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Multiple heparin binding domains of respiratory syncytial virus G mediate binding to mammalian cells

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Summary. Respiratory syncytial virus (RSV) G glycoprotein mediates cell attachment through surface glycosaminoglycans (GAGs). Feldman et al. [10] suggested that specific basic amino acids in residues 184–198 of G defined a critical heparin binding domain (HBD). To further define the G HBD we made a series of truncated G proteins expressed in *Escherichia coli*. G88 (G residues 143–231), bound to HEp-2 cells in a dose dependent manner and binding was inhibited >99% with heparin. Cell binding of G88 was unaltered by alanine substitution mutagenesis of all basic amino acids in Feldman's region 184–198. A G88 variant truncated beyond residue 198, G58, and G58 fully alanine substituted in the region 184–198, G58A6, bound to HEp-2 cells about half as well and 100-fold less well than G88, respectively. G88 and all alanine substitution mutants of G88 inhibited RSV plaque formation by 50% (ID_{50}) at concentrations of ∼50 nM; the ID_{50} of G58 was ∼425 nM while G58A6 had an $ID_{50} > 1600$ nM. These data show that the G HBD includes as much as residues 187–231, that there is redundancy beyond the previously described HBD, and that the cell-binding and virus infectivity-blocking functions of these recombinant G proteins were closely linked and required at least one HBD.

Introduction

Respiratory syncytial virus (RSV) has three integral membrane glycoproteins: G (attachment), F (fusion) and SH (small hydrophobic). Depending on the RSV strain, membrane associated G has 292 to 299 amino acids with an estimated M_r

of 32 K. Virus associated, native G is modified by the addition of *N*- and *O*-linked carbohydrate, resulting in a M_r of between 80–90 K depending on the cell line of origin [16].

RSV G is a type II membrane protein with three functional domains: cytoplasmic region (amino acid residues 1–37), transmembrane region (38–66) and ectodomain (67–298). The ectodomain is further characterised as consisting of two mucin-like domains which flank a central conserved region (residues 156–194). This latter region contains a conserved 13 amino acid sequence (residues 164–176) which is reported to contain a region with a receptor binding function [19] and 4 cysteine residues at positions 173, 176, 182 and 186 which are completely conserved among all naturally occurring isolates of RSV [33] (Fig. 1A). The four cysteines form a disulfide linked noose (cysteine noose) with linkages in the configuration 173–186 and 176–182 [18]. The sequence between cysteine residues 182 and 186 is a CX3C chemokine motif and G protein binds to the fractalkine receptor, CX3CR1, via this motif [40].

The attachment role of RSV G protein was demonstrated by neutralisation of RSV and inhibition of virus binding with G-specific antibody [27]. Despite the suggestion that the cysteine noose of the G protein represents a potential receptor binding domain [25] its deletion from the G protein of recombinant RSV did not adversely affect replication *in vitro* or *in vivo* [39]. However the importance of the G protein in the life cycle of RSV was demonstrated by the reduced ability of a recombinant virus lacking the entire G gene to replicate *in vitro* and *in vivo* [38].

Until recently the cellular molecules that bind G were largely unknown. In 1997 Krusat et al. demonstrated that purified RSV G protein bound to immobilised heparin [24]. Since the *C*-terminus of G protein contains a region of positively charged amino acids (residues 188–217) that resemble consensus heparin binding domains (HBDs) of mammalian heparin binding proteins this region seemed a likely candidate for the G protein to interact with cell surface glycosaminoglycan (GAG) [24].

GAGs are present on the surface of most mammalian cell types, in tissues and in the extracellular matrix, and are known to be involved in a diverse range of biologically important functions such as cell-to-cell signalling and cell adhesion [22]. An increasing number of viral attachment proteins that utilise GAGs as initial or low affinity contacts to facilitate viral infection of host cells are being discovered. Some viruses such as *Sindbis virus* [3] and the flaviviruses, *Tick-borne encephalitis virus* (TBE) [29] and *Murray Valley encephalitis virus* (MVE) [26] viruses appear to utilise one GAG, heparan sulfate (HS), as an attachment receptor only after cell culture passage. However HS is an important receptor for attachment of primary isolates of another flavivirus, *Dengue virus* [7], and a number of herpesviruses, Herpes simplex-1 and 2 [41], Pseudorabies virus (PRV) [34], *Human herpesvirus 6* [8], *Human herpesvirus 7* [35] and *Human herpesvirus 8* [1] and Human cytomegalovirus [2]. GAG binding of RSV also appears to be relevant to the natural course of infection as RSV clinical isolates (both RSV A and B strains) are as susceptible to heparin neutralisation as recombinant A2 laboratory virus (rA2) [38].

