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A validated collection of mouse monoclonal antibodies to human glycosyltransferases functioning in mucin-type O-glycosylation

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Abstract

Complex carbohydrates serve a wide range of biological functions in cells and tissues, and their biosynthesis involves more than 200 distinct glycosyltransferases (GTfs) in human cells. The kinetic properties, cellular expression patterns and subcellular topology of the GTfs direct the glycosylation capacity of a cell. Most GTfs are ER or Golgi resident enzymes, and their specific subcellular localization is believed to be distributed in the secretory pathway according to their sequential role in the glycosylation process, although detailed knowledge for individual enzymes is still highly fragmented. Progress in quantitative transcriptome and proteome analyses has greatly advanced our understanding of the cellular expression of this class of enzymes, but availability of appropriate antibodies for *in situ* monitoring of expression and subcellular topology have generally been limited. We have previously used catalytically active GTfs produced as recombinant truncated secreted proteins in insect cells for generation of mouse monoclonal antibodies (mAbs) to human enzymes primarily involved in mucin-type O-glycosylation. These mAbs can be used to probe subcellular topology of active GTfs in cells and tissues as well as their presence in body fluids. Here, we present several new mAbs to human GTfs and provide a summary of our entire collection of mAbs, available to the community. Moreover, we present validation of specificity for many of our

mAbs using human cell lines with CRISPR/Cas9 or zinc finger nuclease (ZFN) knockout and knockin of relevant GTfs.

Key words: CRISPR Cas9, GalNAc-transferase, immunocytology, immunohistology, western blot

Introduction

The enzymatic glycosylation of proteins and lipids play important roles in almost every biological event at the cell, tissue and organismal level (Varki 2017). Glycosylation is a nontemplate-driven process orchestrated by over 200 distinct glycosyltransferases (GTfs) in mammalian cells that each serve rather unique functions in the sequential biosynthesis of the enormously diverse array of glycans found on proteins and lipids (Hansen et al. 2015; Henrissat et al. 2015; Joshi et al. 2018; Narimatsu et al. 2018; Rini and Esko 2015). The kinetic properties and fine acceptor substrate specificities of expressed GTfs and their distinct subcellular localization are arguably the primary factors orchestrating the glycosylation process and the glycan diversity generated in a cell, although other factors such as availability of substrates and competition between enzymes, cofactors, as well as physiological parameters including pH also play important roles (Stanley 2011). A large number of the identified human (or mammalian) GTfs have been recombinantly expressed and their kinetic properties characterized in detail, and the properties of many others may be inferred from more indirect information. Thus, it is currently possible with a high degree of certainty to assign individual GTfs, or at least families of isoenzymes, to most steps in the biosynthesis of the human glycome (Hansen et al. 2015; Narimatsu et al. 2018). This opens for a more global view of glycosylation capacities and eventually prediction of the glycome of cells by analysis of the expressed repertoire of GTfs and their topology.

Global analysis of the expression levels of GTfs in cells and tissues by comprehensive gene arrays, specialized glycogene arrays including the ones developed by the Consortium for Glycomics (Comelli et al. 2002; Comelli et al. 2006), and more recently RNAseq transcriptome analyses (Human Protein Atlas, GTEx Portal and Expression Atlas EMBL-EBI) have markedly advanced our capabilities of assessing the entire repertoire of expressed glycogenes in cells. There is thus already considerable information available for the community for *in silico* probing of the repertoire of expressed glycogenes in cell lines as well as normal or diseased tissue and cells, and this information is growing exponentially with the decreasing costs of next-generation sequencing. However, the information is still not being widely used, presumably partly because of concerns with quality and reproducibility of data and partly due to concerns with translation of transcriptome data into protein expression and ultimately the glycome. Nevertheless, studies by Moremen and colleagues of transcriptome and glycome profiling of embryonic stem cells clearly demonstrate the potential (Nairn et al. 2012).

Global analysis of the expression of enzyme proteins is less straightforward. Quantitative proteomics by mass spectrometry is beginning to reach sensitivity levels where direct quantification of GTf proteins in cells and tissues may be available with some subcellular localization information (Smirle et al. 2013). However, in general most shotgun proteomics studies report identification of only few GTfs, and it is likely that targeted approaches for this class of proteins are needed. Thus, so far reports using proteomics to probe the global repertoire of GTfs have not appeared, but it is likely just a matter of time before correlative studies of the proteome and glycome will

appear. Currently though the most direct, sensitive and informative approach for probing expression of GTfs is by the use of antibodies and immunostaining. Fluorescence-based immunocytology (IC) and immunohistology (IH) are clearly the most suitable strategies for evaluation of expression and the fine subcellular localization of GTfs. Another fairly novel strategy with future potential is the emerging use of precise gene editing for visualization of endogenous proteins through introduction of, e.g., fluorescence tags in genes or intracellular single-chain antibodies that enable direct monitoring of endogenous expressed GTfs, but this will likely for some time be limited to individual enzymes and cell lines (Fetter et al. 2015; Ma et al. 2012). Thus, antibodies will for a long time be the preferred approach; however, despite community and commercial initiatives for proteome-wide development of antibodies, there is a great void in availability of validated antibodies to GTfs and, in particular, antibodies that have reliable performance in IC and IH required for studies of subcellular localization.

