Regulation of Proteolysis at the Neutrophil-Substrate Interface by Secretory Leukoprotease Inhibitor

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Human neutrophils can initiate the rapid degradation of extracellular matrix macromolecules by localizing the destructive process to sites of cell-substrate contact. Although plasma and its filtrates contain multiple proteinase inhibitors, these inhibitors did not prevent neutrophils from attacking either underlying fibronectin or elastin. However, subjacent substrates could be protected from neutrophils by recombinant secretory leukoprotease inhibitor, a structurally unique serine proteinase inhibitor whose natural counterpart is normally confined to human mucous secretions. The identification of this extravascular proteinase inhibitor as a potent regulator of subjacent proteolysis could lead to the development of a new class of anti-inflammatory therapeutics.

HUMAN PLASMA AND ITS FILTRATES contain a complex mix of proteinase inhibitors that normally protect connective tissues from uncontrolled degradation by endogenous proteolytic enzymes (*1*). However, in inflammatory disease states, stimulated neutrophils can circumvent this defensive barrier by firmly adhering to targeted substrates and forming a sequestered microenvironment wherein the ingress of plasma antiproteinases is restricted (*1, 2*). Within these subjacent zones of tight cell-substrate contact, the neutrophil can then discharge proteolytic enzymes capable of degrading the major components of the extracellular matrix (*1*).

Given the fact that endogenous plasma proteinase inhibitors cannot prevent stimulated neutrophils from degrading underlying substrates (*1, 2*), interest has focused on identifying exogenous proteinase inhibitors that could be used to attenuate pathologic tissue damage in vivo (*3*). However, the possibility that a therapeutically valuable, endogenous inhibitor of subjacent proteolysis might already exist, but in an extravascular site, has received little attention. We now demonstrate that recombinant secretory leukoprotease inhibitor (rSLPI), an unusual serine proteinase inhibitor whose natural counterpart is found concentrated only in human mucous secretions (*4*), can regulate proteolytic degradation at the neutrophil-substrate interface.

In the presence of 20% heat-inactivated plasma, 1×10^6 human neutrophils incubated with phorbol myristate acetate (PMA) rapidly adhered to glass surfaces that had been covalently coated with either [^{3}H]fibronectin or [^{3}H]κ-elastin. Despite the presence of plasma inhibitors, stimulated (but not resting) neutrophils initiated the solubilization of the underlying substrates (Fig. 1). Increasing the heat-inactivated plasma concentration to 100% did not alter these findings [that is, in the presence of 100% plasma, fibronectin and elastin solubilization was 7196 ± 80 and 1662 ± 297 cpm, respectively (n = 3); all reported values are means ± SD].

The inability of plasma to protect the substrates from degradation is consistent with the formation of a privileged environment by the adherent neutrophil for its released proteinases (*1*). However, the degradative process need not be confined solely to subjacent sites (*1*). In sufficient numbers, stimulated neutrophils can overwhelm this barrier either by releasing proteinases in excess of the binding capacity of the surrounding inhibitors or by generating halogenated oxidants that mediate the inactivation of the plasma proteinase inhibitors (*1, 5*). However, when neutrophils were either separated from the protein-coated surfaces by a distance of 0.5 cm or prevented from tightly adhering to the underlying substrates by the addition of a monoclonal antibody (IB4) to the common β_2 chain of the neutrophil CD11/CD18 integrins (*6*), solubilization was almost completely inhibited in the presence of 20% plasma (Fig. 1). Under plasma-free conditions, the degradation of a remote target by stimulated neutrophils was only modestly decreased (Fig. 1) and IB4-treated cells had normal activity (*7*). Thus, in the presence of high plasma concentrations, 1×10^6 neutrophils were unable to attack distant targets, but could degrade those substrates with which they initiated close contact.

