Glycan-binding Properties of Basic Whey Protein Lactoferrin and Its Application in Nerve Regenerative Medicine

Masao Nakamura 1; and Atsushi Sato 2

1 Department of Peptidomics, Sasaki Institute, Sasaki Foundation, 2–2 Kandasurugadai, Chiyoda, Tokyo 101–0062, Japan
2 School of Bioscience and Biotechnology, Tokyo University of Technology, 1404–1 Katakura, Hachioji, Tokyo 192–0982, Japan

© 2022 FCCA (Forum: Carbohydrates Coming of Age)

Abstract

The basic whey protein lactoferrin (LF) is a glycosaminoglycan (GAG)-binding protein that plays a role in innate immunity and is expected to be a potential biopharmaceutical because of its diverse functions. LF binds to heparan sulfate, a type of GAG on the cell surface, and is involved in neuroprotective and anti-cancer activities. Recently, it was found that human LF (hLF) binds to chondroitin sulfate E (CS-E), which inhibits neuronal axon outgrowth and neutralizes CS-E-induced axon outgrowth inhibition. LFs are potential therapeutic targets for GAG-related diseases, such as cancer, neurodegenerative diseases, and infectious diseases. This article outlines the general properties of LF and discusses the binding properties of hLF to CS-E and its application.

A. Introduction

Milk is a biological secretion that is the only source of nutritional support for newborn mammals immediately after birth. It contains various bioactive molecules such as casein, whey proteins (such as α-lactalbumin, lactoferrin [LF], and immunoglobulin), glycolipids, and lactose. Most whey proteins that supplement the physiological functions of immature newborns have an acidic isoelectric point, and very few of them have a basic isoelectric point. The representative basic whey protein is LF. This unique chemical property of LF has been the focus of several studies.

LF is an iron-binding protein with a molecular weight of approximately 80,000 and a structure similar to that of transferrin (1). LF was discovered in the whey fraction of cow’s milk by Sorensen et al. in 1939 and was isolated from breast milk and cow’s milk in 1960 (2, 3). LF is found in milk (5–7 mg/mL in colostrum and 2–3 mg/mL in normal milk in humans) and exocrine fluids such as tears, nasal secretions, saliva, bile, pancreatic juice, cervical mucus, and amniotic fluid (4). LF is also contained in secondary granules as a major cellular component of neutrophils and is released into the blood in response to cytokine stimulation or infection by pathogenic microorganisms, such as gram-negative bacteria. Thus, LFs contained in various exocrine fluids and neutrophils reportedly have diverse functions in vivo (5). Recently, attempts to use LF for medical treatment have been suggested due to its inhibitory effects on carcinogenesis and cancer metastasis, and its protective effects against viral infection. Thus, LF has attracted increased attention as a pharmaceutical seed. This review outlines the general properties of LF, and discusses the binding properties of human LF (hLF) to CS-E. The application of LF that we have recently discovered is also discussed.

B. General Properties of LF: Structure and Function

The structural and biological properties of LFs have been extensively studied by many researchers worldwide.

LF consists of two globular functional domains, the N-lobe and C-lobe, with one iron-binding site in each lobe (6). The degree of iron binding affects the conformation of the N-lobe (7). The N1 domain of the N-lobe contains many amino acid residues with basic side chains, which are involved in interactions with biological macromolecules (Fig. 1B). Unlike other proteins, LF is taken up from the intestine into the peripheral blood by binding to the LF receptor and localized in the liver, kidneys, gall bladder, spleen, and brain (8). LF has the following physiological activities: 1) Modulation of iron metabolism, 2) antimicrobial activity against Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Streptococcus pneumoniae, yeast, human immunodeficiency virus, herpes simplex virus, hepatitis C virus, and Japanese encephalitis virus, 3) modulation of immune and inflammatory responses, 4) antioxidant activity, 5) modulation of cell growth, 6) anti-cancer and cancer metastasis-inhibitory activity in breast cancer, colon cancer, malignant melanoma, and squamous cell carcinoma of the head and neck 7) neuroprotective activity in Alzheimer’s disease and Parkinson’s disease, and 8) growth-promoting activity for Lactobacillus and Bifidobacterium with low iron requirement (9–13). Each of these activities is not necessarily independent of the other and often has a common mechanism of action. In addition, there are many physiological activities, the detailed mechanisms of action for which are unknown.
C. Biological Activities of hLF as a Glycosaminoglycan (GAG)-binding Protein