We have, over the past two decades, generated a number of monoclonal antibodies (mAbs) to GTfs using soluble secreted recombinant enzyme proteins as immunogens. While the generation and validation of specificities of the first developed mAbs were characterized in original publications (Almeida et al. 1999; Bennett et al. 1999; Bennett et al. 1998; Campos et al. 2015; Mandel et al. 1999; Marcos et al. 2011; Rottger et al. 1998; Schwientek et al. 2002; Steentoft et al. 2011; Sutherlin et al. 1997; Vallejo-Ruiz et al. 2001; White et al. 1990), a large number of our more recently developed mAbs have merely been presented as tools, without detailed description of the strategy for their generation and characterization. Moreover, a number of new mAbs have been developed, and new strategies for validation of specificities have been introduced by the opportunities provided with precise gene engineering (Schjoldager et al. 2015; Steentoft et al. 2014). Our current collection includes 43 mouse mAbs to human GTfs primarily acting in the mucin-type O-glycosylation pathway (Figure 1A, Table I and Supplementary data, Table SI). In this report, we provide a summary of these mAbs, selected to ensure performance in a variety of immunoassays.

Results

Generation of mAbs to GTfs

Most GTfs are type II transmembrane proteins with a short cytosolic tail (10–20 amino acids), a hydrophobic alpha-helix transmembrane domain (15–20 amino acids) and a stalk region of different length (20–500 amino acids), often heavily O-glycosylated, that functions as structural spacer for a compact-folded catalytic domain (200–250 amino acids) (Figure 1B). Unique for the polypeptide N-acetylgalactosamine (GalNAc)-transferases (GalNAc-Ts) initiating GalNAc-type O-glycosylation is an additional C-terminal lectin domain (approximately 120 amino acids). Recombinant production of soluble forms of GTfs has been used with great success for many enzymes, albeit not all, by truncating the coding construct to the catalytic domain (Moremen et al. 2018; Paulson and Colley 1989). We have with success used catalytically active recombinant GTfs

Fig. 1. Antibodies to GTfs (Table I) and cancer associated O-Glycans (Table II). **(A)** Graphic rainbow depiction of O-glycosylation pathways that antibodies (annotated by green or orange quartered circle) in the current mAb collection cover. **(B)** Schematic illustration of GTfs. The secreted functionally active GTfs containing the folded catalytic domain and the lectin domain if present were expressed in insect cells, purified and used as immunogens. Monosaccharide symbols according to the “Symbol Nomenclature for Glycans” system (Varki et al. 2015).

expressed in insect or mammalian (CHO) cells as immunogens, to generate mAbs to native enzymes by the traditional hybridoma technology (Mandel et al. 1999; Vester-Christensen et al. 2013).

Recently, a set of guidelines to validate antibody specificity and reproducibility were introduced (Uhlen et al. 2016), where expression of the target protein is recommended to be evaluated by one or more of the following methods: i) genome editing or RNA interference; ii) antibody-independent methods; iii) antibodies with nonoverlapping epitopes; iv) expression of tagged proteins; and v) immunocapture followed by mass spectrometry. This suggested five-pillar validation was proposed to ensure an application-specific validation of individual mAbs, and as a minimum one of the pillars was recommended for each antibody. We adhere to these strategies; however, a key factor for production of valuable antibodies is also to screen with assays related to the intended use of the mAbs to be generated (Baker 2015).

In our workflow for mAb production the primary screening is performed by immunofluorescence on acetone-fixed insect cells infected with baculovirus constructs expressing the soluble, secreted constructs (in some cases the full coding construct) of the GTf used for immunization (Supplementary data, Table SII). By harvesting infected cells early, i.e., 2–2.5 days after infection, sufficient enzyme protein is retained in cells, and as it turns out, this enables detection of antibodies that bind active folded enzyme as well as antibodies that only detect the denatured enzyme

(Mandel et al. 1999). The specificity of selected mAbs is tested by screening a large panel of related GTf isoforms expressed in insect cells, but in fact we have only identified one example of a cross-reactive mAb raised to GalNAc-T13 (1F3) that also reacted with the highly homologous isoenzyme GalNAc-T1 (Supplementary data, Table SII).

