Zn-α_2-glycoprotein, an MHC Class I-Related Glycoprotein Regulator of Adipose Tissues: Modification or Abrogation of Ligand Binding by Site-Directed Mutagenesis†

Lindsay C. McDermott,‡,§,|| June A. Freel,‡ Anthony P. West,⊥ Pamela J. Bjorkman,⊥,# and Malcolm W. Kennedy*,‡

Division of Environmental and Evolutionary Biology, Institute of Biomedical and Life Sciences, and Department of Chemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K., and Division of Biology and Howard Hughes Medical Institute, California Institute of Technology, Pasadena, California 91125

Received September 15, 2005; Revised Manuscript Received December 16, 2005

ABSTRACT: Zn-α_2-glycoprotein (ZAG) is a soluble lipid-mobilizing factor associated with cancer cachexia and is a novel adipokine. Its X-ray crystal structure reveals a poly(ethylene glycol) molecule, presumably substituting for a higher affinity natural ligand, occupying an apolar groove between its α_1 and α_2 domain helices that corresponds to the peptide binding groove in class I MHC proteins. We previously provided evidence that the groove is a binding site for hydrophobic ligands that may relate to the protein's signaling function and that the natural ligands are probably (polyunsaturated) fatty acid-like. Using fluorescence-based binding assays and site-directed mutagenesis, we now demonstrate formally that the groove is indeed the binding site for hydrophobic ligands. We also identify amino acid positions that are involved in ligand binding and those that control the shape and exposure to solvent of the binding site itself. Some of the mutants showed minimal effects on their binding potential, one showed enhanced binding, and several were completely nonbinding. Particularly notable is Arg-73, which projects into one end of the binding groove and is the sole charged amino acid adjacent to the ligand. Replacing this amino acid with alanine abolished ligand binding and closed the groove to solvent. Arg-73 may therefore have an unexpected dual role in binding site access and anchor for an amphiphilic ligand. These data add weight to the distinctiveness of ZAG among MHC class I-like proteins in addition to providing defined binding-altered mutants for cellular signaling studies and potential medical applications.

Zn-α_2-glycoprotein (ZAG)[1] is found in body fluids such as serum, sweat, and seminal and breast cyst fluids (*1*−*3*). It is identical in amino acid sequence to tumor-derived lipid mobilizing factor (LMF), a protein associated with the dramatic loss of adipose body stores in cancer cachexia, and has been shown to stimulate lipolysis by adipocytes in vivo and in vitro (*4*, *5*). A role for ZAG has been proposed in the regulation of body weight, and age-dependent changes in genetically influenced obesity (*6*), and also it regulates melanin production by normal and malignant melanocytes (*7*). It has also recently been classified as a novel adipokine in that it is produced by both white and brown fat adipocytes and may act in a local autocrine fashion in the reduction of adiposity in cachexia (*8*−*10*).

Controlling ZAG/LMF's activity could be life-saving in the management of certain cancers and other cachexia-inducing conditions, and its possible normal role in body fat store homeostasis is deserving of understanding in its own right. ZAG exhibits a class I major histocompatibility complex (MHC) fold but is a soluble protein rather than being anchored to plasma membranes and does not associate with β_2-microglobulin in humans (*11*, *12*). Like antigen-presenting MHC class I proteins, ZAG has an open apical groove, and X-ray crystallography of human-derived ZAG revealed an unidentifiable electron density in a similar position to that occupied by antigenic peptides in classical MHC proteins and glycolipids in isoforms of CD1 (*13*, *14*). This presumptive ligand is not a peptide, and the groove is too small to hold a glycolipid such as is presented by CD1 isoforms.

By analogy with all other MHC class I-related proteins that have an open apical groove [some do not (*15*, *16*)], occupancy by a ligand is probably crucial to ZAG's biological function. Despite all of the structural and biochemical evidence that ZAG binds a ligand, none has so far been found by extraction from protein isolated from biological fluids (*11*, *12*). This difficulty could be because the ligand is labile, heterogeneous, or readily lost during purification procedures. Knowing more about how ZAG interacts with the compounds it has been found to bind, both natural and

† This work was supported by grants from the Wellcome Trust (United Kingdom) to M.W.K.

* To whom correspondence should be addressed. Tel: +44-141-330-5819. Fax: +44-141-330-5971. E-mail: malcolm.kennedy@bio.gla.ac.uk.

‡ Division of Environmental and Evolutionary Biology, Institute of Biomedical and Life Sciences, University of Glasgow.

§ Department of Chemistry, University of Glasgow.

|| Current address: Department of Biochemistry, King's College, Franklin-Wilkins Building, Stamford St., London SE1 9NH, U.K.

⊥ Division of Biology, California Institute of Technology.

Howard Hughes Medical Institute, California Institute of Technology.

[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; CD, circular dichroism; DAUDA, 11-[[[5-(dimethylamino)-1-naphthalenyl]-sulfonyl]amino]undecanoic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MHC, major histocompatibility complex; LMF, lipid mobilizing factor; PUFAs, polyunsaturated fatty acids; WT, wild-type protein; ZAG, Zn-α_2-glycoprotein.

10.1021/bi051881v CCC: $33.50
Published on Web 01/31/2006

artificial, will inform searches for the elusive ligand(s) and its/their role in ZAG's signaling function.

The shape and nature of ZAG's groove are indicative of occupancy by a small hydrophobic molecule, and further work revealed that the unidentified electron density in the original crystal structure was probably poly(ethylene glycol) from the crystallization liquor (*17*). But, our fluorescence- and microcalorimetry-based experiments have shown that ZAG binds hydrophobic ligands in solution, particularly polyunsaturated fatty acids (PUFAs), and there were indirect indications that the binding site for these ligands was in the position suspected (*18*). Moreover, human-derived and bacterial recombinant ZAG proteins behaved identically in the ligand binding assays, although the latter unfolds at a slightly lower temperature than the former, presumably due to the absence of glycosylations in the bacterial recombinant protein.

