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Introduction of germline residues improves the stability of anti-HIV mAb 2G12-IgM

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ABSTRACT

Immunoglobulins M (IgMs) are gaining increasing attention as biopharmaceuticals since their multivalent mode of binding can give rise to high avidity. Furthermore, IgMs are potent activators of the complement system. However, they are frequently difficult to express recombinantly and can suffer from low conformational stability. Here, the broadly neutralizing anti-HIV-1 antibody 2G12 was class-switched to IgM and then further engineered by introduction of 17 germline residues. The impact of these changes on the structure and conformational stability of the antibody was then assessed using a range of biophysical techniques. We also investigated the effects of the class switch and germline substitutions on the ligand-binding properties of 2G12 and its capacity for HIV-1 neutralization. Our results demonstrate that the introduced germline residues improve the conformational and thermal stability of 2G12-IgM without altering its overall shape and ligand-binding properties. Interestingly, the engineered protein was found to exhibit much lower neutralization potency than its wild-type counterpart, indicating that potent antigen recognition is not solely responsible for IgM-mediated HIV-1 inactivation.

1. Introduction

Monoclonal antibodies (mAbs) are increasingly important therapeutics for the treatment of cancer as well as autoimmune and infectious diseases. The clinical success of mAbs is based on their potential to bind their antigens with high affinity and specificity, their long *in vivo* half-life and their effector functions such as antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity. The majority of currently approved mAbs are full-size IgGs (150 kDa), but smaller versions such as minibodies (80 kDa), Fab fragments (50 kDa) or scFv derivatives (27 kDa) are emerging as alternatives [1]. However, many of these new variants suffer from a short half-life or the absence of binding sites for ligands that trigger effector functions.

Another alternative to IgGs are IgMs. These large polymeric antibodies (~970 kDa in their pentameric form) are of increasing importance as therapeutics. IgMs are the first antibodies to be produced during a humoral immune response and thus tend to have low affinity, but their multivalent mode of binding allows for high avidity. Moreover, their complex structure makes them very effective in activating the complement system [2]. It has been demonstrated that IgMs can be employed in anti-cancer therapy [3–6], for combating microbial infections [7,8] or the treatment of graft-versus-host disease [9]. IgMs are considered to be difficult to produce in cell factories or other expression platforms [10,11], and the purified proteins frequently suffer from decreased conformational stability and heterogeneity in oligomeric structure. However, a human IgM has been recently produced in a commercially feasible scale [12].

Here we have investigated how the introduction of germline residues into a human immunodeficiency virus type 1 (HIV-1)-neutralizing IgM modifies its conformational and thermal stability as well as antigen binding and neutralization potency. The IgG version of the broadly neutralizing anti HIV-1 mAb 2G12 [13] exhibits a unique domain-swapped

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structure, which enables it to bind specifically to a highly conserved cluster of high-mannose *N*-glycans exposed on the viral envelope. A class switch from IgG to IgM improved virus neutralization substantially [14]. However, considerable heterogeneity was observed with respect to the oligomeric state of 2G12-IgM, which was attributed to a reduced conformational stability [15]. In the present study, a number of germline residues was selected by rational design and then incorporated into 2G12-IgM to improve the biochemical properties of the protein. Altogether, 17 changes were introduced into the framework of the variable domains of the heavy and light chains, aiming at an increase of protein stability while preserving antigen recognition. A thorough biophysical comparison of 2G12-IgG and its two IgM variants enabled us to determine the conformational and thermal stability of the three recombinant proteins and elucidate their unfolding pathways. Additionally, structural information was derived from small-angle X-ray scattering (SAXS) studies. Finally, the ligand-binding properties of wild-type and variant 2G12-IgMs were assessed by ELISA and neutralization assays.

2. Materials and methods

2.1. Cloning and establishment of cell lines

Highly purified 2G12-IgG was a gift from Polymun Scientific (Immunbiologische Forschung GmbH, Klosterneuburg, Austria). The generation of CHO lines producing wild-type 2G12-IgM (IgM-012) as well as a control antibody (IgM-617) was described previously [16]. The germline variant of 2G12-IgM (IgM-012_GL) was designed according to standard humanization strategies for monoclonal antibodies [17]. Briefly, the germline genes with the highest similarity to vH and vL of IgM-012 were identified as IGHV3-21/JH3 and IGKV1-5/JK1, respectively, using the online tool IMGT/V-QUEST [18,19] and aligned with the variable regions of IgM-012 using CLUSTAL 2.0.8. Suitable replacements were then chosen based on a superimposition of an IgM-012 model with the structure of 2G12-IgG (PDB: 1OM3) using Swiss-PDBViewer [20]. Finally, codon-optimized sequences (Life Technologies) were cloned into bi-cistronic pIRES vectors (Clontech, # 631621) and used for transfection of CHO DG44 cells (Invitrogen, # A10999-01) to establish a CHO cell line producing IgM-012_GL, as described previously [14,16].

