nature genetics

Mutations associated with neutropenia in dogs and humans disrupt intracellular transport of neutrophil elastase

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Cyclic hematopoiesis is a stem cell disease in which the number of neutrophils and other blood cells oscillates in weekly phases. Autosomal dominant mutations of *ELA2*, encoding the protease neutrophil elastase[1], found in lysosome-like granules, cause cyclic hematopoiesis[2] and most cases of the pre-leukemic disorder severe congenital neutropenia (SCN; ref. 3) in humans. Over 20 different mutations of neutrophil elastase have been identified, but their consequences are elusive, because they confer no consistent effects on enzymatic activity[4]. The similar autosomal recessive disease of dogs, canine cyclic hematopoiesis[5], is not caused by mutations in *ELA2* (data not shown). Here we show that homozygous mutation of the gene encoding the dog adaptor protein complex 3 (AP3) β-subunit, directing trans-Golgi export of transmembrane cargo proteins to lysosomes, causes canine cyclic hematopoiesis. C-terminal processing of neutrophil elastase exposes an AP3 interaction signal responsible for redirecting neutrophil elastase trafficking from membranes to granules. Disruption of either neutrophil elastase or AP3 perturbs the intracellular trafficking of neutrophil elastase. Most mutations in *ELA2* that cause human cyclic hematopoiesis prevent membrane localization of neutrophil elastase, whereas most mutations in *ELA2* that cause SCN lead to exclusive membrane localization.

Canine cyclic hematopoiesis (**Fig. 1a**) is known as gray collie syndrome, because it arose in this breed and affected dogs have hypopigmented coats. Canine cyclic hematopoiesis resembles Hermansky–Pudlak syndrome, type 2 (HPS2), a human disorder of partial albinism and neutropenia caused by homozygous mutation of *AP3B1*, encoding the AP3 β-subunit (β3a; refs. 6,7). After excluding canine *ELA2* (data not shown), we tested *AP3B1* as a candidate gene for canine cyclic hematopoiesis. We cloned the dog homolog of *AP3B1* cDNA, comprising 26 exons and 3,964 bp, and mapped the gene to autosome 3. Flanking polymorphic microsatellite markers indicate linkage (lod > 9) and linkage disequilibrium in unrelated pedigrees (**Fig. 1b**). Genomic sequencing identified one coding alteration: insertion of an adenine in a tract of nine adenine residues in exon 20 (**Fig. 1c**), causing frameshift and premature termination. Northern blotting (**Fig. 1d**) indicated that the resultant mRNA is subject to nonsense-mediated decay, with 50% less mRNA in carriers and no mRNA in affected dogs, confirming mutation in *AP3B1* as the cause of canine cyclic hematopoiesis.

AP3 is one of four tetrameric adaptor protein complexes of animal cells that coat the cytoplasmic surfaces of membrane-bound vesicles and direct protein trafficking[8]. AP3 specifically shuttles transmembrane 'cargo' proteins from the trans-Golgi to lysosomes[9]. Luminal cargo proteins extrude a short C-terminal tail through the lipid bilayer of vesicles and associate with μ-subunits in the cytosol through tyrosine-based sorting signals[10] or with β-subunits through dileucine sorting signals[11]. The μ-subunits become degraded when β-subunits are deficient in humans and mice[6,12] with mutations in *AP3B1*. We found that the mutation in *AP3B1* that causes canine cyclic hematopoiesis also leads to absence of both β3a and μ3a (data not shown).

Because mutations in *AP3B1* and *ELA2* cause similar illnesses in humans, we reasoned that AP3 recognizes neutrophil elastase as a cargo protein and normally traffics it to granules. We therefore determined whether human neutrophil elastase interacts with either of the two AP3 subunits, μ3a or β3a, that contact cargo proteins and are deficient in canine cyclic hematopoiesis, using a yeast two-hybrid assay established for this purpose[13]. In addition to N-terminal pre-pro sequences, neutrophil elastase contains a proteolytically processed 20-residue C-terminal extension[14] of previously unknown function but not required for proteolytic activity or granule localization[4,14]. To avoid complexities related to the N-terminal processing of neutrophil elastase and its antifungal antibiotic properties, we developed baits consisting of the distal half of human neutrophil elastase, extending from Pro110 and comprising one of its two cylindrical domains, with (NE-P110) or without (NE-P110ΔC) the C-terminal extension. Lysosomal membrane protein LAMP-2 served as a positive control[13].

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Published online 3 August 2003; doi:10.1038/ng1224

