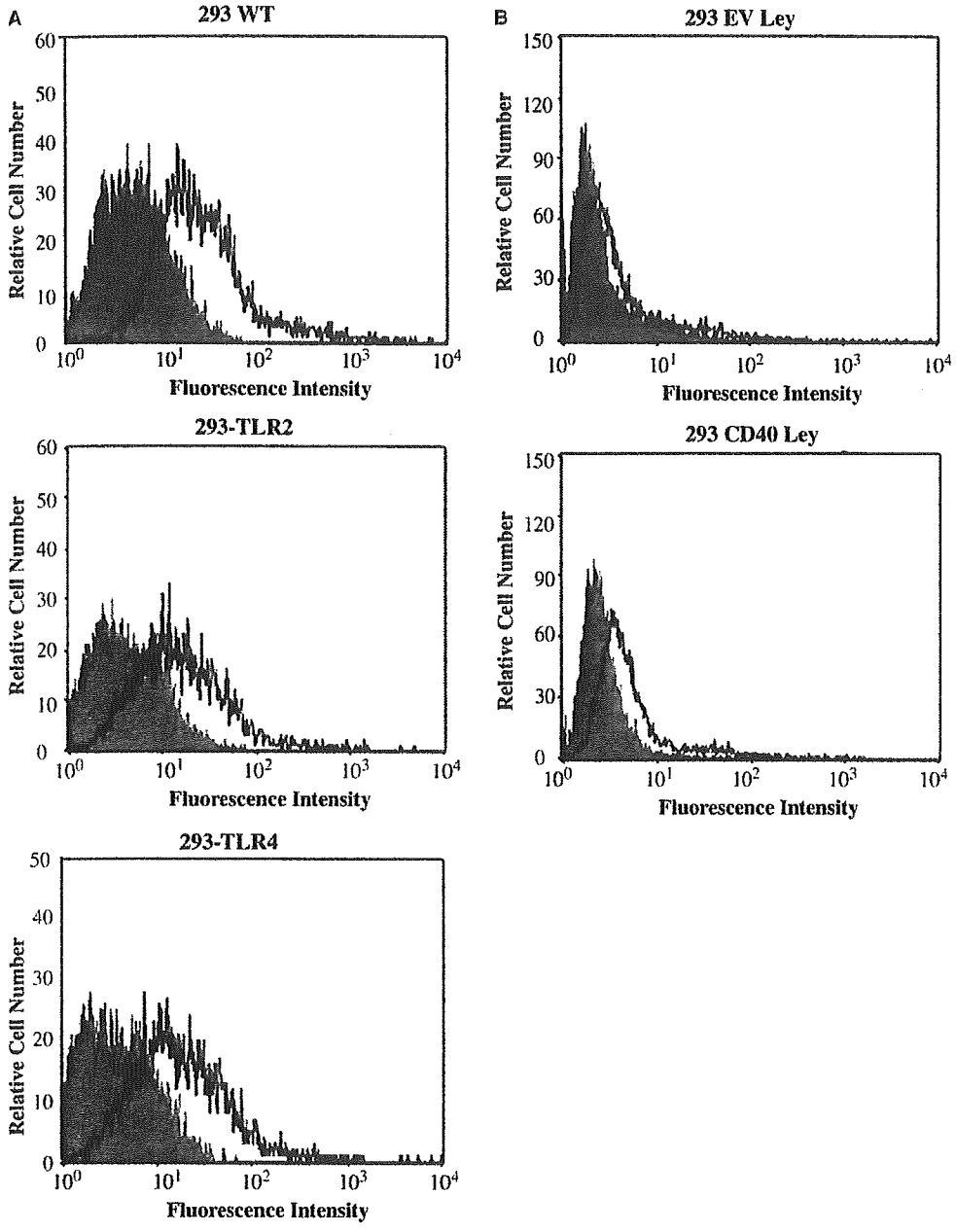

Fig. 3. Absence of association between HSP70.PC and signaling receptors such as TLR 2, 4 and CD40. (A) Wild-type HEK293, HEK293 overexpressing the TLR 2 or 4 (293 TLR2 or TLR4), (B) HEK293 overexpressing an empty vector (293 EV Ley) and HEK293 overexpressing the murine CD40 (293 CD40 Ley) (kindly provided by Dr. Steve C. Ley) were subjected to a HSP70 binding assay as mentioned in Section 2. Shaded gray and thick black line histograms corresponded to BSA and HSP70.PC binding, respectively. Experiments were carried out three times with similar results.

bating them with JTX92 antibody suggesting that these cells may possess a number of potential receptors for HSP70 excluding LOX-1 and these are widely distributed in different cell types (Fig. 5C).

4. Discussion

There has been considerable recent interest in the biological role of extracellular HSP molecular chaperones particularly in the immune response. Such HSP are evidently able to interact with target cells and evince molecular and biological responses. Extracellular HSPs may be released from cells under a range of conditions and travel through the circulation to cellular targets. HSPs are particularly interesting as potential danger signals due to their massive induction by sub-lethal and lethal stresses and their ability to bind to immune effector cells [1,6,15,45]. Previous studies suggest that HSP70 binds to a number of cell surface proteins [12,13,15,32]. Even though specific receptors have shown to

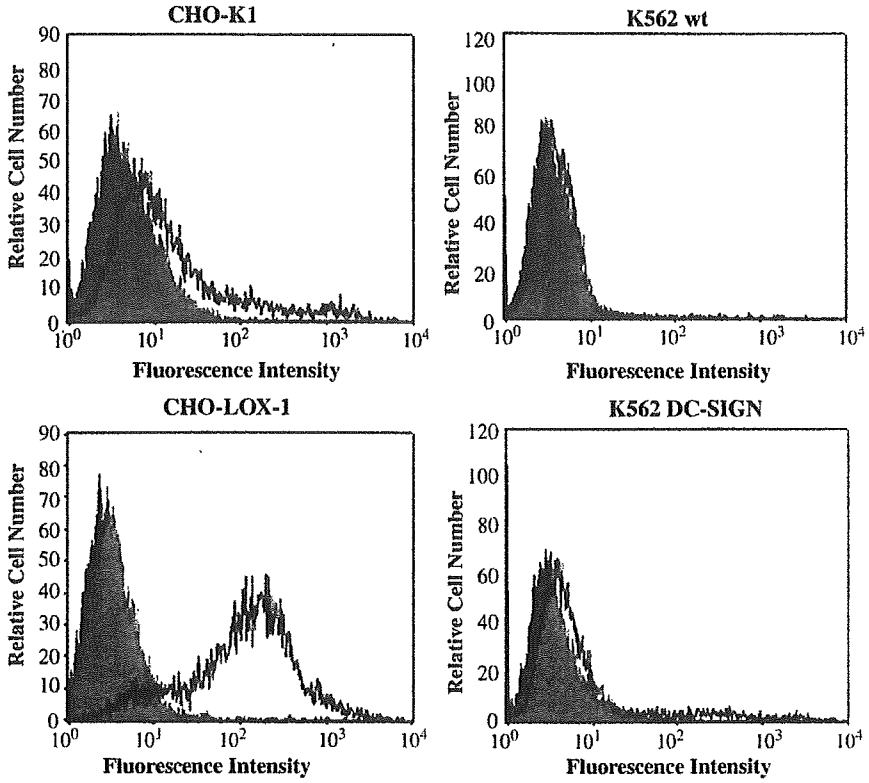

Fig. 4. C-type lectin LOX-1 but not DC-SIGN interacts with HSP70.PC. Wild-type CHO-K1 and CHO overexpressing human receptor LOX-1 (also known as the ox-LDL receptor 1) or wild-type lymphoblastic K562 and K562 overexpressing human DC-SIGN were incubated with Alexa 488-labeled BSA or HSP70.PC as reported in Section 2. Shaded gray and thick black line histograms corresponded to BSA and HSP70.PC binding, respectively. Experiments were carried out three times with similar results.

possess significant HSP70 binding affinity, their relative contribution in cell surface association has not been determined. In this study, we show strong HSP70 binding affinity to SR LOX-1 as first demonstrated by [12]. The specificity of this association seems to be restricted to LOX-1 since no significant binding occurs between HSP70 and a closely related c-type lectin family member known as DC-SIGN or SRs coming from other classes (ex. CLA-1, SR-A1, MARCO and CD36) [12]. As mentioned earlier, the c-type lectin receptor CD94 also shows some binding affinity for HSP70. However, the HSP70 relative affinity of CD94 for HSP70 seems to be weaker than for LOX-1 since more HSP70 (five times more) is needed to see a clear binding to CD94 indicating that conserved residues defining the c-type lectin domain are not the sole determinant necessary for HSP70 interaction [12,13].

LRP/CD91 binds a wide spectrum of ligands and, in every case, ligands interact, in part or entirely, with the second and/or the fourth LRP/CD91 ligand binding site located in the alpha subunit [24,41]. RAP ligand binds exclusively these two domains and can block HSP binding to LRP/CD91 while our studies show that HSP70.PC does not interact with these domains [26,41]. Although, LRP/CD91 does not seem to participate directly in HSP70.PC interaction with cells, it may however be involved at a subsequent stage in HSP70.PC-mediated cross-presentation [26].

From initial experiments showing HSP70-mediated pro-inflammatory cytokine production, HSP70 participation in the innate immune response has been linked to both TLR2 and TLR4 in a CD14-dependent fashion [6–8]. HSP70, HSP90, CXCR4, GDF5 and TLR4 are also mediators of cytokine release in a CD14-independent LPS-activated complex in monocyte [46]. However, no specific interaction is seen between HSP70 and TLR2/4 or with CD14 (Fig. 3A) [42]. It is possible that HSP70-mediated pro-inflammatory cytokine production does not require a direct association between TLRs and HSP70 but involve a putative cross-talk between TLRs and other HSP70 receptors. Supporting this idea, TLR2-mediated inflammatory response can be enhanced through synergic collaboration with endocytic receptors such the c-type lectin receptor DECTIN-1 (also known as the β-glucan receptor) in response against, for example, a yeast cell wall preparation called Zymosan [47,48].

The failure to see HSP70 binding to CD40 was quite unexpected since our HSP70 is associated with ADP and likely contains an array of endogenous mammalian peptide antigens, properties evidently required for HSP70–CD40 interaction [32]. No accurate prediction can be made about the proportion of peptide bound to HSP70. Nonetheless, ADP seems to be the major element required for the direct binding to CD40. However, only one report has proposed mamma-

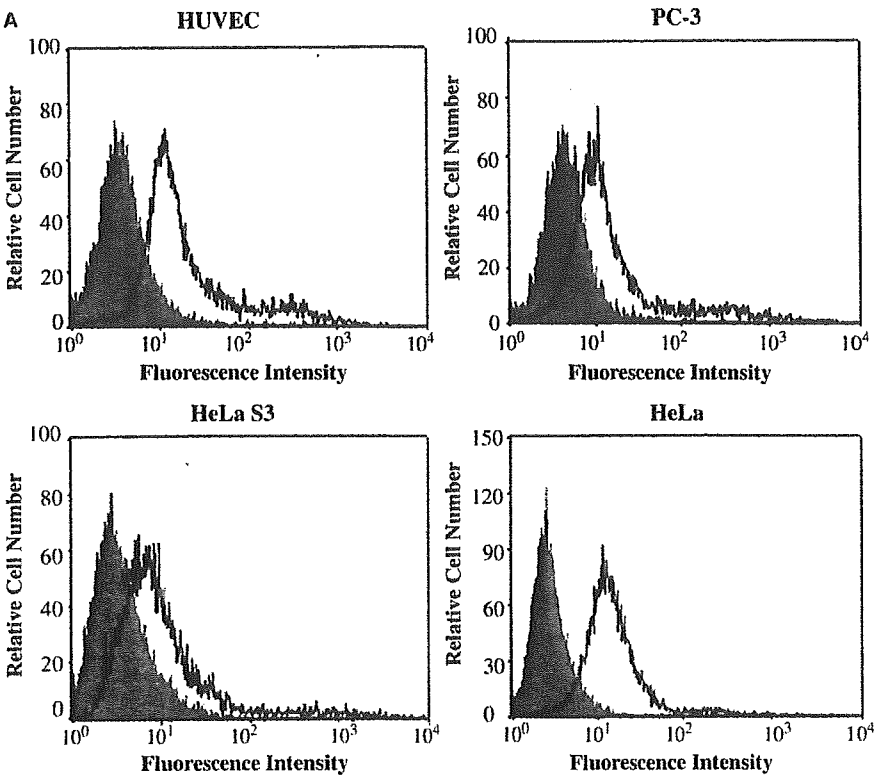

Fig. 5. HSP70.PC interacts with endothelial/epithelial cells but not other non-APC cell types. (A) HSP70 binding assay on human endothelial HUVEC, prostate cancer cell line PC-3 and HeLa from two different backgrounds. (B) No or few interaction between HSP70 and other non endothelial/epithelial cell lines IMR90 (human fibroblasts), MCF-7 (breast cancer cells) and A375 (melanoma cells). Shaded gray and thick black line histograms corresponded to BSA and HSP70.PC binding, respectively. Experiments were carried out three times with similar results. (C) HSP70.PC binding blocking experiments (Section 2) using anti-human LOX-1 (JTX92) (αLOX-1) on CHO-LOX-1, HUVEC, PC-3 and HeLa ($n = 3$). MFI: mean fluoresence intensity.

lian HSP70 as ligand for CD40 whereas other studies suggest instead that only mycobacterial hsp70 is able to transduce signal directly through CD40 while mammalian HSP70 is inactive [49]. The latter study utilized similar experimental conditions used here, involving overexpression of the human CD40 in HEK293 cells.

