Stacy A. Scott,^a Ken Scott^b and
Helen Blanchard^{a*}^aInstitute for Glycomics, Gold Coast Campus,
Griffith University, Queensland 4222, Australia,
and ^bSchool of Biological Sciences, University of
Auckland, Auckland, New ZealandCorrespondence e-mail:
h.blanchard@griffith.edu.au

Received 11 October 2007

Accepted 12 October 2007

Crystallization and preliminary crystallographic
analysis of recombinant human galectin-1

Galectin-1 is considered to be a regulator protein as it is ubiquitously expressed throughout the adult body and is responsible for a broad range of cellular regulatory functions. Interest in galectin-1 from a drug-design perspective is founded on evidence of its overexpression by many cancers and its immunomodulatory properties. The development of galectin-1-specific inhibitors is a rational approach to the fight against cancer because although galectin-1 induces a plethora of effects, null mice appear normal. X-ray crystallographic structure determination will aid the structure-based design of galectin-1 inhibitors. Here, the crystallization and preliminary diffraction analysis of human galectin-1 crystals generated under six different conditions is reported. X-ray diffraction data enabled the assignment of unit-cell parameters for crystals grown under two conditions, one belongs to a tetragonal crystal system and the other was determined as monoclinic $P2_1$, representing two new crystal forms of human galectin-1.

1. Introduction

The carbohydrate moieties of glycoconjugates display enormous and unparalleled structural variability. Once thought of as simply energy stores and cell-structure constituents, emphasis is now placed on understanding how carbohydrate structure translates to function as the specific interplay between carbohydrates and proteins has been widely described to induce various cellular processes. Now thought of as molecules that transfer biological information, the complex array of structurally diverse carbohydrates has commonly been coined the 'sugar code' (Gabiús *et al.*, 2002, 2004). The protein binding partners of the 'sugar code' are called lectins. Galectins are a type of lectin that are defined by a conserved carbohydrate-recognition domain (CRD) of ~130 amino acids that has a specific affinity for glycoconjugates that contain β -galactosides (Barondes *et al.*, 1994). Galectins are endogenous to a wide range of species and 15 mammalian galectins have currently been described. In short, galectins play numerous roles in regulating various cellular processes to achieve homeostasis. They act both extracellularly and intracellularly within most, if not all, tissues (Hernandez & Baum, 2002; Gray *et al.*, 2005). A high conservation of amino-acid sequence exists amongst mammalian galectins. Cattle, rats and humans exhibit above 85% sequence identity in galectin-1. Adult humans express galectin-1 within most tissues and galectin-1-induced activity has been linked to many effects, including apoptosis of thymocytes and T cells (Perillo *et al.*, 1995; Nguyen *et al.*, 2001; Matarrese *et al.*, 2005), differentiation of fibroblasts to the myogenic lineage (Goldring *et al.*, 2002), promotion of host-pathogen (HIV) interaction (Ouellet *et al.*, 2005) and contradictory biphasic concentration-dependent mitogenic and cytostatic growth regulation (Adams *et al.*, 1996; Scott & Zhang, 2002; Vas *et al.*, 2005). The overexpression of galectin-1 by many cancers also suggests that galectin-1 plays a role in tumorigenesis.

Although galectin-1 may have direct effects on tumorigenesis, protection from immune surveillance following lymphocyte apoptosis may also contribute to tumour proliferation (Scott & Weinberg, 2002;