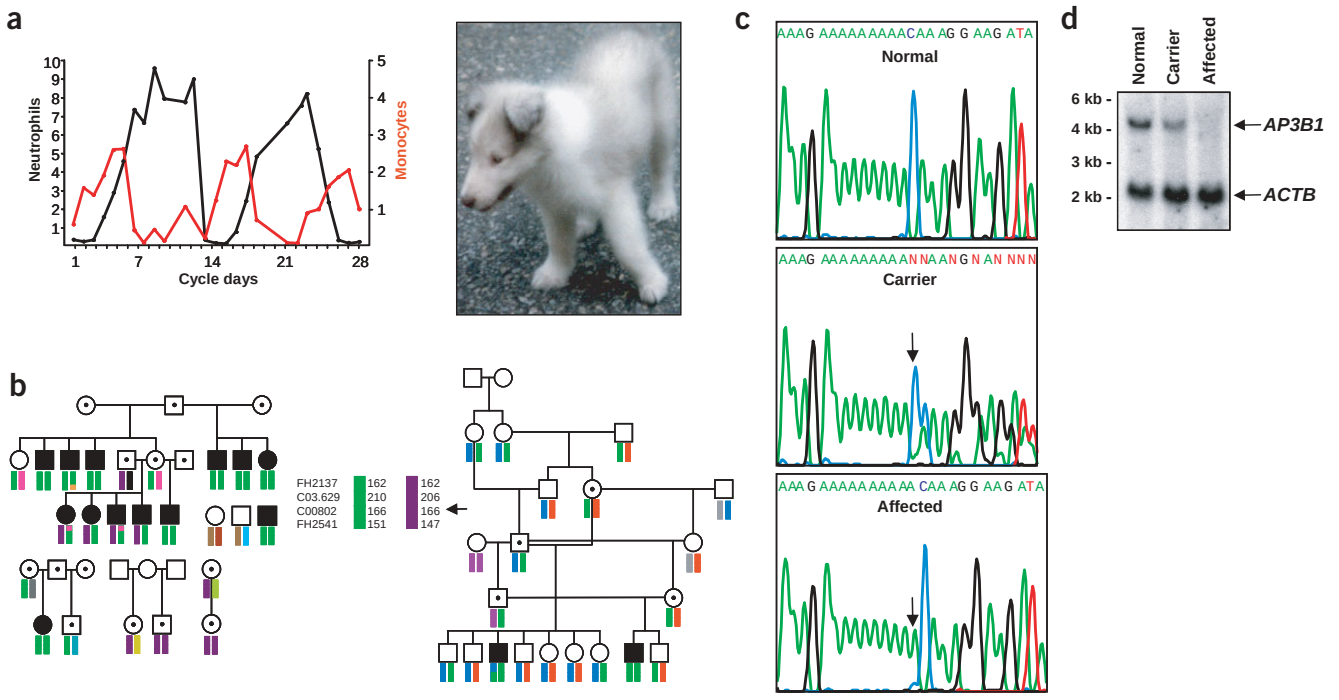

Figure 1 Mutation of *AP3B1* causes canine cyclic hematopoiesis. (**a**) Phenotype of canine cyclic hematopoiesis[5], showing 2-week reciprocal cycles of neutrophil and monocyte production (number of cells × 10^{-3} per μl) and gray coat. (**b**) Canine cyclic hematopoiesis pedigrees. Dots indicate carriers (identified by affected offspring). Each color represents a unique haplotype. Arrow shows region of linkage disequilibrium. Numbers refer to sizes (in nucleotides) of alleles for each marker. (**c**) Genomic DNA electropherogram. Arrows indicate A-insertion mutation. (**d**) Northern blot of total RNA from blood, showing reduced *AP3B1* transcript compared to *ACTB* control.

NE-P110ΔC interacted with μ3a but not β3a (**Fig. 2a**), whereas NE-P110 did not interact with either subunit. As there is only one tyrosine residue in the interacting neutrophil elastase fragment, the sequence LYPDA starting at position 198 is probably a μ3a sorting signal.

To confirm this as an interaction signal, we carried out two experiments. First, we shortened the bait to begin at Pro176 or Ser196 and found that NE-P176ΔC (**Fig. 2a**) and NE-S196ΔC (data not shown) continued to specifically interact, indicating that the signal must be present in the final 23 residues of C-terminally processed neutrophil elastase. Second, we introduced a Y199A substitution and found that it abolished the interaction of either bait, NE-P176ΔC-Y199A (**Fig. 2a**) or NE-S196ΔC-Y199A (data not shown). These results indicate that the processed form of neutrophil elastase interacts *in vitro* with the μ3a subunit of AP3 through a tyrosine-based sorting signal in neutrophil elastase and that the C-terminal extension seems to block interaction with AP3.

