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Scavenger receptor CL-P1 mediates endocytosis by associating with AP-2μ2

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ABSTRACT

Background: Scavenger receptor CL-P1 (*co*llectin *p*lacenta *1*) has been found recently as a first membrane-type collectin which is mainly expressed in vascular endothelial cells. CL-P1 can endocytose OxLDL as well as microbes but in general, the endocytosis mechanism of a scavenger receptor is not well elucidated.
Methods: We screened a placental cDNA library using a yeast two-hybrid system to detect molecules associated with the cytoplasmic domain of CL-P1. We analyzed the binding and endocytosis of several ligands in CL-P1 transfectants and performed the inhibition study using tyrphostin A23 which is a specific inhibitor of tyrosine kinase, especially in μ2-dependent endocytosis and the site-directed mutagenesis in the endocytosis YXXΦ motif in CL-P1 cytoplasmic region. Furthermore, the SiRNA study of clathrin, adaptor AP-2 and dynamin-2 during the endocytosis of OxLDL in CL-P1 transfectant cells was carried out.
Results: We identified μ2 subunit of the AP-2 adaptor complex as a molecule associated with the cytoplasmic region of CL-P1. We demonstrated that AP-2μ2 was essential for CL-P1 mediated endocytosis of OxLDL in CL-P1 transfectant cells and its endocytosis was also mediated by clathrin, dynamin and adaptin complex molecules.
Conclusions: Tyrosine-based YXXΦ sequences play an important role in CL-P1-mediated OxLDL endocytosis associated with AP-2μ2.
General Significance: This might be the first finding of the clear endocytosis mechanism in scavenger receptor CL-P1.

1. Introduction

The scavenger receptor family is a highly heterogenous group of cell surface molecules that commonly bind and uptake modified low density lipoproteins (LDLs), such as acetylated LDL (AcLDL) and oxidized LDL (OxLDL). OxLDLs are considered to be most active in interactions among endothelial cells (EC), macrophages, and smooth muscle cells, and have been implicated in the development of atherosclerosis according to Ross's response-to-injury hypothesis [1,2]. The scavenger receptor family has at least eight different subclasses (Class A–Class H) which bear little sequence homology to each other but recognize common ligands [3]. Vascular endothelial cells express several distinct scavenger receptors, such as SR-BI [4–6], LOX-1 [7], SREC [8], FEEL-1/stabilin-1 and FEEL-2/stabilin-2 [9].

We recently identified collectin placenta 1 (CL-P1) from placental cDNA, which is a C-type lectin containing an inner collagen-like region [10]. CL-P1 is a type II transmembrane protein with a coiled-coil domain, a collagen-like domain, and a carbohydrate recognition domain (CRD). It resembles Class A scavenger receptors (SR-A) in that the scavenger receptor cysteine-rich domain is replaced by a CRD [11]. The ability of CL-P1 to bind and phagocytose Gram-negative and Gram-positive bacteria, as well as to yeast, strongly suggests a role for CL-P1 in host defense. Recently, we demonstrated an important role of scavenger receptor CL-P1 in zymosan phagocytosis by several vascular endothelial cells [12].

Interestingly, CL-P1 can also bind and endocytose OxLDL but not to AcLDL as other modified-LDLs in endothelial cells [10]. LOX-1 is a class E scavenger receptor that was originally considered as the major and only receptor for OxLDL on endothelial cells [7]. Arterial CL-P1 was upregulated in the endothelium after the induction of oxidative stress in vitro as well as in a rat ischemia-reperfusion model [13]. In this report, CL-P1 exhibited a different temporal profile than LOX-1 in that it appeared later in vascular endothelial cells in rat [13]. Furthermore, it showed that the endothelium having CL-P1 overexpressed due to the oxidative stress in rat could endocytose OxLDL there [13].

Clathrin triskelions and the clathrin adaptor complex AP-2 are the major components of the clathrin coats located at the plasma membrane, and are responsible for the endocytosis of various proteins, lipids,

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and viral particles [14]. Recently, LOX-1 was reported to be internalized by a clathrin-independent and dynamin-2-dependent pathway and was thus thought likely to mediate OxLDL trafficking in vascular tissues [15]. Although CL-P1 and Lox-1 might have performed some role as scavenger receptors in vascular endothelial cells, but definitive mechanisms or related molecules related with CL-P1 endocytosis are not elucidated. In our current study, we demonstrated that scavenger receptor CL-P1 bound directly to AP-2μ2 in receptor-mediated endocytosis through a tyrosine-motif-dependent pathway.

2. Materials and methods

2.1. Reagents and antibodies

Ham's F12 medium was obtained from Sigma-Aldrich (Poole, UK). Fetal bovine serum was obtained from Invitrogen Co. (Carlsbad, CA). Alexa Fluor 488-and Alexa Fluor 594-conjugated anti-rabbit, anti-mouse IgG antibody, and Hoechst 33342 were from Invitrogen Co. (Molecular Probes, Eugene, OR). Wortmannin, tyrphostin A23 and genistein were from Calbiochem (San Diego, CA). Monoclonal antibody to β-actin (AC-74) was from Sigma-Aldrich (Poole, UK). Monoclonal antibody to clathrin heavy chain, α-adaptin, β-adaptin, μ2 (AP50) and dynamin-2 were purchased from BD Biosciences (San Jose, CA). Monoclonal and polyclonal antibodies to CL-P1 [10,12] were obtained using the recombinant carbohydrate recognition domain (CRD) of human CL-P1 in *Escherichia coli*. Immunoaffinity purification of antibodies was used to purify antigen-specific antibodies from a preparation of polyclonal antibodies to CL-P1. All restriction enzymes were from New England Biolabs (Ipswich, MA).

