

Characterization of immunoglobulin binding by schistosomes

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SUMMARY

Although controversial, schistosomes are believed to cloak themselves in antibody through non-specific interactions with the immunoglobulin (Ig) molecule. The acquisition of host Ig by the schistosome may mask its foreign status and/or interfere with Fc-dependent functions. We report experiments aimed at characterizing the interaction between Ig-Fc and paramyosin, a schistosome Fc-receptor previously reported to bind human IgG. We show that certain Ig classes, in particular murine IgG2b and IgG3, are not only able to bind recombinant paramyosin, but also associate with other parasite proteins. The Fc region of IgG contains four hydrophobic patches, two of which are known to interact with distinct molecules: one in the C γ 2–C γ 3 interdomain region bound by protein G, mannose binding lectin (MBL), and the neonatal Fc-receptor (FcRn), and one at the top of the C γ 2 domain bound by phagocytic Fc γ Rs and C1q. We provisionally discounted the involvement of these regions, since IgG binding by paramyosin did not inhibit Fc γ R-mediated NADPH respiratory bursts, and protein G was unable to block IgG binding to paramyosin. Given their apparent low affinity, we postulate hydrogen bonding between reactive residues in a hydrophobic patch at the bottom of the C γ 3 domain and negatively charged Glu or Asp amino acids in paramyosin.

Keywords Fc-receptor, immune evasion, immunoglobulin, schistosomiasis

INTRODUCTION

Bacteria and viruses express surface molecules that bind specifically to the Fc portion of immunoglobulins (Ig). For example, *Staphylococcus aureus* expresses protein A and the herpes simplex virus expresses gE–gI. Such proteins play a role in determining the outcome of infection (1). It is proposed that they afford an advantage to the organism through perturbation of Fc-dependent functions, such as the interaction with phagocyte Fc-receptors (FcR) and/or complement, although firm evidence supporting this hypothesis has been obtained only for the IgA-binding proteins from group A and B streptococci (2).

Schistosome receptors for IgG are well documented, although they are less well characterized (3). The acquisition of host Ig by the schistosome tegument presumably masks their foreign status, but may also interfere with Fc-dependent functions. It remains unclear what advantage the binding of Ig-Fc offers a schistosome during an infection. Recently, using Fc derived from human serum IgG, paramyosin was identified as a schistosome FcR (4). Despite this work, neither the regions of interaction between paramyosin and human IgG, nor the mechanisms by which they bind each other are understood. Paramyosin is found in schistosome thick muscle filaments, where it is believed to play a role in worm motility. However, a non-filamentous membrane-bound form of paramyosin has also been described in the tegumental outer layer (5,6) and on the surface (4,7,8) of *S. mansoni*. This form of paramyosin, termed SCIP-1, has the remarkable ability of inactivating the complement membrane attack complex (MAC) by binding both C8 and C9 (8). Although there is still much controversy about the proposed surface location of paramyosin (9), given the ease with which paramyosin can be exposed *in vitro*, it could be argued that in the hostile immunological environment pervading *in vivo*, paramyosin exposure to the host immune response may be very significant. Indeed, *in vivo* binding of Fc by damaged worms could be critical to their survival, buying them time to repair areas of immune-damaged tegument. Proteome analysis of the secretions of schistosome

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larvae (cercariae) has shown that SCIP-1 is a major product of the acetabular glands, validating previous immunolocalization studies (7).

Consistent with surface exposure and/or secretion, paramyosin has been shown to induce protective immunity in vaccination experiments, suggesting it can be seen by the immune system and is immunogenic (10,11). Indeed, paramyosin is one of the World Health Organization's selected priority candidate molecules for further development as a potential vaccine against the disease (11). Although paramyosin has no features to suggest it can exit a cell or end up in the tegument, many examples of anchorless bacterial surface and secreted proteins exist; some even binding the Fc, and suggesting that it is not unreasonable to suspect the existence of such protein forms for paramyosin (12). In addition to defining the biological role paramyosin plays in immune evasion, there is a great deal of empirical interest in defining molecules that interact with the Fc portion of antibodies. Here we report experiments aimed at characterizing the interaction between Ig-Fc and paramyosin. We show that certain antibody classes, in particular mouse IgG3, IgG2b, and rat IgG2c, are able to bind not only paramyosin but other uncharacterized parasite membrane proteins. These interactions are of low affinity and are postulated to involve hydrogen bonding between residues in a hydrophobic patch at the bottom of the C γ 3 domain and negatively charged Glu or Asp amino acids in paramyosin.

EXPERIMENTAL PROCEDURES

Parasite and antigens

The life cycle of a Puerto Rican strain of *Schistosoma mansoni* was maintained in outbred mice and the snail *Biomphalaria glabrata*. Adult worms were recovered from mice by perfusion of the hepatic portal system using phosphate-free buffers as described previously (13). From these worms, a soluble worm antigen preparation (SWAP) containing adult worm antigens was made according to established protocols (14). These experiments were performed using an identical batch of recombinant paramyosin as previously used to investigate human IgG binding (kindly provided by Chris King, Case Western Reserve University, Cleveland, OH) (4).

Antibodies used to examine non-specific binding

Affinity-purified monoclonal human chimeric IgA1 κ , IgG1 κ and mouse IgG2b or IgG3 (anti-MSP1₁₉ hybridoma clones B10 or B6, respectively) and mouse IgG1 κ anti-human Fc γ RI/M22 were available from a previous study (2,15). Human chimeric IgE κ and mouse IgE κ (both anti-DNS) were kindly provided by Professor Sherie Morrison,

UCLA, CA, and Professor Barbara Baird, Cornell University, Ithaca, NY, respectively. Purified human IgG2 κ , IgG3 κ , IgG4 κ , and mouse IgG2a κ , mouse IgE κ anti-TNP (Pharmingen), biotinylated rat IgG2b anti-mouse CD11b and rat IgG2a anti-mouse CD13 (Serotec), rat IgG2c anti-mouse CD3 (Southern Biotechnology Associates, Birmingham, AL) and monoclonal anti-poly histidine/HIS-1 (Sigma) with which to examine Ig binding were purchased from commercial sources as indicated.

Analysis of Ig binding by ELISA, Western blotting and immunofluorescence

Wells of Nunc Maxisorp microtitre plates were coated overnight with 0.5 μ g of recombinant paramyosin or 2 μ g of SWAP in coating buffer (0.05 M sodium carbonate buffer, pH 9.6), and blocked the following day with PBS containing 0.25% gelatine and 0.1% (v/v) Tween-20. After five washes with PBS/0.1% Tween-20 (PBST), 100 μ L of appropriate antibody diluted to 50 μ g/mL ($\sim 3 \times 10^{-7}$ M) in PBST was added per well and incubated overnight at room temperature. Protein G (Calbiochem, UK) at 1×10^{-4} M was co-incubated with the primary antibodies in blocking experiments aimed at defining the interaction site of the Fc for paramyosin. Following five further washes in PBST, 100 μ L of goat F(ab')₂ anti-mouse IgG-biotin conjugate or anti-human kappa-peroxidase (Caltag Laboratories, Burlingame, CA) diluted 1 : 2000 in PBST were added to each well and incubated for 1 h at room temperature. F(ab')₂ antibodies were chosen to preclude non-specific binding by the detecting antibody. A further wash step and incubation with streptavidin-peroxidase (Serotec, UK) diluted 1 : 2000 was carried out for the mouse ELISA. After further washing, reactions were developed by incubating with substrate (Sigma, 3,3',5,5'-tetramethylbenzidine tablets made up according to manufacturer's instructions). Binding of mIgG3 to recombinant hexa-histidine tagged paramyosin and SWAP was analysed on 10% SDS-polyacrylamide electrophoresis gels under reducing and non-reducing conditions. Anti-histidine blots were analysed on 4–15% gradient gels. For Western blotting, proteins were transferred onto nitrocellulose membranes, which were then blocked by incubating at room temperature for 1 h in PBST and 5% non-fat milk powder. After three washes in PBST, blots were incubated in the refrigerator overnight in 50 μ g/mL of mIgG3 anti-MSP1₁₉ diluted in PBST. After three washes in PBST, blots were incubated for 1 h in goat F(ab')₂ anti-mouse IgG-biotin conjugates diluted 1 : 500 in PBST. After a further wash step the blot was incubated with streptavidin-peroxidase diluted 1 : 2000 in PBST for 1 h. After three washes in PBST followed by two with PBS, substrate (a mixture of 10 mg diaminobenzidine, 3 mg of NiCl₂, and 100 μ L of 30%

