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Targeting of proteins to granule subsets is determined by timing and not by sorting: The specific granule protein NGAL is localized to azurophil granules when expressed in HL-60 cells

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ABSTRACT **The mechanism of protein targeting to individual granules in cells that contain different subsets of storage granules is poorly understood. The neutrophil contains two highly distinct major types of granules, the peroxidase positive (azurophil) granules and the peroxidase negative (specific and gelatinase) granules. We hypothesized that targeting of proteins to individual granule subsets may be determined by the stage of maturation of the cell, at which the granule proteins are synthesized, rather than by individual sorting information present in the proteins. This was tested by transfecting the cDNA of the specific granule protein, NGAL, which is normally synthesized in metamyelocytes, into the promyelocytic cell line HL-60, which is developmentally arrested at the stage of formation of azurophil granules, and thus does not contain specific and gelatinase granules. Controlled by a cytomegalovirus promoter, NGAL was constitutively expressed in transfected HL-60 cells. This resulted in the targeting of NGAL to azurophil granules as demonstrated by colocalization of NGAL with myeloperoxidase, visualized by immunoelectron microscopy. This shows that targeting of proteins into distinct granule subsets may be determined solely by the time of their biosynthesis and does not depend on individual sorting information present in the proteins.**

The regulated release of proteins from cells is dependent on stimulation-induced mobilization of storage granules to which the proteins have been targeted. Human neutrophils contain a variety of different storage granules, known as azurophil, specific, and gelatinase granules (1, 2), which are mobilized in a sequential and highly regulated manner (3, 4). These granules differ greatly in their profile of characteristic proteins (1, 5). Correct targeting of granule proteins into these granule subsets is critical for neutrophil function. However, little is known about signals that direct proteins into storage granules, although a hypothesis was recently presented that this may be dependent on hydrophobic sequences of the mature proteins (6).

A complex sorting system would have to be invoked if targeting of proteins into the distinct subsets of human neutrophil granules was dependent on sorting signals present in each individual protein. We and others (7–9) have shown that proteins, characteristic of the different granule subsets of neutrophils, are synthesized at different stages of maturation of neutrophil precursors, and that proteins localized to the same subset of granules are synthesized simultaneously (9). We have therefore suggested that the protein profile of the various granule subsets may be determined solely by the profile of proteins, synthesized at the time of formation of each individual granule subset, and that no specific signals are needed for sorting into different granule subsets, since these are formed sequentially. Although all available data on biosynthesis and granule composition of myeloid cells is consistent with this hypothesis (10), a crucial test of the hypothesis requires the demonstration that the localization of a given protein would change predictably if the expression of the protein were changed from one stage of differentiation to another.

To test this we changed the time of expression of a granule protein from the myelocyte stage to the promyelocyte stage by transfecting the cDNA of the specific granule protein NGAL (11) into the human promyelocytic cell line HL-60 under control of a constitutively active cytomegalovirus promoter. HL-60 cells are arrested in their maturation at the early promyelocyte stage and contain azurophil granules with their characteristic proteins myeloperoxidase, cathepsin G, elastase, and defensins, but do not express any matrix proteins of specific granules (12, 13). NGAL was chosen because this is a well-established constituent of specific granules (14), synthesized in myelocytes and metamyelocytes (9), and particularly resistant to proteases (11), and therefore potentially able to resist the protease milieu of azurophil granules, if targeted to these.

MATERIALS AND METHODS

Transfection of HL-60 Cells. The expression vector pcDNA3 (Invitrogen) was chosen for introducing NGAL cDNA into HL-60 cells. The NGAL expression vector was constructed as follows: the entire coding region of NGAL, including the signal peptide, was amplified with the primers 5′-CGC GGA TCC GCC ACC ATG CCC CTA GGT CTC CTG TGG-3′ and 5′-CGG AAT TCT CAG CCG TCG ATA CAC TGG TCG ATT GGG-3′ from a human chronic myelogenous leukemia cDNA library (15). The PCR product was digested with the restriction enzymes *Bam*HI and *Eco*RI and cloned in the expression vector pcDNA3, restricted with the same restriction enzymes. The NGAL insert was sequenced in both directions and found to be in complete agreement with the cDNA sequence of NGAL (16). The NGAL amplification product was inserted in pcDNA3 such that it is under the control of the powerful cytomegalovirus promoter. The pcDNA3 vector also contains the gene for neomycin resistance, allowing the selection of stably transfected clones. HL-60 cells were stably transfected by electroporation with either the pcDNA3 vector, in which cDNA for NGAL was inserted (pcDNA3-NGAL), or with the vector alone, previously linearized with the restriction enzyme *Pvu*I (Boehringer Mannheim). HL-60 cells (American Type Culture Collection) were grown in RPMI-1640 medium (GIBCO/BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/liter L-glutamine, and penicillin/streptomycin (complete medium) under standard cell culture conditions (37°C, humidified 5% CO_2/95% air atmosphere). The electrotransfection of HL-60 cells was designed by modification of previously published methods (see refs. 17 and 18). HL-60 cells (0.5 ml at 1×10^7 cells/ml) in RPMI-1640 medium, supplemented with 20% FCS, were mixed with 20 μg of plasmid DNA and subjected to a single electric pulse of 240 V

