

Identification of Critical Residues of an Immunodominant Region of *Echinococcus granulosus* Antigen B*[§]

Received for publication, December 13, 2002, and in revised form, March 20, 2003
Published, JBC Papers in Press, March 26, 2003, DOI 10.1074/jbc.M212724200

Gualberto González-Sapienza^{‡§} and Raul E. Cachau[¶]

From the [‡]Cátedra de Inmunología, Facultad de Química, Universidad de la República, Instituto de Higiene, Av. A. Navarro 3051, Montevideo 11600, Uruguay and the [¶]Advanced Biomedical Computing Center, NCI, National Institutes of Health, Science Applications International Corp., Frederick, Maryland 21702

Immune evasion strategies often shape the immunogenicity of parasite components. We recently found that the N-terminal extension of the major subunit of *Echinococcus granulosus* antigen B (AgB), the causative agent of hydatid disease, concentrates the immunoreactive B cell epitopes of the native molecule. The nature of this immunodominance was analyzed using four monoclonal antibodies (mAbs) defining overlapping epitopes in this region of the AgB molecule. The minimal epitope requirements of these mAbs were determined using phage display peptide libraries. The consensus sequences isolated with the mAbs, and alanine replacement analysis with synthetic peptides mapped the relevant molecular contacts within a short stretch corresponding to residues 17–24 of the AgB major subunit. Substitution of two critical residues within this stretch produced a dramatic loss of antigenicity, as determined by using patient sera. The circular dichroism spectra of the antigen, together with the distribution of the contact residues, suggest that this region adopts an amphipathic α -helix structure that clusters the contact residues on its polar side. To provide further insight in the interpretation of the structure activity relationships for this immunoreactive region of *E. granulosus* AgB, we developed a model for the N-terminal extension of the AgB major subunit, which helps to rationalize our data.

Antigen B (AgB),¹ the major antigens of *Echinococcus granulosus*, the causative agent of cystic hydatid disease, is a 120-kDa protein composed of multimers of homologous but different 8-kDa subunits (AgB8/1, AgB8/2, and AgB8/3) (1–3). The antigen is present in large amounts in the metacestode cyst fluid, and its biological functions suggest that it may play a relevant role in the host/parasite interaction. AgB has been reported as a protease inhibitor that impairs the recruitment of polymorphonuclear cells and skews the Th1/Th2 cytokine ratio toward

an immunopathology-associated Th2 polarization (4, 5). In human infections, AgB is highly immunogenic, which has prompted its use as a diagnostic reagent. In fact, AgB possesses the highest diagnostic value among the major *E. granulosus* antigens (6), and we have found that p176, a 38-mer synthetic peptide representing the N-terminal extension of AgB8/1, exhibits higher diagnostic sensitivity and specificity than native AgB (7).

This is a singular finding considering that, in general, synthetic peptides are poor mimics of the parent epitope and, accordingly, exhibit low diagnostic sensitivities. There are several reasons for this. First, the full group of contact atoms of a given antibody/antigen interface are seldom derived from a short linear stretch of the antigen (8); second, the structural conformation of peptides is not restricted in solution, and there is therefore an unfavorable entropic component to the binding (9). In addition, a single peptide mimics a partial region of the parent protein and, consequently, only a fraction of the polyclonal antibody response against the native antigen can be detected. Considering these arguments, the observed elevated antigenicity of p176 can only be explained on the basis of structural elements that favor the mimicking and by the fact that the N-terminal extension of AgB8/1 constitutes a major immunodominant region of the AgB molecule in human infections with *E. granulosus*. The existence of this immunoreactive region in the AgB molecule may involve an evasion strategy to preserve the biological function of this parasite component, as it has been found for components of other parasites (10).

Herein we report the analysis of the antigenic determinants of this region of the AgB molecule by using four monoclonal antibodies, synthetic peptides, and phage display peptide libraries. We were able to map key molecular contacts within residues 17–24 of the AgB8/1 subunit, which are part of the major B-cell epitopes of the antigen. The information provided by the families of phage-borne peptides is in line with the modeling of the N-terminal extension of the AgB8/1.

MATERIALS AND METHODS

Antibodies—The monoclonal antibodies used in this study, mAbs EB7, AB10, EG9, and BG10, have been described before (2). All four mAbs react with the AgB8/1 subunit of AgB or the native antigen but not with the AgB8/2 subunit. The mAbs were separated from culture supernatants using Protein G-Sepharose and were affinity-purified on an AgB-Sepharose column.

AgB and Peptides—AgB was purified to homogeneity from hydatid cyst fluid as described before (2). Recombinant AgB8/1 was a kind gift from Dr. Arnaldo Zaha. The following AgB8/1-derived peptides were used in this study: p176, p65, and p13 corresponding to residues 1–38, 13–38, and 13–26 of AgB8/1, respectively. In addition, alanine replacement variants of these peptides were also synthesized to examine the individual contribution of selected residues. The peptides were synthesized, purified by reverse-phase high performance liquid chromatography, and analyzed by mass spectrometry at the Molecular Biology Unit,

* This work was supported by the Zaffaroni Foundation, Palo Alto, CA; Grant D43 TW005718 from the Fogarty International Center-NIH; the Comisión Sectorial de Investigación Científica, Universidad de la República; and Contract NO1-CO-12400 from the NCI, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[§] The on-line version of this article (available at <http://www.jbc.org>) contains additional text and Supplemental Fig. S1.

