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Post-translational Regulation of Expression and Conformation of an Immunoglobulin Domain in Yeast Surface Display

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Abstract
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Post-translational Regulation of Expression and Conformation of an Immunoglobulin Domain in Yeast Surface Display

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Running Title: Yeast Surface Display of CD47

ABSTRACT

Display of heterologous proteins on the surface of *Saccharomyces cerevisiae* is increasingly being exploited for directed evolution because of straightforward cell screens. However, yeast post-translationally modifies proteins in ways that must be factored into library engineering and refinement. Here, we express the extracellular immunoglobulin domain of an ubiquitous mammalian membrane protein, CD47, which is implicated in cancer, immunocompatibility, and motility. CD47 has multiple sites of glycosylation and a core disulfide bond. We assess the effects of both of these post-translational modifications on expression and antibody binding. CD47's extracellular domain is fused to the yeast mating protein Aga2p on the cell wall, and the resulting fusion protein binds several key antibodies, including a conformation-sensitive antibody. Site-by-site mutagenesis of CD47's five N-linked glycosylation sites progressively decreases expression levels on yeast, but folding appears stable. Cysteine mutations disrupt the expected core disulfide, and also decrease protein expression levels, though not to the extent seen with complete deglycosylation. However, with the core disulfide mutants, antibody binding proves to be lower than expression levels might indicate and glycosylation is clearly reduced compared to wild-type. The results indicate that glycosylation regulates heterologous display on yeast more than core disulfides do and thus suggest bounds on directed evolution by post-translational processing. (201 words)

Keywords*:* yeast display, glycosylation, disulfide, immunoglobulin, CD47

INTRODUCTION

Yeast display of recombinant proteins is an increasingly popular technique for the study of structure-function relationships (Boder and Wittrup, 2000). A large part of this appeal is the potential for rapid screening of a library using techniques such as flow cytometry or magnetically assisted cell sorting (Yeung and Wittrup, 2002; Siegel *et al*., 2004; Feldhaus and Siegel, 2004). However, a number of questions arise with the yeast display of mammalian proteins. The extent and type of glycosylation, for example, is one particularly well-recognized difference between these single cell eukaryotes and higher organisms (Gerngross, 2004) and recognition of the problems that variant glycosylation can pose prompted a humanization of the glycoprocessing in *Pichia pastoris* (Hamilton *et al*., 2003). In the context of directed evolution of mammalian proteins displayed on *Saccharomyces cerevisae,* it is especially important to understand post-translational bounds to functional expression.

One of the largest families of cell surface proteins is the immunoglobulin (Ig) superfamily, whose members share an Ig-fold and several other structural features. They tend to be N-glycosylated. Beyond protecting partially-folded proteins from aggregation, N-glycosylation in cell surface proteins has been implicated in providing protection against hydrolysis, ligand channeling to increase affinity, scaffold formation for an ensemble of interacting proteins or even providing a handle whereby a receptor may discriminate ligand from non-ligand (Rudd *et al*., 1999; 2004). At least one disulfide bond is found in the vast majority of the Ig domains of these proteins, and, based on many available structures, the disulfide is invariably buried in the core near the center of the structure. Ig domains also occur in a large number of intracellular proteins (eg. titin, filamin), but these intracellular Ig proteins lack the core disulfides. Disulfides are therefore not needed in general for folding of an Ig domain.

Whether S-S bond formation and N-linked glycosylation are coupled is an interesting, if difficult, question. Some hints are available in the literature: glycosylation was found to assist folding of the β-subunit of human chorionic gonadotrophin in CHO cells by enabling disulfide bond formation (Feng *et al*., 1995)**.** The adenosine receptor is N-glycosylated in mammalian systems and has a disulfide bond, but, when expressed in yeast, the human adenosine receptor was not N-glycosylated, although it did contain the disulfide bond and was functional (Butz et al, 2003). The mouse substance P receptor was not N-glycosylated either, but it was retained intracellularly and was found to lack a disulfide bond (Niebauer *et al*., 2004). It must be emphasized that the disulfide bond in this case was not a core disulfide, as it is in the many Ig domains.

CD47 or Integrin Associated Protein (IAP) is a prototypical membrane glycoprotein that is ubiquitous in multicellular eukaryotes (Brown and Frazier, 2001) and possesses a single, extracellular Ig domain. Some of the earliest reports showed CD47 to be over-expressed in ovarian carcinomas (Campbell *et al*., 1992), making it a target for *in vivo* imaging and drug delivery (Merdan *et al*., 2003). Closely related if not identical forms of CD47 were also shown to regulate cytoskeletal architecture and motility in concert with integrins (Brown and Frazier, 2001). More recently, CD47 has been postulated to mediate apoptosis of leukemic cells in mice (Kikuchi *et al*., 2004) and chronic lymphocytic leukemia B-cells in humans (Mateo *et al*., 2002). It is also reported to have a role on red blood cells as a 'marker of self' (in mice) that inhibits phagocytosis (Oldenborg *et al*., 2000). Glycosylation has been implicated as a determinant of binding specificity in at least one binding partner of CD47 (van den Nieuwenhof *et al*, 2001; Ogura *et al*., 2004), but analogous information is lacking for CD47 itself. Using yeast surface display as a platform, we describe here the roles that variant glycosylation and disulfide bond

formation play in determining surface expression levels and conformation of CD47's Ig domain (*Ig*CD47).

MATERIALS & METHODS

Plasmids

The extracellular domain of human CD47 (amino acids 1-122 ; *Ig*CD47) was cloned from a mammalian expression vector, pIAP45. We added a c-myc epitope (EQKLISEEDL) and the synthetic pre signal peptide (Arnold *et al.*, 1998) (N-terminal to epitope) to the N-terminus and the FLAG epitope (DYKDDDDK) to the C-terminus of *Ig*CD47. This fragment was cloned into pCT4, a yeast display vector (Boder *et al.*, in press), using the restriction sites for *Aat II* and *Nhe I* introduced into the 5' and 3' primers. The epitope-flanked *Ig*CD47 was linked at its carboxy terminus to Aga2p with an intervening $(Gly_4Ser)_3$ linker to yield the sequence c-myc-*Ig*CD47-FLAG-linker-Aga2p. The sequence, DDDDK, also constitutes an enterokinase (EK) recognition site, enabling the use of enterokinase to cleave *Ig*CD47from Aga2p. Plasmid manipulations were performed in DH5α *Escherichia coli* (Invitrogen Corporation, Carlsbad, California). All enzymes and reagents used for cloning were obtained from New England Biolabs (Beverly, MA). Sequencing was done at the DNA Sequencing Facility of the Biomedical Core Facilities, Department of Genetics, University of Pennsylvania.

