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Multiple Site-Specific *in vitro* Labeling of Single-Chain Antibody

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Abstract

For multiple site-specific conjugations of bioactive molecules to a single-chain antibody (scFv) molecule, we have constructed a human anti HER2 receptor, scFv, with a C-terminal fusion polypeptide containing 1, 3, or 17 threonine (Thr) residues. The C-terminal extended fusion polypeptides of these recombinant scFv fusion proteins are used as the acceptor substrate for human polypeptide- α -N-acetylgalactosaminyltransferase II (h-ppGalNAc-T2) that transfers either GalNAc or 2-keto-Gal, a modified galactose with a chemical handle, from their respective UDP-sugars to the side-chain hydroxyl group of the Thr residue(s). The recombinant scFv fusion proteins are expressed in *E. coli* as inclusion bodies and *in vitro* refolded and glycosylated with h-ppGalNAc-T2. Upon protease cleavage, the MALDI-TOF spectra of the glycosylated C-terminal fusion polypeptides showed that the glycosylated scFv fusion protein with a single Thr residue is fully glycosylated with a single 2-keto-Gal, whereas the glycosylated scFv fusion protein with 3 and 17 Thr residues is found as an equal mixture of 2–3 and 5–8 2-keto-Gal glycosylated fusion proteins, respectively. These fusion scFv proteins with the modified galactose are then conjugated with a fluorescence probe, Alexa488, that carries an orthogonal reactive group. The fluorescence labeled scFv proteins bind specifically to a human breast cancer cell line (SK-BR-3) that overexpresses the HER2 receptor, indicating that the *in vitro* folded scFv fusion proteins are biologically active and the presence of conjugated multiple Alexa488 probes in their C-terminal end does not interfere with their binding to the antigen.

INTRODUCTION

Conjugation of monoclonal antibodies with bioactive molecules such as toxins, drugs, and radioisotopes is an emerging strategy in the treatment and diagnosis of cancer (1–5). Not only is the conjugation of a multiple number of bioactive molecules to a single antibody molecule desired, but also, site-specific conjugation of these molecules is important. The former enhances the sensitivity of detection and better treatment of cancer, while the latter enables the production of a homogeneous antibody–drug complex that ensures the full activity of both the antibody and the bioactive molecule in an antibody–drug conjugate. Multiple site-specific conjugation has been achieved by engineering the target protein, either by the introduction of free Cys residues (6, 7) or by introducing genetically encoded aldehydes at either terminal end of the protein or by site-directed introduction of azido/

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alkynyl-tagged methionine analogues into proteins (8–10). However, protein with free Cys residue(s) needs special handling to prevent its free Cys residues from undergoing undesired oxidation while in the process of introducing the methionine analogue all the methionine residues in the protein are modified. Not many site-specific conjugation methods offer multiple site conjugations.

We have developed a unique, site-specific conjugation method using the mutant galactosyltransferase enzymes, where we first enzymatically transfer 2-acetonil-2-deoxy-galactose (2-keto-Gal) sugar with a unique chemical handle to a specific sugar moiety, β GlcNAc or β Gal1-4GlcNAc, present at the nonreducing end of the glycan of the glycoprotein; then, the chemical handle present in the modified sugar is used for site-specific conjugation with biologically important molecules having a corresponding orthogonal chemical group (11–14). Using this method, we have recently shown that the biantennary *N*-glycans of a therapeutic IgG molecule have been used as the substrate for the mutant Y298L-Gal-T1 enzyme to transfer 2-keto-Gal sugar and further conjugated with bioactive molecules such as biotin to both arms of the biantennary *N*-glycans, thus producing the native IgG molecule with four biotin molecules site-specifically conjugated (12, 14). However, this method requires the presence of a specific sugar moiety of a glycan chain in the glycoprotein and cannot be used for nonglycosylated proteins, such as single-chain antibodies or bacterial toxins expressed in *E. coli*.