Recombinant cell lines were routinely cultivated in chemically defined ProCHO5 medium (Lonza, # BE12-766Q) supplemented with 4 mM L-glutamine (Biochrom, # K0302), 15 μg/mL phenol red (Sigma-Aldrich, # P0290), 0.5 mg/mL G418 (PAA, # P11-012) and 100 nM methotrexate (Sigma-Aldrich, # M8407) [15].

2.2. Purification of IgMs

Collected cell-culture supernatants were concentrated by ultradiafiltration using a Millipore Labscale™ TFF System (Millipore, # XX42LSS12). In the case of IgM-617, the concentrate was stored overnight at 4 °C [21] followed by centrifugation at 8000 *g* for 30 min at 4 °C. Precipitated IgM-617 was dissolved in 0.2 M $NaHCO_3$, 0.15 M NaCl (pH 8.5) containing 3 M urea and then dialyzed against 0.2 M $NaHCO_3$, 0.15 M NaCl (pH 8.5).

IgM-012 and IgM-012_GL concentrates were subjected to affinity chromatography using IgM CaptureSelect Affinity Matrix (Life Technologies, # 289010). 0.1 M glycine (pH 3.0) was used as elution buffer. Eluted IgMs were immediately neutralized to pH 7.0 using Tris–HCl (pH 9.5). Finally, dialysis against 0.2 M $NaHCO_3$, 0.15 M NaCl (pH 8.5) was performed.

Purified protein samples were loaded onto NuPage® gradient 3–12% Bis–Tris gels (Life Technologies, # BN1001BOX) and run at 200 V for 60 min in Tris-Acetate SDS buffer (Life Technologies, # LA0041). Gels were stained either with silver [22] or Sypro® Ruby (Bio-Rad Laboratories, # 170-3126) [15,23]. NativeMark™ unstained protein standard (Life Technologies, # LC0725) was used to estimate the molecular mass of the IgM bands. Densitometric analysis of silver-stained gels was done using Quantity One (Bio-Rad).

Table 1a
Germline IGHV3-21/JH3 residues introduced into the variable regions of the heavy chain of IgM-012 leading to IgM-012_GL. Amino acids previously identified as structurally or functionally important [32–37] were kept unchanged.

Position	2G12	IGHV3/JH3	Change	Reasoning
14	A	P	No	Crucial for domain exchange [34]
19	I	R	No	Hydrophobic patch in VH/VH′ interface [33]
23	G	A	G > A	
24	V	A	V > A	
26	N	G	No	N might contribute to conformation
28	R	T	No	Vernier zone [37]
29	I	F	No	Vernier zone [37]
31	A	S	No	Contact with antigen [33,34]
32	H	Y	No	Contact with antigen [33,34]
33	T	S	No	Contact with antigen [33,34]
39	R	Q	No	Crucial for domain exchange [34]
40	V	A	V > A	
43	G	K	No	Positioned in VH/VL interface [34]
49	A	S	No	Vernier zone [37]
52A	T	S	No	Contact with antigen [33]
55	T	S	No	Contact with antigen [34]
57	R	I	No	Important in VH/VH′ interface [33]
58	D	Y	No	Important according to [33]
62	A	S	No	Both residues small and uncharged
69	V	I	No	Vernier zone [37]
73	D	N	No	Vernier zone [37]
74	L	A	L > A	
75	E	K	No	Crucial for domain exchange [34]
76	D	N	D > N	
77	F	S	No	Hydrophobic patch in VH/VH′ interface [33]
78	V	L	No	Vernier zone
82A	H	N	H > N	
82B	K	S	K > S	
82C	M	L	M > L	
84	V	A	No	V stabilizes structure [33]
89	I	V	I > V	
105	P	Q	No	Unique structural properties of P
108	V	M	V > M	
113	P	S	No	Important for domain swap [33]

2.3. Electronic circular dichroism spectroscopy

Overall secondary structure composition as well as temperature-mediated unfolding was investigated by electronic circular dichroism (ECD) spectroscopy (Chirascan, Applied Photophysics). The instrument

Table 1b
Germline IGKV1-5/JK1 residues introduced into the variable regions of the light chain of IgM-012 leading to IgM-012_GL. Amino acids previously identified as structurally or functionally important [32–37] were kept unchanged.

Position	2G12	IGKV1-5/JK1	Change	Reasoning
2	V	I	I > V	
3	V	Q	Q > V	
18	T	R	T > R	
19	I	V	I > V	
30	E	S	No	Vernier zone [37]
31	T	S	No	Canonical residue [37]
53	T	S	No	Specificity-determining residue [37]
55	K	E	No	Specificity-determining residue [37]
56	T	S	No	Specificity-determining residue [37]
77	G	S	G > S	
80	F	P	F > P	
87	H	Y	No	H unusual at this position
90	H	Q	No	Canonical residue [37]
92	A	N	No	Specificity-determining residue [37]
93	G	S	No	Contact with antigen [33]
96	A	W	No	Specificity-determining residue [37]
103	R	K	R > K	

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