[¶] To whom correspondence should be addressed. Tel.: 5982-480-1196; Fax: 5982-487-4320; E-mail: ggonzal@fg.edu.uy.

¹ The abbreviations used are: AgB, antigen B; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; BSA, bovine serum albumin; CD, circular dichroism.

University of Newcastle-upon-Tyne (UK).

Phage Display Libraries—The phage display peptide libraries used in the initial panning experiments were a kind gift from Dr. William Dower and consisted of: (i) a constrained library (CX₇₋₁₂C-pIII) constructed in the phagemid vector pAFF2 (11) expressing a random sequence of 7–12 amino acids flanked by two invariant cysteine residues at the N-terminal region of the coat protein pIII and (ii) a non-constrained library (X₁₁-pVIII) constructed in the p8V2 vector (12) expressing a linear random sequence of 11 residues fused to the N-terminal end of the coat protein pVIII. The mutational library was constructed in the pAFF2 vector as described previously (13), and the total number of independent transformants obtained was 2×10^8 .

Biopanning Experiment—For panning and ELISAs, the mAbs were diluted in phosphate-buffered saline (PBS) (20 µg/ml) and immobilized on microtiter plates (Maxisorb, NUNC, Denmark) (50 µl/well) overnight at 4 °C. The plates were then blocked with PBS, pH 7.2/1% bovine serum albumin (BSA) for 1 h at room temperature and washed twice with PBS/0.05% Tween 20 (PBS/T). Phage libraries were screened as described (14). Briefly, a phage number equal to 100–1000 library equivalents was diluted in 600 µl of PBS/T, 1%BSA and dispensed into six antibody-coated wells for the first round of panning. The total number of phage added to wells in further rounds of panning was 10^{10} transducing units. The phages were incubated for 2 h at 4 °C, followed by extensive washing with PBS, and acid elution. The phage eluate was titrated and amplified in *Escherichia coli* ARI 292 (Affymax Research Institute).

Phage ELISA—After three or four rounds of enrichment, individual phage clones were isolated and amplified, and their specificity assayed in an ELISA format (15) in wells coated with the mAb of interest, BSA or an non-related mouse IgG1 mAb. Briefly, phage (10^9 infectious particles) in 100 µl of PBS/1%BSA, or 100 µl of 1 µM AgB PBS/1%BSA were added to each well and incubated for 1 h at 37 °C. After extensive washing, horseradish peroxidase-conjugated anti-M13 phage antibody (Amersham Biosciences) was added and incubated for 1 h at 37 °C. After washing, peroxidase activity was determined using H₂O₂/tetramethylbenzidine, measuring absorbance at 405 nm. DNA from immunopositive clones, which were negative in wells containing AgB, was prepared and sequenced as described (11).

Competition Binding Assay—Biotinylated AgB8/1 was used as a tracer to measure the median inhibitory concentration (IC₅₀) of peptides binding to the mAbs adsorbed on ELISA plates. Peptides were diluted in Me₂SO to prepare stock solutions at 2 mM. Serial dilution of the peptides in PBS/1%BSA were added to mAb (50 ng)-coated microtiter wells containing a fix amount of biotinylated AgB8/1 and incubated for 1 h at room temperature. The final concentration of Me₂SO was adjusted to 5% in all wells, which had previously been shown to have no influence in the mAb recognition. After washing, the amount of bound AgB8/1 was measured by the addition of a streptavidin-peroxidase conjugate (Sigma, St. Louis, MO). Nonspecific binding was determined in the presence of 1 µM AgB. Standard curves were obtained by plotting absorbance against the logarithm of inhibitor concentration, which were fitted to a four-parameter logistic equation.

Circular Dichroism Spectra—Native AgB CD spectra were collected using an AVIV 202 CD spectrometer (Aviv Instruments). The sample was diluted 40× with Milli-Q water to a final concentration ~0.1 mg/ml and centrifuged at 3000 rpm ($r = 7$ cm). An aliquot (100 µl) was suctioned from the middle section of the vial after centrifugation and loaded in a methanol-cleaned and air-dried cuvette (Uvonic ZC20). Data were collected in the region 180–260 nm (Wavelength Step, 1.00 nm; averaging time, 1.000 s; settling time, 0.330 s; multi-scan wait, 20.00 s). A 20-way average is presented in the figure.

