### Sample R01 Proposal (New Format)

<table>
<thead>
<tr>
<th>PI: Parrish, Colin R.</th>
<th>Title: Structural controls of functional receptor and antibody binding to viral capsids</th>
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<td>Received: 03/04/2010</td>
<td>FOA: PA10-067</td>
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<td>Council: 10/2010</td>
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<td>IPF: 1614802</td>
<td>Organization: CORNELL UNIVERSITY ITHACA</td>
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<td>Former Number:</td>
<td>Department: Baker Inst. for Animal Health</td>
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<td>IRG/SRG: ZRG1 IDM-R (33)M</td>
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<td>Year 5: 250,000</td>
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<td>Senior/Key Personnel:</td>
<td>Organization:</td>
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<tr>
<td>Colin Parrish</td>
<td>Cornell University</td>
</tr>
<tr>
<td>Susan Hafenstein</td>
<td>Pennsylvania State University - Hershey Medical Center</td>
</tr>
<tr>
<td>Moonsoo Jin</td>
<td>Cornell University</td>
</tr>
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<td>Role Category:</td>
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<td></td>
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<td>Other Professional-Consortium PI</td>
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<tr>
<td></td>
<td>Other Professional-Collaborator</td>
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APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

1. * TYPE OF SUBMISSION
   - Pre-application
   - Application
   - Changed/Corrected Application

2. DATE SUBMITTED

3. DATE RECEIVED BY STATE

4. a. Federal Identifier

4. b. Agency Routing Identifier

5. APPLICANT INFORMATION
   - Legal Name: Cornell University
   - Department: Office of Sponsored Programs
   - Division:
   - Street1: 373 Pine Tree Road
   - City: Ithaca
   - County / Parish: Tompkins
   - State: NY
   - Country: USA
   - * ZIP / Postal Code: 14850-2820
   - * Phone Number: 607-255-5066
   - * Fax Number: 607-255-5058
   - Email: tjb3@cornell.edu
   - * Organizational DUNS: 822612445

6. * EMPLOYER IDENTIFICATION (EIN) or (TIN): x

7. * TYPE OF APPLICANT:
   - Public Institution of Higher Education
   - Private Institution of Higher Education
   - Other (Specify):
   - Small Business
   - Women Owned
   - Socially and Economically Disadvantaged

8. * TYPE OF APPLICATION:
   - New
   - Resubmission
   - Renewal
   - Continuation
   - Revision
   - If Revision, mark appropriate box(es).
   - A. Increase Award
   - B. Decrease Award
   - C. Increase Duration
   - D. Decrease Duration
   - E. Other (specify):

9. * NAME OF FEDERAL AGENCY:

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER:

11. * DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:
    - Structural controls of functional receptor and antibody binding to viral capsid

12. PROPOSED PROJECT:
   - Start Date: 12/01/2010
   - Ending Date: 11/30/2015

13. CONGRESSIONAL DISTRICT OF APPLICANT

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION
   - Prefix: Dr.
   - * First Name: Colin
   - Middle Name: R.
   - Last Name: Parrish
   - Position/Title: John M. Olin Professor of Virology
   - Organization Name: Cornell University
   - Department: Baker Inst. for Animal Health
   - Division: College of Veterinary Medicine
   - Street1: Hungerford Hill Road
   - City: Ithaca
   - County / Parish: Tompkins
   - State: NY
   - Country: USA
   - * ZIP / Postal Code: 14853-6401
   - Phone Number: 607-256-5649
   - Fax Number: 607-256-5688
   - Email: crp3@cornell.edu

Tracking Number: GRANT059156
Funding Opportunity Number: PA-10-067
Received Date: 2010-03-04T17:35:29-04:00
15. ESTIMATED PROJECT FUNDING

<table>
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<th>Fund Type</th>
<th>Amount</th>
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<tbody>
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<td>a. Total Federal Funds Requested</td>
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<td>b. Total Non-Federal Funds</td>
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<td>c. Total Federal &amp; Non-Federal Funds</td>
<td>1,944,550.00</td>
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<tr>
<td>d. Estimated Program Income</td>
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</table>

16. * IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?

- a. YES  
  - This preapplication/application was made available to the state executive order 12372 process for review on: 
  - Date: 
- b. NO  
  - Program is not covered by E.O. 12372; or 
  - Program has not been selected by state for review

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances* and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

* The list of certifications and assurances, or an internet site where you may obtain this list, is contained in the announcement or agency specific instructions.

18. SFLLL or other Explanatory Documentation

Add Attachment  Delete Attachment  View Attachment

19. Authorized Representative

<table>
<thead>
<tr>
<th>Prefix</th>
<th>First Name</th>
<th>Middle Name</th>
<th>Last Name</th>
<th>Suffix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ms.</td>
<td>Judith</td>
<td>r.</td>
<td>Wood</td>
<td></td>
</tr>
</tbody>
</table>

* Position/Title: Grt. Grant and Contract Officer
* Organization: Cornell University
* Department: Office of Sponsored Programs
* Street 1: 371 Pine Tree Road
* City: Ithaca
* County / Parish: Tompkins
* State: NY: New York
* Country: USA: UNITED STATES
* ZIP / Postal Code: 14850-2820

* Phone Number: 607-253-3759  Fax Number: 607-253-3756

* Email: jrw@cornell.edu

* Signature of Authorized Representative: Judy Wood

* Date Signed: 03/04/2018

20. Pre-application

Add Attachment  Delete Attachment  View Attachment
Project/Performance Site Location(s)