ZAG's putative ligand binding site is lined predominately by apolar amino acid side chains, and all but one of those within 4.5 Å of the presumptive ligand are apolar. The exception is a prominent arginine (Arg-73) that projects from the side of the groove and could act as a strut to hold the groove open. It could also anchor the charged headgroup of an amphiphilic ligand, as do arginines in cytosolic lipid binding proteins (*19*, *20*), prostaglandin synthase (*21*), and human serum albumin (*22*, *23*). We now find, using site-directed mutagensis, that these amino acid positions fall into two groups, those whose substitution modifies ligand binding to a greater or lesser extent and those that are essential to binding. This new information provides a framework for further investigation of ZAG's natural ligand, how the ZAG−ligand complex interacts with a putative cell surface signaling receptor, and how ligand occupancy is controlled in this unusual member of the MHC class I-like proteins.

MATERIALS AND METHODS

Mutagenesis and Recombinant Protein Production. Mutagenesis was carried out on the ZAG pET23a clone using a Stratagene (La Jolla, CA) QuikChange II site-directed mutagenesis kit. Two primers (complementary to opposite strands of the vector) were designed to encode the intended mutation, and mutated DNA was synthesized following the manufacturer's instructions. Briefly, amplification of mutated DNA was carried out by PCR; parental DNA was removed by incubation of the combined plasmid pool with the restriction enzyme *Dpn*I, followed by transformation of the mutated DNA into XL1-Blue supercompetent cells. The Arg-73 → Ala (R73A) mutant was prepared using a Clontech (Mountain View, CA) transformer site-directed mutagenesis kit. Two mutagenic primers were designed to anneal to the same DNA strand: one primer containing the desired mutation and the other altering a unique restriction enzyme site (*Pvu*II → *Sac*I). Mutated DNA was transformed into strain mutS *Escherichia coli*. The success of the mutagenesis reactions was confirmed by sequencing individual inserts using the T7 promoter primer, and mutated plasmids were transformed into BL21(DE3)pLysE for expression of recombinant proteins. All mutants are named with the original amino acid, the amino acid position, and the new amino acid, using the single letter codes as exemplified; thus, Tyr-117 mutated to Ala is designated Y117A.

Protein Purification. An *E. coli* clone producing ZAG (or a mutant form) as a His-tag fusion protein was purified to apparent homogeneity by nickel affinity chromatography using a Novagen (San Diego, CA) His-bind kit. The purification was carried out under denaturing conditions (8 M guanidine hydrochloride) to release recombinant ZAG from inclusion bodies. Natively folded proteins were produced using a hyperdilution method whereby unfolded protein was progressively added to refolding buffer (0.1 M Tris, pH 8, 2 mM EDTA, pH 8, 0.4 M L-arginine hydrochloride, 0.5 mM oxidized glutathione, 5 mM reduced glutathione) and left at 4 °C overnight to equilibrate. Proteins were concentrated and dialyzed into 50 mM sodium phosphate, pH 7. Before use in analytical experiments, all proteins were passed down an Extracti-Gel D column (Pierce, Rockford, IL) to deplete any intrinsically bound fatty acids acquired during the purification process and subsequently stored at −20 °C. Protein concentrations were determined spectrophotmetrically using theoretical ϵ_{280} values (68650 M^{-1} cm^{-1} for ZAG, R73A, R73E, I76A, and F77A; 62870 M^{-1} cm^{-1} for W115A, W134A, and W148A; 67280 M^{-1} cm^{-1} for Y14A, Y117A, Y154A, and Y161A) calculated from the amino acid content (*24*).

Circular Dichroism (CD). CD spectra were recorded at 20 °C in a JASCO J-600 spectropolarimeter using quartz cells of path length 0.02 cm. Protein concentrations were in the range 0.1−0.5 mg mL^{-1} as estimated by absorbance at 280 nm. Mean residue weights for each recombinant protein were calculated from their amino acid sequence, and these were in turn used to calculate molar ellipticity values.

Fluorescence Spectrofluorometry. Fluorescence measurements were recorded at 25 °C in a Spex FluorMax fluorometer (Jobin Yvon Horiba, Edison, NJ) using 2 mL samples in phosphate-buffered saline (PBS; 171 mM NaCl, 3.3 mM KCl, 17 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2) in a silica cuvette. Intrinsic tryptophan fluorescence emission spectra were recorded at $\lambda_{exc} = 290$ nm of 0.5 μM protein solutions. Raman scattering by solvent was corrected where necessary by subtraction of spectra obtained using appropriate buffer solutions. The dansyl-labeled fatty acid 11-[[[5-(dimethylamino)-1-naphthalenyl]sulfonyl]amino]undecanoic acid (DAUDA; Molecular Probes, Eugene, OR) was stored as a stock solution of 1 mg mL^{-1} in ethanol in the dark at −20 °C. Titration involved nine successive additions of 0.1 μM protein aliquots to a 0.5 μM DAUDA solution in buffer. Data collected at $\lambda_{exc} = 345$ nm were corrected for dilution and fitted by standard nonlinear regression techniques to a single noncompetitive binding model (*25*−*27*) providing an estimate of the dissociation constant for DAUDA binding (K_d). A 10 μM solution of ANS (8-anilino-1-naphthalenesulfonic acid) was excited at 390 nm and the change in fluorescence emission recorded upon the addition of 0.5 μM protein. To assess whether the ANS and fatty acid binding sites were coincident or at least interactive, 0.15, 1.5, and 15 μM additions of docosahexaenoic acid (DHA; Sigma-Aldrich, Poole, Dorset, U.K.) were made to the ANS−protein complex, and changes in ANS fluorescence emission intensity were recorded. A control experiment revealed that addition of DHA to ANS alone did not alter the probe's fluorescence emission properties.