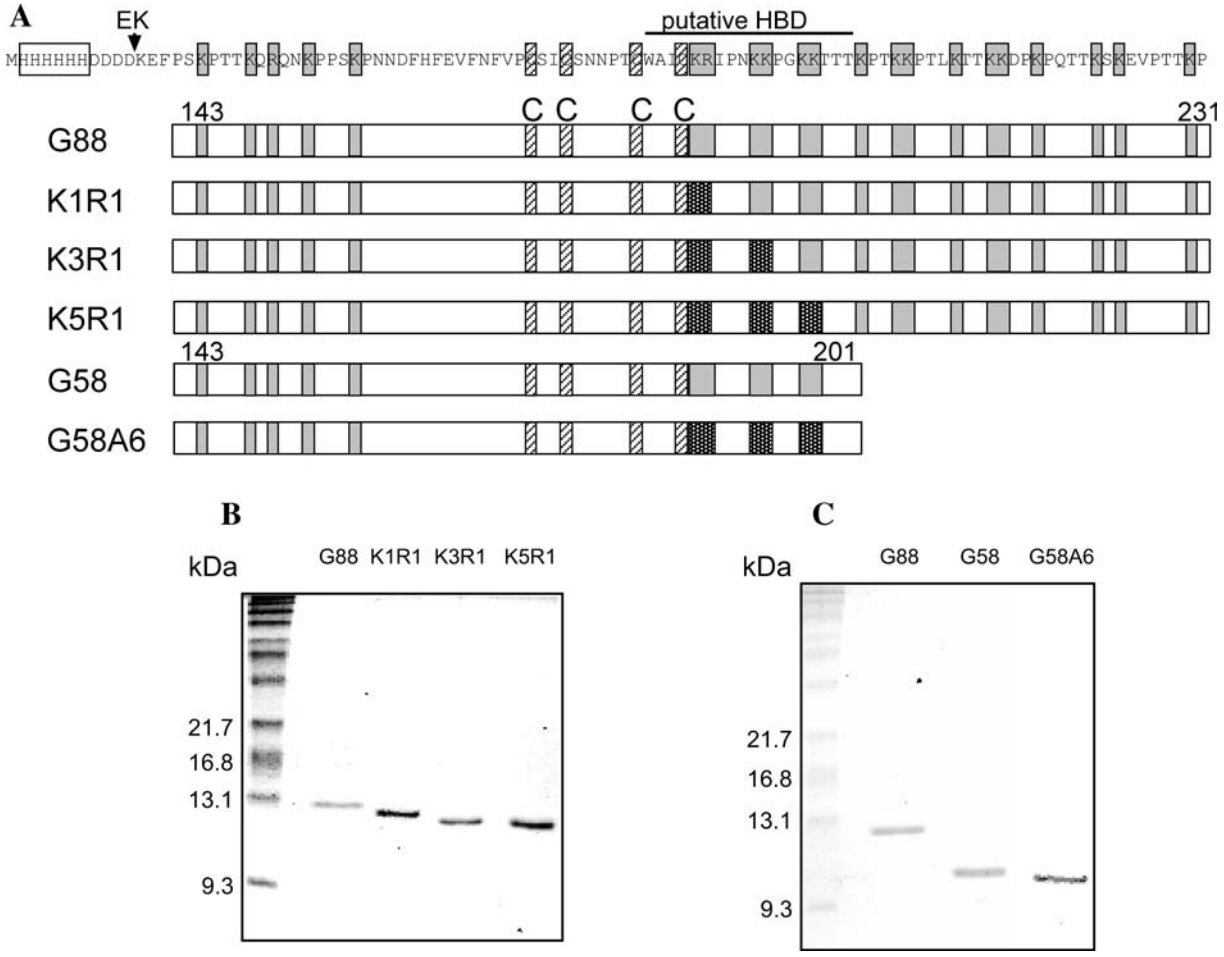

Fig. 1. Constructs prepared for expression of recombinant G proteins in this study. **A**. Schematic diagram of G protein constructs. RSV A2 G protein amino acid sequence (residues 143–231) used to construct G88 and derivatives, the *N*-terminal hexa histidine tag (▭) and enterokinase cleavage (EK) site common to all constructs are shown in the upper amino acid sequence. The putative HBD defined by Feldman et al. [10] encompassing residues 184–198, is indicated by the bar. Positively charged residues (lysine and arginine) are highlighted by grey boxes (▯), lysine and arginine residues mutated to alanine are shown as spotted boxes (▮) and cysteine residues as hatched boxes (▨). **B**. SDS-PAGE and Coomassie brilliant blue staining of purified G88 and sequential alanine substitution mutants of G88, K1R1, K3R1 and K5R1. **C**. SDS-PAGE and Coomassie brilliant blue staining of purified G88 and deletion and alanine substitution mutants of G88, G58 and G58A6. G protein mutants were purified from soluble fractions of *E. coli* BL21 *trxB* cell lysates by Ni^{2+}-NTA affinity chromatography. Fractions containing individual G protein mutants were pooled and dialysed against TBS at 4 °C overnight. Sample protein concentration was adjusted to 3 μM prior to loading of 20 μL (~1 μg total protein) on a 15% acrylamide gel and SDS-PAGE. Proteins were stained *in situ* with Coomassie brilliant blue and the gel was photographed

Heparin binding proteins interact with cell surface GAGs via ionic interactions between basic amino groups of lysine and arginine and the negatively charged sulfates on GAGs. Heparin binding proteins contain clusters of basic amino acids

interspersed with predominantly hydrophobic amino acids known as heparin binding domains (HBDs) which have the consensus sequences XBBBXXBX, XBBXBX or TXXBXXTBXXXTBB, where X is any amino acid, B is a basic residue and T is an amino acid in a turn [6, 22].

The receptor specificity of the different viruses which utilise GAGs, and specifically HS, for attachment appear to involve differences in the composition of GAG monosaccharides, the type of sulfation of carbohydrates (*N*, 6-*O* or 2-*O* linked) and the length of HS chains [36]. HS and chrondroitin sulfate-B, GAGs which contain iduronic acid, have been shown to block infection of susceptible host cells with RSV and the minimum requirements for neutralisation of RSV include a chain with a length of at least 10 saccharide units, *N*-sulfation and the presence of iduronic acid [20, 21].

Feldman et al. [10] further characterized the HBD of the RSV G protein by showing that binding of G homologous peptides to immobilised heparin and GAG expressing cells was almost entirely dependent on the highly basic sequence overlapping residues 184–198, suggesting that this represented the sole HBD of RSV G. Although deletion of residues 187–197 from the G protein of recombinant RSV did not reduce the susceptibility of this virus to neutralisation by heparin or its replication competence *in vitro* or *in vivo* [38], interpretation of these data is confounded by the GAG-binding capacity of the RSV F protein [11].

