The binding specificity of the marker antibodies Tra-1-60 and Tra-1-81 reveals a novel pluripotency-associated type 1 lactosamine epitope

Suvi Natunen1, Tero Satomaa3, Virve Pitkänen2, Hanna Salo3, Milla Mikkola2,4, Jari Natunen3, Timo Otonkoski4,5, and Leena Valmu1,2

1To whom correspondence should be addressed: Tel: +358-50-547-4219; Fax: +358-9-5801310; e-mail: leena.valmu@veripalvelu.fi
2Finnish Red Cross Blood Service, Kivihaantie 7, 00310 Helsinki, Finland;
3Glykos Finland Ltd, 00790 Helsinki, Finland; 4Biomedicum Stem Cell Center, Program of Molecular Neurobiology, University of Helsinki, 00014 Helsinki, Finland; and 5Hospital for Children and Adolescents, Helsinki University Central Hospital, 00029 HUS Helsinki, Finland

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The expression of the epitopes recognized by the monoclonal antibodies Tra-1-60 and Tra-1-81 is routinely used to assess the pluripotency status of human embryonic stem cells (hESCs) and induced pluripotent stem (iPS) cells. Although it is known that the epitopes recognized by Tra-1-60 and Tra-1-81 are carbohydrates, the exact molecular identity of these epitopes has been unclear. Glycan array analysis with more than 500 oligosaccharide structures revealed specific binding of Tra-1-60 and Tra-1-81 to two molecules containing terminal type 1 lactosamine: Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAc and Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc. The type 1 disaccharide in itself was not sufficient for binding, indicating that the complete epitope requires an extended tetrasaccharide structure where the type 1 disaccharide is β1,3-linked to type 2 lactosamine. Our mass spectrometric analysis complemented with glycosidase digestions of hESC O-glycans indicated the presence of the extended tetrasaccharide epitope on an O-glycan with the likely structure Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6(Galβ1-3)GlcNAc. Thus, the present data indicate that the pluripotency marker antibodies Tra-1-60 and Tra-1-81 recognize the minimal epitope Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAc, which is present in hESCs as a part of a mucin-type O-glycan structure. The exact molecular identity of Tra-1-60 and Tra-1-81 is important for the development of improved tools to characterize the pluripotent phenotype.

Keywords: O-glycosylation / pluripotency / stem cells / Tra-1-60 / Tra-1-81

Introduction

Human embryonic stem cells (hESCs) from the inner cell mass of the blastocyst, and the more recently described somatic cell-derived induced pluripotent stem (iPS) cells reprogrammed from somatic cells share the property of being able to grow indefinitely while maintaining pluripotency (Thomson et al. 1998; Takahashi et al. 2007). Extensive research is being carried out on these cell types to develop disease models, methods for drug screening and ultimately regenerative therapies. Embryonic stem cells express certain surface markers that are thought to be associated with pluripotency and are widely used to characterize the cells. These markers include the protein antigens CD9, Thy1 (CD90), tissue-nonspecific alkaline phosphatase (Tra-2-49 and Tra-2-54), class-1 human leukocyte antigen, and podocalyxin (GCTM2), the globoseries glycosphingolipid antigens stage-specific embryonic antigen (SSEA)-3 and SSEA-4, and the carbohydrate epitopes recognized by the monoclonal antibodies Tra-1-60 and Tra-1-81 (International Stem Cell Initiative 2007; Wright and Andrews 2009). Many of the marker antibodies have originally been raised against embryonal carcinoma cells, which were used as a model for human pluripotent cells before hESCs were introduced (Wright and Andrews 2009). iPS cells express many of the same antigens, at least SSEA-3, SSEA-4, Tra-1-60, Tra-1-81 and tissue-nonspecific alkaline phosphatase (Yamanaka 2009).

Many of the stem cell marker antibodies, such as SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81, recognize carbohydrate epitopes (Lanctot et al. 2007; Wright and Andrews 2009). We and others have shown that embryonic stem cells display a characteristic glycosylation profile that distinguishes them from differentiated cell types (Draper et al. 2002; Venable et al. 2005; Wearne et al. 2006, 2008; Satomaa et al. 2009). Examples of typical glycosylation features of hESCs revealed by structural analysis include Lewis x and H type 2 epitopes on N-glycans (Satomaa et al. 2009). The Tra-1-60 and Tra-1-81 pluripotency marker antibodies have been suggested to recognize keratan sulfate epitopes on podocalyxin (Badcock et al. 1999; Schopperle and DeWolf 2007).