Within the confines of the subjacent environment created by the neutrophil, underlying substrates are exposed to proteinases as well as a flux of generated oxidants (*1, 8*). To identify the major class of proteinase involved in subjacent degradation, we stimulated neutrophils in the presence of metallo-, aspartate, cysteine, or serine proteinase inhibitors (*9*). Neither inhibitors of metalloproteinases (phosphoramidon, NP-20, or tissue inhibitor of metalloproteinases), aspartate proteinases (pepstatin A), nor cysteine proteinases (E-64 or leupeptin) interfered significantly with proteolysis when added alone (Table 1) or in combination (*7*). However, after neutrophils were treated

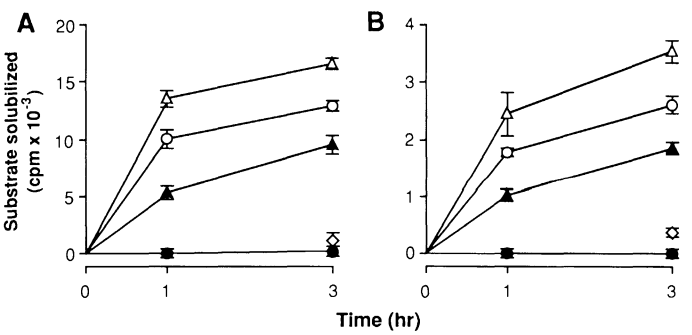

Fig. 1. Degradation of radiolabeled fibronectin (**A**) or elastin (**B**) by human neutrophils. Purified neutrophils were isolated (*5*) and suspended in Dulbecco's phosphate-buffered saline (with or without 25 mM Hepes) that contained glucose (1 mg/ml) at a final pH of 7.4. Substrate-coated surfaces were prepared by cross-linking human plasma fibronectin (Gibco) or κ-elastin (Elastin Products) that had been radiolabeled with [^{3}H]formaldehyde to glass cover slips (*28*). Treated cover slips, which bound 0.9 ± 0.1 μg of fibronectin (specific radioactivity of 18.0×10^3 cpm/μg) or 0.6 ± 0.1 μg of elastin (specific radioactivity of 6.7×10^3 cpm/μg) (n = 10), were then placed in 12-well Limbro plates in a final volume of 1 ml. Less than 5% of the bound substrates were released from cover slips after exposure to 1% boiling SDS, high salt (1 M NaCl), acidic conditions (pH 2.0), or chaotropic agents (6 M guanidinium isothiocyanate). To initiate cell-substrate contact, neutrophils were stimulated with PMA (50 ng/ml) atop the cover slips in the presence (▲) or absence (△) of 20% autologous, heat-inactivated plasma (n = 5). Similar results were obtained with plasma, serum, or heat-inactivated serum. To prevent cell-substrate contact, cover slips were inverted and floated above a layer of neutrophils that had been stimulated atop uncoated cover slips in the presence (●) or absence (○) of heat-inactivated plasma (n = 4). In three experiments, neutrophils were incubated with 10 μg/ml of the murine monoclonal antibody, IB4 (◇). At the indicated times, a sample of the cell-free supernatant was removed, and solubilized radioactivity was determined by β-scintillation counting. Resting neutrophils solubilized 459 ± 196 and 156 ± 67 cpm of fibronectin or κ-elastin, respectively, after a 3-hour incubation in plasma (n = 5).

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with diisopropylfluorophosphate (DFP), a general but specific inhibitor of serine proteinases (*10*), degradation was almost completely blocked (Table 1). Although neutrophil serine proteinases have been localized to the plasma membrane, the specific granules as well as the primary granules (*1, 8*), neither the plasma membrane nor the specific granules were major sources of proteolytic activity, because cytoplasts [granule-depleted membrane vesicles derived from intact neutrophils (*11*)] degraded only small amounts of the subjacent substrates, whereas neutrophils isolated from an individual with specific granule deficiency (SGD) (*12*) proteolyzed fibronectin comparably to normals (Table 1). In contrast, however, Chédiak-Higashi syndrome (CHS) neutrophils that contain only small amounts of active serine proteinases in their primary granules (*13*) had minimal proteolytic activity (Table 1). Oxidants were not required in the degradative process because neutrophils obtained from individuals with chronic granulomatous disease (CGD), an inherited disorder in which the isolated cells did not generate detectable quantities of oxidants (*14*), effectively proteolyzed both substrates (Table 1).