Previous reports have exclusively attributed surface HSPs binding affinity to immune effector such monocytes, macrophages and DC [6,15,39]. Indeed, we show here HSP70.PC interaction with pre-monocytic THP-1, mature macrophages RAW 267.4 and thioglycollate-elicited macrophages (Fig. 1). Surprisingly, we also found that HSP70.PC interacts with non-APC cells especially those from endothelial/epithelial background (Fig. 5A). LOX-1 does not however play a major role in HSP70.PC binding to HUVEC, PC-3 and HeLa suggesting the involvement of, at least, another HSP70 receptor. Concerning the HUVEC cells, one possible explanation is that LOX-1 levels were insufficient to mediate HSP70 binding. The significance of the HSP70 interaction with non-APC cells remains undefined but HSP70 binding to these cells may mediate other non-immune purposes or applications. In fact, HSP70 uptake may participate in the response to external stresses in HSP70-deficient cell types unable to mount a heat shock response such as neurons [43]. Indeed, it has been found that glial cells can release HSP70 in normal conditions or during stress such as heat shock [43]. HSP70 released from glial cells is taken up by adjacent neuronal cells, which in turn develop a higher resistance to stress-induced apoptosis. Interestingly, HSP60 has been shown to induce endothelial cytokine production and adhesion molecule expression [50]. Moreover, HSP70 is aberrantly expressed at the surface on DC and/or secreted in a rheumatoid arthritis model suggesting a role of HSPs in autoimmune diseases [51]. Thus, identification and characterization of endocytic and signaling HSP70 receptors involved in HSP70-mediated tumor antigen cross-presentation will not only help to decipher the role of HSP70 as a self-adjuvant but may pave the way to elucidate the involvement of extracellular HSP70 in other non anti-tumor immune functions such as atherosclerosis and arthritis.

Our experiments therefore indicate that LOX-1 binds with high affinity to HSP70, but other cell surface structures that appear to mediate immune effects of HSP70, such as LRP/CD91, CD40, CD14, TLR2 and TLR4 do not show significant binding to HSP70. It is, however, evident that other perhaps unidentified HSP70 receptors exist as binding to HUVEC, PC-3 and HeLa cells does not involve a major contribution from LOX-1 and our future studies will address this question.

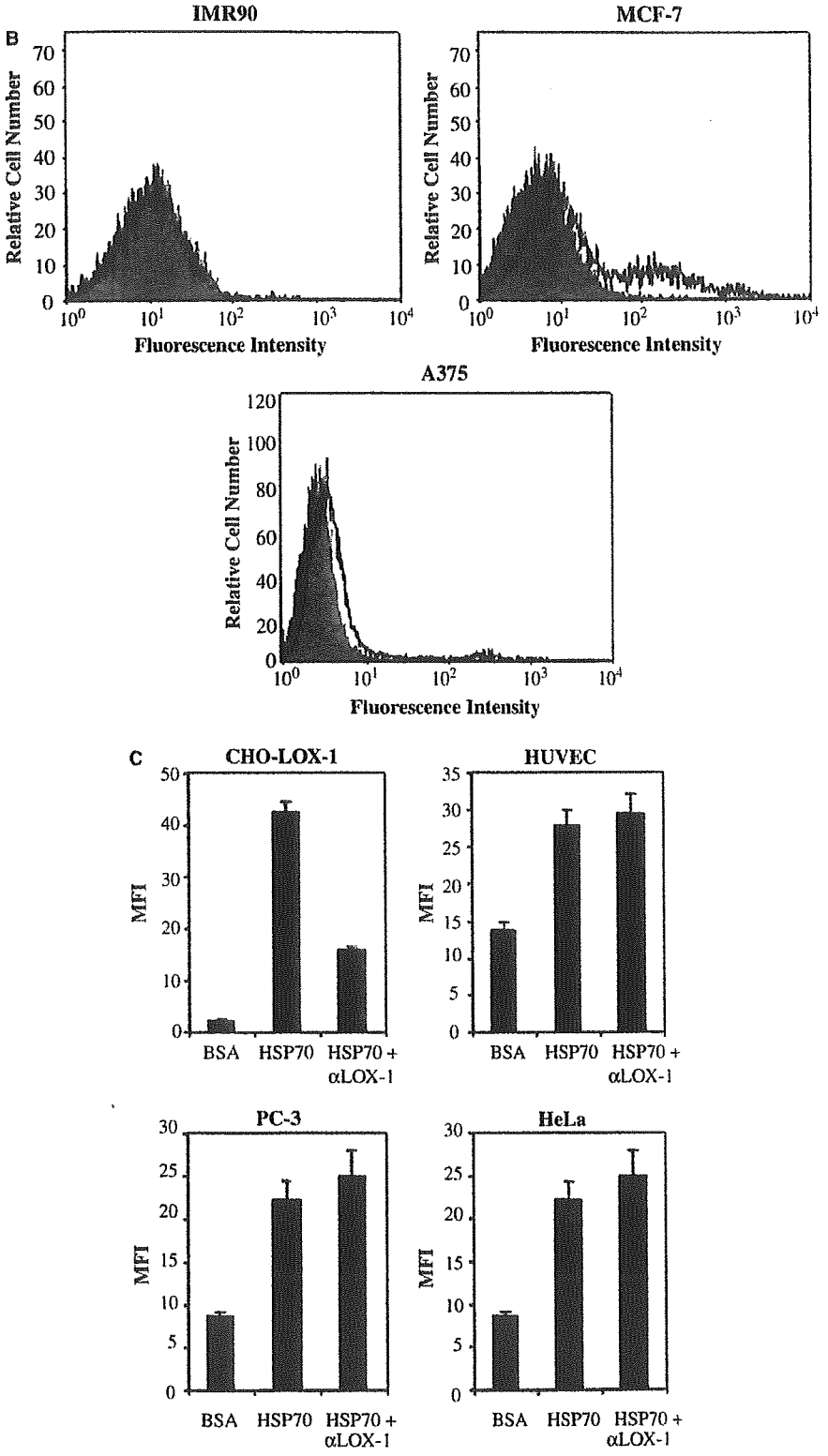

Fig. 5 (*continued*)

Acknowledgements: We thank Dr. Yasahiro Ito (HUVEC), Dr. Guojun Bu (CHO LRP null, LRP 2 and LRP 4), Dr. Vladimir Gabai (IMR90), Dr. Steve C. Ley (293 EV and murine CD40) and Dr. Angel L. Corbi (K562 human DC-SIGN) from kindly provided these cells. We thank Dr. Robin E. Bachelder (Dr. Arthur M. Mercurio) for the utilization of their Flow cytometer. This study was supported by the NIH Grant R01CA047407.

References

[1] Lindquist, S. and Craig, E.A. (1988) The heat-shock proteins. Annu. Rev. Genet. 22, 631–677.
[2] Georgopoulos, C. and Welch, W.J. (1993) Role of the major heat shock proteins as molecular chaperones. Annu. Rev. Cell Biol. 9, 601–634.
[3] Udono, H. and Srivastava, P.K. (1993) Heat shock protein 70-associated peptides elicit specific cancer immunity. J. Exp. Med. 178, 1391–1396.
[4] Srivastava, P. (2002) Roles of heat-shock proteins in innate and adaptive immunity. Nat. Rev. Immunol. 2, 185–194.
[5] Baker-LePain, J.C., Reed, R.C. and Nicchitta, C.V. (2003) ISO: a critical evaluation of the role of peptides in heat shock/chaperone protein-mediated tumor rejection. Curr. Opin. Immunol. 15, 89–94.
[6] Asea, A., Kraeft, S.K., Kurt-Jones, E.A., Stevenson, M.A., Chen, L.B., Finberg, R.W., Koo, G.C. and Calderwood, S.K. (2000) HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. Nat. Med. 6, 435–442.
[7] Vabulas, R.M., Ahmad-Nejad, P., Ghose, S., Kirschning, C.J., Issels, R.D. and Wagner, H. (2002) HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. J. Biol. Chem. 277, 15107–15112.
[8] Asea, A., Rehli, M., Kabingu, E., Boch, J.A., Bare, O., Auron, P.E., Stevenson, M.A. and Calderwood, S.K. (2002) Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. J. Biol. Chem. 277, 15028–15034.
[9] Kuppner, M.C., Gastpar, R., Gelwer, S., Nossner, E., Ochmann, O., Scharner, A. and Issels, R.D. (2001) The role of heat shock protein (hsp70) in dendritic cell maturation: hsp70 induces the maturation of immature dendritic cells but reduces DC differentiation from monocyte precursors. Eur. J. Immunol. 31, 1602–1609.
[10] Singh-Jasuja, H., et al. (2000) Cross-presentation of glycoprotein 96-associated antigens on major histocompatibility complex class I molecules requires receptor-mediated endocytosis. J. Exp. Med. 191, 1965–1974.
[11] Castellino, F., Boucher, P.E., Eichelberg, K., Mayhew, M., Rothman, J.E., Houghton, A.N. and Germain, R.N. (2000) Receptor-mediated uptake of antigen/heat shock protein complexes results in major histocompatibility complex class I antigen presentation via two distinct processing pathways. J. Exp. Med. 191, 1957–1964.
[12] Delneste, Y., et al. (2002) Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. Immunity 17, 353–362.
[13] Gross, C., Hansch, D., Gastpar, R. and Multhoff, G. (2003) Interaction of heat shock protein 70 peptide with NK cells involves the NK receptor CD94. Biol. Chem. 384, 267–279.
[14] Berwin, B., Hart, J.P., Rice, S., Gass, C., Pizzo, S.V., Post, S.R. and Nicchitta, C.V. (2003) Scavenger receptor-A mediates gp96/GRP94 and calreticulin internalization by antigen-presenting cells. EMBO J. 22, 6127–6136.
[15] Basu, S., Binder, R.J., Ramalingam, T. and Srivastava, P.K. (2001) CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. Immunity 14, 303–313.
[16] Binder, R.J., Han, D.K. and Srivastava, P.K. (2000) CD91: a receptor for heat shock protein gp96. Nat. Immunol. 1, 151–155.
[17] Krieger, M. (2001) Scavenger receptor class B type I is a multiligand HDL receptor that influences diverse physiologic systems. J. Clin. Invest. 108, 793–797.
[18] Gough, P.J. and Gordon, S. (2000) The role of scavenger receptors in the innate immune system. Microbes Infect. 2, 305–311.
[19] Peiser, L., Mukhopadhyay, S. and Gordon, S. (2002) Scavenger receptors in innate immunity. Curr. Opin. Immunol. 14, 123–128.
[20] Cambi, A. and Figdor, C.G. (2003) Dual function of C-type lectin-like receptors in the immune system. Curr. Opin. Cell Biol. 15, 539–546.
[21] Figdor, C.G., van Kooyk, Y. and Adema, G.J. (2002) C-type lectin receptors on dendritic cells and Langerhans cells. Nat. Rev. Immunol. 2, 77–84.
[22] Hayashida, K., Kume, N., Minami, M. and Kita, T. (2002) Lectin-like oxidized LDL receptor-1 (LOX-1) supports adhesion of mononuclear leukocytes and a monocyte-like cell line THP-1 cells under static and flow conditions. FEBS Lett. 511, 133–138.
[23] Chen, M., Masaki, T. and Sawamura, T. (2002) LOX-1, the receptor for oxidized low-density lipoprotein identified from endothelial cells: implications in endothelial dysfunction and atherosclerosis. Pharmacol. Ther. 95, 89–100.
[24] Herz, J. and Strickland, D.K. (2001) LRP: a multifunctional scavenger and signaling receptor. J. Clin. Invest. 108, 779–784.
[25] Li, Y., Wood, N., Parsons, P.G., Yellowlees, D. and Donnelly, P.K. (1997) Expression of alpha2-macroglobulin receptor/low density lipoprotein receptor-related protein on surfaces of tumour cells: a study using flow cytometry. Cancer Lett. 111, 199–205.
[26] Binder, R.J. and Srivastava, P.K. (2004) Essential role of CD91 in re-presentation of gp96-chaperoned peptides. Proc. Natl. Acad. Sci. USA 101, 6128–6133.
[27] Gardai, S.J., Xiao, Y.Q., Dickinson, M., Nick, J.A., Voelker, D.R., Greene, K.E. and Henson, P.M. (2003) By binding SIRPalpha or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation. Cell 115, 13–23.
[28] Shepherd, J.C., Schumacher, T.N., Ashton-Rickardt, P.G., Imaeda, S., Ploegh, H.L., Janeway Jr., C.A. and Tonegawa, S. (1993) TAP1-dependent peptide translocation in vitro is ATP dependent and peptide selective. Cell 74, 577–584.
[29] Schumacher, T.N., et al. (1994) Peptide length and sequence specificity of the mouse TAP1/TAP2 translocator. J. Exp. Med. 179, 533–540.
[30] Neefjes, J.J., Momburg, F. and Hammerling, G.J. (1993) Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter. Science 261, 769–771.
[31] Asea, A., Kabingu, E., Stevenson, M.A. and Calderwood, S.K. (2000) HSP70 peptide bearing and peptide-negative preparations act as chaperokines. Cell Stress Chaperones 5, 425–431.
[32] Becker, T., Hartl, F.U. and Wieland, F. (2002) CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes. J. Cell Biol. 158, 1277–1285.
[33] Millar, D.G., Garza, K.M., Odermatt, B., Elford, A.R., Ono, N., Li, Z. and Ohashi, P.S. (2003) Hsp70 promotes antigen-presenting cell function and converts T-cell tolerance to autoimmunity in vivo. Nat. Med. 9, 1469–1476.
[34] Relloso, M., et al. (2002) DC-SIGN (CD209) expression is IL-4 dependent and is negatively regulated by IFN, TGF-beta, and anti-inflammatory agents. J. Immunol. 168, 2634–2643.
[35] Coope, H.J., et al. (2002) CD40 regulates the processing of NF-kappaB2 p100 to p52. EMBO J. 21, 5375–5385.
[36] Moriwaki, H., et al. (1998) Ligand specificity of LOX-1, a novel endothelial receptor for oxidized low density lipoprotein. Arterioscler. Thromb. Vasc. Biol. 18, 1541–1547.
[37] Mambula, S.S., Sau, K., Henneke, P., Golenbock, D.T. and Levitz, S.M. (2002) Toll-like receptor (TLR) signaling in response to *Aspergillus fumigatus*. J. Biol. Chem. 277, 39320–39326.
[38] Peng, P., Menoret, A. and Srivastava, P.K. (1997) Purification of immunogenic heat shock protein 70-peptide complexes by ADP-affinity chromatography. J. Immunol. Methods 204, 13–21.
[39] Noessner, E., et al. (2002) Tumor-derived heat shock protein 70 peptide complexes are cross-presented by human dendritic cells. J. Immunol. 169, 5424–5432.
[40] Berwin, B., Hart, J.P., Pizzo, S.V. and Nicchitta, C.V. (2002) Cutting edge: CD91-independent cross-presentation of GRP94 (gp96)-associated peptides. J. Immunol. 168, 4282–4286.
[41] Obermoeller-McCormick, L.M., Li, Y., Osaka, H., FitzGerald, D.J., Schwartz, A.L. and Bu, G. (2001) Dissection of receptor folding and ligand-binding property with functional