H₂O₂ in 10 mL of PBS) was added, and the blot developed for 1–5 min. For immunofluorescence microscopy experiments, whole adult worms were washed in phosphate-free buffers prior to mild fixation in Mirsky's reagent (Becton Dickinson, UK) for 30 min followed by further washes in PBS. Worms were then incubated in PBS/0.2% gelatin for 1 h, prior to incubation in 200 µL of PBS/0.2% gelatin containing mouse IgG3 (B6) or human IgG1 (B10) at 100 µg/mL and allowed to bind in the refrigerator overnight. After three 1 min washes in PBS/gelatin (gentle swirling of the tube by hand), 200 µL of goat anti-mouse IgG-FITC (Sigma) at 1 : 100 in PBS was added to each worm for 1 h. After three further washes in PBS/0.2% gelatin, worms were mounted on slides and antibody binding was detected using fluorescence microscopy.

Respiratory burst assays to determine if IgG binding by paramyosin inhibits FcγR function

The inhibition assay was a modification of a previously described chemiluminescence assay of respiratory bursts (2). Wells of a chemiluminescence microtitre plate (Dynex Technologies, Ashford, UK) were coated with recombinant MSP-1₁₉ with subsequent incubation with anti-*P. yoelii* hIgG1, mIgG2b, or mIgG3 in PBS (100 µL/well at 50 µg/mL) for 1–2 h at room temperature as previously (15). After washing, recombinant paramyosin at 5×10^{-6} M in Hanks' buffered saline solution (HBSS) containing 20 mM HEPES and 0.1% (w/v) globulin-free BSA (HBSS/BSA) was added. After incubation for 1 h at room temperature to allow pre-binding of the paramyosin to the IgG, neutrophils in HBSS/BSA containing 260 µg/mL luminol (Sigma) were added to give a final suspension of 0.25×10^6 cells/mL, the plate was transferred to a Microumat LB96P luminometer, and the chemiluminescence measured at regular intervals.

Assay to investigate IgG phosphorylation by parasite kinases

Analysis of antibody phosphorylation was typically performed in a reaction mixture containing 19 µL buffer A (50 mM Tris, pH 7.4, 200 mM NaCl, 12 mM MgCl₂), 1 µL [γ -³²P]-ATP (Amersham: 3000 Ci/mmol, 10 mCi/mL), 10 µL of SWAP (12.8 mg/mL) or a membrane preparation derived from 20 µL of SWAP that had been washed five times in Buffer A with intermittent centrifugation at 14 980 *g*. for 5 min. The reactions were incubated for 1 h, after which microfuge tubes were centrifuged as above. The supernatant containing labelled proteins and antibody was transferred to fresh microfuge tubes containing 10 µL of washed protein G-sepharose slurry (Amersham) and allowed to bind for 5 min. The sepharose beads were then washed five times in

buffer A with intermittent centrifugation as above. After the final wash, 40 µL of SDS gel-loading buffer was added to the beads and the samples were boiled for 5 min prior to analysis on 10% SDS-polyacrylamide electrophoresis gels under reducing and non-reducing conditions. The gel was then fixed, stained with Coomassie Brilliant Blue, destained, and dried at 80°C under vacuum. The dried gel was then imaged with a Phosphorimager (Biorad) to quantify the intensity of the bands, or put under film.

Purification of protein G-sepharose binding proteins and analysis by mass spectrometry

1 mg of SWAP preparation was allowed to bind over 1 h to 100 µL slurry of protein G-sepharose beads (Amersham Biosciences). Beads were washed five times in PBS to remove unbound proteins prior to pelleting and running on 4–15% acrylamide gradient gels under reducing conditions. Proteins contained within an excised band were reduced with DTT, alkylated by IAA and digested with trypsin (16). Peptide mixtures eluted from each gel band were analysed by MALDI-TOF-MS (Waters/Micromass), and protein identification was obtained from peptide mass fingerprints (PMF), by searching the relevant databases using Aldente (<http://www.expasy.org/tools/aldente/>).

Statistics

ELISA results are presented as group mean values $\pm$ standard deviations (SD). Repeated assays involving human or murine antibodies were analysed with general linear models (ANOVA) specifying assay as a random factor. Multiple comparisons amongst antibody treatments or with a control treatment were, respectively, analysed using Tukey's or Dunnett's tests.

RESULTS

Antibody class determination of binding to schistosomes

Earlier work exploring the existence of schistosome Fc-receptors has made use of animal Ig as ligand, and only one study used human antibodies (4). To address this issue we initially assayed the ability of live schistosomes to bind to human IgG1 and IgA1 (in recombinant or natural forms) when presented on the surface of red blood cells (RBCs). We found that these Ig-coated RBCs did not appear to interact with cercariae, mechanically transformed schistosomula or adult stage parasites. This lack of binding was not due to dysfunctional antibody since the same sensitized RBCs were able to rosette to FcγR and FcαR present on human neutrophils (17).

