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Mechanistic understanding of the cysteine capping modifications of antibodies enables selective chemical engineering in live mammalian cells

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ABSTRACT

Protein modifications by intricate cellular machineries often redesign the structure and function of existing proteins to impact biological networks. Disulfide bond formation between cysteine (Cys) pairs is one of the most common modifications found in extracellularly-destined proteins, key to maintaining protein structure. Unpaired surface cysteines on secreted mammalian proteins are also frequently found disulfide-bonded with free Cys or glutathione (GSH) in circulation or culture, the mechanism for which remains unknown. Here we report that these so-called Cys-capping modifications take place outside mammalian cells, not in the endoplasmic reticulum (ER) where oxidoreductase-mediated protein disulfide formation occurs. Unpaired surface cysteines of extracellularly-arrived proteins such as antibodies are uncapped upon secretion before undergoing disulfide exchange with cystine or oxidized GSH in culture medium. This observation has led to a feasible way to selectively modify the nucleophilic thiol side-chain of cell-surface or extracellular proteins in live mammalian cells, by applying electrophiles with a chemical handle directly into culture medium. These findings provide potentially an effective approach for improving therapeutic conjugates and probing biological systems.

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1. Introduction

Covalently attaching diverse functional groups to polypeptide chains following ribosomal translation dramatically enhances the diversity of protein structure and function (Krall et al., 2016; Spicer and Davis, 2014; Walsh et al., 2005). Naturally-occurring protein modifications confer proteins with chemical functionalities that are not provided by the 19 standard amino acids (plus selenocysteine for a few selenoproteins) and one imino acid. Installing small modifications synthetically and selectively on biological species in native environments represents both an important tool and a challenging task for interrogating diverse cellular processes (Chin et al., 2003; Elliott et al., 2014; Krall et al., 2016; Laughlin et al., 2008; Mahal et al., 1997; Rhee et al., 2013; Saxon and Bertozzi, 2000; Spicer and Davis, 2014; van Kasteren et al., 2007; Wang et al., 2001), owing to the complexity of living systems and limitations of current methods for protein modification. With a robust nucleophilic side chain and low abundance in protein sequences (Marino and Gladyshev, 2010, 2012; Poole, 2015), the α-amino acid Cys has been exploited extensively in nature and chemistry for protein modification, catalytic, cofactor-binding, and regulatory functions. The ability of sulfur to alter its oxidation state is utilized for disulfide bond formation between Cys pairs. Introducing Cys residues into proteins has long been a strategy for protein engineering. While Cys pair engineering has been utilized to introduce disulfide bonds to stabilize proteins or for redox sensing (Dooley et al., 2004; Gale and Pellequer, 2003; Hanson et al., 2004; Ostergaard et al., 2001;

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Perry and Wetzel, 1984; Sauer et al., 1986; Villafranca et al., 1983; Wells and Powers, 1986), unpaired surface Cys residues have been engineered into antibody for site-specific labeling and drug conjugation (Junutula et al., 2008a,b; Lyons et al., 1990; Shen et al., 2012; Stimmel et al., 2000; Voynov et al., 2010).

Engineering unpaired surface Cys into antibodies renders intrinsic complexity when these Cys-mutant proteins are produced in mammalian cells. The thiol group of these Cys residues has been found disulfide-bonded with Cys (cysteinylation) or glutathione (GSH, glutathionylation) (Chen et al., 2009; Junutula et al., 2008b). These so-called Cys-capping modifications are also detected in naturally-existing solvent-exposed unpaired Cys residues of extracellular proteins such as antibodies and serum albumin in culture or plasma (Banks et al., 2008; Buchanan et al., 2013; Gadgil et al., 2006; Nagumo et al., 2014; Peters and Davidson, 1982). The biological function of these post-translational modifications is unclear and possible roles in Redox sensing have been implied (Banerjee, 2012; Gallogly and Mieyal, 2007), but their presence has created a technical obstacle for conjugation processes. The reactive thiol group needs to be regenerated through a tedious *in vitro* reduction and re-oxidation manipulation (Junutula et al., 2008b), which therefore prohibits direct protein labeling in biological environments. In addition, this *in vitro* treatment involves reducing and then reoxidizing the antibody inter-chain disulfides, which frequently introduces disulfide shuffling (also called disulfide scrambling). This scrambling can adversely affect protein folding and quality, and also cause issues such as poor pharmacokinetics in the resulting therapeutic conjugates.

It has been speculated that the Cys-capping modification may take place in the ER lumen (Banks et al., 2008; Buchanan et al., 2013; Gadgil et al., 2006), where disulfide bond formation occurs (Herrmann and Riemer, 2014; Hudson et al., 2015; Ruddock, 2012; Sevier and Kaiser, 2006). The ER lumen is known to be more oxidizing than the cytosol according to the early data on the thiol-disulfide state of a small glycopeptide (Hwang et al., 1992) as well as a recent data with glutathione-specific redox-sensitive green fluorescent protein (Birk et al., 2013), due to the oxidation Ero1 pathways (Frand et al., 2000; Frand and Kaiser, 1998; Margittai and Banhegyi, 2010; Pollard et al., 1998; Sevier, 2010; Sevier and Kaiser, 2006; Tu et al., 2000; Tu and Weissman, 2004) and several redundant backup systems, including peroxiredoxin 4, vitamin K oxidoreductase, glutathione peroxidases 7&8, and oxidized glutathione (Bulleid and Ellgaard, 2011; Herrmann and Riemer, 2014). GSH or Cys are present in the ER lumen owning to either a transporter (Banhegyi et al., 1999; Hwang et al., 1992) or pores (Le Gall et al., 2004) in the membrane, making the ER lumen a reasonable place for Cys-capping modification. Nonetheless, no conclusive evidence in support of this hypothesis has been reported.