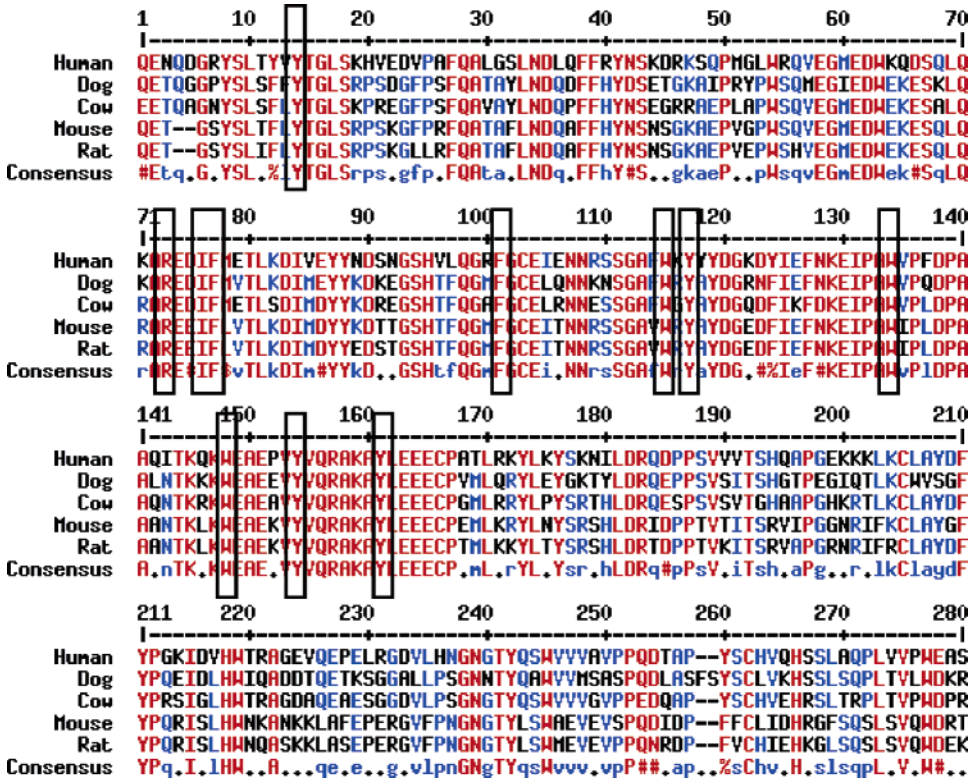

FIGURE 1: Amino acid sequence alignment of human ZAG with that of rat, mouse, dog, and cow (GenBank Accession Codes BC005306, BC105822, NM013478, XM536867, and NM001034331, respectively). Conserved positions are in red and nonidentical but similar amino acids also in red using the groupings defined by the Dayhoff evolutionary substitution matrix, indicated by the symbols $ for L or M, % for F or Y, and # for anyone of N, D, Q, or E. Positions of intermediate consensus are indicated in blue, and black indicates no discernible similarities. The positions of amino acids whose side chains lie within 4.5 Å of the unidentified electron density in the crystal structure of ZAG (*11*), and where the site-directed mutagenesis was carried out, are indicated in the boxes. Leader/signal peptide sequences have been removed, as have short C-terminal sequences that are highly divergent and variable in length. Alignment was carried out using the MultAlin program set for the Dayhoff substitution matrix.

RESULTS AND DISCUSSION

Regional Conservation within Mammalian ZAG Sequences. Figure 1 shows an amino acid alignment of five mammalian ZAG proteins drawn from species in four major clades of placental mammals, using sequences from the dog and cow only very recently available from genome projects. This clearly identifies regions of the proteins that are highly conserved across the mammals and those that are divergent. It is particulary notable that the amino acids immediately adjacent to the putative ligand (boxed) in crystal structures (*11*, *17*) are absolutely conserved among the mammals sampled. All of these lie in highly conserved regions of the protein that map to the immediate region of the putative binding pocket to one end of the space between the helices in the structure (not shown), and regions away from this immediate area show substantial relative diversity. This is strongly indicative of the functional importance of the groove in ZAG's biological function and evolutionary constraints on this region of the protein that does not apply elsewhere. ZAG's ligand binding activity, like that of the peptide (classical class I) and glycolipid (CD1) binding MHC immunity proteins, is likely to be closely linked to biological activity, although ZAG's groove is clearly distinct in terms of its apparent affinity for small amphiphilic ligands and greater exposure to solvent.

Mutant Proteins and Their Structural Integrity. Single site-directed mutant forms of human ZAG were produced as described in Materials and Methods. Each of the amino acids whose side chains lie within 4.5 Å of the unidentified electron density in the crystal structure of human ZAG (*11*, *17*) were changed to alanine, and the prominent Arg-73 was additionally changed to an amino acid of similar size but opposite charge, glutamic acid. All of the mutant proteins gave circular dichroism spectra similar to that of the wild-type protein, with the notable exception of the Arg-73 → Glu (R73E) mutant, which exhibited a substantial change in CD spectrum (Figure 2).

The intrinsic (tryptophan) fluorescence emission spectra of the proteins were also recorded (Figure 3). The mutants showing the greatest changes in emission characteristics were those in which tryptophans had been replaced, each of which gave a significant drop in peak emission intensity attributable to the loss of a Trp fluorophore. Also, the alteration in wavelength of peak fluorescence emission intensity by the Trp substitutions appears to reflect the degree of exposure to solvent of the respective deleted Trp side chains in the wild-type protein (*11*, *28*). The other mutants varied in the extent to which they differed from the wild-type protein with respect to peak wavelength and emission intensities, indicative of consequent alterations to the environments of their Trp side chains. Surprisingly, in light of the changes in CD spectra, neither of the Arg-73 mutants showed substantial changes in their fluorescence emission spectra. Overall, changes in the fluorescence characteristics of the mutants were not predictive of changes in their ligand binding characteristics (see below).