Site-directed Mutagenesis

Mutagenesis essentially followed the Quikchange protocol outlined by Stratagene (Stratagene Corporation, La Jolla, California). After PCR with the template plasmid and two complementary mutagenic primers, the restriction enzyme *Dpn I* was used to digest template (unmutated). A

portion of the PCR product was then transformed into DH5α (Invitrogen). Several single colonies were screened for the desired mutation by sequencing.

Transformation and induction of protein expression in yeast

The *S. cerevisiae* strain, EBY 100, described elsewhere (Boder and Wittrup, 2000), was used for display. Yeast were transformed using the lithium acetate method (Gietz and Wood, 2004) and plated onto selective medium (SD with Casamino acids, SDCAA). Expression under the control of the *GAL1* promoter is induced by galactose. Single colonies were then inoculated into liquid SDCAA and allowed to grow to an optical density between 1 and 2, following which they were switched to liquid SGCAA (where galactose replaces dextrose) supplemented with 10% glycerol and incubated overnight at 30º. Omitting the glycerol had no discernible effect on the expression of *Ig*CD47 (data not shown).

Immunofluorescence, microscopy and flow cytometry

Indirect immunofluorescent staining was used with a secondary polyclonal goat anti-mouse antibody conjugated to R-phycoerythrin (Sigma, St Louis, MO). The sources of the mAbs used in this study are as follows: B6H12 was from BD Biosciences Pharmingen (San Diego, California); 9E10 was from Covance (Princeton, New Jersey); 2D3 was from Calbiochem (San Diego, California); 6H9 was a kind gift from Dr. Mary Telen at Duke University. Cells were labeled with antibodies at room temperature unless otherwise stated: typically, about 10^7 cells were washed twice in PBS with 0.1% bovine serum albumin (BSA) and incubated with primary antibody for 30 minutes with gentle agitation. After two more washes, the cells were incubated with secondary antibody for 30 minutes in the dark. The cells were then washed once more and

suspended in PBS +0.1% BSA on ice for analysis. Microscopic Images were acquired on a Nikon TE300 inverted microscope with a 60 X (oil, 1.4 NA) objective using a Cascade CCD camera (Photometrics, Tuscon, AZ). Image acquisition was performed with Image Pro software (Media Cybernetics, Silver Spring, MD). Flow cytometry experiments were performed on the Becton-Dickinson (San Jose, CA) FACScan or FACSCalibur machines at the Flow Cytometry Shared Facility in the School of Medicine, University of Pennsylvania.

Protein stripping and western blotting

Pelleted cells were stripped of displayed protein by gentle shaking with 1 mM DTT in 50 mM Tris-HCl, pH 8, for 1 hour at 4°C in one-tenth the volume of the original culture (Watzele *et al*., 1988). Digestion with enterokinase (New England Biolabs) was performed overnight at room temperature in the same buffer, which was supplemented for this purpose with 50 mM NaCl and 2 mM $CaCl_2$. Digestion with PNGase F closely followed the instructions of the manufacturer (New England Biolabs). Thus, the sample was heated with denaturing buffer for 10 minutes at 90º C, followed by the addition of NP-40 and the enzyme. The mixture was incubated overnight at 37ºC. SDS-PAGE was performed using the XCell Surelock module (Invitrogen) and NuPAGE gels with MES as the running buffer (Invitrogen). The sample was heated in loading buffer (Invitrogen) with reducing agent, following the manufacturer's directions. Anti-oxidant was added to the running buffer as well. Following electrophoresis, protein was transferred to a 0.2 μm PVDF membrane (BIORAD, Hercules, CA) using the XCell II Blot Module (Invitrogen). The membrane was blocked with 5% non-fat milk in TTBS (Tris-buffered saline with 1% Tween 20) by shaking for 1 hour at room temperature or overnight at 4ºC, washed with TTBS and incubated with primary antibody (9E10 against c-myc tag) in TTBS for 1 hour with gentle

shaking. After repeated washing, the membrane was incubated with secondary antibody (goat anti-mouse conjugated with alkaline phosphatase, Sigma, St Louis, Missouri) in TTBS for 30 minutes. The membrane was washed again and incubated with the alkaline phosphatase substrate, NBT-BCIP (Sigma). Quantification of scanned images was performed with Image J (NIH) after scanned blots were converted to 8-bit black-and-white images.

Thermal denaturation

Heat denaturation experiments were carried out on 40 µl aliquots of yeast suspension (about 10^7 cells) that were washed twice and then suspended in PBS with 0.1% BSA (Orr *et al*., 2003). The suspension was transferred to a thin walled PCR tube and held at the desired temperature in a Sprint thermal cycler (Hybaid International) for 20 minutes. The sequence in which the different temperatures were reached was scrambled between replicate runs. (In time-variant experiments, the duration of incubation in the cycler was controlled). The tube was then removed quickly from the cycler and cooled on ice for between 5 and 10 minutes after which the yeast suspension was diluted into 1 ml ice-cold PBS/BSA . After all of the samples had been heat-treated, labeling with primary and secondary antibodies for flow cytometric analysis was carried out on ice .

RESULTS

*Yeast surface display of Ig*CD47

*Ig*CD47 expression was induced on the surface of *S cerevisiae* (Fig. 1A) as a fusion with the mating protein, Aga2p. The *Ig*CD47 domain is flanked at the amino terminal by the epitope tag, c-myc, and at the carboxyl end by the FLAG tag which is itself separated from Aga2p by a flexible, $(Gly_4Ser)_3$ linker (Figure 1A). Aga2p is linked to Aga1p on the yeast the cell wall by a pair of disulfide bonds, which can be readily reduced for purification and analysis of the yeast displayed protein (Watzele *et al*., 1988; Boder and Wittrup, 1997).