In the present study, we carried out a mass spectrometric analysis of hESC O-glycans complemented with specific exoglycosidase digestions. To get more insight into pluripotency-associated glycosylation, the specificities of the Tra-1-60 and Tra-1-81 antibodies were characterized on a large glycan array developed by the Consortium for...
Functional Glycomics. Our data indicate that the embryonic stem cell marker antibodies Tra-1-60 and Tra-1-81 recognize a specific type 1 lactosamine epitope, which is present in hESCs as a part of a mucin-type O-glycan structure.

Results
The monoclonal antibodies Tra-1-60 and Tra-1-81 stain undifferentiated hESCs in a β1,3-galactosidase sensitive manner
FES29 hESCs were treated with either β1,3-galactosidase (Streptococcus pneumoniae), β1,4-galactosidase (Xanthomonas manihotis) or buffer only, stained with Tra-1-60 and Tra-1-81 antibodies and analyzed by flow cytometry (Figure 1). β1,4-Galactosidase had no effect on the binding of the antibodies, whereas β1,3-galactosidase treatment drastically reduced the binding of both Tra-1-60 and Tra-1-81, suggesting that terminal β1,3-linked galactose is an essential part of the epitope. The glycosidase activities were controlled by using appropriate oligosaccharides as substrates as described in Supplementary data, Supplement SII.

The monoclonal antibodies Tra-1-60 and Tra-1-81 bind specifically to a type 1 lactosamine epitope
The glycan-binding specificity of Tra-1-60 and Tra-1-81 was tested against version 4.2 of the glycan array of the Consortium for Functional Glycomics. Among more than 500 oligosaccharide ligands covalently immobilized on glass, Tra-1-60 and Tra-1-81 specifically bound to Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAc and Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc (Figure 2, Table I). Average RFU values and standard deviations of 6 printed spots are shown in Table I. An irrelevant mouse immunoglobulin M(IgM) was used as control to show that the secondary antibody alone does not bind the array. Complete results of the array are provided in Supplementary data, Table SI. The array was analyzed at 25 and 100 µg/mL of the antibodies; 25 µg/mL was enough to saturate the binding. Nonbinding structures related to the binding structures include Galβ1-3GlcNAc, Galβ1-3GlcNAcβ1-3Galβ1-4Glc, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc, Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAc, Galβ1-3(Fucα1-4) GlcNAcβ1-3Galβ1-4GlcNAc and Galβ1-3GlcNAcβ1-3Galβ1-3GlcNAc (Table I). It can be concluded that the minimal binding epitope for the monoclonal antibodies Tra-1-60 and Tra-1-81 is Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAc, where a β1,3-linkage is required between the nonreducing end galactose and N-acetylgalcosamine, and a β1,4-linkage is required between the reducing end galactose and N-acetylgalcosamine. The N-acetylgalcosamine group of the reducing end N-acetylgalcosamine is required, since an identical structure with reducing end glucose did not bind. α2,3-Sialylation or α1,4-fucosylation of the structure abrogated binding. Both of the antibodies showed strong binding to the same two glycans with minimal background binding, indicating that the binding is specific for the extended type 1 lactosamine epitope.

hESC O-glycans carry the Tra-1-60- and Tra-1-81-defined glycan epitope
Isolated neutral reducing O-glycans from hESC were analyzed by matrix-assisted laser desorption-ionization time-of-flight
The major glycan signals that diminished was the peak observed at \( m/z \) 1136.32 (Hex,HexNAc; Figure 3B). The signal intensity was compared with the intensities of signals at \( m/z \) 1079.30 and 1095.30, which arise from contaminating \( N \)-glycan structures not digested by the enzyme. \( \text{Endo-}\beta\text{-galactosidase} \) specifically cleaves unbranched polylactosamine sequences. The signal at \( m/z \) 1136.32 was also sensitive to \( \beta_1,3\text{-galactosidase} \) exoglycosidase digestion (Figure 3D), but not to \( \beta_1,4\text{-galactosidase} \) digestion (Figure 3C), and therefore, was determined to contain a nonreducing end \( \text{Gal}\beta_1-3\text{GlcNAc}\beta_1-3\text{Gal}\beta_1-4\text{GlcNAc}\beta \) sequence (polylactosamine chain terminating with type 1 lactosamine epitope). The glycosidase activities were controlled by using appropriate oligosaccharides as substrates as described in Supplementary data, Supplement SII. In the present experiments, we did not chemically characterize the reducing end saccharide, but a candidate is the mucin-type \( O \)-glycan core structure \( \text{Gal}\beta_1-3\text{GlcNAc}\alpha \) that has been described in hESC (Weame et al. 2008). In conclusion, the \( O \)-glycan signal at \( m/z \) 1136.32 arises from the glycan structure \( \text{Gal}\beta_1-3\text{GlcNAc}\beta_1-3\text{Gal}\beta_1-4\text{GlcNAc}\beta \), most likely \( \text{Gal}\beta_1-3\text{GlcNAc}\beta_1-3\text{Gal}\beta_1-4\text{GlcNAc}\beta_1-6(\text{Gal}\beta_1-3)\text{GlcNAc} \), which contains the minimal epitope recognized by the Tra-1-60 and Tra-1-81 antibodies.