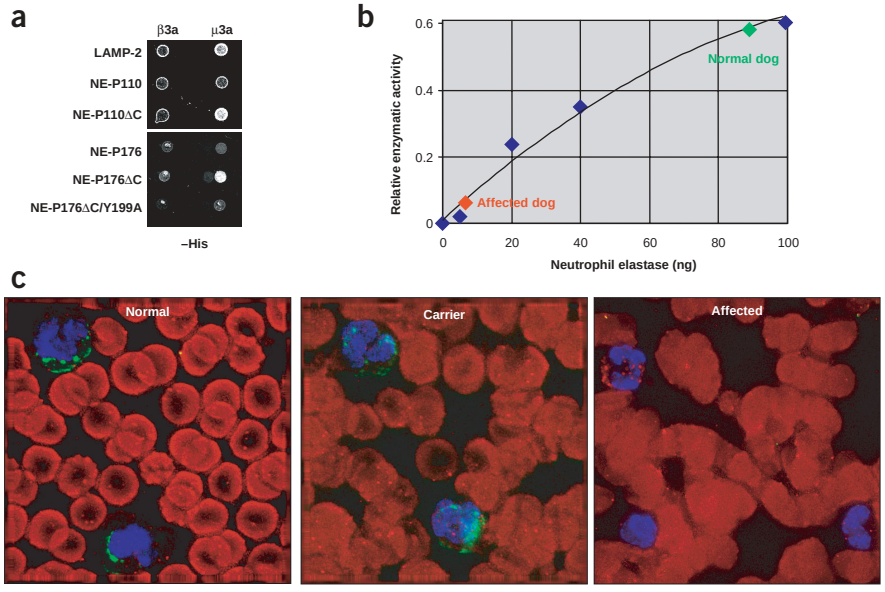

Figure 2 Interaction of AP3 and neutrophil elastase. (**a**) Yeast two-hybrid analysis on selective (–His) medium, showing μ3a interaction with the tyrosine-based sorting signal in neutrophil elastase. (**b**) Near absence of neutrophil elastase enzymatic activity in affected dog (red) compared to normal (green) on a standard curve (blue). (**c**) Absence of neutrophil elastase in dog blood films. Restoration three-dimensional microscopy of indirect immunofluorescence detection of neutrophil elastase (fluorescein, green), with counterstaining of granules and nuclei by LAMP-1 (phycoerythrin, red) and DAPI (blue), respectively. (Uniform staining of erythrocytes results from cross-reaction with the phycoerythrin-conjugated secondary antibody.)

To determine if neutrophil elastase and μ3a associate intracellularly, we tested for deficiency of neutrophil elastase in neutrophils from dogs with canine cyclic hematopoiesis. We measured neutrophil elastase activity on a specific peptide substrate (**Fig. 2b**) and found that neutrophils from affected dogs have only 8% of the enzymatic activity of normal dogs, despite having a normal *ELA2* genotype. Immunofluorescence staining (**Fig. 2c**) of neutrophils from dogs with canine cyclic hematopoiesis showed a corresponding paucity of neutrophil elastase (green) and reduced granular staining for lysosomal membrane protein LAMP-1 (red). (LAMP-1 is also an AP3 cargo protein[6], but granules containing LAMP-1 are distinct from granules containing neutrophil elastase[15].) These observations suggest that AP3 influences the subcellular trafficking of neutrophil elastase.

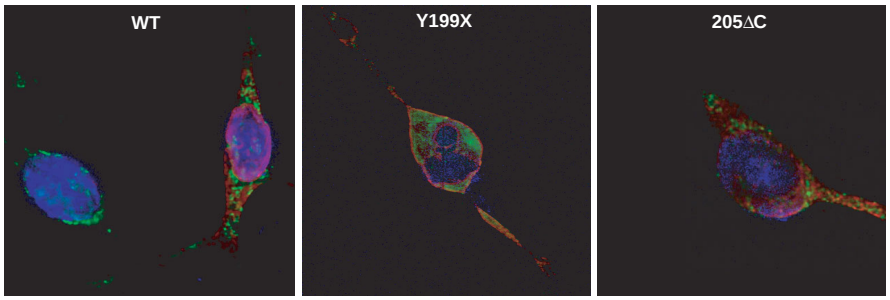

Figure 3 Mislocalization of neutrophil elastase in mutants lacking the μ3a sorting signal and C-terminal extension (Y199X) or just the C-terminal signal (205ΔC) relative to wild-type (WT). Restoration three-dimensional microscopy of transiently transfected RBL cells, imaged by indirect immunofluorescence of neutrophil elastase (rhodamine, red) and LAMP-1 (fluorescein, green), with DAPI (blue) counterstaining of nuclei.

The most common class of mutations in *ELA2* associated with SCN prematurely truncate the protein near the C terminus just ahead of the proposed tyrosine-based μ3a sorting signal[16], suggesting that they could disrupt interactions with AP3. To examine the effect of these mutations, we used immunofluorescence to detect neutrophil elastase with intact N and C termini in RBL cells (rat basophils containing granules lacking endogenous neutrophil elastase[4]) transiently transfected with *ELA2* cDNA. We stained neutrophil elastase (red) and counterstained LAMP-1 (green) and nuclei (with DAPI, blue). Wild-type neutrophil elastase appeared predominately in granules and also in a perinuclear distribution (**Fig. 3**). We also tested two mutant constructs: the Y199X mutation removes the proposed sorting signal and C-terminal extension, and the engineered mutation 205ΔC, corresponding to a neutropenic frameshift mutation, retains the predicted sorting signal but lacks the C-terminal extension. Y199X was absent in granules and appeared instead in the plasma membrane and nuclear envelope, whereas 205ΔC had an accentuated granular pattern (**Fig. 3**). These results are consistent with observations in AP3-deficient human and mouse cells[6,17], in which lysosomal cargo proteins are incorrectly routed to the plasma membrane. We conclude that AP3 directs neutrophil elastase to granules through the μ3a tyrosine-based sorting signal and that the most commonly occurring

