Lysosome-associated membrane protein 1 is a major SSEA-1-carrier protein in mouse neural stem cells

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Abstract

Stage-specific embryonic antigen-1 (SSEA-1) is a well known carbohydrate antigenic epitope of undifferentiated cells, including neural stem cells (NSCs). However, the exact nature of the carrier proteins has not been fully characterized. Using proteomics analyses, we herein report that a lysosomal protein, LAMP-1, is a major carrier protein of SSEA-1 in NSCs, despite the common belief that SSEA-1 is mainly expressed on the cell surface and constitutes a component of the extracellular matrix. Furthermore we found that SSEA-1 on LAMP-1 is completely ablated in differentiated cells derived from NSCs. Our finding raises the possibility that the expression of SSEA-1-positive LAMP-1 is associated with the “stemness” of NSCs.

Introduction

Neural stem cells (NSCs) are undifferentiated neural cells characterized by their high proliferative potential and the capacity for self-renewal with retention of multipotency to differentiate into neurons and glial cells. NSCs have been isolated from basal forebrain (Temple 1989), cerebral cortex (Davis and Temple 1994), hippocampus (Johe et al. 1996), and spinal cord (Kalyani et al. 1997) in embryos as well as in the subventricular zone of lateral ventricles (Doetsch et al. 1999; Nakatani et al. 2010) and hippocampal dentate gyrus (Seri et al. 2001) in adult brains. To identify and isolate NSCs in these regions, certain marker molecules, such as nestin (Lendahl et al. 1990), Sox2 (Zappone et al. 2000), CD133 (prominin-1) (Uchida et al. 2000), stage-specific embryonic antigen-1 (SSEA-1) (Capela and Temple 2002; Klassen et al. 2001), CD24a, peanut agglutinin ligand (Rietze et al. 2001), Musashi-1 (Sakakibara et al. 2002), syndecan-1, Notch-1, β1 integrin (Nagato et al. 2005), biantennary complex-type N-glycans recognized by Phaseolus vulgaris erythroagglutinating lectin (Hamanoue et al. 2009), and GD3 ganglioside (Nakatani et al. 2010), have been utilized. Most of these NSC marker molecules are glycoconjugates, including glycoproteins, glycolipids, and proteoglycans, that are expressed on the cell surface. In NSCs, glycoconjugates serve as excellent biomarkers at various stages of cellular differentiation, and also play important functional roles in determining cell fate such as self-renewal, proliferation, and differentiation (Yanagisawa and Yu 2007).

The carbohydrate antigen, SSEA-1 [Galβ1-4(Fucα1-3)GlcNAcβ-], is a well-known stage-specific marker of undifferentiated cells, including mouse embryonic stem cells (Muramatsu and Muramatsu 2004). SSEA-1 is also expressed in human embryonic NSCs
(Klassen et al. 2001) and mouse embryonic, postnatal, and adult NSCs (Capela and Temple 2002; Kim and Morshead 2003; Klassen et al. 2001; Yanagisawa et al. 2005). Because of its expression pattern and cell-surface localization, SSEA-1 has been widely used as a marker molecule to isolate NSC populations from mouse brains by fluorescence-activated cell sorting (Capela and Temple 2002; Corti et al. 2005; Kim and Morshead 2003). So far, the SSEA-1 epitope in NSCs has been found to be associated with a glycosphingolipid (Yanagisawa et al. 2005), chondroitin sulfate proteoglycans (Kabos et al. 2004), and glycoproteins including β1 integrin (Yanagisawa et al. 2005) and Wnt-1 (Capela et al. 2006). However, since the identification of these molecules was performed by immunocytochemical analysis using specific antibodies, bona fide carrier molecules of SSEA-1 in NSCs have not yet been biochemically identified. Herein, we identified a major SSEA-1 carrier protein in NSCs by proteomic analysis based on mass spectrometry (MS). Unexpectedly this SSEA-1-positive protein was a lysosomal protein, although it had been considered that SSEA-1 is expressed primarily as a cell-surface marker molecule. This is the first study to positively identify an SSEA-1-carrier protein expressed in stem cells.