Further validation of the specificity is achieved by IC with mammalian cell lines with and without known expression of the GTf used as immunogen and finally by IH. IH data for 15 of the mAbs were already published, and 8 of the new mAbs were similarly found to react in tissues by juxta-nuclear staining suggestive of Golgi-localization (Table I) (not shown). A substantial proportion of the selected mAbs fail to bind the endogenously expressed enzymes by IC, but when tested by western blot (WB) analysis with purified recombinant enzymes and/or total cell lysates of mammalian cells or insect cells expressing the relevant enzyme many of these mAbs do bind the target proteins in a denatured form (Almeida et al. 1999; Rottger et al. 1998; Sutherlin et al. 1997). As a result, we have most often generated two different classes of mAbs for use in different assays, recognizing either the native or denatured GTf (Bennett et al. 1999; Marcos et al. 2011) as is illustrated in Figure 2 by our new antibodies to GalNAc-T12 and -T14. Thus 13 of our mAbs bound the native enzymes by IC and did not bind the denatured enzymes by WB, i.e., UH3 4D8 (GalNAc-T1), UH4 4C4 and 2E10 (GalNAc-T2), UH5 2D10 (GalNAc-T3), 4G2 (GalNAc-T4), 5F11 (GalNAc-T5),

Table 1. Summary of the current collection of mAbs to human GTfs

Gene name	Protein	Immunogen ^a	Antibody ^b	Validation ^d					Reference ^f	
				Sf9 ^c	IP	IC	IH	WB		KO/KI ^e
<i>GALNT1</i>	GalNAc-T1	519/41–559	UH3 4D8	+	+	+	+	–	● *	Mandel et al. (1999)
<i>GALNT2</i>	GalNAc-T2	520/52–571	UH4 4C4 2E10	+	+	+	+	–	● *	Mandel et al. (1999)
			UH2 6B7	+	ND	–	–	+	ND	Schjoldager et al. (2012)
<i>GALNT3</i>	GalNAc-T3	582/52–633	UH5 2D10	+	+	+	+	–	● ^	Rottger et al. (1998)
			UH1 2F6	+	–	–	ND	+	ND	Mandel et al. (1999)
<i>GALNT4</i>	GalNAc-T4	547/32–578	UH6 4G2	+	+	+	+	–	◦	Sutherland et al. (1997)
			5B3	+	ND	–	ND	+	ND	Bennett et al. (1998)
<i>GALNT5</i>	GalNAc-T5	592/349–940	5F11	+	ND	+	+	–	#	This report
			1C5	+	ND	–	ND	+	ND	Campos et al. (2015)
<i>GALNT6</i>	GalNAc-T6	570/53–622	UH7 2F3	+	+	–	+	–	^	This report
			2E11	+	–	–	ND	+	ND	Bennett et al. (1999)
<i>GALNT7</i>	GalNAc-T7	620/38–657	8B8	+	ND	+	+	ND	●	Bennett et al. (1999)
<i>GALNT10</i>	GalNAc-T10	542/62–603	6D5	+	ND	+	ND	ND	ND	Steenfot et al. (2019)
			5G7	+	ND	–	ND	ND	ND	Norden et al. (2015)
<i>GALNT11</i>	GalNAc-T11	578/31–608	UH8 1B2	+	ND	+	+	ND	●	This report
<i>GALNT12</i>	GalNAc-T12	Full protein	1F9	+	ND	+	+	–	ND	Schwientek et al. (2002)
		549/33–581	1F5	+	ND	–	ND	+	ND	Norden et al. (2015)
<i>GALNT13</i>	GalNAc-T13	517/40–556	1F3 ^g	+	ND	+	ND	+	ND	This report
			1D4 ^g	+	ND	–	ND	+	ND	This report
<i>GALNT14</i>	GalNAc-T14	525/28–552	3D2	+	ND	+	+	–	ND	This report
			1F6	+	ND	–	ND	+	ND	Laursen et al. (2018)
<i>GALNT16</i>	GalNAc-T16	514/45–558	4C6	+	ND	+	ND	ND	●	This report
			3C2	+	ND	–	ND	+	ND	Laursen et al. (2018)
<i>C1GALT1</i>	C1GalT1	319/45–363	5F8	ND	ND	+	+	ND	◦	Steenfot et al. (2011)
			5B6	ND	ND	+	+	ND	◦	Steenfot et al. (2011)
			2G8	ND	ND	+	+	ND	◦	Steenfot et al. (2013b)
<i>B3GNT6</i>	β 3GnT6	357/27–384	5F2	+	ND	ND	ND	ND	◦	This report
<i>B4GALT1</i>	β 4GalT1	355/44–398	2F5	+	ND	+	+	+	● ◇	Hoffmeister et al. (2003)
<i>B4GALT5</i>	β 4GalT5	336/53–388	6D4	+	ND	+	+	ND	●	This report
			1E10	+	ND	–	ND	+	ND	This report
			5E8	+	ND	+	+	ND	◦	This report
<i>B3GALT5</i>	β 3GalT5	286/25–310	6F8	+	ND	–	ND	+	ND	Castro et al. (2012)

