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G glycoprotein amino acid residues required for human monoclonal antibody RAB1 neutralization are conserved in rabies virus street isolates

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ABSTRACT

Replacement of polyclonal anti-rabies immunoglobulin (RIG) used in rabies post-exposure prophylaxis (PEP) with a monoclonal antibody will eliminate cost and availability constraints that currently exist using RIG in the developing world. The human monoclonal antibody RAB1 has been shown to neutralize all rabies street isolates tested; however for the laboratory-adapted fixed strain, CVS-11, mutation in the G glycoprotein of amino acid 336 from asparagine (N) to aspartic acid (D) resulted in resistance to neutralization. Interestingly, this same mutation in the G glycoprotein of a second laboratory-adapted fixed strain (ERA) did not confer resistance to RAB1 neutralization. Using cell surface staining and lentivirus pseudotyped with rabies virus G glycoprotein (RABVpp), we identified an amino acid alteration in CVS-11 (K346), not present in ERA (R346), which was required in combination with D336 to confer resistance to RAB1. A complete analysis of G glycoprotein sequences from GenBank demonstrated that no identified rabies isolates contain the necessary combination of G glycoprotein mutations for resistance to RAB1 neutralization, consistent with the broad neutralization of RAB1 observed in direct viral neutralization experiments with street isolates. All combinations of amino acids 336 and 346 reported in the sequence database were engineered into the ERA G glycoprotein and RAB1 was able to neutralize RABVpp bearing ERA G glycoprotein containing all known combinations at these critical residues. These data demonstrate that RAB1 has the capacity to neutralize all identified rabies isolates and a minimum of two distinct mutations in the G glycoprotein are required for abrogation of RAB1 neutralization.

1. Introduction

Approximately 55,000 people, mostly children in many countries of Asia and Africa, die each year of rabies, making human rabies a significant public health problem (Knobel et al., 2005). Rabies virus infection causes a nearly 100% fatal acute encephalitis, however mortality is preventable by prompt post-exposure prophylaxis (PEP). PEP consists of wound care and prompt administration of both rabies vaccine and rabies immune globulin (RIG). Administration of vaccine, in combination with RIG, results in nearly universal protection from disease, while vaccine alone is not as efficacious, especially when exposures are severe (Hemachudha et al., 1999; Wilde et al., 1989, 1996). Limited supply of RIG in the developing world due to the expense involved in producing large quantities of a fractionated blood product has resulted in the use of "vaccine-only" PEP, which is not recommended (WHO, 2005). Replacement of RIG with monoclonal antibody would provide a cost effective alternative resulting in appropriate delivery of PEP to exposed individuals.

Numerous mouse and human monoclonal antibodies directed against the G glycoprotein that neutralize rabies virus both *in vitro* and *in vivo* have been isolated and extensively characterized (Bakker et al., 2005; Bunschoten et al., 1989; Coulon et al., 1982; Dietzschold et al., 1990, 1987; Enssle et al., 1991; Flamand et al., 1980; Hanlon et al., 2001; Lafon et al., 1984, 1983; Marissen et al., 2005; Ni et al., 1995; Prehaud et al., 1988; Prosniak et al., 2003; Schumacher et al., 1989; Seif et al., 1985; Wiktor and Koprowski, 1978). These neutralizing antibodies recognize one of five distinct antigenic sites on the rabies virus G glycoprotein (Antigenic sites I, II, III, IV and Minor Site a) (Benmansour et al., 1991; Dietzschold et al., 1990; Lafon et al., 1983; Seif et al., 1985). The vast majority of neutralizing antibodies are directed to either antigenic site II or III. Antigenic site II is a discontinuous, conformational region consisting of amino acids 34–42 and 198–200 (Prehaud et al., 1988) whereas antigenic site III is predicted to be a continuous, conformation-dependent epitope spanning amino acids 330–338 (Seif et al., 1985). These antigenic regions were defined by identification of specific G glycoprotein mutations found in mAb-resistant escape virus. It is important to note that all neutralizing antibodies previously identified, when used alone, are unable to neutralize all available rabies virus isolates, presumably due to the diversity found in the amino acid content of the

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G glycoprotein. However, combinations of monoclonal antibodies are able to broadly neutralize a diverse group of rabies virus isolates (Bakker et al., 2005; Montano-Hirose et al., 1993; Prosniak et al., 2003; Schumacher et al., 1989). This has led to the widely held belief that a single monoclonal antibody would not have the breadth of neutralization (equivalent to RIG) required to protect against all viral isolates.

Using HuMAb mouse technology (Medarex, Inc.), we have identified the rabies-neutralizing human monoclonal antibody RAB1 (17C7) (Sloan et al., 2007) that was able to neutralize a comprehensive panel of 44 rabies street viruses from the Americas, Africa and Asia isolated from a diverse range of species in the Rapid Fluorescent Focus Inhibition Test (RFFIT) (Babcock et al., 2008; Sloan et al., 2007). A large proportion of neutralized isolates were obtained from the Centers for Disease Control and Prevention, USA and although the amino acid sequence of the G glycoprotein for all tested isolates has yet to be determined, all were obtained from distinct animals distributed globally and thus represent unique viruses. RAB1 was shown to protect hamsters from a lethal challenge of rabies virus either alone or in combination with vaccine (Sloan et al., 2007). RAB1 binds to the rabies virus G glycoprotein in both cell surface staining and ELISA. In Western blot, RAB1 could recognize the rabies virus G glycoprotein under non-reducing, denaturing conditions but binding was abolished when reducing conditions were employed. Also, virus derived from the fixed strain, CVS-11 containing a mutation in the G glycoprotein at amino acid position 336 of asparagine to aspartic acid (N336D) was resistant to RAB1 neutralization (Sloan et al., 2007). These data suggest that RAB1 recognizes a continuous, conformation-dependent epitope in the region of amino acid 336 defining it as an antigenic site III-directed antibody based on antigenic regions denoted in the literature. However, unlike other antigenic site III antibodies, RAB1 has the capacity to neutralize all rabies virus isolates that have been tested including street isolates containing the N336D mutation in the G glycoprotein.