We have investigated the ability of various mutated G proteins expressed in *Escherichia coli* to bind to mammalian cells and to block RSV infectivity by a plaque inhibition assay. Deletion and alanine-scanning mutagenesis data show that there are multiple potential HBDs in the *C*-terminus of the RSV G protein (residues 187–231) and confirm the work of Teng et al. [38] that the HBD is not confined to residues 184–198 as proposed previously by Feldman et al. [10]. The functions of cell binding and blocking of virus infection by truncated G proteins are linked and require at least one functional HBD.

Materials and methods

Cells and viruses

HEp-2 cells were grown in medium 199 (Gibco-BRL) supplemented with 5% foetal calf serum (FCS) (199 + 5%). Wild type Chinese hamster ovary cells (CHO-K1 wild type) and glycosaminoglycan deficient CHO derivatives *pgsA*-745 [9] and *pgsD*-677 [28] were kindly supplied by Dr. Ralph Tripp (Centres for Disease Control, USA) and were grown in BME + 10% FCS. The enzyme activity of xylosyltransferase, an enzyme required for linking HS and CS chains to proteoglycan core protein, in CHO *pgsA*-745 cells is reported to be 15 fold less than in CHO-K1 wild type and hence these cells produce about 1% of wild type glycosaminoglycans on the cell surface [9]. The absence of HS on the surface of CHO *pgsD*-677 cells is a consequence of the lack of enzyme activity of *N*-acetylglucosaminyl and glucuronosyl transferases which polymerise HS chains [28].

HEp-2 cells were infected with RSV A2 strain and maintained in 199 + 2% FCS until extensive cytopathic effects (CPE) were observed. Virus was liberated from infected cells by two freeze-thaw cycles and clarified supernatants prepared by centrifugation at 3500 RPM for

10 min. The infectivity titre of RSV stocks was determined in plaque forming units (pfu)/ml using HEp-2 cells following the immunoplaque procedure described below.

Cloning and expression of RSV G protein constructs

A series of truncated G constructs were made in a pET32a expression vector with a modified multi-cloning site. Insert DNA was generated by PCR amplification of cDNA from the RSV A2 G gene previously cloned in pCR-Script SK(+) [32]. These constructs were expressed as fusion proteins with an *N*-terminal hexa-histidine tag (Fig. 1A, B and C) in *E. coli* BL21 *trxB* cells (Novagen) and purified from the soluble fraction by Ni^{2+}-NTA chromatography as described by the manufacturers instructions (Qiagen). Approximately 1 μg of each purified protein was analysed by SDS-PAGE followed by Coomassie blue staining and immunoblotting with monoclonal antibodies to G protein (021-19G, 021-2G, kind gifts from Dr. Jose Melero, Centro Nacional de Biologia Fundamental-Instituto de Salud Carlos III, Spain) and hexa-histidine antibody (Sigma) as described previously [17]. Prior to use in mammalian cell binding assays and plaque inhibition assays proteins were >95% pure and dialysed against Tris-HCl buffered saline (TBS) pH 7.5, overnight at 4 °C.

Mammalian cell binding assays

For binding assays cells were prepared by detaching the cell monolayer with 10 mM EDTA in Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS). In some experiments, monolayers were treated with trypsin-versene (Gibco-BRL) for 5 min to detach cells. Cells were washed twice with 199 + 5% FCS, resuspended in PBS + 1% FCS and counted. To assess binding, 3×10^5 cells in 100 μl were mixed with varying concentrations of purified truncated G protein mutants in designated concentrations in 100 μl TBS and the mixture incubated for 1 h on ice. For heparin inhibition studies, G protein mutants were pretreated with heparin for 1 h prior to addition to washed cells and incubation on ice for 1 h. The cells were then pelleted in a centrifuge at 1000 RPM for 5 min and washed twice with PBS + 1% FCS after which hexa-histidine antibody (1:300) was added followed by another 1 h incubation on ice. Cells were again washed and anti-mouse-FITC (Selenius) (1:40) added and incubated with cells for 1 h on ice. Following a final washing step, cells were resuspended and fixed in PBS + 1% formaldehyde.

Cell associated fluorescence of a minimum of 10^5 cells from each sample was quantitated using a FACStar^{Plus} flow cytometer (Becton Dickinson). Mean fluorescence values were converted to molecules of equivalent soluble fluorescence (MESF) from a Quantum^{TM} 25 FITC (FCSC) microbead standard curve. For each data point, MESF values were calculated for duplicate experiments performed in separate wells. Background MESF values attributed to non-specific antibody binding to cells was determined and subtracted from test MESF values.

Plaque assays

Six-well plates (Nunc) were seeded with HEp-2 cells to achieve 70–80% confluency after 48 h. Protein samples in TBS were filtered to remove any residual microbial contaminants prior to addition to HEp-2 cell monolayers. Serial one ml dilutions of truncated G protein mutants were added to individual wells and incubated with monolayers on ice for 1 hour after which unbound protein was removed by washing once with PBS. 100 pfu RSV A2 was then added to each well and the virus allowed to adsorb on ice for 1 hour. Unbound virus was removed by washing with 199 + 2% FCS and infection was allowed to proceed by incubating at 37 °C in 5% CO_2 for 48 h in the presence of maintenance medium.

At 48 h post-infection monolayers were fixed with 100% cold methanol for 10 min and residual fixative removed by washing with PBS. RSV plaques were then visualised by an immunoplaque procedure. Briefly, the fixed monolayers were blocked with 5% skim milk in

PBS for half an hour at room temperature, washed with PBS and then incubated with 0.5 ml goat anti-RSV (Biodesign) (1:2000 in PBS) for 1 h on ice. The monolayers were then washed once with PBS and incubated with 0.5 ml anti-goat-horseradish peroxidase (HRP) (Silenius) (1:2000 in PBS) for 1 h on ice. The monolayers were washed again and plaques stained with 0.5 ml immunoperoxidase stain (Dako) for 10–20 min at 37 °C. After plaques were visible using a light microscope, residual immunoperoxidase stain was removed by washing with PBS and plaques counted. Only experiments in which at least 60 plaques were counted in control wells were utilised.

