BioMetals (2006) 19:211–215
DOI 10.1007/s10534-005-3251-7

Neutrophil gelatinase-associated lipocalin, a siderophore-binding eukaryotic protein

Niels Borregaard* & Jack B. Cowland
*The Granulocyte Research Laboratory, Department of Hematology, University of Copenhagen, Rigshospitalet-4042, 9 Blegdamsvej, Copenhagen Ø, DK 2200, Denmark; *Author for correspondence: (Phone: +45 3545 4371; Fax: +45 3545 4295; E-mail: borregaard@rh.dk)*

Received 12 September 2005; accepted 20 September 2005

Abstract

NGAL (neutrophil gelatinase-associated lipocalin) also known as lcn2 or siderochalin is constitutively expressed in myelocytes and stored in specific granules of neutrophils. It is highly induced in a variety of epithelial cells during inflammation. Analysis of the crystal structure of NGAL expressed in *E.coli* showed that NGAL has the ability to bind catecholate type siderophores and in this way prevent bacteria from acquisition of siderophore-bound iron. NGAL (or 24p3 as the highly homologous murine orthologue is named) knock out mice have a profound defect in defense against *E.coli* after intraperitoneal injection. This defect can be mimicked in wild-type mice by providing siderophore iron, which cannot be sequestered by NGAL, testifying to the specific role of NGAL as a siderophore binding protein in innate immunity. Megalin, a scavenger receptor functions as a receptor for NGAL and mediates uptake into endosomes, but other NGAL receptors are likely to exist.

Introduction

The lipocalin protein family is extremely widespread with members in bacteria, insects and mammals including man (Ganfornina *et al.* 2000). The members share little overall sequence homology (approx. 20%), but all confine to a common tertiary structure determined by highly conserved segments of the individual lipocalin proteins, termed the lipocalin folds. These organize the lipocalins with eight anti-parallel β-sheets that surround a hydrophobic pocket, central to the function of lipocalins as transport or carrier proteins (Flower 1996).

Lipocalins are known to function as transporters of pheromones (Marchese *et al.* 1998), secreted in urine and responsible for behavioral changes (Beynon & Hurst 2003) including sexual arousal (Briand *et al.* 2004). Lipocalins bind color pigments and give lobsters their coloration (Cianci *et al.* 2002). Lipocalins bind NO and keep it stable in saliva of ticks and exchange NO with histamine during their blood feast, thus providing vasodilatation and inhibition of inflammation in the host (FuentesPrior *et al.* 1997; Andersen *et al.* 1998; Montfort *et al.* 2000).

Several human lipocalins are known, retinol-binding protein (RBP) is an important plasma protein, responsible for transport of retinol (vitamin A) (Cowan *et al.* 1990; Newcomer & Ong 2000). Despite this important function of a lipocalin, it is still not known whether the cellular uptake of vitamin A is mediated by a specific receptor that takes up the RBP–retinol complex or just picks retinol out of the lipocalin pocket of RBP.

Acid glycoprotein (AGP), also known as orosomucoid, is an acute phase protein synthesized by the liver in response to inflammation. It most likely plays a role in down-modulation of the inflammatory response by sequestering endotoxin and TNFα (Logdberg & Wester 2000; Kopecky, Jr. *et al.* 2003).

Results and discussion

We discovered a novel human lipocalin as a contamination of human neutrophil gelatinase and termed it neutrophil gelatinase-associated lipocalin (Kjeldsen *et al*. 1993; Bundgaard *et al*. 1994; Cowland & Borregaard, 1997). This is a 25 kD glycosylated protein with a free cysteine that mediates formation of a heterodimeric complex with gelatinase when these two are expressed together, as is the case in the later stages of myeloid cell maturation in the bone marrow (the myelocyte stage), or a homo-dimeric complex. NGAL is stored in the specific granules of neutrophils as a monomer, homodimer, or as a heterodimer with gelatinase as the binding partner (Kjeldsen *et al*. 1994).