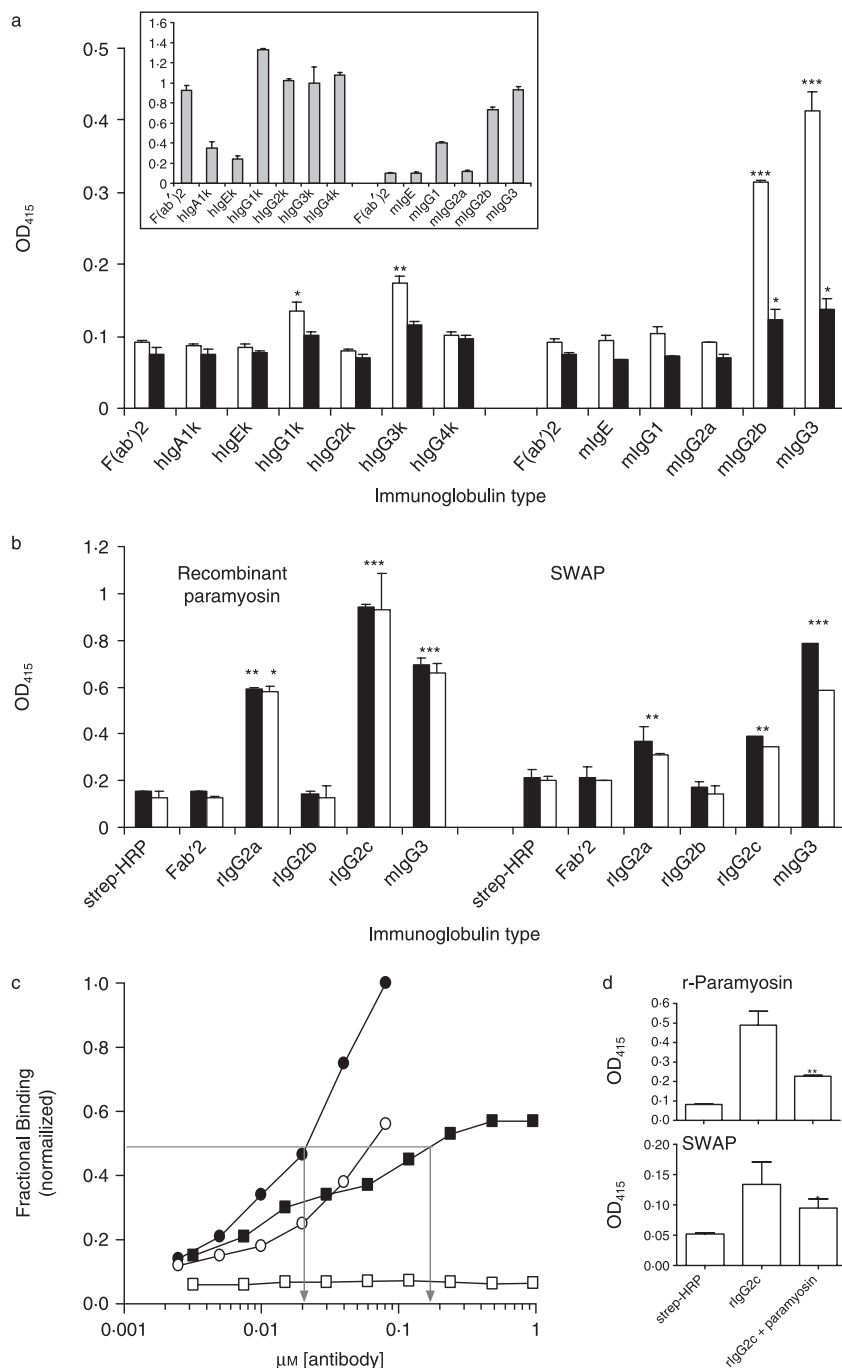


Figure 1 Non-specific binding of human and mouse antibodies to schistosome proteins. (a) Human or mouse antibody at 3×10^{-7} M was analysed by ELISA for binding to recombinant schistosome paramyosin (■), adult worm antigens (□) or staphylococcal protein G (inset) immobilized in microtitre wells.

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ when compared with control F(ab')₂ only.

(b) Non-specific binding of rat IgG2c and IgG2a to schistosome proteins. Biotinylated rat IgG subclasses all at 3×10^{-7} M were analysed by ELISA for binding to both recombinant paramyosin and SWAP immobilized in microtitre wells. Antibody was pre-incubated for 2 h either on its own (■) or in the presence of saturating quantities of protein G (1×10^{-4} M) from *Streptococcus* sp. (□) prior to adding to antigen-coated wells. Intermediate washing steps were performed in PBS in the absence of Tween-20. Mean absorbances ($\pm$ standard deviations) from duplicate wells of at least two experiments are shown. *** $P < 0.001$, ** $P < 0.01$ when compared with control F(ab')₂ only.

(c) Titration of rat IgGc binding to paramyosin (■) or protein G (●) and rat IgG2b binding to paramyosin (□) or protein G (○). Results were normalized by expressing ELISA absorbance as a fraction of mean absorbance seen with rat IgG2c binding by protein G. This experiment was performed at least two times with similar results. (d) Binding of rat IgG2c (0.1 μM) to either recombinant paramyosin (top panel) or SWAP (bottom panel) coated down on ELISA plates can be reduced significantly with a 10-fold molar excess of recombinant paramyosin in competition experiments.

** $P < 0.01$, * $P < 0.05$ when compared with control rat IgG2c in the absence of recombinant paramyosin.

Since Fc derived from pooled human serum IgG has been shown to bind schistosomes via paramyosin, we postulated that a subclass other than IgG1 or IgA1 may be responsible for the observed binding seen by others (4). To test this hypothesis we examined binding of all four human IgG subclasses by ELISA on plates coated either with recombinant paramyosin, adult worm antigens or protein G as an inter-

nal control (Figure 1). We also examined binding by rodent IgG subclasses, since these antibodies have been documented as binding to the schistosome tegument in a non-Fab-dependent manner (18,19). A particular role for murine IgG2b was suggested by these earlier experiments (20,21).

Although human IgG3 could bind weakly to a soluble worm antigen preparation (SWAP), none of the human

antibodies was able to interact with recombinant paramyosin (Figure 1a). In contrast to the human antibodies, mouse IgG2b and IgG3 were very capable of binding adult worm antigens, and weak binding to recombinant paramyosin was also evident with these murine subclasses (Figure 1a). Since the murine IgG2b and human IgG1/IgA1 recognize identical epitopes in *Plasmodium yoelii* merozoite surface protein 1 (MSP1₁₉), binding to paramyosin or SWAP most likely occurs outside the antigen-combining site and via the Fc (15). Intriguingly, although both F(ab')₂-detecting antibodies were developed in goats, only the anti-human kappa reagent was able to bind protein G, presumably due to genetic diversity in the goat Ig gene repertoire (Figure 1a inset).

That binding of the Fc to schistosome proteins, including paramyosin, was of low affinity was confirmed in further ELISA experiments, where detergent was omitted from the wash steps. We reasoned that the use of detergents in the wash buffers would significantly disrupt weak non-covalent bonds known to be important in reversible molecular interactions of biological importance. In these experiments very significant binding of mouse IgG3, rat IgG2c and rat IgG2a could be detected to both SWAP and recombinant paramyosin when compared with binding by equimolar concentrations of rat IgG2b (Figure 1b). Under conditions where detergent was omitted from the wash steps both human IgG3 and human IgE could bind significantly to SWAP, although these values never reached the levels detected with rodent subclasses (data not shown). Co-incubation with saturating quantities of protein G was unable to inhibit binding of the rodent antibodies to paramyosin or SWAP (Figure 1b, white bars).

Curve fitting on the rat IgG2c allowed the *K_d* of the interaction to be estimated at 200–300 nM, approximately $\times 10$ lower affinity than the 20 nM value observed for rat IgG2c binding by protein G, whilst confirming the inability of rat IgG2b to bind (Figure 1c). Furthermore, binding of rat IgG2c to either paramyosin or SWAP coated onto ELISA plates could be significantly reduced when co-incubated with a 10-fold molar excess of paramyosin in competition experiments (Figure 1d).

Murine IgG3 binds to discrete surface structures on fixed adult worms but not live worms

To analyse whether the findings using recombinant paramyosin or SWAP give a true reflection of the reactivity of the proteins *in situ*, we performed binding studies with whole worms. Immunofluorescence microscopy on fixed adult worms demonstrated very bright binding of mIgG3 to the surface and to material that had blebbed off during the fixing process (Figure 2). We also observed binding to elon-

gated lines and discoid areas (arrowed). Since fluorescence staining was not of a weak diffuse and uniform nature, we made the assumption that binding was specific and not due to surface damage during the isolation process, as has previously been reported. However, this pattern of fluorescence was not observed with live unfixed worms where no specific staining was seen, the implication being that Fc binding occurs only when the tegument has been compromised in some way; either through cross-linking during the isolation/fixing process or via immune or drug-mediated damage.