Project/Performance Site Primary Location

☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: Cornell University

DUNS Number: 8726124450000

* Street1: Rungerford Hill Road

Street2: Baker Institute for Animal Health

* City: Ithaca  County: Tompkins

* State: NY: New York

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: 14853-6401  * Project/Performance Site Congressional District: NY-022

---

Project/Performance Site Location 1

☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: The Pennsylvania State University - Hershey Medical Center

DUNS Number: 1293481860000

* Street1: 500 University Drive

Street2: Division of Infectious Diseases, Dept. of Medicine

* City: Hershey  County: Dauphin

* State: PA: Pennsylvania

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: 17033-0850  * Project/Performance Site Congressional District: PA-017

---

Additional Location(s)  Add Attachment  Delete Attachment  View Attachment
RESEARCH & RELATED Other Project Information

1. * Are Human Subjects involved?  □ Yes  □ No
1.a If YES to Human Subjects
   Is the Project Exempt from Federal regulations?  □ Yes  □ No
   if yes, check appropriate exemption number.  1  2  3  4  5  6
   If no, Is the IRB review Pending?  □ Yes  □ No
   IRB Approval Date:  
   Human Subject Assurance Number:  

2. * Are Vertebrate Animals Used?  □ Yes  □ No
2.a. If YES to Vertebrate Animals
   Is the IACUC review Pending?  □ Yes  □ No
   IACUC Approval Date:  10/05/2009
   Animal Welfare Assurance Number:  A3347-01

3. * Is proprietary/privileged information included in the application?  □ Yes  □ No
4.a. * Does this project have an actual or potential impact on the environment?  □ Yes  □ No
4.b. If yes, please explain:  
4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed?  □ Yes  □ No
4.d. If yes, please explain:  

5. * Is the research performance site designated, or eligible to be designated, as a historic place?  □ Yes  □ No
5.a. If yes, please explain:  
6. * Does this project Involve activities outside of the United States or partnerships with international collaborators?  □ Yes  □ No
6.a. If yes, Identify countries:  
6.b. Optional Explanation:  

7. * Project Summary/Abstract  1239-ABSTRACT.pdf
8. * Project Narrative  1238-NARRATIVE.pdf
9. Bibliography & References Cited  1239-BIBREF.pdf
10. Facilities & Other Resources  1240-PACILRREF.pdf
11. Equipment  1241-EQUIPMENT.pdf
12. Other Attachments  

Other Information

Tracking Number: GRANT10549156  Funding Opportunity Number: PA-10-067  Received Date: 2010-03-04T17:35:29-04:00
Here we seek to understand how structural flexibility and variation in parvoviral capsids control their ability to bind receptors leading to cell infection and also to variation in host range, and also how capsid structures control antibody binding and neutralization. Those areas of study are significant because they are features of all animal and human viruses. While parvovirus capsids appear structurally simple, they are clearly sophisticated biomolecular machines that carry out many functions using variants of a single capsid protein, and the features controlling many functions have now been mapped to specific mutations and capsid structures, presenting an opportunity to gain a complete understanding of how virus-host interactions occur in fine detail. Parvoviruses include the B19 virus, human bocavirus, and Parv4, all of which cause disease in humans. Here we use feline and canine parvoviruses as models to build on our previous studies showing that cell infection and animal host ranges are controlled by specific interactions of the capsids with the transferrin receptors type-1 (TfR) of different hosts. There are also distinct outcomes for viral infection of antibody binding, depending on the binding site and angle of attachment.

The three overlapping areas to be investigated in this project are:

1. Define the functional effects of variant and flexible structures of the parvovirus capsids. There is both flexibility and structural variation in the viral capsids which we will further characterize in detail. Some of that variation is asymmetric, only occurring in a small proportion of the capsid subunits, while other variation occurs in most viral sites. Many of those sites are known to affect viral functions.

2. Characterize how different interactions between parvovirus capsids and TfR or other receptors control infection. Capsid binding to different TfRs is controlled by the protein structure, and flexibility, and potentially by protease cleavages. To define the functional TfR binding to the capsids we will use a variety of approaches, including cryoEM analysis and analysis of capsid mutants affecting binding. To define the TfR interaction we will use receptors containing amino acid changes or added glycans within the interaction sites.

3. Use antibody binding to capsids to define their structures, and also to explain the mechanisms of antibody neutralization. We have many antibodies that bind the capsid structures. Some neutralize as Fabs, and others do not. We will use those antibodies as probes for structural variation in the capsids. The antibodies and their domains will be used in competition assays to further define the binding of different TfRs to the capsids, and to detect cleaved subunits or other capsid modifications. Antibodies with altered binding affinities, different attachment sites, or different angles of attachment would be tested for effects on the virus functions involved in cell infection.
NARRATIVE:

This project addresses several issues of central importance to the success of all viruses infecting humans and other animals. Those include: how viral proteins vary in structure over their life cycles, the mechanisms of binding to different receptors, how differences in viral structural proteins can control their host ranges, the processes of cell infection, and how antibody binding can neutralize the virus in some cases but not others. These studies will show how altered receptor binding can lead to the emergence of new epidemic or pandemic viruses, and will also reveal new ways to make more effective vaccines or therapeutics. The specific model viruses being examined infect animals, but they are similar to a variety of important viruses of humans. The small sizes and simple genetic and capsid structures of the parvoviruses make them excellent models for defining the most basic aspects of the viral-host interaction, and the results and broad conclusions will be directly relevant to the better understanding of many different viruses of humans.
FACILITIES AND OTHER RESOURCES:

Parrish, Colin R., Cornell University

Laboratory: 1200 sq. ft. of laboratory space includes a 174 sq. ft. tissue culture room and a second smaller shared tissue culture room for propagation of clean cells. The laboratory can accommodate up to 10 people, and is equipped with two 4-ft biohazard hoods for tissue culture and virus work, and two tissue culture incubators. A shared cold room and a warm room are available. A separate 300 sq. ft. laboratory is used for growing cells in roller bottles and shaker flasks for large-scale production of viral capsids and proteins (such as transferrin receptor and antibody domains) in mammalian, insect or yeast cells.

Animal: The Institute’s Rodent Facility (4200 sq. ft.) is shared by five investigators and houses a closed colony of inbred rats and mice that are maintained under S.P.F. conditions. Mice are also obtained from vendors able to meet the highest standards of S.P.F. designation.

Computer: The laboratory is equipped with Mac and Windows desktop and laptop computers, which are sufficient for the word processing, molecular modeling, and other computing needed for this project. All computers are networked to the campus system for internet access. We have access to all of the software packages required for our work.

Office: Dr. Parrish has dedicated office space of 160 sq. ft. adjacent to the laboratory. There is office space for the students and fellows who work on the projects proposed here. The Institute and College have a variety of expert support personnel, including secretarial services, purchasing, and accounts agents.

Clinical: N/A

Other: A central glassware facility and a shared dark room are available in the building. Conference room facilities and a lecture theater are available within the Institute.

Scientific Environment: The laboratory is within the Baker Institute, in the College of Veterinary Medicine at Cornell University. There are healthy communities of virologists, molecular microbiologists and biochemists in the Institute, the College, and the University. Dr. Parrish has been instrumental in organizing a campus-wide research group of researchers interested in virology, and is PI on a T32 pre-doctoral training grant that supports virology students throughout the campus. Other investigators at Cornell include those interested in infectious disease, immunology, protein structure, and the ecology and evolution of infectious disease agents. Dr. Moonsoo Jin is assisting with the studies involving protein engineering and binding analysis using yeast expression, and he is in the Department of Biomedical Engineering. Dr. Parrish has been heavily involved in all of the efforts to date. The Parrish laboratory already has the materials required to undertake the studies proposed, including panels of native and cloned monoclonal antibodies, viruses that can be prepared as viable capsids or as expressed VLPs, receptors that can be expressed on cells or in insect cells for purification. All of the equipment required for the studies is either in the laboratory or readily available in shared facilities.

Biohazards: This work involves the study of paroviruses that infect dogs and cats. Both the viruses are extremely widespread in the environment and found wherever dogs and cats are found around the world. The studies are of two strains that differ in host ranges for their natural hosts. The work that we are conducting will examine the underlying basis of those host range differences resulting from receptor binding, but the mutations we are making would make the viruses less infectious for animals. The viruses are handled using appropriate containment, and with the appropriate approvals from the Cornell University IBC.
Hafenstein, Susan L., Pennsylvania State University-Hershey Medical Center

**Laboratory:** Dr. Hafenstein has 800 sq. ft. of newly renovated laboratory space within two rooms in the Penn State University College of Medicine. Those laboratories are well equipped for biochemical studies and cell culture work. One is specifically set up for the preparation and purification of samples for structural analysis, and has a unit for controlled freezing of grids for cryoEM.

**Animal:** Animals are not being used as part of the Penn State component of the project, but the Penn State College of Medicine has approved animal housing for rodents and other animals.

**Computer:** The laboratory has a variety of Mac, Windows, and Linux computers for the preparation of manuscripts and grants, for the analysis of data, and for processing and analysis of structural data. Those are all connected to the Penn State high speed network.

**Office:** Dr. Hafenstein has a 130 sq. ft. office for her use, and there is another shared office for the use of graduate students and post-doctoral associates. The Department has expert secretarial support for the preparation of grants and manuscripts and accounting.

**Clinical:** Not applicable

**Other:** There are many shared facilities in the Department, including darkroom spaces and common equipment such as high speed and ultracentrifuges with various rotors.

**Scientific Environment:** Dr. Hafenstein is an Assistant Professor at Penn State University – Hershey Medical Center. Although her laboratory has only recently been established, she has been a close collaborator with the Parrish laboratory for several years while a post-doc and research scientist in Michael Rossmann’s laboratory. She was instrumental carrying out the previous structural studies of both receptor and antibody binding, some of which are to be continued in the proposed studies. Her laboratory is recently renovated, and is on the same floor as 4 other laboratories that are engaged in the studies of molecular virology. She has a variety of equipment required for freezing cryoEM grids under the precisely controlled conditions that are required for the proposed studies, and the computing resources for determining the structures. Access to cryoEM will primarily involve microscopes at Purdue University, which she has used extensively for data collection in previous studies and which are well suited to the studies proposed.
EQUIPMENT:

**Parrish, Colin R., Cornell University**

**Major Equipment:** Equipment includes 4 laminar-flow biological hoods for cell culture and virus culture, −80°C and −20°C freezers, a Sorvall table-top centrifuge, an ABI StepOne real-time PCR machine, two Bio-Rad thermocyclers, and small equipment for gel electrophoresis, electroporation, sonication, and other procedures. Two Amersham ACTA FPLC systems are used for purifying proteins and viruses. A microscopy suite includes a Nikon Eclipse Ti-E microscope with an Andor Luca-R cooled monochrome EM-CCD camera, while a Nikon Eclipse TE300 microscope has a Hammamatsu OrcaER digital camera and is set up for microinjection and live cell imaging applications. A Millipore-Guava flow cytometer is available for the studies of cells binding capsids or infected with virus, and also for analyzing proteins expressed on yeast. A Cary Eclipse spectrofluorometer with a temperature controlling unit and polarization capabilities are available for analytical studies of proteins and complexes.