In this study we unexpectedly found that a significant portion of an engineered unpaired Cys residue in an antibody's CH3 domain was uncapped as a free thiol when expressed in human embryonic kidney (HEK293) cells. Adding excess GSH or cystine (Ctn) into the culture medium generated fully glutathionylated or cysteinylated species. Interestingly, complete removal of Cys, Ctn, and GSH from the culture medium of HEK293 or Chinese Hamster Ovary (CHO) cells produced homogeneously uncapped Cys mutant antibody. In addition, we found that the Cys mutant antibody can be homogeneously capped with small thiol reactive reagents, such as thionitrobenzoate (TNB) when Ellman's reagent (Ellman, 1959), dithionitrobenzoate (DTNB), is added to the medium during culturing. These data together have demonstrated that the source of native glutathionylation and cysteinylation modifications is likely from culture medium, and that these modifications likely take place outside of the cells, not in the ER lumen. These findings also allowed us to develop a straight forward process for the generation of novel Cys-capping modifications of Cys-mutant antibodies and cell-surface membrane proteins. By adding electrophiles such as maleimide PEG_2-biotin (MPB) directly into culture medium, we were able to selectively label the antibodies at the introduced cysteines during expression and avoided the need for an uncapping step prior to conjugation. These findings provide an effective and improved approach for generating therapeutic conjugates.

2. Materials and methods

2.1. Cell culture, transfections, and cell line development

Mammalian cell lines were grown and maintained in a humidified incubator with 5% or 7% CO_2 at 37 °C. HEK293F cells were cultured in FreeStyle^{TM} 293 medium or EXP293^{TM} medium (ThermoFisher, Waltham, MA). A large-scale transient HEK293 transfection process as described previously (Zhong et al., 2013) was used for antibody production. CHO-DUKX cells were grown in alpha medium supplemented with adenosine (10 mg/L), deoxyadenosine (10 mg/L), and thymidine (10 mg/L). The CHO-DUKX cells were stably transfected with DNAs encoding a Cys mutant recombinant antibody protein HAB08 and subjected to selection with 100 nM methotrexate and 1 mg/mL G418, as described (Zhong et al., 2012). The stable pools were allowed to undergo selection for 3 weeks and then seeded at 2×10^5 cells/mL into serum-free suspension at 37 °C. Stable CHO-DUKX cells were maintained in alpha medium supplemented with 100 nM methotrexate and 1 mg/mL G418. During production, cells were seeded in a normal CHO medium AS1 and conditioned media was harvested at the end of production and cleared by centrifugation prior to purification. The Triple-free media is a proprietary chemical defined media containing insulin, amino acids (without cysteine, or cystine, or glutathione), vitamins, Inorganic salts, glucose, fatty acids, minerals and trace elements as well as a shear protectant.

2.2. Protein purification

rmpProtein A resin (GE Healthcare, Piscataway, NJ) was pre-equilibrated with 50 mM Tris, 150 mM NaCl, pH 7.5 (TBS) overnight at 4 °C. The resin was filtered using a 0.2 PES filter and packed into a column where it was washed with 2 column volumes (CV) of TBS, 5CVs of $CaCl_2$, pH 7.5, 3CVs of 10 mM Tris, 10 mM NaCl, pH 7.5 before the protein was eluted using 100% step of 150 mM Glycine, 40 mM NaCl, pH 3.5. The protein was titrated to pH 3.5 using 2 M Glycine, pH 2.7 before adjusting the pH to 7.0 using 2 M HEPES, pH 8.0. The protein was dialyzed into PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 2.7 mM KH_2PO_4, pH 7.2) before being concentrated and loaded onto a Superdex 200 column equilibrated with PBS, pH 7.2. Peak fractions were pooled dialyzed into 20 mM Histidine, 8.5% Sucrose, pH 5.8, and then concentrated to 10 mg/mL using a 50 kDa MWCO centrifugal device.

2.3. Liquid chromatography mass spectrometry

Liquid chromatography mass spectrometry analysis was performed using a Waters Xevo Q-TOF G2 mass spectrometer (Waters, Milford, MA) coupled to an Agilent (Santa Clara, CA) 1200 capillary HPLC. For deglycosylating antibody, protein samples were treated with PNGase F (NE BioLabs, Ipswich, MA) at room temperature for 2 h. Protein samples were acidified by diluting 1:1 with 0.05% TFA (Sigma-Aldrich, St Louis, MO), followed by liquid chromatography mass spectrometry analysis. The samples were separated over a Waters BEH300 C4, 1.7 μm (1.0 × 50 mm) column maintained at 80 °C with a flow rate of 65 μl/min. Mobile phase A was water with 0.05% TFA, and mobile phase B was acetonitrile with 0.05% TFA. Proteins were eluted from the column using a gradient: 2%–20% B

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