Lipid rafts are important for the association of RANK and TRAF6

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Abbreviations: MAPK, mitogen-activated protein kinase; MCD, methyl-β-cyclodextrin; RANK, receptor activator of NF-κB; TLR, Toll-like receptor; TNFR, TNF receptor; TRAF, TNF receptor-associated factor

Abstract

Rafts, cholesterol- and sphingolipid-rich membrane microdomains, have been shown to play an important role in immune cell activation. More recently rafts were implicated in the signal transduction by members of the TNF receptor (TNFR) family. In this study, we provide evidences that the raft microdomain has a crucial role in RANK (receptor activator of NF-κB) signaling. We found that the majority of the ectopically expressed RANK and substantial portion of endogenous TRAF2 and TRAF6 were detected in the low-density raft fractions. In addition, TRAF6 association with rafts was increased by RANKL stimulation. The disruption of rafts blocked the TRAF6 translocation by RANK ligand and impeded the interaction between RANK and TRAF6. Our observations demonstrate that proper RANK signaling requires the function of raft membrane microdomains.

Keywords: membrane microdomains; receptors; signal transduction; tumor necrosis; tumor necrosis factor

Introduction

Osteoclasts are multinucleated giant cells responsible for bone resorption. These cells are differentiated from hematopoietic myeloid precursors of the monocyte/macrophage lineage (Suda et al., 1992). For the differentiation of osteoclast precursors into mature osteoclasts, a cell-to-cell interaction between osteoclast precursors and osteoblasts/stromal cells are required (Udagawa et al., 1990). Recently, many studies have provided ample evidences that the TNF family member RANKL (receptor activator of NF-κB ligand; also known as ODF, OPGL, and TRANCE) is expressed on the surface of osteoblasts/stromal cells and essential for osteoclast differentiation (Anderson et al., 1997; Yasuda et al., 1998; Takahashi et al., 1999). When its receptor RANK was stimulated by RANKL, several TNF receptor-associated factors (TRAFs), especially TRAF6, can be directly recruited into RANK cytoplasmic domains and may trigger downstream signaling molecules for the activation of NF-κB and mitogen activated protein kinases (MAPKs) (Darnay et al., 1998; Wong et al., 1998; Kim et al., 1999). The essential role of RANKL, RANK, and TRAF6 were clearly demonstrated in gene-deficient mice that displayed osteopetrotic phenotype due to defective osteoclastic bone resorption (Dougall et al., 1999; Kong et al., 1999; Lomaga et al., 1999; Naito et al., 1999). In addition to the study of TRAF6$^{-/-}$ mice, the biological significance of the TRAF6-Src signaling pathway in osteoclast differentiation and activation was showed that the association of TRAF6 with Src family kinases and subsequent stimulation of the Src kinase activity mediated phosphoinositide 3-kinase (PI3K)/Akt activation (Wong et al., 1999).

Lipid rafts are specialized membrane microdomains in the plasma membranes. They are enriched in glycosphingolipids and cholesterol, and incorporate specific proteins, among which are many glycosylphosphatidylinositol (GPI)-anchored proteins (Brown and London, 1998; Simons and Toomre, 2000) and Src family kinases. As lipid rafts resist solubilization in non-ionic detergent, following solubilization such non-solubilized membranes can be isolated from the soluble material based on their buoyant density, usually on sucrose gradient (Brown and London, 1998). Raft microdomains are most abundant at the plasma membrane, but may also be present in endocytic and secretory pathways. Membrane lipidation with saturated acyl chain groups of the raft-associated proteins,

such as GPI-anchored proteins and double acylated proteins, have been found to participate in their pre-ferential membrane lipid raft localization (Brown and London, 1998; Simons and Toomre, 2000). However, certain transmembrane proteins can also be enriched in rafts through a mechanism still unclear. The in-volvement of rafts has been implicated in many impor-tant cellular processes, which include generation and maintenance of cellular polarity, chemotactic migra-tion, and cell surface receptor signaling. For T cell and B cell antigen receptors, lipid rafts play a key role in receptor signaling, in which selective signaling molecules are recruited or segregated away (Cherukuri *et al.*, 2001). Antigen or antibody-mediated cross-link-ing of the receptor facilitates its translocation into raft microdomains containing myristate- and palmitate-modified Src family kinases, which initiate signaling cascades by phosphorylating tyrosine residues on the nonenzymatic receptor complexes.