Many adult worm molecules can bind murine IgG3

Reasoning that the poor binding by recombinant paramyosin in the ELISA format may have arisen due to differences between the native and recombinant protein, we examined binding of mouse IgG3 to adult worm antigens by immunoblotting (Figure 3a). The identical batch of recombinant paramyosin that had previously been used to demonstrate human IgG binding was used throughout this study (4). Intriguingly, murine IgG3 not only bound recombinant paramyosin (lanes 1 and 2), but interacted strongly with numerous adult worm molecules that clearly ran at different molecular weights to recombinant paramyosin (lanes 3 and 4), implying that paramyosin is not the only molecule capable of binding mouse IgG3. The increased binding to SWAP over recombinant paramyosin confirmed the results seen in the ELISA experiments. Immunoblotting with monoclonal anti-poly histidine-HRP allowed us to detect the recombinant fusion protein which runs as a monomer at a slightly higher molecular weight than the 101 kDa predicted (http://us.expasy.org/tools/pi_tool.html). Intriguingly, the recombinant protein also formed homodimers (red arrows), as expected for associating muscle proteins, but the higher molecular weight homodimer did not bind mouse IgG3 by blotting.

Paramyosin shares heptad periodicity with many coiled coil proteins known to bind IgG

Prosite searches (<http://us.expasy.org/prosite/>) revealed the presence of a leucine zipper motif in paramyosin, offering an explanation as to why the molecule runs as an apparent homodimer on SDS-PAGE (Figure 3a). Work from our laboratory had previously shown that a 50-residue IgA-binding peptide derived from the streptococcal protein Sir22 could bind IgA in the C α 2–C α 3 interdomain region (2). We observed that this peptide shared heptad periodicity with leucine in position *a*, and that full-length Sir22 protein also contained a coiled coil leucine zipper arrangement, in common with paramyosin and many other alpha-helical coiled coil proteins known to bind immunoglobulins in the Fc

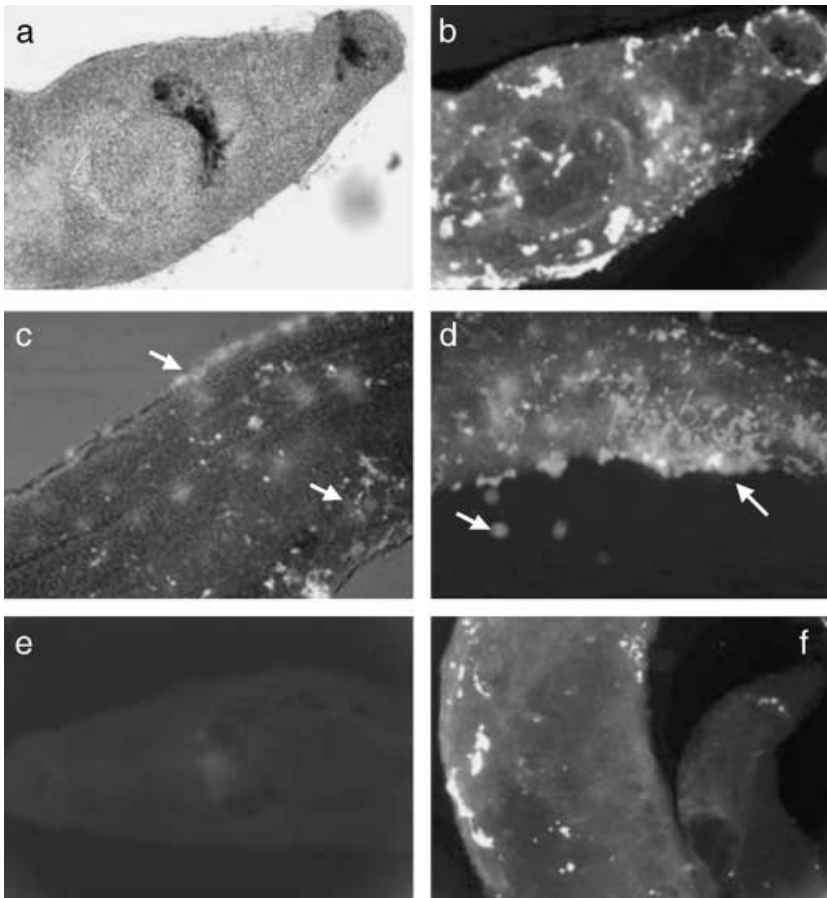


Figure 2 Fluorescence microscopy of antibody binding to the surface of *S. mansoni* adult worms. Worms were incubated with mouse IgG3 (panels a, b, c, and d), human IgG1 (panel f) or no antibody (panel e) at 100 µg/mL in PBS/0.2% gelatin. Bound antibody was detected with goat anti-mouse IgG-FITC. Note binding at the worm's surface and to areas of blebbed off tegument. All $\times 40$ magnification.

(Figure 3b). These heptad repeats are found in two regions within the paramyosin molecule at residues 168–203 and 330–414 (Figure 3c).

Paramyosin does not inhibit binding of IgG to human Fc-receptors

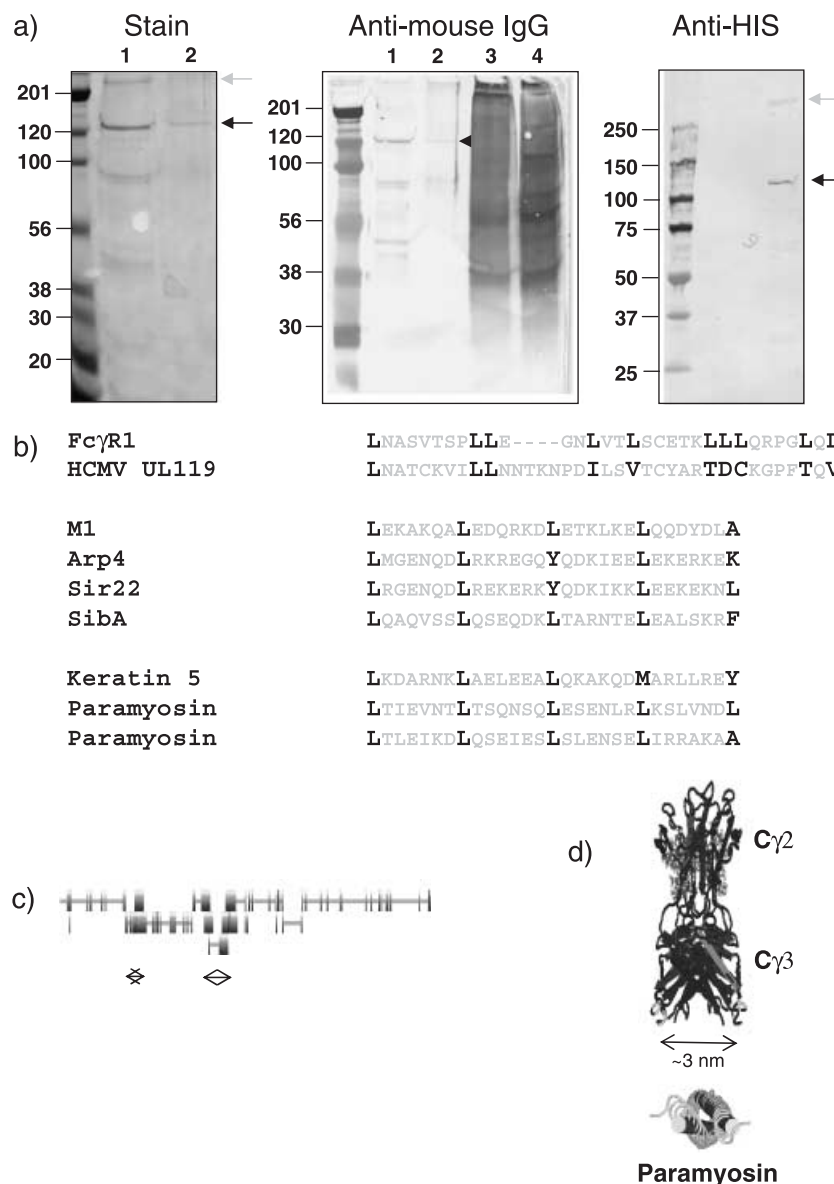
The data reported above revealed that paramyosin together with unknown adult molecules could bind to rodent IgG. Therefore, we investigated the capacity of paramyosin to inhibit the ability of IgG to bind and activate Fc γ R found on human neutrophils. As a physiologically relevant test of function, we assessed the ability of recombinant paramyosin to inhibit the IgG-mediated triggering of a respiratory burst in neutrophils, characterized by NADPH oxidase activation and degranulation, which can be assessed in a chemiluminescence assay (2). The oxidative burst is a biological response of major importance in host defence, and an ability to inhibit it might be advantageous to the worm. Recombinant paramyosin was unable to inhibit hIgG1-, mIgG2b- or mIgG3-triggered oxidative bursts using excess protein to 5×10^{-6} M (Figure 4), molarities known for bacterial Fc-binding proteins