### Discussion

The Tra-1-60 and Tra-1-81 markers are widely used in human stem cell research as positive indicators of a true pluripotent human stem cell (International Stem Cell Initiative 2007). The present study redefines the molecular identity of the epitopes recognized by the Tra-1-60 and Tra-1-81 antibodies. The staining of hESCs by Tra-1-60 and Tra-1-81 was found to be sensitive to \( \beta_1,3\text{-galactosidase} \) treatment of the cells, indicating that terminal \( \beta_1,3\text{-galactose} \) is an essential part of the epitope. On a glycan array of 511 oligosaccharides, the Tra-1-80 and Tra-1-61 antibodies specifically bound to two oligosaccharide structures containing the type 1 lactosamine disaccharide \( \beta_1,3\text{-linked to type 2 lactosamine: Gal}\beta_1-3\text{GlcNAc}\beta_1-3\text{Gal}\beta_1-4\text{GlcNAc}\beta_1-6(\text{Gal}\beta_1-3)\text{GlcNAc} \). An \( O \)-glycan containing the same \( \text{Gal}\beta_1-3\text{GlcNAc}\beta_1-3\text{Gal}\beta_1-4\text{GlcNAc} \) epitope was found to be one of the major \( O \)-glycans of hESCs by mass spectrometric and enzymatic analyses.

The present results are somewhat contradictory to earlier published data indicating that the Tra-1-81 and Tra-1-60 antibodies recognize keratan sulfate epitopes (Badcock et al. 2003).
1999). It is notable that the Glycan Array does not contain polymeric keratan sulfate epitopes. Therefore, without direct comparison with keratan sulfate, the possibility cannot be ruled out that the type 1 lactosamine epitopes represent cross-reacting structures. However, Tra-1-60 and Tra-1-81 did not bind the repeating structural units of keratan sulfate, [6OSO₃]Galβ1-4[6OSO₃]Galβ1-4[6OSO₃]GlcNAc, or Galβ1-4[6OSO₃]GlcNAc on the array. Nor did the antibodies bind any type 2 polylactosamines, the nonsulfated form of keratan sulfate, which are abundantly represented on the array. It is possible that the keratanase enzyme used by Badcock et al. to demonstrate the association of the epitopes with keratan sulfate contained impurities, such as β-galactosidase, that reacted with Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAc. Another possible explanation for the contradictory data is that a type 1 epitope similar to the one found in the structural analysis of O-glycans here is present in hESCs as a terminal modification of keratan sulfate. To our knowledge, type 1 modifications on keratan sulfate glycans have not been reported, but they are biosynthetically feasible. Badcock et al. (1999) also demonstrated that the Tra-1-60 epitope is destroyed by sialidase, whereas the structure indicated in the present study to be the epitope for Tra-1-60 is not sialylated. In fact, the same epitope in its sialylated form did not bind Tra-1-60. Further studies are required to resolve these controversies and to establish whether the type 1 lactosamine epitopes recognized by Tra-1-81 and Tra-1-60 on hESCs are modifications of keratan sulfate or mucin-type O-glycans.