In the current study, employing cell surface staining and lentivirus pseudotyped with rabies virus G glycoprotein (RABVpp) we determine that the N336D mutation is insufficient to confer resistance to RAB1 neutralization and an additional amino acid alteration, in conjunction with N336D, is required. Two specific G glycoprotein amino acid positions, residues 336 and 346 are critical to RAB1 neutralization, neither of which, when mutated, lead to escape from RAB1 neutralization on its own.

2. Materials and methods

2.1. Cells and cell culture

HOS and HEK-293T/17 cells were obtained from the American Type Culture Collection (ATCC) and were grown in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 IU penicillin–streptomycin (PS). All cells were grown at 37 °C in air supplemented with 5% CO_2. CHO cells were grown in CD-CHO medium (Invitrogen) supplemented with 25 μM methionine sulfoximine (MSX, Sigma).

2.2. Antibody expression and purification

CHO cells stably transfected with DNA encoding the RAB1 antibody were expanded and supernatant harvested by centrifugation. Culture supernatants were incubated with protein A Sepharose resin (GE Healthcare) for 2 h at room temperature while rocking. Beads were removed by column filtration, washed with PBS and antibody eluted with 100 mM glycine pH 2.8. Eluate was dialyzed against PBS and concentrated using an Amicon YM-30 centriprep concentrator as described by the manufacturer. Purified antibody was filter sterilized and protein concentration determined by spectrophotometry. For large scale antibody purification, HiTrap (Amersham) protein A column chromatography was employed and purified antibody was concentrated as described above.

2.3. Cloning of recombinant wild-type, chimeric and point mutated rabies virus G glycoproteins

The amino acid sequence of the rabies virus G glycoprotein for the ERA strain (GenBank: AF406693) and CVS-11 strain (GenBank: AF085333) was used to design codon-optimized versions of each gene spanning amino acids 1–524, as described elsewhere (Babcock et al., 2004). The synthetic genes were cloned into pcDNA3.1 (Invitrogen) in frame with an inserted C-terminal 6-histidine ($(His)_6$) epitope tag.

Chimeric CVS-11/ERA G glycoprotein genes were synthesized using overlapping PCR. Briefly, PCR was performed using a forward oligonucleotide (CVS-F) that was complimentary to 5′ end of the codon-optimized CVS-11 G glycoprotein gene (with a 5′ overhang incorporating a HindIII restriction site) and a reverse oligonucleotide (CVS-R1 through CVS-R6) corresponding to the desired junction between the CVS-11 and ERA genes. In this reaction, the 3′ end of the reverse oligonucleotide would anneal to the CVS-11 glycoprotein gene, leaving an overhang corresponding to ERA sequence. For the second PCR, a forward oligonucleotide complimentary to the chosen CVS-R oligonucleotide (ERA-F1 through ERA-F6) in combination with a reverse oligonucleotide (ERA-R) complimentary to the 3′ end of the ERA gene (with a 5′ overhang incorporating an XbaI restriction site) were used to generate amplicons from the codon-optimized G glycoprotein gene. Amplicons from both PCRs were mixed at equimolar ratios and additional PCR performed employing the CVS-F and ERA-R oligonucleotides. The resulting product encoding the entire chimeric G glycoprotein gene(s) was cloned into pcDNA3.1 containing a $(His)_6$ C-terminal epitope tag.

Site-directed mutagenesis was performed using the QuickChange II Site-Directed Mutagenesis kit (Stratagene) as described by the manufacturer. Briefly, overlapping primers containing the desired point mutations were used to amplify full-length mutant glycoprotein genes from the codon-optimized ERA G glycoprotein gene. The amplified DNA was digested with DpnI to remove the template DNA, transformed into bacteria, and screened for the intended mutation by sequencing. All constructed G glycoprotein sequences, including synthetic genes, chimerics and point mutants were sequenced by standard DNA sequencing technology to insure the fidelity of the PCR reactions.

2.4. Transfection and cell surface staining

All constructs were transfected into HEK-293T/17 cells using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. Cells were grown to 85% confluence in T-75 tissue culture flasks in 12 ml of DMEM/10% fetal calf serum (FCS). Fifteen microgram of DNA mixed with 37.5 μl of Lipofectamine 2000 was added to the cells, and plates were incubated overnight at 37 °C. Cells were incubated for 24 h at which time the transfection media was replaced with 15 ml of fresh DMEM/FCS supplemented with 100 IU PS. Incubation continued for an additional 24 h and cells were detached from the flask using PBS/5 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0 to preserve membrane protein structures.

Detached cells were incubated with varying concentration of RAB1 for 1 h at room temperature. As a control for cell surface expression, cells were incubated with 100nM of an antigenic site II directed HuMAb antibody (2B10, generated in-house). Cells were washed twice in PBS/0.5% BSA/0.01% sodium azide (PBS-BA) and

incubated with goat anti-human IgG-phycoerythrin (1:100, Jackson Immunoresearch) for 1 h at room temperature. Cells were again washed twice with PBS-BA and fluorescence analysis performed using a FACScan instrument with CellQuest software (Becton Dickinson).

2.5. RABVpp neutralization assays

Pseudovirus was generated employing an HIV backbone that contained a mutation to prevent HIV envelope glycoprotein expression and a luciferase gene to direct luciferase expression in target cells (pNL4-3.Luc.R–E–, obtained through the AIDS Research and Reference Program, Division of AIDS, NIAID, NIH from Dr. Nathaniel Landau). Rabies virus G glycoprotein was provided *in trans* by cotransfection of HEK-293T/17 cells with pcDNA-G with pNL4-3.Luc.R–E–. Supernatant containing virus particles was harvested 48–72 h post-transfection, concentrated using Centricon 70 concentrators, aliquoted and stored frozen at −80 °C. Before assessing antibody neutralization, a titration of RABVpp was performed on Hep3B cells to determine what volume of virus generated 50,000 cps in the infections assay. The appropriate volume of RABVpp was pre-incubated with varying concentrations of RAB1 for 1 h at room temperature before adding to HOS cells. After incubation for 72 h, infection was quantified by luciferase detection with BrightGlo luciferase assay (Promega) and read in a Victor3 plate reader (Perkin Elmer) for light production.