to be very effective at inhibiting respiratory bursts (2). We have previously shown that mIgG2b is able to elicit a respiratory burst through interactions with human Fc γ R (15).

Identification of potential binding sites on rodent IgG

For the binding mouse IgG2b, epitope-matched human IgG1 and IgA1 (available as chimeric human/mouse antibodies recognizing identical epitopes in MSP1₁₉), were available to us from a previous study (15). We therefore made the assumption that differences in the Fab were unlikely to contribute to the non-specific reactivity observed. Since paramyosin was incapable of inhibiting Ig-mediated respiratory bursts (Figure 4), we also hypothesized that binding of parasite proteins was unlikely to involve the hinge proximal region of Ig-Fc, already known to determine binding of Fc γ -receptors (22). Earlier work had shown that binding of paramyosin to Ig-Fc was not inhibited by soluble protein A, a result we confirmed in the current study with protein G (Figure 1b), implying that paramyosin binding is also unlikely to involve the CH2–CH3 interdomain region and the Fab CH1 domain (4). We therefore made amino acid

Figure 3 Comparison of adult worm IgG3-binding proteins. (a) Western blot analysis of recombinant HIS-tagged paramyosin (lanes 1 and 2) and adult worm antigen (lanes 3 and 4) binding by mouse IgG3 under non-reducing (lanes 1 and 3) and reducing (lanes 2 and 4) conditions (10% acrylamide). An equivalent 10% acrylamide Coomassie-stained SDS gel (furthest left) and 4–15% acrylamide gradient gel blotted with anti-Histidine-HRP (furthest right) for recombinant paramyosin are shown. The recombinant protein runs as a monomer (black arrows) and as a homodimer (grey arrows) that stains for the anti-histidine mAb but not for the anti-mouse IgG. 10 µg of protein was run in each lane. (b) Amino acid alignments comparing the heptad periodicity regions of schistosome paramyosin against known viral, bacterial and human Ig-binding proteins. Those amino acids present in heptad position *a* are shown in boldface. (c) Location of heptad periodicity regions to two hotspots on the paramyosin molecule (arrowed). (d) Molecular model of human IgG1 Fc, highlighting the location of two exposed loops at the bottom of the Cγ3 domain postulated to be involved in low affinity binding to alpha-helical coiled coil proteins. The models of human IgG1 and the alpha-helical coiled coil protein tropomyosin are adapted from refs. (23) and (55), using atomic coordinates and the macromolecular structure viewer Cn3D 4.1 available from NCBI under accession numbers 1HZH and 1IC2.



alignments of the rodent IgGs against those Ig that we knew did not to bind, in an attempt to identify residues unique to these antibodies that might explain their differential capacity for specific worm binding. The only residues found to be unique to mIgG3, mIgG2b and rIgG2c were found in the CH3 domain. They were Ser³⁵⁹, Ser³⁷⁸, Glu/His³⁸⁶, Thr⁴¹⁴, Ser/Lys⁴¹⁶, and Glu/Asp⁴²¹. Intriguingly, the majority of these residues mapped to two exposed loops at the bottom of the CH3 domain, previously shown to provide a solvent-accessible hydrophobic patch with potential for protein–protein interactions, when modelled on the known crystal structure of human IgG1 (Figure 3d) (23). Thus, this region of the rodent Igs may have a charge distribution that is

appreciably different to nonbinding Ig classes. Since 20% of paramyosin comprises Glu or Asp amino acids, we postulate that these residues could potentially act as hydrogen-bond acceptors or donors with the Ser/Thr residues identified in the Cγ3 domain of the rodent antibodies. Although these residues are not identical in rat IgG2a, which also binds paramyosin, there are unique Ser, Thr and other charged residues that lie nearby, including Ser³⁶¹, Thr³⁷⁹, Gln³⁸⁸, Lys⁴¹³, and Thr⁴¹⁶. Analysis of crystal structure and NMR data highlighted that the distance between the two loops in the Cγ3 domain approximated the distance between four repeats on models of alpha-helical coiled coil proteins (~1 nm × 3 nm) (Figure 3d).

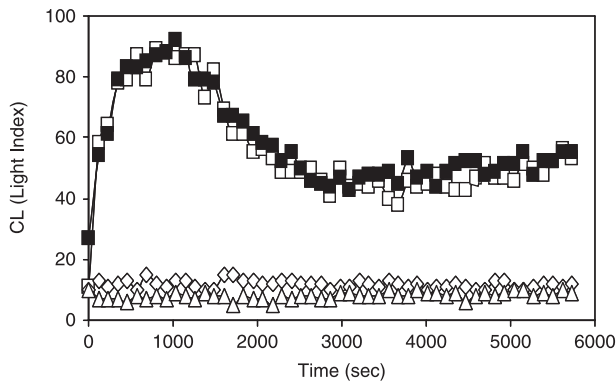


Figure 4 Analysis of inhibition of an IgG-mediated neutrophil respiratory burst by recombinant paramyosin. Chemiluminescence (CL, arbitrary units) was induced by IgG alone ($\square$) or in the presence of recombinant paramyosin ($\blacksquare$). The negative controls show chemiluminescence in the absence of antibody and inhibitor (Δ) or wells coated in paramyosin alone ($\diamond$). Data from two separate experiments are shown for hIgG1 but similar results were obtained for mIgG2b, also known to bind human Fc γ R.

