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COMMUNICATIONS

Published on Web 05/16/2006

A Designed Glycoprotein Analogue of Gc-MAF Exhibits Native-like Phagocytic Activity

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Glycoproteins play a key role in many biological processes, such as mediating the immune response and inflammation. At the molecular level, the composition and geometry of the carbohydrate moieties displayed on glycoproteins are crucial in determining the specificity of these processes.[1,2] One such protein is vitamin D binding protein (VDBP) (Figure 1A), also known as group-specific component (Gc) globulin, a 52 KDa serum glycoprotein with functions including the transport of vitamin D and its metabolites and the binding of actin as part of the extracellular actin scavenger system.[3] The enzymatic processing of its carbohydrate moiety results in a potent macrophage activating factor (Gc-MAF/DBP-MAF) that stimulates the Fc-receptor-mediated phagocytic activity of macrophages.[4a,b] This process requires the participation of B and T lymphocytes during inflammation. Specifically, a loop in domain III of VDBP is physiologically O-glycosylated at threonine 420 with a trisaccharide composed of a *N*-acetyl-D-galactosamine (GalNAc) core and dibranched galactose and sialic acid residues.[4b−d] In vivo, the oligosaccharide is cleaved stepwise by membrane-bound *β*-galactosidase and Neu-1 sialidase on B and T lymphocytes, respectively, to expose a single GalNAc residue that is essential for activity: hydrolysis of the Thr−GalNAc bond by secreted α-*N*-acetyl-D-galactosaminidase (GalNAcase) results in Gc-MAF inactivation.[4e,f]

The macrophage activating function of Gc-MAF suggests its possible use in therapy as an immunostimulatory agent;[3] several studies demonstrated its effectiveness in murine tumor models.[4e−g] However, its provenience from mammalian serum and the multiplicity of its functions restrict its application in therapy. One possible approach to facilitate the use of Gc-MAF consists of the design of a miniaturized protein analogue on which the active site of Gc-MAF could be displayed. The grafting of a binding epitope on a folded, stable protein is a well-established approach to the design of biologically active miniature protein.[5−10] In this paper, we are applying this approach to the display of post-translationally modified epitopes. Ideally, a scaffold should be stable and monomeric at physiological conditions, soluble, and structurally characterized. The requirement for glycosylation introduces additional constraints: the size must be within the limits for solid-phase synthesis of peptides, to facilitate the direct introduction of the necessary GalNAc−Thr. To allow for the preparation of large quantities of analogue, the scaffold should also be amenable to expression in organisms capable of carrying out the correct glycosylation.[11]

A visual inspection of the crystal structure of Gc-MAF[12] shows that the glycosylation site, Thr 420, is part of a helical hairpin that abuts the main structure (Figure 1A). An analysis of the backbone dihedral angles shows that Thr 420 is the first helical residue, immediately following a Pro residue that signals the end of the loop. We hypothesized that the glycosylated loop would contain the minimal primary and secondary structure elements necessary for activity. According to the criteria listed above, the three-helix bundle is a suitable system for the display of an inter-helix loop. In particular, de novo designed three-helix bundles derived from Coil-Ser[13] rely on the assembly of the helices for proper folding, while the loops serve essentially to stabilize the topology, suggesting the possibility of swapping these linkers with the more structured loop of Gc-MAF. Two of them, α3D[14] and α3W,[15] were structurally characterized. Both proteins were modeled using the procedure described in the Supporting Information. Briefly, the loop was overlaid onto the structures of α3W and α3D, using the position of apolar residues in the sequence and the coordinates of the helices' backbone atoms as a guide; the glycosylated threonine and its flanking residues were then spliced onto the structure of the scaffold bundle. The process was repeated for the second loop. On the basis of the modeling studies, we selected an analogue derived from α3W,[15] MM1 (Figure 1).

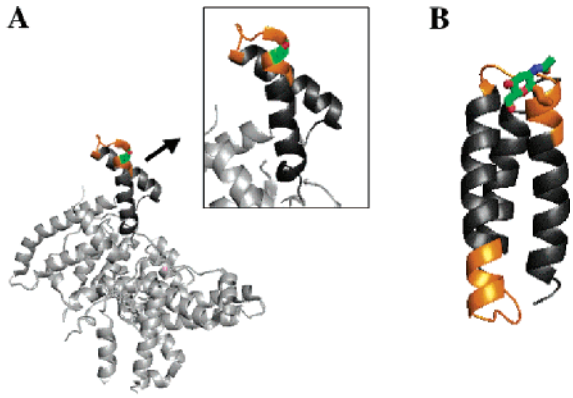

Figure 1. Design of MM1, a Gc-MAF mimic based on a de novo designed three-helix bundle, α3W. Ribbon representation (Pymol, DeLano Scientific) of Gc-MAF (A) and of GalNAc-MM1 (B); the glycosylated threonine is shown in red. Sequence alignment of α3W, residues 14−35, Gc-MAF/G13 (blue), residues 410−424, corresponding to the glycosylated loop, and of MM1, loops 1 and 2 (residues 14−35 and 36−53); the residues grafted from VDBP are highlighted in blue. In this study, only loop 1 was glycosylated.