minireceptors of LDL receptor-related protein. J. Cell Sci. 114, 899–908.
[42] Lipsker, D., et al. (2002) Heat shock proteins 70 and 60 share common receptors which are expressed on human monocyte-derived but not epidermal dendritic cells. Eur. J. Immunol. 32, 322–332.
[43] Guzhova, I., Kislyakova, K., Moskaliova, O., Fridlanskaya, I., Tytell, M., Cheetham, M. and Margulis, B. (2001) In vitro studies show that Hsp70 can be released by glia and that exogenous Hsp70 can enhance neuronal stress tolerance. Brain Res. 914, 66–73.
[44] Sawamura, T., et al. (1997) An endothelial receptor for oxidized low-density lipoprotein. Nature 386, 73–77.
[45] Broquet, A.H., Thomas, G., Masliah, J., Trugnan, G. and Bachelet, M. (2003) Expression of the molecular chaperone Hsp70 in detergent-resistant microdomains correlates with its membrane delivery and release. J. Biol. Chem. 278, 21601–21606.
[46] Triantafilou, K., Triantafilou, M. and Dedrick, R.L. (2001) A CD14-independent LPS receptor cluster. Nat. Immunol. 2, 338–345.
[47] Brown, G.D., Taylor, P.R., Reid, D.M., Willment, J.A., Williams, D.L., Martinez-Pomares, L., Wong, S.Y. and Gordon, S. (2002) Dectin-1 is a major beta-glucan receptor on macrophages. J. Exp. Med. 196, 407–412.
[48] Gantner, B.N., Simmons, R.M., Canavera, S.J., Akira, S. and Underhill, D.M. (2003) Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. J. Exp. Med. 197, 1107–1117.
[49] Wang, Y., et al. (2001) CD40 is a cellular receptor mediating mycobacterial heat shock protein 70 stimulation of CC-chemokines. Immunity 15, 971–983.
[50] Kol, A., Bourcier, T., Lichtman, A.H. and Libby, P. (1999) Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages. J. Clin. Invest. 103, 571–577.
[51] Martin, C.A., Carsons, S.E., Kowalewski, R., Bernstein, D., Valentino, M. and Santiago-Schwarz, F. (2003) Aberrant extracellular and dendritic cell (DC) surface expression of heat shock protein (hsp)70 in the rheumatoid joint: possible mechanisms of hsp/DC-mediated cross-priming. J. Immunol. 171, 5736–5742.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular and Cellular Cardiology 39 (2005) 553–561
Journal of Molecular and Cellular Cardiology
www.elsevier.com/locate/yjmcc

Original article

Essential role of cytoplasmic sequences for cell-surface sorting of the lectin-like oxidized LDL receptor-1 (LOX-1)

Mingyi Chen [a,b], Tatsuya Sawamura [a,*]

[a] *Department of Vascular Physiology, National Cardiovascular Center Research Institute, Suita, Osaka 565-8565, Japan*
[b] *Department of Pathology and Laboratory Medicine, Howard Hughes Medical Institute, University of California, Los Angeles, CA 90095, USA*

Received 7 March 2005; received in revised form 19 April 2005; accepted 4 May 2005

Available online 01 June 2005

Abstract

Lectin-like oxidized LDL receptor-1 (LOX-1) is an oxidized low-density lipoprotein (OxLDL) receptor found in endothelial cells and a member of the natural killer (NK) receptor gene complex. Here, we demonstrate that the ability of LOX-1 binding to OxLDL distinguishes it from other NK receptors. Domain swapping of the lectin-like domain between LOX-1 and the NK cell receptors CD94, NKG2D, and LY-49A demonstrated the crucial role of this domain for recognition of OxLDL by LOX-1, but not for the correct cell-surface sorting of LOX-1. Using LOX-1 GFP fusion constructs, we find that the combination of cytoplasmic and transmembrane domains of LOX-1 is sufficient to target the chimeric protein to the cell-surface. Using N-terminal deletions we determined that the correct cell-surface localization is dependent on a positively charged motif present in the cytosolic juxtamembrane region of LOX-1. Furthermore, the extracellular localization of the LOX-1 C-terminus is disrupted when we mutated the cytoplasmic basic amino acids, Lys-22, Lys-23 and Lys-25 to Glu. Collectively, these results indicate that the N-terminal cytoplasmic domain of LOX-1 determines the correct expression of the lectin domain on the cell-surface.

Keywords: Lectin-like oxidized LDL receptor-1; Cell-surface sorting; Integral membrane protein topology

1. Introduction

LOX-1 is an oxidized low-density lipoprotein (OxLDL) receptor that is found in endothelial cells [1]. Multiple lines of evidence support the important role of LOX-1 in the pathogenesis of atherosclerosis [2]. LOX-1 mediates recognition and endocytosis of oxidized LDL [1], advanced glycation end (AGE) products [3], aged/apoptotic cells [4], activated platelets [5], and some bacteria species [6], emphasizing its important (patho)physiological roles [2]. Binding of OxLDL to LOX-1 triggers a pathological process that results in activation of NF-κB and increased reactive oxygen species production [7,8] that induces monocyte adhesion to endothelial cells [9] and endothelial apoptosis [10].

In addition to vascular endothelial cells, LOX-1 is also detected in monocyte-derived macrophages [11], smooth muscle cells [12]. The expression of LOX-1 is induced by many proinflammatory cytokines and oxidized LDL in cultured cells [2]. In vivo, LOX-1 is enhanced in several proatherogenic settings such as hypertension, hyperlipidemia and diabetes, and is indeed accumulated in atherosclerotic lesions [13]. All of these findings support the relevance of LOX-1 in the pathogenesis of atherosclerosis [2].

LOX-1 belongs to the C-type lectin protein family [1]. The LOX-1 protein consists of four domains: an intracellular domain, a single transmembrane-spanning domain, a connecting neck domain, and an extracellular C-type lectin domain [14]. Studies by several groups, including ours, demonstrated that the lectin-like domain of LOX-1 was required for OxLDL recognition [14–16].

LOX-1 is a type II membrane protein that has a short N-terminal intracellular polypeptide chain (34 amino acids). The function of this intracellular domain is currently un-

Abbreviations: BSA, bovine serum albumin; CHO cell, Chinese hamster ovary cell; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; GFP, green fluorescent protein; HLOX-1, human LOX-1; LOX-1, lectin-like oxidized LDL receptor-1; OxLDL, oxidized low-density lipoprotein; PBS, phosphate-buffered saline.

* Corresponding author. Tel.: +81 66 833 5012 ext. 2518; fax: +81 66 835 5329.
E-mail address: t-sawamura@umin.ac.jp (T. Sawamura).

0022-2828/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.yjmcc.2005.05.001

known. The present study was performed to characterize the detailed structure–functional relationship of this domain for LOX-1 targeting to the plasma membrane. We investigated the distinct function of the lectin-like domain of LOX-1 in ligand binding specificity by lectin domain swapping between LOX-1 and structurally related NK receptor such CD94, NKG2D, and LY-49A. Additionally, we have identified an important role of the cytoplasmic domain of LOX-1, which determines the correct cell-face sorting of the lectin domain of LOX-1.

2. Materials and methods

2.1. Plasmid constructs

The wild type and deletion mutants of human LOX-1, mouse CD94, human NKG2D, and mouse LY-49A cDNAs were amplified by PCR and cloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen). This results in an *in-frame* fusion of the V5 epitope at the C-terminus. All of the constructs were verified by sequencing.

2.1.1. Construction of chimeric receptors

The lectin-like domains of mCD94 (amino acids 53–179), hNKG2D (amino acids 95–216) and mLY-49A (amino acids 143–262) were swapped with the lectin-like domain of human LOX-1 (amino acids 138–274). The chimeric CD94-LOX-1-Lectin, NKg2D-LOX-1-Lectin, and Ly-49A-LOX-1-Lectin were generated and cloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen) by standard molecular biological techniques. Expression of the different chimeric constructs was determined by Western blotting using an anti-V5 or an anti LOX-1 antibody.

2.1.2. Generation of LOX-1-green fluorescent protein (GFP) fusion proteins

Wild type and deletion mutations of human LOX-1 were amplified by PCR with flanking primers containing *XhoI* and *BamHI* overhangs and cloned into pEGFP-N3 vector (Clontech).

2.1.3. Site-directed mutagenesis

Coding sequences for specific amino acids of human LOX-1 protein were substituted using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Briefly, using pcDNA3.1-hLOX-1-V5 vector as template, two complementary primers (125 ng each) containing the desired mutation and 50 ng of template in 1× reaction buffer were denatured at 95 °C for 30 s and annealed at 55 °C for 1 min, and DNA synthesis was carried out by *Pfu* polymerase at 68 °C for 14 min; this cycle was repeated 18 times. The methylated template was removed by incubation with 10 units of *Dpn*I at 37 °C for 1 h. cDNA constructs containing a combination of mutations were constructed by carrying out serial mutagenesis reactions. The mutated cDNAs were sequenced completely to verify the mutations.

2.2. Cell culture and transfection

Cells were incubated at 37 °C in 95% air and 5% CO_2. Wild type CHO-K1 cells were maintained in Ham's F-12 medium (Gibco) with 10% fetal calf serum. For transfections, 1.5×10^6 of cells in 100 mm dishes were transiently transfected with 20 μg plasmids using Lipofectamine 2000 (Gibco). After 24 h the cells were plated in 12-well dishes or 2-well chamber slide. Cells were used for experiments 48 h after transfection.