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van der Brule *et al.*, 2004). In accordance with galectin classification, *in vitro* galectin-1 is able to bind to glycoconjugates that contain β -galactosides, but significantly only in the presence of a reducing agent such as β -mercaptoethanol (β -ME). The X-ray crystal structure of human galectin-1 reveals that when in the presence of β -ME it is a non-covalently bound homodimer consisting of two 14.5 kDa subunits each comprised of antiparallel β -strands that fold into a β -sandwich structure (Lopez-Lucendo *et al.*, 2004). Each subunit possesses a β -galactoside-binding site; these are positioned at opposing ends of the dimer (Lopez-Lucendo *et al.*, 2004). Considering this X-ray structure, the bivalent lectin ability of a dimeric form may mediate galectin-1 activity as it would enable the cross-linking of cells, *via* interaction with cell-surface β -galactosides, (i) to each other, (ii) to the basal lamina or (iii) to the extracellular matrix, which would initiate downstream reaction pathways and ultimately achieve a phenotypic affect (Brewer, 2002). However, this hypothesis can only be part of the picture, as lectin-independent activity has also been described for galectin-1. Non-lectin activity has been attributed to galectin-1-induced growth inhibition (Adams *et al.*, 1996; Vas *et al.*, 2005), the interaction of galectin-1 with activated oncoprotein H-Ras12V (GTP bound; Paz *et al.*, 2001; Prior *et al.*, 2003) and the B-cell-specific transcriptional coactivator OCA-B (Yu *et al.*, 2006) and also the interaction of galectin-1 with small synthetic peptides (Andre *et al.*, 2005). A non-lectin active site has been suggested to be located near the region of the dimeric interface (Adams *et al.*, 1996; Scott & Zhang, 2002; Rotblat *et al.*, 2004), but a monomeric form has also been shown to be devoid of lectin activity and to act as a transforming growth regulator. Circular dichroism indicates the formation of disulfide bridges in the monomeric form, suggesting that a significant change in subunit structure occurs compared with that exhibited in the dimer (Yamaoka *et al.*, 1996; Inagaki *et al.*, 2000; Kadoya & Horie, 2005). Currently, there is no knowledge of the location of the non-lectin active-site within this monomeric form and further investigation into which conformation of galectin-1 mediates which galectin-1 activity is required. Despite this ambiguity, a well documented positive correlation exists between reduced galectin-1 activity *in vitro* and inhibition of the carbohydrate-binding site (*via* addition of excess lactose) and has driven the production of β -galactoside derivatives as a means of galectin-1-specific drug design in the fight against cancer.

A problem intrinsic to the design of carbohydrate-based drugs is that carbohydrate-protein interactions are notoriously weak. The K_d of the disaccharide lactose binding to galectin-1 has been reported as 800 μ M (Giguere, Patnam *et al.*, 2006; Giguere, Sato *et al.*, 2006). The multivalency of larger natural glycoconjugate ligands such as asialofetuin circumvent low affinity to some degree (Andre *et al.*, 1999). Chemically synthesized multivalent compounds in the form of linearly repeated lactose units (Di Virgilio *et al.*, 1999), larger wedge-like (Andre *et al.*, 2001) and starburst (sugar-ball) glycodendrimers (Andre *et al.*, 1999) and lactosamine derivatives composed of substitutions at non-critical hydroxyl groups (Ahmed *et al.*, 1990; Andre *et al.*, 1997) demonstrate variable degrees of increased affinity to galectin-1, but it is to be noted that increasing ligand size does not translate into linearly increasing affinity to galectin-1 (Andre *et al.*, 1999). Conversely, multivalent aglycon compounds have demonstrated K_d values as low as 3.2 μ M against galectin-1 (Giguere, Patnam *et al.*, 2006; Giguere, Sato *et al.*, 2006; Tejler *et al.*, 2006). The development of potent galectin-1-specific therapeutics is a rational field of research in the fight against cancer and possibly HIV infection because galectin-1 knockout mice are fertile and appear normal (Poirier & Robertson, 1993; McGraw *et al.*, 2005; Sakaguchi *et al.*, 2006).

In silico investigations of human and bovine galectin-1 and interactions with aglycon, trisaccharides and tetrasaccharides predict that part of these ligands extend outside the characterized galectin-1 carbohydrate-binding cleft. This suggests the importance of regions adjacent to this site that possibly could provide additional and/or alternative areas to be targeted in the advancement of the design of more potent and specific galectin-1 therapeutics, particularly utilizing modifications of ligands larger than glycoside disaccharides (Ford *et al.*, 2003; Giguere, Sato *et al.*, 2006). Asialofetuin, a large endogenous ligand of galectin-1, is a glycoprotein containing terminating tri-antennary glycosides that demonstrates variation in binding affinity to human galectin-1 across a pH range (Ahmed *et al.*, 1990). It has not yet been established for galectin-1 whether pH-induced structural rearrangements occur in the carbohydrate-binding site and/or adjacent regions that could be responsible for such variation. The crystal structure of wild-type human galectin-1 was obtained at pH 5.6 and several mutant structures have also been determined (Lopez-Lucendo *et al.*, 2004). Determination and subsequent analysis of further X-ray structures of human galectin-1 obtained under different crystallization conditions, along with comparison to the previous wild-type structure, will enable the assessment of structural differences arising from various conditions such as pH that may affect interaction with carbohydrates. This will provide further understanding of how the galectin-1 protein structure adapts and interacts with endogenous ligands in different environments.