Continued

(Continued)

Table I. Continued

Gene name	Protein	Immunogen ^a	Antibody ^b	Validation ^d				Reference ^f
				Sf9 ^c	IP	IC	IH	
<i>ST3GAL1</i>	ST3Gal-1	315/26-340	4B10	+	+	+	+	Vallejo-Ruiz et al. (2001)
<i>ST6GALNAC1</i>	ST6GalNAc-1	307/294-600	2C3	+	ND	+	+	Marcos et al. (2011)
			1C9	+	ND	–	ND	Marcos et al. (2011)
<i>B4GALT7</i>	β 4GalT7	265/63–327	URH2 3G6	+	ND	+	+	This report
			URH1 2C3	+	ND	–	ND	Almeida et al. (1999)
<i>POMGNT1</i>	POMGnT1	595/44-638	5C6	+	ND	+	ND	This report
			1G4	+	ND	–	ND	This report
ABO	ABenzyme	Full protein	WKH1	ND	+	+	+	White et al. (1990)
ABO	ABenzyme	Full protein	WKH2	ND	+	+	+	White et al. (1990)
ABO	ABenzyme	Full protein	WKH3	ND	+	+	+	White et al. (1990)

^aThe size and the position in the GTf (Uniprot isoform-1) is shown for the recombinant-expressed immunogen.

^bThe current collection of mAbs is produced as described in the Materials and methods and is isotype IgG1 except mAbs to ST6GalNAc-1 (2C3) and POMGnT1 (5C6), which are isotype IgG2a.

^cmAbs selected and specificity analyzed by IC on Sf9 cells infected with baculovirus expressing the target enzyme and one to three other closest isoforms as negative control.

^dmAbs with (+) for IC (acetone-fixed cell lines) and IH (on frozen sections) staining show distinct perinuclear punctuate staining in cell lines and tissues suggestive of Golgi localization, (–) no reaction. ND, not determined.

^eKO/KI presented in this report in HEK293 (●) (paraformaldehyde-fixed, permeabilized cells), expression in CHO (○) or KO/KI in other reports in HepG2 (*, Schjoldager et al. 2015), LST174T (*, Lavrsen et al. 2018), CHO-GS (◇, Schulz et al. 2018) or K562 (#, Campos et al. 2015). ND, not determined

^fThe first publication where the mAb has been developed and/or applied. A list of all remaining publications is presented in Supplementary data, Table S1.

^gmAbs to GalNAc-T13 shows cross reactivity to GalNAc-T1.

Fig. 2. Figure illustrating that the presented mAbs in general react either with the native conformation of the enzyme by IC or with denatured enzyme by WB. **(A)** Immunofluorescence staining on acetone-fixed HT-29 cancer cells and **(B)** WB analysis with purified recombinant enzymes. mAbs 1F9 to GalNAc-T12 and 3D2 to GalNAc-T14 showed Golgi-like staining in IC (positive reaction is green in opposite to yellow autofluorescence from the cells) **(A)** and no bands by WB **(B)**, not even at longer exposure time (not shown). In contrast, mAbs 1F5 to GalNAc-T12 and 1F6 to GalNAc-T14 showed antigen-specific labeling in WB (approximately M_r 60 kDa) and did not label cells.

UH7 2F3 (GalNAc-T6), 1F9 (GalNAc-T12), 3D2 (GalNAc-T14), 2C3 (ST6GalNAc-I) and WKH1-3 (ABenzyme). Quite oppositely 14 of our mAbs bound the denatured enzyme antigen in WB and did not bind the native enzyme by IC, i.e., UH2 6B7 (GalNAc-T2), UH1 2F6 (GalNAc-T3), 5B3 (GalNAc-T4), 1C5 (GalNAc-T5), 2E11 (GalNAc-T6), 1F5 (GalNAc-T12), 1D4 (GalNAc-T13), 1F6 (GalNAc-T14), 3C2 (GalNAc-T16), 1E10 (β 4GalT5), 6F8 (β 3GalT5), 1C9 (ST6GalNAc-1), URH1 2C3 (β 4GalT7) and 1G4 (POMGnT1) (Table I).

Importantly, among mAbs showing IC reactivity, only clones with a Golgi-like staining pattern of cell lines were found to be specific (Figures 2 and 3). Except for a few examples where we detect reactivity in both WB and IC (2F5 to β 4GalT1 (Hoffmeister et al. 2003; Schulz et al. 2018), 1F3 to GalNAc-T13/T1 and 4B10 to ST3Gal-1 (Vallejo-Ruiz et al. 2001)) the majority of our mAbs were assay specific, suggesting that in general caution should be taken

when using mAbs and in particular polyclonal antibodies (pAbs) reactive in both assays.