Fluorescence microscopy of *Ig*CD47-expressing yeast cells using the mAb 6H9 confirmed the edge-bright surface display of the construct (Figure 1B). Although a fraction of the induced population remained dark (white arrow, Figure 1B) and was therefore considered to be non-expressing, all of the control wild-type or uninduced yeast remained dark when similarly probed. The same immunofluorescence scheme was then used to quantitate relative expression levels by flow cytometry (FC). Immunostaining with B6H12, anti-FLAG, or 9E10, a c-myc antibody, followed with R-phycoerythrin-conjugated secondary, showed two broad populations of expressing and non-expressing cells (Figure 1C), as generally reported by others with yeast display (Boder and Wittrup, 2000). Similar bimodal patterns were obtained with 6H9 and 2D3, other CD47 mAbs (data not shown). The non-expressing fraction at lower fluorescence levels represents less than 40% of total cell number, and the strong signals elicited by the anti-FLAG, anti-c-myc, and many CD47 mAbs clearly suggest that full-length *Ig*CD47 is expressed. It should be noted that B6H12 is conformationally sensitive (Rosales *et al*., 1992) and reportedly blocks binding of CD47's physiological ligand, signal regulating protein α (SIRPα), which

makes B6H12 a reasonable, high affinity surrogate for SIRPα (Seiffert *et al*., 1999), in contrast to 2D3 which is reportedly a non-blocking antibody (Seiffert *et al*., 1999).

The fusion protein was dissociated from the yeast surface in 1 mM DTT with gentle shaking in the cold for one hour. FC using 9E10 on DTT-treated cells showed a greatly diminished positive peak (data not shown), consistent with liberation of the fusion protein. Subsequent cleavage of the DTT-treated supernatant by EK was used to separate Aga2p from *Ig*CD47, and an aliquot of this reaction mixture was then denatured and treated with the endoglycosidase, PNGase F, to cleave N-linked sugars from the asparagine. SDS-PAGE was performed on EK-treated protein samples both before and after PNGase treatment; and immunoblotted with the c-myc mAb 9E10 (Figure 2A).

DTT-liberated protein is heterogeneous in molecular weight. Major species are glycosylated isoforms of *Ig*CD47, with the highest molecular weight diffuse bands in lane 2 likely to represent hyper-mannosylated *Ig*CD47. Such post-translational modification is common to many species of yeast, and has motivated re-engineering of yeast glycosylation pathways in *Pichia* (Hamilton *et al*., 2003), since it often interferes with desired function. Treatment with PNGase, however, collapses the bands into single major band around 17 kDa, (the calculated molecular weight for the amino acid sequence of *Ig*CD47 flanked by the c-myc and FLAG epitopes is 16 kDa). The molecular weight of O-glycosylated Aga2p is ca. 18 kDa, while it appears close to 23 kDa on SDS-PAGE (Capellaro et al, 1991; Huang and Shusta 2005). Minor bands constituting less than 10% of the protein are likely to be degradation products resulting from excessive EK-treatment

since they were not observed with an alternative cleavage construct employing tobacco etch virus protease as the cleaving reagent in place of EK (data not shown).

Glycosylation mutants and expression levels

In order to explore the effect of glycosylation on expression and binding function, we constructed and expressed a spectrum of glycosylation mutants of *Ig*CD47. *Ig*CD47 is predicted to be N-glycosylated at five possible sites: N5, N16, N32, N55 and N93. Putative N-linked glycosylation sites were disrupted by an asparagine to glutamine mutation at one or more desired locations. The fusion protein has an additional N-linked glycosylation site near the C-terminus of Aga2p.

When blots of protein released by DTT + EK treatment of yeast expressing wild-type, N16/55/93Q (Δ3), N16/32/55/93Q (Δ4), and N5/16/32/55/93Q (Δ5) *Ig*CD47 mutants were probed with 9E10, hypergylcosylated protein (bands at >80kDa) was observed (Figure 2). Bands around 25, 20, and 17 kDa are displayed by the Δ3, Δ4, and Δ5 mutants, respectively. Unglycosylated protein is evident with the Δ4 mutant and, of course, in the Δ5 mutant, although higher molecular weight bands are present as well (see below). Strikingly, the Aga2p-wild-type *Ig*CD47 fusion seems to be most heavily hyperglycosylated since the bands at high MW are stronger than they are for the other mutants. Figure 2B depicts intensity traces over the relevant lanes, with the vertical hash mark denoting the median molecular weight in each case. As might be expected, the median molecular weight shifts lower as N-linked glycosylation is progressively reduced (Figure 2B). The hyperglycosylation in Δ3 and Δ4 must be contributed by N5, the glycosylation site that the two have in common. A possible contribution from glycosylation at

N32 in Δ3 is likely to be smaller than that from N5 since silencing this site (going from Δ3 to Δ4) has a comparatively smaller effect on the hyperglycosylated bands than when going from wild-type to Δ3. The bands at 20 and 25 kDa (boxed) for Δ4 and Δ3 *Ig*CD47 may reflect the emergence of protein that is exclusively core-glycosylated. Unglycosylated protein can also be seen in the Δ4 mutant. The multiple bands between 31 and 40 kDa for the Δ5 and Δ4 mutants may reflect incomplete EK digestion of the fusion protein, as suggested by the appearance of nearly identical bands in absence of EK digestion of the Δ4 mutant Aga2p-*Ig*CD47 (data not shown).

When treated with PNGase F, the recombinant *Ig*CD47 band collapses to about 17 kDa in all cases. The effect of the glycosidase on the Δ5 mutant – with no N-linked glycosylation sites – was negligible, as expected. The relative proportion of the approximately 17 kDa protein between PNGase F-treated and untreated Δ5 mutant (Figure 2A) was nearly 1:1. This same ratio for the other constructs is virtually 1:0, which indicates that at least N5 is universally occupied, based on the mutants studied.