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at a capacitance setting of 960 μF with a time constant of 22 ms in a 0.4-cm electroporation cuvette (Bio-Rad Gene Pulser with capacitance extender) at room temperature. After 30 min on ice, cells were transferred to 5 ml of RPMI-1640 medium containing 20% FCS and allowed to recover at 37°C. After 3 days the cells had doubled in number. G418 (Geneticin) was then added to a final concentration of 1.5 mg/ml and the cells were grown under G418 selection pressure for 10 days. Clonal sublines were selected by plating in semi-solid methyl cellulose Iscove's medium (Stem Cell Technologies, Paisley, Scotland) containing 1.5 mg/ml G418. The stably transfected sublines were continuously grown in standard liquid medium supplemented with 1 mg/ml G418.

Northern Blotting. Northern blotting was performed as described in ref. 19. Equal amounts (30 μg) of RNA were size separated by formaldehyde agarose gel electrophoresis and blotted to nylon membrane (Hybond N, Amersham). The blot was probed first with NGAL cDNA extracted from the pcDNA3 construct and labeled by random priming. Subsequently, the blot was reprobed with β-actin cDNA as a control of RNA loading.

Western Blotting. Wild-type HL-60 cells (2×10^5) and stably G418-resistant subclones, transfected with either pcDNA3 vector alone or pcDNA3-NGAL, were pelleted, washed once in PBS, lysed in Laemmli SDS sample buffer (20), electrophoresed in a 10% polyacrylamide gel, and transferred to nitrocellulose membranes (21). Membranes were blotted with 2.5 μg/ml rabbit anti-NGAL antibodies (11) and visualized with enhanced chemiluminescence (Amersham).

Biosynthetic Labeling and Immunoprecipitation of NGAL. NGAL transfected HL-60 cells, suspended at 2.5×10^6 cells/ml in methionine-free medium (GIBCO/BRL) containing 10% dialyzed heat-inactivated FCS, were incubated for 60 min at 37°C. The cells were then pulsed by addition of 50 μCi of [^{35}S]methionine/ml (1175 Ci/mmol; 1 Ci = 37 GBq; DuPont/NEN). The pulse was stopped after 60 min by pelleting the cells and washing once. The biosynthetically-labeled NGAL was then chased by resuspending the cells at 10^6 cells/ml in complete medium and withdrawing 5-ml samples for immunoprecipitation at timed intervals. Cells in the samples were pelleted and solubilized at 10^7 cells/ml in RIPA buffer [150 mM NaCl/30 mM Hepes, pH 7.3/1% (vol/vol) Triton X-100/1% (wt/vol) sodium deoxycholate/0.1% (wt/vol) SDS] supplemented with 1 mM phenylmethylsulfonyl fluoride. After 60 min, the lysates were cleared from DNA by centrifugation at 32,000 × *g* for 30 min. Rabbit anti-NGAL antibodies (2.5 μg/ml) (11) were added both to the lysate and to the medium from which the cells had been pelleted. These samples were incubated for 60 min. Then 4 mg/ml of protein A-Sepharose CL4-B (Pharmacia) was added. After incubation for 60 min, the Sepharose was pelleted and washed four times in RIPA buffer, three times in PBS, and then resuspended in Laemmli SDS sample buffer (20), boiled, and subjected to electrophoresis in 10% polyacrylamide gels. Fluorography using Amplify (Amersham) was performed by exposing the dried gels to Kodak X-Omat AR film at −80°C for 5 days.

Immunocytochemistry. Cytospin preparations of wild-type and transfected cells containing NGAL cDNA were fixed in 4% formaldehyde in 0.1 mol/liter of phosphate buffer, permeabilized with 1% Triton X-100, and labeled with a monoclonal IgG_1 anti-NGAL antibody (2.5 μg/ml) essentially as described (9). The antibody was visualized by the alkaline phosphatase/anti-alkaline phosphatase detection kit (Dako).