In agreement with the suggestions of Uhlen et al. (2016), we have used both selective immunoprecipitation (IP) of active enzymes from total cell extracts (Bennett et al. 1998, 1999; Mandel et al. 1999; White et al. 1990) as well as more recently gene knock-outs (KO) or zinc finger nuclease (ZFN) knock-ins (KI), in cells to confirm specificities (Campos et al. 2015; Lavrsen et al. 2018; Narimatsu et al. 2018; Schjoldager et al. 2015). We have generated a large library of HEK293 and CHO cell lines with targeted KO/KI of GTf genes (Narimatsu et al. 2018, 2019a, 2019b; Yang et al. 2015b), and here we tested our mAb collection on a library of HEK293 isogenic cell lines (Figure 3, Table I). MAbs to GalNAc-T1 (UH3 4D8), GalNAc-T2 (UH4 4C4), GalNAc-T3 (UH5 2D10), GalNAc-T7 (8B8), GalNAc-T11 (UH8 1B2), GalNAc-T16 (4C6) (not shown), β 4GalT1 (2F5), β 4GalT5 (6D4), β 4GalT7 (URH2 3G6) and POMGnT1 (5C6) all produced Golgi-like staining in WT HEK293 cells, whereas staining was completely abolished after KO of the relevant GTf gene. Conversely, for mAbs not reactive with HEK293 WT cells, such as mAb 2C3 generated to ST6GalNAc-1, we used KI of *ST6GALNAC1* to demonstrate induction of a clear Golgi-like staining. Antibodies to five GTfs were validated by expression in CHO cells and all confirmed specific induction of Golgi-like staining (GalNAc-T4 (4G2) (Bennett et al. 1998), C1GalT1 (5F8/5B6/2G8), β 3GnT6 (5F2), β 3GalT5 (5E8) and ST3Gal-1 (4B10)) (Table I).

We also correlated IC reactivity on a panel of 28 human cell lines with predicted expression patterns based on RNAseq transcriptome analyses as an orthogonal validation method (Supplementary data, Figure S1 and Tables SIII and SIV). Correlation of IC reactivity and mRNA expression (classified as no, low, medium and high) was 90–100% for six GTf genes (*GALNT1*, *GALNT2*, *GALNT7*, *C1GALT1*, *B4GALT1* and *B4GALT7*), 60–90% for 14 GTf genes (*GALNT3*, *GALNT4*, *GALNT5*, *GALNT6*, *GALNT11*, *GALNT12*, *GALNT13*, *GALNT14*, *GALNT16*, *B3GNT6*, *B3GALT5*, *ST3GAL1*, *ST6GALNAC1* and *POMGNT1*) and less than 60% for 2 GTf genes (*GALNT10* and *B4GALT5*). Poor correlations were associated with low expression levels, but other factors such as clonal variations and growth conditions of cells may contribute as well.

Collection of mAbs for the GalNAc-type O-glycosylation pathway

The current mAb collection includes most of the polypeptide GalNAc-Ts that initiate O-glycosylation, as well as the core1 synthase (C1GalT1) and the α 2,3sialyltransferase (ST3Gal-I) functioning in the most common core1 pathway (Figure 1A, Table I). The collection also includes mAbs to the α 2,6sialyltransferase (ST6GalNAc-I) responsible for the cancer-associated truncated STn glycosylation (Marcos et al. 2011), and mAbs to the core3 synthase (β 3GnT6) and to the core3 extension enzyme (β 3GalT5) are also available. IC-applicable mAbs to GalNAc-Ts (1F3 to GalNAc-T1/13) and β 3GalTs (5E8 to β 3GalT5) have not been reported before, and a longer list of WB-applicable mAbs have also not been reported (Table I). Finally, our collection also contains six mAbs to the cancer-associated O-glycan antigens including Tn (GalNAc α 1-O-Ser/Thr), STn (NeuAc α 2-6GalNAc α 1-O-Ser/Thr) and T (Gal β 1-3GalNAc α 1-O-Ser/Thr) (Table II), which we have further validated with a combination of neuraminidase treatment and KO studies (Steentoft et al. 2011, 2013a, 2013b).

Fig. 3. Immunofluorescence staining of GTfs using mAbs of the current selection in WT paraformaldehyde fixed and permeabilized HEK293 and CRISPR/Cas9 KO or KI of the respective GTf antigen. mAbs were validated by loss of staining in the KO in relation to positive staining (green) in the appropriate WT HEK293 for (A) GalNAc-transferases (GalNAc-T1 (UH3 4D8), -T2 (UH4 4C4), -T3 (UH5 2D10), -T7 (8B8), -T11 (UH8 1B2)) and (B) β 4GalT1 (2F5), β 4GalT5 (6D4), β 4GalT7 (3G6) and POMGnT1 (5C6). (C) mAb to ST6GalNAc-1 (2C3) was validated by KI in HEK293 WT. Blue color shows DAPI nuclear staining.