Plaque inhibition by each G protein mutant was determined by the following formula: RSV plaque reduction = No. of plaques in G protein mutant treated well/No. of plaques in untreated well × 100%.

Results

Expression of recombinant G proteins and mutants

A series of G protein constructs were prepared for this study (Fig. 1A). The purity of expressed and purified proteins was estimated to be >95% on Coomassie stained gels (Fig. 1B and 1C). G88 migrated at an apparent M_r of 12 K and migrated more slowly than the alanine substitution mutants, K1R1, K3R1 and K5R1 (Fig. 1B). G58 migrated at an apparent M_r of 10 K and the alanine substituted form of G58, G58A6 migrated faster than G58 (Fig. 1C). Differences in apparent M_r of alanine substitution mutants may be explained by the removal of the large basic side chains of lysine (K = 128 Da) and arginine (R = 156 Da) and replacement with the smaller neutral side chain of alanine (A = 71 Da). A further-truncated G58A6 protein was also constructed, G42A6, comprising residues 159–201 but lacking 16 amino acids (143–158) proximal to the cysteine noose (residues 173–186); these 16 amino acids include a cluster of 5 positively charged residues but not in the configuration of a consensus HBD [6, 22]. We were unable to express and purify enough of G42A6 for analysis.

The reactivity of truncated G protein mutants with monoclonal antibodies 021-19G and 021-2G, which have been shown previously to react with the central conserved region of the native G protein [14], was determined by western blotting (data not shown). G protein mutants (K1R1, K3R1, K5R1 and G58A6) with K187A and R188A substitutions bound 021-19G but not 021-2G. Hexa-histidine antibody detected all six G protein mutants and was therefore used for immunoflow cytometry assays.

Characteristics of G88 binding to mammalian cells

Using a flow cytometry assay we investigated the ability of the truncated G protein, G88, to bind to mammalian cells. Trypsin/versene- and EDTA-treated HEp-2 cell populations were of uniform in size and granularity and were made up almost entirely of single cells (Fig. 2A). Background fluorescence attributable to binding of primary (anti-hexa-histidine) and secondary (FITC-anti-mouse) antibodies to trypsin and EDTA treated HEp-2 cells was determined for each experiment (Fig. 2A). Typically we observed a uniform and marked shift in the fluorescence histogram of cell populations on addition of G88, which suggests