With site-by-site removal of glycosylation, display of *Ig*CD47 decreased, as quantified by FC measurements of *Ig*CD47 (Figure 3A and B). At saturating mAb levels (for wild-type), FC using B6H12 showed that all five single mutants (N5Q, N16Q, N32Q, N55Q, N93Q) differed little from wild-type in staining level: the ratio of B6H12 binding to mutant normalized with respect to wild-type was 1.16 ± 0.20 in all cases (data not shown). Evidently, no single glycosylation site modulates binding of this ligand-surrogate mAb. The triple mutant Δ3 (N16/55/93Q) showed a decrease in immunostaining compared to wild-type, and for both Δ4 and Δ5 the signal was even

lower. Assuming B6H12 is sensitive to *Ig*CD47 conformation but 9E10 is not, the fidelity of folded *Ig*CD47 expression seems not to be undermined by loss of glycosylation (the intensity ratio remains around 1). Thus the parallel decrease in staining intensity for both mAbs strongly suggests overall loss of expression rather than epitope rearrangement. Not only is no single glycosylation site needed for binding of B6H12, but neither is any of the tested combinations.

Thermal effects and glycosylation

The effect of mutations on thermal stability of yeast-displayed protein has been measured by incubating recombinant yeast at selected temperatures and measuring the remnant binding of a conformation-sensitive antibody to the protein (Esteban and Zhao, 2004; Orr et al , 2003; Shusta et al, 2000). We used B6H12 as the antibody and wild-type and the Δ5 mutant as samples, since they represent the extremes of glycosylation. To determine an appropriate duration of incubation, a preliminary experiment was run wherein wild-type was held isothermally at 55 °C (Figure 3A) for different durations. The binding of the c-myc antibody 9E10 was found to be relatively temperature invariant (suggesting that the scaffold was stable in this temperature range) whereas the binding of B6H12 decreased with time of incubation, yielding a $t_{1/2}$ of 20 minutes. The variable-temperature study therefore used incubation durations of 20 minutes.

Data obtained from this experiment in two independent replicates are shown in Figure 4B. The error bars represent standard deviations and the fit was drawn with a simple two-state model. The leveling-off at high temperature seen in the Δ5 case probably stems from a conflation of expressor and non-expressor populations (see the reduced separation even at room temperature in this mutant vis-à-vis wild-type in Figure 3A) of the two peaks. Notwithstanding the substantial

change in glycosylation between wild-type and Δ5 (Figure 2) the temperature dependence of B6H12 binding and thus conformational stability of the two samples seems very similar. The temperature at which half the binding is retained ($t_{1/2}$) is around 60 °C in each case.

Cysteine mutants and the core disulfide

The disulfide bond between a pair of core cysteine residues, usually about 50-70 amino acids apart in primary sequence, is a defining feature of almost any extracellular Ig domain. In *Ig*CD47, Cys23 and Cys96 form a disulfide bond (Rebres *et al*., 2001; see Fig.1). A "free" cysteine (Cys15) is believed to partner in a long-range disulfide bond with a cysteine presumed to be located on an extracellular loop between transmembrane segments (Rebres *et al*., 2001), but this latter cysteine is absent in the truncated construct expressed here. The role of the cysteine residues was investigated by displaying the three single mutants: C15S, C23S and C96S in the same fusion protein construct defined above.

Western blots were run on protein stripped as before from yeast expressing C23S and C96S *Ig*CD47 (Figure 5A). It is immediately apparent that the mutations lead to (1) a decrease in hyperglycosylation with a shift toward lower molecular weight and (2) a weak band to appear around 25 kDa, for both C96S and C23S. Upon treatment with PNGase F, the dominant band at 40 kDa resolves into a doublet above 31 kDa that is most likely due to incomplete deglycosylation, whereas the band at 25 kDa is no longer seen. This observation leads us to conclude that these bands are differentially glycosylated products with the 25 kDa band possibly being core-glycosylated *Ig*CD47. As with the glycosylation mutants, the median molecular weight of the hyperglycosylation band is lower in the mutants compared to wild-type, suggesting

decreased hyperglycosylation in these cys mutants (Figure 5B). Removing the disulfide bond thus *decreases* the average molecular weight of the added sugars, whether by limiting the length of each chain or by sequestering some of the asparagines, suggesting that hyperglycosylation and disulfide formation are coupled.

9E10 and B6H12 were used as before, to deconvolve the effects of the mutations on expression level and structure, respectively (Figure 6A). The C15S mutant has staining intensities similar to wild-type for both antibodies (Figure 6B), signifying that this mutation has little effect on epitope and expression levels. When the core disulfide bond is broken by mutating either of the two remaining cysteines to serine (C23S or C96S), the staining intensity with 9E10 and B6H12 falls off sharply, reaching disproportionately lower levels with B6H12 than with 9E10. The C15S mutant has B6H12/9E10 staining intensity ratios of 0.8, which is within the range of noise 0.7-1.3 established as uncompromised expression of the glycosylation mutants (Figure 3). In contrast, B6H12 binding falls off sharply relative to 9E10 to values of 0.3 and 0.1, for C23S and C96S, respectively. The 9E10 intensity by itself falls only to around 30% of wild-type values for C23S and C96S, whereas it remains around 1 for C15S (Figure 6B). We conclude that the effect of breaking the core disulfide bond is to decrease the expression level by about one-third, while also effectively abrogating B6H12 binding. In contrast, abolishing glycosylation (Δ5) was shown above to reduce surface expression by more than 90%, while still maintaining B6H12 reactivity.

Removal of N-glycosylation sites affects the level of expression of the protein, but not its ability to fold. However, the effect on expression levels of removing either Cys23 or Cys96, which are

likely to form a core disulfide bond, is symmetric, with expression levels dropping to 30% of wild-type in either case. Whether this drop is *mediated* by the evident reduction in glycosylation levels is not clear. Nonetheless, the effect of these two Cys mutations on folding of the B6H12 epitope appears *asymmetric*, with C96S destabilizing native-type folds somewhat more than C23S.

DISCUSSION

Display of proteins on the cell wall of *S. cerevisiae* is a lively topic of research and has attracted considerable attention since it combines ease of display with access to a broad ensemble of post-translational modifications in an eukaryotic single cell host (Boder and Wittrup, 2000). There are important issues to consider, however, with the expression of individual heterologous proteins in this system. The N-linked glycosylation process in yeast differs considerably from that in mammals in the sugars used, the types of linkage present and, most importantly, in the number of residues attached (Gerngross, 2004; Hamilton *et al*., 2003). Hypermannosylation by the yeast secretory pathway is a hurdle that must be overcome for functional expression of heterologous proteins. CD47 is a ubiquitous cell-surface protein with a wide-ranging functional repertoire (Mawby *et al*., 1994; Brown and Frazier, 2001; Mateo *et al*., 2002; Merdan *et al*., 2003) that should be useful in future structure-function studies, provided the roles of glycosylation and determinants of stability can first be carefully qualified.