Table II. mAbs defining cancer-associated O-glycans

Antibody	Isotype	Immunogen ^a	Specificity	Applications ^b	Reference
5F4	IgM	aOSM	Tn-antigen (GalNAc α 1-O-Ser/Thr)	IC, IH, WB	Steentoft et al. (2011)
IE3	IgG2a	aOSM	Tn-antigen (GalNAc α 1-O-Ser/Thr)	IC, IH, WB	Takahashi et al. (1988)
3F1	IgG1	OSM	STn-antigen (NeuAc α 2-6GalNAc α 1-O-Ser/Thr)	IC, IH, WB	Steentoft et al. (2011)
TKH2	IgG1	OSM	STn-antigen (NeuAc α 2-6GalNAc α 1-O-Ser/Thr)	IC, IH, WB	Kjeldsen et al. (1988)
3C9	IgM	Galactosyl-A-glycolipid	T-antigen (Gal β 1-3GalNAc α 1-O-Ser/Thr)	IC, IH, WB	Bohm et al. (1997)
HH8	IgM	Galactosyl-A-glycolipid	T-antigen (Gal β 1-3GalNAc α 1-O-Ser/Thr)	IC, IH, WB	Clausen et al. (1988)

^aaOSM, asialo ovine submaxillary mucin; OSM, ovine submaxillary mucin.

^bIH on frozen or paraffin embedded tissue sections.

Collection of mAbs to essential GTfs in other biosynthetic pathways

Our collection also includes unreported mAbs to the early extension enzyme of the O-Man (POMT-directed) core M1 glycosylation pathway (5C6 and 1G4 to β 2GlcNAc-T POMGnT1), the second enzyme

in assembly of the proteoglycan linker region (3G6 to β 4GalT7) and the most dominant assembly of glycosphingolipids (6D4 and 1E10 to β 4GalT5) (Table I). Finally, mAbs to the dominant N-acetyl-Lactosamine synthase (2F5 to β 4GalT1) and the human blood group A/B transferase (WKH1-3 to α 3Gal(NAc)-T) are available.

Discussion

Antibodies are among the most frequently used tools in basic and biomedical research as well as in clinical tests and other applications. Usage of these important reagents is most often combined with interpretations of presence or absence of the corresponding antigen, and it is obvious that specificity, assay design and quality are key parameters for meaningful results. There are numerous potential pitfalls with Abs, and community efforts to improve and standardize characterization and use of these reagents have recently been proposed (Edfors et al. 2018; Hewitt et al. 2014; Uhlen et al. 2016). We have generated a comprehensive set of mAbs for use in probing the complex biosynthesis of mucin-type O-glycosylation and the truncated O-glycans characteristically found in cancers, and here we provided a summary of these reagents with added validation studies in compliance with the recent proposed guidelines. An important lesson from these studies is that most mAbs to GTfs selectively recognize the native folded enzyme protein or the denatured protein and only rarely recognize both. This has implications for generation of mAbs to GTfs as well as for use of existing mAbs and likely also pAbs.

Several of the first antibodies to human GTfs suffered from specificity issues and often recognized glycans associated with the immunizing protein preparation (Berger et al. 1986; Childs et al. 1986; Feizi and Childs 1987; Feizi et al. 1987; White et al. 1990). The latter part is of particular concern when data from different experiments using pAbs were correlated. With increasing abilities to purify GTf enzymes to homogeneity and the advent of recombinant production of GTfs, specific pAbs (affinity purified) and mAbs have emerged (Iwai et al. 2005; Mandel et al. 1999; Taatjes et al. 1988; White et al. 1990). Academic and commercial efforts for high-throughput production of pAbs and/or mAbs to all human proteins are quite advanced, and today one can, in general, find a source for antibodies to any human protein including most known GTfs (Thul et al. 2017). These initiatives invariably take advantage of existing capabilities for high-throughput synthesis of peptides and/or recombinant peptides/fragments of proteins expressed in *Escherichia coli* for eliciting immunity in animals (Lindskog et al. 2005; Nilsson et al. 2005). These immunogens obviously do not present the correctly folded GTfs, and this strategy for producing Abs may not be generally applicable to the class of GTf enzymes, and care with specificity must be taken as discussed here. This can be illustrated by many of the pAbs produced to GTfs by The Human Protein Atlas consortium (Thul et al. 2017; Uhlen et al. 2010) using *E. coli*-produced protein fragments (<https://www.proteinatlas.org/>). For example, in the GalNAc-T family of isoenzymes only pAbs to three human GalNAc-T isoforms (GalNAc-T2, -T3 and -T4) were indicated to produce Golgi-like staining in human cells, while pAbs to other GalNAc-T isoforms (GalNAc-T5, -T6, -T7, -T8, -T9, -T10, -T14 and -T16) were shown to exhibit staining patterns not restricted to the Golgi or with completely different staining patterns without apparent Golgi reactivity (Thul et al. 2017). In contrast, we have only observed Golgi-like staining patterns in cells with all the mAbs in the collection presented here to the GalNAc-T isoenzymes (Figure 3, Table I, Supplementary data, Table SI). We therefore suggest that it is likely that most of the pAbs to GalNAc-Ts developed by the Human Protein Atlas consortium, despite the specificity analyses performed, do not reliably identify these GalNAc-Ts in cells and tissues by the applied IC/IH methods. Use of such antibodies may have consequences and provide conflicting results, as recently illustrated by an interesting study suggesting localization of the GalNAc-T3 isoenzyme in the nucleus of HeLa cells (Cejas et al.