Further understanding of the structural features that pertain to protein-ligand binding will advance galectin-1-specific inhibitor design and analysis of the X-ray crystal structures of human galectin-1 will be invaluable. Here we report the cloning, expression, purification, crystallization and preliminary diffraction analysis of human galectin-1 crystals grown under six novel conditions. These preliminary data provide new directions toward structural investigation of this protein that is important in cancer and immunology.

2. Experimental procedures and results

2.1. Cloning of human galectin-1

pProEX HTb-h-gal-1 encodes human galectin-1 fused to a hexahistidine tag and was extracted from h-gal-1 recombinant *Escherichia coli* DH5 α cells (Scott & Zhang, 2002). In order to facilitate crystallization, molecular biology techniques were utilized to generate non-tagged protein. The polymerase chain reaction (PCR) was used to amplify the human galectin-1 gene sequence, LGALS1, using pProEX HTb-h-gal-1 as the template, the forward primer 5'-**CATATGGCTTGTGGTCTGGTCGCCAGC**-3' (GeneWorks) and the reverse primer 5'-**GGATCCTCAGTCAAAGGCCACACATTGATC**-3'. The PCR product was ligated into the blunt-ended pCR-Blunt vector (Invitrogen), producing the pCR-Blunt-gal-1 plasmid. The primers engineered restriction-enzyme sites at regions flanking the LGALS1 gene sequence (bold). Digestion of pCR-Blunt-gal-1 plasmid with *Nde*I and *Bam*HI restriction enzymes allowed sticky-end ligation of LGALS1 into a similarly cut pET-3a vector (Novagen), thus yielding the pET-3a-gal-1 plasmid which encodes non-tagged human galectin-1. The integrity of the LGALS1 gene sequence inserted into the pET-3a vector was assessed by DNA sequencing.

2.2. Expression and purification of human galectin-1

E. coli strain BL21 DE3 was transformed with pET-3a-gal-1 plasmid for overexpression of human galectin-1. Recombinant *E. coli* BL21 DE3 cultures were grown at 303 K in Luria-Bertani (LB)