3. Results

3.1. Resistance to RAB1 neutralization is context-dependent

To better understand the relationship between the N336D mutation and RAB1 neutralization in the context of G glycoproteins isolated from different viral strains, we employed a novel *in vitro* infection system that allowed the introduction of point mutations into the G glycoprotein for assessment of neutralization of viral entry. Rabies virus G-pseudotyped viral particles (RABVpp) were generated from replication-defective HIV-1 that are devoid of native glycoproteins and engineered to contain the rabies virus G glycoprotein. The low level of native ERA G glycoprotein expression complicated the production of high titer RABVpp (data not shown). To overcome this limitation, we generated a codon-optimized gene encoding the entire G glycoprotein of the ERA strain of rabies (CO-ERA_{524}). Expression of ERA G glycoprotein was significantly increased allowing the production of high titer RABVpp (ERA_{WT}-RABVpp) (data not shown). RABVpp was generated by co-transfection of HEK-293T/17 cells with the codon-optimized rabies virus G glycoprotein expression plasmid and the lentiviral backbone containing the luciferase reporter gene. ERA_{WT}-RABVpp was harvested from the culture supernatants, concentrated and stored frozen in aliquots. The volume of RABVpp-containing supernatant that generated approximately 50,000 counts per second (CPS) light output after infection of HOS cells was determined for each virus. This constant amount of virus was incubated with a range of dilutions (0.001–1000 nM) of RAB1 and the results normalized to luciferase activity in the absence of antibody. A representative experiment is shown in Fig. 1. As predicted from work with native virus, RAB1 potently neutralized ERA_{WT}-RABVpp. Using site-directed mutagenesis we generated ERA-RABVpp containing the N336D mutation in the ERA envelope glycoprotein (ERA_{N336D}-RABVpp) and assessed RAB1 neutralization capacity. An antibody directed against the G glycoprotein antigenic site II (with binding distant from the N336 residue) neutralized both ERA-derived pseudotypes equivalently and an irrelevant human antibody showed no effect on pseudovirus infectivity (data not shown). Surprisingly, RAB1 was able to neutralize ERA_{N336D}-RABVpp although neutralization capacity was reduced (500-fold) as compared to ERA_{WT}-RABVpp (Fig. 1). This result was in contrast with the generation of CVS-11-derived virus containing the N336D mutation that was isolated from viral infection in culture.

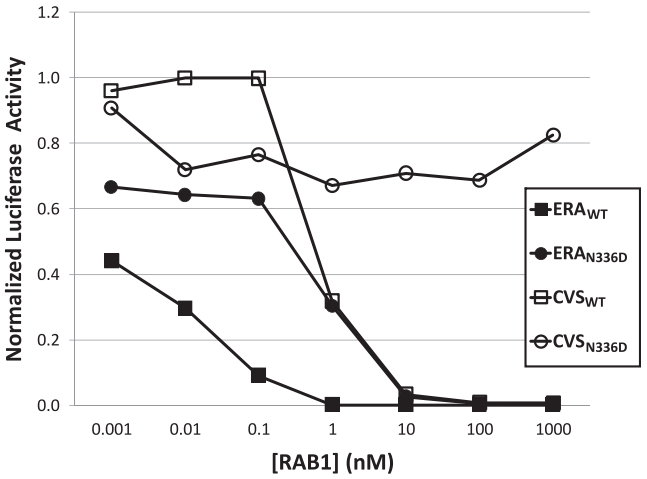

Fig. 1. RAB1 neutralizes ERA_{N336D}- but not CVS-11_{N336D}-RABVpp. Tenfold dilutions of RAB1 were incubated for 1 h at room temperature with RABVpp pseudotyped with ERA_{WT} (solid squares), ERA_{N336D} (solid circles), CVS-11_{WT} (open squares) or CVS-11_{N336D} (open circles) G glycoprotein. The various RABVpp:antibody mixtures were added to HOS cells followed by incubation at 37 °C for 72 h. Cells were lysed and infection assessed with Brightglo luciferase reagent followed by analysis using a Victor3 multilabel reader. Light output, normalized to a control sample for each RABVpp containing no antibody, was plotted.

We reasoned there were two possible explanations for the difference in RAB1 neutralization results obtained using native rabies virus as compared to RABVpp. Either RABVpp was not representative of natural rabies virus infection measured using RFFIT analysis or there were differences between the ERA and CVS-11 envelope glycoproteins that differentially affected RAB1 neutralization in the presence of an N336D mutation. To address this question, we synthesized a codon-optimized gene encoding the G glycoprotein of the CVS-11 rabies strain (CO-CVS_{524}) and generated RABVpp containing this glycoprotein (CVS_{WT}-RABVpp). Varying concentrations of RAB1 were tested for neutralization of CVS_{WT}-RABVpp and as expected RAB1 was able to neutralize (Fig. 1). CVS_{WT}-RABVpp containing an N336D mutation was constructed (CVS_{N336D}-RABVpp) and RAB1 was unable to neutralize even at the highest concentration tested (Fig. 1). However, the antigenic site II directed antibody could neutralize all ERA and CVS-11 pseudotypes equivalently (data not shown). These results confirm the data obtained by generating RAB1-resistant CVS-11 virus in culture suggesting that differences between the CVS-11 and ERA G glycoproteins are responsible for the differential neutralization capacity of RAB1.