2.3. Immunofluorescent staining and confocal microscopy

Binding and uptake of DiI-labeled OxLDL to LOX-1 transfected CHO cells were determined by fluorescent microscopy. Briefly, the cells were incubated with 10 μg/ml of DiI-labeled OxLDL at 4 °C for 30 min (binding) or 37 °C for 3 h (binding and uptake). After three washed with phosphate-buffered saline (PBS), the cells were fixed by treatment with 4% paraformaldehyde for 20 min at room temperature. Some of cells were permeabilized by 0.1% Triton X-100 in PBS at room temperature for 10 min. Cells were blocked with 0.1% bovine serum albumin in PBS containing non-immune goat serum. Cells were stained with anti-V5 monoclonal antibody (Invitrogen) for 60 min, and then with fluorescein isothiocyanate-conjugated (FITC) goat anti-mouse IgG for 60 min in 10% FCS in PBS. Staining for LOX-1 expression was stained with a monoclonal antibody (JTX-92), followed by Cy5-conjugated goat anti-human IgG (Amersham Life Science). EGFP or EGFP fusion proteins were detected with polyclonal anti-GFP antibody (Molecular Probes) followed by Cy5-labeled goat anti-rabbit antibody (Amersham Life Science). The coverslips were mounted using 80% glycerol supplemented with 2% triethylenediamine and the cells were examined on an Olympus confocal microscope.

2.4. Immunoblot analysis

Cells were lyzed in 62.5 mmol/l Tris–HCl (pH 7.4), 2% SDS, and 10% glycerol, and protein concentrations were determined by using a BCA protein assay kit (Pierce Chemical) with bovine serum albumin as standard. Samples containing 60 μg of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore). Membranes were probed with an anti LOX-1, or anti-V5 (Invitrogen) or anti-GFP antibody (Molecular Probes), followed by horseradish peroxidase (HRP)-conjugated horse anti-mouse antibody. Bands were visualized by Vectastain Elite ABC kit (Vector). Data shown are representative of three independent experiments with similar results.

2.5. N-glycosidase digestion

Fifty micrograms of cell lysate protein was dissolved in denaturation buffer (1% SDS/1 M Tris–HCl pH 6.8) contain-

ing 0.2 M 2-mercaptoethanol, and then boiled for 5 min. The volumes were adjusted to final detergent concentrations of 0.1% SDS and 1.25% Nonidet P-40 and incubated for 20 h at 37 °C in the presence of 5 munits/ml N-glycosidase F (Takara). After digestion, cell lysates were subjected to SDS-PAGE followed by Western blot analysis with an anti-LOX-1 monoclonal antibody (5-2).

3. Results

3.1. The lectin-like domain of LOX-1 enables it to bind oxidized LDL

LOX-1 is a homologue of the lectin-like NK cell receptors that include NKG2, CD94, CD69, NKR-P1A in humans and Ly-49A in mouse. Our previous studies using deletion and mutations of the lectin-like domain of LOX-1 demonstrated that it is required for ligand binding [14,15]. Here, we generated chimeric receptors in which the lectin domain of LOX-1 was used to replace the lectin domain of CD94 (Fig. 1A). All constructs were expressed and detected on the plasma membrane of transfected CHO cells (Fig. 1B, C). CHO cells transfected with LOX-1 bound to OxLDL, in contrast to CHO cells transfected with the CD94 cDNA (Fig. 1C). However, swapping of the lectin domain of CD94 with the lectin domain of LOX-1 was sufficient to allow this chimera to bind OxLDL. Chimeric constructs of hNKg2D and mLY-49A in which the lectin domain was replaced with the counterpart of LOX-1 showed similar results (data not shown).

3.2. Characterization of LOX-1 cell-surface sorting by LOX-1-GFP fusion proteins

In order to visualize the LOX-1 receptor directly, we generated LOX-1-GFP fusion proteins in which the C-terminus of LOX-1 was fused to the enhanced GFP (designated as LOX-1-EGFP (Fig. 2A)). All the GFP fusion proteins were expressed and detected in transfected CHO cells (Fig. 2B). The localization of the fusion proteins was monitored by GFP fluorescence, and the cell-surface expression was analyzed by staining with an anti-GFP antibody without cell-surface permeabilization.

Fusion of GFP to WT LOX-1 does not impair its ability to be expressed on the cell-surface (Fig. 2C). Deletion of the

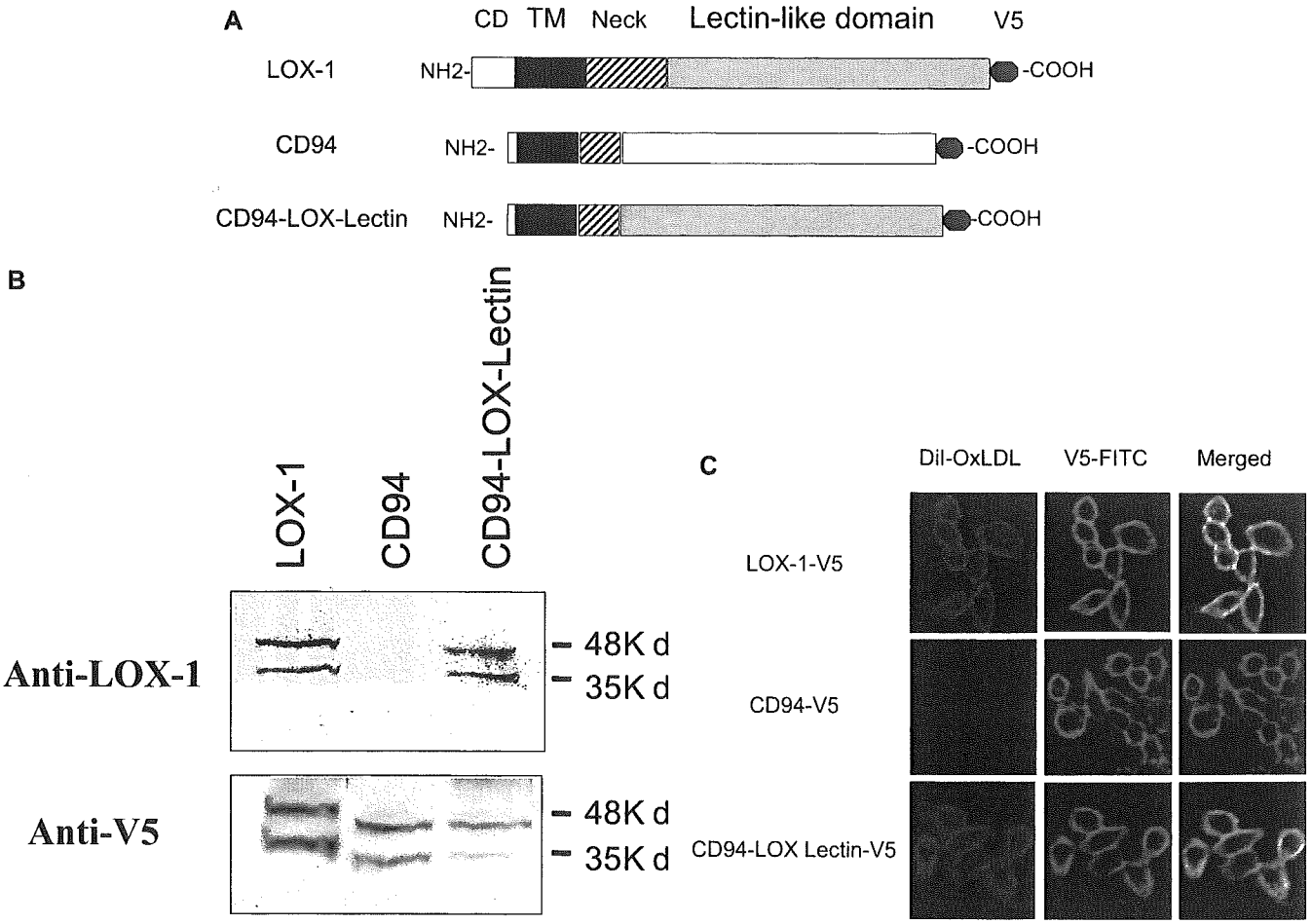

Fig. 1. Swapping the lectin domain of LOX-1 to NK cell receptor CD94. (**A**) Schematic representation of the structure and domain organization of native and chimeric proteins of human LOX-1, mCD94, and CD94-LOX-1. (**B**) Immunoblot analysis confirms the expression of chimeric proteins in CHO cells with anti-LOX-1 and anti-V5 antibodies. (**C**) Replacement of lectin domain of the NK cell receptor CD94 with the counterpart of LOX-1 results in binding to oxidized LDL binding at 4 °C.

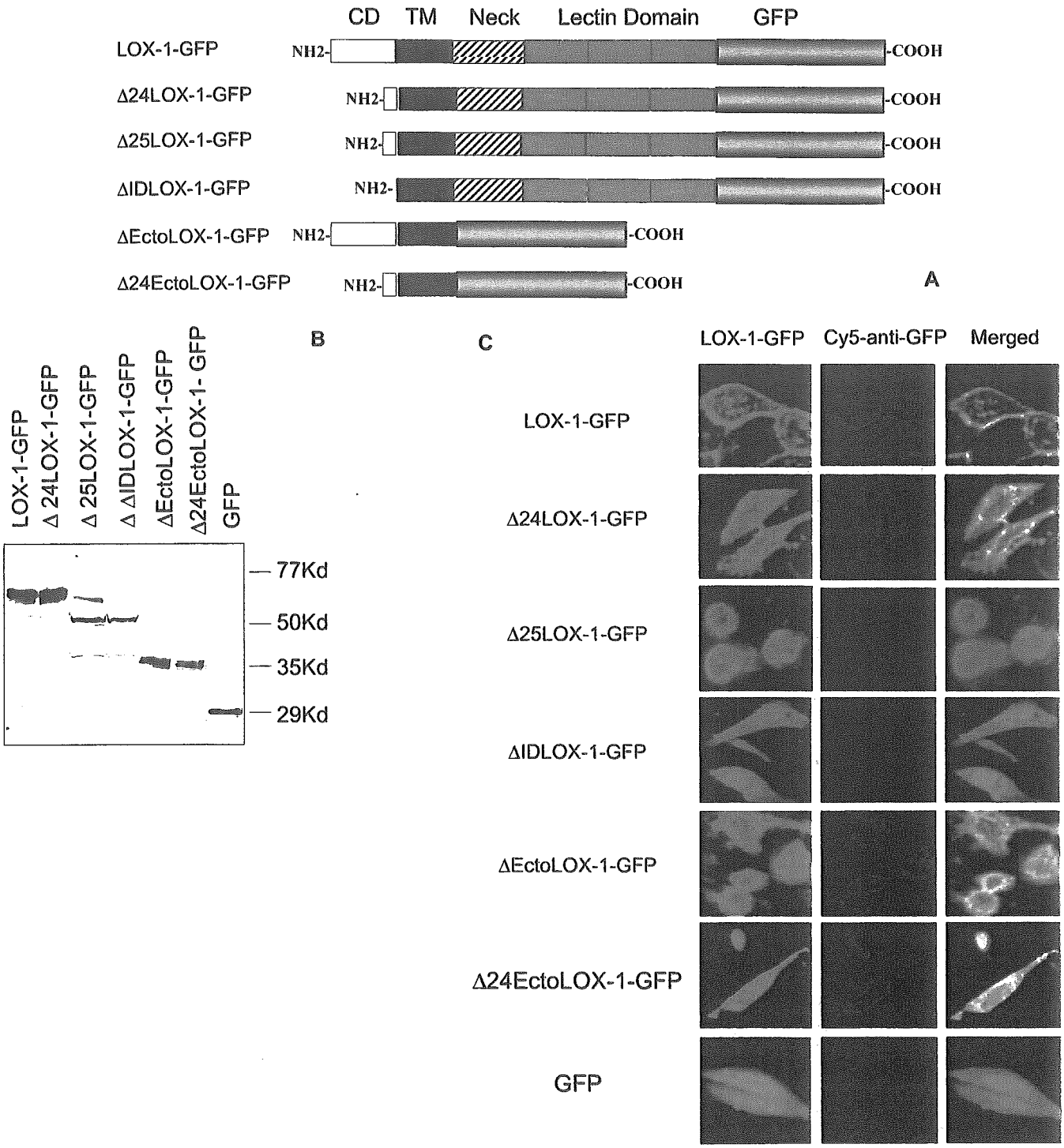

Fig. 2. Intracellular localization LOX-1-GFP fusion proteins. (**A**) Schematic illustration of the LOX-1-GFP fusion proteins. Wild type or different domains of LOX-1 were fused with GFP. WT, wild type human LOX-1; Δ24, deletion N-terminal 24 amino acids; Δ25, deletion N-terminal 25 amino acids; ΔID, deletion intracellular domain; ΔEcto, deletion extracellular domain. (**B**) CHO cells were transfected with LOX-1-GFP or truncated constructs and the protein expression was confirmed by immunoblot analysis with anti-GFP antibody. (**C**) GFP fluorescence of LOX-1-GFP fusion proteins (green). The extracellular surface localization of GFP was further confirmed by staining with an anti-GFP antibody (red) without permeabilization.

first 24 cytoplasmic amino acids (Δ24LOX-1-GFP) or deletion of the extracellular domain of LOX-1 (ΔEctoLOX-GFP) exhibited similar localization as wild type LOX-1-GFP. In contrast, deletion of the whole cytoplasmic sequence (ΔIDLOX-1-GFP), leads to intracellular accumulation of the receptor. Deletion of the first 25 cytoplasmic amino acids (Δ25LOX-1-GFP) results in partially localized LOX-1 on the cell-surface; however, little GFP expression could be detected on the cell-surface by staining with an anti-GFP antibody without cell-surface permeabilization. Strikingly, the cytoso-

lic juxtamembrane sequence of 10 amino acids combined with the transmembrane domain of LOX-1 (Δ24/EctoLOX-GFP) was enough to target GFP to cell-surface (Fig. 2C).

