The endocytic receptor megalin binds the iron transporting neutrophil-gelatinase-associated lipocalin with high affinity and mediates its cellular uptake

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Abstract Neutrophil-gelatinase-associated lipocalin (NGAL) is a prominent protein of specific granules of human neutrophils also synthesized by epithelial cells during inflammation. NGAL binds bacterial siderophores preventing bacteria from retrieving iron from this source. Also, NGAL may be important in delivering iron to cells during formation of the tubular epithelial cells of the primordial kidney. No cellular receptor for NGAL has been described.

We show here that megalin, a member of the low-density lipoprotein receptor family expressed in polarized epithelia, binds NGAL with high affinity, as shown by surface plasmon resonance analysis. Furthermore, a rat yolk sac cell line known to express high levels of megalin, endocytosed NGAL by a mechanism completely blocked by an antibody against megalin.

Keywords: NGAL; Lipocalin; Megalin; Siderophore; Iron

1. Introduction

Lipocalins constitute a family of more than 30 proteins, found in species as separate as insects and man [1,2]. Lipocalins share a common structural fold, often in the absence of significant sequence homology, that forms a ligand-binding site (calyx) that is typically lined with hydrophobic residues [3,4]. Lipocalins function in general as transport proteins, e.g., odorant-binding protein, bilin-binding protein, and retinol-binding protein, but the physiological ligands have not been identified for all lipocalins. Lipocalins are extracellular proteins but some are involved in transport of substances that must be delivered intracellularly to exert a function, e.g., retinol. Very little is known about the receptors that mediate cellular uptake of lipocalins and their ligands. It is not known whether the ligands are presented by the lipocalins to ligand receptors on cells or whether the lipocalins are taken up by receptors and then deliver their cargo intracellularly [5]. It is assumed that most lipocalins are taken up by membrane receptors but only two have been identified so far. One is a novel 51 kDa protein which binds tear-lipocalin (lipocalin-1 (Lcn-1)) and consequently is known as Lcn-1-Interacting Membrane Protein [6,7]. The other receptor is megalin [8], a multi-ligand endocytosis receptor that is expressed on a variety of epithelia, primarily such that have a high absorptive capacity such as tubular epithelial cells of kidneys, ileum, choroid plexus, and yolk sac [9]. Megalin belongs to the low density lipoprotein receptor family [8]. Megalin has been shown to bind the mouse lipocalins retinol-binding protein, α_1-microglobulin, mouse major urinary protein, and odorant-binding protein [5,10].

We discovered a major human lipocalin, neutrophil-gelatinase-associated lipocalin (NGAL) (also known as lipocalin-2 (lcn-2) or siderocalin), and have characterized its tissue expression [11]. NGAL is constitutively expressed in human neutrophils but is also induced in epithelial cells when these are engaged in inflammation [12–14]. This tissue expression indicates that NGAL is involved in innate immunity. This notion was supported when siderophores were identified as NGAL ligands [15] and followed by the recent demonstration of decreased survival of mice with a targeted disruption of the NGAL (lcn-2) after intraperitoneal challenge with *Escherichia coli* [16]. Siderophores are extremely strong iron chelators secreted by microorganisms when iron is limiting [17]. Siderophores can extract iron out of iron-binding proteins such as transferrin and lactoferrin for subsequent uptake by specific siderophore receptors on the microorganisms. In this way, microorganisms can secure a supply of this essential nutrient at the expense of their host [17]. It has been shown that apo-NGAL is able to prevent siderophore producing *E. coli* from dividing and that this restrain on bacterial growth is alleviated by supplying iron in excess of the iron-siderophore binding capacity of NGAL [15]. However, such antimicrobial activity can be expected to be temporary because several microorganisms secrete proteases that may degrade NGAL even though NGAL is known to be a very protease resistant molecule [18]. NGAL would therefore be expected to have a more sustained antimicrobial effect, if NGAL is taken up by host cells.

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Abbreviations: BN, Brown Norway; Lcn-1, lipocalin-1; Lcn-2, lipocalin-2; LRP, LDL-receptor related protein; NGAL, neutrophil-gelatinase-associated lipocalin; RAP, receptor associated protein; SPR, surface plasmon resonance

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This has been further indicated by the recent observation that the mouse ortholog of NGAL, 24p3, is expressed in the fetal kidney and delivers iron to cells at the stage where differentiation of the budding kidney epithelia takes place during organogenesis of the kidneys [19,20]. The rapid clearance of NGAL from the circulation indicates a mechanism of cellular uptake [21]. In addition, NGAL has been inferred as a factor inducing apoptosis through a receptor mediated mechanism in IL-3 dependent cells such as bone marrow cells in the mouse [22], but this has not been confirmed [23].

Since none has yet been identified, we decided to test whether megalin could also function as a receptor for NGAL.

2. Materials and methods

Recombinant human NGAL was generated either as apo-NGAL, i.e., without bacterial siderophore, in Sf9 insect cells using the Baculovirus technique as previously described [24], or as NGAL with *E. coli* siderophore as detailed in [15,25], with the modification that 50 ml overnight culture of *E. coli* was added to 500 ml LB medium and incubated for 90 min. Then, 0.1 mM IPTG and 0.1 mg/ml desferoxamine (Desferal, Roche) were added to induce bacterial growth (IPTG) and secure iron deprivation and enterobactin production by the bacteria. After 3 h, 0.1 mg/ml $FeCl_3$ was added and the bacteria incubated for another 90 min. The bacteria were then pelleted by centrifugation and formed a bright red pellet. The bacteria were lysed and rhNGAL was isolated as described [25]. The isolated NGAL was scanned as described from 250 to 800 nm, and showed the characteristic peaks at 330 and 500 nm indicative of enterobactin–Fe^{3+} complex in the lipocalin pocket [15]. These were absent in the apo-NGAL from Sf9 cells (Fig. 1). It was ascertained that desferoxamine did not bind to apo-NGAL by incubating apo-NGAL with desferoxamine and $FeCl_3$.