Surface display of the extracellular Ig domain of human CD47, *Ig*CD47, as a fusion protein with Aga2p-Aga1p, a mating protein, was clearly accomplished here under the control of the *GAL*1 promoter. It binds common antibodies such as 2D3, 6H9 as well as the widely used SIRPα-blocking antibody, B6H12 (Figure 1). Although a dozen or so proteins have been displayed on

yeast, there is relatively little information on the specific effect of glycosylation on expression and function. A lack of information on the occupancy of glycosylation sites in the native protein generally complicates attempts to ascribe differences in activity seen in recombinant protein to variant glycosylation. Thus, possible hyperglycosylation was suggested as a cause for the observed diminution in affinity seen with a surface-displayed epidermal growth factor receptor (EGFR) (Cochran *et al*., 2004), which has 12 consensus N-linked glycosylation sites.

Several early steps in the process of N-linked glycosylation in the ER are conserved amongst eukaryotes (Gerngross, 2004). The $GlcNAc_2Man_9Glc_3$ unit added to an asparagine in the ER has the three glucose units and a mannose trimmed when it leaves the ER *en route* to the Golgi. Further processing of the sugars differs greatly amongst species and even between different proteins in the same cell. In *S. cerevisiae*, some of the carbohydrate chains are poorly elaborated with up to 14 mannoses, whereas some others are 'hyperglycosylated" with the addition of 200-300 mannoses (Conde *et al*., 2004). It should be noted that there are important differences between glycosylation in the ER and the Golgi: ER glycosylation ("core glycosylation") proceeds co-translationally and is coeval with folding, enabling aspargines that will later be buried to be modified with sugar residues, whereas hyperglycosylation in the Golgi takes place on the fully-folded protein (Allen *et al*., 1995; Holst *et al*., 1996; Conde *et al*., 2004). Hyperglycosylation in the Golgi can only occur with asparagines that are solvent-accessible in the final structure.

There are reports that the NXT sequon is more likely to be glycosylated than the NXS sequon (Gavel and von Heijne, 1990). Only N55 is part of an NXS sequon in *Ig*CD47. Mellquist *et al*

(1998) found that the amino acid immediately carboxy to the sequon also influences glycosylation, but the modulation is much weaker for NXT than for NXS sequons in rabies virus glycoprotein expressed in a cell-free system. The immediate environment of the sequon as well as elements of tertiary structure would also be expected to modulate glycosylation (Kasturi *et al*., 1995; Shakin-Eshleman *et al*., 1996). Studying mutations in an NXS sequon in a model exoglucanase in *S. cerevisiae,* Conde *et al* (2004) found that hyperglycosylation is favored when X is a basic amino acid than an acidic one, or an aromatic amino acid such as tryptophan or tyrosine but not phenylalanine. Only N16 has an acidic neighbor in *IgCD47*. The N-linked glycosylation sequons in *Ig*CD47 are compatible with these molecular determinants of glycosylation/hyperglycosylation and one might therefore expect that the protein will be (hyper-) glycosylated in *S. cerevisiae*.

Such is indeed the case. Abolishing the N-glycosylation sequon in stages with an N-to-Q mutation, results in redistribution of glycosylation with a shift toward lower molecular weight, so much so that core-glycosylated *Ig*CD47 starts to appear in the Δ3 mutant, suggesting that N5 and N32 are variably glycosylated. The Δ3, Δ4 and (of course) the Δ5 mutants contain the N16,55,93Q triple mutation. Considering the Δ3 case first, the marked decrease in high molecular weight bands suggests that N16, N55 and N93 are hyperglycosylated, although N16 may be less hyperglycosylated than the others since this sequon has an acidic amino acid in the second position. Core- and hyperglycosylation at N5 and N32 would account for the remaining bands seen. In Δ4, N32 is removed as a glycosylation site. The Δ3 and Δ4 lanes differ little at high molecular weights suggesting that N32 may not be hyperglycosylated, but the band

assigned to core glycosylation now decreases by around 5 kDa from 25 to 20 kDa, suggesting that N32 has a core glycosylation unit of about 25 residues.

N5 is additionally lost as a glycosylation motif in the Δ5 mutant and the band at 20 kDa in Δ4 moves to a limiting value around 17 kDa, implying occupancy of about 15 core residues at N5. The entire high molecular weight signal is absent for Δ5, suggesting that the faint bands seen in the Δ3 and Δ4 cases arose from N5. In summary, our data indicate that N5, N16, N55 and N93 are hyperglycosylated. It is not clear whether N32 is hyperglycosylated. N32 and N5 seem to have core glycosylation motifs of ~25 and ~15 mannose residues, respectively. Loss of N32 leads to anomalous mobility on the gel, but this is not exacerbated by the loss of glycosylation at N5. This analysis assumes that the level of glycosylation at each sequon is independent of that at other sequons but this remains to be proved. ~~Loss of glycosylation may affect mobility on the gel to a different extent than can be predicted by the resulting change in molecular weight~~.

The binding of a conformation-sensitive CD47 mAb, B6H12, parallels expression levels (as measured by the mAb to the c-myc epitope tag, 9E10) in the case of the glycosylation mutants, indicating that the expressed protein is folded correctly. The thermal profiles of the Δ5 mutant and wild-type *Ig*CD47 are very similar suggesting that their stabilities are likewise similar under the conditions of the experiment. To set our data in perspective, swings in the $t_{1/2}$ as large as 20 to 30 °C have been observed amongst mutants by Orr et al (2003) and Shusta et al., (2000). Although the structural ramifications of the mutations engineered by these workers is not clear, our mutations have been directed at reducing glycosylation and it is striking that removal of five glycosylation sites results in negligible shift of the thermal profile. Wittrup and associates