3.3. The cytoplasmic domain of LOX-1 is indispensable for cell-surface targeting

These experiments with the LOX-1-GFP constructs suggest the role of the N-terminal domain for proper targeting of the receptor. To further investigate this, a series of N-terminal deletion mutations were constructed (Fig. 3A). After transfection into CHO cells, all the proteins were detected by immunoblot analysis (Fig. 3B). To study the functional consequence of these deletions, we tested the ability of transfected cells to bind OxLDL (Fig. 3C). The cell-surface expression of the mutant forms of LOX-1 was confirmed by staining with an anti-V5 antibody without permeabilization of the

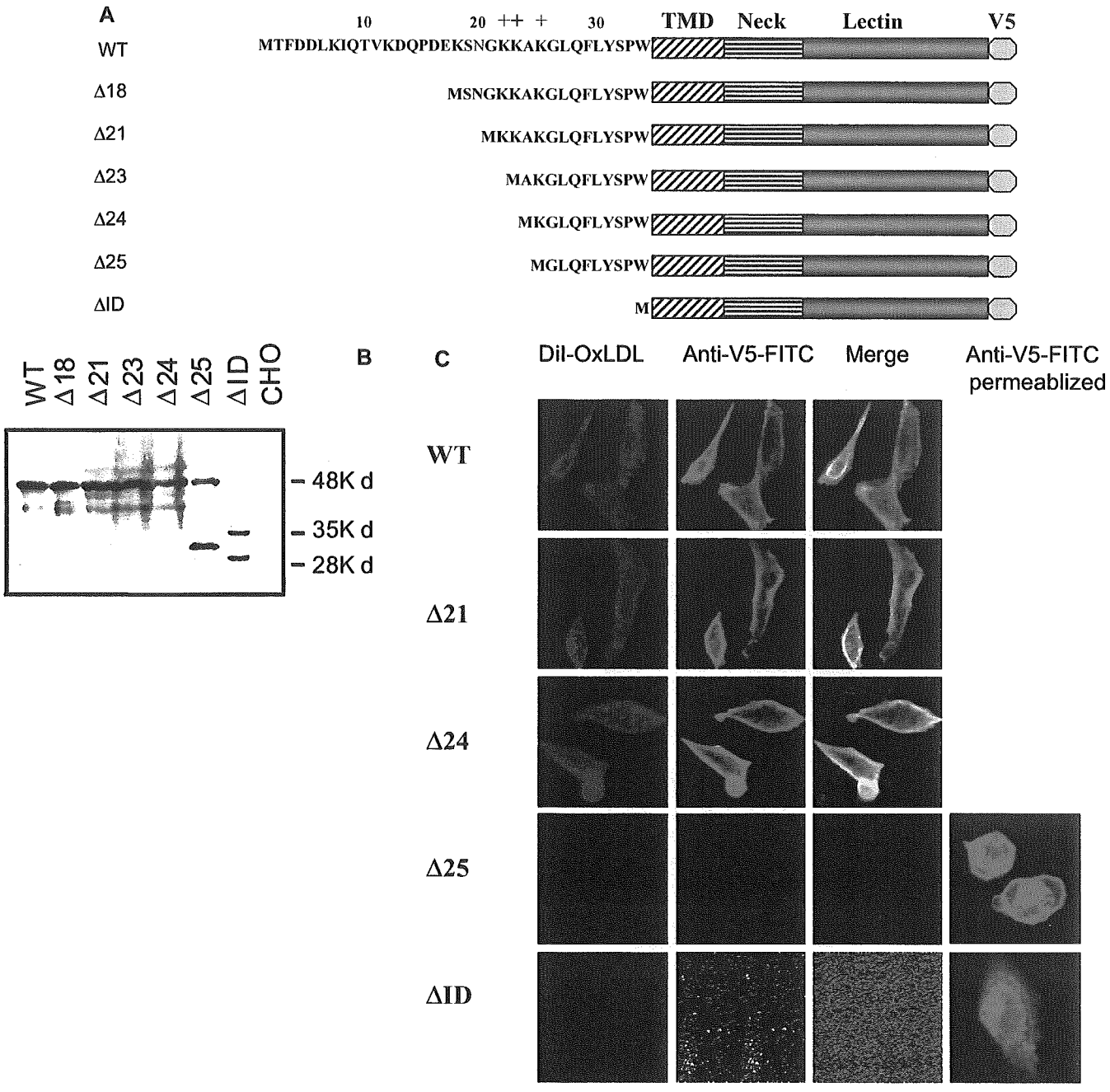

Fig. 3. The N-terminal cytoplasmic domain of LOX-1 is required for cell-surface targeting. (**A**) Schematic representation of the N-terminal deletion mutants of human LOX-1. Wild type and a series of N-terminal deletion mutants of human LOX-1 were constructed and transfected into CHO cells. WT, wild type human LOX-1; ΔID, deletion intracellular domain. (**B**) Confirmation of the protein expression by immunoblot with an anti-LOX-1 antibody. (**C**) Confocal fluorescence microscope images of CHO cells expressing wild type or deletion mutants of human LOX-1. The cell-surface expression was detected by anti-V5 antibody (green), and the activity of LOX-1 was measured as binding and internalization of DiI-labeled OxLDL (red) at 37 °C. Merged images demonstrate the co-localization of LOX-1 protein with the ligand (DiI-OxLDL) on the cell-surface (yellow). For the loss of function mutations of Δ25 and ΔID, the intracellular accumulation of LOX-1 protein was confirmed by staining with anti-V5 antibody after permeabilization.

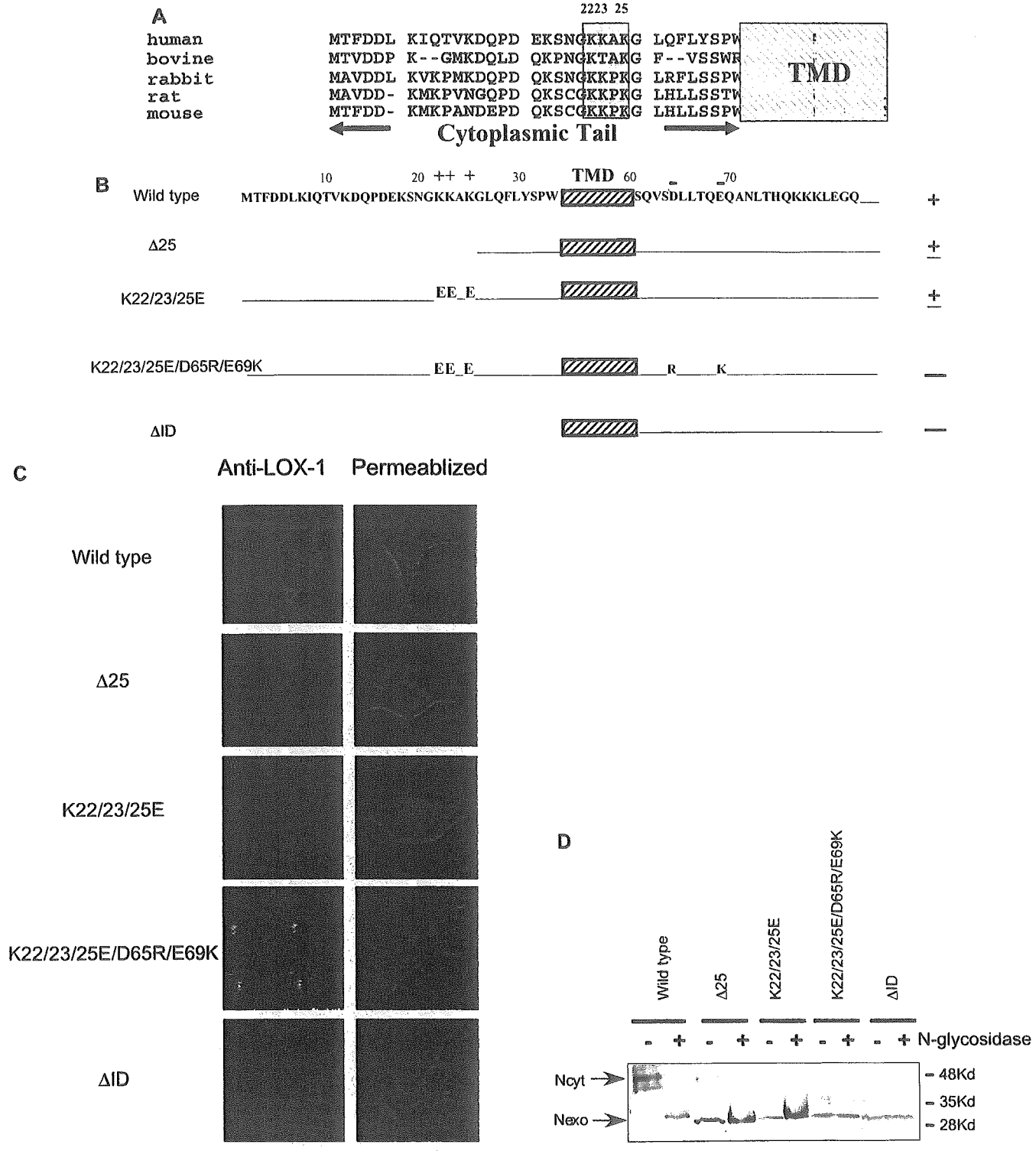

Fig. 4. Analysis of the cytosolic juxtamembrane positively charged residues. (**A**) Alignment of the cytoplasmic amino acid sequence of LOX-1 proteins. A positively charged motif (KKXK) that is conserved among different species of LOX-1 protein is highlighted by a shaded box. (**B**) Schematic representation of the point mutation constructs in the N-terminal cytoplasmic sequences of human LOX-1. Detection of the lectin domain on the cell-surface was summarized as positive (+), negative (–), and decreased degree (±), respectively. (**C**) Wild type and multiple point mutations of N-terminal positively charged residues of human LOX-1 were transfected into CHO Cells. The subcellular localization of LOX-1 protein was detected by immunostaining with a specific anti-LOX-1 monoclonal antibody (JTX-92) with, or without permeabilization. (**D**) Identification of the potential glycosylation changes by mutations of LOX-1. The cell lysates were treated with (+) or without (–) N-glycosidase and then studied by immunoblot analysis with anti-LOX-1 antibody. N_{cyt} and N_{exo} denote N-terminal in the cytoplasmic side and N-terminal in the extracellular side, respectively. Wild type and deletion of the cytoplasmic amino acid up to 24 of LOX-1 are detected as a major band of 48 kDa, which represents the fully glycosylated proteins. In contrast, ΔID and K22/23/25E/D65R/E69K mutants are detected as a major band around 32 kDa, which represents the unglycosylated proteins.

plasma membrane. Deletion of the complete cytoplasmic domain, the ΔID mutant, resulted in the absence of cell-surface expression and OxLDL binding. Mutants with deletions up to Ala-24 (Δ24) could still be detected on the cell-surface and retain the ability to bind OxLDL, similar to the wild type receptor (Fig. 3C). However, deletion of 25 amino acids (Δ25) in the N-terminal abolished cell-surface staining in the absence of permeabilization. After the cells were permeabilized, the Δ25 mutants could be detected on the cell-surface, albeit at a low level. The ΔID was only detected intracellularly (Fig. 3C).

3.4. Mutational analysis of the N-terminal sequences of LOX-1

The studies described above suggest that there is a cell-surface sorting signal present at the intracellular N-terminal of LOX-1. Comparison of the LOX-1 sequences from different species shows that a positively charged motif (KKXK) is conserved in the cytoplasmic domain of LOX-1 (Fig. 4A). To study the role of this motif, we generated constructs that contain mutations as depicted in Fig. 4B. The cell-surface expression of wild type human LOX-1 in CHO cells can be detected by a specific monoclonal antibody for LOX-1 (shown in Fig. 4C). In non-permeabilized cells, LOX-1 protein was detected solely on the cell-surface. Whereas after permeabilization, some of the signal was detected intracellularly. All the other mutants used in this experiment could not be detected on the cell-surface without permeabilization. The K22/23/25A mutant still efficiently bound OxLDL, while in the K22/23/25E mutant; the binding was severely reduced similar to what was observed with the Δ25 mutants (data not shown).

We further analyzed the affect of point mutations of N-terminal, positively charged, residues of human LOX-1 on glycosylation. Treatment of whole cell lysates with N-glycosidase resulted in higher mobility of the wild type receptor indicating a removal of N-linked oligosacchride chains from the polypetide chain (Fig. 4D). The Δ25 and K22/23/25E mutant receptors showed similarly results. In contrast, the K22/23/25E/D65R/E69K mutant and deletion of the whole cytoplasmic domain (ΔID) were detected only as a 32 kDa band which is similar to the size of unglycosylated LOX-1 (Fig. 4D).