2019). Although additional data to support the presence of enzyme activity and O-glycoproteins in nucleocytoplasmic preparations were presented in this study, we note that the mAb to GalNAc-T3 included in our collection has only produced Golgi-like staining in cells including in HeLa (Rottger et al. 1998). Moreover, extensive sensitive O-glycoproteomics studies performed with the SimpleCell strategy has also largely negated the presence of GalNAc glycosylation of nucleocytoplasmic proteins (Campos et al. 2015; Steentoft et al. 2011, 2013b). Currently, there are only few commercial mAbs to GTfs available comparable to the collection presented here (antibodypedia.com) (Björling and Uhlen 2008), and only few of these have to our knowledge been reported with IC or IH data.

Antibodies directed to denatured proteins are likely to react with linear continuous peptide epitopes representable by short synthetic peptides and/or recombinant peptide fragments, and they most often show reactivity in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) WB analysis, where correct estimated molecular weight of reactive bands provides assurance. A number of common immunoassays are available for analyzing specificities for such antibodies including peptide and protein arrays (Nilsson et al. 2005; Uhlen et al. 2005); although, it is important to consider if proteins are folded or not in protein arrays, for it is a potential error that can be misleading in the validation of the antibody specificity. When using antibodies directed to denatured proteins it is also important to consider how the antigen-retrieval strategies with fixed sections affect the interpretation of the antibody specificity and reactivity pattern.

Antibodies exclusively reactive with folded active enzymes present different obstacles in evaluating specificities. For example, the use of protein arrays may be misleading if proteins are not correctly folded. In our hands IP of active enzymes from total cell extracts is one of the early and good measures of specificity (Bennett et al. 1999; Bennett et al. 1998; Berger et al. 1986; Mandel et al. 1999; White et al. 1990), and although convincing results can be obtained, this approach requires considerable efforts. With the introduction of precise gene-editing technologies, it is now possible and simple to generate isogenic cell lines with and without any given GTf and conclusively establish binding specificities (Figure 3, Table I).

A limitation with our collection of mAbs that can be used for IC/IH applications is that the majority of these are sensitive to fixation, and most do not tolerate the routine paraformaldehyde fixation with paraffin embedding used for histopathological archives. Attempts to use different antigen-retrieval methods have also failed. We have found that most of these mAbs tolerate fixation in ice-cold acetone and paraformaldehyde but not methanol (Vester-Christensen et al. 2013). Only a few mAbs to GTfs have been shown to give Golgi-like staining in formalin-fixed paraffin-embedded sections, including mAbs to the core3 synthase (β 3GnT6) (Iwai et al. 2005) and mAbs to GalNAc-T14 and FUT3/6 (Stern et al. 2010). In the latter study, denatured enzymes were used as immunogens, and this strategy may be applicable to other enzymes as well.

In conclusion, the presented collection of mAbs represents a comprehensive collection of mAbs to GTfs involved in mucin-type O-glycosylation. The mAbs are extensively validated and available to the community.

Materials and methods

mAb generation

mAbs generation has been extensively described by Vester-Christensen et al. (2013). In short recombinant, secreted, soluble, active

human enzymes produced in insect cells were purified by simple purification methods suitable for mg batches and used as immunogen. Balb/c mice were immunized three times with a single subcutaneous injection of 10 µg of undenatured purified enzyme in Freund's adjuvant and finally an intravenous boost without adjuvant.