Mouse monoclonal anti-NGAL antibody was used for Western blotting [26]. NGAL was quantitated as described by Kjeldsen et al. [26].

Megalin was purified by receptor-associated protein (RAP) affinity chromatography from human kidney cortex according to standard procedures [27]. Human LDL-receptor related protein (LRP) was purified by α_2 macroglobulin-affinity chromatography as described [28]. Purified sheep polyclonal antibodies against rat megalin, previously described [29], were used to block uptake of NGAL by megalin. Purified sheep non-immune IgG served as negative control.

3. Surface plasmon resonance analysis

The binding to megalin and LRP was studied by surface plasmon resonance (SPR) analysis on a Biacore 2000 instrument (Biacore, Sweden). The procedure was as follows: Biacore sensor chips type CM5 were activated with a 1:1 mixture of 0.2 M *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide and 0.05 M *N*-hydroxysuccimide in water according to the manufacturer's instructions. Megalin and LRP were immobilized at a concentration of 10 μg/ml in 10 mM sodium acetate, pH 4.5, and the remaining binding sites were blocked with 1 M ethanolamine, pH 8.5. A control flow cell was made by performing the activation and blocking procedures only. The resulting receptor densities were 25–30 fmol receptor/mm^2. Samples were dissolved in 10 mM HEPES, 150 mM NaCl, 1.5 mM $CaCl_2$, 1.0 mM EGTA, and 0.005% Tween 20, pH 7.4, or in 10 mM HEPES, 150 mM NaCl, 20 mM EGTA, and 0.005% Tween 20, pH 7.4. Sample and running buffer were identical. Regeneration of the sensor chip after each analysis cycle was performed with 1.6 M glycine–HCl buffer, pH 3.0. The Biacore response is expressed in relative response units (RU), i.e., the difference in response between protein and control flow channel (an activated but uncoupled flow cell). Kinetic parameters were determined by BIAevaluation 4.1 software using a Langmuir 1:1 binding model and simultaneous fitting of all curves in the concentration range considered (global fitting).

4. Cellular uptake of NGAL

Megalin-expressing Brown Norway (BN) rat yolk sac epithelial cells transformed with mouse sarcoma virus (BN cells) [30] were grown on two-chamber Permanox slides (Nunc, Roskilde, Denmark) and washed in PBS, pH 7.4. Cells were then incubated for 1 h at 37 °C in HyQ-CCM5 serum-free medium (Hyclone, Logan, Utah) containing 1% BSA (w/v) and ≈60 μg/ml rhNGAL labeled with Alexa-488 (Molecular Probes, Leiden, The Netherlands). To some cells was also added sheep polyclonal anti-rat megalin IgG antibody (200 μg/ml) or sheep non-immune IgG antibody (200 μg/ml). After incubation, cells were washed in PBS, pH 7.4, and fixed in 4% formaldehyde for 1 h at 4 °C. Subsequently, cells were washed in PBS, pH 7.4, containing 0.05% Triton X-100 and incubated with rabbit anti-rat cubilin antibody [31] (10 μg/ml) in this buffer for 1 h at room temperature in order to visualize the megalin-expressing structures of the cells. After washing in PBS, 0.05% Triton X-100, cells were incubated for 1 h at room temperature with Alexa-594-conjugated secondary anti-rabbit IgG (Molecular Probes) diluted 1:200 in the same buffer. Slides were washed in PBS, pH 7.4, mounted in Dako fluorescent mounting medium (DakoCytomation, Glostrup, Denmark) and analyzed

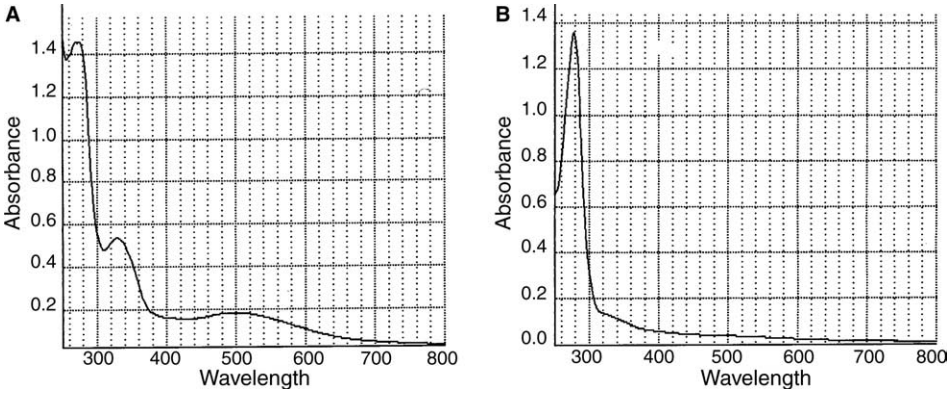

Fig. 1. Absorption spectrum of NGAL. NGAL (1.2 mg/ml) with siderophore (A) and without siderophore (B) in PBS was scanned against PBS.