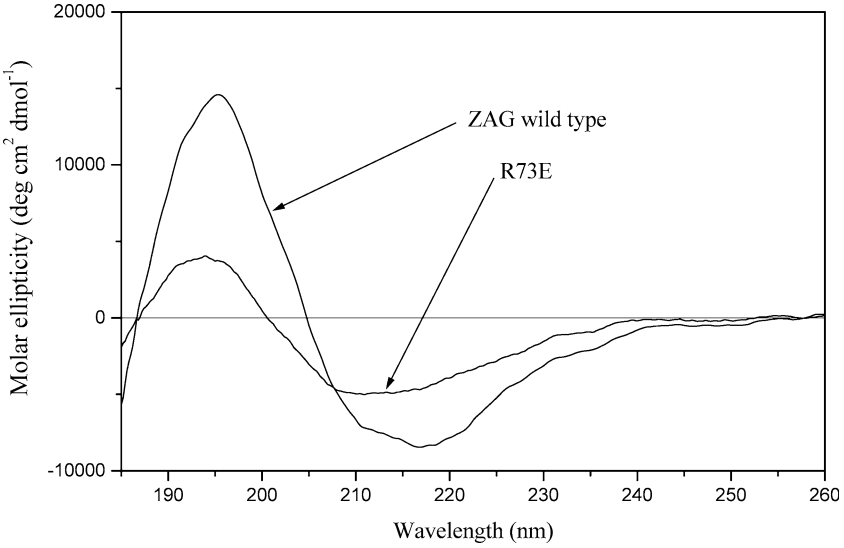

FIGURE 2: Far-UV circular dichroism analysis of wild-type ZAG and R73E mutant. Spectra were collected using a 0.02 cm path length cell with protein solutions in 50 mM sodium phosphate buffer, pH 7. The proteins were measured at a concentration range of 0.1−0.5 mg mL^{-1}, and the spectra were corrected for protein concentration.

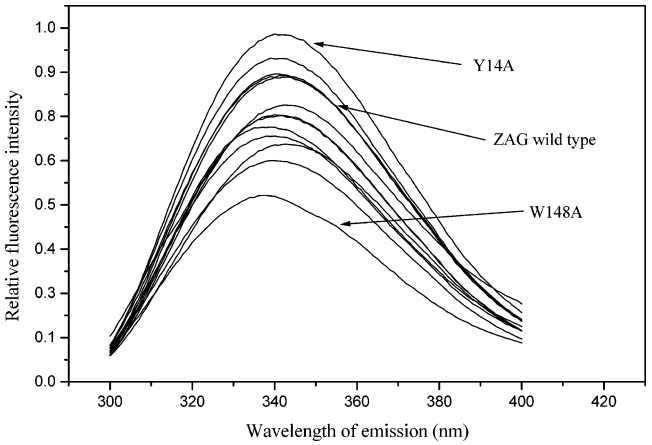

FIGURE 3: Intrinsic tryptophan fluorescence of wild-type and mutant ZAG proteins. Intrinsic fluorescence emission spectra were recorded at $\lambda_{exc} = 290$ nm using wild-type ZAG and mutant proteins at 0.5 μM protein in phosphate-buffered saline, pH 7.3. Wild-type ZAG contains eight tryptophans, the collective emission spectra for which peaks at 342 nm, and which is altered slightly upon addition of fatty acid ligands (*18*). The spectra in descending order of emission intensity recorded at 342 nm are Y14A > R73E > F101A ≈ ZAG wild type ≈ R73A >Y154A > F77A ≈ I76A > Y161A >Y117A > W134A > W115A > W148A. All spectra were corrected for dilution and Raman scatter.

Taken together, these data indicate that, with the particular exception of R73E, the mutant proteins did not depart substantially from the structure of the wild-type ZAG, although this requires confirmation with more definitive structural analysis.

Effects of Mutations on Ligand Binding. Wild-type ZAG has been shown to bind the fluorophore-tagged fatty acid, DAUDA, which undergoes a substantial increase in emission intensity, plus a blue shift in peak wavelength of emission, upon binding to either human-derived or recombinant wild-type ZAG (*18*). The degree of blue shift is less than that found when DAUDA is fully enclosed in the binding sites of specialized lipid transporter proteins (*25*, *27*, *29*) but is consistent with occupancy of a binding site that is not fully segregated from the solvent water. DAUDA is displaced by a range of fatty acids, most efficiently by polyunsaturated forms such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). DAUDA can therefore reasonably be used as a probe for a specific ligand binding site, despite the addition of the dansyl fluorescence reporter group.

The mutant ZAG proteins fell into two distinct groups with respect to DAUDA binding: those in which binding was relatively unaffected or even enhanced and those in which binding was ablated or virtually so (Figure 4, Table 1). DHA displaced DAUDA efficiently in all mutants that retained DAUDA binding (data not shown), indicating that the specificity of binding had not been affected.

Those mutants in which binding was only slightly reduced were W115A, W134A, and W148A, and binding by Y117A was notably enhanced in comparison to the control ZAG wild-type protein. The former three were all substitutions of Trps that contribute to a nest of apolar side chains to one end of the binding cleft (*11*), and replacement with the small aliphatic Ala was seemingly relatively inconsequential to the immediate apolar environment. The Y117A mutant showed enhanced DAUDA binding that might be due to an increase in the volume or accessibility of the binding site, without the loss of binding specificity. Such a conclusion is supported by the results of similar experiments with a nonspecific probe, ANS (see below), and by the fact that Y117A exhibited the greatest decrease in intrinsic (Trp) fluorescence intensity among the non-Trp substitutions, consistent with quenching of remaining solvent-accessible Trps because of increased exposure of their side chains to water.