For proteins that lack glycosylation motifs, we have recently developed a method using the human polypeptide- α -*N*-acetyl-galactosaminyltransferase II enzyme (h-ppGalNAc-T2), which transfers *N*-acetylgalactosamine sugar (GalNAc) from UDP-GalNAc to Thr/Ser residues on an acceptor polypeptide that is at least 11 amino acids long (15, 16). In this method, we have engineered the acceptor polypeptide substrate of the h-ppGalNAc-T2 with one Thr residue as a fusion peptide at the C-terminus of a bacterial glutathione-*S*-transferase (GST), which is not normally glycosylated. We used this fusion polypeptide moiety as an acceptor substrate for the h-ppGalNAc-T2 to transfer modified Gal with chemical handle such as 2-keto-Gal or GalNAz, thus enabling us to site-specifically conjugate bioactive molecules with a corresponding orthogonal chemical group (15). Using this method, in the present study we demonstrate that an anti human HER2 receptor single-chain antibody (anti HER2 scFv) with a fusion peptide containing more than one Thr residue at its C-terminal end can be successfully glycosylated with 2-keto-Gal and then conjugated with Alexa488, a fluorescence probe. Furthermore, our cell surface immunostaining analysis of HER2-receptor expressing SK-BR-3 cells (by fluorescent-activated cell sorting [FACS]) shows that antibody binding to the HER2 receptor is not affected by the one or more Alexa488 molecules present at the C-terminal end of these scFv molecules.

EXPERIMENTAL PROCEDURES

Anti HER2 Single-Chain Antibody Gene Construction and Expression in *E. coli*

The antihuman HER2 scFv gene was constructed from a fully human Fab isolated from a large native phage display Fab library, using HER2 ectodomain as a target (Zhu, Z., and Dmitrov, D., unpublished data). For the construction of H1 and H3 scFv proteins, the anti HER2 scFv gene was repeatedly amplified with the 5-primer, 3-primer-1, and 3-primer2, which introduces a 17-amino-acid peptide as a C-terminal extension following a Tev protease cleavage site. A final PCR amplification with either 3-H(1) or 3-H(3) primer results in a product with a C-terminal O-glycosylation tag without a stop codon, having *Eco*RI and *Xho* I restriction enzyme sites at their 5' and 3' termini, respectively. This construction uses the stop codon at the 3' terminal in the vector after the His-tag.

5-primer: GCC CCG *GAA TTC* GGG CGC GGC GAA GTG CAG CTG GTG CAG
TCT GGA GCA GAG GTG AAA AAG.

3-Primer 1: GCC TTG GAA GTA AAG GTT TTC TAG GAC GGT GAC CTT GGT
CCC AGA TCC GAA GAC AGC AAT.

3-Primer 2: TTT AGC TGC CGG TGC GGG AGT AGC TGC AGC GCC TTG GAA
GTA AAG GTT TTC.

3-H1 Primer: GTG GTG GTG *CTC GAG* TTT AGC TGC CGG TGC GGG ACT AGC
TGC AGG GCC.

3-H3 Primer: GTG GTG GTG *CTC GAG* TTT AGC TGC CGG TGT GGG AGT AGG
TGT AGG GCC.

The PCR-amplified product was digested with *Eco*RI and *Xho* I restriction enzymes and ligated with the pET23a vector digested with the same enzymes. The ligated vector was transfected into XL2 supercompetent cells. The ampicillin-resistant clones were screened for the presence of the anti HER2 scFv gene in their plasmid DNA between their *Eco*RI and *Xho* I restriction sites. The N-terminal T7-tag, which enhances the protein expression in *E. coli* and the C-terminal His × 6 tag, followed by a stop codon, comes from the pET23a vector DNA. For the construction and expression of H17 scFv protein, the cDNA for the scFv protein was first cloned into the pET23a vector between the *Eco*RI and *Hind* III restricted enzyme sites without the stop codon. This cloning was followed by inserting between the *Hind* III and *Xho* I site a cDNA sequence coding for a Tev protease cleavage site and the 50 amino-acid-long polypeptide of the O-glycosylation region of the human small breast epithelial mucin protein (BC111421) (17) without the stop codon. This construction uses the stop codon at the 3' terminal in the vector after the His-tag, similar to H1 and H3 scFv gene constructs. The positive clones were sequenced and transfected into Rosetta (DE3) LysS competence cells for expression of the protein.

