Identification of a Linear Heparin Binding Domain for Human Respiratory Syncytial Virus Attachment Glycoprotein G

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Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract disease in infants and young children worldwide. Infection is mediated, in part, by an initial interaction between attachment protein (G) and a highly sulfated heparin-like glycosaminoglycan (Gag) located on the cell surface. Synthetic overlapping peptides derived from consensus sequences of the G protein ectodomain from both RSV subgroups A and B were tested by heparin-agarose affinity chromatography for their abilities to bind heparin. This evaluation identified a single linear heparin binding domain (HBD) for RSV subgroup A (156A-159K21) and B (156K-258K26). The binding of these peptides to Vero cells was inhibited by heparin. Peptide binding to two CHO cell mutants (pgsD-677 and pgsA-745) deficient in heparan sulfate or total Gag synthesis was decreased 50% versus the parental cell line, CHO-K1, and decreased an average of 87% in the presence of heparin. The RSV-G HBD peptides were also able to inhibit homologous and heterologous virus infectivity of Vero cells. These results indicate that the sequence 184A/183K189P/K197 for RSV subgroups A and B, respectively, defines an important determinant of RSV-G interactions with heparan.

Human respiratory syncytial virus (RSV), a member of the genus Pneumovirus within the family Paramyxoviridae, is the leading cause of lower respiratory tract infection in infants and young children worldwide (8). Currently, there are no effective licensed vaccines. During clinical trials in the 1960s, children inoculated with a formalin-inactivated RSV vaccine were left unprotected and developed exacerbated disease associated with cosinophilia upon subsequent exposure to wild-type virus (7, 23, 25). Since that time, many investigators have worked to gain a better understanding of the mechanisms involved in the development of severe bronchiolitis sometimes observed during the course of natural infection. One important aspect of this process is identifying the steps required for attachment and infection of target cells.

RSV-G is one of three glycoproteins found on the surface of the virion and is synthesized as a core protein of 298 amino acids. RSV-G then undergoes extensive N- and O-linked glycosylation prior to expression on the cell surface as a type II integral membrane protein (8, 9, 38, 50). RSV-G has been shown to function as an attachment protein (29). Many have speculated that the receptor-binding domain of RSV-G may be located between amino acids 156H-157C26. The principal evidence supporting this speculation is based on the observation that this region is exactly conserved among all wild-type RSV isolates sequenced to date (21). While no specific receptor has been described that recognizes the G glycoprotein, it was recently shown that RSV could bind to immobilized heparin (27). In vivo, heparin is primarily located in the granules of mast cells and basophils. However, heparan sulfate, a related compound, is found on the surface of most mammalian cell types and in the extracellular matrix (17). Many viruses, including herpesviruses (16, 28, 31, 51), human immunodeficiency viruses (33, 36, 37), flaviviruses (6), picornaviruses (20), and alphaviruses (3, 26), utilize heparan sulfate to mediate attachment and infection of target cells. Heparin binding proteins are known to interact with heparin via electrostatic charge interactions generated between the negatively charged sulfate groups on heparin and the positively charged amino acids within the protein’s heparin binding domain (HBD) (5, 16, 45). Interestingly, the ectodomain of the RSV-G protein contains a cluster of positively charged amino acids (150P-K253) (27) which falls within an immunodominant region of RSV-G. It has been postulated that RSV-G–heparin binding interactions are mediated via this clustering of basic amino acids within the RSV-G ectodomain (27). However, there has been no experimental evidence to corroborate this assumption. Therefore, it is the purpose of this study to identify potential linear HBDs within the ectodomain of the RSV-G protein and to determine if the clustering of positively charged amino acids is involved in RSV-G-Gag interactions.