Because the most abundant serine proteinases found in azurophilic granules are neutrophil elastase, cathepsin G, and proteinase 3 (*8, 15*), the ability of small molecular sized, synthetic inhibitors of these enzymes to regulate proteolysis was tested. In combination, inhibitors of neutrophil elastase and proteinase 3 [either Ala-Ala-Pro-Val-chloromethylketone or the cephalosporin derivative, L-659,166 (*16, 17*)] and cathepsin G [methoxysuccinyl-Val-Pro-Phe-trifluoromethylketone (*18*)] were able to completely block proteolysis in the absence of cell contact under plasma-free conditions (*7*), but these same inhibitors only modestly attenuated subjacent proteolysis (Table 1) (*19*).

Although neither plasma nor synthetic serine proteinase inhibitors protected subjacent substrates, extravascular fluids contain additional proteinase inhibitors whose physiologic roles remain unclear (*4*). Mucous fluids contain a number of unusual serine proteinase inhibitors, but the major inhibitory species of these secretions is secretory leukoprotease inhibitor (SLPI), a boomerang-shaped 11.7-kD, basic polypeptide whose local concentration (~8.5 μM) can be more than 1000 times as high as that in plasma (*4*). To assess directly the ability of SLPI to inhibit contact-dependent degradation, we initially examined the effect of the rSLPI (*20*) on the solubilization of the underlying fibronectin substrate by indirect immunofluorescence (*2*) (Fig. 2). Whereas resting neutrophils did not degrade the fi-

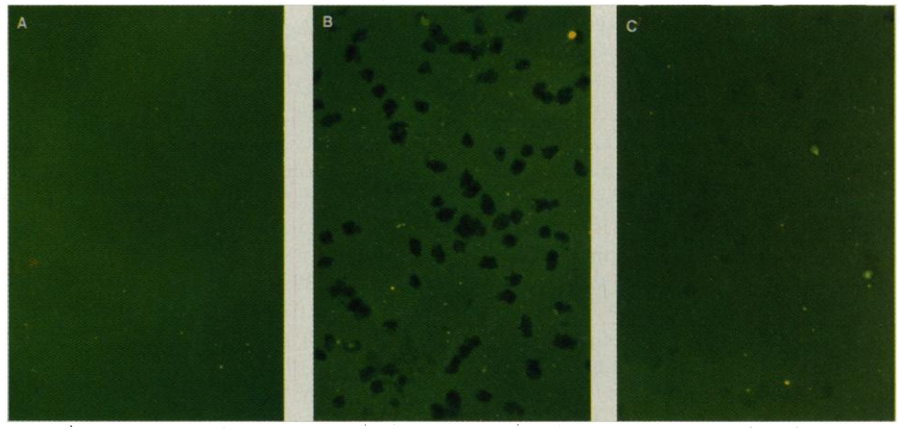

Fig. 2. Effect of rSLPI on fibronectin proteolysis by PMA-stimulated neutrophils as visualized by indirect immunofluorescence. Fibronectin-coated cover slips were incubated for 3 hours in 20% heat-inactivated plasma 37°C with (**A**) neutrophils alone, (**B**) neutrophils stimulated with PMA, or (**C**) neutrophils stimulated in the presence of rSLPI (100 μg/ml). The samples were then fixed and permeabilized with a solution of 3% paraformaldehyde and 0.5% Triton X-100, incubated with rabbit antiserum to human fibronectin (Collaborative Research) then with fluorescein-conjugated goat antibodies to rabbit immunoglobulin G (Boehringer Mannheim), and viewed by immunofluoresence microscopy. Phase-contrast microscopy revealed that areas of degradation were localized subjacent to triggered neutrophils (*2*). Identical results were obtained with unlabeled substrates. Magnification ×200.