Immunoelectron Microscopy. Ultrathin cryosections were prepared from cells fixed in 0.5% glutaraldehyde/4% paraformaldehyde. The sections were then immunolabeled with rabbit anti-NGAL and then incubated with goat anti-rabbit IgG linked to 10 nm gold. Double immunolabeling was performed with a mixture of rabbit anti-NGAL and mouse monoclonal anti-MPO (22) and then incubated with a mixture of goat anti-rabbit IgG linked to 10 nm gold and goat anti-mouse IgG linked to 5 nm gold. Ultrathin cryosections incubated with irrelevant control antibodies under the same conditions produced negligible background labeling.

RESULTS AND DISCUSSION

The specific granule protein NGAL was absent from HL-60 cells, as demonstrated both by Western blotting and immunocytochemistry, and no mRNA for NGAL was detected by Northern blotting (Fig. 1 and 2). This is in agreement with the noted absence of other specific granule proteins from HL-60 cells (13). This reflects the maturational arrest of HL-60 cells at the early promyelocytic stage of differentiation where azurophil granules and their associated proteins, such as myeloperoxidase, are formed, and before the synthesis of specific granule proteins has been unleashed, which normally occurs at the myelocyte/metamyelocyte stage of maturation (1, 9). NGAL was stably transfected to HL-60 cells to test whether the protein profile of granules is determined by specific sorting information present in the individual proteins or by the profile of proteins synthesized at the time of formation of the individual granules. It is observed from Fig. 1 that transfection of HL-60 with NGAL cDNA inserted behind a constitutively active cytomegalovirus promoter re-

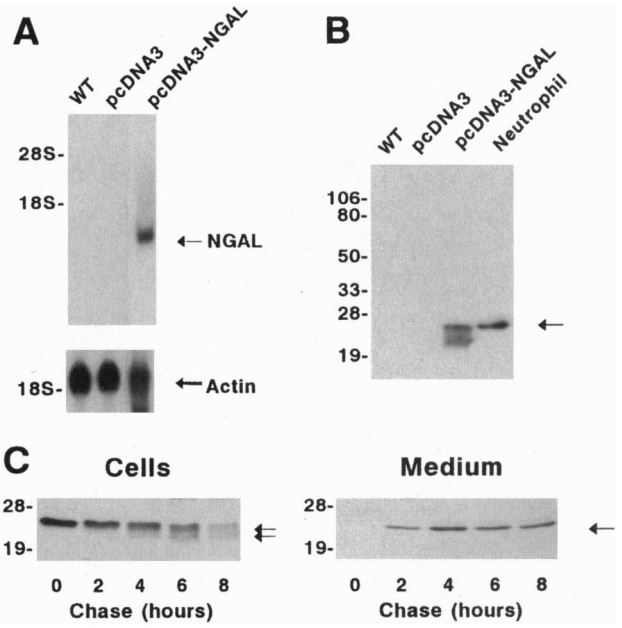

FIG. 1. NGAL expression in wild-type and transfected HL-60 cells. (*A*) Northern blot of total RNA from wild-type HL-60 cells (WT), HL-60 cells transfected with the vector alone (pcDNA3), and HL-60 cells transfected with the vector containing the NGAL cDNA (pcDNA3-NGAL). The blot was probed first with NGAL cDNA (*Upper*). Subsequently, the blot was reprobed with β-actin cDNA as a control RNA (*Lower*). Positions of 28S and 18S RNA are shown on the left. (*B*) Western blot of proteins from lysate of 2×10^5 wild-type HL-60 cells (WT) and stably G418-resistant subclones transfected with either pcDNA3 vector alone (pcDNA3) or cDNA for NGAL (pcDNA3-NGAL). A control lysate of 10^5 neutrophils, isolated from peripheral blood, was included. M_r standards (in kDa) are shown on the left. Essentially identical results were obtained in all three clones transfected with pcDNA3-NGAL and two clones transfected with pcDNA3 alone. (*C*) Proteolytic processing of NGAL in transfected HL-60 cells. Cells were pulse-labeled with [^{35}S]methionine for 60 min followed by pulse-chase for up to 8 hr. Immunoprecipitates with anti-NGAL antibodies of cell lysates and medium withdrawn at indicated time points were electrophoresed and fluorographed. The positions of the two forms of NGAL are indicated on the right. M_r markers are shown on the left.