Recently, the association with lipid rafts of some members of the tumor necrosis factor receptor (TNFR) family, including CD40, has been reported (Vidalain *et al.*, 2000). The tumor necrosis factor re-ceptor-associated factor (TRAF) proteins are key signaling adaptor molecules utilized by many TNFR family receptors. Among the six mammalian TRAF family proteins, TRAF2 and TRAF3 were shown to be recruited to raft microdomains during CD40 signaling (Hostager *et al.*, 2000; Vidalain *et al.*, 2000). Similarly, the association of TRAF2 with caveolin-1, a compo-nent that along with rafts constitutes caveolae, has been reported (Feng *et al.*, 2001). The association of CD40 and TRAF in raft microdomains raises the pos-sibility that rafts may function as the signaling plat-form for the TNFR group of transmembrane proteins as in the case for the immune cell antigen receptors.

Based on that TRAF6 molecules are essential for osteoclast function and RANK signaling, we sought to address the potential role of membrane rafts for signaling by RANK and TRAF6. We found that the majority of the ectopically expressed RANK and substantial portion of endogenous TRAF6 were detec-ted in the low-density raft fractions and TRAF6 was recruited to rafts by RANKL stimulation. And disrup-tion of rafts impeded interaction between RANK and TRAF6.

Materials and Methods

Reagents

DMEM and FBS were purchased from Invitrogen Life Technologies. Anti-TRAF2 (H-249) and anti-TRAF6 (H-274) were purchased from Santa Cruz Biotech-nology. Anti-flotillin was obtained from BD Biosciences and anti-T7 was from Novagen.

293 Cell transfection

293 cells, the human embryonic kidney cell line, were maintained in DMEM containing 10% FBS. For transient transfection, 2×10^5 cells were plated onto a well of 6-well plates. The next day, transfection was carried out with SuperFect reagent (Qiagen) following the manufacturer's instruction. 40-48 h after trans-fection, cells were harvested. The mammalian expres-sion plasmid encoding T7-tagged full-length human RANK was described previously (Kim *et al.*, 1999).

Isolation of rafts

Rafts were isolated by a discontinuous sucrose den-sity gradient ultracentrifugation. Cells were washed with ice-cold PBS and lysed in 2 ml of ice-cold TNE buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM EDTA with protease and phosphatase inhi-bitors) containing 0.5% Brij 58. The lysate was in-cubated on ice for 30 min and mixed with an equal volume of 80% wt/vol sucrose in TNE. The mixture was overlaid with 4 ml of 35% sucrose, which in turn was topped with 4 ml of 5% sucrose. The gradient was subjected to ultracentrifugation at 38,000 rpm in an SW41 rotor (Beckman Instruments) for 18 h at 4°C. After centrifugation, 1 ml fractions were collected from the top of the gradient. Fractions were analyzed for the raft marker protein flotillin.

Cell fractionation

Cells were washed with ice-cold PBS and lysed in ice-cold TNE buffer containing 0.5% Brij 58 followed by incubation on ice for 30 min. Insoluble fractions were pelleted by microcentrifugation at 14,000 rpm for 20 min. The supernatant was removed and con-sidered soluble (S) fraction. The insoluble pellet was resuspended in the lysis buffer supplemented with 60 mM N-octyl-β-D-glucopyranoside and 0.3% deoxy-cholic acid, incubated for 1 h on ice, and microcen-trifuged for 20 min at 14,000 rpm. The supernatant from this step was referred to as insoluble (I) fraction. The whole process was performed below 4°C.

Western blotting analysis

Total cell lysates were prepared by lysing cells in TNE/0.5% Brij 58 buffer supplemented with 60 mM N-octyl-β-D-glucopyranoside and 0.3% deoxycholic acid for 1 h on ice and obtaining the supernatants by micro-centrifugation at 14,000 rpm for 20 min. Total cell lysates or the fractionated cellular proteins described above were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The mem-brane was probed with a primary antibody followed by incubation with an appropriate secondary antibody conjugated to horseradish peroxidase. The immune

complexes were detected with an enhanced chemi-luminescence system.