Immunoglobulins are not phosphorylated by surface parasite kinases but are cleaved by parasite proteases

Given the close proximity of these antibodies to the worm surface by nature of their low affinity interactions with parasite FcR, we speculated that these exposed Ser and Thr residues were open to phosphorylation by known parasite tegumental surface kinases. Post-translational modification of proteins by phosphorylation can dramatically alter protein function, and we hypothesized that phosphorylation of antibodies in the Fc region by parasites may alter the function of antibodies in such a way as to benefit the parasite. Using the NetPhos 2.0 neural network-based method (<http://www.cbs.dtu.dk/services/NetPhos/>), we identified numerous Ser, Thr and Tyr residues in both the C γ 2 (Ser²⁶⁷, Thr³³⁵, Ser³³⁷ and Tyr²⁹⁶) and C γ 3 (Ser³⁵⁹, Ser³⁶⁴, Thr⁴¹⁴, Ser⁴¹⁶, Ser⁴⁴² and Ser⁴⁴⁴) domains that were potentially amenable to phosphorylation. Many of these putative phosphorylation sites lie close to functionally important zones in the Fc.

To test this hypothesis we incubated [γ -³²P]-ATP with mIgG3, mIgG2b or mIgG1 and looked for phosphorylation of these antibody classes by SWAP or tegumental membrane preparations from adult parasites (Figure 5). Protein G-sepharose columns provided a convenient way to purify and wash the labelled antibody away from the reaction mix. Purified mIgG3 (Figure 5a, lanes 2–4) and mIgG1 (Figure 5a, lanes 5–7) were run on 10% SDS-PAGE gels, stained, and exposed to film. Overlaying the film onto the stained gel clearly demonstrated that neither the immunoglobulin heavy nor the light chains were amenable to phosphorylation by either the membrane (Figure 5a, lanes 3

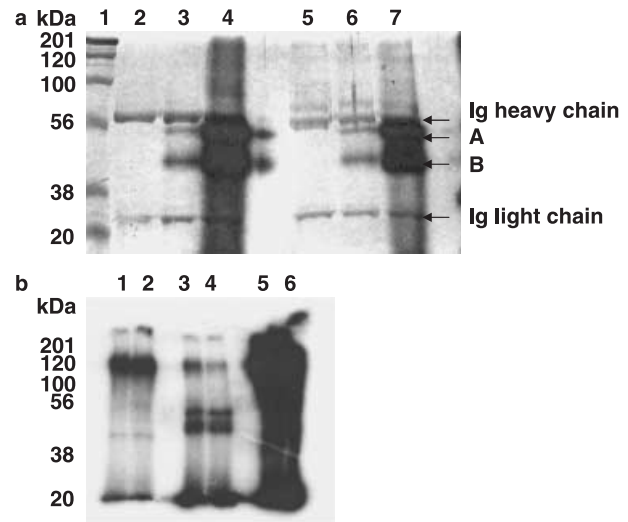


Figure 5 Immunoglobulins are not phosphorylated by surface parasite kinases. (a) To ascertain if antibodies are post-translationally modified through parasite-mediated phosphorylation, mouse IgG3 or IgG1 was incubated in the presence of [γ -³²P]-ATP with adult worm antigens (lanes 4 and 7) or a tegumental membrane preparation derived therefrom (lanes 3 and 6). Mouse IgG3 (lanes 2–4) and IgG1 (lanes 5–7) were run on 10% SDS-PAGE gels, stained, and exposed to film. Overlaying the autoradiograph onto the stained gel clearly shows that antibody heavy and light chains are not phosphorylated when intact. Lanes 2 and 5, controls where antibody was incubated in the absence of parasite material. (b) Autoradiograph of pull down experiments with mouse IgG2b to identify the nature of the phosphorylated proteins was performed under both non-reducing (lanes 1, 3, and 5) and reducing conditions (lanes 2, 4, and 6). Controls where either parasite membranes (Figure 5a, lanes 2 and 5 and Figure 5b, lanes 1 and 2) or antibody (Figure 5b, lanes 5 and 6) were omitted from the reaction mix clearly show that the phosphorylated fragments are derived from parasites and bind directly to protein-G-sepharose when excess material is loaded into individual lanes.

and 6) or the SWAP preparation (Figure 5a, lanes 4 and 7). However, two phosphorylated proteins with native molecular weights of approximately 45 and 50 kDa, were co-purified by the protein G-sepharose from both the SWAP and membranes derived from the SWAP preparation.

Controls where either parasite membranes (Figure 5a, lanes 2 and 5; Figure 5b, lanes 1 and 2) or antibody (Figure 5b, lanes 5 and 6) were omitted from the reaction mix clearly show that these two proteins do bind directly to the protein-G-sepharose and are therefore of parasite origin. Since the phosphorylated proteins ran at identical molecular weights under both reducing and non-reducing conditions they are unlikely to represent phosphorylated IgG fragments, whose heavy and light chains are held together by numerous inter-heavy chain disulphide bridges. Further controls using excreted/secreted proteins from the

nematode *Trichuris muris* confirmed that these observations were specific to schistosomes (data not shown). We performed larger scale pull-down experiments with protein-G-sepharose in order to identify the phosphorylated proteins by MALDI-TOF-MS. Although we identified schistosome heat shock protein-70 (sHSP-70) as a novel ligand for protein G, we were unable to identify the other two phosphorylated proteins (Figure 6a, lane 1).

To exclude the possibility that these phosphorylated proteins were derived from degraded IgG, we ran control pull-downs of mIgG2b that had been incubated with SWAP for 1 h (Figure 6a, lane 2). This revealed the presence of two peptides that ran below 37 kDa. Peptide sequencing by nano-ESI-Q-TOF MS/MS showed that these bands represented cleaved Fc, degraded in the lower hinge region (A↓PNLEGGPSV, represented by mass peak 1825.90) by unidentified parasite proteases (Figure 6a,c). We were therefore confident that cleaved antibody fragments did not represent the phosphorylated proteins, which all ran above 40 kDa and must therefore represent novel phosphorylated parasite proteins.

DISCUSSION

Adult schistosomes, which are millimetres in length, can survive in the human bloodstream for decades (24). One of the most striking immune evasion strategies displayed by these parasites is the acquisition of host products onto their surface tegument in order to mask their foreign status. These molecules include the immunoglobulins (4,18–21,25,26), β 2-microglobulin (4,19), α 2-macroglobulin (27), blood group antigens (28), MHC products (29), and numerous complement components (8,30,31). Although these interactions are known to occur, the underlying molecular mechanisms remain obscure and many of the parasite ligands involved remain to be identified. The recent publication of the *S. mansoni* transcriptome will undoubtedly help resolve some of these outstanding issues (32).

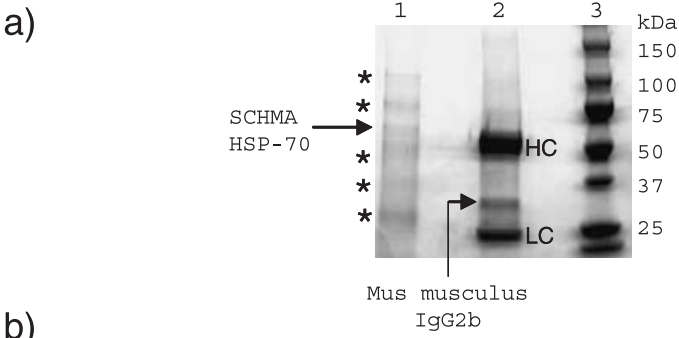
Here we confirm that *S. mansoni* paramyosin is a FcR for IgG, albeit of low affinity. We show that proteins derived from adult parasites or cercarial stages (data not shown) preferentially bind rodent Ig, in particular mouse IgG2b/IgG3 and rat IgG2c. Fc-binding is not exclusive to paramyosin and other parasite proteins must contribute to the binding of IgG. Recently, the IL-4-inducing principle of *S. mansoni* eggs (IPSE) has been cloned and shown to bind human IgE in a non-antigen-specific manner involving both the Fc and Fab regions (33). IPSE is also known to bind IgG, and a related molecule in adult worms may explain our observation that hIgG3 and hIgE can bind SWAP in the absence of detergent (Helmut Haas, pers. comm.). The inference is that more than one Fc-binding protein is present in

schistosomes and that binding is determined as much by conformation as by primary sequence.