We soon discovered that NGAL was not solely expressed in myelocytes of the bone marrow and stored in neutrophils. NGAL turned out to be highly expressed in gut epithelial cells at sites of inflammation. Immunohistochemistry showed massive staining for NGAL protein in epithelial cells in both diverticulitis, appendicitis, ulcerative colitis and morbus Crohn, as well as in neoplastic conditions (Nielsen *et al*. 1996). These are all characterized by significant inflammation and in some cases also by neutrophil infiltration, but the latter does not explain the presence of NGAL in epithelial cells, and *in situ* hybridization has demonstrated a marked upregulation of NGAL mRNA in the epithelial cells in the aforementioned conditions (Nielsen *et al*. 1996). Infection with virus also induces significant upregulation of NGAL (Vijay-Kumar *et al*. 2005). NGAL is highly upregulated in the epithelial cells of the respiratory tract. Some constitutive expression is seen in goblet cells of the bronchial mucosa, and small amounts are synthesized by type II pneumocytes of the lungs. Type II pneumocytes make up for 60% of the cells lining the alveoli, but as they are cuboidal in shape, they constitute only 1–5% of the respiratory surface. The type II pneumocytes also generate surfactant protein A and C (Cowland *et al*. 2003). NGAL is induced in keratinocytes of the skin during wounding (Sorensen *et al*. 2003). This massive induction of NGAL in epithelial cells made us suspect that NGAL serves a role in innate immunity, and we initiated a collaboration with Roland K. Strong to obtain insight into the possible function by analysis of the crystal structure of NGAL. We also conducted a series of experiments to identify the pathways leading to induction of NGAL synthesis in epithelial cells.

Induction of NGAL synthesis in epithelial cells

In a model system of type II pneumocytes (A549 cells), NGAL is induced by IL-1β, but not by TNFα. This cell line does not contain the TLR4 receptor for endotoxin, but the cells do respond to endotoxin with a brisk induction of NGAL transcription and synthesis when transfected with TLR4 (Cowland *et al*. 2003). Since all three stimuli are known to activate the NF-κB transcription factor by inducing phosphorylation and proteosomal degradation of the NF-κB inhibitor I-κB, it was puzzling why TNFα was unable to induce NGAL transcription, more so since the cells responded well to TNFα by induction of IL-8 transcription, which is also NF-κB-dependent.

Studies of the NGAL promoter identified an NF-κB consensus-binding site at −179 to −170. When this was mutated, IL-1β was no longer able to induce expression of NGAL (or of a reporter gene). Thus, the expression of NGAL in epithelial cells (at least in type II pneumocytes) and skin is completely dependent on NF-κB but also depends on an additional signal that is generated by IL-1β but not by TNFα (Cowland *et al*. 2003). This was recently found to be due to the induction of an NF-κB-binding co-factor, which is specifically induced by IL-1β and not by TNF-α and is required for transcription of the NGAL gene (Cowland *et al*. manuscript in preparation).

In colon epithelial cells, double-stranded RNA has been shown to induce NGAL via Protein Kinase R activation (Vijay-Kumar *et al*. 2005).

It seems that some major differences exist between mouse and man with respect to regulation of NGAL expression. In mouse the orthologue of NGAL, 24p3 or uterocalin, is induced as an acute phase protein synthesized by the liver. This does not seem to be the case in man. In studies of isolated liver cells from mouse and man, TNFα induces NGAL in mouse cells, but not in human liver cells (Klausen *et al*. in press).

The regulation of expression of NGAL and its upregulation in epithelial cells fits well with a putative role of NGAL in innate immunity. The clue to its direct function came from crystallography carried out by Dr. Roland K. Strong and collabo-

rators who noticed that recombinant NGAL generated by *E. coli* was deeply red in color in contrast to recombinant NGAL generated by insect cells. It was shown that this red color was due to an iron molecule that was trapped in the siderophore of *E.coli*, enterochelin, indicating that NGAL is a siderophore binding protein (Strong *et al.* 1998; Goetz *et al.* 2000). Many such are of course known that mediate the uptake of siderophores in bacteria (Neilands 1995), but no eukaryotic, let alone mammalian protein with siderophore binding properties was known. The binding of enterochelin by NGAL is strong with dissociation constants of 0.4 nM, and the siderophores were significantly more stable when bound in the lipocalin pocket of NGAL (Goetz *et al.* 2002). This of course means that the ability of NGAL to sequester iron via siderophores is durable. This was demonstrated by experiments where apo-NGAL, i.e. NGAL generated without siderophore was added to *E.coli*. NGAL was able to completely block growth of *E.coli* under iron poor conditions but NGAL with siderophore iron was not.

The affinity of NGAL for different classes of siderophores was investigated in a series of experiments using either change in plasmon resonance or quenching of fluorescence of a tryptophan residue deep in the bottom of the lipocalin pocket, and extended with structural analysis. This showed that the best fit is obtained by the siderophores of mycobacteria, whereas the siderophores from another major human pathogen, *Pseudomonas aeruginosa* are not bound (Holmes *et al.* 2005).