In vitro* Folding of Inclusion Bodies of Anti-HER2 scFv Protein from *E. coli

The Rosetta (DE3) LysS cells containing the anti HER2 scFv cDNA sequence in the pET23a vector were grown to an optical density of 0.7–0.8 and then induced with IPTG. The inclusion bodies were purified from the bacterial pellet as described earlier (18). From 1 L of bacterial culture, 60 to 70 mg of protein were obtained as inclusion bodies. The *in vitro* folding of anti HER2 scFv was carried out in a way similar to that of β 4Gal-T1 (18). Typically, 100 mg of sulfonated protein was folded for 48 h in 1 L of folding solution that contains oxido-shuffling agents and 500 mM arginine HCl. After refolding the protein, the folding solution was extensively dialyzed against water. During dialysis, the misfolded protein precipitated out, while the folded protein remained soluble. The soluble protein was concentrated to a concentration of 1 mg/mL on an Amicon stirred cell, using a YM-10 membrane, and further purified on a Ni-column. There was no significant loss of protein observed during concentration or during Ni-column purification steps, as estimated by measuring the protein concentration using the Bradford method (BioRad). Nearly 20 mg of folded anti HER2 scFv protein was obtained from 1 L of folding solution. The C-terminal glycosylation peptide tag was released from a 5 μ g protein by Tev protease cleavage and analyzed by mass spectrometry.

Glycosylation and Alexa488 Conjugation of Anti HER2 scFv

The cloning, expression, and refolding of the h-ppGal-NAc-T2 enzyme has been previously published (15). UDP-GalNAc was purchased from Sigma chemicals while the UDP-2-keto-Gal was synthesized in-house. Forty micrograms of anti HER2 scFv fusion proteins—H1, H3, or H17—were incubated overnight at room temperature 20 °C with 20 μ g of h-

ppGalNAc-T2 in the presence of 25 mM Tris-HCl (pH 8.0), 10 mM MnCl₂, and 0.5 mM UDP-sugar (UDP-GalNAc, or UDP-2-keto-Gal,) in a total volume of 100 μ L (Figure 2). The amount of h-ppGalNAc-T2 enzyme used in these experiments to fully glycosylate the fusion peptide using UDP-GalNAc as the donor substrate was first determined in separate experiments. To remove the entire reaction buffer, the glycosylated anti HER2 scFv protein was precipitated with 40% ammonium sulfate and desalted three times by repeated dilution to 500 μ L with water and concentrated each time to 40 μ L using microcon centrifuge filters with 10K molecular weight cutoff. The salt-free glycosylated anti HER2 scFv protein was reconstituted in 40 μ L water, and an aliquot (2 μ L) was cut with Tev protease and analyzed by mass spectrometry (MALDI-TOF).

The entire 40 μ g of the desalted 2-keto-Gal glycosylated anti HER2 ScFv protein was conjugated in 60 μ L with 75 mM sodium acetate buffer (pH 5.0) and 8 μ L *N*-aminoxymethyl-carbonylhydrazino-Alexa488 (1 mg/mL in DMSO). Nearly 1000-fold excess of Alexa-488 molecules, more than the manufacturer (Invitrogen) suggested amount, was used in this conjugation reaction. The reaction was carried out in the dark, overnight, at room temperature. The conjugated protein was precipitated with 40% ammonium sulfate, redissolved in water to a 1 μ g/ μ L concentration and used for cell binding. The presence of Alexa488 was confirmed by visualizing as little as 10 ng of protein in SDS-PAGE. The mass spectroscopic analyses were carried out in the Protein Chemistry Laboratory, NCI-Frederick, Frederick, MD.