Immunoprecipitation

Association of RANK with TRAF6 is dependent on the integrity of lipid rafts. RANK-T7 transfected 293 cells were incubated for 4 h in serum-free media, pretreated with or without MCD (15 mM) for 30 min, then stimulated with RANKL (500 ng/ml) for 15 min. Total cell lysates were immunoprecipitated with anti-T7 Ab, followed by immunoblotted with anti-T7, TRAF6, and TRAF2 antibodies.

Results

RANK and TRAF6 are localized in fractionationated rafts in RANK-overexpressing 293 cells

Despite the growing appreciation of the importance of lipid raft microdomains in transducing external stimulatory signals by membrane receptors, the involvement of and the role played by rafts in RANK signaling pathway have not been fully elucidated. We therefore investigated whether lipid rafts have any importance for the RANK signaling. To explore this possibility, we first examined the potential of RANK and TRAF6 for being associated with rafts in cells overexpressing RANK. The human embryonic kidney cell line 293 was transfected with a T7-tagged RANK expression plasmid. Raft fractions were separated by sucrose density gradient centrifugation. The majority of the ectopically expressed RANK (Figure 1, top panel) and substantial portion of endogenous TRAF6 (Figure 1, 2nd panel) were detected in the low-density raft fractions where the raft marker protein flotillin was exclusively present (Figure 1, bottom panel). A greater portion of endogenous TRAF2 was localized in raft fractions than in non-raft fractions (Figure 1, 3rd panel).

RANK and TRAF6 are localized in detergent-insoluble fractions in RANK-overexpressing 293 cells

Lipid rafts were initially defined by their solubility in cold, non-ionic detergents. When RANK-T7-transfected 293 cells were treated with 0.5% Brij 58 (a mild nonionic detergent) below 4°C, the flotillin, marker protein for raft, was exclusively localized in the insoluble fraction (Figure 2, bottom panel). This means that the experimental conditions were valid for raft separation. Most of the ectopically expressed RANK was resistant to detergent solubilization in 293 cells (Figure 2, top panel, lanes 3 vs. 4). The endogenous TRAF6 and TRAF2 proteins were almost
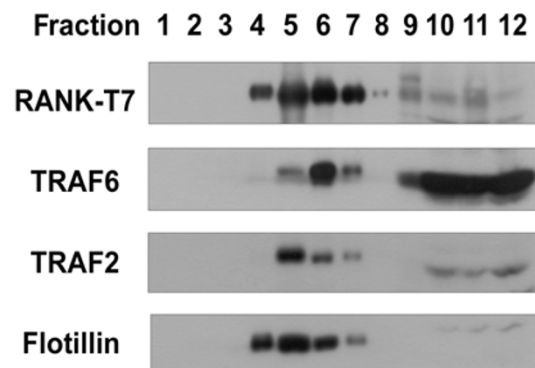

Figure 1. Localization of RANK and TRAF6 in fractionated rafts. 293 cells were transfected with RANK-T7 and cultured for 40 h. RANK-T7 transfected 293 cells were lysed in a buffer containing 0.5% Brij 58 and the lysates fractionated on sucrose density. Fractions were collected from the top of the gradients and the distribution of indicated proteins was assessed by Western blotting.

Figure 2. Localization of RANK and TRAF6 in detergent-insoluble fractions in RANK-overexpressing 293 cells. Mock or RANK-T7 transfected 293 cells were starved for 4 h in serum-free medium. Cells were stimulated with RANKL (500 ng/ml) for 15min and lysed in a buffer containing 0.5% Brij 58. 30 mg of proteins from the Brij 58-soluble (S) and Brij 58-insoluble (I) fractions were analyzed by immunoblotting.