The biological significance of NGAL's ability to inhibit bacterial growth by sequestering siderophore iron was investigated in a knock-out mouse generated by Flo *et al.* (2004). The mice were healthy with normal hematopoiesis and normal kidney function. This argues against a major role of NGAL in regulation of myeloid development as has been presented by Devireddy *et al.* (2001) and also lends little support to NGAL as an important iron carrier of significance for kidney development as suggested (Yang *et al.* 2002). At least other systems must be able to compensate for the lack of NGAL in these situations. Also, the mice suffered no major infections. However when challenged by intraperitoneal injection of *E.coli*, the knock out mice succumbed rapidly, in contrast to wild-type mice. It was elegantly shown that the resistance of wild-type mice was indeed due to their ability to induce NGAL synthesis and reduce the amount of siderophore iron available to bacteria, since providing the wildtype mice with siderophore iron from a source which NGAL cannot bind, made the mice as vulnerable to infection as knock-out mice (Flo *et al.* 2004). Since there are major differences between regulation of NGAL expression in man and mice, it will be particularly important to explore whether the protection offered by NGAL is provided by NGAL expressed by the liver as an acute phase protein in mice but not man or by the upregulation in epithelial cells (well investigated in man but not in mice) or the constitutive expression in neutrophils.

It was thus demonstrated that NGAL works as a bacteriostatic agent by sequestering siderophore bound iron, but NGAL is not bactericidal, and prevents bacterial growth only as long as NGAL is able to sequester siderophores. This raises two questions. Is the ability of NGAL to bind siderophores influenced by whether the siderophores themselves have chelated iron or not? This has not been completely addressed. The binding of siderophores to the pocket of NGAL is largely mediated by ionic strength where positively charged amino acids (Arg81, Lys125, and Lys134) interact with the negatively charged side chains of siderophores (Holmes *et al.* 2005).

The next question is what happens to the NGAL siderophore complex? If bacterial proteases were able to degrade NGAL, its bacteriostatic effect would be to short. NGAL is known to be very protease resistant (Kjeldsen *et al.* 1993), but it can be degraded (Le Cabec *et al.* 1996, 1997), and its long term effect in protection against bacterial infections would depend on the ability of epithelial cells not only to synthesize NGAL in response to bacterial infection, but also to take up NGAL and thus protect it from microbial or other proteases in the extracellular milieu.

We recently showed that megalin, the multifunctional scavenger receptor highly expressed in kidney epithelial cells and epithelial cells of the ileum, lungs ependyma, epididymis and other tissues (Moestrup & Verroust 2001), binds NGAL with high affinity as documented by changes in plasmon resonance. Megalin is also able to mediate uptake of NGAL in cells that express megalin (Hvidberg *et al.* 2005). We were not able to observe any difference in the uptake of apoNGAL and siderophore loaded NGAL.

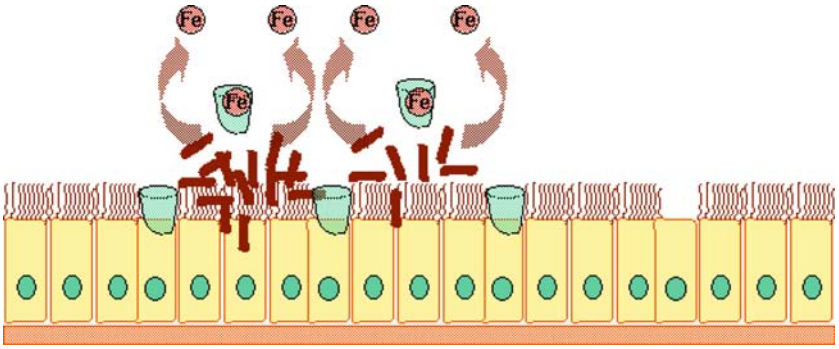

Figure 1. Epithelial cells synthesize and release NGAL in response to inflammation. Microorganisms synthesize and release siderophores that capture iron and provide a mechanism for supplying this essential nutrient to the bacteria. NGAL binds siderophores and prevent their uptake in microorganisms and thus deprive the microorganisms of this essential nutrient.