(1998) have described a model for secretion level by yeast with a quality control mechanism in the endoplasmic reticulum: if the folding rate is not limiting, the relative stability of folded versus unfolded forms (which are in pseudo-equilibrium) determines secretion level. The reduction in the amount of protein displayed as glycosylation sites decrease could arise from a kinetic or a thermodynamic bottleneck, or some combination of the two. Noting that (a) the B6H12 epitope remains unaffected with loss of glycosylation (Figure 3B) and (b) the thermal stability of the mutant protein appears to be similar to that of wild-type (Figure 4), it is clear that progressively smaller amounts of "correctly folded" protein emerge on the surface as the number of glycosylation sites decreases. Thus, the similar stability of the mutants argues for a kinetic bottleneck brought about by limiting folding rates. In contrast to our results, a prior study found no effect on display levels in response to mutation of N-linked glycosylation sites of a mutant single-chain T cell receptor (Shusta et al, 2000). In this case, the mutant studied demonstrated an unfolding transition midpoint of nearly 70°C, as opposed to the ~55°C midpoint observed for CD47. These contrasting results suggest that the effects of glycosylation on display efficiency might not apply to proteins of very high stability, although this hypothesis has not been tested.

Reports in the literature exploring the link between glycosylation and disulfide formation have treated cells with a reducing agent to retain the reduced form of cysteine (Braakman *et al*., 1991; Jamsa *et al*., 1994), an approach not feasible here where it would have resulted in cleavage of the displayed protein from its anchor (Aga2p-S-S-Aga1P). This has generally led to an extension of the duration spent by the protein in the ER. When CHO cells expressing t-PA (a protein richly endowed with cysteines) were treated with DTT, reduced folding rates led to the glycosylation of an asparagine that was not normally glycosylated (Allen *et al*., 1995). The authors suggest that

folding rates and glycosylation rates could compete, occasionally affording a normally buried residue access to the glycosylation machinery. Upon DTT washout, the functionality of the secreted protein was found to be unaffected. A recombinant carboxypeptidase in *S. cerevisiae* was found to have a greater degree of glycosylation at newly-introduced N-linked glycosylation sites with the details depending on folding conditions (Holst *et al*., 1996). Clearly, folding does not precede glycosylation. In the present case, removal of each of the two cysteines decreases the level of hyperglycosylation to the extent that a fraction of the protein is unglycosylated. The effect is asymmetric between the two core cysteines. Disruption of the disulfide bond in an Ig domain may thus hinder access to the glycosylating machinery and reduce the average molecular weight of the carbohydrate chain.

The level of B6H12 binding does not reflect expression levels when the core cysteines (C23 and C96) involved in the disulfide bond are mutated. The effect is asymmetric with the B6H12/9E10 intensity ratio decreasing far more for C96S than for C23S. The disulfide bond may serve to bring together a patch of non-contiguous residues, thereby forming an epitope for B6H12, but the epitope appears to partially survive the loss of the disulfide bond.

Evidently glycosylation, rather than the disulfide bond, plays a larger role in determining whether an Ig domain can be robustly displayed on yeast. Proba *et al*. (1998) found that an scfv fragment of an anti-HER2 antibody secreted into the periplasm of *E. coli* could fold correctly even when the core disulfide cysteines were replaced by valine and alanine. Replacing cysteines with serines had earlier been found to be more destabilizing than replacements with alanine or valine in the case of bovine pancreatic trypsin inhibitor (Liu *et al*., 1996): although cysteine and

serine differ only in the substitution of an –OH bond for the –SH bond, hydrogen-bonding does not ameliorate the effect of burying polar groups in the interior of the molecule. The consensus, corroborated by the present data, therefore seems to be that disulfide bonds are not indispensable to the integrity of Ig domains and their display or secretion in prokaryotic or eukaryotic hosts. In light of the anti-HER2 finding, it remains an open question whether other replacements of cysteine in the present case would have retained functional integrity as reflected in an ability to bind B6H12.

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REFERENCES

Allen S, Naim HY, Bulleid NJ. 1995. Intracellular folding of tissue-type plasminogen activator. Effects of disulfide bond formation on N-linked glycosylation and secretion. J Biol Chem. **270**: 4797-804.

Arnold CE, Parekh RN, Yang W, and Wittrup KD. 1998. Leader peptide efficiency correlates with signal recognition particle dependence in Saccharomyces cerevisiae. Biotechnol Bioeng. **59**: 286-93.

Boder ET, Bill JR, Nields AN, Marrack PC, Kappler JW. Biotechnol Bioeng, in press.

Boder ET and Wittrup, KD. 2000. Yeast surface display for directed evolution of protein expression, affinity, and stability. Methods Enzymol. **328**:430-44.

Boder ET and Wittrup, KD. 1997. Yeast Surface Display for Screening Combinatorial Polypeptide Libraries. Nature Biotechnol. **15**: 553-7.

Braakman I, Hoover-Litty H, Wagner KR, Helenius A. 1991. Folding of influenza hemagglutinin in the endoplasmic reticulum. J Cell Biol. **114**: 401-11.

Brown EJ, Frazier WA. 2001. Integrin-associated protein (CD47) and its ligands. Trends Cell Biol. **11**:130-5.

Butz J, Niebauer RT and Robinson AS. 2003. Co-expression of molecular chaperones does not improve the heterologous expression of mammalian G-protein coupled receptor expression in yeast. *Biotechnol. Bioeng.* **84**: 292–304.

Campbell IG, Freemont PS, Foulkes W, Trowsdale J. 1992. An ovarian tumor marker with homology to vaccinia virus contains an IgV-like region and multiple transmembrane domains. Cancer Res. **52**: 5416-20.

Capellaro C, Hauser K, Mrsa V, Watzele M, Watzele G, Gruber C and Tanner W. 1991. *Saccharomyces cerevisiae* a- and α-agglutinin:characterization of their molecular interaction. EMBO J, **10**, 4081-4088 (1991).

Cochran JR, Kim YS, Olsen MJ, Bhandari R, Wittrup KD. 2004. Domain-level antibody epitope mapping through yeast surface display of epidermal growth factor receptor fragments. J Immunol Methods. **287**: 147-58.

Conde R, Cueva R, Pablo G, Polaina J, Larriba G A 2004. Search for hyperglycosylation signals in yeast glycoproteins. J Biol Chem. **279**: 43789-98.

