces their affinity, especially for PR3, so that the K_i is ~2 orders of magnitude greater (i.e., affinity decreased). Nevertheless, the K_i values of 0.3−20 nM and dissociation rate constants (k_{diss}) in the 10^{-4} s^{-1} range (Table 1) indicate that both fibronectin-bound inhibitors contribute to the regulation of protease activity in vivo, along with soluble inhibitors. This makes elafin and/or trappin-2 attractive for use as serine protease inhibitors in aerosols for treating inflammatory lung diseases. The extracellular matrix is altered in response to inflammation, and is thus exposed (becomes able to trap inhibitors) during the process of tissue remodeling and repair. TGase-mediated conjugation of elafin or trappin-2 could be enhanced under inflammatory conditions, when the extracellular concentrations of elastase and proteinase 3 are dramatically increased as a consequence of massive neutrophil recruitment. TGase activity is increased in inflammatory situations (*24*, *32*, *33*), and soluble fibronectin and other ECM components are synthesized and secreted by various cell types prior to matrix assembly in wound repair under both normal and pathological conditions. Thus, the question of whether elafin or trappin-2 may also be involved in healing arises, since SLPI, another elastase inhibitor, homologous to elafin, is thought to be essential for wound healing (*34*). We examined the reactivity of SLPI to type 2 TGase under the same conditions that were used for elafin or trappin-2 but detected no significant cross-linking of SLPI to the ECM proteins that were tested (not shown). We also examined the capacity of the TGase activity of plasma factor XIIIa to cross-link elafin or trappin-2, since elastase and possibly other neutrophil serine proteases can inhibit tissue repair, including the remodeling of vessels and pathogenesis of arterial diseases (*35*, *36*). Preliminary experiments revealed that factor XIIIa can catalyze the conjugation of both elafin and trappin-2 to fibronectin and fibrinogen (not shown). We are now evaluating further the capacity of factor XIIIa to catalyze the cross-linking of elafin or trappin-2. Two serpin family protease inhibitors, α_2-antiplasmin (α_2-AP) and plasminogen activator inhibitor 2 (PAI-2), are cross-linked by factor XIIIa or type 2 TGase to fibrinogen (*37*) or fibrin (*38*). Other cystatin family protease inhibitors, cystatin α and cystatin M, are also TGase substrates (*39*, *40*). This suggests that the transglutaminase-catalyzed targeting of a protease inhibitor to tissue surfaces or to insoluble structures not only stabilizes the protein components but also locally regulates endogeneous or exogeneous proteases. The most appealing property of trappin-2 conjugated to extracellular matrix proteins by transglutaminase(s) is that it may be of crucial importance for reducing elastase-induced lung hemorrhage in hamsters (*41*) or lipopolysaccharide-induced acute lung inflammation in mice (*42*), whereas elafin has a weaker effect (*41*, *42*).

We have shown that bound trappin-2 is still inhibitory and that the conjugated inhibitor is less susceptible to proteolysis. Thus, cross-linking trappin-2 dramatically increases its bioavailability, making it much better than that of the soluble inhibitors. These findings highlight the therapeutic potential of elafin and trappin-2 for inhibitor-based anti-inflammatory therapies of lung diseases.

ACKNOWLEDGMENT

The English text was checked by Dr. Owen Parkes.