Modeling of the AgB8/1 Subunit N-terminal Extension—The simulations were carried out using X-plor version 3.451 (16) using the CHARMM param19x set, including all atoms. A slow temperature ramp, from $T = 150$ K to $T = 400$ K (1 ns) and a very large cooling trajectory (10 ns, $T_{\text{final}} = 300$ K) was used to ensure that the model was properly relaxed. The model was subsequently minimized. All calculations were done using the Berendsen thermostat with FBETA = 10. An independent model for the peptide and the mimotopes was built using a technique called Feedback Restrained Molecular Dynamics (17). No significant differences are observed between the two peptide models. The detailed analysis of the Feedback Restrained Molecular Dynamics models is presented elsewhere.²

TABLE I
mAb AB10 and EG9 binding peptides isolated from the CX₇₋₁₂C-pIII library

mAb AB10	mAb EG9
<u>CEKYIMRTGDEIYC</u> (3)	<u>CEKYIMRTGDEIYC</u>
<u>CKYVGRTEGTNYNC</u> (2)	<u>CKYVHRTGTNYNC</u>
CGLMAKYLLERC	CKYLHRRFFDTSEEC
<u>CLKYQAGKDVSVFC</u> (2)	<u>CKYYTERIGVATFC</u> (2)
<u>CLKYRSDKDVTVNC</u>	<u>CLKYQAGKDVSVFC</u>
	<u>CLKYRSDKDVTVNC</u>
	<u>CLKYQAGKDVSLVC</u>
KYX ₂₋₃ (K/R)	

RESULTS

Identification of the mAb Contact Residues with Phage Display Libraries—Using mAbs immobilized on ELISA plates, two phage display peptide libraries were screened for peptides that bind to the antibodies: a library with a variable number of random amino acids (ranging from 7 to 12) flanked by two cysteine residues and a random 11-mer linear library. After three rounds of affinity selection, clones that bound specifically to the selector antibody, but not to negative control wells (BSA or a non-related mAb), were selected by phage ELISA. The specificity of the selected clones was confirmed by studying the inhibitory effect of varying concentrations of AgB on their binding (not shown).

Table I shows the deduced peptide sequences of 17 clones isolated with mAbs AB10 and EG9 from the CX₇₋₁₂C-pIII library. Many of the clones selected with mAb AB10 were also selected with the mAb EG9. Moreover, all of these 17 clones were positive in phage ELISA with both mAbs (not shown). All isolated sequences contained the motif KY followed by a positively charged amino acid after the second or third residue. The stretch KYFFER found in the N-terminal extension of AgB8/1 subunit is a likely match for this motif (Fig. 1).

Twenty-four clones were selected with mAb BG10 from the CX₇₋₁₂C-pIII library, which were grouped in two families (Table II). Family I was composed of 14 clones containing the KYX₂₋₃(R/K) consensus, which was the same as that obtained with mAbs AB10 and EG9. In addition, the deduced sequences of three members of this family were identical to that of counterpart clones isolated with mAbs AB10 and EG9. However, a second family composed of 10 clones sharing the (R/S)XLH motif was also isolated with mAb BG10, indicating that this mAb defines a more complex epitope. To evaluate the relevance of these two families in the conformation of the binding epitope of mAb BG10, the X₁₁-pVIII library was panned with mAb BG10. All eight selected clones encoded peptide sequences that contained the KY consensus.

Selection of the CX₇₋₁₂C-pIII library with mAb EB7 yielded a different picture (Table III). Twenty-one clones were selected, with the consensus sequence EXXPXRY. To further define the role of the different consensus residues and the relevance of the disulfide bridge, we built a mutagenesis library with the structure: (C/S)xxexxxry(C/S). Where C/S stands for equal probability of finding Cys or Ser in that position. Amino acids shown in lowercase letters indicate a 50% probability of finding that residue in that position, whereas it is randomly occupied in the remaining 50%. Finally, x indicates a fully random position. All twenty-two binding peptides isolated from this mutagenesis library were flanked by Cys residues and had the expected amino acids in the biased positions (except for the final Tyr in two of the clones) (Table III). Markedly, all clones had Pro

² R. E. Cachau, G. González-Sapientza, S. K. Burt, and O. N. Ventura, submitted for publication.

I	KYXXXR
II	EG-YP-ERY
AgB8/1	DDGLTSTSRVSMKMFGEV KYFFER DLPGQKVVDLLKELEEVFQLLRKKLRMALRSHLRGLIAEGE
p176	DDGLTSTSRVSMKMFGEV KYFFER DLPGQKVVDLLKEL
p65	KMFGEV KYFFER DLPGQKVVDLLKEL

FIG. 1. Alignment of the AgB8/1 subunit and two synthetic peptides (p176 and p65) with the consensus sequences isolated from the peptide libraries. *I*, consensus sequence selected with mAbs AB10, EG9, and BG10; *II*, consensus sequence isolated with mAb EB7. The residues assumed to be part of the antibody binding site are shown in **boldface**.