All of the remaining mutations (Y14A, F101A, F77A, I76A, Y154A, Y161A, R73A, R73E) exhibited near or complete loss of binding. Most of these substituted positions are at the opposite end of the binding groove from the group of Trps, and three are close to Arg-73. This points to a central role for this arginine, or adjacent side chains in ligand binding, in which an interacting network of amino acids may coordinate with a charged headgroup of an amphiphilic ligand.

The disruptive effect of replacing Tyr-154 is more difficult to explain given that its side chain projects at an angle from the inside of helix α2, but upward and away from the ligand's

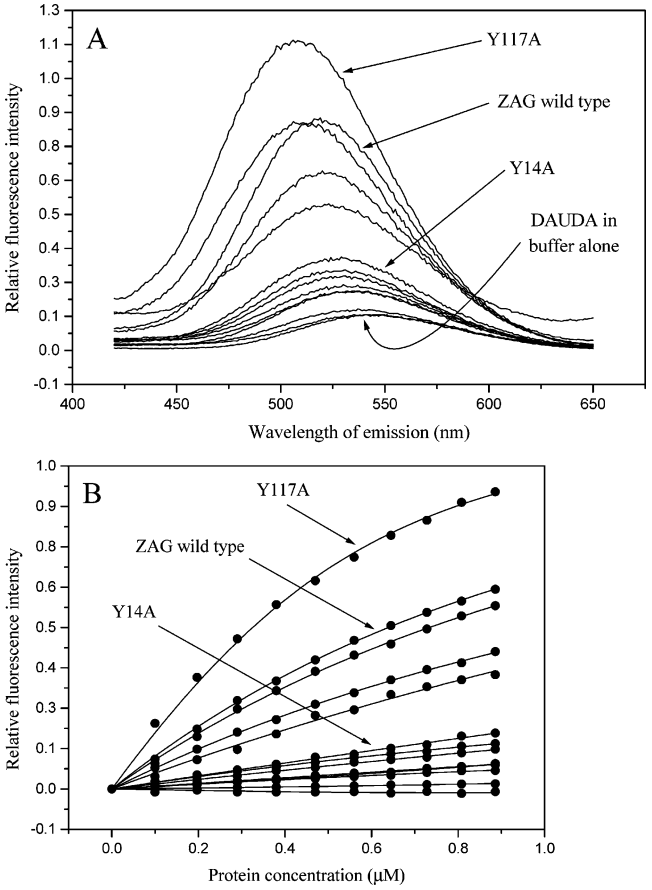

FIGURE 4: Fluorescent fatty acid binding by wild-type ZAG and 12 mutant forms. (A) Fluorescence emission spectra obtained upon addition of 1 μM ZAG wild-type or mutant protein to 0.5 μM DAUDA in PBS (λ_{exc} = 345 nm). Wild-type ZAG produces a shift from 542 nm (DAUDA in buffer) to 519 nm upon addition of protein. R76A and R76E failed to bind DAUDA while the remaining mutants showed diverse binding activities. At an emission wavelength of 519 nm, the intensities for DAUDA in complex with equivalent amounts of each protein were Y117A > ZAG wild type > W115A > W134A > W148A > Y14A > F101A > F77A > Y154A > I76A > Y161A > R73A ≈ R73E. (B) Progressive additions of 0.1 μM protein were made to 0.5 μM DAUDA solution, and the fluorescence emission intensity was measured at the maximum wavelength of fluorescence emission for DAUDA bound to wild-type ZAG (519 nm). The resulting fluorescence titration curves were corrected for dilution and fitted using standard nonlinear regression techniques to a single noncompetitive binding model as previously described (*25*, *26*), providing an estimate of the dissociation constant for DAUDA binding (K_d; Table 1). The rank order of fluorescence maximum emission achieved for each protein in the experiment was as for (A) above.

position. The substitution for Ala may alter the disposition of the α_2 helix, or Tyr-154's aromatic ring may play a role in ligand binding by packing against and shielding it from solvent in the wild-type protein.

Effects of Mutations on Conformation of the Binding Site. The mutations may either corrupt the gross structure of the binding site or leave it intact but without an amino acid side chain that directly controls its ligand specificity. We therefore also examined interactions between ZAG mutants and the nonspecific hydrophobic probe ANS (Figure 5, Table 1), which is widely used to detect exposed hydrophobic regions of misfolded proteins and will also bind to hydrophobic ligand binding sites of correctly folded proteins. In complex with all of the mutants that retained fatty acid binding

Table 1: Effects of Mutations on Ligand Binding by ZAG

protein[a]	DAUDA binding K_d (μM^{-1})[b]	protein + DAUDA relative emission intensity[c]	protein + ANS relative emission intensity[d]	displacement of ANS by DHA[e]
Y117A	0.19 ± 0.05	142	668	+
ZAG WT	0.73 ± 0.08	100	100	+
W115A	0.92 ± 0.15	88	203	+
W134A	1.23 ± 0.14	70	191	+
W148A	3.05 ± 1.72	57	216	+
Y14A	−	30	22	−
F101A	−	24	52	±
F77A	−	20	246	+
Y154A	−	17	42	±
I76A	−	13	22	−
Y161A	−	13	82	+
R73A	−	4	0	−
R73E	−	0	17	−

[a] The mutant and wild-type (WT) forms of ZAG are listed in order of maximal emission intensities produced in the titration experiments shown in Figure 4B. [b] Dissociation constant (K_d) estimates from fluorescence titration curves (Figure 4B), corrected and calculated as described in Materials and Methods. Cases where the binding function had been compromised to such a degree that no reliable K_d value was obtainable are indicated (−). [c] Fluorescence emission intensities of 1 μM DAUDA with 2 μM each of the mutant proteins, expressed as a percentage of intensity with wild-type ZAG. Data were taken from the spectra given in Figure 4A, corrected for dilution and Raman scatter, and subtracted for emission intensity of DAUDA in buffer alone. All values used were taken at the wavelength of peak fluorescence emission by the wild-type ZAG−DAUDA mixture (514 nm) corrected as above. [d] As for footnote *c* but with 10 μM ANS, and the values used were taken at a fluorescence emission wavelength of 480 nm. [e] Displacement of ANS from the binding site of protein−ANS complexes by subsequent addition of 15 μM DHA, classified as displacement observed (+), no displacement observed (−), or slight or questionable displacement observed (±).