Cell Binding Assays

The human cancer cell lines SK-BR-3 (HER2 receptor-positive) and MDA-MB-468 (HER2 receptor-negative) were used to test receptor binding activity of the scFv fusion proteins. HER2 receptor expressing human breast adenocarcinoma cells (SKBR-3) and HER2 receptor negative human breast adenocarcinoma cells (MDA-MB-468) were purchased from the American type Culture Collection (ATCC, Manassas, VA). The cells were cultured in McCoy's 5A medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin as antibiotics at 37 °C in a humidified atmosphere containing 5% CO₂. Other culture reagents were bought from Invitrogen (Carlsbad, CA). In a typical experiment, cells were suspended in enzyme-free cell dissociation buffer (Invitrogen Corp, Carlsbad, CA) and pelleted. The cells were resuspended in phosphate buffer saline (PBS) supplemented with 5% fetal calf serum (PBS-FCS) at a concentration of 10⁷ cells per mL. The samples were incubated at room temperature for 15 min to block nonspecific binding sites. The cells were washed twice with cold PBS with 1% bovine serum albumin (PBS-BSA), resuspended in PBS-FCS (10⁷ cells/ml), and aliquoted in 0.1 mL samples. Various amounts (0.2, 0.5, and 1 μ g) of Alexa-conjugated scFv were added to the cells, and incubations were continued for 45 min on a rotator at 4 °C in the dark. The cells were washed twice with cold PBS-BSA, suspended in PBS-BSA, and analyzed using a FACS caliber flow cytometer (Becton Dickinson, San Jose, CA), analyzing 10 000 events per sample. Herceptin was used as a positive control in these experiments to confirm HER2 expression on SK-BR-3 cells (HER2-positive) and MDA-MB-468 cells (HER2 receptor-negative).

RESULTS AND DISCUSSION

We have previously developed a novel site-specific glyco-conjugation of glutathion-S-transferase, by engineering a C-terminal fusion peptide containing one Thr residue that can be glycosylated by h-ppGalNAc-T2 with a modified sugar having a chemical handle (15). This site-specific glycan moiety can then be conjugated with bioactive molecules carrying an orthogonal chemical group. Here, we have extended this strategy to engineer, not just one glycosylation site at its C-terminal end, but multiple sites on an antihuman HER2 receptor

single-chain antibody (anti HER2 scFv) (Figure 1a). The anti HER2 scFv fusion protein is designed to have a N-terminal T7-tag, which enhances its protein expression in *E. coli* and at the C-terminal end a Tev protease cleavage site, an O-glycosylation peptide followed by a His $\times$ 6 tag (Figure 1b). For the present study, three forms of anti HER2 scFv proteins have been constructed: the H1, H3, and H17, with C-terminal extensions having 1, 3, and 17 Thr residues in their O-glycosylation tag, respectively (Figure 1b). The two C-terminal extension peptides, H1 and H3, were designed based on the preferred acceptor peptide sequence for h-ppGalNAc-T2 enzyme, as described earlier (15,16).

Expression of the Anti Her2 scFv Proteins in *E. coli* and Their *in vitro* Folding

Although many scFv proteins have been expressed as soluble active proteins in *E. coli*, the anti HER2 scFv proteins, H1, H3, and H17, mostly formed inclusion bodies; and only less than 100 μ g of soluble active protein could be obtained from 1 L of bacterial culture. Previously in our laboratory, we have used the *in vitro* S-sulfonation and refolding method to produce large quantities of active β -1,4-galactosyl-transferase (β 4Gal-T1) enzyme from inclusion bodies (18). Using the same method, a large quantity of a soluble form of anti HER2 scFv proteins H1, H3, and H17, with C-terminal extension, has been obtained. The scFv fusion protein has four Cys residues and has two disulfide bonds. The *in vitro* folded protein, nonreduced and reduced with β -mercaptanol, shows in SDS-PAGE a significant mobility difference between the protein bands confirming the presence of disulfide bond(s) in the soluble protein. The yield of the *in vitro* folded protein for H1, H3, and H17 proteins corresponds to 10, 10, and 2.5 mg per liter of bacterial culture. Thus, it seems that the presence of the 17 amino acid C-terminal tag does not interfere with the folding of scFv molecules. Nevertheless, the 50 amino-acid-long tag significantly reduces the folding efficiency. All the refolded proteins can be concentrated to at least 10 to 15 mg/mL concentrations, without any precipitation.