TABLE II
mAb BG10 binding peptides isolated from random peptide libraries

Amino acid sequences of clones are grouped according to sequence homology. Numbers in parentheses indicate the occurrence of each clone. **Boldface letters** identify the amino acids that were constant or frequent. Two peptide sequences that were also isolated with mAbs AB10 and EG9 are underlined. The consensus motif is shown at the bottom of each column.

Library: CX ₇₋₁₂ C-pIII		Library: (X) ₁₁ -pVIII	
Family I	Family II		
CKYALRQQLC	CEAYWRP		KYLAKSLFEGQ
CKYSLRQGLC	CDRTWRP	GPVE	KYCLRL
CDKYLLREGSEMRC	CEYDHRP	GGLE	KYCVERM
CEKYIMRTGDEIYC	CGDHQGRV	SSEM	KYLIEIV
CKYWDREFRGLHWC	CEGHTGRV	SPAL	KYLLMTF
CKYAMRQLSDWC	CQFYGWRV	FSAEGLLV	KYC
CKYYCRVEDTLHTC	CSA	SSFGTFL	KYWT
CVKYLVERDFFGRC	CSA	TPAQ	KYAEIC
CGSMAYVMERC	CSS		
CYKYASEKNLGFGC			
CLKYQAGKDVSVFC (2)			
CEKYTNEVEPLFRC			
CAKYRETMDNYKC			
KY (X) ₂₋₃ (K/R)	(R/S) XLH		

TABLE III
mAb EB7 binding peptides isolated from the C(X)₇₋₁₂C-pIII library (first column) and the mutagenesis library (second column)

Amino acid sequences of clones are grouped according to sequence homology. Numbers in parentheses indicate the occurrence of each clone. **Boldface letters** identify the amino acids that were invariant or frequent. The consensus motif is shown at the bottom of each column.

Library: C(X) ₇₋₁₂ C-pIII	Library: CxxxxxxxC-pIII
CMVENYPERYC (5)	CAR EGYPARYC (6)
CSQEGWPERYC (4)	CMV ENYPERYC (4)
CSWEGWPERYC	CRL EGWPERYC
CSVEGYPARYC (3)	CAR EGWPERYC
CSVEGYPARYC (2)	CFM ENHPERYC
CAREGWPARYC (2)	CEI EGCPERYC (2)
CAAEYGPVRYC	CEI EGWPERYC
CMVEKNPVWYC	CDQ EGWPERYC
CAYEERPIWYC	CQL EGWPPRYC
CYWERNGVVIQC	CAR EGFPPRYC
	CAREGYPPRHC
	CAMEGWPERFC
	CEIEGWPERFC
-EXXPXRY-	-EG(Y/W) PERY-

in position seven, which had not been biased in the library. In addition, there was a noticeable tendency for Gly at position five, a bulky aromatic residue (Tyr or Trp) at position six and Glu at position eight, corresponding to the **EG(Y/W)PERY** motif, where boldface letters indicate highly conserved residues. The presumed alignment of this sequence with AgB8/1 is shown in Fig. 1.

Delimiting the Role of Critical Residues with Synthetic Peptides—To delimit the location of the epitopes defined by our mAbs we first used two synthetic peptides (p176 and p65) derived from the N-terminal region of the AgB8/1 subunit, which have been described as diagnostic reagents for hydatid disease. The relative affinity of the mAbs against peptides p176 and p65 was ranked by determining their IC₅₀ in plates coated with the mAbs where the peptides competed for binding sites with the biotinylated AgB8/1 subunit (Table IV).

All mAbs reacted with native AgB in the subnanomolar range and with the peptides in the sub- to micromolar range. Because native AgB has several copies of the AgB8/1 subunit,

the loss of the antibody bivalent binding explains the weaker interaction observed with the peptides. The reactivity with p176 and p65 narrowed down the location of the four mAb epitopes to residues 13–38 of AgB8/1 and is in agreement with the postulated alignment of the consensus sequences shown in Fig. 1. The stretch **EVKYFFER**, corresponding to residues 17–24, appeared to concentrate the relevant contact residues of the epitopes defined by the four mAbs (shown here in **boldface**). To confirm this assumption, we synthesized p13 (KMFGEVKYFFERDP), a truncated form of peptide p65, and systematically replaced the conserved positions with alanine to determine the effect on binding (Table V). Alanine substitutions were chosen to minimize unfavorable steric contacts and to avoid imposing new charge interactions or hydrogen bonds from the substituted side chains. In general, the mAbs bound to the truncated form of p65 with lower affinity, showing the relevance of the additional residues of p65 in the stabilization of the peptide structure that reproduces the antigenic determinants of AgB. The binding of the mAbs was highly sensitive to alanine replacement of the consensus residues. In that regard, mAbs AB10, EG9, and BG10, which produced the consensus sequence KYXXXR, were inactive when assayed with peptides p13–7, p13–8, and p13–12, corresponding to substitutions of these residues in the stretch **EVKYFFER**. On the other hand, they still showed significant residual binding to peptide p13–5, which has the non-essential Glu residue at position 5 of p13 substituted by Ala. In the case of mAb EB7, it reacted with p13–7, which has a substitution of the Lys residue, that is absent in the consensus residues isolated with this mAb, but not with p13–5, p13–8, or p13–12, where critical amino acid were substituted. Due to its scant solubility, no information was obtained about inhibition with p13–11.