The study of NGAL has disclosed a hitherto unknown mechanism of innate immunity. It is well known that the ability to reduce the availability of iron for microbial growth is important for defense against microbial infections. Lactoferrin is a highly abundant protein expressed by the same myeloid cells as NGAL (Borregaard & Cowland 1997) and this chelates iron directly. NrampI, also expressed in the granules of neutrophils that harbor NGAL and lactoferrin (Canonne-Hergaux *et al*. 2002), is believed to reduce the availability of iron in the phagocytic vacuole by pumping iron into the cytosol, and its importance for defense against mycobacterial infections was the basis for its discovery and its name. Hepcidin an antibacterial protein highly induced in liver cells in response to IL-6 stimulation shuts down the release of iron from intestinal cells and macrophages and in this way provides an iron deficiency state (Loreal *et al*. 2005). Microorganisms have developed several mechanisms to secure their supply of iron, which is very scarce as a soluble molecule. A major mechanism is the ability to synthesize siderophores that are the strongest iron chelators known, well able to recover iron from transferrin and lactoferrin (Neilands 1995). NGAL binds siderophores and prevents its uptake by microorganisms (Fig. 1).

The immediate questions are whether other members of the lipocalin family share the ability of NGAL to bind siderophores. Tear lipocalin which is quite closely related to NGAL has been shown to bind siderophores and to prevent bacterial growth but the molecular details have not been worked out yet (Fluckinger *et al*. 2004). The question is whether there are other receptors for NGAL than megalin and whether NGAL is recycled, liberated from its siderophore after cellular uptake.

References

Andersen JF, Weichsel A, Balfour CA, Champagne DE, Montfort WR. 1998 The crystal structure of nitrophorin 4 at 1.5 angstrom resolution: transport of nitric oxide by a lipocalin-based heme protein. *Structure* **6**, 1315–1327.

Beynon RJ, Hurst JL. 2003 Multiple roles of major urinary proteins in the house mouse, *Mus domesticus*. *Biochem Soc Trans* **31**, 142–146.

Borregaard N, Cowland JB. 1997 Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* **89**, 3503–3521.

Briand L, Trotier D, Pernollet JC. 2004 Aphrodisin, an aphrodisiac lipocalin secreted in hamster vaginal secretions. *Peptides* **25**, 1545–1552.

Bundgaard JR, Borregaard N, Kjeldsen L. 1994 Molecular cloning and expression of a cDNA encoding NGAL: a lipocalin expressed in human neutrophils. *Biochem Biophys Res Commun* **202**, 1468–1475.

Canonne-Hergaux F, Calafat J, Richer E, *et al*. 2002 Expression and subcellular localization of NRAMP1 in human neutrophil granules. *Blood* **100**, 268–275.

Cianci M, Rizkallah PJ, Olczak A, *et al*. 2002 The molecular basis of the coloration mechanism in lobster shell: beta-crustacyanin at 3.2-A resolution. *Proc Natl Acad Sci USA* **99**, 9795–9800.

Cowan SW, Newcomer ME, Jones TA. 1990 Crystallographic refinement of human serum retinol binding protein at 2 Å resolution. *Proteins: Struct Function Genet* **8**, 44–61.

Cowland JB, Borregaard N. 1997 Molecular characterization and pattern of tissue expression of the gene for neutrophil gelatinase-associated lipocalin from humans. *Genomics* **45**, 17–23.

Cowland JB, Sorensen OE, Sehested M, Borregaard N. 2003 Neutrophil gelatinase-associated lipocalin is up-regulated in human epithelial cells by IL-1beta, but not by TNF-alpha. *J Immunol* **171**, 6630–6639.

Devireddy LR, Teodoro JG, Richard FA, Green MR. 2001 Induction of apoptosis by a secreted lipocalin that is transcriptionally regulated by IL-3 deprivation. *Science* **293**, 829–834.

Flo TH, Smith KD, Sato S, *et al*. 2004 Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature* **432**, 917–921.

Flower DR.. 1996 The lipocalin protein family: structure and function. *Biochem J* **318**, 1–14.

Fluckinger M, Haas H, Merschak P, Glasgow BJ, Redl B. 2004 Human tear lipocalin exhibits antimicrobial activity by scavenging microbial siderophores. *Antimicrob Agents Chemother* **48**, 3367–3372.

FuentesPrior P, NoeskeJungblut C, Donner P, Schleuning WD, Huber R, Bode W. 1997 Structure of the thrombin complex with triabin, a lipocalin-like exosite-binding inhibitor derived from a triatomine bug. *Proc Natl Acad Sci USA* **94**, 11845–11850.

Ganfornina MD, Gutierrez G, Bastiani M, Sanchez D. 2000 A phylogenetic analysis of the lipocalin protein family. *Mol Biol Evol* **17**, 114–126.

Goetz DH, Holmes MA, Borregaard N, Bluhm ME, Raymond KN, Strong RK. 2002 The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. *Mol Cell* **10**, 1033–1043.