It has been reported that GAGs interact with various biological macromolecules to regulate cell proliferation, motility, and adhesion (14). GAGs are roughly classified into compounds such as heparin (HP), heparan sulfate (HS), and chondroitin sulfate (CS), according to the differences in the disaccharide repeating units (Fig. 2). In addition, several subtypes depend on the position of the attached sulfate group. For example, CS, whose disaccharide skeletal structure consists of glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc), is classified into several subtypes: E unit (CS-E), where GalNAc is sulfated at two positions (C4 and C6); A unit (CS-A), where GalNAc is sulfated only at C4; C unit (CS-C), where GalNAc is sulfated only at C6 (Fig. 2). So far, more than 700 molecules, including antithrombin III, fibronectin, laminin, interferon, hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF), are known to interact with GAGs (15, 16). The interactions of GAGs with biological macromolecules allow them to maintain their stability and enhance their binding to the relevant receptors, mediating many biological events (17–20).

In a previous study, hLF was shown to bind to HP-Sepharose and was identified as a GAG-binding protein (21). hLF has two GAG-binding sites in the N1 domain (Arg2–Arg3–Arg4–Arg5, and Arg28–Lys29–Val30–Arg31) and one in the linker region (Arg342–Arg343–Ala344–Arg345) (22, 23). The binding of hLF to HP neutralizes the inhibition of the thrombin-serpin reaction (24). hLF binding to HS is effective in 1) exerting anti-proliferative activities against human breast cancer cell lines, 2) protecting from dopaminergic neuronal damage in a Parkinson’s disease model, and 3) blocking the entry of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) into host cells (Table 1) (25, 29, 30). LFs are
expected to be used as therapeutic targets for GAG-related diseases in the field of cancer, neurodegeneration, and infection

D. CS-binding Properties of hLF

The binding of hLF to HP/HS is relevant to the regulation of cancer, neurodegenerative diseases, and infectious diseases. However, the binding of hLF to GAGs other than HP/HS has remained largely unexplored. We have analyzed the binding of hLF to CS in detail, which regulates neural function. First, we analyzed the changes in the secondary structure of hLF and the N-lobe in the presence of four CS subtypes (CS-A, CS-C, CS-D, or CS-E). The results showed that secondary structures of hLF and N-lobe were significantly changed in the presence of CS-C, CS-D, or CS-E. The binding of hLF and the N-lobe to CS-E was then evaluated using a solid-phase binding assay; consequently, hLF and the N-lobe bound directly to the CS-E (Fig. 3A, B). This binding was inhibited by excess HP and CS-E, but not by CS-C (Fig. 3A, B) (31). The chemical structures of CS-C and CS-E indicate that hLF and the N-lobe do not get attracted to the negative charge of CS-E due to their positive charges, but rather recognize the position of the sulfate group on CS-E with high specificity.

E. Physiological Activity of hLF as a CS-E-binding Protein: Neutralization of the Neuronal Axon Growth Inhibition

CS is expected to be a therapeutic target for central nervous system injuries, such as neurological intractable diseases including spinal cord injury (SCI). Among CS subtypes, CS-E is a potent inhibitor of axonal regeneration (32–34). It is assumed that severed axons cannot regenerate. However, if a molecule blocking CS-E-induced neurodegeneration by directly binding to CS-E is discovered, it is expected to be a new therapeutic candidate for SCI. Currently, the administration of large doses of steroids, HGF, granulocyte colony-stimulating factor (G-CSF), and FGF within 72h after injury has been attempted, but a therapeutic method has not yet been established (35–39). As a new therapeutic method,

Table 1. Interaction hLF with GAG and its biological roles.