**Shared Equipment at Baker Institute:** A Becton-Dickinson FACScalibur flow cytometer (4 channels); and an Auto MACS cell separator; a Zeiss LSM Confocal Unit with an inverted microscope that has lasers with allowing multicolor imaging; an ABI 7500 FAST real-time PCR machine; two Syngene digital gel imaging stations; two Beckman ultracentrifuges and high speed centrifuges with a variety of rotors; a Beckman scintillation counter and a gamma counter; Tecan fluorescence/ absorbance microplate reader; a cuvette spectrophotometer; nanodrop spectrophotometer; phospholimager; autoclaves; ELISA plate washer and reader, and Millipore ultra-pure water.

**Other Cornell University Resources:** Core facilities at Cornell University’s BioResource Center (fee for service): HPLC, mass spectrometry (MALDI and ESI), DNA sequencing and fragment analysis, protein sequencing, and protein expression profiling.

**Hafenstein, Susan L., Pennsylvania State University-Hershey Medical Center**

**Major Equipment:** Within personal lab space of the Hafenstein laboratory are located EMITECH carbon coater/glow discharge unit, humidity and temperature controlled chamber, BL2 level safety cabinet with plunge freezing unit for vitrification of samples, liquid N2 storage dewars, and N2 transport dewars. For data processing the Hafenstein laboratory has four networked Dell Precision T5500 workstations and additional access to computing clusters and image processing labs at Pennsylvania State University Hershey and University Park campuses.

**Shared Equipment/Facilities:** JEOL 1400 TEM is available in the CORE facility for use to screen samples and assess samples by negatively stained specimen preparation.
BIOGRAPHICAL SKETCH
Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2. Follow this format for each person. DO NOT EXCEED FOUR PAGES.

NAME
Parish, Colin R.

E-mail USER NAME
CRP3CORNELL

POSITION TITLE
John M. Olin Professor of Virology

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

<table>
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<th>INSTITUTION AND LOCATION</th>
<th>DEGREE (if applicable)</th>
<th>YEAR(s)</th>
<th>FIELD OF STUDY</th>
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<tr>
<td>Auckland Technical Institute, New Zealand</td>
<td>NZCS</td>
<td>1976</td>
<td>Microbiology/Biochem.</td>
</tr>
<tr>
<td>Massey University, New Zealand</td>
<td>BSc (Hon)</td>
<td>1979</td>
<td>Microbiology</td>
</tr>
<tr>
<td>Cornell University, Ithaca, NY, USA</td>
<td>Ph.D.</td>
<td>1984</td>
<td>Virology</td>
</tr>
<tr>
<td>Monash University, Melbourne, Australia</td>
<td>Post Ph.D.</td>
<td>1986-1988</td>
<td>Arbovirus Research</td>
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A. Personal Statement
In this project we will investigate the connections between the parvovirus capsid structures and their binding to host specific transferrin receptors (TFRs) and to antibodies. I have many years of experience investigating the structures and functions of parvovirus capsids, both during a 20 year collaboration with Michael Rossmann, and more recently along with researchers who did their post-doctoral training with him, Mavis Agbandje-McKenna and Susan Hafenstein. Over that period the studies in my laboratory have focused on understanding the viral and host controls of infection, and in particular the ways in which viruses can transfer and adapt to new hosts. We study two well documented examples of these processes - the feline and canine parvoviruses (being examined here), and the equine and canine influenza viruses. I have written several reviews about parvovirus and influenza viral host range controls, as well as reviews of the more general features of viruses. I have long experience overseeing research of the type proposed here, and in guiding projects as they evolve in different directions. Those include studies of the viral controls of host range, of capsid structures and functions, identification of variant receptors as host barriers, the processes of cell binding, entry and infection, and structural studies of TFR-capsid and antibody-capsid interactions. Many of our studies have involved successful and sustained collaborations with researchers elsewhere at Cornell and in other institutions, as will be required for some projects proposed here. In summary, I am well qualified to undertake the studies proposed, have the materials and skills to carry out the work, and the experience to deal with unforeseen opportunities or problems as they arise during this work.

B. Positions and Honors
Positions and Employment
1973-1975 Technician, Department of Scientific and Industrial Research, Auckland, New Zealand.
1980-1984 Research Assistant, College of Veterinary Medicine, Cornell University, Ithaca, New York.
1984-1986 Assistant Professor, James A. Baker Institute for Animal Health, Cornell University.
1986-1988 Research Officer, Dept. of Microbiology, Monash University, Clayton, Victoria, Australia.