4. Discussion

Oxidized LDL exerts diverse atherogenic effects via binding to scavenger receptors [17]. LOX-1 was initially identified as the major OxLDL receptor in endothelial cells. Considerable evidence supported the role of LOX-1 in the pathogenesis of atherosclerosis [2]. LOX-1 is different from other known OxLDL receptors, including class-A and -B scavenger receptors because of its unique C-type lectin structure [1]. LOX-1 is a type II glycoprotein consisting of a short intracellular domain, a transmembrane-spanning region, and an extracellular C-type lectin domain [14].

Human LOX-1 gene is located within the natural killer (NK) cell receptor gene complex on chromosome 12p12-p13 between AICL and CD94/NKG2 [18]. LOX-1 is a close homologue to C-type lectin NK cell receptors by sharing identical gene organization and protein architecture [19,20]. The expression pattern are different, the NK cell receptors are preferentially expressed on NK cells and some T cells, however LOX-1 is only expressed in vascular rich organs [20].

In this study, we further demonstrated that LOX-1 is functionally distinct from its homologues of the C-type lectin NK cell receptors. The NK cell receptors including NKG2A, B, C, D, E, CD94, CD69, NKR-P1A in humans and Ly-49A in mouse are implicated in antigen recognition and presentation [21]. We generated chimeric receptors in which the lectin-like domain of the NK cell receptors was replaced with the counterpart from LOX-1. We found that the chimeric proteins exhibited oxidized LDL binding features as the *bona fide* LOX-1 receptor. The swapping experiments further confirmed the sufficient role of the lectin-like domain of LOX-1 for OxLDL recognition. It provides a new structural rationale that might explain the unique function of LOX-1's feature as an OXLDL receptor. Collectively, these results demonstrate that despite a 50–60% amino acid sequence similarity LOX-1 is distinct from other members of the NK gene complex, not only with respect to its unique expression in vascular tissues, but also due to its unique ability of binding to OxLDL.

In addition, we analyzed the role of different domains of LOX-1 by constructing a series of deletion mutants of LOX-1 that are C-terminally fused with GFP. Deletion of the whole extracellular domain of LOX-1 did not change the correct cell-surface sorting of LOX-1. These results suggest that the combination of the short cytoplasmic sequence and transmembrane domain of LOX-1 is a sufficient signal for cell-surface sorting, while the extracellular lectin-like domain is dispensable for this process.

We further investigated the structural requirements involved in the cell-surface sorting of LOX-1. Truncation of the cytoplasmic domain of LOX-1 (ΔID) resulted in a complete loss of cell-surface expression and a consequent failure to bind OxLDL. A similar finding was seen when the first 25 amino acids of LOX-1 were deleted, whereas a construct

Supplementary: Wild type and multiple point mutations of charged residues of human LOX-1 were transfected into CHO cells. The cells were incubated with cell-surface biotinylation regents (Pierce) for 30 min. After three washes with PBS, the cells were lyzed in RIPA buffer containing proteinase inhibitor cocktails (Sigma) and then immunoprecipitated with anti-LOX-1 antibody (JM-92). The protein samples were separated by SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membrane (Immobilon, Millipore). Signals were amplified by Vectastain Elite ABC kit (Vector) and detected by enhanced chemoluminescence (ECL) reagents.

with deletion of the first 24 amino acids was targeted properly. These results suggest that N-terminal residues up to 25 are essential, and residues up to 24 are sufficient for correct surface localization of human LOX-1. In fact, the cytoplasmic sequences are highly conserved among LOX-1 species; including a positively charged motif (KKXK) is present in the cytosolic juxtamembrane region.

Previous studies have shown that the lectin-like domain of LOX-1 is required for ligand binding [15,16]. The N-terminal sequence of LOX-1 is located in the cytosolic side, while the C-type lectin structure is exposed to extracellular space, which enables it to bind OxLDL [2]. Integral membrane proteins destined for the plasma membrane are processed via the classical ER-Golgi pathway and are inserted into the plasma membrane via hydrophobic stretches of amino acids encompassing transmembrane domains [22]. Other posttranslational modifications, such as N-glycosylation, further imparts features to the membrane protein in the extracellular domain versus intracellular domain [23]. The orientation of the single pass integral membrane protein is determined by the charge difference of the amino acids flanking the hydrophobic sequence in which the more positively charged end always remains in the cytosol (positive-inside rule) [22].

In general, the type II proteins use the hydrophobic transmembrane residues as the anchor domain, which serves the dual function of targeting and anchoring the polypeptide in the endoplasmic reticulum (ER) membrane [24,25]. In eukaryotes, insertion of integral proteins occurs cotranslationally. The N-terminal signal-anchor sequences interact with the signal-recognition particle (SRP) receptors [24]. It is well established that the charged residues preceding the transmembrane domain determine the membrane protein topology in either $N_{cyt}C_{exo}$ or $N_{exo}C_{cyt}$ orientation [25,26]. Actually, a cluster of basic amino acids KKAK at sequence of 22–25 are present in the N-terminal cytosolic juxtamembrane region of human LOX-1 protein. This motif is also conserved in the sequence of LOX-1 in other species. We observed a striking correlation between the cell-surface sorting process and the length and the positive charge potential in the N-terminal cytoplasmic domain of LOX-1. This strongly suggests that the LOX-1 protein cell-surface sorting is governed by the presence of an N-terminal cytoplasmic signal, which is in part composed of positively charged residues.

We further studied the role of the charged residues flanking both sides of the transmembrane domain of LOX-1 by site-directed mutagenesis of the basic amino acid located at Lys-22, Lys-23 and Lys-25, as well as the negatively charged residues at Glu-65 and Asp-69. Substitutions of Lys-22, Lys-23, and Lys-25 to Alanine (K22A/K23A/K25A) did not change surface expression and binding to OxLDL (data not shown). However, changing these positively charged residues to negative ones (K22/K23E/K25E) greatly reduced the cell-surface expression of the C-terminus of LOX-1. Furthermore, the combined charge reversal of intracellular positive residues along with extracellular negative ones that reside on both sides of the membrane (K22E/K23E/K25E/D65R/E69K) almost completely abolished the cell-surface expression of the C-terminus of LOX-1 in non-permeabilized state.

All together these results further confirm that the N-terminal cytoplasmic sequence of LOX-1 is critical for ER-processing, consistent with the "positive-inside rule" [22]. The N-terminal mutants are quite likely to affect the whole processing by the ER-Golgi system including potential N-glycosylation of LOX-1 [23].

In summery, we performed detailed structure–function analysis of LOX-1. In combination with our previous studies, we can conclude that [1] the extracellular lectin domain of LOX-1 is not only essential but also sufficient for OxLDL binding activity, while it is not required for cell-surface sorting [2]; the hydrophobic transmembrane domain serves the dual functions of targeting and anchoring LOX-1 in the cell-surface membrane [3]; the cytosolic domain of LOX-1 contains a positively charged motif that is an important determinant for the correct cell-surface sorting of the lectin domain of LOX-1. Since LOX-1 has been characterized as a major OxLDL receptor on the cell-surface of vascular endothelial cells, and is implicated in the pathogenesis of atherosclerosis, our present study contributes to the detailed understanding of the structure–function relationship of this important receptor.

Acknowledgements

We thank Dr. W. Yokoyama in Washington University who provided the mCD94, hNKG2D, and mLY-49A vectors. We thank Dr. N. Zelcer and Dr. S. French for advice and critical reading of the manuscript. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Ministry of Health, Labor and Welfare of Japan, the Organization for Pharmaceutical Safety and Research.

References

[1] Sawamura T, Kume N, Aoyama T, Moriwaki H, Hoshikawa H, Aiba Y, et al. An endothelial receptor for oxidized low-density lipoprotein. Nature 1997;386:73–7.

[2] Chen M, Masaki T, Sawamura T. LOX-1, the receptor for oxidized low-density lipoprotein identified from endothelial cells: implications in endothelial dysfunction and atherosclerosis. Pharmacol Ther 2002; 95:89–100.

[3] Jono T, Miyazaki A, Nagai R, Sawamura T, Kitamura T, Horiuchi S. Lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) serves as an endothelial receptor for advanced glycation end products (AGE). FEBS Lett 2002;511:170–4.

[4] Oka K, Sawamura T, Kikuta K, Itokawa S, Kume N, Kita T, et al. Lectin-like oxidized low-density lipoprotein receptor 1 mediates phagocytosis of aged/apoptotic cells in endothelial cells. Proc Natl Acad Sci USA 1998;95:9535–40.

[5] Kakutani M, Masaki T. Sawamura T. A platelet–endothelium interaction mediated by lectin-like oxidized low-density lipoprotein receptor-1. Proc Natl Acad Sci USA 2000;97:360–4.

[6] Shimaoka T, Kume N, Minami M, Hayashida K, Sawamura T, Kita T, et al. LOX-1 supports adhesion of Gram-positive and Gram-negative bacteria. J Immunol 2001;166:5108–14.

[7] Cominacini L, Pasini AF, Garbin U, Davoli A, Tosetti ML, Campagnola M, et al. Oxidized low density lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells induces the activation of NF-kappaB through an increased production of intracellular reactive oxygen species. J Biol Chem 2000;275:12633–8.

[8] Cominacini L, Rigoni A, Pasini AF, Garbin U, Davoli A, Campagnola M, et al. The binding of oxidized low density lipoprotein (ox-LDL) to ox-LDL receptor-1 reduces the intracellular concentration of nitric oxide in endothelial cells through an increased production of superoxide. J Biol Chem 2001;276:13750–5.

[9] Li D, Mehta JL. Antisense to LOX-1 inhibits oxidized LDL-mediated upregulation of monocyte chemoattractant protein-1 and monocyte adhesion to human coronary artery endothelial cells. Circ 2000;101: 2889–95.

[10] Li D. Mehta JL. Upregulation of endothelial receptor for oxidized LDL (LOX-1) by oxidized LDL and implications in apoptosis of human coronary artery endothelial cells: evidence from use of antisense LOX-1 mRNA and chemical inhibitors. Arterioscler Thromb Vasc Biol 2000;20:1116–22.

[11] Moriwaki H, Kume N, Kataoka H, Murase T, Nishi E, Sawamura T, et al. Expression of lectin-like oxidized low density lipoprotein receptor-1 in human and murine macrophages: upregulated expression by TNF-alpha. FEBS Lett 1998;440:29–32.

[12] Aoyama T, Chen M, Fujiwara H, Masaki T, Sawamura T. LOX-1 mediates lysophosphatidylcholine-induced oxidized LDL uptake in smooth muscle cells. FEBS Lett 2000;467:217–20.

[13] Chen M, Kakutani M, Minami M, Kataoka H, Kume N, Narumiya S, et al. Increased expression of lectin-like oxidized low density lipoprotein receptor-1 in initial atherosclerotic lesions of Watanabe heritable hyperlipidemic rabbits. Arterioscler Thromb Vasc Biol 2000;20: 1107–15.

[14] Chen M, Narumiya S, Masaki T. Sawamura T. Conserved C-terminal residues within the lectin-like domain of LOX-1 are essential for oxidized low-density-lipoprotein binding. Biochem J 2001;355:289–96.

[15] Chen M, Inoue K, Narumiya S, Masaki T, Sawamura T. Requirements of basic amino acid residues within the lectin-like domain of LOX-1 for the binding of oxidized low-density lipoprotein. FEBS Lett 2001;499:215–9.

[16] Shi X, Niimi S, Ohtani T, Machida S. Characterization of residues and sequences of the carbohydrate recognition domain required for cell surface localization and ligand binding of human lectin-like oxidized LDL receptor. J Cell Sci 2001;114:1273–82.

[17] Terpstra V, van Amersfoort ES, van Velzen AG, Kuiper J, van Berkel TJ. Hepatic and extrahepatic scavenger receptors: function in relation to disease. Arterioscler Thromb Vasc Biol 2000;20:1860–72.

[18] Bull C, Sobanov Y, Rohrdanz B, O'Brien J, Lehrach H, Hofer E. The centromeric part of the human NK gene complex: linkage of LOX-1 and LY49L with the CD94/NKG2 region. Genes Immun 2000;1: 280–7.

[19] Aoyama T, Sawamura T, Furutani Y, Matsuoka R, Yoshida MC, Fujiwara H, et al. Structure and chromosomal assignment of the human lectin-like oxidized low-density-lipoprotein receptor-1 (LOX-1) gene. Biochem J 1999;339(Pt 1):177–84.

[20] Yamanaka S, Zhang XY, Miura K, Kim S, Iwao H. The human gene encoding the lectin-type oxidized LDL receptor (OLR1) is a novel member of the natural killer gene complex with a unique expression profile. Genomics 1998;54:191–9.

[21] Lanier LL. Natural killer cell receptors and MHC class I interactions. Curr Opin Immunol 1997;9:126–31.