Table 1. Effect of antiproteinases on fibronectin and elastin degradation. Neutrophils (1×10^6) or cytoplasts prepared without heat (*11*) were stimulated with PMA (50 ng/ml) atop cover slips coated with radiolabeled fibronectin or κ-elastin in the presence of 20% heat-inactivated plasma for 3 hours at 37°C. Some cells were also incubated in the presence of 5×10^{-6} M phosphoramidon (Sigma), 3×10^{-4} M NP-20 [$ClCH_2CO$ (N-OH)-DL-Phe-L-Ala-L-Ala-NH_2; Enzyme Systems Products], recombinant tissue inhibitor of metalloproteinases (50 μg/ml; Synergen), 3×10^{-4} M pepstatin A, 5×10^{-4} M leupeptin, 3×10^{-4} M E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane; Sigma], 2×10^{-5} M Ala-Ala-Pro-Val-chloromethylketone (AAPVCK; Enzyme Systems Products), 2×10^{-5} M methoxysuccinyl-Val-Pro-Phe-trifluoromethylketone (MeO-Val-Pro-Phe-FMK), or 2×10^{-5} M L-659, 166 (Merck Sharp & Dohme). DFP-treated neutrophils were prepared by incubating cells with 5 mM DFP for 60 min at 25°C, then washed five times. Treated cells generated normal amounts of hypochlorous acid, oxidatively activated collagenase, and gelatinase and migrated normally (*7*). In the cytoplast experiments, 2×10^6 cells were used, to account for their smaller surface area (*11*). Results are expressed as the mean counts per minute solubilized ± SD of the number of experiments listed in parentheses. ND; not determined.

Cells	Inhibitor	Substrate solubilized (cpm)			
		Fibronectin	(*n*)	κ-Elastin	(*n*)
Control neutrophils	None	7857 ± 503	(10)	1883 ± 234	(5)
	Phosphoramidon	8126 ± 488	(3)	1846 ± 188	(3)
	NP-20	8857 ± 192	(3)	1742 ± 139	(3)
	Tissue inhibitor of metalloproteinases	7667 ± 199	(3)	1902 ± 229	(3)
	Pepstatin	8235 ± 441	(3)	1619 ± 172	(3)
	Leupeptin	8244 ± 346	(3)	1810 ± 133	(3)
	E-64	8019 ± 617	(3)	1731 ± 217	(3)
	DFP	993 ± 617	(5)	213 ± 41	(5)
	AAPVCK and MeO-Val-Pro-Phe-FMK	7114 ± 507	(5)	1606 ± 161	(5)
	L-659,166 and MeO-Val-Pro-Phe-FMK	7243 ± 886	(5)	1639 ± 203	(5)
Cytoplasts	None	1347 ± 58	(3)	259 ± 81	(3)
SGD neutrophils	None	9852	(1)	ND	
CHS neutrophils	None	1890	(1)	ND	
CGD neutrophils	None	8209 ± 644	(3)	1814 ± 209	(3)

bronectin in the presence of 20% plasma (Fig. 2A; the immunoreactive fibronectin appears as a green lawn of fluorescent material), stimulated neutrophils rapidly solubilized the subjacent substrate in discrete zones, leaving darkened "holes" (Fig. 2B). In contrast, when plasma was supplemented with rSLPI (100 μg/ml or ~8.5 μM), the PMA-stimulated neutrophils adhered to the fibronectin-coated surface, but were almost completely prevented from clearing the underlying substrate (Fig. 2C). Similar results were obtained with κ-elastin. These qualitative findings were corroborated when degradation was monitored as solubilized radioactivity. At a concentration of 100 μg/ml, rSLPI inhibited both fibronectin and elastin degradation by 93.0 ± 5.2% (n = 22) and 88.5 ± 2.6% (n = 10), respectively, after a 3-hour incubation. Neither lysing the adherent neutrophils with 1% Triton X-100 nor boiling the recovered cover slips in 1% SDS released additional radioactivity. When the incubation period was lengthened to 18 hours, the inhibitory effect of rSLPI remained constant at 85.3 ± 4.7% for fibronectin and 89.3 ± 2.0% for elastin (n = 5) (*21*). Proteolysis was not inhibited when either heat-inactivated rSLPI (100 μg/ml) or lysozyme (100 μg/ml), a basic control protein whose size and pI are similar to those of SLPI (*20*), was substituted for the native inhibitor (n = 5). Finally, rSLPI did not interfere with the ability of stimulated neutrophils to generate oxidants, discharge granule-associated contents, or migrate in response to a chemotactic gradient (*22*).