Glycosylation of H1, H3, and H17 anti-HER2 scFv Proteins

As a first step, the H1, H3, and H17 scFv fusion proteins were glycosylated overnight with h-ppGalNAc-T2, using UDP-GalNAc as the donor sugar substrate. The C-terminal fusion polypeptide from these proteins was released by Tev protease digestion and analyzed by mass spectrometry (MALDI-TOF) (Figure 3). The MS analysis of the glycosylated peptide tags from H1 and H3 proteins showed that they carry 1 and 3 GalNAc sugars, respectively (Figure 3a,b), suggesting that the H1 protein has been fully and homogeneously glycosylated while the H3 proteins is mostly glycosylated with three GalNAc sugars. However, the polypeptide released from H17 scFv protein by Tev protease was glycosylated with a maximum of 11 sugars instead of 17 sugars (Figure 3c). The catalytic reaction using UDP- α -2-keto-Gal as donor substrate was carried out under the same conditions as the one used for UDP-GalNAc. After the glycosylation, a small amount of the glycoprotein was subjected to the Tev digestion, and the released C-terminal fusion glycopeptides was analyzed by MALDI-TOF analysis. The glycopeptide from the H1 protein was found fully glycosylated with a single 2-keto-Gal sugar, whereas the glycopeptide from H3 and H17 was found to be a mixture of peptides with 2 to 3 and 5 to 8 2-keto-Gal sugars, respectively (Figure 3d,e,f). These results show that all the Thr residues present in the O-glycosylation tag of the H3 and H17 scFv proteins are not homogeneously glycosylated by the h-ppGalNAc-T2, particularly with the modified sugar.

In humans, the h-ppGalNAc-T2 enzyme exists as a family of 17 enzymes, namely, ppGalNAc-T1 to ppGalNAc-T17 (19). It is well-known that h-ppGalNAc-T2 efficiently glycosylates nonconsecutive Thr residues, while ppGalNAc-T10 uses glycopeptide as the substrate and efficiently glycosylates the consecutive Thr residues if either one is glycosylated (16, 20). The O-glycosylation tag of the H17 fusion protein has 10

nonconsecutive Thr residues. Thus, it is not surprising to observe that its tag is glycosylated by h-ppGalNAc-T2 with only 11 GalNAc sugars, and to fully glycosylate this tag, it may be essential to use the ppGalNAc-T10 enzyme, in addition to h-ppGalNAc-T2. Even though the 2-keto moiety in the 2-keto-Gal resembles the N-acetyl moiety in GalNAc, due to its conformational flexibility, the 2-keto-Gal sugar may not be as preferred a donor sugar substrate as is GalNAc by the h-ppGalNAc-T2 enzyme; therefore, the catalytic efficiency of h-ppGalNAc-T2 is expected to be slightly poorer for the 2-keto-Gal transfer than for GalNAc.

Preparation of Alexa488-Anti-Her2ScFv Conjugates and Their Binding to HER2-Receptor-Expressing Cells

To demonstrate a site-specific conjugation, the H1, H3, and H17 scFv proteins with 2-keto-Gal sugars were conjugated with a fluorescence probe, Alexa488, with an orthogonal chemical group, the aminoxy group. The Alexa488-conjugated H1, H3, and H17 scFv proteins can be detected on an SDS-PAGE by their fluorescence emission, even at nanogram amounts (Figure 4a). Interestingly, the H3 and H17 scFv proteins that carry more than one Alexa488 molecule are observed as sharp protein bands in the SDS-PAGE. This is in contrast to limited but random conjugated proteins, where the conjugated proteins are often observed as a smeared broad band in SDS-PAGE. For example, the streptavidin-Alexa488 conjugate protein from Invitrogen is observed as a broad band in SDS-PAGE (Figure 4b). Thus, observation of a sharp protein band on the gel indicates conjugation of the fluorophores to unique site(s) on the protein molecules. The observation that the fluorescence emission from H17 protein was not significantly enhanced, compared to the H1 scFv protein, may be due to self-quenching of conjugated Alexa488 molecules. It is known that multiple fluorescence probes conjugated to protein molecules show self-quenching. It is interesting to observe that even though the H3 and H17 protein-Alexa488 conjugates are heterogeneous, arising from their glycosylation with 2-keto-Gal, the protein bands in the SDP-PAGE gel remain sharp. Although the actual number of Alexa-488 molecules that are conjugated to the 2-Keto-galactosylated scFv molecules could not be determined by conventional MALDI-TOF analysis, in our previous studies it was shown that when, instead of Alexa488, biotin molecule was used for conjugation under similar reaction condition, all the 2-keto-Gal sugar molecules were completely biotinylated as determined by MALDI-TOF analysis (14, 15). Since we have used nearly 1000-fold excess of Alexa488 molecules in the conjugation reaction, all the 2-keto-Gal sugar molecules are expected to be conjugated with Alexa488 molecules.