A variant of p65 (p65-er) was also synthesized to assess the contribution of the **EVKYFFER** stretch in the polyclonal response against AgB. For this, the initial Glu and last Arg residues of this stretch (fifth and twelfth residues of p65) were replaced by Ala. In agreement with the previous alanine replacement experiments, the reactivity of mAbs AB10 and EB7 (as well as that of mAbs EG9 and BG10, not shown) was

TABLE IV
Values of IC_{50} (M) for peptides p65 and p176 and native AgB determined by ELISA on microtiter plates coated with the mAbs and using biotinylated AgB8/1 subunit as reporter

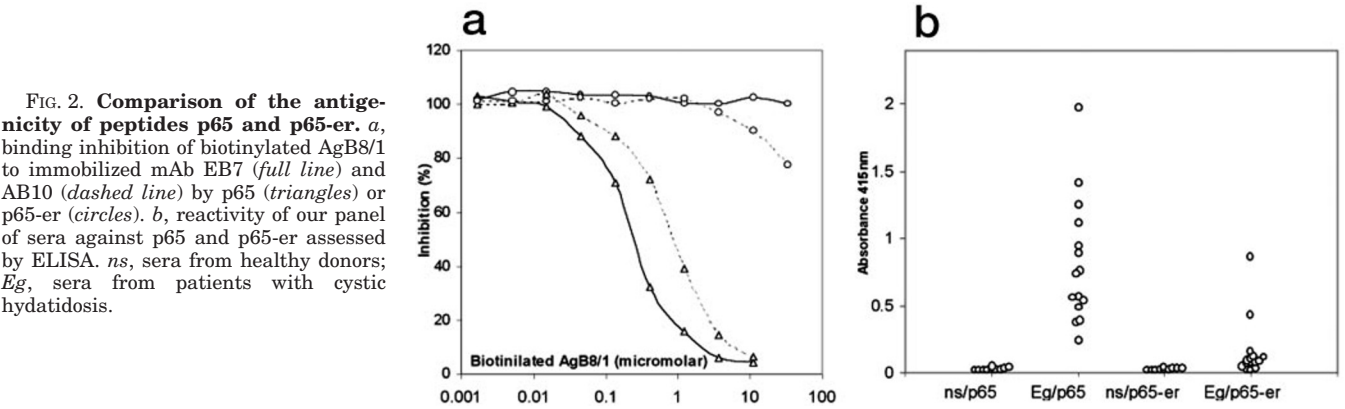
Antigen	mAb AB10	mAb EG9	mAb BG10	mAb EB7
P65	8.8×10^{-7}	9.2×10^{-7}	8.3×10^{-6}	2.3×10^{-7}
P176	1.2×10^{-7}	1.4×10^{-7}	2.6×10^{-6}	9.1×10^{-8}
AgB	7.7×10^{-10}	6.0×10^{-10}	3.2×10^{-10}	4.2×10^{-10}

TABLE V
Alanine replacement analysis

Values of IC_{50} (μ M) for the p13-based substitution series determined by ELISA on microtiter plates coated with the mAbs and using biotinylated AgB8/1 subunit as reporter.

Peptide	Sequence	AB10	EG9	BG10	EB7
p13	KMFGEVKYFFERDP	8.8	10	14	5.5
p13-5	KMFGAVKYFFERDP	10	16	33	>500
p13-7	KMFGEVAYFFERDP	>500	>500	>500	35
p13-8	KMFGEVKAFFERDP	>500	>500	>500	>500
p13-11	KMFGEVKYFFAARDP	>50 ^a	>50 ^a	>50 ^a	>50 ^a
p13-12	KMFGEVKYFFEAADP	>500	>500	>500	>500

^a Solubility limit.



impaired by the modifications introduced in p65-er (Fig. 2a). The loss of p65 antigenicity by substitution of these two residues was also evident when the immunoreactivity of p65-er was examined using fifteen serum samples from patients with surgically confirmed hydatid disease and 10 serum samples from healthy donors (Fig. 2). The single modification of two residues in p65 affected the overall antibody response to the peptide, showing that the antigenic determinants built by the stretch EVKYFFER are major B cell epitopes in the polyclonal response to AgB in human infections with *E. granulosus*.

Secondary Structure Analysis—The region of the AgB8/1 subunit that contains the EVKYFFER stretch is strongly predicted to have an α -helix secondary structure (18). This is in consistent with the data of the native AgB CD spectrum (Fig. 3), which suggest that over 65% of the secondary structure of the AgB molecule corresponds to an α -helix organization (see Supplementary Material for a detailed analysis). Interestingly, such a structure would bring together, on the polar side of an amphipathic helix, the contact residues found in the consensus sequence isolated with the mAbs, which would thus be exposed to the solvent in the native AgB molecule (see Fig. 3, inset).