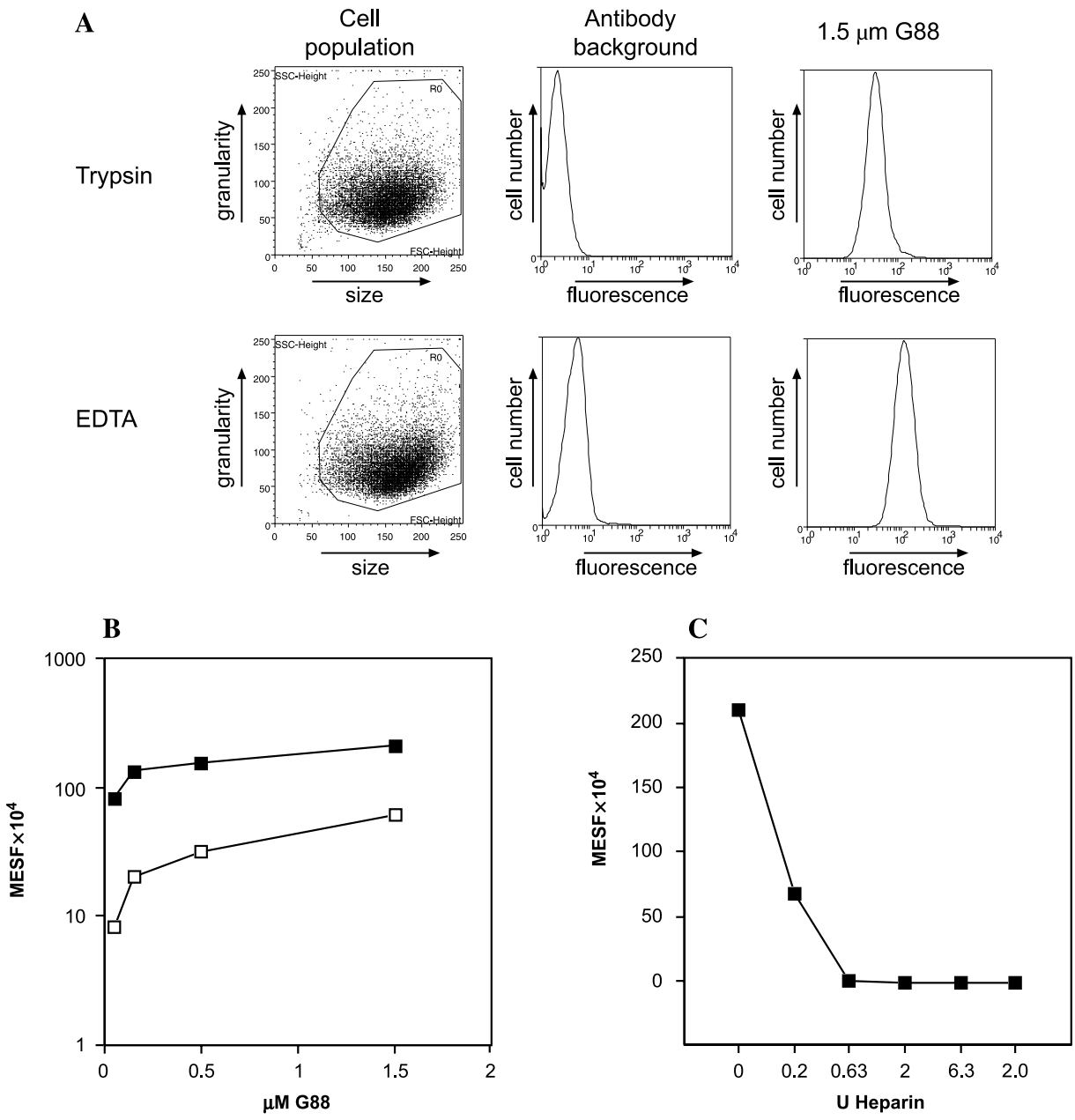

Fig. 2. Characteristics of G88 binding to HEp-2 cells. **A**. Flow cytometric analysis of G88 binding to trypsin/versene- and EDTA-treated HEp-2 cells. HEp-2 cell monolayers were treated with 10 mM EDTA in PBS for 20 min at 37 °C or trypsin/versene for 5 min at 37 °C and the released cells dispersed. The first panel column shows the distribution of size and granularity of trypsin and EDTA treated HEp-2 cell populations. In the second column of panels ("Antibody background") the fluorescence histogram attributed to non-specific binding of hexa-histidine and mouse-FITC antibodies is shown using the binding assay described in the Materials and methods; in the last column of panels the fluorescence histogram of G88 (at 1.5 μM) binding is shown. **B**. Graphic analysis of the flow cytometric data in (**A**). EDTA treatment (■); trypsin/versene treatment (□). **C**. Heparin inhibits G88 binding to HEp-2 cells. A plateau concentration of G88 used in the binding assay in **A** (1.5 μM in a volume of 200 μl), was preincubated with half log dilutions of heparin for 1 h at 4 °C. The mixtures were added to HEp-2 cells treated with 10 mM EDTA and G88 binding quantitated as described in the Materials and methods

that G88 bound to all cells present in the population with approximately equal affinity (Fig. 2A).

G88 binding to HEp-2 cells was studied in eight separate experiments. Binding was dose dependent and appeared to reach a plateau at a concentration of about 1.5 μM (range, 1–3 μM); a typical result is shown in Fig. 2B. We believe that the plateau is a function of the assay system, perhaps due to limiting concentrations of the secondary antibody, not saturation of the very abundant cell surface GAGs [22] by G88. Previously Walsh et al. (1984) demonstrated that anti-G affinity chromatography purified I^{125}-labelled G protein did bind to HEp-2 cells, however the level of binding did not reach saturation, suggesting that G receptors were very abundant on the surface of HEp-2 cells [42].

Trypsin treatment of HEp-2 monolayers to detach cells from the substrate resulted in a 5–10 fold reduction in G88 binding compared to detaching cells with EDTA (Fig. 2B). The trypsin effect may be a consequence of proteolysis of the proteoglycan core of GAGs removing them from the cell surface [31]. For subsequent assays cells were detached with EDTA rather than trypsin. We have also documented binding of purified native RSV G glycoprotein (a gift from Dr. Speelman, Lederle Praxis Biochemicals, USA, prepared as a by-product of F glycoprotein purification) to HEp-2 cells by the same assay system (data not shown).

To assess the affect of heparin on G88 binding to HEp-2 cells the plateau concentration of G88 determined previously (1.5 μM) was incubated with increasing concentrations of heparin and binding was determined as described above. Heparin (0.2 U per 3×10^5 cells) was sufficient to inhibit G88 binding by ~70% and binding was undetectable (<1% of control) in the presence of ≥0.63 U per 3×10^5 cells (Fig. 2C).

We next investigated the ability of G88 to bind to Chinese hamster ovary (CHO) cells deficient in production of cell surface glycosaminoglycans. G88 bound to CHO *pgsA*-745, CHO *pgsD*-677 and CHO-K1 wild type in a dose dependent manner. However at the maximal concentration of G88 (2.12 μM), binding to CHO *pgsA*-745 and CHO *pgsD*-677 was reduced by approximately 90% and 70% respectively, compared to the binding to CHO-K1 wild type cells (Fig. 3). G88 binding to CHO cell mutants was also inhibited with increasing concentrations of heparin (data not shown) indicating that although these cells are GAG deficient they still express residual GAG.

Mutational analysis of the G88 heparin binding domain

We first introduced sequential alanine substitutions of basic amino acids in the putative HBD as defined by Feldman et al. [10]. G88 and the G88 alanine-substituted G protein mutants (K1R1, K3R1 and K5R1; Fig. 1A) bound to HEp-2 cells in a dose-dependent manner, however the level of binding of the alanine-substituted mutants was indistinguishable from that of G88 (Fig. 4A). In particular the mutant K5R1, which completely lacks positively charged residues in the putative HBD, had unaltered binding to HEp-2 cells. Binding of G58 (Fig. 1A) to HEp-2 cells was

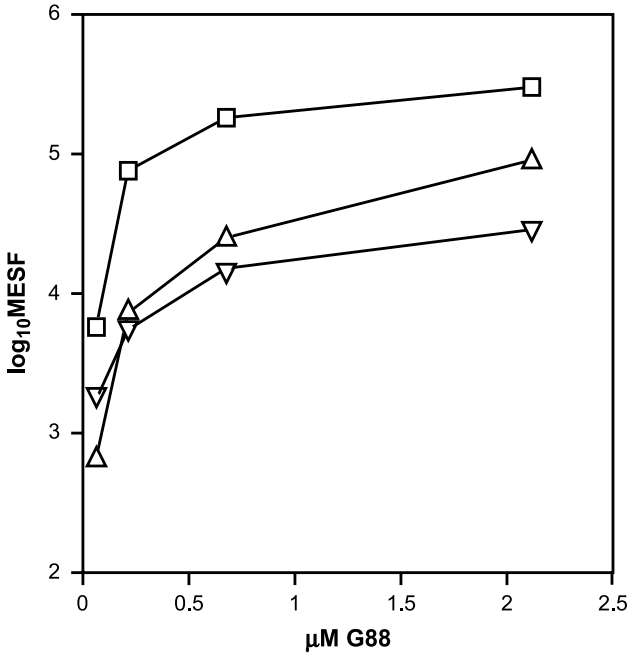

Fig. 3. Binding of G88 to CHO cells deficient in glycosaminoglycan synthesis. CHO K1 wild type, heparan sulfate deficient CHO *pgsD*-677 and glycosaminoglycan deficient CHO *pgsA*-745 cells were prepared in the same way as EDTA treated HEp-2 cells. G88 binding to CHO K1(□), CHO *pgsD*-745 (▽) and CHO *pgsA*-677 (△) was quantitated as described in the Materials and methods