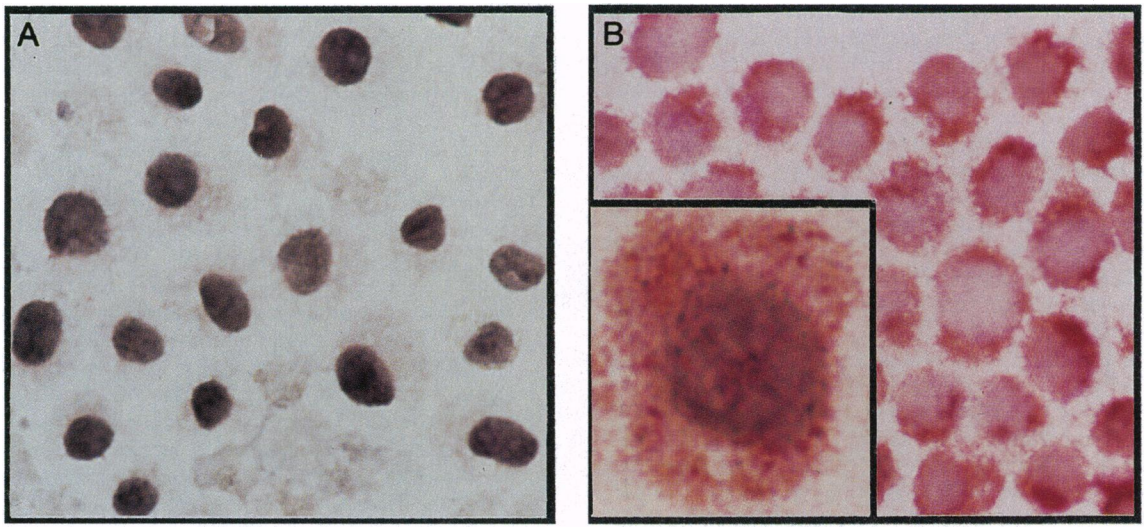

FIG. 2. Immunocytochemistry of wild-type and transfected HL-60 cells. Cytospin of wild-type (*A*) and transfected cells containing NGAL cDNA (*B*) were fixed in 4% formaldehyde in 0.1 mol/liter of phosphate buffer, permeabilized with 1% Triton X-100, and labeled with a monoclonal IgG_1 anti-NGAL antibody (2.5 μg/ml). (*A* and *B*, ×400; insert in *B*, ×1000).

sults in generation of NGAL mRNA. More importantly, the stably transfected cells synthesize NGAL as shown by Western blotting.

As shown in Fig. 1*B*, two forms of NGAL are detected in equal amounts in cell lysates; one with an apparent molecular mass of 25 kDa (corresponding to NGAL in mature neutrophils) and one at 21 kDa. This was observed in all subclones tested (not shown). Although NGAL is highly protease resistant, as are other members of the lipocalin family (23), the 21-kDa form is most likely a proteolytic product of the 25-kDa protein. This was substantiated by pulse-chase experiments (Fig. 1*C*) in which degradation to the 21 kDa occurred after several hours of chase. Both forms were then further degraded, resulting in diminution of the signal. The kinetics of this