3.2. N336D-mutation in CVS-11 but not ERA G glycoprotein abolishes RAB1 binding

It is clear that RAB1 is unable to neutralize CVS-11_{N336D}-RABVpp. To determine the mechanism by which mutant RABVpp resists RAB1 neutralization, we assessed binding of RAB1 to the G glycoproteins employed in the RABVpp assay. CVS-11 and ERA G glycoproteins, with or without the N336D mutation, were expressed in HEK-293T/17 cells. Transfectants were incubated with varying concentrations of RAB1 followed by a phycoerythrin (PE)-conjugated goat anti-human polyclonal antibody and fluorescence intensity was determined by flow cytometry. A representative experiment

is shown in Fig. 2. RAB1 binding was very strong for ERA_{WT}. RAB1 bound ERA_{N336D} and CVS_{WT} well albeit significantly less than that seen for ERA_{WT}. However, RAB1 binding was essentially nonexistent for CVS_{N336D} G glycoprotein. ERA_{N336D} binding in this assay is consistent with the observed neutralization capacity of RAB1 to ERA_{N336D}-RABVpp. Staining with a control human antibody directed against antigenic site II of the G glycoprotein demonstrated that all G glycoproteins had similar expression levels and an irrelevant human antibody did not bind to transfectants even at the highest concentration tested (data not shown). Given that expression of all proteins was essentially equivalent, the variability in mean fluorescence intensity most likely represents RAB1 affinity differences for each specific G glycoprotein. These data demonstrate that the inability of RAB1 to neutralize CVS_{N336D}-RABVpp is due to a lack of binding to CVS-11_{N336D} G glycoprotein and we predict that residues distinct from N336 that vary between the ERA and CVS-11 G glycoprotein are responsible for the lack of RAB1 binding and neutralization of CVS_{N336D}.

3.3. CVS-11 G glycoprotein contains amino acid residues in the context of the N336D mutation that disrupt RAB1 binding

The CVS-11 and ERA G glycoprotein amino acid sequences were aligned and it was determined that of the 439 extracellular residues, 31 amino acids were not conserved between the two proteins. To determine if one or a combination of the 31 residues from CVS-11 was conferring resistance to RAB1 in the context of the N336D mutation we generated chimeric full-length G glycoproteins that were partially CVS-11_{N336D} sequence and partially ERA_{N336D} amino acid sequence. All constructs contained the N336D mutation and consisted of the N-terminus of CVS-11 G glycoprotein fused to the C-terminus of ERA at various positions within the protein: amino acids 101, 221, 321, 341, 361 and 381 (Fig. 3). To assess the impact of combining CVS-11_{N336D} and ERA_{N336D} G glycoproteins on RAB1 binding a cell surface staining experiment was performed. Chimeric G glycoprotein-encoding constructs were transfected into HEK-293T/17 cells; cells were harvested and incubated with either 100 nM RAB1 or an antigenic site II antibody (expression control). Cells were subsequently incubated with anti-human IgG-phycoerythrin (PE) and bound fluorescence analyzed by flow cytometry. All chimeric constructs expressed well

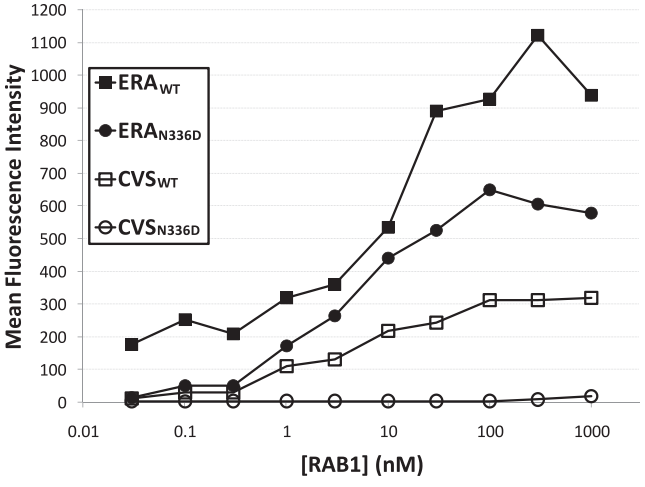

Fig. 2. RAB1 binds to ERA_{N336D} but not CVS-11_{N336D}. HEK-293/17 cells were transfected with ERA_{WT} (solid squares), ERA_{N336D} (solid circles), CVS-11_{WT} (open squares) or CVS-11_{N336D} (open circles). Transfectants were incubated with varying concentrations of RAB1 followed by anti-human PE-conjugated antibody. Bound fluorescence calculated as mean fluorescence intensity was determined using flow cytometry and the results plotted.

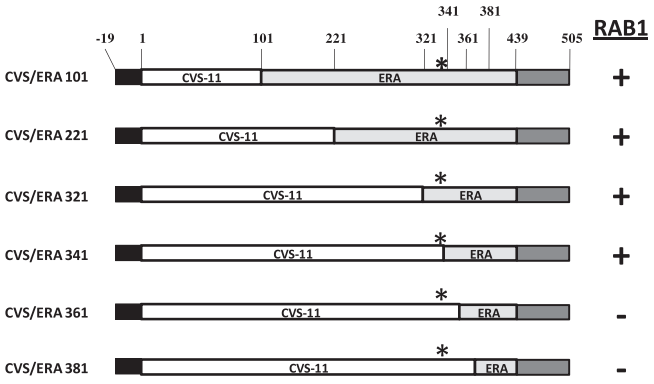

Fig. 3. RAB1 recognition of chimeric CVS-11/ERA glycoproteins containing the N336D mutation. Chimeric G glycoproteins are represented with junction amino acid positions defined at the top of the figure. The asterisk represents the position of the N336D mutation present in all chimeric proteins. The black bar (amino acids −19 to 0) represents the leader peptide and the dark gray (amino acids 440–505) delineates the transmembrane and intracellular domain. White bars define CVS-11_{N336D}-derived amino acid sequence and the light gray area is sequence donated by ERA_{N336D} G glycoprotein. Chimerics contained the first 101, 221, 321, 341, 361 or 381 amino acids from CVS-11_{N336D} with the remaining amino acid sequence to amino acid 505 obtained from ERA_{N336D}. Expression constructs encoding the chimeric proteins were transfected into HEK-293T/17 cells and binding of RAB1 to transfectant cell surfaces assessed by flow cytometry. Results of RAB1 recognition of the various G glycoproteins is represented at the right with a (+) or a (−).

as determined by the control antibody (data not shown). Chimeric constructs CVS/ERA_{101}, CVS/ERA_{221}, CVS/ERA_{321} and CVS/ERA_{341} (all containing the N336D mutation) were recognized by RAB1 similarly to ERA_{N336D} suggesting that residues upstream of amino acid 341 in the CVS-11 glycoprotein were not responsible for resistance to RAB1 binding. However, CVS/ERA_{361} and CVS/ERA_{381} were not recognized by RAB1 (Fig. 3, right side) indicating the importance of residues in this region for recognition by RAB1. Given the recognition of CVS/ERA_{341} but not CVS/ERA_{361}, it can be concluded that, in the context of an N336D mutation, residues unique to the CVS-11 G glycoprotein located between residues 341 and 361 are responsible for disruption of RAB1 binding.