Other Experiences, Federal Review or Advisory Service, and Professional Memberships
Panel member “Experimental Virology,” NIH (1999-2002); Reviewer, intramural programs, NIH (2001, 2002, 2004); Steering Committee Chair, NIH-NIAID workshop on emerging epidemic viruses (2005); ad hoc member of Virology A Study Section (2005, 2007, 2008); ad hoc reviewer, National Science Foundation (2006); ad hoc member, Biological Chemistry and Macromolecular Biophysics Study Section (2006); ad hoc member, NIAID Microbiology and Infectious Diseases B - training grants (2007); National Center for Research Resources
C. Selected Peer-Reviewed Publications (from 125 total publications)

Five recent articles most relevant to current application.


5. xxxxxxx

Additional recent or particularly relevant publications of important to the application (in chronological order)


**D. Research Support**

xxxxxxx
**BIOGRAPHICAL SKETCH**

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2. Follow this format for each person. DO NOT EXCEED FOUR PAGES.

**NAME**
Hakenstein, Susan L.

**POSITION TITLE**
Assistant Professor of Medicine (Infectious Diseases) and Microbiology and Immunology

| eRA COMMONS USER NAME | XXXXXXX |

**EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)**

<table>
<thead>
<tr>
<th>INSTITUTION AND LOCATION</th>
<th>DEGREE (if applicable)</th>
<th>YEAR(s)</th>
<th>FIELD OF STUDY</th>
</tr>
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<tbody>
<tr>
<td>Louisiana State University, Baton Rouge, LA</td>
<td>B.Sc.</td>
<td>1992</td>
<td>Animal Sciences</td>
</tr>
<tr>
<td>University of Arizona, Tucson, AZ</td>
<td>Ph.D.</td>
<td>2003</td>
<td>Pathobiology</td>
</tr>
<tr>
<td>Purdue University, West Lafayette, IN</td>
<td>Postdoc</td>
<td>2003-2007</td>
<td>Structural Virology</td>
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</table>

**A. Personal Statement**

As Consortium PI of this application, Dr. Hakenstein brings a background in molecular biology combined with training in the laboratory of Michael Rossman in cryo-electron microscopy (cryoEM), including single particle reconstruction, and also training in viral and protein X-ray crystallography. Her recent work includes icosahedrally-averaged reconstructions of viruses interacting with receptors or antibody domains, as well as asymmetric reconstructions of the transferrin receptor-capssid complex. The Hakenstein laboratory is actively developing innovative approaches to visualize viruses during biologically significant events that cause changes in structure, or breaks from icosahedral symmetry. A variety of these approaches are required during the studies of the capsid structural variation, binding to receptors and antibodies (including immobilized ligands), and she has the background and training to carry out the work proposed here.

**B. Positions and Honors**

<table>
<thead>
<tr>
<th>Period</th>
<th>Position</th>
<th>Institution</th>
<th>Location</th>
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<tbody>
<tr>
<td>5/97-8/97</td>
<td>Research Technician</td>
<td>Department of Biological Sciences</td>
<td>Univ. of Arkansas, Fayetteville, AR.</td>
</tr>
<tr>
<td>9/97-5/98</td>
<td>Research Associate</td>
<td>Department of Biological Sciences</td>
<td>University of Arkansas, Fayetteville, AR.</td>
</tr>
<tr>
<td>5/98-7/03</td>
<td>Graduate Research Associate</td>
<td>Department of Veterinary Science and Microbiology</td>
<td>University of Arizona, Tucson, AZ.</td>
</tr>
<tr>
<td>1/99-5/99; 1/00-5/00</td>
<td>Microbiology Lab Instructor (Spring semesters only)</td>
<td>University of Arizona, Tucson, AZ.</td>
<td></td>
</tr>
<tr>
<td>7/03-7/07</td>
<td>Postdoctoral Research Associate Fellow</td>
<td>Department of Biological Sciences</td>
<td>Purdue University, West Lafayette, IN.</td>
</tr>
<tr>
<td>8/07-8/09</td>
<td>Assistant Research Scientist</td>
<td>Department of Biological Sciences</td>
<td>Purdue University, West Lafayette, IN.</td>
</tr>
<tr>
<td>8/09-present</td>
<td>Assistant Professor</td>
<td>Pennsylvania State University, Department of Medicine, Division of Infectious Diseases</td>
<td>Hershey, PA.</td>
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**Honors**

2001 Travel grant to attend International Virus and Phage Conference, Helsinki, Finland, to present a talk and act as chairperson of section on DNA virus assembly, June 2001.

2003 Veterinary Science and Microbiology Departmental Outstanding Graduate Student Award for graduate research, University of Arizona, 2003.

2004 National Science Foundation Postdoctoral Research Fellowship in Microbial Biology for 2004 (declined in order to accept NIH fellowship).

2006 Travel grant to attend the International School of Crystallography course on "Structure and Function of Large Molecular Assemblies" in Erice, Sicily, Italy, June 9-18, 2006.

C. Selected Peer-Reviewed Publications

Five recent articles most relevant to the current application.