[22] von Heijne G, Gavel Y. Topogenic signals in integral membrane proteins. Eur J Biochem 1988;174:671–8.

[23] Kataoka H, Kume N, Miyamoto S, Minami M, Murase T, Sawamura T, et al. Biosynthesis and post-translational processing of lectin-like oxidized low density lipoprotein receptor-1 (LOX-1). N-linked glycosylation affects cell-surface expression and ligand binding. J Biol Chem 2000;275:6573–9.

[24] Singer SJ. The structure and insertion of integral proteins in membranes. Annu Rev Cell Biol 1990;6:247–96.

[25] Parks GD, Lamb RA. Topology of eukaryotic type II membrane proteins: importance of N-terminal positively charged residues flanking the hydrophobic domain. Cell 1991;64:777–87.

[26] Hartmann E, Rapoport TA, Lodish HF. Predicting the orientation of eukaryotic membrane-spanning proteins. Proc Natl Acad Sci USA 1989;86:5786–90.

LOX-1 Unlocked

The solution of the crystal structure of LOX-1, originally identified as the endothelial receptor for oxidized LDL (Sawamura et al., 1997), has been reported by the Tate (Ohki et al., 2005) and Boyington groups (Park et al., 2005).

Oxidized low-density lipoprotein (OxLDL) is implicated in the pathogenesis of atherosclerosis, including both the formation of foam cells and the induction of endothelial dysfunction (Ross, 1993). Several kinds of receptors that are able to mediate the action of oxLDL in the vascular wall have collectively come to be termed "scavenger receptors," although no conserved sequences are present in the primary structures of the different receptor classes. Among these receptors, LOX-1 is unique for its C-type lectin-like ligand binding domain and structurally belongs to the natural killer (NK) cell receptor family (Chen et al., 2002). The human LOX-1 gene is located within the NK cell receptor gene complex on chromosome 12p12-p13 between AICL and CD94/NKG2.

The expression of LOX-1 is increased in hypertension, diabetes, and hyperlipidemia, and high expression of LOX-1 is found in atherosclerotic lesions. Interestingly, enhanced expression of LOX-1 has also been observed in the endothelium of the prelesion areas of hyperlipidemic rabbits, suggesting that this expression precedes the changes in vascular function induced by hyperlipidemia. In endothelial cells, activation of LOX-1 by OxLDL induces the upregulation of MCP-1, ICAM-1, and VCAM-1 expression, the release of the superoxide anion, and a reduction in the release of NO, all of which are known characteristics of "endothelial dysfunction." Since endothelial dysfunction enhances the recruitment of leukocytes into the subendothelial space and leads to inflammation, oxidative stress, and vasoconstrictive changes, this pathological step is recognized as the potential basis of the vascular diseases, including atherosclerosis.

Besides OxLDL, LOX-1 also binds other ligands, including poly(I), acidic phospholipids, apoptotic cells,

Gemin 6 and 7 Lend a Hand to snRNP Assembly

A structure of the Gemin 6 and 7 heterodimer (Ma et al., [2005], this issue of *Structure*) suggests how the survival of motor neuron (SMN) complex might facilitate the assembly of snRNPs that play important roles in pre-mRNA splicing.

Assembly of RNAs and proteins into large functional particles, such as the ribosome and the spliceosome, is a formidable task for cells. In the case of the spliceosome, five large RNA protein complexes (U1, U2, U4, U5 and U6 snRNPs) are responsible for the removal of the non-coding sequences (introns) from protein-encoding eukaryotic genes. The RNA components (U1, U2, U4, and U5 snRNAs) of four of these snRNPs are first transported from the nucleus to the cytoplasm where the set of seven Sm proteins (B/B′, D1, D2, D3, E, F, and G) binds to each snRNA forming a ring-like core domain around the Sm site (a short uridine-rich sequence present in these snRNAs) (Kambach et al., 1999) (Figure 1). Subsequently, the 7-methyl-G cap of the snRNA is converted to a 2,2,7-trimethyl-G cap. Sm protein association and 5′-cap hypermethylation are both required for the import of the snRNP into the nucleus where the final maturation of snRNPs occurs.

In vitro, the U1 and U2 snRNPs spontaneously assemble when the appropriate RNA and protein components are combined (Will et al., 1996; Muto et al., 2004). However, the formation of spliceosomal snRNPs in vivo is accomplished by an elaborate assembly system involving the 6S pICln complex, the 20S methylosome, and the hetero-disperse 30-70S SMN complex (Meister et al., 2002; Yong et al., 2004b). The Sm proteins do not exist freely in the cytoplasm, but instead associate with the protein pICln in a 6S complex (Friesen et al., 2001). pICln interacts with the Sm domain of the Sm proteins and possibly prevents non-specific binding of Sm proteins to other RNAs. pICln may also play a role in recruiting Sm proteins to the 20S methylosome, which contains pICln, MEP50 (WD40 protein), and JBP1 or PRMT5 (arginine methyl transferase) (Meister et al., 2002; Yong et al., 2004a, 2004b). Three of the Sm proteins (B/B′, D1, and D3) contain Arg-Gly repeats in their extended C-terminal tails. These arginines are converted to symmetric dimethyl arginines within the 20S methylosome (Friesen et al., 2001). Methylation increases the affinity of the Sm proteins for the SMN complex, the final complex formed in the assembly pathway (Brahms et al., 2001; Friesen et al., 2001). The SMN complex is composed of the SMN protein and Gemin proteins 2 to 7 (Meister et al., 2002; Yong et al., 2004b; and references therein). Associating with all seven Sm proteins and the four snRNAs, the SMN complex facilitates core domain formation in an ATP-dependent and step-wise manner (Meister et al., 2002; Yong et al., 2004b). The high-affinity SMN binding regions of U1, U2, U4, and U5 snRNAs have been identified (Yong et al., 2004b). In U1 snRNA, stem-loop I is sufficient whereas the minimal SMN binding regions of U2, U4, and U5 snRNAs contain the Sm site and at least one well-defined stem-loop structure. Therefore, in addition to providing a structural platform for the Sm proteins and snRNAs to interact, SMN confers specificity to the process (Pellizzoni et al., 2002).

Ma et al. report in this issue the crystal structure of the Gemin 6/7 heterodimer, a subcomplex within the SMN complex. The structure gives important insight into how Sm proteins might be recruited to the SMN complex and how the core domain may be assembled. Although Gemin 6 and Gemin 7 exhibit no significant sequence similarity to Sm or Sm-like proteins, they show strong structural resemblance to the Sm-fold, a highly bent 5-stranded anti-parallel β sheet flanked by an N-terminal helix. Reminiscent of the human Sm protein heterodimers D1D2 and D3B (Kambach et al., 1999), Gemin 6 and Gemin 7 dimerize head-to-tail through their outermost β strands to form a continuous 10-stranded anti-parallel β sheet. Ma et al. also show that the Gemin

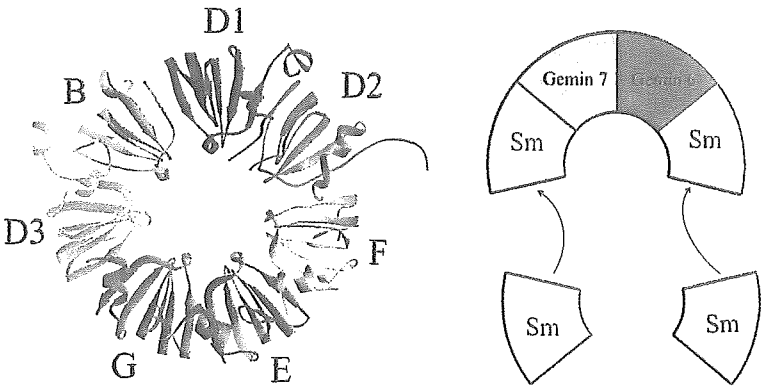

Figure 1. A Possible Mechanism for snRNP Assembly in the SMN Complex

A model for the snRNP core domain (left) consisting of seven Sm proteins (Kambach et al., 1999). Gemin 6 and Gemin 7 found in the SMN complex have the Sm protein fold and form a heterodimer through paring of their two outermost β strands as in an Sm protein dimer (right) (Ma et al., 2005). In the SMN complex, Sm proteins may be added onto the Gemin 6/7 heterodimer to nucleate the core domain assembly.

6/7 dimer can interact with B, D2, D3, and E in GST pull-down experiments. The dimeric arrangement of Gemin 6/7 immediately suggests that individual Sm proteins or a subcomplex of them might interact on platelets, leukocytes, and bacteria. (Honjo et al., 2003; Kakutani et al., 2000; Oka et al., 1998). This suggests a versatility of functions for LOX-1 in both pathological and physiological conditions in vivo. In some animal models of inflammation, LOX-1 promotes leukocyte infiltration as a cell-adhesion molecule and enhances inflammation. The broad range of ligand specificity in part overlaps with other scavenger receptors and Toll-like receptors (TLRs). In this sense, LOX-1 might be involved in innate immunity. In point of fact, LOX-1 is expressed in dendritic cells and is involved in antigen presentation (Delneste et al., 2002), although its precise roles in this immunological context have yet to be determined.

On the other hand, LOX-1 has an interesting ligand specificity. LOX-1 binds OxLDL and acetylated LDL, but not native LDL. During oxidation, various changes occur to LDL, i.e., degenerative fragmentation of apolipoprotein and its covalent modification by lipids. Therefore, OxLDL may have several structural features distinct from native LDL. Acetylated LDL is generated by acetylation of the epsilon-amino group of apolipoprotein in LDL. The major consequence is increased negative charge, which also occurs with OxLDL. Similarly, negatively charged phospholipids such as phosphatidylserine are recognized by LOX-1, but neutral phospholipids such as phosphatidylcholine are not. These findings suggest that the negative charge on ligands is of crucial importance for ligand recognition by LOX-1. In fact, substitution of basic with neutral amino acids greatly decreases the ligand binding capacity of LOX-1. It is not clear whether there are additional common structural features among modified LDL, acidic phospholipids, and poly(I).

From the crystal structures of LOX-1 reported by two groups, we now have some clues toward understanding the ligand-receptor interaction of LOX-1 (Ohki et al., 2005; Park et al., 2005). In the structural models, the ligand binding interface is hydrophobic except for the basic spine, which is composed of arginine residues. Ohki et al. (2005) pointed out that this is a structure unique to LOX-1 compared with the related C-type lectin-like molecules NKG2D and Ly49A. Furthermore, they performed expression experiments of mutated LOX-1 and observed that substitution of a single amino acid in the basic spine, R208N, resulted in the greatest loss of binding affinity for acetylated LDL, although other arginine residues in the basic spine also contributed to the binding of acetylated LDL (Ohki et al., 2005). Together with the hydrophobic surface, the positive charge of the basic spine seems to provide the ligand specificity of LOX-1 for anionic lipids/lipoproteins.

The crystal structure of human LOX-1's ligand binding domain has been successfully solved as a homodimeric structure with an intermolecular disulfide bond (Ohki et al., 2005). Interestingly, a single amino acid change, W150A, located in the homodimer interface resulted in the loss of binding affinity for acetylated LDL in their expression study. This suggests that an appropriate form of dimerization is needed for ligand recognition. It was also shown that the dimeric structure changes depending on pH. As an endocytotic receptor, decreasing pH in the endosome may change the dimeric structure of LOX-1 and thus lead to the dissociation of ligands and a recycling of the receptor to the cell surface.

The structure solved in the reported study (Ohki et al., 2005) is not the ligand-receptor complex but the receptor alone. Therefore, it has yet to be determined how the structure of LOX-1 changes upon interaction with ligands. However, a characteristic "empty cavity" formed between the two LOX-1 molecules in a dimer might be the key to understanding why a particular variety of molecule is recognized by LOX-1.

Among the scavenger receptors, LOX-1 is the first molecule examined crystallographically. It has been a mystery how these different classes of receptors recognize the same molecules, including OxLDL, despite the absence of a common sequence in primary structure. The solution of the structure of LOX-1 may help unravel of how the scavenger receptors/pattern recognition molecules are able to bind a broad range of molecules, and may also help in designing receptor antagonists which might be useful in diseases related to endothelial dysfunction, such as inflammation and atherosclerosis.

Additionally, genome analysis revealed a cluster of C-type lectin-like molecules on the short arm of chromosome 12, increasing the number of distinct members of this interesting protein family. These proteins may offer a good model for understanding molecular evolution from both genomic and structural perspectives.

Tatsuya Sawamura
Department of Vascular Physiology
National Cardiovascular Center Research Institute
Japan

Selected Reading

Chen, M., Masaki, T., and Sawamura, T. (2002). Pharmacol. Ther. *95*, 89–100.

Delneste, Y., Magistrelli, G., Gauchat, J., Haeuw, J., Aubry, J., Nakamura, K., Kawakami-Honda, N., Goetsch, L., Sawamura, T., Bonnefoy, J., and Jeannin, P. (2002). Immunity *17*, 353–362.