3.4. Amino acid 346 is a critical residue for RAB1 binding and neutralization

Analysis of the amino acid sequence of the G glycoprotein of both CVS-11 and ERA revealed a single amino acid difference located between amino acids 341 and 361. CVS-11 possessed a lysine (K) whereas ERA contained an arginine (R) at position 346 suggesting that lysine at this position in combination with an aspartic acid (D) at position 336 resulted in ablation of RAB1 binding and neutralization. Given that these chimeric proteins contained multiple amino acid differences outside of amino acids 341–361 and distant amino acids could possibly contribute to RAB1 binding, we created multiple ERA and CVS-11 G glycoproteins containing point mutations at amino acids 336 and 346. Specifically, G glycoproteins derived from ERA containing N336D, N336D/R346K and R346K as well as CVS-11 with N336D, N336D/K346R and K346R were cloned and transfected into HEK-293T/17 cells for assessment of RAB1 binding. Mutations at these two positions in either CVS-11 or ERA did not result in reduction of surface expression as determined by staining with an antibody that bound the unrelated antigenic site II (data not shown). Also, an irrelevant HuMAb did not nonspecifically interact with transfectant cell surfaces (data not shown). Binding of a range of concentrations of RAB1 to transfectants was assessed using flow cytometry and mean fluorescence intensity of a representative experiment was plotted (Fig. 4A (ERA) and 4B (CVS-11)). ERA_{N336D} and ERA_{R346K} were recognized

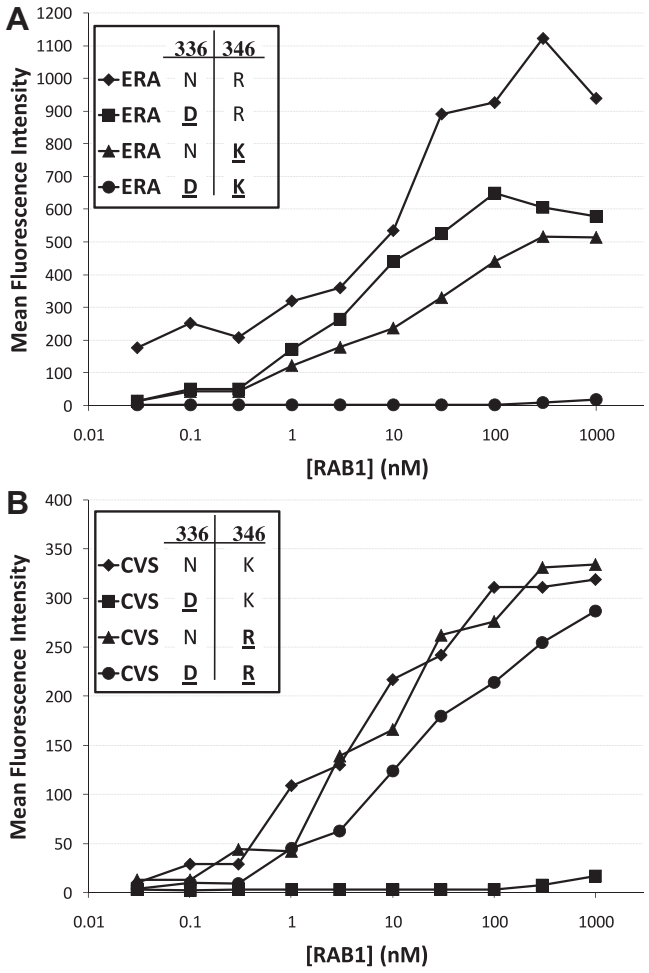

Fig. 4. RAB1 binding to CVS-11 and ERA G glycoproteins containing alterations at amino acids 336 and 346. (A) Constructs encoding ERA_{WT} (N336/R346, diamonds), ERA_{N336D} (squares), ERA_{R346K} (triangles) or $ERA_{N336D/R346K}$ (circles) were transfected into HEK-293T/17 cells and transfectants were incubated with varying dilutions of RAB1. Mean fluorescence intensity was determined using flow cytometry and the results were plotted for each dilution. Underlined and bolded amino acid residues in the legend represent mutations in the ERA G glycoprotein at the position listed at the top of the legend. Standard font amino acid residues in the legend represent natural amino acids. The amino acid position is listed in the top of the legend. (B) As in (A) but transfectants were expressing CVS-11_{WT} (N336/K346, diamonds), CVS-11_{N336D} (squares), CVS-11_{K346R} (triangles) or CVS-$11_{N336D/K346R}$ (circles).

by RAB1 albeit less well than ERA_{WT} (Fig. 4A). In combination, the N336D/R346K mutation abolished RAB1 binding to the ERA glycoprotein (Fig. 4A). CVS-11 G glycoprotein was strongly bound by RAB1 while CVS-11_{N336D} was not recognized (Fig. 4B). When lysine at position 346 was replaced with an arginine in the context of CVS-11_{N336D} (CVS-$11_{N336D/K346R}$), RAB1 binding was restored demonstrating the importance of both amino acids for RAB1 binding (Fig. 4B). Not surprisingly, CVS-11_{K346R} was strongly recognized by RAB1. These data confirm the results obtained using chimeric G glycoproteins and demonstrate that alterations at both amino acid 336 and 346 are required for disruption of RAB1 binding. Specifically, resistance to RAB1 binding requires an aspartic acid at position 336 in combination with a lysine at position 346. These data also suggest that other residues not conserved between CVS-11 and ERA do not adversely impact RAB1 binding.