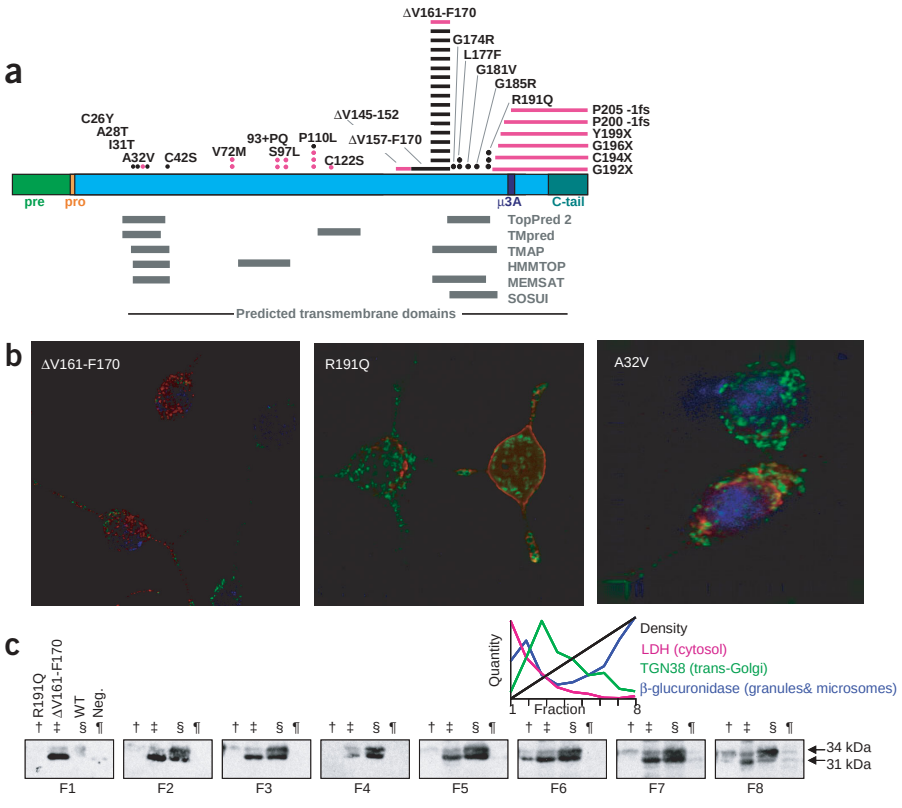

Figure 4 Mutations of neutrophil elastase that cause cyclic hematopoiesis generally accentuate granule accumulation, whereas those that cause SCN generally accentuate membrane accumulation. (**a**) Primary neutrophil elastase sequence, showing general alignment of mutations that cause cyclic hematopoiesis and SCN (in black and pink, respectively) with transmembrane domains and with the μ3a sorting signal. Dots and lines represent amino acid missense substitutions and deletions, respectively. Each marker represents the independent occurrence of the mutation. Predicted transmembrane segments are aligned with corresponding algorithm. (**b**) Subcellular distribution of proteins with transmembrane domain mutations showing reduced or accentuated granule accumulation depending on the effect of the proposed transmembrane domain. Restoration three-dimensional microscopy of transiently transfected constructs, imaged by indirect immunofluorescence of neutrophil elastase (red) and LAMP-1 (green), with DAPI (blue) counterstaining of nuclei. (**c**) Retention of C terminus of neutrophil elastase in mutants localizing to membranes and preferential processing of C terminus in mutants localizing to granules. Subcellular localization of mutant neutrophil elastase detected by western blotting of stably transfected RBL cells fractioned by density-gradient centrifugation. Negative control (Neg) was transfected with wild-type *ELA2* (WT) but uninduced. Inset shows density increasing across fractions with cellular compartment identification.

class of mutations associated with SCN deletes the sorting signal, causing mislocalization of neutrophil elastase.

Neutrophil elastase is a soluble disulfide-bonded protein primarily found in the lumen of granules[1], and AP3 coats the cytoplasmic surface of vesicles[8]. Because the two proteins reside on opposite sides of a membrane, interaction between the two—as our results suggest—requires neutrophil elastase to cross the membrane. We therefore searched the human neutrophil elastase sequence with eight transmembrane prediction algorithms[18] (**Fig. 4a**). Most, but not all, of the programs agree on the placement of two transmembrane domains—one just before the proposed sorting signal and another near the N terminus (but distinct from the signal sequence). This is not completely unexpected, because numerous biochemical and immunolocalization studies[19–24] have convincingly detected neutrophil elastase in the plasma membrane, granule membrane, nuclear envelope and trans-Golgi membranes.

Notably, most mutations in *ELA2* that are primarily associated with human cyclic hematopoiesis align with the predicted transmembrane domains (**Fig. 4a**). Therefore, we determined whether the mutations in the predicted transmembrane domains alter subcellular trafficking. We expressed wild-type neutrophil elastase and the mutants R191Q and ΔV161-F170 in RBL cells. We expected these two mutants to have opposite effects on the putative distal transmembrane domain, because ΔV161-F170 deletes part of the predicted transmembrane domain, whereas R191Q replaces a charged sidechain, improving its hydrophobicity. Immunofluorescence staining of transiently transfected cells showed that ΔV161-F170 tracked exclusively to granules, whereas R191Q localized only to the plasma membrane (**Fig. 4b**). We also examined mutations in the other proposed transmembrane domain; the neutrophil elastase mutation A32V, causing cyclic hematopoiesis, similarly had an exclusively granular distribution (**Fig. 4b**).