Test bleeds were taken 7 days after the third immunization, and the titer and specificity of antibodies evaluated. Spleen cells were fused to NS-1 myeloma cells, and the cloning procedure was performed as described previously (Mandel et al. 1999; White et al. 1990). Hybridomas were selected by IC on air-dried, acetone-fixed Sf9 cells infected with various full-coding or secreted GTf baculovirus constructs as previously described (Vester-Christensen et al. 2013). In some cases, selection of hybridomas was confirmed by ability to differentially IP active recombinant enzymes (GalNAc-T1, -T2, -T3, -T4, -T6, ST3Gal-1 and ABenzyme) (Bennett et al. 1998, 1999; Mandel et al. 1999; Vallejo-Ruiz et al. 2001; White et al. 1990). Further characterization of the hybridoma clones was done on human cell lines expressing full-length coding enzymes (Steentoft et al. 2011). For IH mAbs (undiluted hybridoma supernatants) were added overnight at 4°C to fresh frozen sections either unfixed or fixed in acetone or formaldehyde and detected with rabbit anti-mouse-Ig-FITC (Dako, Denmark) or goat anti-mouse IgG γ chain specific, (1030-02, SouthernBiotech, AL) (Mandel et al. 1999).

Hybridomas are kept at -150°C and can be revived for repeated antibody production. Antibodies can be stored at 4°C and usually kept for years. Most antibodies tolerate freezing, but generally repeated freezing cycles are not recommended. Our antibodies can be supplied in reasonable amounts together with cell lines with and without enzyme targets for validation.

Multiplex GTf-gene targeting (KI/KO)

GTf KO in HEK293 cells were obtained by CRISPR/Cas9 using validated gRNA encoding plasmids (Narimatsu et al. 2018) co-transfected with Cas9-PBKS plasmid (Addgene, MA, USA). Cells were harvested after 1 d, and a fraction sorted on GFP expression by FACS. After 1 week of culture the FACS-sorted cell pool was single sorted into 96-well plates and screened by Indel Detection by Amplicon Analysis as previously described (Lonowski et al. 2017; Yang et al. 2015a).

For ST6GalNAc-I KI we used a modified ObLiGaRe-targeted KI strategy with two inverted ZFN binding sites flanking the ST6GalNAc-I full open reading frame in the donor plasmid as previously described (Narimatsu et al. 2019b; Pinto et al. 2017; Steentoft et al. 2019). KI clones were screened by the IC staining with anti STn (3F1) antibody, and the targeted integration into the AAVS1 site was confirmed with 5' and 3' junction polymerase chain reaction.

IC

HEK293 cells were grown on sterile cover slides for 1 d, fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and blocked with 3% bovine serum albumin. Human cancer cells were trypsinized and seeded on slides, air-dried at room temperature and fixed for 5-10 min in ice-cold acetone. Slides were incubated with mAbs (undiluted hybridoma supernatants) to human GTfs overnight followed by rabbit anti-mouse Ig FITC-conjugated antibodies (Dako) for 1 h at RT and mounted with Vectashield with DAPI (Vector Labs, CA, USA) as described previously (Steentoft et al. 2011). Fluorescence

microscopy was performed using a Zeiss Axioskop 2 plus with an AxioCam MR3.

WB

WB analysis with purified recombinant enzymes were also performed as previously described (Almeida et al. 1999; Bennett et al. 1999; Sutherlin et al. 1997). In short, approximately 8 µg of recombinant enzymes was after denaturation loaded into a NuPAGE Bis-Tris 4-12% gel (Invitrogen, CA, USA) and transferred onto a nitrocellulose membrane. The membrane was blocked in 5% skim milk incubated with undiluted hybridoma supernatant at 4°C overnight, washed and incubated with the secondary layer at room temperature for 1 h (rabbit anti-mouse Ig-HRP (Dako, Denmark, 1:4000 dilution) and developed using the Thermo Scientific Pierce ECL Western Blotting Substrate kit.

Transcriptome analysis

The RNAseq data were extracted from public sources (EMBL Expression Atlas, The Human Protein Atlas and GEO NCBI) (Supplementary data, Table SIV). RNAseq was not available for three cell lines; 18 data sets were from the Expression Atlas, Genentech (Klijn et al. 2015), 4 from Expression Atlas, Cell Lines CCL (Barretina et al. 2012), 2 from The Human Protein Atlas (Uhlen et al. 2015) and 1 from NCBI (Papadimou et al. 2015). The RNAseq data were subdivided into four expression levels presented in transcripts per million (TPM); TPM < 1, no expression; TPM between 1 and 20, low expression; TPM between 20 and 50, medium expression and TPM > 50, high expression (Supplementary data, Table SIII).

Supplementary data

Supplementary data is available at *Glycobiology* online.

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Conflict of interest statement

None declared.

Abbreviations

GalNAc, N-acetylgalactosamine; GTf, glycosyltransferase; IC, immunocytology; IH, immunohistology; IP, immunoprecipitation; KI, knockin; KO, knockout; mAb, monoclonal antibody; pAb, polyclonal antibody; TPM, transcripts per million; WB, Western blot; ZFN, zinc finger nuclease

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