Three-dimensional Model of the N-terminal Extension of the AgB8/1 Subunit—The CD spectra of native AgB and the two-dimensional prediction of the region corresponding to residues 1–38 of the AgB8/1 subunit (which we will call the p38 peptide), indicate that this region is predominantly α -helical with a short coil region determined by the Pro-X-Gly pair (DPLG region, Wisconsin Package Version 10.3, Accelrys Inc., San Diego, CA). The structure of this peptide was thus initially modeled as an α -helix. The model was then relaxed using

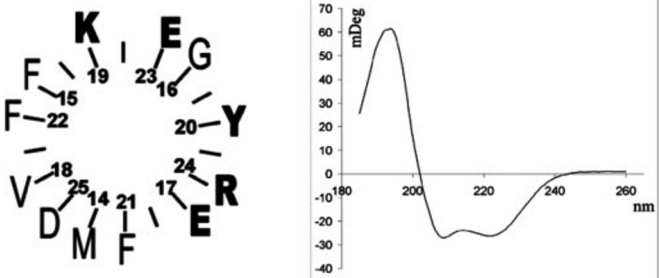


FIG. 3. Native AgB CD spectrum and helical wheel representation of residues 14–25 of the AgB8/1 subunit. Notice that the predicted α -helix organization of this antigenic region would cluster the contact residues that built the antibody binding sites on the hydrophilic side of an amphipathic helix.

molecular dynamics simulations (see “Materials and Methods”). The structure of the final model is shown in Fig. 4. According to the model, the disposition of the hydrophilic and hydrophobic residues segregated in regions disposed in opposite faces of an elongated α -helix above and below the region where the immunogenic residues are found (Fig. 4). This disposition is the result of Pro²⁶ disrupting the progression of the α -helical structure and the ability of Lys¹⁸ to directly interact with the backbone carbonyl of Gly¹⁶. This interaction is disrupted in some model simulations, including discrete water molecules (not shown). The most important modification of the model resulting from the inclusion of explicit solvent in the simulation is the relative higher flexibility of the region involving Pro²⁶, Leu²⁷, and Gly²⁸. The lack of information regarding

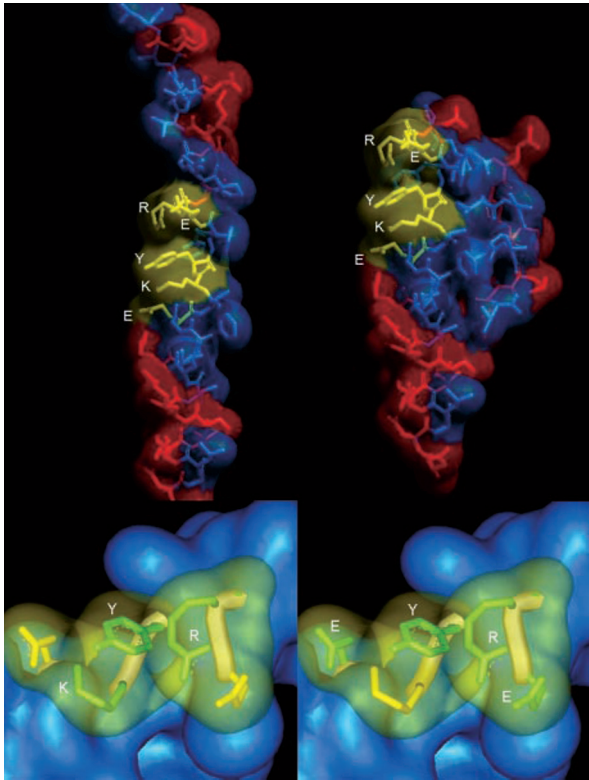


FIG. 4. **p38 model.** *Top left*, side view of the extended p38 model with its surface colored by the residue hydrophobicity (in red hydrophilic residues and in blue hydrophobic residues). The surface defined by the preserved residues in the consensus sequences (see Fig. 1) is indicated in gold. Notice the surface defined by these residues is contiguous. The hydrophobic region is on opposite sides of the α -helix above and below these antigenic residues. The disposition of the hydrophobic residues suggests a bent conformation of the antigen. *Top right*, putative model of the p38 bent structure based in the clustering of the hydrophobic region and the preservation of the geometry of the immunogenic region. Note the interlock of the hydrophobic residues in this conformation. *Bottom*, enlarged view of the modeled structure of residues 17–24 of p38. *Bottom left*, the consensus residues isolated with mAbs AB10, EG9, and BG10 denoted in green (consensus motif: **KYX₂₋₃(R/K)**). *Bottom right*, consensus residues isolated with mAb EB7 denoted in green (consensus motif **EG(Y/W)PERY**).