2.2. Yeast two-hybrid screening

The *Saccharomyces cerevisiae* strain AH109 was maintained on YPD agar plates. Transformation was carried out using the lithium acetate procedure described in the instructions for the MATCHMAKER two-hybrid kit (Takara Bio). An adult human placental cDNA library (Takara Bio) was directly cloned into pGADT7 as an in-frame fusion with Gal4 AD. This library was co-transformed with the pGBKT7/CL-P1NT construct as a bait into the yeast strain AH109, with the integrated growth selection reporter gene *HIS3* using the polyethylene glycol/lithium acetate procedure. Positive clones were isolated by growth on histidine–free medium. After 5 days at 30 °C, their ability to grow on plates lacking histidine and the level of β-galactosidase (X-α-gal) activity were tested. To examine direct interaction, the yeast stain AH109 was co-transformed with the indicated constructs and analyzed by growth in selective medium lacking histidine. In the liquid β-galactosidase assay, cultures of transformants were prepared according to the instructions supplied with the MATCHMAKER two-hybrid kit. β-galactosidase activity was measured using a chemiluminescent β-galactosidase assay kit (Takara Bio) and a luminometer. Briefly, yeast cells were resuspended at 10 A_{600}/mL in lysis buffer and disrupted by vortexing in the presence of glass beads. After centrifugation at 4 °C for 15 min in a microcentrifuge, 10–50 μl of the lysate was used for the measurements. Results were normalized by the protein concentration and expressed as the mean ± standard deviation of three independent determinations.

2.3. Plasmids and their construction

The GAL4 DNA-binding domain constructs (GAL4BD)-CL-P1NT/Wild, GAL4BD-CL-P1NT/E7,8A, GAL4BD-CL-P1NT/E9,10A, GAL4BD-CL-P1NT/

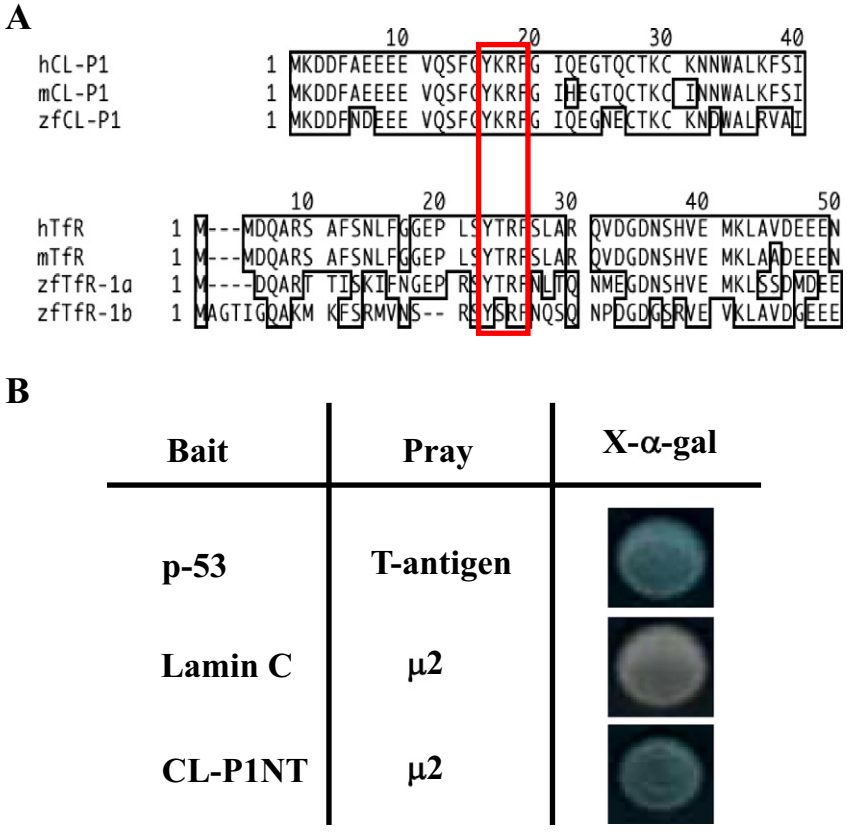

Fig. 1. Yeast two-hybrid screening of the cytoplasmic domain of CL-P1. A, Multiple sequence alignment of known amino acids of CL-P1 and transferrin receptor (TfR) cytoplasmic domains (h, human; m, mouse; z, zebrafish). The YKRF endocytosis motif in red frame is completely conserved in endocytosis motif among different animal CL-P1s. B, Yeast two hybrid analysis revealed that AP-2μ2 interacts with the cytoplasmic domain of CL-P1 (CL-P1NT). The blue colonies containing the β-galactosidase reporter activities (X-alpha-gal), demonstrated the positive interaction in its assay. p53 (murine) and T-antigen (SV40 large T-antigen) used as a positive control but lamin C (human) as a negative one.