Cell-binding assay by FACS methodology analysis shows that Alexa488-conjugated scFv fusion proteins H1, H3, and H17 bind specifically to cells that express HER2 receptors on the cell surface (Figure 5). We did not observe any binding of these modified scFv molecules above background levels when MDA-MB-468 cells (HER2 receptor-negative) were used (Figure 5b). In these experiments, FACS spectra using various amount of scFv fusion proteins ranging from 200 ng to 2.5 μ g showed no significant difference, suggesting that even a nanogram quantity of the H1, H3, or H17 protein was sufficient for the detection of HER2 receptor. Although there is enhanced fluorescence emission (FL1-H) with H3 and H17, compared to H1, it is not linearly proportional to the number of additional fluorescence probes conjugated to these proteins (Figure 5a, insert table). This kind of observation for the multiple conjugations of protein probes to proteins is known due to the internal quenching. However, our technology is likely to yield similar conjugates with multiple numbers of MRI image contrast agents (on a single protein molecule), since there is no quenching effect in MRI. The present results suggest that the *in vitro* folded scFv is biologically active and the incorporation of the sugar and the Alexa488 moieties at the C-terminal end had no or little effect on their affinity for the HER2 receptor, thus demonstrating that even a large fusion

protein with many conjugated fluorescence probes does not affect the affinity of the scFv to its antigen.

CONCLUSION

Our current method offers a unique possibility not only for site-specific conjugation but also for multiple site conjugations of bioactive molecules at the C-terminal end of the scFv molecule through modified sugars. The proteolysis of the fusion peptide is an important factor for *in vivo* studies. The presence of glycosylation is known to hinder the proteolysis of peptides, and furthermore, the modified sugars are conjugated with bulky bioactive molecules; thus, their susceptibility to proteolysis is expected to be much more limited compared to that of nonglycosylated fusion peptide. Similarly immunogenicity due to the fusion peptide and the modified sugars may be concern. However, it is possible that the fusion peptide may not be well exposed to the environment due to the presence of bulky conjugated bioactive molecules that may reduce the *in vivo* immunogenic effect. Such concerns are associated with most conjugated molecules that are being used or planned for use in *in vivo* studies.

Previously, scFv protein with large protein-like green fluorescence protein fused at its C-terminal end has been found to bind to the antigen without compromising its affinity (21). Also, a large mucin protein like, muc6, has been expressed as a soluble protein in *E. coli* and has been *in vitro* glycosylated with more than 50 sugars with GalNAc, using ppGalNAc-T1 (22). Therefore, using our present site-specific and multiple site conjugating method, scFv proteins with a C-terminal muc6 fusion protein can be glycosylated with modified sugars and conjugated with bioactive molecules. Such complexes are expected to carry not just a few but several tens of bioactive molecules conjugated to scFv molecules. The methodology described here can generate site-specific and multiple site-conjugated antibody-bioactive molecules that are in great need for the development of targeted MRI image contrast agents and a targeted drug delivery system.

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Figure 1.