also found to be dose-dependent although it appeared to bind about half as well as G88 over a concentration range of 0.15–1.5 μM (Fig. 4B). However the G protein mutant that completely lacks any consensus HBDs (G58A6; see Figure 1A) bound more than 100-fold less well than G58 (Fig. 4B).

Virus blocking activity of G protein mutants

G88 and G88 mutants in which the positively charged residues of the putative HBD [10] were progressively substituted with alanine (K1R1, K3R1 and K5R1) inhibited RSV A2 plaque formation equivalently, with 50% inhibition achieved using concentrations of protein between 25 and 65 nM (Fig. 5A). Notably, K5R1, which completely lacks positively charged residues within the region 184–198, had RSV blocking activity equivalent to G88. G58 inhibited RSV plaque formation about 10-fold less well than G88 however, the blocking activity of G58A6 was at least 4 times less potent than G58 and more than 30 times less potent than G88 (Fig. 5B). The weak RSV blocking ability of G58A6 requires further investigation; however, it may be due either to GAG binding via the cluster of positively charged amino acids proximal to the cysteine noose (lacking a known consensus HBD motif), or to binding to CX3CR1 [40].

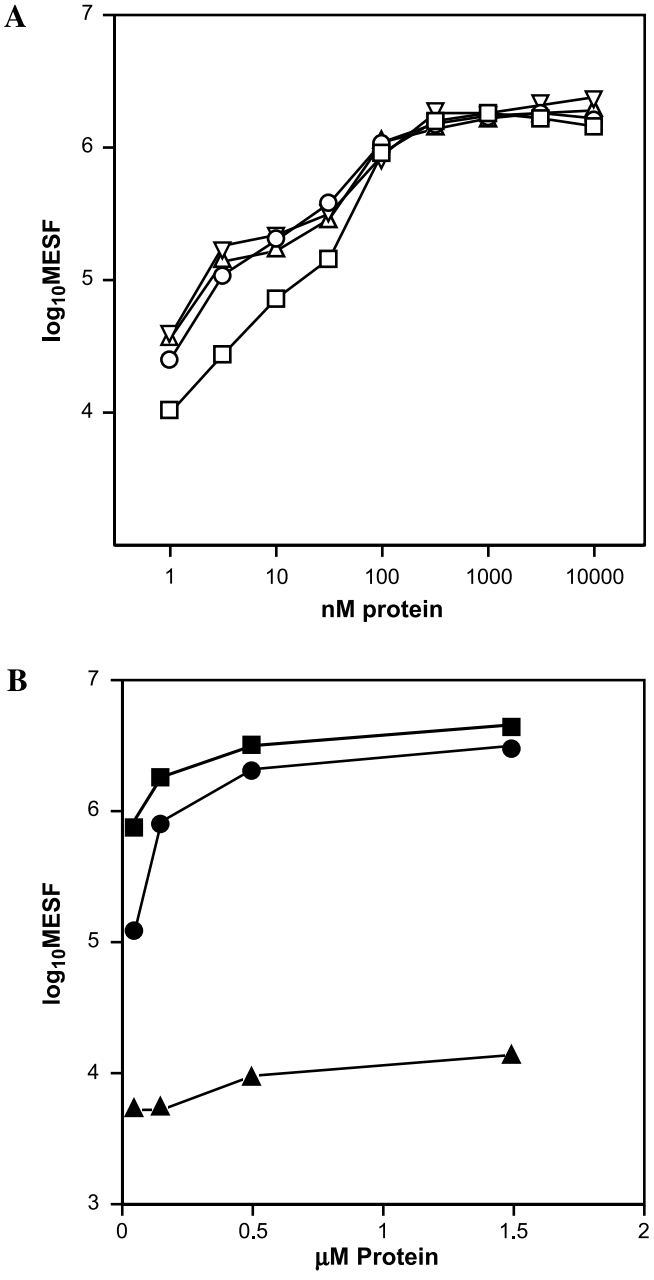

Fig. 4. Binding of alanine substitution and deletion mutants of G88 to HEp-2 cells. **A**. Binding of G88 and G88 alanine substitution mutants to HEp-2 cells. Site-directed mutagenesis of pairs of positively charged residues within the putative HBD [10] was performed as described in the text. Protein samples were adjusted to equimolar concentrations and binding of G88 (□), K1R1 (○), K3R1 (△) and K5R1 (▽) to HEp-2 cells was quantitated as described in the Materials and methods. **B**. Binding of G88, G58 and G58A6 to HEp-2 cells. Protein samples were adjusted to equimolar concentrations and binding of G88 (■), G58 (●) and G58A6 (▲) to HEp-2 cells quantitated as described in the Materials and methods