Goetz DH, Willie ST, Armen RS, Bratt T, Borregaard N, Strong RK. 2000 Ligand preference inferred from the structure of neutrophil gelatinase-associated lipocalin. *Biochemistry* **39**, 1935–1941.

Holmes MA, Paulsene W, Jide X, Ratledge C, Strong RK. 2005 Siderocalin (Lcn 2) also binds carboxymycobactins, potentially defending against mycobacterial infections through iron sequestration. *Structure (Camb)* **13**, 29–41.

Hvidberg V, Jacobsen C, Strong RK, Cowland JB, Moestrup SK, Borregaard N. 2005 The endocytic receptor megalin binds the iron transporting neutrophil-gelatinase-associated lipocalin with high affinity and mediates its cellular uptake. *FEBS Lett* **579**, 773–777.

Kjeldsen L, Bainton DF, Sengeløv H, Borregaard N. 1994 Identification of neutrophil gelatinase-associated lipocalin as a novel matrix protein of specific granules in human neutrophils. *Blood* **83**, 799–807.

Kjeldsen L, Johnsen AH, Sengeløv H, Borregaard N. 1993 Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. *J Biol Chem* **268**, 10425–10432.

Kopecky V Jr, Ettrich R, Hofbauerova K, Baumruk V. 2003 Structure of human alpha-1 acid glycoprotein and its high-affinity binding site. *Biochem Biophys Res Commun* **300**, 41–46.

Le Cabec V, Calafat J, Borregaard N. 1997 Sorting of the specific granule protein, NGAL during granulocytic maturation of HL-60 cells. *Blood* **89**, 2113–2121.

Le Cabec V, Cowland JB, Calafat J, Borregaard N. 1996 Targeting of proteins to granule subsets determined by timing not by sorting: the specific granule protein NGAL is localized to azurophil granules when expressed in HL-60 cells. *Proc Natl Acad Sci USA* **93**, 6454–6457.

Logdberg L, Wester L. 2000 Immunocalins: a lipocalin subfamily that modulates immune and inflammatory responses. *Biochim Biophy Acta* **1482**, 284–297.

Loreal O, Haziza-Pigeon C, Troadec MB, *et al*. 2005 Hepcidin in iron metabolism. *Curr Protein Pept Sci* **6**, 279–291.

Marchese S, Pes D, Scaloni A, Carbone V, Pelosi P. 1998 Lipocalins of boar salivary glands binding odours and pheromones. *Eur J Biochem* **252**, 563–568.

Moestrup SK, Verroust PJ. 2001 Megalin- and cubilin-mediated endocytosis of protein-bound vitamins, lipids, and hormones in polarized epithelia. *Annu Rev Nutr* **21**, 407–428.

Montfort WR, Weichsel A, Andersen JF. 2000 Nitrophorins and related antihemostatic lipocalins from Rhodnius prolixus and other blood-sucking arthropods. *Biochim Biophys Acta* **1482**, 110–118.

Neilands JB. 1995 Siderophores: structure and function of microbial iron transport compounds. *J Biol Chem* **270**, 26723–26726.

Newcomer ME, Ong DE. 2000 Plasma retinol binding protein: structure and function of the prototypic lipocalin. *Biochim Biophys Acta* **1482**, 57–64.

Nielsen BS, Borregaard N, Bundgaard JR, Timshel S, Sehested M, Kjeldsen L. 1996 Induction of NGAL synthesis in epithelial cells of human colorectal neoplasia and inflammatory bowel disease. *Gut* **38**, 414–420.

Sorensen OE, Cowland JB, Theilgaard-Monch K, Liu L, Ganz T, Borregaard N. 2003 Wound healing and expression of antimicrobial peptides/polypeptides in human keratinocytes, a consequence of common growth factors. *J Immunol* **170**, 5583–5589.

Strong RK, Bratt T, Cowland JB, Borregaard N, Wiberg FC, Ewald AJ. 1998 Expression, purification, crystallization and crystallographic characterization of dimeric and monomeric human neutrophil gelatinase associated lipocalin (NGAL). *Acta Crystallogr D Biol Crystallogr* **54**(Pt 1), 93–95.

Vijay-Kumar M, Gentsch JR, Kaiser WJ, *et al*. 2005 Protein kinase R mediates intestinal epithelial gene remodeling in response to double-stranded RNA and live rotavirus. *J Immunol* **174**, 6322–6331.

Yang J, Goetz D, Li JY, *et al*. 2002 An iron delivery pathway mediated by a lipocalin. *Mol Cell* **10**, 1045–1056.