In vivo, neutrophils can be stimulated to mediate tissue damage by a variety of soluble or particulate stimuli (*1, 8*). Although rSLPI was able to inhibit proteolysis initiated by a soluble stimulus, more intimate cell-substrate contact is believed to occur when neutrophils specifically adhere to an antibody-coated surface (*1, 2*). Nonetheless, rSLPI was almost equally effective with either stimulus (Fig. 3). Although complete protection of the subjacent substrates was not obtained, only 0.6 to 1.25 μg/ml of rSLPI (50 to 100 nM) was required for half-maximal inhibition of fibronectin or elastin degradation (Fig. 3, A and B) (*23*). Incubating the neutrophils with rSLPI for up to 30 min before the addition of either stimulus did not increase the effectiveness of the inhibitor, whereas cells incubated with SLPI, and then washed, expressed normal degradative activity. However, rSLPI could inhibit ongoing proteolysis. That is, when rSLPI (100 μg/ml) was added 15, 30, or 60 min after proteolysis was initiated, subsequent degradation was inhibited by 87.1 ± 4.3, 93.9 ± 4.6, and 87.6 ± 10.5%, respectively, for PMA-stimulated cells and 89.2 ± 8.0, 86.1 ± 3.6, and 89.6 ± 10.7%, respectively, for antibody-stimulated cells (n = 3). Thus, nanomolar concentrations of rSLPI exerted reversible and fast-acting inhibition with either PMA- or antibody-stimulated neutrophils.

To date, attempts to control neutrophil proteolysis have focused on efforts to identify nontoxic proteinase inhibitors that have been either isolated from various nonhuman sources or chemically synthesized (*3*). However, we have shown that neutrophil degradation of subjacent substrates in a plasma milieu can be regulated by the mucus-derived proteinase inhibitor, SLPI. Although SLPI bears no sequence similarity to other human proteins, it can reversibly inhibit a number of tissue-destructive serine proteinases, including the neutrophil enzymes, elastase and cathepsin G (*4*). Paradoxically, however, attempts to duplicate the protective effects exerted by SLPI with small molecular sized, synthetic inhibitors of neutrophil elastase, cathepsin G, or proteinase 3 have been unsuccessful. These results raise the possibility that rSLPI exerted a protective effect independently of its ability to inhibit proteinases, but in additional studies, active site variants of SLPI have been identified [prepared by site-specific mutagenesis (*24*)] that have completely lost their ability to regulate subjacent proteolysis (*25*). Because elastase, cathepsin G, and proteinase 3 are the only serine proteinases found in the primary granule (*26*) and SLPI does not inhibit proteinase 3 (*17*), we postulate that SLPI gains access to proteolytic zones inaccessible to other fluid-phase inhibitors, and binds elastase and cathepsin G at rates sufficient to protect subjacent substrates (*4, 19*). However, the possibility that SLPI inhibits one or more of the uncharacterized serine proteinases reported to be present in neutrophils cannot be ruled out (*27*). Although further studies are required to identify the proteinases targeted by SLPI, it appears that evolutionary pressures have conspired to generate a unique inhibitor of contact-dependent proteolysis whose activity cannot be mimicked by other plasma proteinase inhibitors. The availability of large quantities of rSLPI for in vivo testing and the potential to engineer even more effective mutant inhibitors could lead to the development of a new class of therapeutics capable of attenuating the tissue destruction associated with acute inflammatory disease states.