completely soluble in mock-transfected cells (Figure 2, 2nd and 3rd panels, lanes 1 and 2). The over-expression of RANK caused a translocation of significant portions of the TRAF proteins into the detergent-insoluble fraction (Figure 2, 2nd and 3rd panels, lanes 2 vs. 4). The translocation of TRAF6, and by a less extent that of TRAF2, was further increased upon stimulation with RANKL (Figure 2, 2nd and 3rd panels, lanes 4 vs. 6). As gene transfection in these cells often results in a constitutive activation of the gene product, thought to be caused by over-expression-induced protein aggregation, it is possible that the
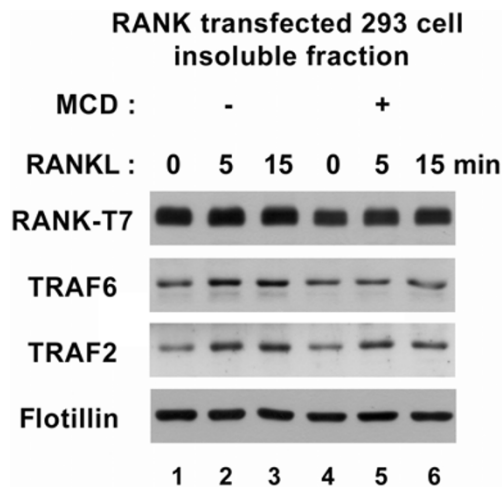
RANK transfected 293 cell insoluble fraction

Figure 3. MCD treatments block TRAF6 translocation. RANK-T7 transfected 293 cells were incubated for 4 h in serum-free medium, pretreated with or without MCD (15 mM) for 30 min, then stimulated with RANKL (1 g/ml) for the indicated time. Cells were lysed in a buffer containing 0.5% Brij 58 and the insoluble fractions were subjected to Western blotting with antibodies for the indicated proteins.

presence of TRAF6 and TRAF2 in the insoluble fraction in the unstimulated RANK-transfected cells (Figure 2, lane 4) was dependent on the basal activation of RANK. Therefore, these results suggest that activated RANK, localized in rafts, can recruit TRAF6 and TRAF2 to form a signaling complex.

Raft-disrupting agents block TRAF6 translocation Induced by RANKL

To evaluate the significance of TRAF6 translocation into rafts, which occurs in a manner dependent on RANK activation (Figure 2), we next assessed effects of methyl-β-cyclodextrin (MCD) on TRAF6 distribution and RANK signaling. MCD and filipin have been reported to extract and sequester, respectively, cholesterol and thereby disrupt raft microdomains (Kilsdonk *et al*., 1995; Vereb *et al*., 2000). MCD treatment of 293 cells transfected with RANK resulted in a slight decrease in detergent-insoluble fraction of RANK (Figure 3, top panel). More significantly, MCD blocked the RANKL-stimulated translocation of TRAF6 into the detergent-insoluble fraction in these cells (Figure 3, 2nd panel). The translocation of TRAF2 was also affected by MCD, albeit by a less extent (Figure 3, 3rd panel).

Raft disruption blocks the association of RANK with TRAF6 but not with TRAF2

We next examined effects of raft disruption on RANK signaling. The interaction of RANK with TRAF adaptor molecules is the initial step in RANK signaling. The
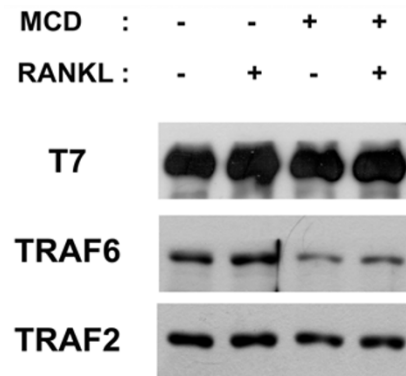

Figure 4. RANK interacts with TRAF6 in rafts. RANK-T7 transfected 293 cells were incubated for 4 h in serum-free medium, pretreated with or without MCD (15 mM) for 30 min, then stimulated with RANKL (500 ng/ml) for 15 min. Total cell lysates were immunoprecipitated with anti-T7 Ab, followed by Western blotting with antibodies for the indicated proteins.

association of RANK with TRAF6 has been shown to result in the activation of c-Src, Akt, and PI3K and be important for osteoclast function and survival. RANK, when over-expressed in 293 cells, coimmuno-precipitated with endogenous TRAF6 and TRAF2 (Figure 4). However, the RANK/TRAF6 interaction was significantly reduced by MCD pretreatment whereas the RANK/TRAF2 interaction was not affected (Figure 4). The reduced RANK/TRAF6 interaction may be due to the decreased TRAF6 translocation into raft microdomains by MCD treatment (Figure 3). This result indicates that raft integrity is necessary for RANK/TRAF6 association.