the folding of AgB8/1 subunit hinders our ability to uniquely identify the conformation of this region. The large flexibility of the region around Pro²⁶ and the disposition of the hydrophobic residues are suggestive of a folded conformation similar to that first observed in staphylococcal Protein A, where an α -helix is bent over itself resulting in two α -helical segments anti-aligned (19). Thus, we explored ways to fold p38 that cluster the hydrophobic regions following this pattern. We did this by resorting to the intercalation rules frequently used for the interpretation of the disposition of side-chains in coil-coiled structures and by subjecting the putative models to simulated annealing, solvating the model with 2200 water molecules (20). The lowest energy structure obtained, where the geometry of the immunogenic region has been preserved, is shown in Fig. 4. It must be noticed that this disposition of the p38 peptide makes the immunogenic region fully exposed by placing it at the elbow of the bent peptide model. It must also be noticed that the proper intercalation of the hydrophobic residues, now clustered at the core of the p38 model, almost entirely occluded and inaccessible to the solvent.

DISCUSSION

Although the current knowledge on the biological role of AgB is limited, there is some evidence that strongly suggests that this is a crucial molecule for parasite survival. AgB is the major

component of *E. granulosus* metacestode fluid and is highly abundant. In addition, the antigen is able to pass across the cyst wall and reach the host internal milieu. In evidence of the latter, patients affected by hydatid disease often elicit a strong antibody response against this antigen. The analysis of this response, using synthetic peptides and human sera, has revealed that the specificity of this antibody response is highly polarized to the N-terminal region of the AgB8/1 subunit (7, 21). In this work we utilized four previously characterized mAbs defining epitopes in this region of the AgB molecule as probes to analyze the nature of this immunogenicity. The mAbs were chosen on the basis of their capacity to inhibit the reactivity of human sera with native AgB (not shown).

The alignment of the consensus sequences isolated with the four mAbs indicate that they define three overlapping antibody binding sites within a short region (EVKYFFER, residues 17–24) of the N-terminal extension of the AgB8/1 subunit. One of these sites is defined by antibodies AB10 and EG9, which produced the **KYX₂₋₃(R/K)** consensus sequence (putative contact residues in boldface). All phage clones isolated with AB10 cross-reacted with EG9 and *vice versa*; therefore, these two mAbs, which share the IgG1 isotype, are most likely derived from the same original B-cell clone and have identical antibody binding sites. The second site, defined by mAb BG10, is closely related to the former, but in addition to the KYX₂₋₃(R/K) motif, this mAb also selected a phage family containing the (R/S)XLH consensus, which did not cross-react with mAbs AB10 or EG9. This indicates that the mAb BG10 binding site is more complex and may extend beyond the N-terminal region of the AgB8/1 subunit. In any case, the major contribution to the binding energy comes from the KY motif, as suggested by the fact that this motif was present in all clones isolated from the unconstrained X₁₁-pVIII library, where, due to multivalent binding, selection is driven by avidity.

The third antigenic site is delineated by mAb EB7 and was aligned to residues 17–24 of AgB/1. This antibody selected a strong consensus sequence that was further refined using a mutagenesis library. This fine-tuning demonstrated the relevance of the structural constraint imposed by the formation of the disulfide bond, and produced the **EG(Y/W)PERY** consensus (putative contact residues in boldface), which evidenced a preference for particular residues at positions other than those biased in the library. The deduction of the putative contact residues of mAb EB7 binding site was done on the basis that antibodies pick those amino acids out of the library with which they normally make molecular contacts, whereas other amino acids of the consensus sequence are necessary to build the scaffold that exposes the contact residues in the proper orientation (22). This seems to be the role of the consensus glycine and proline residues, whose effect on polypeptides is almost entirely steric, markedly so in the case of proline, that being a ring greatly limits the number of possible peptide conformations.

The proposed alignment of the four mAbs consensus sequences with residues 17–24 of the AgB/1 subunit was confirmed, first by analysis of the reactivity of the mAbs using long peptides and then by the alanine replacement strategy with short peptides. This region of the AgB8/1 subunit not only demonstrably contained the binding sites of the four mAbs, but it also proved to be a major immunodominant region of the AgB molecule during the course of human infections, as evidenced by the significant drop in the diagnostic value of p65 when the flanking residues of the stretch EVKYFFER were replaced by Ala.

To provide further insight in the interpretation of the structure-activity relationships for this immunoreactive region of

E. granulosus AgB, we developed a model for this subunit. We restricted our analysis to the N-terminal half of AgB8/1 (residues 1–38), which, based on the outcome of two-dimensional predictive algorithms and the AgB CD spectra, was modeled as an α -helix by standard modeling techniques (Fig. 4). The model 1 is very much in line with the information obtained with the panning experiments and the alanine replacement analysis. According to our model, the main contact residues of the four mAbs form a contiguous surface, which is also in agreement with the observation that these mAbs compete to each other. The central Tyr²⁰ residue is flanked by two salt bridges, Glu¹⁷/Lys¹⁹ and Glu²³/Arg²⁴; with the exception of the aromatic pairing of Tyr²⁰ with Phe²¹ and the interaction of Glu¹⁷ with Lys¹³ forming a salt-bridge triad, all other main interactions occur within this region. Based, therefore, on the p38 model, this establishes a favorable situation to promote antibody recognition, because the weak bond reorganization that takes place at the antigen-antibody interface would not propagate to the rest of the AgB molecule thus producing a thermodynamically favored binding. In addition, the contiguous Pro-X-Gly motif suggests that the model may actually be bent in the folded conformation, which may add to our interpretation of the data by even further exposing the immunogenic residues at the elbow of the bent region (Fig. 4).