Dorner AJ, Bole DG, Kaufman RJ. 1987. The relationship of N-linked glycosylation and heavy chain-binding protein association with the secretion of glycoproteins. J Cell Biol. 105: 2665-74.

Esteban O and Zhao H. 2004. Directed evolution of soluble single-chain human class II MHC molecules. J Mol. Biol., **340**, 81-95 (2004).

Feldhaus M and Siegel R. 2004. Flow cytometric screening of yeast surface display libraries. Methods Mol Biol. **263**: 311-32.

Feng W, Matzuk MM, Mountjoy K, Bedows E, Ruddon RW, Boime I. 1995. The asparagine-linked oligosaccharides of the human chorionic gonadotropin beta subunit facilitate correct disulfide bond pairing. J Biol Chem. **270**:11851-9.

Gavel Y, von Heijne G. 1990. Sequence differences between glycosylated and non-glycosylated Asn-X-Thr/Ser acceptor sites: implications for protein engineering. Protein Eng. **3**: 433-42.

Gerngross TU. 2004. Advances in the production of human therapeutic proteins in yeasts and filamentous fungi. Nat Biotechnol. **22**:1409-14.

Gietz, R.D. and R.A. Woods. 2002. Transformation of yeast by the LiAc/ss carrier DNA/PEG method. Methods in Enzymology. **350**: 87-96.

Hamilton SR, Bobrowicz P, Bobrowicz B, Davidson RC, Li H, Mitchell T, Nett JH, Rausch S, Stadheim TA, Wischnewski H, Wildt S, Gerngross TU. 2003. Production of complex human glycoproteins in yeast. Science. **301**:1244-6,

Holst B, Bruun AW, Kielland-Brandt MC, Winther JR. 1996. Competition between folding and glycosylation in the endoplasmic reticulum. EMBO J. **15:** 3538-46.

Huang D and Shusta EV. 2005. Secretion and surface display of green fluorescent protein using the yeats *Saccharomyces cerevisiae*. Biotechnol. Prog. **21**, 349-357 (2005).

Jamsa E, Simonen M, Makarow M. 1994. Selective retention of secretory proteins in the yeast endoplasmic reticulum by treatment of cells with a reducing agent. Yeast. **10**: 355-70.

Kasturi L, Eshleman JR, Wunner WH, Shakin-Eshleman SH. 1995. The hydroxy amino acid in an Asn-X-Ser/Thr sequon can influence N-linked core glycosylation efficiency and the level of expression of a cell surface glycoprotein. J Biol Chem. **270**: 14756-61.

Kikuchi Y, Uno S, Yoshimura Y, Otabe K, Iida S, Oheda M, Fukushima N, Tsuchiya M. 2004. A bivalent single-chain Fv fragment against CD47 induces apoptosis for leukemic cells Biochem Biophys Res Commun. **315**: 912-8.

Kowalski JM, Parekh RN, Mao J and Wittrup KD. 1998. Protein folding stability can determine the efficiency of escape from endoplasmic reticulum quality control. J Biol Chem. **273**: 19453-8.

Liu Y, Breslauer K, Anderson S. 1997;. Designing out disulfide bonds: thermodynamic properties of 30-51 cystine substitution mutants of bovine pancreatic trypsin inhibitor. Biochemistry. **36**: 5323-35.

Martina Seiffert, Charles Cant, Zhengjun Chen, Irene Rappold, Wolfram Brugger, Lothar Kanz, Eric J. Brown, Axel Ullrich, and Hans-Jörg Bühring. 1999. Human signal-regulatory protein is expressed on normal, but not on subsets of leukemic myeloid cells and mediates cellular adhesion involving its counterreceptor CD47. Blood. **94**: 3633-3643.

Mateo V, Brown EJ, Biron G, Rubio M, Fischer A, Deist FL, Sarfati M. 2002. Mechanisms of CD47-induced caspase-independent cell death in normal and leukemic cells: link between phosphatidylserine exposure and cytoskeleton organization. Blood. **100**: 2882-90.

Mawby WJ, Holmes CH, Anstee DJ, Spring FA, Tanner MJ. 1994. Isolation and characterization of CD47 glycoprotein: a multispanning membrane protein which is the same as integrin-associated protein (IAP) and the ovarian tumour marker OA3. Biochem J. **304**: 525-30.

Mellquist JL, Kasturi L, Spitalnik SL, Shakin-Eshleman SH. 1998. The amino acid following an asn-X-Ser/Thr sequon is an important determinant of N-linked core glycosylation efficiency. Biochemistry. **37**: 6833-7.

Merdan T, Callahan J, Petersen H, Kunath K, Bakowsky U, Kopeckova P, Kissel T, Kopecek J. 2003. Pegylated polyethylenimine-Fab' antibody fragment conjugates for targeted gene delivery to human ovarian carcinoma cells. Bioconjug. Chem. **14**: 989-96.

Niebauer RT, Wedekind A, Robinson AS. 2004. Decreases in yeast expression yields of the human adenosine A2a receptor are a result of translational or post-translational events. Protein Expr Purif. **37**:134-43.

Ogura T, Noguchi T, Murai-Takebe R, Hosooka T, Honma N, Kasuga M. 2004. Resistance of B16 melanoma cells to CD47-induced negative regulation of motility as a result of aberrant N-glycosylation of SHPS-1. J Biol Chem. **279**:13711-20.

Oldenborg PA, Zheleznyak A, Fang YF, Lagenaur CF, Gresham HD, Lindberg FP. 2000. Role of CD47 as a marker of self on red blood cells. Science. **288**: 2051-4.

Orr BA, Carr LM, Wittrup KD, Roy EJ, Kranz DM. 2003. Rapid method for measuring scFv thermal stability by yeast surface display. Biotechnol Prog. **19**: 631-8.

Proba K, Worn A, Honegger A, Pluckthun A. 1998. Antibody scFv fragments without disulfide bonds made by molecular evolution. J Mol Biol. **275**: 245-53.

Rebres RA, Vaz LE, Green JM, Brown EJ. 2001. Normal ligand binding and signaling by CD47 (integrin-associated protein) requires a long range disulfide bond between the extracellular and membrane-spanning domains. J Biol Chem. **276**: 34607-16.

Rosales C, Gresham HD, Brown EJ. 1992. Expression of the 50-kDa integrin-associated protein on myeloid cells and erythrocytes. J Immunol. **149**: 2759-64.

