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

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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.

Keywords: development/LAMP-1/N-glycosylation/neural stem cell/SSEA-1

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) (Klassen et al. 2001; Capela and Temple 2002), 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 T and Muramatsu H 2004). SSEA-1 is also expressed in human embryonic NSCs (Klassen et al. 2001) and mouse embryonic, postnatal and adult NSCs (Klassen et al. 2001; Capela and Temple 2002; Kim and Morshed 2003; 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; Kim and Morshed 2003; Corti et al. 2005). 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 immunocytocchemical 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 (Reynolds and Weiss 1992; Nakatani...
et al. 2010). 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 \([\text{Gal}^\beta_1-4(\text{Fuc}^\alpha_1-3)\text{Glc}^\beta_1-] \) (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 (Figure 1), was detected in the lysates of NSCs (lanes 1, 3, 5 of Figure 1). However, this band completely disappeared in the lysates of cells differentiated from NSCs (lanes 2, 4, 6 of Figure 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 liquid chromatography (LC)–MS/MS analysis. The data indicated that the 80 kDa protein positive for SSEA-1 is lysosome-associated membrane protein 1 (LAMP-1) (Figure 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 Figure 2B (left panel), LAMP-1 was clearly detected in SSEA-1-positive immunoprecipitates. These SSEA-1-positive LAMP-1 (Figure 2B, left panel) and control LAMP-1 in lysates detected by the anti-LAMP-1 antibody (Figure 2B, right panel) were subsequently analyzed by western blot with anti-LAMP-1 antibody and anti-β-actin antibody. In addition, 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.
panel) had a molecular mass of 80 kDa, identical to that of the major SSEA-1-carrier protein in Figure 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 (Figure 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 (Figure 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 Figure 3, SSEA-1 signals were found in intact NSCs treated without 0.1% Triton X-100. The result indicates that SSEA-1 is localized on the cell surface. However, in NSCs permeabilized with 0.1% Triton X-100, 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 (Figure 1), we next examined the expression of LAMP-1 before and after differentiation. As shown in Figure 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 (Figure 4B). It has been reported that α1,3-fucosyltransferase IX (FUT9) is involved in the expression of SSEA-1 in rodent brains (Shimoda et al. 2002; Kudo et al. 2007). In the cells differentiated from NSCs, FUT9 was found to be downregulated; there was no significant difference in the expression levels of other fucosyltransferases, FUT4, FUT10 and FUT11, before and after differentiation (Figure 4B). Pax6 (paired box gene 6), a transcription factor which has been reported to regulate the

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% Triton X-100 (Trx100) and then stained with AK97 and Alexa Fluor 488-conjugated antimouse 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.
expression of FUT9 in rat embryonic forebrain (Shimoda et al. 2002), was also decreased in the differentiated cells (Figure 4B). These results suggest that the lack of SSEA-1-positive LAMP-1 molecules in the differentiated cells (Figure 1) is responsible for the downregulation 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 (Figure 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; Kim and Morshead 2003; Corti et al. 2005; 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 findings have 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 (Reynolds and Weiss 1992; Nakatani et al. 2010). To induce differentiation, the NSCs were cultured in Neurobasal-A medium containing B27, l-glutamine (Invitrogen, Carlsbad, CA) 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 lysis buffer (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid 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 antismouse IgM antibody (Jackson ImmunoResearch, West Grove, PA), antirabbit IgG antibody (GE Healthcare Life Sciences, Piscataway, NJ) or antigoat IgG antibody (GE Healthcare Life Sciences). The protein bands were visualized by Western Lightning 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 antismouse IgM (BD Biosciences Pharmingen, San Diego, CA) or AK97 antibody for 1 h, followed by incubation with protein L-Sepharose (Pierce, Rockford, IL) at 4°C for 3 h. After washing three times with lysis 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 phosphate-buffered saline (PBS) containing 4% paraformaldehyde. The NSCs were treated with PBS containing 3% fetal bovine serum and 0% or 1% Triton X-100 for 2 h and then stained with AK97 and Alexa Fluor 488-conjugated antimouse IgM antibodies (Invitrogen). Nuclei...
were stained with 2 μg/mL of Hoechst 33258 (Sigma-Aldrich). The stained NSCs 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 (Ngamukote et al. 2007; Nakatani et al. 2010). 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 s, 52–55°C for 30 s and 72°C for 30 s. The sequences of primers were as follows (5′–3′): TCTTCAGTGTGCAAGTCCAG and TGGACCAAGAAGATTTTAGGTTTG for LAMP-1; TTGACACCTTATCTGCTG and GTTGAGATCGTCTCCGGAATA for FUT4; TCTGCCAATTTTAAATGTCC and TTGTGCTTACCGTCAAGAG for FUT9; CATGGAAGATCCCCAAAAAA and CCGCTGTGTAAGATCTG for FUT10; AGGCACACGAAAGCTTTCC and TGTACCAACGATAACATAACC and AGGAGTGTGCTGCGCTGTC for Pax6; AAGCCTTCTGATGATGTC and CGGGAAGCGTGTACTAT for Sox2; GAGCCTAGCTGAGTTCC and TCTCAGCTGTTGGTGGAAG 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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Abbreviations
ESI, electrospray ionization; 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


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