(a) Schematic molecular structure of the Alexa488-conjugated scFv fusion protein. In the present study a single-chain antibody against human HER2 receptor (anti HER2 scFv) with x number of Alexa488 molecules conjugated through the modified sugar is named H x . The anti HER2 scFv protein has been designed to have specific 1 to 17 O-glycosylation sites. (b) Schematic diagram of the design of anti HER2 scFv proteins with multiple glycosylation sites. The antihuman HER2 receptor single-chain antibody (anti HER2 scFv) expressed in *E. coli* contains a N-terminal T7-tag, a C-terminal Tev protease-cleavable O-glycosylation tag, and an His tag. The proteins with 1, 3, and 17 Thr residues in their O-glycosylation tags (shown as stars at their bottom) are named H1, H3, and H17, respectively. The Tev protease cleavage site is indicated by an arrow. In the presence of Mn^{2+} , the polypeptide- α -*N*-acetylgalactosaminyltransferase II (h-ppGalNAc-T2) enzyme transfers GalNAc or 2-keto-Gal sugar from their UDP derivatives to the side-chain hydroxyl group of the Thr residue present in the O-glycosylation tag.

Figure 2.

SDS-PAGE gel analysis of the unglycosylated (H1– and H17–) and 2-keto-Gal glycosylated (H1+, H3+, and H17+) proteins: The unglycosylated H1 and H17 proteins are labeled as H1– and H17–, respectively, while the 2-keto-Gal glycosylated H1, H3, and H17 are labeled as H1+, H3+, and H17+, respectively. The h-ppGalNAc-T2 in the glycosylation mixture is also labeled. MALDI-TOF analysis of the H1+, H3+, and H17+ anti HER2 scFv proteins show that these proteins carry 2-keto-Gal (see below). The large difference in the mobility between unglycosylated and glycosylated H17 protein is due to glycosylation, however the microheterogeneity in its glycosylation is not observed (see below). Similarly the mobility difference between the H1 unglycosylated protein and the H1+ and H3+ glycosylated protein may be due to their glycosylation.

Figure 3.

The mass spectrum (MALDI-TOF) of the Tev protease cleaved glycosylated polypeptides with GalNAc (a,b,c) and 2-keto-Gal (d,e,f) from H1, H3, and H17 anti HER2 scFv fusion proteins, respectively. The number of sugar molecules present in the peptide is shown in the parentheses next to the mass value of the corresponding peptide peak. The H1 protein is fully glycosylated with GalNAc sugar (a) while a small trace amount of unglycosylated peptide is observed when 2-keto-Gal was used (d). Although all three Thr residues in the fusion peptide from H3 have been mostly glycosylated with GalNAc, a small trace amount of mono- and diglycosylated forms are observed (b). However, under the same glycosylation condition, a mixture of fusion peptides with two and three 2-keto-Gal has been observed (e)

for the H3 protein. Similarly, the H17 fusion protein is glycosylated to a maximum of 11 Thr residues with GalNAc sugar (c), compared to eight with 2-keto-Gal (f).

Figure 4.

(a) Fluorescence emission of the Alexa488 conjugated H1, H3, and H17 anti HER2 scFv fusion proteins from the nonreduced SDS-PAGE. Lanes 1–3, 3–5, and 7–9 show each 10 ng, 20 ng, and 40 ng of H1, H3, and a mixture of H3 and H17 proteins, respectively. The H17 protein has 5 to 8 conjugated molecules of Alexa488. The fluorescence emission image of the SDS-PAGE gel was scanned using Hitachi FMBIO II fluorescence imaging system. Even 10 ng fusion proteins can be very well detected, and the protein bands are sharp. (b) Fluorescence emission from various amounts of streptavidin-Alexa488 conjugate on nonreduced SDS-PAGE. The protein conjugate from Invitrogen was prepared by a random but limited conjugation method.

Figure 5.

(a) FACS analysis on the binding of Alexa488 conjugated H1, H3, and H17 fusion proteins to SKBR3 cells. 200 ng of the each conjugated protein was used in the assay. (b) Binding of H1, H3, and H17 proteins did not occur with MDAMB-468 cells, since these cells do not express HER2 receptors. The insert table lists the mean FL1-H value for cells without and with bound antibody conjugates. Although the H17 conjugates have nearly seven more fluorescence probes, compared to H1, due to self-quenching there was no linear increase in the fluorescence emission.