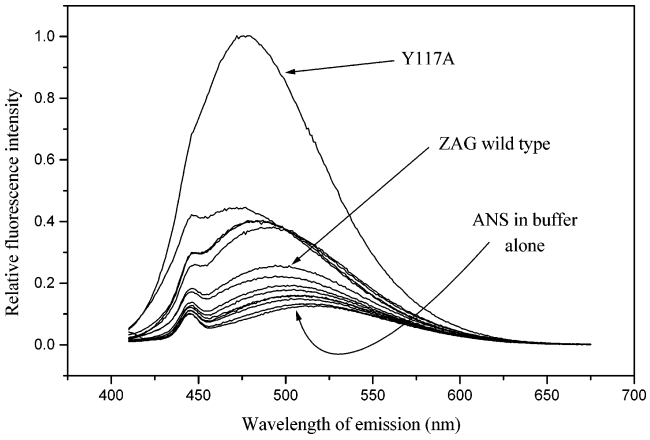

FIGURE 5: Binding of ANS by wild-type ZAG and mutated proteins. Proteins were added to a final concentration of 0.5 μM in a cuvette containing 10 μM ANS solution in PBS, pH 7.3, and fluorescence emission spectra recorded with λ_{exc} = 390 nm. The rank order of decreasing peak fluorescence intensity recorded at 478 nm upon addition of equivalent amounts of each protein was Y117A > F77A > W148A ≈ W115A > W134A > ZAG wild type > Y161A > F101A > Y154A > Y14A, ≈ I76A > R73E > R73A.

(Y117A, W115A, W134A, W148A), ANS's emission was enhanced over that with unmutated ZAG, particularly so for Y117A. In each of these cases, ANS was displaceable with DHA (as was DAUDA), so the specificity of the binding sites was retained. The increased binding by ANS, therefore, could be due to a proportion of misfolded protein, or an

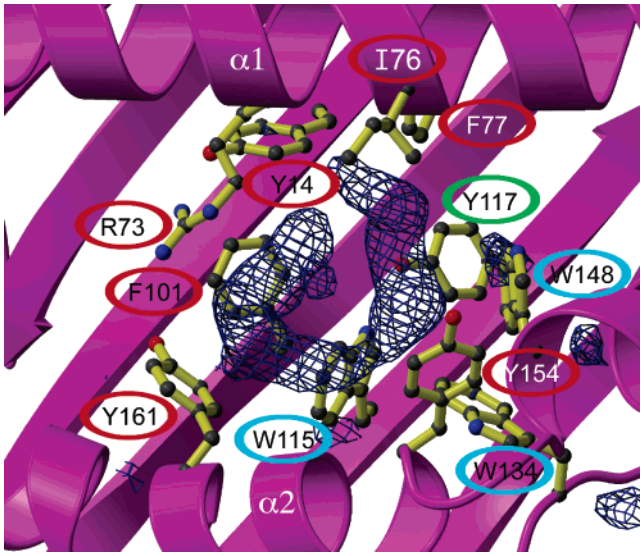

FIGURE 6: Structure of the binding site region of wild-type ZAG as derived by X-ray crystallography and effects of mutation of ligand-proximal side chains (*11*, *17*). The side chains of only those amino acid positions that were mutated are shown and are indicated according to whether the changes abrogated (red), enhanced (green), or had only slight effects (blue) on ligand (DAUDA and fatty acids by competition) binding. The shape, volume, and position of the space occupied by the presumptive ligand is indicated, as defined by occupancy of poly(ethylene glycol) in crystals of ZAG (*17*).

increase in accessibility or volume of the binding site due to loss of bulky side chains, without apparent loss of specificity. This applied particularly to the Y114A mutant, in which the binding site was of enhanced accessibility by the criteria of increased ANS binding and decreased tryptophan emission.

In most of those mutants in which DAUDA binding was abrogated (namely, Y14A, F101A, I76A, Y154A, R73A, and R73E), ANS binding was reduced or lost, indicative of partial or complete closure of the binding site. In F77A and Y161A, however, ANS binding was either increased or only slightly reduced, and these were the only mutants in this group in which ANS was displaceable with DHA, consistent with partial retention of binding specificity. Interestingly, any substitution of Arg-73 had the greatest negative effect on ANS (and DAUDA) binding, in a manner supportive of the idea that this amino acid acts as a strut to hold the binding site open.

Overall, the effects of the mutations are consistent with the binding site being designed for a small amphiphilic molecule whose hydrophobic end would fit into a space surrounded by apolar amino acid side chains and a polar headgroup that would occupy a more charged region adjacent to Arg-73 (Figure 6). Arg-73 has an unexpectedly central role in the structure of the binding site, its substitution resulting in the apparent closure of the binding site, such as is the case for other members of the MHC class I protein family (*15*, *16*). We also speculate that Arg-73 has the additional role of acting as an anchor for the ligand, by interacting with the negatively charged headgroup of, for instance, a fatty acid. We were, however, unable directly to test such an anchoring role since any modification of Arg-73 abrogated both ligand binding and accessibility to the binding site. But, arginines are found to act as anchors of fatty acids or their metabolites in a number of proteins, such as human serum albumin, prostaglandin synthase, and several types of cytosolic fatty acid binding protein (*19*−*23*). Moreover, the charge interaction and hydrogen-bonding networks involved in this tethering frequently involve tyrosines, two of which are close to Arg-73 in ZAG, and the substitution of which ablates ZAG's ligand binding. It would be interesting to replace Arg-73 with an amino acid of similar charge and size, Lys, given that this amino acid can also act to tether fatty acids and other small hydrophobic compounds in binding proteins (*23*, *30*).