Additional recent publications of importance to the Field (in chronological order)


# PHS 398 Modular Budget, Periods 1 and 2

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## Budget Period: 1

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### A. Direct Costs

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Cognizant Agency (Agency Name, POC Name and Phone Number)

DHS, Division of Cost Allocation, Robert Aaronson, 212-264-9613

Indirect Cost Rate Agreement Date

07/30/2009

Total Indirect Costs

89,100.00

### C. Total Direct and Indirect Costs (A + B)

Funds Requested ($)

399,710.00

## Budget Period: 2

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### A. Direct Costs

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<td>Total Direct Costs</td>
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### B. Indirect Costs

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Cognizant Agency (Agency Name, POC Name and Phone Number)

DHS, Division of Cost Allocation, Robert Aaronson, 212-264-9613

Indirect Cost Rate Agreement Date

07/30/2009

Total Indirect Costs

75,600.00

### C. Total Direct and Indirect Costs (A + B)

Funds Requested ($)

386,210.00
# PHS 398 Modular Budget, Periods 3 and 4

## Budget Period: 3

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### A. Direct Costs

- **Direct Cost less Consortium F&A**: $250,000.00
- **Consortium F&A**: $60,610.00
- **Total Direct Costs**: $310,610.00

### B. Indirect Costs

#### Indirect Cost Type

1. Modified total direct costs, consortium exclusion $170,610
   - **Indirect Cost Rate (%)**: 54%
   - **Indirect Cost Base ($)**: $140,000.00
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### C. Total Direct and Indirect Costs (A + B)

- **Funds Requested ($)**: $366,210.00

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### A. Direct Costs

- **Direct Cost less Consortium F&A**: $250,000.00
- **Consortium F&A**: $60,610.00
- **Total Direct Costs**: $310,610.00

### B. Indirect Costs

#### Indirect Cost Type

1. Modified total direct costs, consortium exclusion $170,610
   - **Indirect Cost Rate (%)**: 54%
   - **Indirect Cost Base ($)**: $140,000.00
   - **Funds Requested ($)**: $75,000.00

### C. Total Direct and Indirect Costs (A + B)

- **Funds Requested ($)**: $386,210.00

---

**Cognizant Agency (Agency Name, POC Name and Phone Number)**

- **DHHS, Division of Cost Allocation, Robert Aaronson, 212-264-9613**

**Indirect Cost Rate Agreement Date**: 07/30/2009
# PHS 398 Modular Budget, Periods 5 and Cumulative

## Budget Period: 5

**Start Date:** 12/01/2014  
**End Date:** 11/30/2015

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### B. Indirect Costs

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Division of Cost Allocation, Robert Aaronson, 212-264-9613

**Indirect Cost Rate Agreement Date:** 07/30/2009  
**Total Indirect Costs:** 75,600.00

### C. Total Direct and Indirect Costs (A + B)

**Funds Requested:** 386,210.00

## Cumulative Budget Information

1. **Total Costs, Entire Project Period**

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<tr>
<td>Section A, Total Direct Costs for Entire Project Period</td>
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2. **Budget Justifications**

   - **Personnel Justification:** 1243-Justification.pdf  
   - **Consortium Justification:** 1244-ConsortiumJustification.pdf  
   - **Additional Narrative Justification:** 1244-AdditionalNarrative.pdf  

   [Attachments: Add Attachment, Delete Attachment, View Attachment]
BUDGET JUSTIFICATION:

Personnel: Cornell University

Principal Investigator: Colin R. Parrish (1.8 calendar months), John M. Olin Professor of Virology. He will oversee all of the work in this project, and will directly supervise that which is conducted at Cornell. He will be responsible for the administration of the program and any necessary reporting, and will ensure that the various combined projects are carried out between the workers at Cornell and Penn State, as well as with the laboratories of the collaborators/consultants on this project.

Postdoctoral Fellow: [Redacted] (6.0 calendar months) will be involved in studies of the protease sensitivity of the capsids and receptors as well as carrying out the studies in the laboratory using yeast expression and mutagenesis to identify the residues in the antibodies and receptors that interact with the capsid surface, and to alter the affinities.

Postdoctoral Associate: TBH (7.2 calendar months) will be responsible for the studies that involve the analysis of the capsid structures through mutagenesis, and the biochemical and biophysical analysis of the resulting viruses. This person would also prepare materials for analysis of the receptor and antibody binding to the wild type or mutant capsids that are uncleaved, or treated with proteases.

Research Support Specialist: [Redacted] (3.6 calendar months) supports the various projects in the laboratory, and provides general support for this project including preparation of the tissue culture cells, antibodies, and general laboratory maintenance. She is an expert in the production of monoclonal antibodies from hybridomas, and would carry out those parts of the project.

Research Technician III: [Redacted] (6.0 calendar months) is responsible for the routine production and purification of virus capsids, antibody IgGs and Fabs, and receptor proteins from baculovirus expression. She also spends some time on the general laboratory work that supports this project.

Consultants / Collaborators:

Collaborator: Moonsoo Jin, Cornell University. Dr. Jin is an Assistant Professor in the Department of Biomedical Engineering. He has a background in studies of protein engineering starting from his post-doctoral work in Tim Springer's laboratory at Harvard University, and in particular in the use of yeast display for isolation of proteins with altered binding properties. Studies in his laboratory include preparation of receptors for other viruses (rhinovirus and adenovirus-associated viruses) with this technology, and he is also examining the mouse TfR. This is a natural collaboration, and in the studies proposed, the work involving the selection of altered TfRs and antibodies would be conducted in the Parrish laboratory by Laura Goodman, but would involve the close assistance of Dr. Jin and members of his laboratory. A letter of collaboration is included.
CONSORTIUM BUDGET JUSTIFICATION:

The Pennsylvania State University – Hershey Medical Center, Dr. Susan Hafenstein

Consortium Costs:

Approximately $853,000 Total Costs for all years. ($550,000 direct costs; $303,000 F&A costs)
Consortium with Pennsylvania State University – Hershey Medical Center (X) Domestic ( ) Foreign
Calculations are based on 55.1% F&A rate.