MATERIALS AND METHODS

Cells, virus, and purified viral proteins. Vero cells were grown in Eagle’s medium containing Earle’s salts (EMEM) (Mediatech Inc., Herndon, Va.) and 10% fetal bovine serum (FBS) (Intergen, Purchase, N.Y.). The following Chinese hamster ovary (CHO) cells were grown in Ham’s F12 medium (Mediatech Inc.) containing 10% FBS: K1, the parental CHO cell line; pgsD-677, which contains a defect in GlcNAc and GlcA transferase and is heparan sulfate negative while producing three to four times the normal amount of chondroitin sulfate; and pgsA-745, which is xylosyl transferase deficient, producing approximately 1% of wild-type Gag (13, 14). Human RSV strains A2 and 1837 were prepared by inoculating Vero cells at a multiplicity of infection between 0.1 and 1 (32). Virus was concentrated as previously described (32) or pelleted directly from the tissue culture supernatant and resuspended in EMEM containing 1% FBS, 100 mM MgSO4, and 50 mM HEPES (Bio-Whittaker, Walkersville, Md.). Infectious titers were determined following inoculation of Vero cell monolayers and reported as 50% tissue culture infectious doses (TCID50) or PFU by methods previously described (19).

Purified attachment (G) protein (0.31 mg/ml) from the A2 strain of RSV grown in Vero cells and polyclonal rabbit anti-G antiserum was supplied by Lederle-Praxis Biologicals (West Henrietta, N.Y.) (29).

Synthesis of RSV-G overlapping peptides derived from G protein ectodomain sequence. Consensus sequences were generated for the G protein amino acid sequence deduced from G gene nucleotide sequence data for RSV subgroup A.

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A2
RSVCONSA

18537

I
VTV
TKI
MNTN
RTS
V
PERV
S
KQPP
T
PH
NG
TIS

L

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120

RSVCONSB

I
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FIG. 1. RSV G glycoprotein ectodomain consensus sequences. Fifteen-amino-acid long overlapping peptides were generated for both RSV subgroups. Consensus peptides (RSVCONSA and -SB) are aligned with subgroup A (strain A2) and subgroup B (strain 18537) viruses to demonstrate homology of the consensus peptides with wild-type virus. Peptides are numbered 1 through 44 (subgroup A) or 1 through 45 (subgroup B) starting from the carboxy terminus. The highlighted sequence represents the exactly conserved region (amino acids 164 to 176), and conserved cysteine residues are marked by black dots. The putative heparin binding region of the RSV-G ectodomain (amino acids 187 to 217) (27), characterized by a cluster of basic amino acids, is defined by the black box. Peptides for subgroup A (solid lines) and subgroup B (dashed lines) indicate the positions of the peptides that bound heparin.
RESULTS

RSV synthetic-peptide HAAC. In an attempt to determine regions on the RSV-G protein important for heparin binding, a series of overlapping peptides representing the consensus sequence for Ga and Gb were synthesized (Fig. 1). Using HAAC, the peptide pools were tested for their ability to bind immobilized heparin. Figure 2 demonstrates that the heparin binding activity for subgroup A and B peptide pools resides primarily within pool 3 (A3 and B3), spanning amino acids \( 144S\)\( -2T198 \) for subgroup A and \( 148T\)\( -2P202 \) for subgroup B. The interactions of pools A3 and B3 are considered heparin specific, as the peptides were eluted with heparin and did not bind to unlinked Sepharose CL-4B (Fig. 3). Individual peptides comprising pool 3 for both subgroups were then examined for their abilities to bind immobilized heparin (Fig. 4). The results show that pool A3 peptides a19, a20, and a21 correspond to amino acids \( ^{175}SICSNPTCWAIC \)\( ^{198}KKPGKKT \) (bold letters represent basic residues), of the Ga glycoprotein. Examination of the individual pool B3 peptides shows that peptide b20 \( ^{180}KSICKTIPSNKPKK \) and b27 \( ^{148}TKFPRSKNPPPKPK \) were the only peptides with significant heparin binding activity (Fig. 4).