The Tra-1-60, Tra-1-81, K4, K21 and GCTM2 monoclonal antibodies were originally raised against human embryonal carcinoma cells (Andrews et al. 1984; Retig et al. 1985; Pera et al. 1988). Tra-1-60, Tra-1-81 and GCTM2 are used as markers antigens in the characterization of hESCs (International Stem Cell Initiative 2007). Tra-1-60, Tra-1-81, K4, K21 and GCTM2 all recognize the same high-molecular-weight glycoprotein that has been suggested to be podocalyxin but has not yet been definitively characterized (Badcock et al. 1999; Schopperle et al. 2003; Schopperle and DeWolf 2007). GCTM2 recognizes the core protein (Pera et al. 1988), whereas the other antibodies recognize glycan epitopes (Retig et al. 1985; Badcock et al. 1999). K21 has been shown to bind to lacto-N-tetraose (LNT; Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer), whereas K4 binds to sialosyl-LNT (Neu5Aco2-3LNT; Neu5Aco2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer) in an immuno-TLC assay of isolated glycosphingolipids (Fukuda et al. 1986). In the light of the present results, it seems that Tra-1-60, Tra-1-81 and K21 recognize closely related epitopes, and K4 recognizes a similar epitope in the sialylated form. However, the novel specificity of the Tra-1-60 and Tra-1-81 antibodies is directed to a longer polylactosamine epitope, which may be more specific for pluripotent cells.

The results presented here together with earlier literature support the idea that various type 1 lactosamine structures are typical features of O-glycans and glycosphingolipids of pluripotent human cells. In contrast, the N-glycans of hESCs carry exclusively type 2 lactosamine epitopes (Satoma et al. 2009). Human embryonal carcinoma cells express a LNT-like type 1 epitope recognized by the monoclonal antibody K21 (Retig et al. 1985; Fukuda et al. 1986; Garin-Chesa and Retig 1989; Andrews et al. 1996), the sialylated type 1 lactosamine epitope Neu5Aco2-3LNT, recognized by the monoclonal antibodies K4 and DUPAN-2 (Retig et al. 1985; Fukuda et al. 1986; Andrews et al. 1996; Kamoshida et al. 2002; Fujita et al. 2006) and the fucosylated type 1 epitope sia1yl Lewis a [Neu5Aco2-3Galβ1-3(Fucx1-4)GlcNAc; CA19-9; Tsuruta et al. 1997; Fujita et al. 2006]. The lacto-series glycosphingolipids LNT and Neu5Aco2-3LNT have been detected in human embryonal carcinoma cells by the combination of MS, methylation analysis and glycosidase digestions (Fukuda et al. 1986). We have recently demonstrated the presence of lacto-series glycosphingolipids in hESCs by structural analysis employing tandem MS and glycosidase digestion, as well as the presence various type 1 lactosamine epitopes by immunochromatographic methods (Mikkola et al., in preparation). Thus, various type 1 antigens can be detected in the glycosphingolipids and O-glycosylated proteins of pluripotent human cells by both immunochromatographic and structural analysis. iPS cells express the Tra-1-60 and Tra-1-81 epitopes, but the glycosylation of iPS cells has not been studied as of yet. It will be interesting to see whether the expression of various type 1 antigens on glycosphingolipids and O-glycans is a characteristic feature of iPS cells as well.

The molecular identity of the targets for the widely used pluripotency marker antibodies Tra-1-60 and Tra-1-81 opens up new horizons into the glycostructure of pluripotent cells. The expression and biological function of Tra-1-60, Tra-1-81 and related type 1 lactosamine epitopes in different types of pluripotent and differentiated cells warrants further study. It remains to be elucidated whether the type 1 lactosamine antigens are functionally related to pluripotency, or whether their expression is a secondary phenomenon. In general, few biological functions have been assigned to type 1 lactosamine epitopes, possibly due to their lower abundance when compared with type 2 epitopes in widely studied systems such as immune cells. The biosynthetic switch from type 1 lactosamines to type 2 lactosamines on glycolipids and mucin-type O-glycans may play a role in the recognition and signaling events taking place during the differentiation process of pluripotent cells.

Structural information on embryonic stem cell glycosylation and on the epitopes recognized by marker antibodies will be useful in the development of novel pluripotency markers. Especially with the rapidly growing research in the field of induced pluripotency, there is a need to thoroughly understand the phenotype of a truly pluripotent cell. A number of markers are currently used to characterize embryonic stem cells and iPS cells. However, evidence is accumulating that these cells are heterogenous in morphology, phenotype and function and therefore need to be classified into subpopulations characterized by multiple sets of biomarkers (Enver et al. 2009). Biomarker discovery, as well as gathering structural and functional information on the biomarker molecules, is important for stem cell biology to find tools to classify and isolate pluripotent cells and to monitor their differentiation state by antibody-based techniques, as well as to elucidate the molecular mechanisms of pluripotency and differentiation.
Materials and methods