medium supplemented with $100\ \mu\text{g ml}^{-1}$ ampicillin. On reaching an OD_{600} of approximately 0.5, the cultures were grown for a further 3 h and cells were then harvested by centrifugation at $6000g$ for 15 min and frozen. The expression system is not transcriptionally silent and so requires no inducer for optimal expression of target protein. A previous description of recombinant human galectin-1 purification (Lopez-Lucendo *et al.*, 2004) served as a guide for the following methods. For cell lysis, frozen cell pellets were resuspended in $137\ \text{mM NaCl}$, $2.7\ \text{mM KCl}$, $10\ \text{mM Na}_2\text{HPO}_4$, $2\ \text{mM KH}_2\text{PO}_4$ pH 7.4 (PBS) supplemented with $4\ \text{mM } \beta\text{-ME}$, $1\ \text{mM}$ phenylmethylsulfonyl fluoride and $20\ \mu\text{g ml}^{-1}$ DNaseI to reduce the viscosity upon lysis. The cells were lysed using hen egg-white lysozyme. Cell debris was removed by centrifugation at $20\ 000g$ for 30 min and the supernatant was filtered before being passed through a column of lactosylated Sepharose 4B (Levi & Teichberg, 1981) equilibrated in PBS supplemented with $4\ \text{mM } \beta\text{-ME}$ (PBS/ $\beta\text{-ME}$). The column was then washed with PBS/ $\beta\text{-ME}$ to remove unbound material, followed by elution of the target protein with PBS/ $\beta\text{-ME}$ supplemented with $100\ \text{mM}$ lactose. The eluant was concentrated to $\sim 1\ \text{ml}$ using Ultracel 5k concentrators (Amicon) and further purified by size-exclusion chromatography using Sephacryl S100 resin equilibrated in PBS/ $\beta\text{-ME}$ supplemented with $5\ \text{mM}$ lactose. Elution fractions exhibiting high galectin-1 purity were determined *via* SDS-PAGE analysis and then pooled for concentration and buffer exchange into $20\ \text{mM}$ potassium/sodium phosphate, $5\ \text{mM}$ lactose, $4\ \text{mM } \beta\text{-ME}$ pH 7.0 ('crystallization buffer') using Ultracel 5k concentrators (Amicon). Bradford reagent was used to estimate the final protein concentration and SDS-PAGE analysis (Fig. 1) of $1\ \text{mg ml}^{-1}$ human galectin-1 was then performed as a final check of purity. The non-covalently bound subunits of dimeric human galectin-1 disassociate under SDS-PAGE analysis to yield a band approximately 14.5 kDa in size (lane 2). The high purity of the human galectin-1 protein sample is corroborated by our dynamic light-scattering (DLS) results. Analysis of $10\ \text{mg ml}^{-1}$ human galectin-1 in crystallization buffer ($20\ \text{mM}$ potassium/sodium phosphate, $5\ \text{mM}$ lactose, $4\ \text{mM } \beta\text{-ME}$ pH 7.0) at $293\ \text{K}$ using a CoolBatch 90 T instrument (Precision Detectors) reveals a reproducible single compact peak across 20 experiment repeats (Fig. 2). The low polydispersity or 'spread' of the peaks (average polydispersity 25%) is indicative of a very homogeneous protein sample. The average hydrodynamic radius (R_h) is $1.88\ \text{nm}$, representing the dimeric (the calculated radius of gyration is $1.98\ \text{nm}$; that of the monomer is $1.38\ \text{nm}$) form of human galectin-1.

2.3. Crystallization of human galectin-1

Human galectin-1 in the presence of lactose (ligand) and $\beta\text{-ME}$ was crystallized under six different conditions *via* hanging-drop vapour diffusion at $295\ \text{K}$ using 24-well plates (0.5 ml reservoir solution; crystals A–F; Fig. 3). Crystals A–E were obtained within days and crystal F within three months from drops containing $1\ \mu\text{l}$ protein solution (galectin-1 at a concentration of $10\ \text{mg ml}^{-1}$ in crystallization buffer) and $1\ \mu\text{l}$ reservoir solution as listed in Table 1. Except for crystal A (Fig. 3a), all crystals were reproducible.

2.4. X-ray diffraction analysis

The protein nature of the crystals grown under all six new conditions has been demonstrated *via* X-ray diffraction analysis. Data from crystals grown under two conditions enabled the determination of unit-cell parameters and for one of these enabled determination of the structure by molecular replacement; refinement is in progress of this new crystal structure of human galectin-1. Conditions are currently being further optimized; here, we report the preliminary

findings. Crystals A and B (Figs. 3a and 3b) were mounted in quartz capillaries (0.7–1.0 mm in diameter) and tested for X-ray diffraction at $295\ \text{K}$ using a Bruker SMART 6000 diffractometer equipped with a SMART 6000 CCD detector and a MacScience M06X^{CE} rotating-anode generator ($\text{Cu K}\alpha$; $\lambda = 1.5418\ \text{\AA}$). Crystal A (Fig. 3a) cracked during loading into the capillary; the largest fragment diffracted to $2.4\ \text{\AA}$ but decayed significantly after 20 min. The fragile hexagonal-shaped crystal B (Fig. 3b) diffracted to only $3.5\ \text{\AA}$, with the diffraction pattern indicating internal crystal disorder. Under higher pH conditions (pH 6.5) thin plate-like crystals were produced (Table 1, crystal C; Fig. 3c). One crystal was dipped in cryoprotectant solution comprising 30% PEG 8000, $0.1\ \text{M}$ sodium cacodylate pH 6.5, $0.2\ \text{M}$ ammonium sulfate, 1% $\beta\text{-ME}$, 15% glycerol and flash-frozen using liquid N_2 prior to X-ray diffraction analysis at beamline 8.3.1 at the Advanced Light Source synchrotron (California, USA; wavelength $1.1159\ \text{\AA}$ at $100\ \text{K}$) using an ADSC CCD detector. Crystal C diffracted to $3\ \text{\AA}$; the diffraction data (frames with 1° φ oscillation range and with 1 s exposure) showed high mosaicity and the unit-cell parameters and crystal system were ambiguous. The clustered plate-like crystal D, indicated by an arrow in Fig. 3(d), was dipped in cryoprotectant solution comprising 20% PEG 8000, $0.1\ \text{M}$ sodium cacodylate pH 6.5, $0.2\ \text{M}$ magnesium acetate, 1% $\beta\text{-ME}$, 20% glycerol and flash-frozen with liquid N_2 . X-ray diffraction quality was analysed at $100\ \text{K}$ at beamline BL9-2 at the Stanford Synchrotron Radiation Laboratory (SSRL, California, USA; wavelength $0.97946\ \text{\AA}$) using a MAR325 CCD detector. Crystal D diffracted to $2.7\ \text{\AA}$; the pattern was anisotropic, with very weak diffraction exhibited along one axis, and the unit-cell parameters are currently undetermined. The small cube-like crystal E (Fig. 3e) was dipped in cryoprotectant solution

