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Simulative and experimental investigation on the cleavage site that generates the soluble human LOX-1

Silvia Biocca [a,1], Tania Arcangeli [b,1], Elisa Tagliaferri [b,1], Barbara Testa [a], Giulia Vindigni [a], Francesco Oteri [b], Alessandra Giorgi [c], Federico Iacovelli [b], Giuseppe Novelli [d], Alessandro Desideri [e], Mattia Falconi [e,*]

[a] Department of Systems Medicine, University of Rome "Tor Vergata", Via Montpellier 1, 00133 Rome, Italy
[b] Department of Biology, University of Rome "Tor Vergata", Via della Ricerca Scientifica, 00133 Rome, Italy
[c] Department of Biochemical Science "A. Rossi Fanelli", University of Rome "La Sapienza", P.le Aldo Moro 5, 00185 Rome, Italy
[d] Department of Biomedicine and Prevention, Genetics Section, University of Rome "Tor Vergata", Via Montpellier 1, 00133 Rome, Italy
[e] Department of Biology, University of Rome "Tor Vergata", Interuniversity Consortium, National Institute Biostructure and Biosystem (INBB), Via della Ricerca Scientifica, 00133 Rome, Italy

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ABSTRACT

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is a scavenger receptor that mediates the recognition, the binding and internalization of ox-LDL. A truncated soluble form of LOX-1 (sLOX-1) has been identified that, at elevated levels, has been associated to acute coronary syndrome. Human sLOX-1 is the extracellular part of membrane LOX-1 which is cleaved in the NECK domain with a mechanism that has not yet been identified. Purification of human sLOX-1 has been carried out to experimentally identify the cleavage site region within the NECK domain. Molecular modelling and classical molecular dynamics simulation techniques have been used to characterize the structural and dynamical properties of the LOX-1 NECK domain in the presence and absence of the CTLD recognition region, taking into account the obtained proteolysis results. The simulative data indicate that the NECK domain is stabilized by the *coiled-coil* heptad repeat motif along the simulations, shows a definite flexibility pattern and is characterized by specific electrostatic potentials. The detection of a mobile inter-helix space suggests an explanation for the *in vivo* susceptibility of the NECK domain to the proteolytic cleavage, validating the assumption that the NECK domain sequence is composed of a *coiled-coil* motif destabilized in specific regions of functional significance.

Introduction

Atherosclerosis is characterized by oxidized low density lipoprotein (ox-LDL) accumulation in arterial blood vessel walls [1] that constitutes an early step in the development of atherosclerosis [2]. The lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is the major receptor of ox-LDL in endothelial cells [3] and plays a crucial role in endothelial dysfunction, characterized by reduced vasodilation, proapoptotic and proinflammatory states and prothrombotic properties [4,5].

LOX-1 is a membrane protein with a type II orientation, member of the C-type lectin-like protein family. Proteins in this family are classified into evolutionary group V and share the domain architecture that comprises a stalk region (NECK domain) and a C-type lectin-like ligand-binding domain (CTLD)[2], that are located in the extracellular regions [6] (Fig. 1). Members of this protein family display a high level of sequence conservation in the CTLD and only a limited sequence similarity in the NECK domain, although all of them retain the coiled-coil sequence character [6,7].

In human LOX-1 the CTLD forms a heart-shaped homodimer (Fig. S1A) with an inter-chain disulfide bond at Cys140, not present in other species. Mutation of Cys140 does not affect the ox-LDL-binding activity and does not destabilize the dimeric form of the ligand-binding domain [8,9]. On the top of human LOX-1 there is a linear arrangement of positively charged residues crossing the dimer surface of CTLD, which is called basic spine (Fig. S1B and C). Mutation of the basic spine arginines reduces the binding activity, indicating their significant role in the ox-LDL recognition [10,11]. Mutant analyses, classical molecular dynamics (MD) simulations and X-ray structure analysis, have shown that mutation of the

* Corresponding author. Fax: +39 06 2022798.
E-mail address: falconi@uniroma2.it (M. Falconi).
[1] These authors equally contributed to this work.
[2] *Abbreviations used:* CTLD, C-type lectin-like ligand-binding domain; md, molecular dynamics; DC-SIGN, dendritic-cell-specific intercellular adhesion molecule 1-grabbing nonintegrin; DC-SIGNR, DC-SIGN-related receptor; MBP, mannose-binding protein; PVDF, polyvinylidene difluoride; PMSF, phenyl methylsulfonylfluoride; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; RMSD, root mean square deviation; RMSF, root mean square fluctuation; SAS, solvent accessible surface; CTLD, crystallographic structure.

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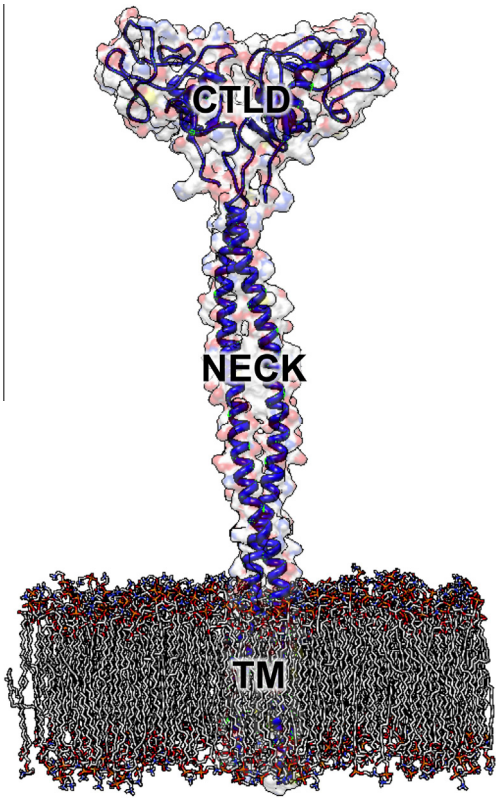

Fig. 1. Entire LOX-1 3D model without cytoplasmic domain. The protein is represented by a blue ribbon inside a transparent surface while membrane is depicted using stick model phospholipids colored by atom type. Labels indicate the domains of the receptor. This image was obtained using program Chimera [33]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

dimer interface residue Trp150 and Lys167 alters the basic spine structure, explaining the severe reduction of LOX-1 binding activity [12–14].