Cell lines and cell culture

Finnish hESC line FES 29 was cultured as described previously (Mikkola et al. 2006). The cells were cultured on mouse embryonic fibroblast feeder cells (mEFs) in a serum-free medium supplemented with Knockout serum replacement (Invitrogen, Carlsbad, CA) for O-glycan analyses, and on Matrigel (BD Biosciences, Franklin Lakes, NJ) with mEF conditioned medium for glycosidase treatments and flow cytometry. The hESC sample purity after collection from the feeder cell layer was at least over 90%, based on both flow cytometry analysis with stem cell marker antibodies and mass spectrometric N-glycan profiling analyses (Satomaa et al. 2009). In the present O-glycan analyses, mouse-derived Neu5Gc sialic acids were undetectable, suggesting that no major O-glycoprotein-specific contamination had occurred.

Monoclonal antibodies

Tra-1-81 and Tra-1-60 monoclonal antibodies were purchased from Millipore (Billerica, MA).

Glycosidase treatment of cells

hESCs were cultured on matrigel, trypsinized and Glycosidase treatment of cells from Millipore (Billerica, MA). Monoclonal antibodies major Neu5Gc sialic acids were undetectable, suggesting that no major O-glycoprotein-specific contamination had occurred.

Flow cytometry

The glycosidase-treated cells were stained with 25 µg/mL of the Tra-1-60 and Tra-1-81 antibodies followed by 15 µg/mL of Alexa488-labeled anti-mouse IgM (Jackson Immunoresearch, West Grove, PA). The cells were analyzed by FACSArria and FACSDiva™ Version 5.0.2 software (Becton Dickinson, Franklin Lakes, NJ).

Glycan array

The antibodies were tested against version 4.2 of the glycan array of the Consortium for Functional Glycomics (www.functionalglycomics.org) using the standard protocol. The antibodies were tested at 25 and 100 µg/mL and detected with Alexa488-labeled anti-mouse IgM.

O-Glycan analysis

Several million FES29 hESCs were grown on mouse embryonic feeder cell layers and subjected to N-glycosidase F digestion, acetone precipitation and methanol extraction to remove the majority of N-glycans as described before (Satomaa et al. 2009). O-Glycans were isolated with nonreductive β-elimination by incubation in ammonium carbonate in concentrated ammonia at +60°C for 2 days as described (Huang et al. 2001). The resulting glycans were purified by solid-phase extraction methods (Hemmoranta et al. 2007). MS and data analysis were performed as described previously (Hemmoranta et al. 2007; Heiskanen et al. 2009; Satomaa et al. 2009). The presence of residual N-glycans in the O-glycan fraction indicated that de-N-glycosylation had not been complete.

The endo-β-galactosidase (0.25 mU/µL; 1 U = 1 µmol/min, E. freundii, Seikagaku Corporation, Tokyo, Japan), β1,4-galactosidase (0.09 mU/µL; 1 U = 1 µmol/min, S. pneumoniae, Calbiochem/Merck) and β1,3-galactosidase (2.7 mU/µL; 1 U = nmol/h, X. manihotis, Calbiochem/Merck) reactions were carried out in 50 mM Na-acetate buffer pH 5.5 at +37°C for 20 h. After the incubation, the reactions mixtures were boiled for 3 min to stop the reactions. The substrate glycans were purified using chromatographic methods and analyzed with MALDI-TOF MS (Heiskanen et al. 2009). The glycosidase activities were controlled by using appropriate oligosaccharides as substrates as described in Supplementary data, Supplement SII.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest

The authors declare potentially competing interests: T.S. and J.N. are shareholders of Glykos Finland Ltd.

Abbreviations

Fuc, 1-fucose; Gal, β-galactose; GalNAc, N-acetyl-β-galactosamine; Glc, β-glucose; GlcNAc, N-acetyl-β-glucosamine; hESC, human embryonic stem cell; Hex, hexose; HexNAc, N-acetylhexosamine; iPSC, induced pluripotent stem; LNT, lacto-N-tetraose; MALDI-TOF, matrix-assisted laser desorption-ionization time-of-flight; mEF, mouse embryonic fibroblast feeder cell; MS, mass spectrometry; Neu5Ac, β-acetylneuraminic acid; SSEA, stage-specific embryonic antigen.

References