Figure 1
SDS-PAGE (13%, stained with Coomassie blue) analysis of human galectin-1 after purification. Lane 1, ladder. Lane 2, band ($\sim 14.5\ \text{kDa}$) representing disassociated subunits of the human galectin-1 homodimer.

Figure 2
Dynamic light-scattering analysis of a $10\ \text{mg ml}^{-1}$ human galectin-1 protein sample under reducing conditions and in the presence of lactose. A single 'compact' peak is reproduced throughout 20 experimental repeats. Shown here is a peak from one experiment. The average of 20 repeats reveals a homogeneous protein sample of dimeric human galectin-1 that has a hydrodynamic radius (R_h) of $1.88\ \text{nm}$ (calculated radius of gyration is $1.98\ \text{nm}$).

Table 1

Crystallization conditions (reservoir solutions), crystal dimensions and maximum observed diffraction resolution for crystals A–F.

Crystal	Reservoir solution	Crystal dimensions (mm)	Maximum diffraction observed (Å)
A	20% 2-propanol, 0.1 M sodium citrate pH 5.6, 20% PEG 4000, 1% β -ME	1.63 $\times$ 0.25 $\times$ 0.25	2.4
B	1.8 M (NH ₄) ₂ SO ₄ , 0.1 M sodium citrate pH 5.6, 0.2 M sodium tartrate, 1% β -ME	0.5 $\times$ 0.5 $\times$ 0.1	3.5
C	30% PEG 8000, 0.1 M sodium cacodylate pH 6.5, 0.2 M (NH ₄) ₂ SO ₄ , 1% β -ME	0.75 $\times$ 0.25 $\times$ 0.025	3.0
D	20% PEG 8000, 0.1 M sodium cacodylate pH 6.5, 0.2 M magnesium acetate, 1% β -ME	0.75 $\times$ 0.25 $\times$ 0.08	2.7
E	20% PEG 550, 0.1 M MES pH 6.0, 0.01 M ZnSO ₄ , 1% β -ME	0.13 $\times$ 0.13 $\times$ 0.13	2.8
F	30% PEG 4000, 0.1 M Tris–HCl pH 8.0, 0.2 M Li ₂ SO ₄ , 1% β -ME	0.35 $\times$ 0.063 $\times$ 0.063	2.0

comprising 20% PEG 550, 0.1 M MES pH 6.0, 0.01 M ZnSO₄, 1% β -ME, 5% glycerol and flash-frozen with liquid N₂ before X-ray diffraction analysis at SSRL BL9-2. Crystal *E* diffracted anisotropically to a maximum resolution of 2.8 Å. Data were collected (frames with 0.5° φ oscillation and 20 s exposure time), indexed and processed using *MOSFLM* (Leslie, 1992) and scaled using *SCALA* (CCP4 suite; Collaborative Computational Project, Number 4, 1994), giving a data set that was complete to 3.9 Å resolution (Table 2). Crystal *E* represents a new crystal form of human galectin-1 that belongs to a tetragonal crystal system, with unit-cell parameters $a = b = 113.5$, $c = 62.6$ Å. Further optimization of this crystallization condition is in progress. Crystal *F* (Fig. 3f) was tested for X-ray diffraction at 295 K using our in-house X-ray source (described above for crystals A and B). Crystal *F* diffracted to a maximum resolution of 2.0 Å. Data were collected (frames with 0.3° φ oscillations and 60 s

Table 2

Diffraction data statistics for crystals *E* and *F* (depicted in Figs. 3e and 3f).