Binding of RSV G heparin binding peptides to cells. Each of the peptide pools was then examined for their ability to bind Vero cells. Peptide pools A3, B3, and the positive control peptide Vn, bound to Vero cells, as determined by ELISA (Fig. 5), whereas all the other peptide pools for both subgroups as well as the negative-control peptide, 3vnm, were not able to bind. Furthermore, the reactivity of pools A3, B3, and Vn with cell surface molecules was inhibited by the addition of soluble heparin (2 mg/ml), suggesting that this interaction was likely mediated via cellular, heparin-like Gags (Fig. 5). Reactivity with Vero cells was inhibited by 84, 76, and 84% for pool A3, pool B3, and Vn, respectively.

Individual subgroup A (a19, a20, and a21) and B (b20) peptides were also able to bind to Vero cells. All the other individual pool A3 peptides were negative. Likewise, all the remaining subgroup B3 peptides were considered negative, with the exception of peptide b27, which bound only weakly to Vero cells (Fig. 6). The binding of peptides a19, a20, and a21 was inhibited by 87, 85, and 83%, respectively, by the addition of 2 mg of heparin/ml (Fig. 6). Heparin decreased the reactivity of peptide b20 and b27 by 90 and 48%, respectively. However, even though peptide b27 binding was decreased 48% in the presence of heparin, a direct comparison of the overall reactivity of untreated b27 peptide with Vero cells was approximately 90% less than that of b20. Interestingly, the degree of
binding inhibition of purified RSV G glycoprotein in a similar assay as well as heparin inhibition of whole-virus binding to uninfected Vero cells was approximately 50% (unpublished data). However, considering the extensive glycosylation and second-structure nature of G on the native virion, we cannot rule out non-heparin-mediated attachment that might account for the incomplete inhibition. Of note, peptides spanning the conserved region \((164^H-C176)\) or the entire cysteine noose region \((16^H-I189)\) did not appear to react with Vero cells.

In an attempt to determine the specific Gag requirements for the RSV-G peptides, we measured the reactivity of the peptide pools with three CHO cell lines, two of which contained defects in their abilities to express particular Gags. The results shown in Fig. 7 demonstrate that the peptide pools A3 and B3 reacted with each of the cell lines examined while all the other pools did not bind (data not shown). The reactivity of pools A3 and B3 with both the heparan sulfate-deficient (pgsD-677) and Gag-deficient (pgsA-745) cell lines was approximately 60 and 47% that of the parental (K1) cell lines for pools A3 and B3, respectively. In addition, heparin reduced the reactivity of pool A3 by 70% for each of the three CHO cell lines and reduced pool B3 reactivity with CHO-K1 cells by a total of 60%, reduced that with pgsD-677 cells by 92%, and reduced that with pgsA-745 cells by 84% (Fig. 7). Interestingly, the positive-control peptide, Vn, also reacted strongly with all three CHO cell lines and did not exhibit a significant decrease in binding to either of the Gag-deficient CHO cell lines (Fig. 7). The binding of the Vn peptide was decreased by 84, 74, and 82% for the K1, pgsD-677, and pgsA-745 CHO cell lines, respectively, when heparin was added.

Examination of the individual peptides from pools A3 and B3 with CHO-K1, pgsD-677, and pgsA-745 cell lines revealed that only peptides a19 to a21 and b20 bound (Fig. 8). All the other individual peptides within pools A3 and B3 were unable to bind (data not shown). On average, the reactivities of a19 to a21 and b20 were reduced by 60 and 50% for pgsD-677 and pgsA-745 cells, respectively, compared to the parental CHO K1 cell line. Furthermore, the reactivity of a19 to a21 and b20 decreased an average of 92, 80, and 88% in the presence of heparin for CHO-K1, pgsD-677, and pgsA-745 cells, respectively (Fig. 8). In contrast to the Vero cell data (Fig. 6), peptide b27 did not react with any of the CHO cell lines tested.