REFERENCES

1. Guyot, N., Zani, M. L., Berger, P., Dallet-Choisy, S., and Moreau, T. (2005) Proteolytic susceptibility of the serine protease inhibitor trappin-2 (pre-elafin): Evidence for tryptase-mediated generation of elafin, *Biol. Chem. 386*, 391−9.
2. King, A. E., Critchley, H. O., Sallenave, J. M., and Kelly, R. W. (2003) Elafin in human endometrium: An antiprotease and antimicrobial molecule expressed during menstruation, *J. Clin. Endocrinol. Metab. 88*, 4426−31.
3. Pfundt, R., van Ruissen, F., van Vlijmen-Willems, I. M., Alkemade, H. A., Zeeuwen, P. L., Jap, P. H., Dijkman, H., Fransen, J., Croes, H., van Erp, P. E., and Schalkwijk, J. (1996) Constitutive and inducible expression of SKALP/elafin provides anti-elastase defense in human epithelia, *J. Clin. Invest. 98*, 1389−99.
4. Tremblay, G. M., Sallenave, J. M., Israel-Assayag, E., Cormier, Y., and Gauldie, J. (1996) Elafin/elastase-specific inhibitor in bronchoalveolar lavage of normal subjects and farmer's lung, *Am. J. Respir. Crit. Care Med. 154*, 1092−8.
5. Mihaila, A., and Tremblay, G. M. (2001) Human alveolar macrophages express elafin and secretory leukocyte protease inhibitor, *Z. Naturforsch. 56*, 291−7.
6. Thompson, R. C., and Ohlsson, K. (1986) Isolation, properties, and complete amino acid sequence of human secretory leukocyte protease inhibitor, a potent inhibitor of leukocyte elastase, *Proc. Natl. Acad. Sci. U.S.A. 83*, 6692−6.
7. Zani, M. L., Nobar, S. M., Lacour, S. A., Lemoine, S., Boudier, C., Bieth, J. G., and Moreau, T. (2004) Kinetics of the inhibition of neutrophil proteinases by recombinant elafin and pre-elafin (trappin-2) expressed in *Pichia pastoris*, *Eur. J. Biochem. 271*, 2370−8.
8. Nara, K., Ito, S., Ito, T., Suzuki, Y., Ghoneim, M. A., Tachibana, S., and Hirose, S. (1994) Elastase inhibitor elafin is a new type of proteinase inhibitor which has a transglutaminase-mediated anchoring sequence termed "cementoin", *J. Biochem. 115*, 441−8.
9. Bairoch, A., Bucher, P., and Hofmann, K. (1997) The PROSITE database, its status in 1997, *Nucleic Acids Res. 25*, 217−21.
10. Wiedow, O., Schroder, J. M., Gregory, H., Young, J. A., and Christophers, E. (1990) Elafin: An elastase-specific inhibitor of human skin. Purification, characterization, and complete amino acid sequence, *J. Biol. Chem. 265*, 14791−5.
11. Schalkwijk, J., Chang, A., Janssen, P., De Jongh, G. J., and Mier, P. D. (1990) Skin-derived antileucoproteases (SKALPs): characterization of two new elastase inhibitors from psoriatic epidermis, *Br. J. Dermatol. 122*, 631−41.
12. Sallenave, J. M., and Ryle, A. P. (1991) Purification and characterization of elastase-specific inhibitor. Sequence homology with mucus proteinase inhibitor, *Biol. Chem. Hoppe-Seyler 372*, 13−21.
13. Sallenave, J. M., and Silva, A. (1993) Characterization and gene sequence of the precursor of elafin, an elastase-specific inhibitor in bronchial secretions, *Am. J. Respir. Cell Mol. Biol. 8*, 439−45.
14. Molhuizen, H. O., Alkemade, H. A., Zeeuwen, P. L., de Jongh, G. J., Wieringa, B., and Schalkwijk, J. (1993) SKALP/elafin: An elastase inhibitor from cultured human keratinocytes. Purification, cDNA sequence, and evidence for transglutaminase cross-linking, *J. Biol. Chem. 268*, 12028−32.
15. Saheki, T., Ito, F., Hagiwara, H., Saito, Y., Kuroki, J., Tachibana, S., and Hirose, S. (1992) Primary structure of the human elafin precursor preproelafin deduced from the nucleotide sequence of its gene and the presence of unique repetitive sequences in the prosegment, *Biochem. Biophys. Res. Commun. 185*, 240−5.
16. Zeeuwen, P. L., Hendriks, W., de Jong, W. W., and Schalkwijk, J. (1997) Identification and sequence analysis of two new members of the SKALP/elafin and SPAI-2 gene family. Biochemical properties of the transglutaminase substrate motif and suggestions for a new nomenclature, *J. Biol. Chem. 272*, 20471−8.
17. Schalkwijk, J., Wiedow, O., and Hirose, S. (1999) The trappin gene family: Proteins defined by an N-terminal transglutaminase substrate domain and a C-terminal four-disulphide core, *Biochem. J. 340*, 569−77.
18. Tamechika, I., Itakura, M., Saruta, Y., Furukawa, M., Kato, A., Tachibana, S., and Hirose, S. (1996) Accelerated evolution in inhibitor domains of porcine elafin family members, *J. Biol. Chem. 271*, 7012−8.
19. Brown, T. I., Mistry, R., Collie, D. D., Tate, S., and Sallenave, J. M. (2004) Trappin ovine molecule (TOM), the ovine ortholog of elafin, is an acute phase reactant in the lung, *Physiol. Genomics 19*, 11−21.
20. Steinert, P. M., and Marekov, L. N. (1995) The proteins elafin, filaggrin, keratin intermediate filaments, loricrin, and small proline-rich proteins 1 and 2 are isodipeptide cross-linked components of the human epidermal cornified cell envelope, *J. Biol. Chem. 270*, 17702−11.
21. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227*, 680−5.
22. Zani, M., Brillard-Bourdet, M., Lazure, C., Juliano, L., Courty, Y., Gauthier, F., and Moreau, T. (2001) Purification and characterization of active recombinant rat kallikrein rK9, *Biochim. Biophys. Acta 1547*, 387−96.
23. Korkmaz, B., Attucci, S., Moreau, T., Godat, E., Juliano, L., and Gauthier, F. (2004) Design and use of highly specific substrates of neutrophil elastase and proteinase 3, *Am. J. Respir. Cell Mol. Biol. 30*, 801−7.
24. Griffin, M., Casadio, R., and Bergamini, C. M. (2002) Transglutaminases: Nature's biological glues, *Biochem. J. 368*, 377−96.
25. Bieth, J. G. (1995) Theoretical and practical aspects of proteinase inhibition kinetics, *Methods Enzymol. 248*, 59−84.
26. Fersht, A. (1985) *Enzyme Structure and Mechanism*, W. H. Freeman and Co., New York.
27. Bonnefoy, A., and Legrand, C. (2000) Proteolysis of subendothelial adhesive glycoproteins (fibronectin, thrombospondin, and von Willebrand factor) by plasmin, leukocyte cathepsin G, and elastase, *Thromb. Res. 98*, 323−32.
28. Yoshida, N., Egami, H., Yamashita, J., Takai, E., Tamori, Y., Fujino, N., Kitaoka, M., Schalkwijk, J., and Ogawa, M. (2002) Immunohistochemical expression of SKALP/elafin in squamous cell carcinoma of human lung, *Oncol. Rep. 9*, 495−501.
29. Nakane, H., Ishida-Yamamoto, A., Takahashi, H., and Iizuka, H. (2002) Elafin, a secretory protein, is cross-linked into the cornified cell envelopes from the inside of psoriatic keratinocytes, *J. Invest. Dermatol. 119*, 50−5.
30. Suzuki, Y., Furukawa, M., Abe, J., Kashiwagi, M., and Hirose, S. (2000) Localization of porcine trappin-2 (SKALP/elafin) in trachea and large intestine by in situ hybridization and immunohistochemistry, *Histochem. Cell Biol. 114*, 15−20.
31. Sumi, Y., Inoue, N., Azumi, H., Seno, T., Okuda, M., Hirata, K., Kawashima, S., Hayashi, Y., Itoh, H., and Yokoyama, M. (2002) Expression of tissue transglutaminase and elafin in human coronary artery: Implication for plaque instability, *Atherosclerosis 160*, 31−9.
32. Verderio, E. A., Johnson, T., and Griffin, M. (2004) Tissue transglutaminase in normal and abnormal wound healing: Review article, *Amino Acids 26*, 387−404.
33. Chen, R., Gao, B., Huang, C., Olsen, B., Rotundo, R. F., Blumenstock, F., and Saba, T. M. (2000) Transglutaminase-mediated fibronectin multimerization in lung endothelial matrix in response to TNF-α, *Am. J. Physiol. 279*, L161−74.
34. Ashcroft, G. S., Lei, K., Jin, W., Longenecker, G., Kulkarni, A. B., Greenwell-Wild, T., Hale-Donze, H., McGrady, G., Song, X. Y., and Wahl, S. M. (2000) Secretory leukocyte protease inhibitor mediates non-redundant functions necessary for normal wound healing, *Nat. Med. 6*, 1147−53.
35. Dollery, C. M., Owen, C. A., Sukhova, G. K., Krettek, A., Shapiro, S. D., and Libby, P. (2003) Neutrophil elastase in human atherosclerotic plaques: Production by macrophages, *Circulation 107*, 2829−36.
36. Fontaine, V., Touat, Z., Mtairagel, M., Vranckx, R., Louedec, L., Houard, X., Andreassian, B., Sebbag, U., Palombi, T., Jacob, M.