To determine if the binding results correlate with neutralization, RAB1 inhibition of RABVpp was assessed for virus pseudotyped with all ERA and CVS-11 glycoproteins containing a combination of the above described mutations at amino acids 336 and 346. For all RABVpp, an irrelevant HuMAb demonstrated no neutralization activity in the assay (data not shown). RAB1 potently neutralized ERA_{WT}-RABVpp and neutralization was potent yet significantly reduced for ERA_{R346K}- and ERA_{N336D}-RABVpp (Fig. 5). As expected from the cell surface binding data, $ERA_{N336D/R346K}$-RABVpp could not be neutralized by RAB1. CVS-11_{WT}-RABVpp was neutralized by RAB1 and neutralization was greatly enhanced for CVS-11_{K346R}-RABVpp (Fig. 5). As previously shown, RAB1 could not neutralize CVS-11_{N336D}-RABVpp but neutralization was restored when the CVS-11 G glycoprotein contained both the N336D and K346R mutations (Fig. 5). Mean EC_{50} values for RAB1 neutralization of all wild type and mutant pseudoviruses from multiple experiments were calculated and the results are shown in Table 1 (upper panel). These results confirm that resistance to RAB1 neutralization requires amino acid mutations at both amino acids 336 and 346.

3.5. Amino acids 336 and 346 are highly conserved in rabies virus isolates

The results of an extensive search of GenBank to identify amino acid diversity found at positions 336 and 346 of the rabies virus G glycoprotein are summarized in Table 2. Of the 469 identified sequences, 360 (76.8%) contained N336/R346, the optimal sequence for RAB1 neutralization. Approximately 12% of the sequences were N336/K346 (29/469, 6%) or D336/R346 (28/469, 6%) which are expected to be neutralized by RAB1 based on the binding and RABVpp data. Other identified sequences contained S336/R346 (42/469, 9%), N336/S346 (2/469, 0.4%), N336/T346 (5/469, 1.1%), K336/R346 (1/469, 0.2%) and N336/E346 (2/469, 0.4%). Interestingly, the sequence combination that would be expected to resist RAB1 neutralization, D336/K346, was not identified. It is important to note that a GenBank search was also performed specifically searching for any sequence (not only rabies sequences) that contained the D336/K346 combination. Sequences were found that matched at these positions, but all belonged to non-rabies lyssaviruses which RAB1 is known not to neutralize (Sloan et al., 2007).

A number of 336/346 G glycoprotein amino acid combinations were identified in our GenBank search that had not been tested

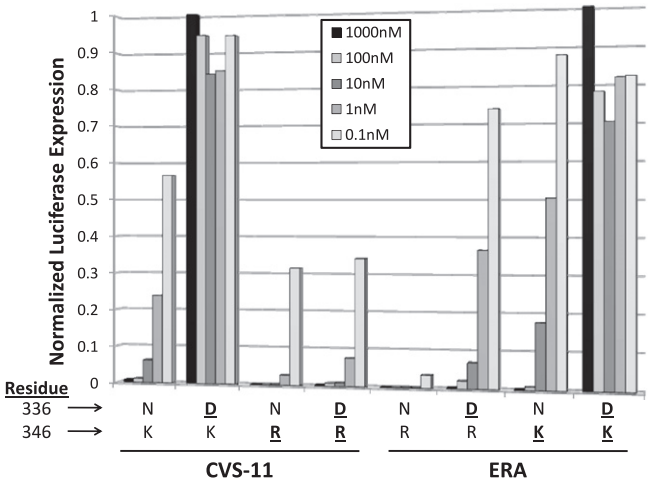

Fig. 5. RAB1 neutralization of RABVpp harboring mutations at amino acids 336 and 346. RABVpp were generated containing CVS-11 or ERA glycoproteins with or without alterations at amino acids 336 and 346. The specific amino acids contained within each RABVpp at positions 336 and 346 are listed below the *x*-axis with mutated residues underlined/bolded and naturally occurring amino acids in standard font. The background G glycoprotein used is listed below the *x*-axis. Tenfold dilutions of RAB1 were incubated with the various RABVpp and infection determined as a measure of light output. Luciferase activity as compared to the RABVpp-only control (no antibody) was plotted as a function of the RAB1 concentration (shown in the center legend).

Table 1
RAB1 neutralization potency of wild type and mutant RABVpp.

G glycoprotein backbone	Mutation(s)	Number of assays	EC_{50} mean (nM)	EC_{50} range (nM)
CVS-11	None	3	0.77	0.40–1.6
CVS-11	N336D	5	>1000	>1000
CVS-11	K346R	2	<0.01	<0.01
CVS-11	N336D/ K346R	2	<0.01	<0.01
ERA	None	5	<0.01	<0.01
ERA	N336D	4	0.29	0.14–0.39
ERA	R346K	2	1.3	1.1–1.5
ERA	N336D/ R346K	5	>1000	>1000
ERA	R346S	2	<0.01	<0.01
ERA	R346E	2	2.6	2.0–3.2
ERA	R346T	2	<0.01	<0.01
ERA	N336S	2	<0.01	<0.01
ERA	N336K	3	25.1	16.3–34.3

Table 2
Conservation of G glycoprotein residues critical to RAB1 neutralization.