The first major implication of our findings is that the binding site for fatty acid ligands has been correctly identified as that occupied by the unidentified electron density in the crystal structure of ZAG; it is unlikely that another site or sites exist that would have been affected by the mutations we created. While it is now known that poly(ethylene glycol) artifactually cocrystallizes in the apolar groove (*17*), the shape and volume of the groove so delineated are consistent with occupancy by a fatty acid-like molecule. Our previous and present findings using a fluorophore-tagged fatty acid and competitive displacement by natural lipids point strongly to a polyunsaturated fatty acid being a natural ligand for ZAG. However, no ligand has yet been successfully isolated and identified from human-derived ZAG, leaving open the possibility that other classes of small amphiphilic compounds are pertinent. Other such molecules include retinoids and steroids, although we have found no evidence for their binding to ZAG (*18*). It is possible, however, that ZAG's natural ligands are heterogeneous, and the balance of them modulates ZAG's natural regulatory function; DHA and EPA, for instance, both bind but can have different effects in other physiological contexts (*31*, *32*).

Given ZAG/LMFs' dramatic effects in inducing adipolysis in certain pathological states, it is probable that it operates by interaction with a cell surface receptor that is connected to an intracellular signaling pathway. Currently, the only candidate for such a receptor for which ZAG-associated cell activation has been found is a β-adrenergic receptor on adipocytes (*33*). If the ligands bound to ZAG are physiologically significant, then it would be expected that preloading the protein homogeneously with selected natural ligands or drugs should modulate ZAG's biological activity. Similarly, the availability of mutants that are binding negative, enhanced, or modified in specificity could be used experimentally to test whether ligand occupancy is a requirement for induction of adipolysis in vivo or in vitro. Finally, binding-defective or modified mutant ZAGs may compete with endogenous ZAG for activation of a cell surface receptor, even in the context of the postulated local autocrine pathway (*8*−*10*). This could in turn open up opportunities for the application of ZAG mutants in clinical use to control ZAG-associated adipose loss in cancers or other pathological states or to correct inborn or otherwise caused imbalances in body adipose stores.

ACKNOWLEDGMENT

We are indebted to Alan Cooper for insightful advice and discussions and to Thomas Jess, Sharon Kelly, and Nick Price for invaluable help in the CD analysis.