Rudd PM, Wormald MR, Dwek RA. 2004. Sugar-mediated ligand-receptor interactions in the immune system. Trends Biotechnol. **22**:524-30.

Rudd PM, Wormald MR, Stanfield RL, Huang M, Mattsson N, Speir JA, DiGennaro JA, Fetrow JS, Dwek RA, Wilson IA. 1999. Roles for glycosylation of cell surface receptors involved in cellular immune recognition. J Mol Biol. **293**:351-66.

Shakin-Eshleman SH, Spitalnik SL, Kasturi L. 1996. The amino acid at the X position of an Asn-X-Ser sequon is an important determinant of N-linked core-glycosylation efficiency. J Biol Chem. **271**: 6363-6.

Shusta EV, Holler PD, Kieke MC, Kranz DM And Wittrup KD 2000. Directed evolution of a stable scaffold for T cell receptor engineering. Nature Biotech. **18**, 754-759.

Siegel RW, Coleman JR, Miller KD, Feldhaus MJ. 2004. High efficiency recovery and epitope-specific sorting of an scFv yeast display library. J Immunol Methods. **286**:141-53.

Starwalt SE, Masteller EL, Bluestone JA, Kranz DM. 2003. Directed evolution of a single-chain class II MHC product by yeast display. Protein Eng. **16**:147-56.

Ticchioni M, Deckert M, Mary F, Bernard G, Brown EJ, Bernard A. 1997. Integrin-associated protein (CD47) is a comitogenic molecule on CD3-activated human T cells. J Immunol. **158**: 677-84.

van den Nieuwenhof IM, Renardel de Lavalette C, Diaz N, van Die I, van den Berg TK. 2001. Differential galactosylation of neuronal and haematopoietic signal regulatory protein-alpha determines its cellular binding-specificity. J Cell Sci. **114**: 1321-9.

Watzele M, Klis F and Tanner W. 1988. Purification and characterization of the inducible *a* agglutinin of *Saccharomyces cerevisiae*. The EMBO J. **7**:1483-1488.

Yeung YA and Wittrup KD. 2002. Quantitative screening of yeast surface-displayed polypeptide libraries by magnetic bead capture. Biotechnol Prog. **18**: 212-20.

FIGURE LEGENDS

Figure 1. (A) A schematic of the *Ig*CD47-Aga2p fusion protein displayed on the surface of the yeast cell. The molecular weights of Aga2p and unglycosylated *Ig*CD47 (with the epitope tags) are ~23 and 16 kDa, respectively. (B) Phase contrast (top) and immunofluorescence (bottom) photographs of yeast cells after induction in galactose-containing selective medium. A CD47 monoclonal antibody, 6H9, was used to stain cells in the bottom panel followed by a polyclonal TRITC-conjugated goat anti-mouse secondary. (C) Flow cytograms of induced yeast stained with monoclonal anti c-myc , CD47 and anti-FLAG antibodies as indicated in each panel. The secondary reagent was goat anti-mouse conjugated with R-phycoerythrin. Cells that do not display the recombinant protein are evident in (B) and (C) and constitute typically 30-40% of the total number.

Figure 2. (A) Western blots, under reducing conditions, of N-linked glycosylation mutants. (B) Wild type and mutant IgCD47 was treated with enterokinase overnight, followed by PNGase F. The contents of the lanes are as follows: marker, wild-type (wt), N16/55/93Q (Δ3), N16/32/55/93Q (Δ4), N5/16/32/55/93Q (Δ5) and marker. Blotted protein was probed with the antibody, 9E10, to the c-myc epitope tag at the amino terminus of *Ig*CD47.(B) Lane intensity profiles for the recombinant proteins in panel (A) with the molecular weight at median intensity indicated in each case by the vertical mark .

Figure 3. (A) Flow cytograms of yeast displaying glycosylation mutants of *Ig*CD47, stained with the c-myc antibody, 9E10 (filled) or the *Ig*CD47 antibody, B6H12 (open), followed by R phycoerythrin-conjugated goat anti-mouse secondary. The symbols denoting the samples in each panel are described in the legend to Figure 3. (B) Normalized median intensities of the positive peak (wild-type =100) measured with an antibody to c-myc (9E10, filled bar) and to CD47 (B6H12, open bar) for the different mutants. The error bar reflects the standard deviation of data resulting from independent experiments. The B6H12/9E10 intensity ratio does not significantly vary in the mutants reflecting a systematic drop in expression level, rather than epitope rearrangement.

Figure 4. (A) Staining levels (normalized to a value of 1 at time zero) measured after different intervals of time at 55ºC, using antibodies either to c-myc (9E10) or to CD47 (B6H12) with yeast expressing the *Ig*CD47 variants indicated. The 9E10 data were fit to a straight line, the other lines shown are visual guides. (B) Staining levels of wild-type (closed) and Δ5 (open) *Ig*CD47 with B6H12 after the yeast was heated for 20 minutes at the temperatures shown. Data are normalized with respect to the staining intensity at 30 °C. Error bars represent the standard deviation over two independent replicates. The line represents a two-state model fit to wild-type data.

Figure 5. (A) Western blots of C96S *Ig*CD47 or C23S *Ig*CD47 under reducing conditions. Protein stripped from the yeast surface with 1 mM DTT (see text) was subsequently treated with enterokinase to cleave the *Ig*CD47 from Aga2p and followed with the endoglycosidase, PNGase F, to remove all N-linked sugars. Blotted protein was probed with the *c-myc* antibody, 9E10, to

the epitope tag at the amino terminus of *Ig*CD47. Lanes at extreme ends of the gels contained molecular weight marker. (B)) Lane intensity profiles for the recombinant proteins in panel (A) with the molecular weight at median intensity indicated in each case by the vertical mark

Figure 6. (A) Flow cytograms of wild-type and cysteine-to-serine mutants stained with the c-myc antibody, 9E10 (filled) or the *Ig*CD47 antibody, B6H12 (open), followed by R phycoerythrin-conjugated goat anti-mouse secondary. (B) Normalized median intensities of the positive peak (wild-type =100) measured with an antibody to c-myc (9E10, filled bar) and to CD47 (B6H12, open bar) for the different mutants.