	Crystal <i>E</i>	Crystal <i>F</i>
Resolution (Å)	54.9–3.9 (4.1–3.9)	60.3–2.4 (2.5–2.4)
Total No. of observations	53867 (7586)	79390 (10825)
Total No. of unique observations	7432 (1058)	21146 (3006)
Redundancy	7.2 (7.2)	3.8 (3.6)
Completeness (%)	99.7 (100)	97.8 (96.4)
$I/\sigma(I)$	6.8 (4.9)	8.8 (3.2)
$R_{\text{merge}}^{\dagger}$ (%)	7.9 (11.5)	6.4 (24.2)
Crystal system/space group	Tetragonal 4/m (Laue group)	Monoclinic $P2_1$
Unit-cell parameters (Å, °)	$a = b = 113.5$, $c = 62.6$	$a = 43.58$, $b = 60.34$, $c = 106.07$, $\beta = 93.52$

$$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \overline{I(hkl)}|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

exposure time), indexed and processed using *MOSFLM* (Leslie, 1992) and scaled using *SCALA* (CCP4 suite; Collaborative Computational Project, Number 4, 1994), giving a data set that was complete to 2.4 Å resolution (Table 2). Crystal *F* represents another new crystal form of human galectin-1 that belongs to a monoclinic crystal system, with unit-cell parameters $a = 43.58$, $b = 60.34$, $c = 106.07$ Å, $\beta = 93.52^\circ$. Using a human galectin-1–lactose complex (PDB code 1gzv; Lopez-Lucendo *et al.*, 2004) as a search model and *AMoRe* (Navaza, 1994), a clear molecular-replacement solution in space group $P2_1$ was determined. Refinement of this new crystal structure is ongoing for its ultimate use in *in silico* structure-based drug design of novel human galectin-1 inhibitors.

The role of galectin-1 in severe human ailments is well established and the consequent need for the development of potent specific galectin-1 inhibitors is recognized as becoming more urgent. Currently, one X-ray structure of wild-type human galectin-1 and two mutant galectin-1 structures (with a number of different ligands bound) are available for *in silico* structure-based drug design (Lopez-Lucendo *et al.*, 2004). We focus on generating further X-ray crystallo-

Figure 3

Human galectin-1 co-crystallized with lactose in the presence of β -ME. Crystals A–F shown in (a)–(f) were produced *via* hanging-drop vapour diffusion at 295 K. The scale bar represents 0.5 mm (all photographs are on the same scale).

graphic structures of wild-type human galectin-1 obtained under conditions that differ from those reported, incorporating variations in pH, and also structures determined under different crystal systems. These will provide valuable insight into the ability of this protein to interact with both endogenous receptors as well as small molecules, providing unique structural information that can be utilized for structure-based drug design.

HB gratefully acknowledges the receipt of a Byrne family PhD scholarship awarded to SS. HB acknowledges financial support from the Access to Major Research Facilities Programme, a component of the International Science Linkages Programme established under the Australian Government's innovation statement Backing Australia's Ability. We thank the staff at beamline 8.3.1 at the Advanced Light Source (ALS) for assistance in crystallographic data collection. Beamline 8.3.1 at the Lawrence Berkeley National Laboratory, Advanced Light Source was funded by the NSF, the University of California and Henry Wheeler. Portions of this research were carried out at the Stanford Synchrotron Radiation Laboratory, a national user facility operated by Stanford University on behalf of the US Department of Energy, Office of Basic Energy Sciences. The SSRL Structural Molecular Biology Program is supported by the Department of Energy, Office of Biological and Environmental Research and by the National Institutes of Health, National Center for Research Resources, Biomedical Technology Program and the National Institute of General Medical Sciences. Specifically, we thank Mr Xing Yu for data collection performed at SSRL and for assistance by BL9-2 staff. SS thanks Ms Anna Gangl for assistance in the crystallization of human galectin-1.

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