Effects of RSV-G heparin binding peptides on virus infectivity. To determine if the RSV-G HBD peptides could inhibit virus infectivity by blocking the interaction between infectious virus and cellular Gags, an infectivity inhibition assay was carried out. Three RSV-G\(_G\) peptides (a19, a20, and a21) inhibited the homologous subgroup A virus (strain A2) infectivity by 60 to 90% (Fig. 9). Peptide b20 was able to inhibit the infectivity of heterologous A2 virus by 60%. In the reciprocal experiment, peptide b20 reduced homologous subgroup B virus (strain 18537) infectivity by 81%, and a19, a20, and a21 were able to inhibit 18537 infectivity by 70, 75, and 76%, respectively (Fig. 7). Peptide b27 or the Vn peptide, both of which contain the mammalian consensus HBD motif (XBBXBX), did not inhibit A2 or 18537 infectivity. Furthermore, the peptide pools 1, 2, 4, and 5 for each subgroup did not inhibit A2 or 18537 infectivity, nor did the remainder of the peptides in pool A3 or B3 (data not shown). It should be noted that pools A3 and B3 (data not shown) or a mixture of peptides a19 to a21 did not further decrease A2 or 18537 virus infectivity over that of the individual peptides (a19, a20, a21, or b20). The results of this assay were then tested for significance based on the analysis of vari-
Figure 7. Reactivities of pooled biotinylated peptides with various CHO cell lines. Pooled peptides were reacted with various CHO cells, and after fixation, bound peptides were detected as described in the legend to Fig. 5. Values at or above the dashed line (twice background) are considered positive. The data are from a representative experiment of at least three experiments, with the error bars indicating the standard error of the mean of quadruplicate wells.

Figure 8. Reactivities of individual biotinylated peptides with various CHO cell lines. Individual peptides from pools A3 and B3 shown previously to interact with cellular receptors (18) were reacted with CHO cells. Assuming this peptide folded correctly, these experiments did not support the idea that this region interacts with cellular receptors. However, an important limitation of these experiments was that they may not represent native proteins in terms of conformation or glycosylation. Thus, further binding studies among all sequences examined, with the exception of two subgroup A viruses, each of which contained a single conservative change (192N→S or 196K→R). Examination showed that the RSV-Ga HBD, 183K→K197, was 100% conserved for all but one strain B virus, wV10010, which contained the mutation 192P→S. The homology between the subgroup A and B HBDS was approximately 60%.

DISCUSSION

In order to map regions of RSV-G important for heparin binding, we used two sets of synthetic overlapping peptides representing the ectodomains of both RSV subgroups. We identified two linear sequences, 184AICKRRIPKPKK189 for subgroup A viruses and 183KSIKCTIPSNKPKK197 for subgroup B viruses, as being important for RSV heparin binding. When subgroup A and B HBD peptide sequences were compared with G glycoprotein sequences from their respective subgroups, the HBD region was conserved among the majority of sequences examined. Although RSV-G A and B HBD sequences have only 60 to 70% sequence homology, the HBDS spatially mapped to nearly identical locations on their respective proteins (42). Interestingly, the HBDS are proximal to the conserved region (I189→H→C193) including the cysteine noose, which suggests that this site might play a critical role in the function of RSV-G HBDS. In our assays a peptide representing the conserved region (I189→H→C193) or the entire cysteine noose region (I189→H→C193) of RSV-G did not appear to bind to Vero cells. Assuming this peptide folded correctly, these experiments did not support the idea that this region interacts with cellular receptors (18). However, an important limitation of these experiments was that they may not represent native proteins in terms of conformation or glycosylation. Thus, further binding studies...
will be necessary to determine if the proximity of the linear HBD to the cysteine noose region is important for heparin binding and whether there are conformational determinants involved in RSV-heparin interactions.