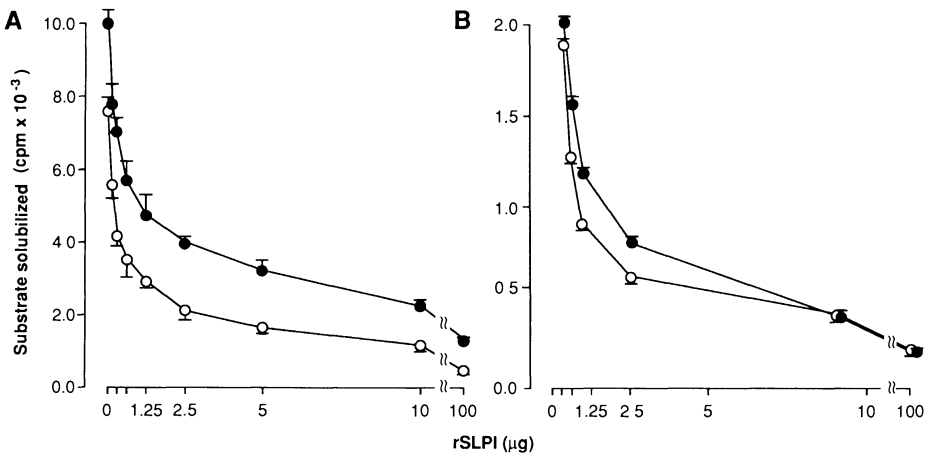

Fig. 3. Effect of rSLPI on fibronectin and elastin degradation by stimulated neutrophils. (**A**) Neutrophils (1×10^6) were suspended in 20% heat-inactivated plasma and stimulated atop fibronectin-coated cover slips with either PMA (○) or rabbit antiserum to human fibronectin (●) in the presence of the indicated dose of rSLPI for 3 hours at 37°C (n = 3). (**B**) Neutrophils were suspended in plasma as described above and stimulated atop elastin-coated cover slips with either PMA (○) or rabbit antibodies to bovine insoluble elastin (Elastin Products) (●) in the presence of the indicated dose of rSLPI (n = 3). Increasing the rSLPI concentration to 200 μg/ml did not inhibit proteolysis further (*29*).