Results

Detection of a glycoprotein carrying SSEA-1 in NSCs

NSCs were isolated from the striata of mouse embryos (embryonic day 14.5) via neurospheres, floating aggregates formed by NSCs in vitro (Nakatani et al. 2010; Reynolds and Weiss 1992). To detect glycoproteins carrying SSEA-1 in NSCs, we performed Western blot analysis using AK97 mouse monoclonal antibody (Yanagisawa et al. 1999). AK97 was originally established for a spirometo-series parasitic glycosphingolipid having a characteristic trisaccharide structure [Galβ1-4(Fucα1-3)Glcβ-] (Kawakami et al. 1993) but strongly reacts also with SSEA-1 (Yanagisawa et al. 1999). Because this parasitic trisaccharide structure is not expressed in mammals, AK97 can specifically detect glycoproteins carrying SSEA-1 in mouse NSCs. In addition to a few minor bands, one major protein band reactive with AK97 anti-SSEA-1 antibody, corresponding to an apparent molecular mass 80 kDa (Fig. 1), was detected in the lysates of NSCs (lanes 1, 3, 5 of Fig. 1). However, this band completely disappeared in the
lysates of cells differentiated from NSCs (lanes 2, 4, 6 of Fig. 1). This major SSEA-1-carrier protein, with the molecular mass 80 kDa, expressed in NSCs, was further analyzed.

**Identification of a glycoprotein carrying SSEA-1 in NSCs**

To isolate this SSEA-1-carrier protein, SSEA-1-positive immunoprecipitates prepared from secondary neurospheres with an anti-SSEA-1 monoclonal antibody, AK97, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and followed by staining with Coomassie Brilliant Blue G-250. A piece of the polyacrylamide gel containing the 80 kDa protein was excised, digested with trypsin, and then subjected to LC-MS/MS analysis. The data indicated that the 80 kDa protein positive for SSEA-1 is lysosome-associated membrane protein 1 (LAMP-1) (Fig. 2A). For confirmation, SSEA-1-carrier proteins in NSCs were immunoprecipitated with AK97 and analyzed by Western blot with an anti-LAMP-1 antibody. As shown in Fig. 2B (left panel), LAMP-1 was clearly detected in SSEA-1-positive immunoprecipitates. This SSEA-1-positive LAMP-1 (Fig. 2B, left panel) and control LAMP-1 in lysates detected by the anti-LAMP-1 antibody (Fig. 2B, right panel) had a molecular mass of 80 kDa, identical with that of the major SSEA-1-carrier protein in Fig. 1. Furthermore, to determine whether the SSEA-1 epitope on LAMP-1 is carried by N- or O-glycans, the cell lysates from NSCs were treated with peptide N-glycanase F (PNGase F) and then analyzed by Western blot with AK97 and anti-LAMP-1 antibodies. After PNGase F treatments, the 80 kDa protein band reactive with AK97 disappeared (Fig. 2C, left panel). Likewise, the protein band reactive with anti-LAMP-1 antibody, corresponding to the 80 kDa protein disappeared and a new band with a molecular mass of 40 kDa appeared (Fig.2C, center panel). These data clearly indicate that LAMP-1 is highly N-glycosylated with the SSEA-1 epitope.

**Localization of SSEA-1 in NSCs**

To evaluate the localization of SSEA-1 in NSCs, we stained NSCs prepared from neurospheres with the AK97 anti-SSEA-1 antibody. As shown in Fig. 3, SSEA-1 signals were found in intact NSCs treated without 0.1% TritonX-100. The result indicates that SSEA-1 is localized on the cell surface. However, in NSCs permeabilized with 0.1% Triton-X100, SSEA-1 signals were intensely detected. This result suggests that SSEA-1 is localized not only on the cell surface, but also in the intracellular regions of NSCs. This result is consistent with the lysosomal localization of LAMP-1.
LAMP-1 expression in differentiated cells