To independently corroborate these results, we carried out western blotting of subcellular fractions purified by density-gradient centrifugation from stably transfected cells (**Fig. 4c**). We found that wild-type neutrophil elastase existed in two isoforms, of 34 and 31 kDa, representing neutrophil elastase with and without the C-terminal extension[14], respectively. Wild-type neutrophil elastase lacking the C-terminal extension predominated in fraction 2, where the soluble contents of granules sediment. Wild-type neutrophil elastase retaining the C-terminal extension was in excess in fraction 8, which is the most dense and contains the membrane. Thus, the isoform retaining the C terminus preferentially localizes to the membrane. Presumably, the intact C terminus blocks the interaction between the sorting signal and AP3 ordinarily required for diversion of cargo proteins to the granule from their default destination to membrane compartments. The distribution of the proposed transmembrane domain mutations further supports this conclusion. The ΔV161-F170 mutant showed a preponderance of neutrophil elastase lacking the C-terminal extension across all fractions (**Fig. 4c**), with the greatest concentration in the first two fractions, composed of soluble granule contents. In contrast, R191Q mostly retained the C-terminal extension and appeared in the densest, membrane-containing fractions (**Fig. 4c**). Disruption of a proposed transmembrane domain therefore seems to favor both the processing of the C terminus and its consequent distribution to the granule, whereas improvement of the proposed

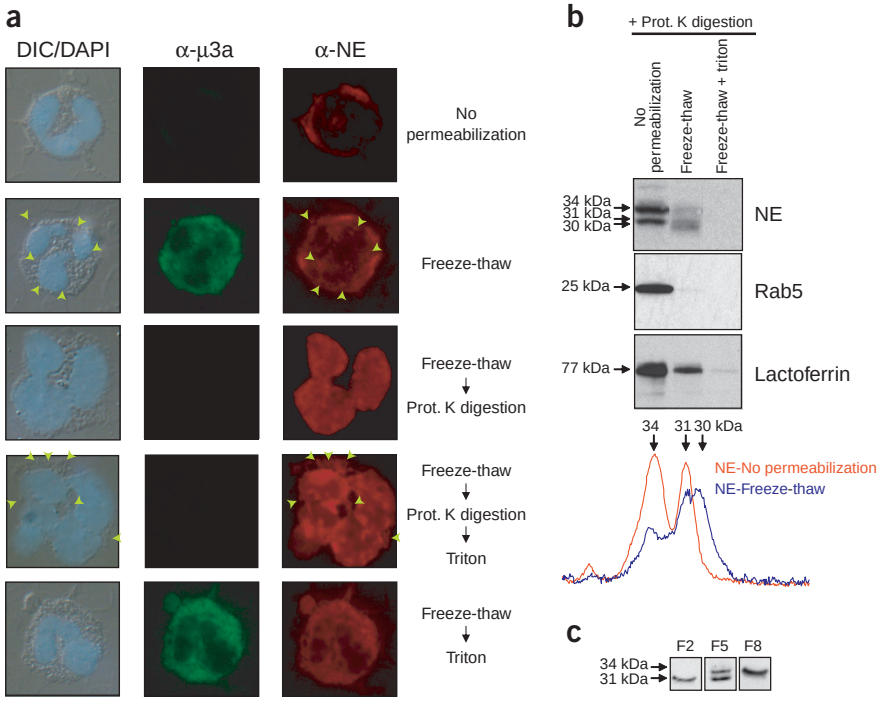

Figure 5 Transmembrane properties of neutrophil elastase identified by protease protection assays in selectively permeabilized cells. (**a**) Cytoplasmic accessibility of neutrophil elastase by antibodies or proteinase K. DIC images with DAPI staining of nuclei and indirect immunofluorescence of μ3a (fluorescein, green) and neutrophil elastase (NE; rhodamine, red) are shown in columns. Normal human neutrophils in peripheral blood films are shown without permeabilization, after selective freeze-thaw permeabilization of the plasma membrane with or without digestion by proteinase K, and after complete permeabilization with freeze-thaw fracture succeeded by treatment with Triton detergent, with or without proteinase K as an intervening step are shown in rows. Cytoplasmic, especially granular, neutrophil elastase is stained (second row, arrows showing granules) when antibodies are applied through the cytoplasm and destroyed by proteinase K treatment through the cytoplasm (third row). But neutrophil elastase in the lumen of granules is protected from proteinase K digestion, and staining (arrows, fourth row) becomes evident once more with complete permeabilization in Triton detergent. (**b**) Selective cleavage of the 34-kDa isoform of neutrophil elastase (NE) containing the C terminus by proteinase K in the cytoplasm. Western blots of normal human neutrophils treated with proteinase K after no permeabilization, selective freeze-thaw fracture of the plasma membrane or complete membrane permeabilization with freeze-thaw treatment followed by treatment with Triton detergent. Inset shows densitometric analysis of two left lanes in top panel. Antibody detection of neutrophil elastase shows greater vulnerability to proteinase K of the 34-kDa isoform containing the C terminus (the proposed transmembrane form), yielding a 30-kDa digestion product in cells in which the plasma membrane is selectively permeabilized. In contrast, the non-transmembrane, but membrane-associated, cytoplasmic protein Rab5 is completely degraded and the granule protein lactoferrin is protected from digestion in cells in which the plasma membrane is selectively permeabilized. Intervening lanes edited from upper panel. (**c**) Subcellular localization of the two neutrophil elastase isoforms in U937 cells fractionated by density centrifugation. Fraction numbering corresponds to **Figure 4c**.

transmembrane domain properties favors preservation of the C terminus and its retention in membranes.