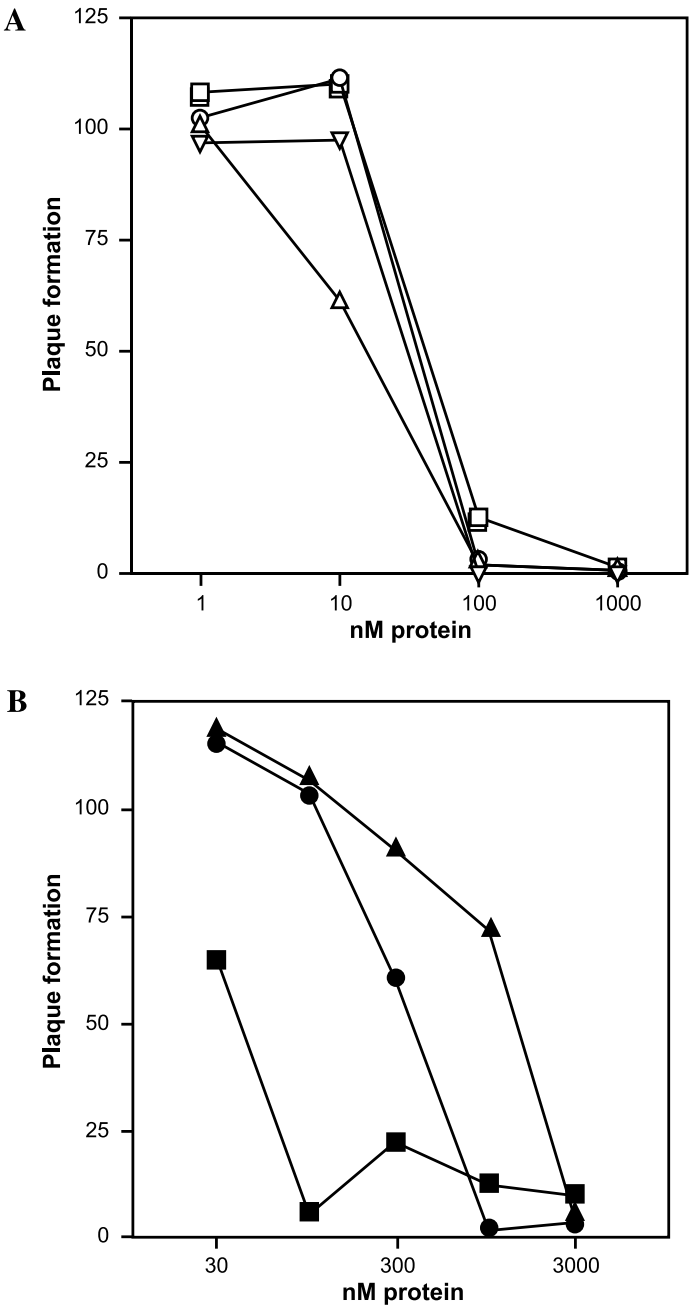

Fig. 5. RSV blocking activity of G protein mutants. Six-well plates were seeded with HEp-2 cells to achieve 70–80% confluency after 48 h. Dilutions of G protein mutants were added to each well and incubated with cells for 1 h on ice. Each experiment comprised a mock infected well and an RSV only well as controls. After washing with PBS, 100 pfu RSV A2 was added to each well except the mock well and after 1 h on ice unbound virus was washed off. Adsorbed virus was then allowed to infect cells and plaques visualised and counted as described in the Materials and methods. **A**. Inhibition of RSV A2 plaque formation with G88 (□) and alanine substitution mutants K1R1 (○), K3R1 (△) and K5R1 (▽). **B**. Inhibition of RSV A2 plaque formation with G88 (■), G58 (●) and G58A6 (▲)

Discussion

The work reported here shows for the first time that the HBD of RSV G protein extends well beyond the HBD (residues 184–198) previously defined by peptide mapping studies [10] and by biological studies [38] and that it may encompass most or all of the positively charged region (residues 184–231) initially suggested for the RSV Long strain [24]. Our studies further show that the ability of G protein mutants to bind GAGs on the surface of HEp-2 cells correlates well with their ability to block of RSV infection.

The use of truncated mutant G proteins expressed in *E. coli* allowed investigation of the importance of all the potential HBDs of the G protein in isolation from other virus glycoproteins present in whole viral particles. Although 50% of RSV attachment has been attributed to G protein interactions with GAGs, the remainder appears to be due to F protein (25% attributed to F protein-GAG interactions and 25% to non-GAG F interactions) [37]. Therefore Teng's recombinant virus lacking the putative HBD of G protein (G HBD-mut) [38] could bind to glycosaminoglycans either through the F protein (which also binds GAGs [11, 37]) and/or via additional HBDs in the G protein other than the putative HBD (residues 184–198). Our data suggest strongly that the retained susceptibility to heparin neutralisation of Teng's G HBD-mut virus is at least in part attributable to residual binding of G to cell associated GAGs. In our studies, G88 at concentrations of >100nM completely blocked RSV plaque formation, suggesting that these concentrations of G88 were sufficient either to block all of the glycosaminoglycan sites, including the GAGs used by the F protein for attachment, or to sterically hinder virus attachment or entry.

The coding sequence of the *C*-terminus of the RSV G gene is adenosine rich and it therefore has an abundance of codons for the positively charged amino acids lysine (AAA) and arginine (AGA) as well as for serine and threonine (potential sites for *O*-linked glycosylation). It has been reported that growth of RSV Long in neutralising monoclonal antibody 63G selects for polymerase slippage mutations in poly-adenosine tracts in the *C*-terminus of the G gene [15], resulting in changes in the length of the *C*-terminus of the protein and alterations in *O*- and *N*-linked glycosylation sites [4]. Interestingly, the number and position of potential HBDs in these antibody escape mutants, based on the consensus sequences of mammalian heparin binding proteins [6], remain unchanged (Fig. 6). Changes in either potential *O*- and *N*-linked glycosylation sites or epitopes for existing neutralising antibodies are unlikely to affect the ability of progeny viruses to bind to GAGs. The fact that both laboratory and clinical RSV isolates are equally sensitive to heparin neutralisation [38] and the observation that variation in the G gene is not attributed to propagation in tissue culture [5] argue that, unlike *Sindbis virus* [3] TBE [29] and MVE [26] that have acquired a GAG binding phenotype through cell culture passage, the G-GAG interaction is an authentic feature of the RSV virus life cycle.