The resulting protein and its monoglycosylated form, MM1 and GalNAc-MM1, were synthesized by standard solid-phase methods. The circular dichroism (CD) spectra of the two proteins overlap almost completely and exhibit minima at 222 and 208 nm typical of highly helical proteins. In contrast, the CD spectrum of GalNAc-G13, a peptide derived from VDBP synthesized as a control (Figure 1), is typical of a random coil structure. MM1 was shown to be

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10.1021/ja0604212 CCC: $33.50

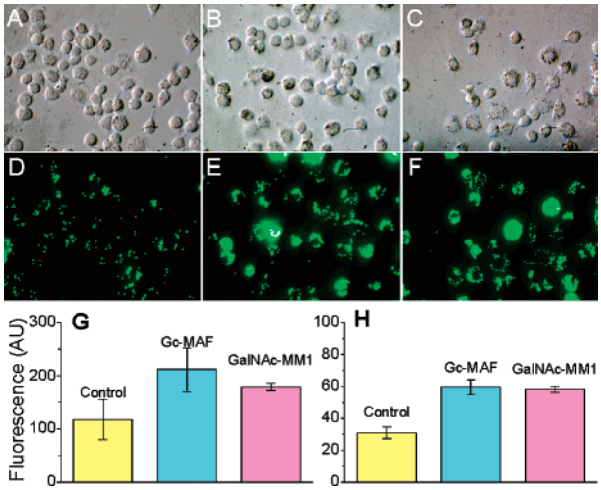

Figure 2. Enhancement of phagocytosis in macrophages. (A−C) Phase contrast and (D−F) fluorescent micrographs of phagocytosis of opsonized latex beads (fluorescein labeled) by cultured macrophages. (A and D) Negative control; (B and E) and (C and F) cells treated with Gc-MAF or GalNAc-MM1, respectively. (G−H) Plot of intracellular fluorescence following phagocytosis of *E. coli* particles in (G) secondary macrophages or (H) primary macrophages (negative control, yellow; Gc-MAF, cyan; GalNAc-MM1, magenta). Identical concentrations were used for the assay (see Supporting Information).

monomeric by equilibrium sedimentation analysis (Supporting Information), which yielded an apparent molecular weight in solution of 7790 ± 130 Da (calculated: 7891 Da). MM1 and GalNAc-MM1 are stable to thermal denaturation in the 20−90 °C range. The thermodynamic stability of MM1 was further evaluated by chemical denaturation (Supporting Information, Figure S3). The protein undergoes a reversible, cooperative denaturation transition with a free energy of folding, ΔG_{H_2O}, of −3.23 ± 0.1 kcal mol^{-1}; for comparison, de novo designed three-helix bundles in the α3 family,[14,15] which includes the original scaffold protein, exhibit values of ΔG_{H_2O} in the −4.6 to −6.2 kcal mol^{-1} range.

Taken together, these data indicate that the spliced loop is well tolerated by and does not interfere significantly with the secondary and tertiary structure of the three-helix bundle scaffold.

The ability of GalNAc-MM1 to enhance Fc-receptor-mediated phagocytosis was assessed on cultivated macrophages. Immunoglobulin-coated fluorescent latex beads were incubated with RAW 264.7 cells previously stimulated with Gc-MAF or GalNAc-MM1 and were observed with an epifluorescent microscope after a short incubation (30 min). The results are illustrated in Figure 2 (top panel); micrographs of different fields randomly selected show a strong increase of fluorescence over the control (panel D) in cells exposed to GalNAc-MM1 (F) at concentrations comparable with those of Gc-MAF (E).

Gc-MAF, GalNAc-MM1, and GalNAc-G13 were compared more quantitatively by monitoring the total fluorescence measured after uptake of fluorescein conjugated *E. coli* K-12 particles by the cells; the extracellular fluorescence due to a specific absorption is quenched before measurements.[16] On RAW 264.7 cells (Figure 2G), GalNAc-MM1 had about the same effect as Gc-MAF. GalNAc-G13 showed minimal residual activity (only 14% over the control, see Supporting Information), while nonglycosylated MM1, VDBP, and BSA had no effect. The experiment was repeated on freshly harvested murine peritoneal macrophages (Figure 2H); the phagocytic stimulation effect was more significant (90% increase over the control) and showed lower variability, possibly because cultivated macrophages have a higher degree of intrinsic phagocytic activity.

The displaying of a short loop containing a GalNAc-modified threonine on a stable helical scaffold results in a miniature glycoprotein, which exhibits a biological activity comparable to that of the naturally occurring protein. Future studies involving generation and analysis of a library of mutants could clarify the sequence and structural requirements for the activation of macrophages. Further, an optimized protein could lead to Gc-MAF analogues with increased activity for use in therapy.

Acknowledgment. We thank Dr. James D. Lear for access to the analytical ultracentrifuge, Tong Fu for maintaining the RAW 264.7 cell line, and Olga Ananieva for help with mice cells harvest. This work was supported in part by ADCRC, The Wallace Foundation, and the Biodesign Institute at ASU.

Supporting Information Available: Computer modeling details, peptide sequence and synthesis, and experimental conditions for CD spectroscopy, analytical ultracentrifugation, and biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA0604212