In general, the immunoreactivity of a given protein is the result of intrinsic features of its structure as well as factors intrinsic to the host encountering the antigen (8). According to our model, residues 17–24 of AgB8/1 fulfill the basic structural requirements that may determine their antigenicity, however, the observed immunodominance of this region is surely a more complex phenomenon, which was shaped under the selective pressure imposed by the need to preserve the functionality of the AgB molecule against the host immune response (10). Thus, the information of the structural characteristics of the AgB immunodominant antigenic sites not only has potential value for further development of peptidomimetics for diagnosis but it also provides an interesting case of protein immunogenicity. This detailed knowledge may con-

tribute to a better understanding of the biological role of this parasite component.

Acknowledgments—G. G.-S. thanks Dr. Bill Dower, Dr. Peter Schatz, and the Affymax Research Institute, Palo Alto, CA, for hosting the stay that allowed the work with the phage display libraries. R. E. C. thanks Sergei Tasarov and the Biophysics Resource of the Structural Biology Laboratory of the Center for Cancer Research, NCI, National Institutes of Health, for help with CD measurements.

REFERENCES

- Oriol, C., and Oriol, R. (1975) *Am. J. Trop. Medic. Hyg.* **24**, 96–100
- Gonzalez, G., Nieto, A., Fernandez, C., Orn, A., Wernstedt, C., and Hellman, U. (1996) *Parasite Immunol.* **18**, 587–596
- Chemale, G., Haag, K. L., Ferreira, H. B., and Zaha, A. (2001) *Mol. Biochem. Parasitol.* **116**, 233–237
- Shepherd, J., Aitken, A., and McManus, D. (1991) *Mol. Biochem. Parasitol.* **44**, 81–90
- Rigano, R., Profumo, E., Bruschi, F., Carulli, G., Azzara, A., Ioppolo, S., Buttari, B., Ortona, E., Margutti, P., Teggi, A., and Siracusano, A. (2001) *Infect. Immun.* **69**, 288–296
- Barbieri, M., Fernandez, V., Gonzalez, G., Luaces, V. M., and Nieto, A. (1998) *Parasite Immunol.* **20**, 51–61
- González-Sapienza, G., Lorenzo, C., and Nieto, A. (2000) *J. Clin. Microbiol.* **11**, 3979–3983
- Berzofsky, J. (1985) *Science* **229**, 932–940
- Janin, J., and Chothia, C. (1990) *J. Biol. Chem.* **265**, 16027–16030
- Cazzulo, J. J., and Frasch, A. (1992) *FASEB J.* **6**, 3259–3264
- Martens, C. L., Cwirla, S. E., Lee, R. Y., Whitehorn, E., Chen, E. Y., Bakker, A., Martin, E. L., Wagstrom, C., Gopalan, P., Smith, C. W., Tate, E., Koller, K. J., Schatz, P. J., Dower, W. J., and Barrett, R. W. (1995) *J. Biol. Chem.* **270**, 21129–21136
- Cwirla, S. E., Dower, W. J., and Li, M. (1999) *Methods Mol. Biol.* **128**, 131–141
- Gallop, M. A., Barrett, R. W., Dower, W. J., Fodor, S. P., and Gordon, E. M. (1994) *J. Med. Chem.* **37**, 1233–1251
- Cwirla, S. E., Peters, E. A., Barrett, R. W., and Dower, W. J. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 6378–6382
- Barrett, R. W., Cwirla, S. E., Ackerman, M. S., Olson, A. M., Peters, E. A., and Dower, W. J. (1992) *Anal. Biochem.* **204**, 357–364
- Brünger, A. (1993) *X-PLOR Version 3.1 Manual*, Yale University Press, New Haven, CT
- Cachau, R. E. (1996) in *Advances in Computational Biology* (Villar, H. O., ed) Vol. 2, pp. 66–119, JAI, London
- Rost, R., and Sander, C. (1993) *J. Mol. Biol.* **232**, 584–599
- Starovasnik, M. A., Skelton, N. J., Oconnell, M. P., Kelley, R. F., Reilly, D., and Fairbrother, W. J. (1996) *Biochemistry* **35**, 15558–15569
- Lazaridis, T., and Karplus, M. (1996) *J. Chem. Phys.* **105**, 4294–4316
- Leggatt, G., and McManus, D. (1994) *Parasite Immunol.* **16**, 87–96
- Rodi, D. J., and Makowski, L. (1999) *Curr. Opin. Biotechnol.* **10**, 87–93