Analysis of several mammalian heparin binding proteins has yielded two consensus sequences, XBBBX\text{X} and XBBBXXBX, where B is almost always a basic residue and X is usually an uncharged hydrophobic residue (for a review, see reference 5). Several viral HBDs have been mapped and experimentally shown to bind heparin (15, 16, 30, 35, 39, 46, 52). Many of these viral HBDs do not conform to either of the mammalian consensus HBD linear sequence motifs (Table 1). Interestingly, for RSV-G, peptide b27 is a proline-rich peptide that contains an XBBBX\text{X} mammalian HBD motif, yet this peptide bound 16-fold less than peptide b20 in the HAAC assay. Furthermore, peptide b27 bound only weakly to Vero cells and did not bind to any of the CHO cells assayed, whereas the Vn HBD peptide, also containing an XBBBX\text{X} mammalian HBD motif, reacted strongly with both Vero and CHO cells and was inhibited by heparin. Thus, these data suggest that heparin binding is not limited to linear XBBBX\text{X} or XBBBXXBX motifs (6, 26, 47). Furthermore, factors such as conformation, accessibility of the basic residues to heparin, and possibly proline content may also influence viral protein interactions with cellular Gags. Likewise, our data suggest that our heparin binding peptides preferentially bind heparin; however, the RSV HBD peptide requirements for heparin may not be absolute. Peptide binding to pgsD-677 cells was reduced but not completely abrogated. This finding was not unexpected, as chondroitin sulfate, which is overexpressed on the pgsD-677 cells, was able to partially elute the RSV HBD peptides from heparin agarose (data not shown). In addition, these peptides may interact with an as-yet-unknown cellular protein. Unexpectedly, the RSV HBD peptides also bound to the pgsA-745 CHO cells. Work is currently under way to explain this paradox. Interestingly, preliminary data from gel electrophoresis of cell lysates stained with toluidine blue revealed the presence of a single high-molecular-mass proteoglycan (>250 kDa) that stains in pgsA-745 cells that was also one of multiple bands present in the K1 cell line, suggesting that these cells are Gag deficient but may not be Gag negative. These data could explain, in part, the binding of RSV and Vn HBD peptides to these cells. Little work has been done to model viral HBDs; thus, the minimal sequence requirements for RSV-G heparin binding are as yet unclear (6, 16). Further studies will be required to better characterize RSV peptide interactions with CHO cells in hopes of better understanding virus interactions with cellular glycosaminoglycans.

Initial attempts to understand how RSV interacts with heparin were focused on the mechanism by which heparin inhibits virus infectivity. Recent reports have created some controversy over this mechanism. One report suggests that the heparin inhibition of RSV is the result of heparin binding to RSV-G, thereby blocking attachment and/or infectivity of the virus (27). Our data lend further support to this mechanism of RSV heparin inhibition. In contrast, a second report argues that virus grown in Hep2 cells results in the production of viral heparin-like molecules associated with RSV-G. In this scenario, exogenous heparin binds to the cell surface, blocking interactions with viral proteoglycans (2). Our data could also suggest that cellular Gags might be complexed with RSV-G via interactions with intrinsic HBDs. Many viruses acquire cellular

| Table 1. Linear sequence alignment of various viral and mammalian HBDs |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| HBD*            | Location*       | RSV peptide    | Sequence*       | Consensus* motif| Reference(s)*   |
| Viral           | RSV-A 184–198   | a19–21          | ---AICKRIPKX---PGKK--- | XBBX            |
|                 | RSV-B 183–197   | b20             | ---KSICRKIPKX---FKRR--- | XBBX, XBBX     |
|                 | RSV-B 148–162   | b27             | ---TKPCISPX---FKSXX--- | XBBX           |
|                 | PRV 263–271     | ---PERSXRK---H--- | XBBBXXBX         | 30, 52          |
|                 | HSV1 142–152    | ---IY---CRFRNSTRM--- | XBBX            |
|                 | Mammalian       | Vn 346–358      | ---AKKQFRHRNRRKG--- | XBBX           |