The budget is administered through Cornell University, but the Pennsylvania State University – Hershey
Medical Center portion supports those individuals at Penn State-Hershey who are involved in these parvovirus
projects, as well as the costs of those studies.

Consortium Personnel:

Principal Investigator: Susan L. Hafenstein (3.0 calendar months). As PI of the project, Dr. Hafenstein will
supervise the aspects of the project that involve structural analysis of the capsids and antibodies or receptors.
She has experience with studies using cryoEM of capsids and receptors or antibodies. She will be making
affinity grids, setting up properly controlled experiments, data processing, interpretation of results, and
assistance in writing up manuscripts and progress reports.

Postdoctoral Research Associate: [REDACTED] (9.0 calendar months). [REDACTED] will carry out the cryoEM
reconstruction of viruses and receptors. The project will include making grids, performing experiments,
processing the data and reporting the progress on the asymmetric reconstruction of parvovirus capsids
interacting with...
**Capsid structures, variation and flexibility.** This project brings together the skills of laboratories at Cornell University and at Pennsylvania State University Medical Center to provide a detailed understanding of the roles of structural variation and flexibility in the parvoviral capsid, and their effects on receptor and antibody binding and the controls of cell infection and host range. These are fundamental problems that apply to all animal viruses, where the capsid must protect the genome in the environment, interact with host molecules including cell receptors and antibodies, and undergo a series of regulated structural transitions during cell entry to eventually release the genome for replication. Viral capsid binding to host receptors and antibodies can have varying and often unpredictable effects on infection, and those interactions also control many other replication steps. Where these virus-host interactions are specific they can control the viral host ranges.

**A model for understanding virus cell infection and host range control through differential receptor binding.** We study two viruses that differ in host range due to 3 or 4 capsid protein mutations that control specific receptor binding. Canine parvovirus (CPV) arose around 1976 as a variant of feline panleukopenia virus (FPV), and caused a pandemic of disease during 1978 and 1979. That virus has continued to circulate worldwide as a serious canine pathogen, and has also evolved new antigenic, receptor binding, and host range variants. FPV and CPV both can bind the feline transferrin receptor 1 (TfR) to infect cat cells, and CPV gained the host range for dogs by gaining the ability to bind the canine TfR. This new binding property was associated with increased flexibility of 2 or 3 of the surface loops in the capsid that allowed CPV to accommodate a glycan on the canine TfR binding domain. Flexibility in the capsid is controlled by variation in hydrogen bonds, by cleavages of the VP2, and by ion binding. Later steps in infection also involve changes in the capsid structure that release the viral DNA or protein domains of VP2 and VP1. These viruses are targeted by antibodies that can differ in their binding sites and in their ability to neutralize the virus. We will examine a set of antibodies that detect specific capsid structures, examining the effects of antibodies on TfR binding, and seeking to understand the mechanisms of neutralization.

**Aim 1. To define the structural variation in parvovirus capsids, and to determine the effects on capsid functions and DNA release.** *Hypothesis:* That the capsids of paroviruses undergo structural variation that is important for infection. That occurs through the binding or release of divalent ions, by site-specific proteolysis, or by variation in specific intra- or inter-chain bonds.

a) Further define the structural flexibility in the capsid through analysis of the structures and to identify sources of variation using specific peptide and protease analysis.

b) Determine the functions of specific capsid structures by preparing mutants with altered inter-chain bonds, divalent ion binding sites, or protease cleavage sites.

c) Compare the functions of capsid structures in mutant or naturally variant viruses to reveal the structures and interactions that are critical for capsid stability, TfR binding, and the processes of cell infection.

**Aim 2. To define the structural interactions between various parvovirus capsids and variants of the transferrin receptor or artificial receptors.** *Hypothesis:* That specific binding of capsids to the feline or canine TfRs is required for successful cell infection, and those interactions are controlled by viral structures varying in structure and flexibility.

a) Determine the interactions of the feline and canine TfRs with different parvovirus capsids, examining cryoEM structures of receptor-capsid complexes at moderately high resolution. By correlating residues on the capsid and TfR that affect binding, identify the interacting structures.

b) Identify functional sites on the capsids by selecting for mutants of CPV or FPV by growth on TfRs with mutant binding domains, or on receptors with artificial binding ligands.

c) Prepare capsids with insertions that bind alternative cell receptors, and test for cell infection.

d) Examine how flexibility of capsid loops controls interactions with different host TfR - in particular receptors with additional glycans within the attachment face of the receptor.

**Aim 3. Use antibodies to probe the capsid structure, and also to determine how binding to overlapping sites leads to variable neutralization.** *Hypothesis:* That antibodies can be used to detect variant structures in the viral capsid, and that the specific position and orientation of binding controls the likelihood of competition with the TfR, and neutralization of infection.

a) Examine antibodies with known capsid binding sites for their effects on TfR binding, including the effects of cleavage with proteinases or after other asymmetrically or symmetrically displayed modifications.

b) Determine the effects on viral functions of antibody variants engineered with increased binding affinities. Identify sites on the virus that do not bind antibodies but that bind TfR, for example those subunits with cleavages in surface loops.
A) SIGNIFICANCE.