The NECK domain, connecting the transmembrane portion of the receptor to CTLD, is assumed to be a dimer consisting of two α-helices that wrap in a parallel coiled-coil structure [10,11,15]. This folding design is a prevailing oligomerization motif in proteins, is very versatile and allows for variations in the sequence that lead to different states of oligomerization [16], reflecting the specific function of each member protein [15]. It is known that different coiled-coil sequences result in different assemblies of the ligand-binding domains for dendritic-cell-specific intercellular adhesion molecule 1-grabbing nonintegrin (DC-SIGN), DC-SIGN-related receptor (DC-SIGNR) [17,18], mannose-binding protein (MBP) [19] and Langerhans cells Langerin [20]. All these proteins are members of the C-type lectin-like family. DC-SIGN and DC-SIGNR forms a tetramer through a coiled-coil stalk consisting of a series of conserved but non-identical sequences of 23 residues [17,18], MBP is a trimer comprising a collagen-like NECK of a parallel triple stranded coiled-coil of α-helices [19], while Langerin exists as an oligomer, forming trimers stabilized by a coiled-coil of α-helices in the NECK region [20]. The different assemblies induced by the NECK are closely related to the specific function of each protein and accordingly, LOX-1 NECK, which has a different sequence from that of DC-SIGN, DC-SIGNR, MBP or Langerin, presumably plays a specific functional role during LOX-1 ligand recognition [15]. Among known LOX-1 sequences mouse LOX-1, composed by 363 residues, shows triple repeats of the sequence in the extracellular NECK domain [21]. The sequence of the repeat unit is also present in human and bovine LOX-1 NECK, where appears only once [21]. The human LOX-1 NECK domain has been subjected to structural analyses revealing that one-third of the N-terminal NECK is less structured than the remainder of the protein and is highly sensitive to cleavage by a variety of proteases [15]. Indeed, a soluble form of LOX-1 (sLOX-1) is found in the blood, has been correlated with high level of LOX-1 in the cell membrane of endothelial cells in atherosclerosis [22] and it is currently considered as a biomarker of cardiovascular diseases [23]. In the work of Ishigaki [15] the coiled-coil structure has been mainly localized at the C-terminal part of the NECK domain (i.e. close to the CTLD region), in dynamic equilibrium among multiple conformational states, suggesting that this structural property of the NECK region may enable clustered LOX-1 on the cell surface to recognize ox-LDL. It is worth noting that LOX-1 receptor dimer per se has a low affinity to ox-LDL and multimerization and cluster organization in plasma membrane have been proposed to be important requisites for LOX-1 activity [10,24,25]. In fact, comparing the size of LOX-1 CTLD (~70 Å) with the ox-LDL diameter (~250 Å), it seems realistic that the LOX-1 basic spine binds to the ox-LDL amphipathic α-helices as an assembly of clustered receptors. During the process of ox-LDL recognition a flexible NECK domain structure has been supposed to facilitate the required interactions [15].

This work has been carried out to further investigate the structural and functional role of the NECK domain in the human LOX-1 receptor. Purification of human sLOX-1 from conditioned medium of LOX-1-expressing cells and mass spectrometry have been carried out to experimentally identify the cleavage site region within the NECK domain. The molecular modelling and molecular dynamics techniques have been applied to propose a NECK structural model able to explain the observed cleavage site that generates the sLOX-1. Our results confirm the assumptions indicating the NECK domain as a dimer consisting of two α-helices wound in a parallel *coiled-coil* structure and identify specific residues which functionally modulate the flexibility of this region.

Materials and methods

DNA construct, cell cultures and transfection

For the expression in mammalian cells, human LOX-1 was subcloned into pEF/V5-His vectors (Invitrogen, Inchinnan, Paisley, UK), as previously described [26]. COS cells were grown in DMEM medium (Biowest, Miami, FL, USA) supplemented with 10% foetal bovine serum (GIBCO, Inchinnan, Paisley, UK) and 100 U/ml penicillin/streptomycin (Euroclone, Devon, UK) and transiently transfected with JetPEI (Polyplus Transfection, Illkirch, France), following the manufacturer's instructions, with a DNA/transfectant reagent ratio (w/v) of 1:2.

Western blot analysis

For LOX-1 detection, transfected cells were lysed and extracts prepared as described [25]. Protein concentration was measured by Bradford assay (Sigma–Aldrich, St. Louis, MO). For sLOX-1 detection in the medium, conditioned media from transiently transfected COS cells cultured in Opti-MEM (GIBCO) for different times were centrifuged at 10,000×*g*, precipitated with 10% trichloroacetic acid (Sigma Aldrich, St. Louis, MO), solubilized in SDS–PAGE sample buffer and transferred in polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Chalfont St. Giles, UK). Immunoreactive bands were detected with Mab α-V5 (Invitrogen) and goat anti-mouse IgG HRP (Jackson Immunoresearch Laboratories Inc., West Grove, PA).