* Viral and mammalian sequences were grouped together for alignments.
* Numbers indicate amino acid positions; RSV, respiratory syncytial virus; PRV, pseudorabies virus; HSV1, herpes simplex virus type 1; Vn, vitronectin.
* Comparison of putative RSV HBD sequences to the mammalian HBD consensus motifs, where B is almost always a basic residue and X is usually an uncharged, often hydrophobic residue.
* Consensus HBD motifs (XBBX\text{X} and XBBBXXBX) are derived from mammalian heparin binding proteins (5). Viral consensus motifs are based on homologies to the mammalian consensus sequences.
* Citation listed for sequence identification of HBD.
membrane components while budding from the plasma membrane of the cell (1, 4), and it is possible that RSV acquires cellular heparan sulfate while budding from the plasma membrane. Acquisition of cellular heparan sulfate by RSV could benefit the virus in at least two ways: (i) heparan sulfate binding could act to stabilize glycoprotein conformation (48) and (ii) heparan sulfate may mask an important functional site from the host response (48). Interestingly, evidence was recently presented from studies with RSV B/cp52, a subgroup B G-SH deletion mutant, suggesting that the G glycoprotein is not absolutely required for virus infectivity (24). This finding raises some interesting questions, the first being what the functional role of RSV-G is and the second being how this function relates to RSV-G-heparin interactions.

The most obvious functional role for RSV-G interactions with heparan sulfate involves receptor binding, tissue tropism, and determination of the extent of viral infection within the respiratory tract. Heparan sulfate is the membrane-associated cellular homologue of heparin, and it consists of a heterogeneous population of molecules that differ in chain length, hexuronic acid composition, and degree of sulfation (12). As reported previously for dengue virus (6), RSV seems to bind best to highly sulfated forms of heparan sulfate, as inhibition of RSV infectivity is more sensitive to heparinase treatment than to heparitinase (27). This distinction in G glycan sensitivity implies that the degree of sulfation may be one of the primary factors influencing binding avidity (3). In fact, given the molecular heterogeneity of heparan sulfate and its varied expression on different cell types (47), it seems plausible that RSV could differentially increase the probability of virus attachment to cells based on the form of cellular heparan sulfate expressed. Thus, binding interactions between RSV HBDs and cellular Gags might influence not only viral tropism but virulence as well. It would seem that even though RSV-G is not required for infectivity, it does confer a selective advantage, allowing the virus to spread and easily attach to neighboring cells. It should also be noted that non-heparin-dependent virus-cell interactions might influence attachment and infectivity. Examination of non-heparin-dependent interactions between RSV-G and cell surface molecules is currently under way.

While the primary role for RSV-G interactions appears to involve direct interactions with cell surface molecules, it may also play a secondary role in immunomodulation. Several recent studies clearly demonstrate that G glycoprotein activation of CD4+ Th1 lymphocytes was largely responsible for the enhanced pulmonary eosinophilia seen after RSV challenge in a murine model of RSV infection (10, 11, 22, 34, 49). This adverse response was abrogated by the vaccination of mice with a vaccinia-G construct with amino acids 193 to 205 of the G protein deleted (41). Furthermore, a subgroup A peptide identical to the linear HBD sequence described here, 134A→T198R, was able to prime mice for a CD4+ Th1 response, induce pulmonary eosinophilia, and stimulate a proliferative response in peripheral blood mononuclear cells in some human donors (44). Interestingly, several immune mediators, including cytokines and chemokines, perform their effector functions via an initial interaction with heparan sulfate (40). Thus, it is possible that RSV-G interactions with heparan sulfate on the surface of CD4+ T cells mediate subsequent cytokine or chemokine responses. Obviously, RSV-G interactions with cellular Gags need to be investigated further in order to better understand the mechanism by which the RSV-G HBDs (134A→T198R→K197T/K197) may prime for disease characterized by pulmonary eosinophilia.

While RSV-G may act to enhance attachment of RSV to cells, it does not appear to be absolutely required for infectivity. Interestingly, no wild-type virus has been isolated that does not express the G glycoprotein. Thus, it would seem that this protein must provide RSV with some selective advantage in vivo. To understand the biology of RSV, it will be important to understand not only the involvement of the G glycoprotein HBDs in virus attachment and infectivity but the role these domains play in the development of host immunity as well. By developing this understanding, important steps can then be taken to develop future therapeutic and preventive strategies against RSV-associated disease.

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