Amino acid **336**	Amino acid **346**	Number of isolates of 469	% of total	RAB1 binding/ neutralization
Asparagine (N)	Arginine (R)	360	76.8	+
Serine (S)	Arginine (R)	42	9.0	+
Asparagine (N)	Lysine (K)	29	6.2	+
Aspartic acid (D)	Arginine (R)	28	6.0	+
Asparagine (N)	Threonine (T)	5	1.1	+
Asparagine (N)	Serine (S)	2	0.4	+
Asparagine (N)	Glutamic acid (E)	2	0.4	+
Lysine (K)	Arginine (R)	1	0.2	+
Aspartic acid (D)	Lysine (K)	0	0.0	–

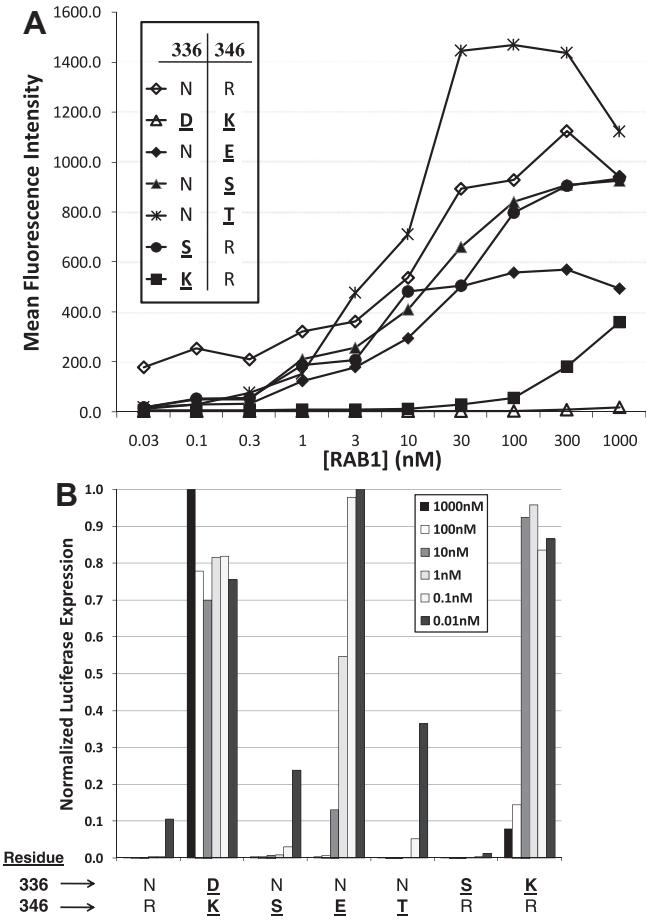

Fig. 6. RAB1 binding and RABVpp neutralization of ERA G glycoprotein containing alterations at amino acids 336 and 346 found in all identified rabies isolates. (A) HEK-293T/17 cells were transfected with constructs encoding ERA G glycoproteins containing either ERA_{WT} (N336/R346, open diamonds), $ERA_{N336D/R346K}$ (open triangles) or alterations identified in the rabies virus G glycoprotein database (solid symbols). In the legend, amino acid position is listed at the top, residues in standard font represent amino acids naturally present in ERA and underlined/bolded amino acids represent introduced mutations. Transfectants were stained with varying concentrations of RAB1, fluorescence determined by flow cytometry and the results plotted. (B) RABVpp bearing the G glycoproteins described in (A) were incubated with tenfold dilutions of RAB1, the mixture applied to HOS cells and light output determined using a Victor3 multilabel reader. Normalized luciferase was determined as a comparison to luminescence generated by RABVpp in the absence of RAB1 antibody and the results plotted. The specific amino acids contained within each RABVpp at positions 336 and 346 are listed below the *x*-axis with mutated residues underlined/bolded and naturally occurring amino acids in standard text.

for RAB1 binding and neutralization. To assess RAB1 activity against these identified 336/346 combinations, all that were found in the database were engineered into the ERA G glycoprotein. ERA G glycoprotein mutants were either expressed in HEK-293T/17 cells and RAB1 binding was assessed (Fig. 6A) or used to construct RABVpp to assess neutralization (Fig. 6B). All five identified alterations at 336/346 identified by GenBank were recognized and neutralized by RAB1 as shown in representative Fig. 6. Serine at either position 336 or 346 had no effect on either RAB1 binding or neutralization. Aspartic acid at position 336 or either lysine or glutamic acid at position 346 reduced relative RAB1 affinity but binding and neutralization was still strong. Threonine at position 346 had no demonstrable effect on either RAB1 binding or neutralization. In addition, RAB1 could bind to and neutralize lysine at position 336 however relative affinity was significantly reduced. Only the N336D/R346K combination, which is not found in the G glycoprotein database, abolished RAB1 interaction with the G glycoprotein and the capacity for RAB1 to neutralize RABVpp. A summary of the EC_{50} results for all GenBank-derived mutations engineered into ERA pseudovirus is shown in Table 1 (lower panel). Based on the sequence analysis, RAB1 antibody is expected to neutralize all rabies viruses in which the G glycoprotein sequence is known. This data is in complete agreement with the results from native rabies virus in which all tested isolates are neutralized by RAB1.

4. Discussion

Mortality due to rabies virus exposure continues to be of significant concern in the developing world. Although vaccine is available, polyclonal antibody (hRIG or eRIG) is in short supply and also quite expensive resulting in incomplete post-exposure prophylaxis (PEP). Generation of an affordable and broadly cross-reactive human monoclonal antibody to replace RIG in PEP is of paramount importance to protect the developing world from rabies-related mortality (WHO, 2002). RAB1 has been shown to neutralize all currently identified isolates *in vitro*, protect hamsters from rabies-associated mortality *in vivo* and is a fully human monoclonal antibody making it an appropriate, cost-effective replacement for RIG in rabies PEP.

Numerous mouse and human monoclonal antibodies, specific for the rabies virus G glycoprotein, have been isolated and extensively characterized. None of these antibodies have the capacity to neutralize all tested rabies viral isolates in a tissue culture-based assessment of viral infection. Conversely, as published previously (Sloan et al., 2007), RAB1 has the unique ability to neutralize a panel of viruses representing isolates from numerous species and geographical locations. It is important to note that not one single isolate has been tested in RFFIT analysis that cannot be neutralized