Given that SSEA-1-carrying LAMP-1 was not expressed in cells differentiated from NSCs (Fig. 1), we next examined the expression of LAMP-1 before and after differentiation. As shown in Fig. 4A, LAMP-1 is similarly expressed in NSCs and cells differentiated from NSCs. This is consistent with the result of reverse transcription-polymerase chain reaction (RT-PCR) analysis indicating that the LAMP-1 mRNA level exhibited no changes during differentiation (Fig. 4B). It has been reported that α1,3-fucosyltransferase IX (FUT9) is involved in the expression of SSEA-1 in rodent brains (Kudo et al. 2007; Shimoda et al. 2002). In the cells differentiated from NSCs, FUT9 was found to be down-regulated; there was no significant difference in the expression levels of other fucosyltransferases, FUT4, FUT10 and FUT11, before and after differentiation (Fig. 4B). Pax6 (Paired box gene 6), a transcription factor which has been reported to regulate the expression of FUT9 in rat embryonic forebrain (Shimoda et al. 2002), was also decreased in the differentiated cells (Fig. 4B). These results suggest that the lack of SSEA-1-positive LAMP-1 molecules in the differentiated cells (Fig. 1) is responsible for the down-regulation of SSEA-1, but not of LAMP-1, probably via change of the glycosyltransferase expression level.

Discussion

In this study, we identified LAMP-1, a lysosomal membrane protein, as a major carrier protein of SSEA-1 in NSCs using LC-MS/MS and Western blot analyses. LAMP-1 is known as a lysosomal marker. It is a highly N-glycosylated lysosomal membrane protein (Chen et al. 1985; Lewis et al. 1985) and has been suggested to involve in lysosome biogenesis and autophagy (Eskelinen 2006). LAMP-1 possesses 17 to 20 N-glycosylation sites as well as 5 O-glycosylation sites (Carlsson et al. 1993; Eskelinen et al. 2003). By PNGase F treatment, we demonstrated that SSEA-1 is carried by N-glycans (Fig. 2C). N-glycans of LAMP-1 have been suggested to protect LAMP-1 from proteolytic digestion, because deglycosylation of LAMP-1 by endoglycosidase H led to its rapid degradation (Kundra and Kornfeld 1999). There is a possibility that SSEA-1 is also involved in this process.

SSEA-1 is also known as Lewis X, which plays important roles in cell-cell communication such as carbohydrate-protein and carbohydrate-carbohydrate interactions. It is generally considered that SSEA-1 is expressed on the cell surface or extracellular matrix in
NSCs. Indeed, NSCs in brain tissues have been reported to be sorted by fluorescence-activated cell sorting using anti-SSEA-1 antibody (Capela and Temple 2002; Corti et al. 2005; Kim and Morshead 2003; Koso et al. 2006). Our proteomics data, however, revealed that SSEA-1 is mainly carried by a lysosomal protein, LAMP-1, in NSCs. This is the first identification of an intracellular molecule that serves as an SSEA-1-carrier in stem cells, including NSCs. In addition to mouse embryonic stem cells, LAMP-1 may also be a carrier protein of SSEA-1 in other SSEA-1-positive stem cells. In support of our contention, Brito et al. recently suspected that SSEA-1 might be attached to LAMP-1 in hippocampus cell cultures resulting from colocalization of SSEA-1 with LAMP-1 by immunofluorescence staining (Brito et al. 2009). Although it is necessary to perform further studies including elucidation of the function of SSEA-1 on LAMP-1 molecules, our finding has raised the possibility that expression of SSEA-1-positive LAMP-1 in NSCs is associated with the “stemness” of stem cells.

Materials and Methods

**NSC culture**

NSCs were prepared from ICR mouse embryos (embryonic day 14.5) in the form of neurospheres according to previously described methods with slight modifications (Nakatani et al. 2010; Reynolds and Weiss 1992). To induce differentiation, the NSCs were cultured in Neurobasal-A medium containing B27, L-glutamine (Invitrogen), and 1% fetal bovine serum for 10 days. The ICR mice (Harlan, Indianapolis, IN) used in this study were treated in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Medical College of Georgia.