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17. Purified proteinase 3 (*15*) was inhibited by either Ala-Ala-Pro-Val-chloromethylketone or L-659,166, but was unaffected by rSLPI at the highest concentration tested (100 μg/ml) (B. Gray, S. Regiani, S. Weiss, unpublished observation).
18. The more commonly used cathepsin G inhibitor, *N*-benzyloxycarbonyl-Gly-Leu-Phe-chloromethylketone, is toxic to neutrophils [Y. Sibille, N. Merrill, J. Cooper, Jr., L. Polomski, J. Gee, *Am. Rev Resp Dis.* **130**, 110 (1984)]. Methoxysuccinyl-Val-Pro-Phe-trifluoromethylketone is a nontoxic cathepsin G inhibitor characterized and synthesized by Merrell Dow Research Institute.
19. Although synthetic elastase inhibitors act irreversibly, their association rate constants ($M^{-1}s^{-1}$) are two to three orders of magnitude slower than that of α_1PI or SLPI [M. Padrines and J. Bieth, *Am Rev Resp. Dis* **139**, 199A (1989); (*4*)].
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21. The ability of rSLPI to inhibit proteolysis was confirmed with insoluble elastin. When 1×10^6 neutrophils were stimulated atop ^{3}H-labeled insoluble elastin [specific activity of 10^3 cpm/μg prepared as described in H A. Chapman, O. L. Stone, Z. Varin, *J Clin Invest* **73**, 806 (1984)] in the presence of 20% heat-inactivated plasma, 7630 ± 641 and 18,483 ± 980 cpm were solubilized at 3 and 18 hours, respectively. In the presence of rSLPI (100 μg/ml), solubilization was reduced to 867 ± 6 cpm at 3 hours and 2193 ± 207 cpm at 18 hours (n = 3). If the remaining elastin was treated with hot alkali to expose more subtle proteolytic damage [P. J. Stone *et al*, *J. Clin. Invest* **82**, 1644 (1988)], no increase in elastin solubility was detected.
22. In the absence or presence of rSLPI (100 μg/ml), PMA-stimulated neutrophils generated 49.9 ± 3.7 and 56 1 ± 7.8 nmol of superoxide per 10^5 cells per hour, respectively [performed as described in S. J. Weiss, S. T Test, C. M. Eckmann, D. Roos, S. Regiani, *Science* **234**, 200 (1986)], released 1.07 ± 0.04 and 1.14 ± 0.18 μg of lactoferrin per 10^6 cells per hour, respectively [performed as described in E. Wilson *et al*, *Arch Biochem Biophys* **259**, 204 (1987)], and cleaved 0.30 ± 0 03 and 0.34 ± 0.03 nmol (per 10^6 cells per hour) of the β-glucuronidase substrate, phenolphthalein, respectively (*ibid*). Similarly, the ability of 10^6 neutrophils to migrate across a type I collagen–coated filter in response to zymosan-activated plasma [performed as described in A. Huber and S. Weiss, *J. Clin Invest.* **83**, 1122 (1989)] was unaffected by the addition of rSLPI (that is, 60.5 ± 1.2 and 60.1 ± 2.3% of the neutrophils migrated through the collagen barrier and accumulated in the lower compartment after an 8-hour incubation in the absence and presence of SLPI, respectively). All values are reported as the means ± SD of three experiments.
23. Although SLPI is sensitive to oxidative damage [J. A. Kramps, Ch. van Twisk, E. C. Klasen, J. H. Dijkman, *Clin Sci* **75**, 53 (1988)], the protective effect exerted by the inhibitor was not enhanced by the addition of antioxidants to the plasma system.
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29. After submission of the manuscript, we found that neutrophils express greater proteolytic activity when suspended in Earle's balanced salt solution and incubated in a 5% CO_2/95% air atmosphere. Under these conditions, 1×10^6 PMA-stimulated neutrophils degraded 11,550 ± 1617 cpm in 20% heat-inactivated plasma and 12,260 ± 2028 cpm in 20% heat-inactivated serum after a 3-hour incubation (n = 5). In the presence of rSLPI (100 μg/ml), proteolysis was inhibited by ~80% (for example, 2395 ± 394 cpm and 2483 ± 477 cpm in heat-inactivated plasma and serum, respectively (n = 4).
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Type 1 Neurofibromatosis Gene: Identification of a Large Transcript Disrupted in Three NF1 Patients

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Von Recklinghausen neurofibromatosis (NF1) is a common autosomal dominant disorder characterized by abnormalities in multiple tissues derived from the neural crest. No reliable cellular phenotypic marker has been identified, which has hampered direct efforts to identify the gene. The chromosome location of the *NF1* gene has been previously mapped genetically to 17q11.2, and data from two NF1 patients with balanced translocations in this region have further narrowed the candidate interval. The use of chromosome jumping and yeast artificial chromosome technology has now led to the identification of a large (~13 kilobases) ubiquitously expressed transcript (denoted *NF1LT*) from this region that is definitely interrupted by one and most likely by both translocations. Previously identified candidate genes, which failed to show abnormalities in NF1 patients, are apparently located within introns of *NF1LT*, on the antisense strand. A new mutation patient with NF1 has been identified with a de novo 0.5-kilobase insertion in the *NF1LT* gene. These observations, together with the high spontaneous mutation rate of NF1 (which is consistent with a large locus), suggest that *NF1LT* represents the elusive *NF1* gene.

THE VARIABLE AND DIVERSE MANIFESTATIONS of neurofibromatosis (NF1) have puzzled clinicians and neurobiologists alike since the condition was first described by von Recklinghausen in 1882 (*1*). With an incidence of about 1 in 3000 in all ethnic groups (*2*), it is one of the most common autosomal dominant disorders of man.

Despite considerable efforts, it has not been possible to define a consistent abnormality in NF1 tissues that would provide sufficient information on the gene product to allow direct cloning of the gene. The remaining alternative has been to identify the *NF1* gene by positional cloning [formerly referred to as "reverse genetics" (*3, 4*), although this may be a misleading designator]. In this process, a gene is identified on

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