by RAB1. CVS-11 variants were selected in the laboratory that contained an amino acid substitution at position 336 (N336D) of the G glycoprotein that were resistant to RAB1 neutralization. This fact suggests that RAB1 binds to the previously defined antigenic site III of the G glycoprotein (Seif et al., 1985). Numerous antigenic site III-directed antibodies have been identified and many are sensitive to alterations at position 336 in the G glycoprotein (Bakker et al., 2005; Dietzschold et al., 1983; Seif et al., 1985). For other antibodies, lack of neutralization of CVS-11_{N336D} virus has correlated with an inability to neutralize rabies street virus that contains an N336D mutation (Bakker et al., 2005). Surprisingly, RAB1 is able to neutralize all viral isolates tested, including those with N336D mutations. Why then does RAB1 neutralize rabies isolates that other antigenic site III-directed mAbs cannot? To answer this question we exploited the fact that RAB1 could neutralize ERA_{N336D}-RABVpp but not CVS-11_{N336D}-RABVpp, our first clue that RAB1 sensitivity to the N336D mutation was G glycoprotein context-dependant. By synthesizing CVS-11/ERA chimeric G glycoproteins and RABVpp bearing these glycoproteins we identified amino acid 346, in conjunction with amino acid 336, as a critical residue for RAB1 binding and neutralization. Our data supports that elimination of RAB1 neutralization requires a combination of two alterations in the rabies virus G glycoprotein at positions 336 and 346. Interestingly, amino acid 346 is outside of the traditional antigenic site III (amino acids 330–338) and it is suggestive that the epitope recognized by RAB1 may represent a unique epitope not bound by other previously identified antibodies, delineating a new region for virus neutralization. We speculate that RAB1 is the only identified antigenic site III-directed human monoclonal antibody that is sensitive to mutations at position 336 only when a second damaging mutation, at position 346, is also present in the G glycoprotein.

Although both amino acids 336 and 346 of the G glycoprotein are highly conserved across all rabies street isolates, an extensive search of GenBank identified a number of alterations at positions 336 and 346 of the rabies virus G glycoprotein. Approximately 95% of all identified sequences contained either N336 or R346 in the G glycoprotein. Our data strongly suggests that isolates that contain N336 or R346 in the G glycoprotein can be neutralized regardless of the amino acids present in other parts of the protein. We generated ERA-RABVpp containing all identified combinations of these two residues and found that only D336/K346 (identified in the CVS-11-derived mutant) could avoid neutralization. Interestingly, of 469 described G glycoprotein sequences analyzed none was identified containing this combination of mutations. Individually, both mutations are rare with D336 and K346 being identified in only 6.0% and 6.2%, respectively, of all currently sequenced isolates. It has been demonstrated that mutations in antigenic site III reduce viral fitness and alter growth properties in culture although this work was performed on highly attenuated fixed strains of rabies virus such as CVS-11 (Bakker et al., 2005; Coulon et al., 1998; Dietzschold et al., 1983; Seif et al., 1985). Given the lack of rabies isolates that contain the D336/K346 G glycoprotein combination, we speculate that individually both the 336 and 346 mutations in the G glycoprotein of street virus reduce viral fitness *in vivo* and a combination of the two mutations drastically impedes the ability of the virus to cause disease.

The epitope recognized by RAB1 is suspected to be a continuous, disulfide-stabilized, conformation-dependent epitope given that RAB1 binding can occur after SDS treatment but only when reducing agents are excluded (Sloan et al., 2007). The importance of amino acids 336 and 346 as critical contacts for binding strongly suggests that this continuous epitope resides in and around amino acids 336–346. The region encompassing amino acids 336–346 is highly conserved across all street isolates. In fact, amino acids 324–347 are highly conserved in all rabies street isolates with amino acids 340–345 being conserved in all lyssaviruses demonstrating the importance of this region for viral entry. It is formally possible that residues distant from amino acids 336–346 contribute to RAB1 binding. We believe this to be unlikely, however, since RAB1 broadly neutralizes all street isolates tested, which contain diverse G glycoprotein sequences, suggesting that alterations at distant residues do not impact RAB1 binding/neutralization. It is also possible that residues distant from 336 to 346 are involved in RAB1 binding and neutralization but these residues are refractory to mutation because of a negative impact on viral fitness.

The current study employs the ERA and CVS-11 viral isolates to determine the importance of amino acids 336 and 346 in RAB1 interaction with G glycoprotein. The unanticipated identification of amino acid 346 as critical to RAB1 binding raises the possibility that other unexpected residues in the G glycoprotein of other isolates (other than CVS-11 and ERA) may be critical to RAB1 neutralization. Although formally possible, we believe this to be unlikely given that RAB1 has been shown to neutralize all identified rabies isolates which is in direct agreement with the lack of the deleterious combination of amino acids at positions 336 and 346 (D336/K346) in the literature. To further address this possibility, we have attempted to generate escape variants from additional rabies isolates without success due to the low culture titer of these viral isolates, as compared to CVS-11. The inability to generate escape virus with other strains of rabies virus also precludes us from directly confirming the correlation between amino acids 336 and 346 of the G glycoprotein and RAB1 neutralization in the setting of natural rabies virus infection.

It is surprising that RAB1 has a unique breadth of cross-reactivity when compared to other antigenic-site III directed antibodies. In general, neutralizing antibodies considered for clinical use have been selected based on potent neutralization of CVS-11 (Bakker et al., 2005; Marissen et al., 2005). Instead, we directly screened our antibodies for the ability to broadly neutralize a diverse group of street isolates, allowing us to identify RAB1, whose potency against CVS-11 is relatively lower than that observed for street isolates. It is clear that using highest potency to CVS-11 as the main criterion for selection would not have led to the identification of RAB1 in our screening. Our current hypothesis is the lab-adapted CVS-11, containing the unusual amino acid K346 in the G glycoprotein, is not an optimal virus for selecting cross-reactive antibodies. We believe that by using a screening approach that focused on breadth of neutralization we were able to successfully identify this unexpectedly broadly cross-reactive antibody.

RAB1 represents the first monoclonal antibody that has the characteristics required to be a replacement for hRIG as a single antibody in PEP. Given the breadth of neutralization of rabies viruses isolated from numerous species and continents and the lack of identified street viruses that are resistant to neutralization allows us to conclude that RAB1 will provide protection in PEP equivalent to hRIG. An open-label phase I safety study of RAB1 has recently been completed in India (Leav et al., 2010) and a phase II/III efficacy study is planned for 2011.

Conflict of Interest

This study was funded by MassBiologics. Drs. Sloan, Ambrosino and Babcock are named as coinventors on relevant patents with all rights or royalties assigned to MassBiologics.

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