**Western blot analysis and immunoprecipitation**

The lysates prepared from NSCs and cells differentiated from NSCs using lysate buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA and 1% Triton X-100) were subjected to Western-blot analysis with AK97 (Yanagisawa et al. 1999), anti-LAMP-1 antibody (Cell Signaling Technology, Boston, MA) or anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO) and horseradish peroxidase-conjugated anti-mouse IgM antibody (Jackson ImmunoResearch, West Grove, PA), anti-rabbit IgG antibody (GE Healthcare Life Sciences, Piscataway, NJ), or anti-mouse IgG antibody (GE Healthcare Life Sciences). The protein bands were visualized by WesternLightning Chemiluminescence Reagent (Perkin Elmer Life and Analytical Sciences,
Waltham, MA). To remove N-glycans on glycoproteins, lysates containing 20 μg of proteins were incubated with PNGase F (50 units, New England BioLabs, Beverly, MA) at 37°C for 3 h before being subjected to SDS-PAGE.

For immunoprecipitation, the cell lysates were gently agitated in the presence of control anti-mouse IgM (BD Biosciences Pharmingen, San Diego, CA) or AK97 antibody for 1h, followed by incubation with protein L-Sepharose (Pierce, Rockford, IL) at 4°C for 3 hours. After washing 3 times with lysate buffer, immunoprecipitates were subjected to SDS-PAGE and then Western blot analysis. AK97 or anti-LAMP-1 antibody was used as primary antibody.

**Identification of glycoprotein by LC/MS/MS analyses**

For LC-MS/MS analyses, glycoproteins were digested as described previously (Yagi et al. 2008). The resultant digested peptides were reconstituted in 0.1% formic acid and analyzed by a Thermo LTQ linear ion-trap mass spectrometer equipped with a nano-electrospray ionization (ESI) source and a Finnigan Surveyor LC system (Thermo Fisher Scientific, Waltham, MA). The peptides were directly infused into the ESI source through a reverse phase-C18 trap column equilibrated in 0.1% formic acid at a flow rate of 100 nL/min, and were sequentially eluted with an acetonitrile gradient from 5 to 40% over 60 min. The spectrometer was operated in data-dependent mode using normalized collision energy of 35%. The temperature of the ion transfer tube was set at 200°C and the spray voltage was at 1.8 kV. MS analysis was performed with one full MS scan followed by five MS/MS scans on the five most intense ions from the MS spectrum. The resultant MS and MS/MS data were searched against NCBI mouse database using the TurboSequest algorithm in the Bioworks software 3.2.

**Immunocytochemistry**

NSCs prepared from neurospheres were plated onto chamber slides (Nalge Nunc International, Naperville, IL) coated with poly-L-ornithine (Sigma-Aldrich) and fibronectin (Sigma-Aldrich), and fixed in PBS containing 4% paraformaldehyde. The NSCs were treated with PBS containing 3% fetal bovine serum and 0% or 0.1% TritonX-100 for 2 hours and then stained with AK97 and Alexa Fluor 488-conjugated anti-mouse IgM antibodies (Invitrogen). Nuclei were stained with 2 μg/ml of Hoechst 33258 (Sigma-Aldrich). The stained NECs were photographed under a Nikon Eclipse TE300 fluorescent microscope (Nikon Instruments,
Melville, NY) equipped with a Magnafire digital charge-coupled device camera (Optronics, Goleta, CA).

**RT-PCR**

RT-PCR was performed as previously described (Nakatani et al. 2010; Ngamukote et al. 2007). Total RNAs were isolated from cells using Trizol reagent (Invitrogen). cDNAs were synthesized from the total RNAs as templates using SuperScript III reverse transcriptase (Invitrogen). PCR was performed with the following settings: 30 (for Pax6 and β-actin), 33 (for LAMP-1, FUT9, FUT10, FUT11 and Sox2) or 38 (for FUT4) cycles of 94°C for 10 sec, 52-55°C for 30 sec, 72°C for 30 sec. The sequences of primers were as follows (5'-3'):