<table>
<thead>
<tr>
<th>Year</th>
<th>Key features</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1980</td>
<td>Discovery that hLF binds to HP-Sepharose</td>
<td>[21]</td>
</tr>
<tr>
<td>1994</td>
<td>R²-R³-R⁴-R⁵ and R²⁸-K²⁹-V³⁰-R²⁵ of hLF are involved in binding to HP</td>
<td>[22]</td>
</tr>
<tr>
<td>1995</td>
<td>hLF neutralizes HP-induced inhibition of the thrombin-serpin reaction</td>
<td>[24]</td>
</tr>
<tr>
<td>1997</td>
<td>Identification of R²-R³-R⁴-R⁵ of hLF as the HP-binding site</td>
<td>[23]</td>
</tr>
<tr>
<td>1998</td>
<td>Reduced levels of HS on the human breast cancer cell lines decreased the binding of hLF to them and its anti-proliferation activities</td>
<td>[25]</td>
</tr>
<tr>
<td>2003</td>
<td>R²⁵ and R²⁸ of hLF neutralize HP function in the blood</td>
<td>[26]</td>
</tr>
<tr>
<td>2008</td>
<td>HS modulates the brain diffusion of hLF in the neocortex</td>
<td>[27]</td>
</tr>
<tr>
<td>2008</td>
<td>hLF inhibits cell-to-cell spread of herpes simplex virus type 1 by interacting with CS</td>
<td>[28]</td>
</tr>
<tr>
<td>2013</td>
<td>Neuroprotective actions of hLF due to its binding to HS on dopaminergic neurons</td>
<td>[29]</td>
</tr>
<tr>
<td>2021</td>
<td>hLF inhibits HS proteoglycan-mediated SARS-CoV-2 infection</td>
<td>[30]</td>
</tr>
<tr>
<td>2021</td>
<td>hLF neutralizes CS-E-induced axon growth inhibition in dorsal root ganglion cells</td>
<td>[31]</td>
</tr>
</tbody>
</table>

*Abbreviation, R: Arginine, K: Lysine, V: Valine.

Fig. 3. hLF neutralizes CS-E-induced axon growth inhibition. A and B. Binding of hLF to CS-E (A) and that of the N-lobe to CS-E (B) were analyzed using a solid-phase binding assay. C. Effects of hLF and N-lobe on CS-E inhibition of axon growth were analyzed using chick embryo dorsal root ganglion cells. D. Effects of hLF and N-lobe neutralization on CS-E inhibition of axon growth are shown in the schematic diagram.

*Fig. 3A–C are modified from the figures published by M. Nakamura et al., Biochem. Biophys. Res. Commun. (2021).
F. Conclusion

According to the Annual Report on the Aging Society in FY2020 published by the Cabinet Office, it is predicted that by 2065, one in every 2.6 people in Japan will be aged 65 or over, and one in every 3.9 people will be aged 75 or over (41). There are more than 100,000 patients with SCI in Japan, and approximately 5,000 new cases occur annually. In Japan, where the population is aging, SCI is one of the intractable diseases for which the number of patients is expected to increase, and the development of therapeutic agents is eagerly awaited.

In summary, hLF binds to CS-E, an acidic carbohydrate, and neutralizes CS-E-induced inhibition of axon elongation. It is expected that scientific findings on the functional recovery of hLF using animal models of SCI will lead to the development of therapeutic agents for SCI that target CS-E.

Acknowledgments

Masao Nakamura acknowledges the support of the Sasakawa Science Research Grant. We would like to thank Prof. Toru Imamura of Tokyo University of Technology (now Hoshi University of Pharmacy and Life Sciences), Dr. Masashi Suzuki of the National Institute of Advanced Industrial Science and Technology, and Shinji Kagaya of S&K BioPharma for their helpful advice and encouragements in carrying out this research. We would like to thank Akira Shiga, Takumi Matsuzaki, Terumasa Saito, Ami Iimori, Nao Tsutsumi, Yuta Hirai, and Syunya Ejima for their cooperation in conducting the experiments in this study.

References


Atsushi Sato is a Professor of School of Bioscience and Biotechnology at Tokyo University of Technology. He received his master of science in engineering in 1989 from Tokyo Institute of Technology. He joined the basic research lab of Toray industries Inc., where he was involved in research and development of diagnosis and biopharmaceuticals. From 1995 to 1998, he worked at Biomolecular Engineering Research Institute (BERI). He received his Ph.D. from the University of Tokyo in 2000 and moved Brain Science Institute, the Institute of Physical and Chemical Research (RIKEN). He then moved to Tokyo University of Technology where he became an associate professor in 2003 and a professor in 2009. Director of the Japanese association for lactoferrin. His main research interest is functional analysis of lactoferrin and development of lactoferrin with an improved plasma half-life as a biopharmaceutical (a potential drug for cancer and spinal cord injury treatment).