To confirm that neutrophil elastase is a transmembrane protein, we carried out two experiments to determine its vulnerability to proteolytic digestion in selectively permeabilized cells. First, we determined the subcellular distribution of neutrophil elastase in normal human neutrophils with indirect immunofluorescence after selective permeabilization of subcellular compartments (**Fig. 5a**). Antibodies detected some neutrophil elastase (red) on the surface of non-permeabilized neutrophils, in accord with previous observations of neutrophil elastase on the plasma membrane, but no μ3a (green), as expected for a cytoplasmic protein (**Fig. 5a**).

To selectively permeabilize only the plasma membrane without disrupting lysosomal membranes, we subjected the cells to an established freeze-thaw fracture method[25] used for determining the topology of transmembrane proteins (**Fig. 5a**). Although this method distorts subcellular architecture more than conventional fixation methods, it shows enhanced staining for both μ3a and neutrophil elastase (including in granules), indicating that both proteins are accessible to antibodies through the exposed cytoplasm. After incubation with proteinase K, however, cytoplasmic staining of μ3a and neutrophil elastase disappeared, but neutrophil elastase persisted on the nuclear envelope (**Fig. 5a**). When these proteinase K–treated cells were then subjected to treatment with Triton detergent, which disrupts all membranous compartments, including granules, granular staining of neutrophil elastase (arrows) was once again evident, but μ3a staining remained absent (**Fig. 5a**). In contrast, freeze-thaw fracture followed by Triton detergent, without the intervening proteinase K treatment, detected neutrophil elastase in both granules and membranes (**Fig. 5a**).

μ3a was only detected using the cytoplasmic approach, as expected for a cytoplasmic protein. But neutrophil elastase in granules, and probably other membrane bound vesicles, was detected by antibodies applied from either the luminal or cytoplasmic surfaces, as expected for a transmembrane protein. Moreover, granular staining of neutrophil elastase disappeared after treatment of the cytoplasm with proteinase K, also as expected for a transmembrane protein.

As a second test of the transmembrane properties of neutrophil elastase, we selectively permeabilized normal human neutrophils, treated them with proteinase K and then analyzed the lysates by western blotting with antibodies directed against neutrophil elastase and other proteins whose locations are representative of different subcellular compartments (**Fig. 5b**). Neutrophil elastase in non-permeabilized cells is protected from cleavage by proteinase K, appearing in isoforms of 34 and 31 kDa, with or without the C terminus, respectively (**Fig. 5b**). But treatment with proteinase K of cells whose plasma membrane was selectively permeabilized by freeze-thawing[25] shows digestion of both the 34-kDa form containing the C terminus and the 31-kDa form lacking the C terminus, as well as the appearance of an additional band of 30 kDa. Because the intensity of the 34-kDa band was more substantially reduced than that of the 31-kDa band, we presume that the new 30-kDa band primarily represents a digestion product of the 34-kDa isoform (**Fig. 5b**). Complete permeabilization, by freeze-thawing, treatment with Triton detergent and then treatment with proteinase K, completely digested both forms of neutrophil elastase (**Fig. 5b**).

The most likely explanation for this is that the 34-kDa isoform containing the C terminus is in a transmembrane configuration in subcellular organelles and exposes a cytoplasmic portion accessible to digestion by proteinase K in cells in which only the plasma membrane is selectively permeabilized. Given apparent digestion of the 31-kDa band, it is also possible that this isoform lacking the C-terminal extension may traverse the membrane, albeit to a lesser extent than the 34-kDa species. Alternatively, the digestion of the 31-kDa band may be non-specific, because the freeze-thawing could unintentionally disrupt granule membranes.

As controls, we analyzed the same lysates by western blotting for the non-transmembrane (but membrane-associating) cytoplasmic protein Rab5 (ref. 26) and the granule protein lactoferrin[27]. Rab5 was completely degraded by proteinase K after selective plasma membrane permeabilization. Contrastingly, although there was some non-specific lactoferrin digestion after freeze-thaw permeabilization (probably arising from unintentional disruption of granules), lactoferrin was largely protected from digestion by proteinase K because of its location in the granule lumen. Lactoferrin was more vulnerable to digestion after complete permeabilization with addition of Triton detergent. These results substantiate the hypothesis that the 34-kDa isoform containing the C terminus is in a transmembrane configuration in subcellular organelles. Subcellular fractionation studies in human monocytic U937 cells further support this conclusion. The 34-kDa species appeared in the densest, membrane-containing fraction, whereas the 31-kDa form resided in the lighter fractions containing soluble granule contents; intermediate fractions contained both forms (**Fig. 5c**).

This model suggests that previous observations of accumulation of neutrophil elastase in membranes is a result of its transmembrane conformation. The two predicted transmembrane domains reside in surface loops pinned at their bases by disulfide bonds. Conceivably, these transmembrane domains could become accessible with alternate folding under reducing conditions. Alternatively, as with membrane forms of cytochrome P450 (ref. 28), these hydrophobic loops could unidirectionally insert themselves into the membrane.