Discussion

Lipid rafts are specialized membrane microdomains which were initially defined by their insolubility in cold, non-ionic detergents and have been postulated to function as a signaling platforms for immune cell surface receptors such as TCT, BCR, and FcεR (Sheets *et al*., 1999; Janes *et al*., 2000; Cheng *et al*., 2001). More recently, an integral role of the membrane lipid rafts in proximal signaling events has been demonstrated for the TNFR family member such as CD40. CD40 engagement leads to a membrane- associated recruitment of TRAF 3 to CD40's cytoplasmic domain (Vidalain *et al*., 2000). The TRAF family molecules are cytoplasmic adaptor proteins known to mediate signaling events specifically for members of the TNFR family (Darnay *et al*., 1998; Kim *et al*., 1999). To date six members of the TRAF family have been identified. Among these proteins, TRAF6 has been suggested to mediate the NF-κB activation by

CD40, IL-1 receptor, RANK, two LPS receptors Toll-like receptor 2 (TLR2) and TLR4 (Bradley and Pober, 2001; Chung *et al.*, 2002). The pivotal role of RANK-TRAF6 signaling for the generation of functional osteoclasts has been evidenced by gene-targeting mouse studies in which TRAF6 deficiency led to osteopetrotic phenotype (Dougall *et al.*, 1999; Lomaga *et al.*, 1999; Naito *et al.*, 1999). The defect in bone modeling is likely due to impaired TRAF6-mediated signal transduction for the RANKL/RANK interaction that initiates either osteoclast activation or osteoclast differentiation (Dougall *et al.*, 1999; Lomaga *et al.*, 1999; Naito *et al.*, 1999). In our previous works, raft disruption with cholesterol sequestering agents selectively impaired the RANKL signaling pathways of Akt activated through TRAF6-Src pathway. Furthermore, raft-disrupting agents destroyed the integrity of actin-ring structure, bone-resorbing activity, and survival of osteoclasts. However, raft disruption did not impaired MAPK and NF-κB activation by RANKL (Ha *et al.*, 2003). In this study, we demonstrated that the majority of the over-expressed RANK was localized in lipid rafts portion (Figure 1). RANK over-expression provoked the translocation of TRAF6 into the detergent-insoluble raft fraction (Figure 2 and 3). Although a significant fraction of TRAF6 was present in raft microdomains, greater portion of TRAF6 was detected outside rafts (Figure 1). It may be possible that TRAF6 has differential roles within and outside lipid rafts. Our previous report that rafts disruption interfered the activation of Akt but not MAPK and NF-κB (Ha *et al.*, 2003) support this possibility. It will be intriguing to investigate whether TRAF6 outside lipid rafts is fully functional for the activation of MAPK and NF-κB.

Raft disruption with a cholesterol-sequestering agent MCD reduced the association of RANK and TRAF6 (Figure 4), possibly due to reduced TRAF6 translocation into rafts, implicating that lipid rafts play a critical role in RANK-TRAF6 signaling. Src is one of the proteins of which expression greatly increases during osteoclast differentiation (Horne *et al.*, 1992). RANKL stimulation of the catalytic activity of Src was reported and the recruitment of TRAF6 to form a tri-molecular complex with RANK and Src was suggested to result in the Src activation (Wong *et al.*, 1999). Given that RANKL induces TRAF6 translocation into the detergent-insoluble fraction (Figures 2 and 3), it is possible that Src resident in rafts functions as a docking site for the RANK signaling complexes encompassing TRAF6. Rafts in osteoclasts may function as the platform for a network of signaling molecules that start to assemble in response to external stimuli such as RANKL. In the assembly process RANKL-induced recruitment of TRAF6 to RANK and to rafts may be an early event. The raft-recruitment may stimulate the interaction of Src, resident constitutively in rafts, with RANK-TRAF6 complexes, leading to the activation of Src.

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