REFERENCES

1. Bürgi, W., and Schmid, K. (1961) Preparation and properties of Zn-α_2-glycoprotein of normal human plasma, *J. Biol. Chem. 236*, 1066−1074.
2. Díez-Itza, I., Sánchez, L. M., Allende, M. T., Vizoso, F., Ruibal, Á., and López-Otín, C. (1993) Zn-α_2-glycoprotein levels in breast cancer cytosols and correlation with clinical, histological and biochemical parameters, *Eur. J. Cancer 9*, 1256−1260.
3. Tada, T., Ohkubo, I., Niwa, M., Sasaki, M., Tateyama, H., and Eimoto, T. (1991) Immunohistochemical localization of Zn-α_2-glycoprotein in normal human tissues, *J. Histochem. Cytochem. 39*, 1221−1226.
4. Russell, S. T., and Tisdale, M. J. (2002) Effect of a tumour-derived lipid-mobilising factor on glucose and lipid metabolism in vivo, *Br. J. Cancer 87*, 580−584.
5. Russell, S. T., Zimmerman, T. P., Domin, B. A., and Tisdale, M. J. (2004) Induction of lipolysis in vitro and loss of body fat in vivo by zinc-alpha(2)-glycoprotein, *Biochim. Biophys. Acta 1636*, 59−68.
6. Gohda, T., Makita, Y., Shike, T., Tanimoto, M., Funabiki, K., Horikoshi, S., and Tomino, Y. (2003) Identification of epistatic interaction involved in obesity using the KK/Ta mouse as a type 2 diabetes model—Is Zn-alpha(2) glycoprotein-1 a candidate gene for obesity?, *Diabetes 52*, 2175−2181.
7. Hale, L. P. (2002) Zinc alpha-2-glycoprotein regulates melanin production by normal and malignant melanocytes, *J. Invest. Dermatol. 119*, 464−470.
8. Bao, Y., Bing, C., Hunter, L., Jenkins, J. R., Wabitsch, M., and Trayhurn, P. (2005) Zinc-alpha(2)-glycoprotein, a lipid mobilizing factor, and is expressed and secreted by human (SGBS) adipocytes, *FEBS Lett. 579*, 41−47.
9. Bing, C., Bao, Y., Jenkins, J., Sanders, P., Manieri, M., Cinti, S., Tisdale, M. J., and Trayhurn, P. (2004) Zinc-{alpha}2-glycoprotein, a lipid mobilizing factor, is expressed in adipocytes and is up-regulated in mice with cancer cachexia, *Proc. Natl. Acad. Sci. U.S.A. 101*, 2500−2505.
10. Bing, C., Bao, Y., Jenkins, J., Sanders, P., Manieri, M., Cinti, S., Tisdale, M. J., and Trayhurn, P. (2004) Zinc-alpha-2-glycoprotein, a lipid-mobilizing factor, is expressed in adipocytes and upregulated in mice with cancer cachexia, *Int. J. Obesity 28*, S92−S92.
11. Sanchez, L. M., Chirino, A. J., and Bjorkman, P. J. (1999) Crystal structure of human ZAG, a fat-depleting factor related to MHC molecules, *Science 283*, 1914−1919.
12. Sanchez, L. M., LopezOtin, C., and Bjorkman, P. J. (1997) Biochemical characterization and crystalization of human Zn-alpha(2)-glycoprotein, a soluble class I major histocompatibility complex homolog, *Proc. Natl. Acad. Sci. U.S.A. 94*, 4626−4630.
13. Niazi, K. R., Porcelli, S. A., and Modlin, R. L. (2002) The CD1b structure: antigen presentation adapts to a high-fat diet, *Nat. Immunol. 3*, 703−704.
14. Im, J. S., Yu, K. O. A., Illarionov, P. A., LeClair, K. P., Storey, J. R., Kennedy, M. W., Besra, G. S., and Porcelli, S. A. (2004) Direct measurement of antigen binding properties of CD1 proteins using fluorescent lipid probes, *J. Biol. Chem. 279*, 299−310.
15. Burmeister, W. P., Gastinel, L. N., Simister, N. E., Blum, M. L., and Bjorkman, P. J. (1994) Crystal structure at 2.2 Å resolution of the MHC-related neonatal Fc receptor, *Nature 372*, 336−343.
16. Bennett, M. J., Lebron, J. A., and Bjorkman, P. J. (2000) Crystal structure of the hereditary haemochromatosis protein HFE complexed with transferrin receptor, *Nature 403*, 46−53.
17. Delker, S. L., West, A. P., McDermott, L., Kennedy, M. W., and Bjorkman, P. J. (2004) Crystallographic studies of ligand binding by Zn-alpha(2)- glycoprotein, *J. Struct. Biol. 148*, 205−213.
18. Kennedy, M. W., Heikema, A. P., Cooper, A., Bjorkman, P. J., and Sanchez, L. M. (2001) Hydrophobic ligand binding by Zn-alpha(2)-glycoprotein, a soluble fat-depleting factor related to major histocompatibility complex proteins, *J. Biol. Chem. 276*, 35008−35013.
19. LaLonde, J. M., Levenson, M. A., Roe, J. J., Bernlohr, D. A., and Banaszak, L. J. (1994) Adipocyte lipid-binding protein complexed with arachidonic acid: Titration calorimetry and X-ray crystallographic studies, *J. Biol. Chem. 269*, 25339−25347.
20. Thumser, A. E. A., Voysey, J., and Wilton, D. C. (1996) Mutations of recombinant rat liver fatty acid-binding protein at residues 102 and 122 alter its structural integrity and affinity for physiological ligands, *Biochem. J. 314*, 943−949.
21. Malkowski, M. G., Ginell, S. L., Smith, W. L., and Garavito, R. M. (2000) The productive conformation of arachidonic acid bound to prostaglandin synthase, *Science 289*, 1933−1937.
22. Petitpas, I., Grune, T., Bhattacharya, A. A., and Curry, S. (2001) Crystal structures of human serum albumin complexed with monounsaturated and polyunsaturated fatty acids, *J. Mol. Biol. 314*, 955−960.
23. Curry, S., Mandelkow, H., Brick, P., and Franks, N. (1998) Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites, *Nat. Struct. Biol. 5*, 827−835.
24. Gill, S. C., and von Hippel, P. H. (1989) Calculation of protein extinction coefficients from amino acid sequence data, *Anal. Biochem. 182*, 319−326.
25. Kennedy, M. W., Brass, A., McCruden, A. B., Price, N. C., Kelly, S. M., and Cooper, A. (1995) The ABA-1 allergen of the parasitic nematode *Ascaris suum*: fatty acid and retinoid binding function and structural characterization, *Biochemistry 34*, 6700−6710.
26. McDermott, L., Moore, J., Brass, A., Price, N. C., Kelly, S. M., Cooper, A., and Kennedy, M. W. (2001) Mutagenic and chemical modification of the ABA-1 allergen of the nematode *Ascaris*: Consequences for structure and lipid binding properties, *Biochemistry 40*, 9918−9926.
27. McDermott, L., Kennedy, M. W., McManus, D. P., Bradley, J. E., Cooper, A., and Storch, J. (2002) How helminth lipid-binding proteins offload their ligands to membranes: Differential mechanisms of fatty acid transfer by the ABA-1 polyprotein allergen and Ov-FAR-1 proteins of nematodes and Sj-FABPc of schistosomes, *Biochemistry 41*, 6706−6713.
28. Eftink, M. R., and Ghiron, C. A. (1976) Exposure of tryptophanyl residues in proteins: Quantitative determination by fluorescence quenching studies, *Biochemistry 15*, 672−679.
29. Wilkinson, T. C. I., and Wilton, D. C. (1986) Studies on fatty acid-binding proteins. The detection and quantification of the protein from rat liver using a fluorescent fatty acid analogue, *Biochem. J. 238*, 419−424.
30. Wu, S.-Y., Perez, M. D., Puyol, P., and Sawyer, L. (1999) β-Lactoglobulin binds palmitate within its central cavity, *J. Biol. Chem. 274*, 170−174.
31. Dutta-Roy, A. K. (2000) Cellular uptake of long-chain fatty acids: role of membrane-associated fatty-acid-binding/transport proteins, *Cell. Mol. Life Sci. 57*, 1360−1372.
32. Wynter, M. P., Russell, S. T., and Tisdale, M. J. (2004) Effect of n-3 fatty acids on the antitumour effects of cytotoxic drugs, *In Vivo 18*, 543−547.
33. Russell, S. T., Hirai, K., and Tisdale, M. J. (2002) Role of beta 3-adrenergic receptors in the action of a tumour lipid mobilizing factor, *Br. J. Cancer 86*, 424−428.

BI051881V