We thus propose that neutrophil elastase may adopt soluble or transmembrane conformations. During normal processing of neutrophil elastase, an intermediate transmembrane form retaining the C-terminal extension traverses the trans-Golgi membrane with the C terminus projecting into the cytosol. After cleavage of the C terminus, the protein remains membrane-associated and binds to AP3. Release of the cleaved protein from the membrane into the lumen occurs on transport to granules. Loss of the sorting signal or failure to remove the 20-residue C-terminal pro-sequence prevents interaction with AP3 and causes routing to membranes, the default destination for cargo proteins in the absence of AP3 (refs. 6,17). Deficiency of AP3 in canine cyclic hematopoiesis prevents normal trafficking of neutrophil elastase to granules and misdirects it to membranes. In most cases of SCN, deletion of the sorting signal from neutrophil elastase prevents its interaction with AP3 and results in similar misrouting. In most cases of human cyclic hematopoiesis, disruption of a transmembrane domain favors a soluble conformation of neutrophil elastase that resides in granules.

METHODS

Genetic linkage, physical mapping and mutational analysis. We extracted DNA from peripheral venous blood of dogs using protocols approved by the Animal Care Committee at the Auburn University College of Veterinary Medicine and the Cornell University College of Veterinary Medicine. We amplified by PCR microsatellite marker oligonucleotides (Operon) fluorescently labeled at the 5′ end with 6-FAM and 5-HEX and analyzed them using capillary electrophoresis (ABI). We ordered markers with respect to the canine cyclic hematopoiesis phenotype by applying the canine-hamster radiation hybrid panel (Research Genetics) using the integrated dog map. We isolated canine *ELA2* and *AP3B1* cDNA by RT–PCR amplification from total RNA from blood using primers to conserved regions along with 5′- and 3′-RACE using GeneRacer (Invitrogen). We extracted RNA from blood using RNeasy (Qiagen) and reverse-transcribed it with Omniscript (Qiagen). We delineated *AP3B1* exon boundaries with the Universal GenomeWalker kit (BD Biosciences). We sequenced both DNA strands using ABI Big Dye terminator chemistry.

Northern-blot analysis. We isolated total RNA from blood, resolved 5 μg of each sample by electrophoresis on a 1.0% agarose formaldehyde-denaturing gel, blot-transferred the RNA to a nylon membrane (BrightStar-Plus, Ambion) and immobilized it by ultraviolet cross-linking. We carried out hybridization in Ultrahyb (Ambion) at 42 °C using an 800-bp ^{32}P-labeled probe corresponding to canine *AP3B1* cDNA nucleotides 3,129–3,927. We rehybridized washed filters with a ^{32}P-labeled probe complementary to *ACTB* to control for loading.

Yeast two-hybrid analysis. We fused *ELA2* cDNA to the Matchmaker (Clontech) Gal4 DNA-binding domain vector pGBKT7. μ3a and β3a Gal4 activation domain and LAMP-2 Gal4 DNA binding domain constructs[13] were gifts of X. Zhu and J. Bonifacino (National Institutes of Health, Bethesda, Maryland, USA). We screened for double transformants in AH109 *S. cerevisiae* as described[13].

Immunofluorescence. We grew RBL cells on cover slips, transiently transfected them with *ELA2* cDNA expression vectors, fixed them with methanol and processed them for indirect immunofluorescence staining of neutrophil elastase as described[4]. We counterstained with LY1C6 mouse monoclonal antibody to rat LAMP-1 (Calbiochem) at 1:250 with secondary detection with goat antibody to mouse conjugated with fluorescein (1:500; Jackson Research). We fixed dog peripheral blood films with methanol and processed them similarly, except we used goat antibody to rabbit conjugated with fluorescein and goat antibody to mouse conjugated with phycoerythrin (both 1:400; Pharmingen) for secondary detection of neutrophil elastase and LAMP-1, respectively, to minimize background cross-reaction. (No antibody among those tested specifically detected canine μ3a.) We used a Deltavision (Applied Precision) restoration three-dimensional microscope with a Zeiss 63× infinity-corrected plan-apochromat objective for imaging.

Subcellular fractionation. We used the Tet-On system (Clontech) to stably express *ELA2* cDNA in RBL cells. We first stably transfected RBL cells with the Tet-On vector using G418 selection and then co-transfected G418-resistant cells with *ELA2* cDNA pTRE2 expression vectors and pTK-hygro under hygromycin selection. We induced *ELA2* expression with 1 μg ml^{-1} doxycycline. We resuspended 10^6 RBL cells or U937 human monocytic cells in 1.5 ml of 0.34 M sucrose, 10 mM HEPES buffer pH 7.3 and 0.3 mM EDTA, applied 50 strokes with a Dounce homogenizer in the presence of 1 mg ml^{-1} Pefabloc (Roche) and then centrifuged at 700*g* for 10 min. We layered the supernatant on top of 6 ml of 20% Percoll (Amersham) in 15 mM HEPES buffer pH 7.3 and 0.25 M sucrose, centrifuged at 32,000*g* and 4 °C for 1 h in a Sorvall SM-24 rotor and then collected fractions of 0.8 ml from the top. We extracted granule contents by treatment with 0.3% hexadecyltrimethyl-ammonium bromide (CTAB), 150 mM NaCl and 5 mM HEPES buffer pH 7.4 on ice for 1 h and cleared them by centrifugation at 14,000*g* and 4 °C for 1 h. We carried out western blotting of neutrophil elastase as described[4]. We measured lactate dehydrogenase activity, to identify cytosolic fractions, using the Sigma LDH assay kit. We measured β-glucuronidase, whose activity has a dual localization in lysosomes and microsomal membranes[29], fluorometrically. To localize trans-Golgi fractions, we carried out western blotting with mouse monoclonal antibody to TGN38 (Pharmingen).