Honjo, M., Nakamura, K., Yamashiro, K., Kiryu, J., Tanihara, H., McEvoy, L.M., Honda, Y., Butcher, E.C., Masaki, T., and Sawamura, T. (2003). Proc. Natl. Acad. Sci. USA *100*, 1274–1279.

Kakutani, M., Masaki, T., and Sawamura, T. (2000). Proc. Natl. Acad. Sci. USA *97*, 360–364.

Ohki, I., Ishigaki, T., Oyama, T., Matsunaga, S., Xie, Q., Ohnishi-Kameyama, M., Murata, T., Tsuchiya, D., Machida, S., Morikawa, K., and Tate, S. (2005). Structure *13*, this issue, 905–917.

Oka, K., Sawamura, T., Kikuta, K., Itokawa, S., Kume, N., Kita, T., and Masaki, T. (1998). Proc. Natl. Acad. Sci. USA *95*, 9535–9540.

Park, H., Adsit, F.G., and Boyington, J.C. (2005). J. Biol. Chem. *280*, 13593–13599.

Ross, R. (1993). Nature *362*, 801–809.

Sawamura, T., Kume, N., Aoyama, T., Moriwaki, H., Hoshikawa, H., Aiba, Y., Tanaka, T., Miwa, S., Katsura, Y., Kita, T., and Masaki, T. (1997). Nature *386*, 73–77.

Overexpression of Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1 Induces Intramyocardial Vasculopathy in Apolipoprotein E–Null Mice

Kazuhiko Inoue, Yuji Arai, Hiroki Kurihara, Toru Kita, Tatsuya Sawamura

Abstract—Endothelial dysfunction induced by oxidized low-density lipoprotein (OxLDL) has been implicated in the pathogenesis of atherosclerosis and vasculopathy. Increased expression of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), the receptor for OxLDL in endothelial cells, has been demonstrated in the atherosclerotic plaques from experimental atherosclerotic animal models and human clinical samples. In vitro, activation of LOX-1 alters the expression of several endothelial cell genes that are involved in endothelial dysfunction. To investigate the role of LOX-1 in terms of both endothelial dysfunction and resultant vascular changes, we generated mice overexpressing LOX-1 (LOXtg) in C57BL/6 and apolipoprotein E–null mice (apoEKO) backgrounds. We found that the expression of the transgene was prominent in coronary vessels and cardiomyocytes. The enhancement of OxLDL uptake in LOXtg mice was consistent with the expression level of LOX-1. Under hyperlipidemic conditions, both OxLDL and 8-hydroxy-2′-deoxyguanosine accumulated in the coronary arteries of LOXtg/apoEKO mice. The expression of ICAM-1 and VCAM-1, as well as the number of macrophages around blood vessels, were significantly increased in LOXtg/apoEKO mice compared with control littermates. There were no differences in either the hemodynamic profile or the plasma lipid profile between the 2 groups of animals. LOXtg/apoEKO mice displayed accelerated intramyocardial vasculopathy, and the atheroma-like lesion area was increased 10-fold in the LOXtg/apoEKO mice compared with nontransgenic littermates after 3-weeks on the high-fat diet. Thus, it is demonstrated that LOX-1 overexpression promotes inflammatory intramyocardial vasculopathy in a hyperlipidemic mouse model, and this effect is probably mediated through the endothelial dysfunction induced by overexpression of LOX-1. (***Circ Res.* 2005;97:176-184.**)

Key Words: endothelial dysfunction ■ lectin-like oxidized low-density lipoprotein receptor-1 ■ oxidized low-density lipoprotein

Endothelial cells respond to chemical factors, mechanical stress, and blood flow by producing molecules that regulate vascular tonus, coagulation, cell proliferation, and leukocyte trafficking to stabilize blood vessels. A number of molecules that alter the normal function of the endothelium have been identified over the last decade. These molecules have facilitated the understanding of both vascular physiology and the vascular diseases caused by the complication of endothelial dysfunction, including atherosclerosis, hypertension, diabetes, inflammation, and various types of organ failure. The diversity of the diseases related to endothelial dysfunction indicates the pivotal role of the endothelium in normal physiologic homeostasis.[1,2]

Oxidatively modified low-density lipoprotein (OxLDL) is believed to be one of the major causes of endothelial dysfunction associated with proatherogenic conditions.[3] OxLDL induces the expression of adhesion molecules, eg, ICAM-1, VCAM-1, and selectins. It also stimulates the release of chemokines and smooth muscle growth factors and impairs endothelium-dependent vasorelaxation. These changes lead to the recruitment of leukocytes into the subendothelial space, proliferation of smooth muscle cells,[4] and an increase in vascular tone. The endothelial dysfunction induced by OxLDL is thought to precede tissue morphological changes and is believed to be one of the initiators of vasculopathy and atherosclerosis.

We recently identified the lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1)[5] as the receptor for OxLDL on endothelial cells. LOX-1 is a type II membrane glycoprotein with an apparent molecular weight of 50 kDa. It has a C-terminal extracellular C-type lectin-like domain. The lectin-like domain is essential for binding to OxLDL.[6] LOX-1 is highly homologous in the lectin-like domain of NKR-P1, which is essential for activation of natural killer cells and is

Original received May 11, 2004; resubmission received April 5, 2005; revised resubmission received June 3, 2005; accepted June 8, 2005.

From the Department of Vascular Physiology (K.I., Y.A., T.S.), National Cardiovascular Center Research Institute, Osaka; the Department of Cardiovascular Medicine (K.I., T.K.), Graduate School of Medicine, Kyoto University; the Department of Physiological Chemistry and Metabolism (H.K.), Graduate School of Medicine, University of Tokyo; and the Department of Molecular Pathophysiology (T.S.), Graduate School of Pharmaceutical Sciences, Osaka University, Japan.

Correspondence to Dr Tatsuya Sawamura, Department of Vascular Physiology, National Cardiovascular Center Research Institute, Suita, 565-8565 Osaka, Japan. E-mail t-sawamura@umin.ac.jp

Circulation Research **is available at http://circres.ahajournals.org** **DOI: 10.1161/01.RES.0000174286.73200.d4**

the NK cell receptor family member in this group of C-type lectin-like receptors.[7,8] LOX-1 expression in endothelial cells is relatively low under normal conditions, but can be induced with proinflammatory cytokines and vasoconstrictive peptides in vitro.[9–14] The expression of LOX-1 is increased in hypertension, diabetes, and hyperlipidemia, and the highest expression of LOX-1 is found in atherosclerotic lesions.[15–19] Interestingly, enhanced expression of LOX-1 has also been observed in the endothelium of the prelesion areas of hyperlipidemic rabbits, suggesting that this expression precedes the changes in vascular function induced by hyperlipidemia.[17] In endothelial cells, activation of LOX-1 by OxLDL induces an upregulation of MCP-1, ICAM-1, and VCAM-1 expression[20,21] and a reduction in the release of NO,[22] all of which are known characteristics of endothelial dysfunction.

LOX-1 has also been demonstrated to bind other ligands in addition to OxLDL, including aged cells, apoptotic cells,[23] platelets,[24] leukocytes,[25] and bacteria,[26] suggesting a diversity of functions for LOX-1. The binding of platelets to LOX-1 enhances the release of endothelin-1 (ET-1) and suppresses the release of NO from endothelial cells,[24,27] suggesting that a platelet–endothelium interaction via LOX-1 may also have a role in the induction of endothelial dysfunction.

These interesting in vitro data on the effects of activation of the LOX-1 receptor prompted us to investigate the role of LOX-1 and OxLDL in vivo by means of a transgenic mouse. In this study, we generated mice overexpressing LOX-1, and determined the role of LOX-1 in the vasculopathy associated with hyperlipidemia in these animals.

Materials and Methods

Generation of LOXtg

Targeted LOX-1 gene expression in endothelial cells was achieved using the expression vector PEP8, which consists of the murine preproendothelin-1 promoter, a *Not*I site, and the SV40 intron/polyA signal, as described previously.[28] Bovine LOX-1 (bLOX-1; GenBank NM_174132) cDNA tagged with a *Not*I adaptor was inserted into the *Not*I site of PEP8 downstream of the preproendothelin-1 promoter. The resultant plasmid was designated PEP8-bLOX-1. PEP8-bLOX-1 was linearized by *Xho*I digestion and injected into fertilized eggs prepared from superovulated C57BL/6 mice (Japan SLC, Shizuoka, Japan). The eggs were transferred into the oviducts of pseudopregnant ICR foster mothers (Japan SLC, Shizuoka, Japan). Founder mice were identified by Southern blot analysis. Genomic DNA obtained from the tails of the mice was digested by *Eco*RI, separated by electrophoresis, and transferred to a nylon membrane (Biodine, Pall). The membrane was probed with a [^{32}P]dCTP-labeled *Eco*RI fragment of PEP8-bLOX-1 and analyzed with a BAS-5000 (Fuji Photo Film). Mice were maintained under controlled temperature and humidity with 12 hours light/dark cycle. They were provided standard chow diet and water ad libitum. All procedures were in accordance with the institutional guidelines for animal research in the National Cardiovascular Center.

Generation of LOXtg/ApoEKO

Heterozygous LOXtg mice lines, carrying 24 and 16 copies of the transgene, were crossbred with homozygous C57BL/6:apoEKO mice. The offspring, which carried the LOX-1 transgene and were obligatorily heterozygous for the apoE gene, were further crossbred with C57BL/6:apoEKO mice to generate LOXtg/apoEKO. Mice were maintained on a standard chow diet and water ad libitum until they were fed the high-fat diet. Homozygous mutation in the apoE gene was confirmed by Southern blot analysis as described.[29]

Total RNA preparation and Northern blot analysis, RT-PCR, Western blot analysis, immunohistochemistry, and analyses of the uptake of DiI-OxLDL were performed as indicated in the online supplement (available at http://circres.ahajournals.org).

Measurement of Hemodynamic and Plasma Lipid Indexes

Blood pressure and heart rates were measured by tail cuff plethysmography (BP-98A; Softron). Total cholesterol, triglycerides, phospholipids, and nonesterified fatty acids (NEFA) in the sera of the mice were measured using ELISA kits (T-Cho E, TG E, PL C, and NEFA C, respectively; Wako). Serum lipoprotein profiles were analyzed with high-performance liquid chromatography (HPLC) (Tosoh) by the online cholesterol oxidase method. Five μL of serum (diluted 100 times with PBS in the case of the apoEKO mice) was applied to 2 serially connected columns for gel permeation chromatography (TSKgel Lipopropak XL, ϕ 7.8 mm×300 mm, Tosoh), and eluted with the TSK eluent LP-1 (Tosoh) at a flow rate of 0.6 mL/min. The postcolumn effluent was mixed on line with enzyme/chromogen solution consisting of cholesterol esterase, cholesterol oxidase, horseradish peroxidase, and sodium 3,5-dimetoxy-N-ethyl-N-(2-hydroxy-3-sulfopropyl)-aniline, and 4-aminoantipyrine from a kit (Cholesterol E-test, Wako), at a flow rate of 0.3 mL/min in the reaction tube (Teflon, ϕ 0.4 mm×7.5 m) at a temperature of 45°C. Then the reaction product was measured on line by the absorbance at 550 nm.[30]

Evaluation of Intramyocardial Vasculopathy

After weaning for 3 weeks, male LOXtg/apoEKO mice and apoEKO mice were maintained on a regular chow diet (CE-2; Clea Japan). From 8 weeks of age, mice were fed with a high-fat diet (1.25% cholesterol, 0.5% cholate, 20% milk casein, 15% cocoa butter, and reduced α-tocopherol) for 3 weeks. After 3 weeks of high fat loading, mice were euthanized and used for analyses. The murine heart was sliced perpendicularly on the long axis into 8-μm thickness. To evaluate atheroma-like intramyocardial vasculopathy, sections at every 40 μm from the level of the mitral valve to the level of the aortic valve were subjected to staining with Oil red O (nacalai tesque) followed by counterstaining with Mayer's hematoxylin. The Oil red O positive area was measured with NIH image software.

Statistical Analysis

Data are expressed as the mean±SEM and processed by Mann–Whitney analysis. A P value of <0.05 was considered statistically significant.

Results

Generation of LOXtg

To obtain transgenic mice with LOX-1 overexpressed in endothelial cells, we used a murine preproendothelin-1 promoter. A LOX-1 expression plasmid was constructed by inserting bovine LOX-1 cDNA into a PEP8 vector, which contains the murine preproendothelin-1 and SV40 intron/polyA signal. The linearized plasmids were microinjected into fertilized eggs from C57BL/6 mice. Four lines of LOXtg in the C57BL/6 background were established, which carry 24, 14, 2, and 2 copies of the transgene, respectively (Figure 1a). Among these, one line that exhibited the highest expression level of the transgene was selected for detailed analysis.

Tissue Distribution of the Transgenic LOX-1 Expression

Northern blot analysis revealed the transgene was most prominently expressed in the heart. However, almost all tissues exhibited moderate expression levels (Figure 1b). The endogenous murine LOX-1 mRNA was only detected in the