P., Meilhac, O., and Michel, J. B. (2004) Role of leukocyte elastase in preventing cellular re-colonization of the mural thrombus, *Am. J. Pathol. 164*, 2077−87.
37. Ritchie, H., Lawrie, L. C., Crombie, P. W., Mosesson, M. W., and Booth, N. A. (2000) Cross-linking of plasminogen activator inhibitor 2 and α2-antiplasmin to fibrin(ogen), *J. Biol. Chem. 275*, 24915−20.
38. Ritchie, H., Robbie, L. A., Kinghorn, S., Exley, R., and Booth, N. A. (1999) Monocyte plasminogen activator inhibitor 2 (PAI-2) inhibits u-PA-mediated fibrin clot lysis and is cross-linked to fibrin, *Thromb. Haemostasis 81*, 96−103.
39. Takahashi, M., Tezuka, T., Kakegawa, H., and Katunuma, N. (1994) Linkage between phosphorylated cystatin α and filaggrin by epidermal transglutaminase as a model of cornified envelope and inhibition of cathepsin L activity by cornified envelope and the conjugated cystatin α, *FEBS Lett. 340*, 173−6.
40. Zeeuwen, P. L., Van Vlijmen-Willems, I. M., Jansen, B. J., Sotiropoulou, G., Curfs, J. H., Meis, J. F., Janssen, J. J., Van Ruissen, F., and Schalkwijk, J. (2001) Cystatin M/E expression is restricted to differentiated epidermal keratinocytes and sweat glands: A new skin-specific proteinase inhibitor that is a target for cross-linking by transglutaminase, *J. Invest. Dermatol. 116*, 693−701.
41. Tremblay, G. M., Vachon, E., Larouche, C., and Bourbonnais, Y. (2002) Inhibition of human neutrophil elastase-induced acute lung injury in hamsters by recombinant human pre-elafin (trappin-2), *Chest 121*, 582−8.
42. Vachon, E., Bourbonnais, Y., Bingle, C. D., Rowe, S. J., Janelle, M. F., and Tremblay, G. M. (2002) Anti-inflammatory effect of pre-elafin in lipopolysaccharide-induced acute lung inflammation, *Biol. Chem. 383*, 1249−56.

BI051418I