Selective cell permeabilization assays. The assay is based on a modification of the method of Mardones and Gonzalez[25]. We freshly collected 5 ml peripheral blood from healthy human donors into EDTA preservative tubes by peripheral venipuncture; red blood cells lysis took place with 1× hemolytic buffer (150 mM NH_4Cl, 12 mM $NaHCO_3$) and white cells pelleted with centifugation for 5 min at 1,000*g*. We resuspended the pellet in 600 μl 1× phosphate-buffered saline and subjected aliquots of 150 μl to one of the following three processes: no treatment, one freeze-thaw cycle (liquid nitrogen for 1 min followed by 37 °C until thawed) or 0.2% Triton X (final concentration) for 5 min at 37 °C. We incubated the cells with proteinase K (100 μg ml^{-1}) for 30 min at 37 °C and then inactivated the proteinase K with phenylmethylsulfonyl fluoride (1 mM). We lysed the cells by adding an equal volume of 2× lysis buffer (100 mM Tris-Cl pH 8.0, 8% glycerol, 2% Triton X, 1% Nonidet-P40 and 3 μg ml^{-1} each of pepstatin and aprotinin) and subjected them to three freeze-thaw cycles and centrifugation (15,000*g* for 15 min at 4 °C).

For western blotting, we first denatured samples at 100 °C for 3 min in 1× sodium dodecyl sulfate (SDS) gel loading buffer (50 mM Tris-Cl, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100 mM dithiothreitol), separated them by SDS–PAGE on a Novex 12% Tris-glycine gel (Invitrogen) at 160 V for 1.5 h, and electroblotted them overnight onto nitrocellulose (Biorad). All immunodetections took place in 5% non-fat milk, 1× Tris-buffered saline and 0.1% Tween with 1 h of blocking, 1 h of incubation with primary antibody (1:1,000) and 1 h of incubation with secondary antibody (1:10,000) followed by two 20-min washes with 1× Tris-buffered saline and 0.1% Tween and detection with the ECL system (Amersham Biosciences). We used the following primary antibodies: rabbit polyclonal antibody to neutrophil elastase (Calbiochem), rabbit polyclonal antibody to lactoferrin (United States Biological) and mouse monoclonal antibodies to μ3A and Rab5 (BD Biosciences Pharmingen). Species-specific secondary antibodies conjugated with horseradish peroxidase were from Jackson Research.

We made blood films on glass slides with 1 μl of normal human peripheral blood and air-dried them. We carried out freeze-thaw treatment of slides, fixation and immunofluorescence staining as described by Mardones and Gonzalez[25]. We carried out proteinase K digestion on cells after freeze-thaw treatment and fixation. After the final washing with phosphate-buffered saline, we mounted samples in Crystal Mount (Biomeda) and imaged them with epifluorescence and differential interface contrast (DIC) on a Zeiss Axioskop microscope with a 63× plan-apochromat objective.

Neutrophil elastase activity assay. We measured neutrophil elastase activity in extracts of 10^5 mononuclear cells from freshly drawn peripheral dog blood purified on Ficoll-Hypaque density gradients spectrophotometrically on the specific substrate suc-Ala-Ala-Ala-pNa as described[4].

Transmembrane domain prediction. Transmembrane prediction algorithms (TopPred, TMpred, TMAP, HMMTOP, MEMSAT, DAS, PRED-TMR2 and SOSUI; ref. 18) used World Wide Web interfaces (see URLs).

GenBank accession numbers. Canine *ELA2*, AY221639; canine *AP3B1*, AY221640.

URLs. Integrated Dog Map is available at http://www-recomgen.univ-rennes1.fr/Dogs/maquette-1800.html. Transmembrane prediction algorithms are available online at the following sites: TopPred 2, http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html; TMpred, http://www.ch.embnet.org/software/TMPRED_form.html; TMAP, http://workbench.sdsc.edu/, HMMTOP at http://www.enzim.hu/hmmtop/; MEMSAT, http://saier-144-37.ucsd.edu/memsat.html; DAS, http://www.sbc.su.se/~miklos/DAS/; PRED-TMR2, http://biophysics.biol.uoa.gr/PRED-TMR2/; SOSUI, http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html.

ACKNOWLEDGMENTS

We thank J. Miller for help with microscopy, E. Ostrander for sample conveyance, G. Cuneo for collie photo, X. Zhu and J. Bonifacino for reagents and advice and M. Gelb, E. Davie and the late H. Neurath for critical discussion. This work was supported by grants from the US National Institutes of Health, Doris Duke Foundation (*in vitro* studies only) and Burroughs-Wellcome (to M.H.).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 12 May; accepted 14 July 2003
Published online at http://www.nature.com/naturegenetics/

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