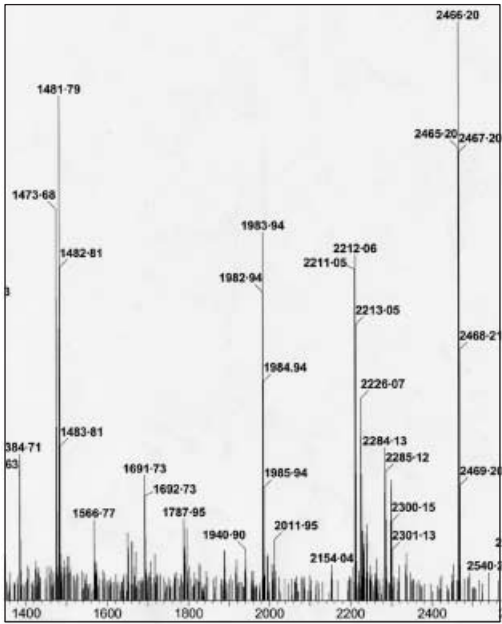
We assume that differences which allowed some Ig classes to bind paramyosin and or SWAP lie in the Fc region and not in the Fab, since many of the monoclonal Ig we used to assess specific binding were epitope matched. For example human IgG1 and IgA1 (non-binders) used in this study were derived by V_H and V_L gene cloning from a murine IgG2b hybridoma (binder) recognizing MSP1₁₉ (15). Aligning the Fc regions of binders with non-binders allowed us to identify residues in exposed loops in the C γ 3 domain putatively involved in this weak association with worm molecules. Intriguingly, these were commonly Ser or Thr residues, amino-acids which may be capable of serving as hydrogen-bond donors and acceptors. No amino acids unique to mouse IgG2b and IgG3 were found in the C γ 2 domains. The Fc region of IgG contains four hydrophobic patches, two of which are known to interact with distinct molecules: one in the C γ 2–C γ 3 interdomain region bound by protein G/A, mannanose binding lectin (MBL), and the neonatal Fc-receptor (FcRn) and one at the top of the C γ 2 domain bound by phagocytic Fc γ Rs (22,34–36). We provisionally discounted the involvement of these regions since IgG binding by paramyosin did not inhibit Fc γ R-mediated NADPH respiratory bursts, and in our hands protein G was incapable of blocking IgG binding to paramyosin.

When comparing domain structures of IgGs from different species, we made the assumption that all would adopt the same structure as the comparable domain for which a crystal structure was available (23). The hydrophobic patch on the outside of the C γ 3 domains is large and would therefore be expected to be accessible to interacting macromolecules. There is significant variability in the size of this patch between IgGs from different species and significant data to suggest, at least for rabbit Fc, that these residues have considerable flexibility in solution and are not all covered by the C-terminus (36).

In common with the streptococcal IgA-binding protein Sir22, paramyosin forms an alpha-helical coiled coil structure. Recently, it was discovered that Sir22 can inhibit IgA function by blocking interaction with human phagocyte Fc α R, although this is clearly not the case with paramyosin (2). Twenty per cent of schistosome paramyosin is made up of Glu and Asp amino acids. Comparative molecular modelling suggests that these Glu and Asp residues may form regular zones of negative charge on the outer surface of the paramyosin helix, which we postulate hydrogen bond with Thr/Ser residues in the Fc (37). Schistosome paramyosin contains a leucine zipper-like motif and heptad periodicity with leucine in position *a*, structural features also seen in a number of reported immunoglobulin-binding M and M-like proteins (Sir22, Arp4, ML2-2, M1, M49 and SibA) from



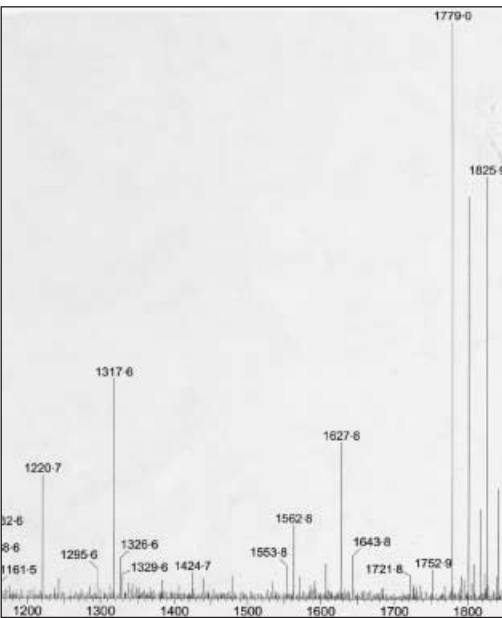
b)



Score: 0.91, 10 matching peptides: P08418 (HS70-SCHMA)
pI: 5.42, Mw: 69875.04

user mass	matching mass	peptide
1787.95	1787.99	IINEPTAAAIAYGLDKK
1691.73	1691.72	STAGDTHLGGEDFDNR
1659.87	1659.89	IINEPTAAAIAYGLDK
1481.79	1481.80	SQIHDIIVLVGGSTR
1473.68	1473.68	TTPSYVAFTDSEK
1384.71	1384.70	HWPFVETQVGGK
1384.71	1384.77	IPESDRQVIISK
1253.63	1253.61	FEELNADLFR
1169.67	1169.6637	DAGAIAGLNVLR
1081.57	1081.56	LLQDFENGK

c)



Score: 0.67, 7 matching peptides: P01867 IgG2b [Mus musculus] Theoretical pI: 8.29, Mw: 49661.62

user mass	matching mass	peptide
1825.90	1825.98	PNLEGGPSVFIFPPNIK
1779.0	1778.97	APQVYILPPPAEQLSR
1627.8	1627.78	STLPIQHQQDWMSGK
1220.7	1219.58	LLPPPAEQLSR
1116.5	1115.58	DVLMLSLTPK
994.5	994.53	PPPAEQLSR
926.47	926.491	DLPSPIER

streptococcal strains reported to bind immunoglobulins (e.g. M49) (2,12,38). Many of these proteins often have a tyrosine substitution in place of leucine in the heptad arrangement, and this is true for schistosome paramyosin, although more commonly there is a glutamic or aspartic acid substitution (38). There is no direct evidence that this heptad periodicity is directly linked to immunoglobulin binding in paramyosin; although the evidence from the M protein family is very suggestive for the importance of this secondary structure (2,12,38).