- TCTTCAGTGTGCAGGTCCAG and TGGACCAGAGATTCCCTTTG for LAMP-1;
- TTGACCACCTTCATCTGCTG and GTTGGATCGCTCCTGGAATA for FUT4;
- TTCGCCCATTTCTAATCGTC and TTGTGCTCACCGTCAAGAAG for FUT9;
- CATGAAGAGTCCCCCCAAAAA and CCGCTGGTTAGAGATCTTGC for FUT10;
- AGGCATCACCAACCAGTTTC and TTCCCAATACAGGGCAAGAG for FUT11;
- CTGTACCAACGATAACATACCC and AGGAGTGGTGCTGGCCTGTC for Pax6;
- AACGCCCTTCATGGTATGGTC and CCGGAAGCGTGTACTTATC for Sox2; AGCCAT GTACGTAGCCATCC and TCTCAGCTGTGGTGGAAG for β-actin. The PCR products were analyzed by agarose gel electrophoresis using 2% agarose gels containing SYBR safe DNA Gel Stain (Invitrogen).

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**Conflict of Interest Statement**

None declared.

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Footnotes

Abbreviations
FUT, fucosyltransferase; LAMP-1, lysosome-associated membrane protein 1; LC, liquid chromatography; MS, mass spectrometry; NSCs, neural stem cells; pax6, Paired box gene 6; PNGase F, peptide N-glycanase F; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SSEA-1, stage specific embryonic antigen-1; SVZ, subventricular zone

References


**Figure Legends**

**Fig. 1. Detection of SSEA-1-carrier proteins in NSCs and differentiated cells.** Proteins from primary (lane 1), secondary (lane 3), and tertiary (lane 5) neurospheres and cells differentiated from primary (lane 2), secondary (lane 4) and tertiary (lane 6) neurospheres were analyzed by
Western blot with AK97 anti-SSEA-1 antibody or anti-β-actin antibody (Blot). β-Actin was detected as a loading control. Arrow indicates a major band positive for SSEA-1 with a molecular mass of 80 kDa. The bands indicated by asterisk were non-specifically detected by the secondary anti-mouse IgM antibody.

**Fig. 2. LAMP-1 as an SSEA-1-carrier protein.** LAMP-1 was identified as a major SSEA-1-carrier protein by LC-MS/MS analysis after enrichment by immunoprecipitation with AK97 antibody. (A) Amino acid sequence of LAMP-1. The sequences of observed peptides are underlined. The putative signal sequence and transmembrane domain are marked by double and dashed underlines, respectively. Twenty potential N-glycosylation sites are indicated by asterisks. (B) Proteins from the tertiary neurospheres were immunoprecipitated with control mouse IgM or AK97 (IP), and then subjected to Western blot analysis with anti-LAMP-1 antibody (left panel). The cell lysate was detected by AK97 as a control (right panel). (C) The lysates of tertiary neurospheres (containing 20 μg of proteins) were incubated with PNGase F (0 or 50 units/20 μl) at 37°C for 3 h to remove N-glycans and then analyzed by Western blot with AK97, anti-LAMP-1 antibody, and anti-β-actin antibody.

**Fig. 3. Localization of SSEA-1 in NSCs.** NSCs prepared from neurospheres were treated with PBS containing 3% fetal bovine serum and 0% or 0.1% TritonX-100 (Trx100) and then stained with AK97 and Alexa Fluor 488-conjugated anti-mouse IgM antibody (green). Nuclei were stained with 2 μg/ml of Hoechst 33258 (H33258; blue).

**Fig. 4 LAMP-1 expressed in NSCs and differentiated cells.** (A) Cell lysates from primary (lane 1), secondary (lane 3), and tertiary (lane 5) neurospheres and cells differentiated from primary (lane 2), secondary (lane 4), and tertiary (lane 6) neurospheres were analyzed by Western blot with anti-LAMP-1 antibody or anti-β-actin antibody. (B) The mRNA expression of LAMP-1, FUT4, FUT9, FUT10, FUT11, Sox2 and β-actin in undifferentiated NSCs (undiffer) and differentiated cells (differ) were analyzed by RT-PCR. Sox2 was detected as a marker gene of undifferentiated NSCs. β-Actin was used as a control.