We have reported that there is redundancy in the function of the positively charged region previously described by Krusat et al. [24]. A similar redundancy

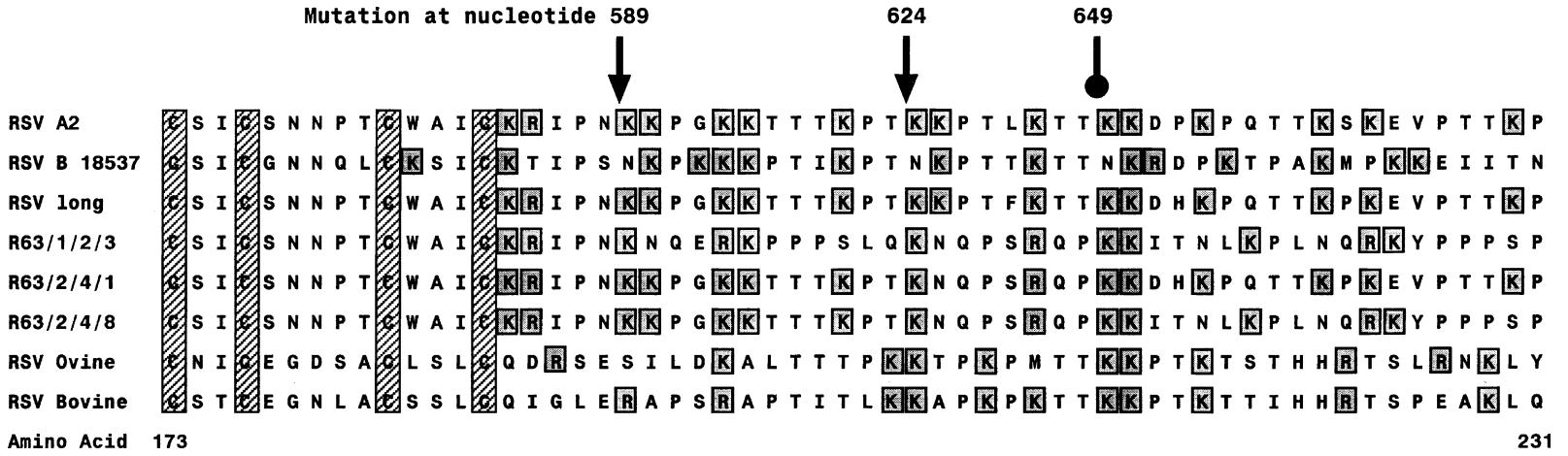

Fig. 6. Amino acid alignment of RSV G proteins in the region encompassing residues 173–231 (172–230 for Ovine RSV G protein). Antibody escape mutants of the RSV Long strain are variants that have escaped neutralisation by the G monoclonal 63G [15]. R63/1/2/3 has a single adenosine deletion in the oligo A region beginning at nucleotide 589 (▾) and R63/2/4/8 has a single adenosine deletion in the oligo A region beginning at nucleotide 624 (▾). As a consequence the open reading frame of these G proteins terminates at 244 amino acids. R63/2/4/1 has a single adenosine deletion in the oligo A region beginning at nucleotide 624 (▾) and a single adenosine insertion in the oligo A region beginning at nucleotide 649 (▾), which restores the open reading frame. Positively charged residues are indicated in grey boxes (□) and conserved cysteine residues in hatched boxes (▨)

has been described for the PRV gC glycoprotein which facilitates HS dependent attachment via one of three independently functioning HBDs [12, 13]. In addition to the putative HBDs present in the region distal to the cysteine noose of the RSV G protein there is a cluster of positively charged residues (145–158) in the proximal region to the cysteine noose that does not have a consensus HBD. The inability of this domain (145–158) to bind GAGs is supported by our studies showing that a G protein with this domain as the sole HBD (G58A6) had no measurable heparin binding, especially as compared with the same protein having a single consensus HBD (G58). However the sparse distribution of positively charged residues in this region (XBXXXBXBXXBXXXB) does resemble the newly-defined HBD of HSV-1 gC which is ^{142}XBXBXBXXXBXXXBXXXXB^{160} (and ^{129}BB^{130}) [30]. We attempted to express a truncated form of G58A6 lacking this putative HBD (G42A6) to investigate the potential role of this region in the weak plaque inhibition activity of G58A6; however we were unable to purify sufficient quantities of this very small protein to perform binding and/or plaque inhibition assays.

It is interesting to note that the arrangement of positively charged residues in the *C*-terminus of ovine and bovine G proteins is dissimilar to group A and B human RSV G protein. However the bovine G (and F) have been shown to bind to immobilised heparin [23], suggesting that the two motifs which resemble the XBBXBX motif between residues 202–216 are sufficient to mediate GAG binding of bovine RSV G (Fig. 6) and most likely the closely related ovine RSV G protein.

The fact that a virus lacking the entire G protein (ΔG) is attenuated for replication *in vivo* and in HEp-2 cells, whilst the G HBD-mut virus replicates normally [38] supports the suggestion that the G protein is very important at least *in vivo* and in HEp-2 cells, despite the role of F in GAG-dependent attachment. Given that cell binding of G58A6 was almost completely abrogated compared to that of G88, it is likely that construction of a recombinant RSV with a G gene mutated such that GAG binding is $<1\%$ of normal would result in a recombinant virus having a markedly reduced ability to bind to GAGs and attenuated replication *in vitro* and *in vivo*, similar to that of a ΔG virus [38].

Taken together our data provide evidence that G protein mutants with intact HBDs bind to host cells through glycosaminoglycans and that this interaction is authentic to RSV virions as these G mutants block the attachment and infectivity of RSV A2 in HEp-2 cells. Clearly further studies are required to investigate the role of glycosaminoglycan binding and the relevance of G protein interactions with CX3CR1 in the virion life cycle.

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