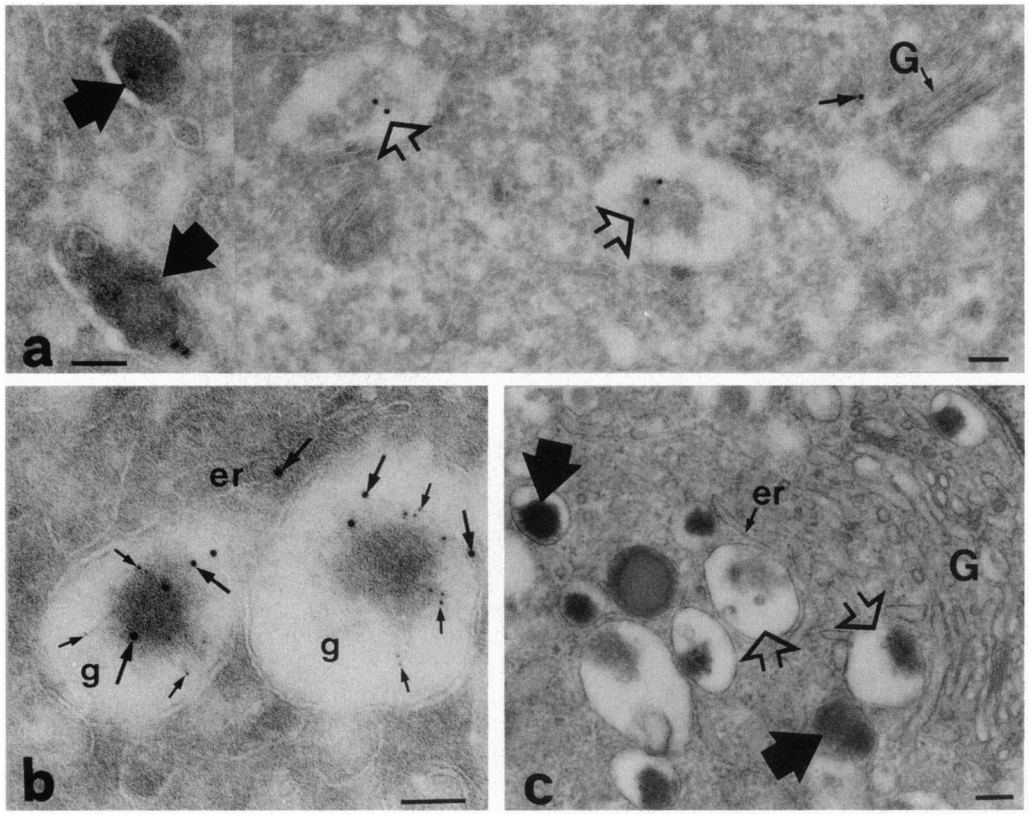

FIG. 3. Immunoelectron microscopy of ultrathin sections of HL-60 cells transfected with NGAL cDNA. (*a*) Cryosection showing expression of NGAL in electron-dense (solid arrows) and electron-lucent (open arrows) granules, and in small vesicles (small arrow) near the Golgi complex (G). (*b*) Cryosection with two granules (g) double immunolabeled for NGAL (10 nm gold particles, large arrows) and myeloperoxidase (5 nm gold particles, small arrows). Presence of NGAL in endoplasmic reticulum (er) is indicated by arrow. Colocalization of NGAL and MPO was observed in both electron-dense and in electron-lucent granules (not shown). (*c*) Thin section of cells fixed with 2.5% glutaraldehyde and 1% osmium tetroxide, and embedded in LX112/araldite. Electron-dense (solid arrows) and electron-lucent (open arrows) granules are seen as in cryosections. (*a* and *b*, bars = 100 nm; *c*, bar = 200 nm)

degradation of NGAL follows the same kinetics as the proteolytic processing of the myeloperoxidase propeptide during targeting to azurophil granules in HL-60 cells, as well as in normal bone marrow cells (24). This suggests that the degradation of NGAL, which is not observed in normal neutrophils, occurs only when the protein is targeted to azurophil granules. In agreement with this, degradation was not observed in the minor part of NGAL that escaped retention in granules and was secreted to the medium (Fig. 1*C*).

Immunocytochemistry, performed on cytospin preparations, showed positive staining for NGAL in granules of cells transfected with NGAL cDNA (Fig. 2), but not in cells that were transfected with plasmid without the NGAL cDNA insert, or in wild-type cells. Localization of NGAL to granules was observed in cells from all three positive clones tested.

Transfection of von Willebrand factor into cells capable of forming storage granules has been shown to induce generation of Weibel–Palade-like structures, the normal storage organelle for von Willebrand factor in endothelial cells (25). Likewise, transfection of VIP21/caveolin can induce formation of caveolae in lymphocytes (26). The possibility that synthesis of NGAL by itself might induce formation of a distinct granule subset was therefore investigated by immunoelectron microscopy. NGAL was localized in granules (Fig. 3*a*). Of 161 granules analyzed, labeling of both NGAL and MPO was observed in 71%, whereas single labeling for MPO was observed in 15% and single labeling for NGAL was observed in 14%. This is consistent with complete colocalization of NGAL (large gold particles) with myeloperoxidase (small gold particles), a normal constituent of azurophil granules, in the granules of transfected cells (Fig. 3*b*). The morphology of granules was investigated by electron microscopy of cells embedded in LX112/araldite. Electron-lucent and electron-dense granules were observed in cells transfected with plasmid containing cDNA of NGAL (Fig. 3*c*), as well as in wild-type cells and in cells transfected with plasmid without NGAL insert (not shown).

This is the first demonstration that a protein, normally present in one type of intracellular granules, will be targeted to another type of granules if the stage of maturation of the cell at which this protein is expressed is changed. This proves that the localization of individual proteins to distinct granule subsets can be determined solely by the time (i.e., stage of maturation of the cell) of their biosynthesis, provided that the proteins or their precursors are retained in storage granules. This therefore indicates that factors controlling the timing of protein expression, and not sorting mechanisms, may be important for controlling the structural heterogeneity of storage granules in cells that contain more than one type of storage granules. The proteolytic degradation of NGAL, which occurs when the protein is targeted to the wrong granule, underscores the significance of correct targeting to subsets of granules.

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