Rodents form the greatest number of individual species (56%) of the seven known extant mammalian orders considered important definitive hosts for the *S. mansoni* group (39), and therefore binding of rodent IgG may be important for these parasites in the wild as a means to immune evasion. Since paramyosin is highly conserved amongst different genera of organisms and between diverse schistosome species, binding of Ig by rodent-specific schistosomes, e.g. *S. rhodaini* would be very likely and thus may represent an important immune evasion strategy employed by animal schistosomes. The recent finding that mouse IgG2b/IgG3 (but not mouse IgG1/IgG2a) are particularly potent at inducing complement-activated autoimmune responses may explain this evolved selectivity, given the critical role of paramyosin in inhibiting the complement membrane attack complex (8,40). Interestingly, rat IgG2c is known to be a potent blocking antibody and has been shown to inhibit potent anti-schistosome immune effector mechanisms conferred by other Ig classes, including rat IgG2a (41), although blocking activity has not been documented with murine IgG2b or IgG3. Intriguingly, human keratin 5, which also forms an alpha-helical coiled coil, has recently been identified as a rabbit IgG Fc-binding protein in human corneal epithelium, although the binding site on the Ig remains unknown (42). Comparative homology modelling shows that rabbit IgG has many more serine and threonine residues in these loops as well as the ones it shares with mIgG2b and mIgG3, and therefore binding via these residues in rabbit Cγ3 may also occur via this domain. We have not tested binding of rabbit IgG to paramyosin, but would advise caution in the use of these antibody classes, due to the very real possibility of non-specific cross-reactivity in immune assays with schistosomes.

A close examination of residues Thr⁴¹⁴ to Glu⁴²¹ revealed that this loop had all the signatures of a potential phosphorylation site for serine/threonine kinases (43). The NetPhos 2.0 neural network-based method allowed us to predict that for mouse IgG3, for example, Ser³⁵⁹, Ser³⁷⁸, Ser⁴¹⁶, Ser⁴⁴² and Ser⁴⁴⁴, but none of the Thr residues were potentially phosphorylated (44). This is notable because marked serine–threonine kinase activity is associated with the parasite surface and two *S. mansoni* receptor kinases (SmRK1 and SmRK2) cloned from adult worms, and members of the receptor serine–threonine kinase (RSTK) family, have been localized to the surface of the parasite syncytial tegument (45,46). Given the location of these Ser/Thr loops, we wondered if mouse Ig was amenable to phosphorylation by known tegumental parasite kinases. Although we could find no evidence to suggest that Ig heavy or light chains could be phosphorylated by the parasite, we did identify a number of parasite-derived phosphorylated proteins that bound readily to protein G-sepharose, including schistosome heat shock protein 70 (SCHMA-HSP70). The 70 kDa heat shock protein DnaK from *Escherichia coli* is known to bind the C-terminus of staphylococcal protein A and does not interfere with IgG binding (47). We assume that the SCHMA-HSP70 interacts in a similar manner with protein G, and this finding may be relevant for researchers wishing to purify the schistosome protein. Of relevance is the finding that schistosome HSP-70 localizes to the surface tegument, is immunogenic, and has been proposed as a vaccine candidate (48). Since bacterial HSP-70 binds tightly to ATP and carries out auto-phosphorylation, we were surprised not to see a phosphorylated product at 70 kDa, although this does suggest that schistosome HSP-70 may function differently from *E. coli* DnaK (49).

To exclude the possibility that these phosphorylated proteins represent degraded Ig, we analysed extensive protein G-sepharose pull downs of mIgG2b after co-incubation with worm proteins. Two major peptides running below 37 kDa (and therefore unlikely to represent the phosphorylated proteins, which run above 40 kDa), were positively identified as mIgG2b Fc, with the most N-terminal cleavage occurring in the lower hinge region. This finding may explain why researchers (including ourselves) do not consistently

Figure 6 Mass spectrometry and sequencing analysis of protein G-sepharose binding proteins. (a) 1 mg of SWAP preparation was incubated either alone or in the presence of mIgG2b (to exclude the possibility that phosphorylated proteins are derived from degraded IgG fragments) for 1 h prior to binding by 100 µL of protein G-sepharose beads. After five washes of the beads in PBS, SWAP (lane 1) or SWAP + mIgG2b (lane 2) were run on 4–15% acrylamide gradient gels under reducing conditions. Gel bands (starred) were excised and analysed by MALDI-TOF-MS. (b). The identity of schistosome HSP-70 (arrowed) was confirmed by analysis of 10 matching mass peaks. (c). Two peptides running below 37 kDa, and therefore unlikely to represent the phosphorylated parasite proteins, were positively identified as cleaved mIgG2b Fc fragments (Figure 6a; lane 2 arrowed). Their observed molecular weight of ~30 kDa together with the identification of mass peak 1825.90 representing peptide (PNLEGGPSVFIFPPNIK) implied cleavage in the lower hinge after Ala²³¹, although lower abundance contaminating mass peaks of 994.5 and 1627.8 suggested further cleavage sites C-terminal to the hinge at Val³⁰³/Ser³⁰⁴ and Leu³⁵¹/Pro³⁵². Peptide sequencing by nano-ESI-Q-TOF MS/MS of all the major mass peaks confirmed their identity as arising from the Fc constant region of mIgG2b.

detect antibody binding by live schistosomes, bound either to paramyosin or to other FcRs. Since metabolically active worms are actively secreting proteases, they may also cleave the detecting antibody itself, thus making such assays on live worms difficult to interpret. For example, after 3 h in culture, schistosomula have lost their ability to rosette IgG-coated erythrocytes, implying either the loss of Fab following a proteolytic cleavage or the shedding of the IgG molecule and/or Fc receptor (50). Such an explanation appears particularly attractive and fits with observations that antibody binding could only be detected by indirect methods, e.g. immunofluorescence, and not following direct rosetting assays with antibody-coated erythrocytes (data not shown) (3,25). It is also known that schistosomula and adult worms can ingest fluorescently labelled IgG which is fragmented to 10–15 amino acid peptides by gut proteases (Thors *et al.* pers. comm.) (26). These peptide fragments rapidly find their way to the acetabular glands and presumably from there are excreted onto the surface of the parasite (26). We cannot rule out the possibility that cleavage of IgG3 also occurs at the level of the gut, with cleaved Fc being secreted in the vomitus, or incorporated by diffusion into tegumental structures, as observed by Thors *et al.* (pers. comm.). The identity of the protease remains unknown. It is unlikely to be cathepsin D, the only characterized schistosome protease with IgG-cleaving ability, since this enzyme cleaves human IgG at Phe²⁴¹/Leu²⁴² and Leu³⁰⁶/Thr³⁰⁷, the homologous residues being intact in our mass spectrometric and sequencing analysis with IgG2b (51). Since cathepsin D and other aspartic proteases are responsible for haemoglobin digestion in the schistosome gut, it would be reasonable to suspect a tegumental origin for this particular protease (52). The recent finding that the Mac-1 and Mac-2 (also known as IdeS) proteins from group A *Streptococcus* can both bind and cleave IgG simultaneously, presents the exciting possibility that such proteins may well exist for schistosomes (53). Although schistosomes do not appear to have a Mac-1 homologue, the cleavage site for IgG identified by mass spectrometry lies within a sequence postulated to contain the cleavage and binding site for Mac-1 (54).

We therefore propose the following model. Antibodies that come close to the worm surface through charge interactions can be cleaved in the hinge region. The cleaved Fc fragments may remain associated with the membrane, diffuse into the main body of the worm, or given the low affinity binding, diffuse away into the host's immune circulation. Binding and cleavage of antibodies is likely to be important for worms at a number of levels; including immune evasion, nutrient supply and tegumental repair. Future work is aimed at defining the proteases involved in antibody cleavage and elucidating the role of cleaved Fc fragments in immune evasion by schistosome parasites.

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