A1) Importance: understanding fundamental aspects of virus infection processes controlled by receptor or antibody binding, and the controls of viral host ranges. The paroviruses include many different human and animal pathogens, including the long-known B19 virus which causes the childhood fifth disease and more severe diseases of adults, as well as the recently identified human bocavirus and Parv4. The adeno-associated viruses (AAVs) are paroviruses that are not associated with disease, but are being developed as human gene therapy vectors and the same issues of receptor and antibody recognition are important for vector optimization. The viruses we are studying in this model are the canine parvovirus (CPV) and its close relative feline panleukopenia virus (FPV), which bind to the host transferrin receptor type-1 (TfR) to infect cells (53). The paroviruses have a 25 nm diameter T=1 capsid that is assembled from 60 copies of two or three versions of a single capsid protein, and the single stranded DNA of the virus is packaged into the pre-formed capsid by the action of the larger non-structural protein (NS1). Although those capsids are remarkably robust and survive in the environment, structural variation results in viruses with different properties, and those also show structural changes during the process of cell entry and nuclear trafficking. The simple and well defined structures of the parovirus capsids, the known properties of the TfR, and the well characterized antibodies available for these studies allow us to examine several processes of viral infection. Variant viruses with extended host ranges can cause new outbreaks or epidemics of infectious disease. The viruses that we are examining include the comparison of such a system, where one variant arose as a pandemic pathogen in a new host through the acquisition of mutations in the capsid protein that altered its structure to change host-specific receptor binding, and also to change its antigenic structure.

A2) Critical barriers: to antiviral therapy and vaccination success. Animal viruses are complex biological machines that engage host cell receptors and undergo a series of varying structural and functional changes to allow cell penetration and release of the genome for replication. Those infection processes are key to the success of any virus, and are targets of various anti-viral drugs. A better understanding of the details of the general processes involved will likely allow the development of more effective and broadly acting antiviral drugs. Although antibodies are critical components of immune responses of all vertebrates, in many cases they are poorly effective so that viruses maintain persistent infections or vaccines do not work well. Understanding the underlying rules that determine how antibodies bind to viruses and block the processes of cell infection will reveal how effective antibody responses might be elicited against different viruses.

A3) Improvement of scientific knowledge: understanding fundamental viral mechanisms and clarifying textbook knowledge of virus structures and functions. This project addresses several mechanisms important for all viruses of humans and other animals. In general terms those include understanding viral recognition of cell receptors, how changes in receptor binding sites lead to alterations of binding and host range, capsid and receptor structures and the interactions that control uptake and trafficking within cells. During each of these steps the viral proteins must assume the correct conformations, bind receptors with the correct contacts, and in the process undergo a variety of structural transitions to release internal peptides, protein domains, and the viral DNA. Here we will investigate the roles of flexible and variable structures in the parovirus capsid and show how receptor and antibody binding control cell infection.

B) INNOVATION.

This is an unusually complete model for understanding virus structures and functions involved in cell infection and host range control, as there are few other viruses where the ancestors and descendents of a host-switching virus that caused a pandemic of disease in its new host are available for analysis. The laboratories presenting this proposal are the only ones working with this model in any detail, but we have been able to explain many aspects of the process, from the evolutionary processes allowing emergence, to the identification of specific receptor binding as a key step that lead to the extended host range of the virus [reviewed (52)]. There are several fundamental issues being investigated in these studies, including being able to obtain a detailed understanding of the variation and dynamic properties of viral proteins, how those structural changes control receptor attachment and cell infection, and that analysis is complemented by studies of antibody binding and its effects on receptor binding and infection. By analysis of naturally variant or mutant viruses, receptors, and antibodies we will gain a better understanding of the functional properties of the capsid-ligand structures and alterations in binding properties. We use a variety of advanced methods and structural, biophysical, biochemical and functional assays in these studies. While many of the methods we propose are being used in viral studies (including by our laboratory), the proposed studies include combinations of those methods that are being used in novel ways to gain a complete understanding of the mechanisms involved.
C) APPROACH.
The three related and overlapping specific aims are directed at understanding: (1) the roles of structural variation and flexibility in controlling capsid functions; (2) the interactions of viral capsid structures with different receptors, and the mechanisms that lead to infection; and (3) the antibody binding to capsids and resulting competition with receptors and mechanisms of neutralization

C1) Aim 1. To define the structural variation in parvovirus capsids, and to determine the effects on capsid functions and DNA release. Here we seek to further define the structural variation of the capsid, including sources of asymmetry. In these studies (and also in those of Aims 2 and 3) we also define the effects of those changes on the functions of the virus, in particular its interactions with receptors or antibodies.

C1a) Background – brief summary of literature and preliminary results.

C1a1) Functional variation and flexibility of parvoviral protein structures. The structures of parvovirus capsids vary between different viruses, and in our previous work we have shown that small changes control significant variation in the viral properties including receptor binding, host range, and antigenicity [some recent examples are: (19, 21, 22, 24, 26, 31, 45, 46, 49)]. Among the functional flexible or variable structures seen are loops with varying numbers of intra-chain and inter-chain bonds, loops which bind or release 2 or 3 divalent ions, the possible formation or disruption of disulfide bonds, protease cleavage of a proportion of the proteins, or opening and closing of cylindrical pores at the fivefold axes which allow VP2 N-termini and the viral DNA 5'-termini to pass to the outside of the capsid (14, 15).

In the studies proposed, we are using information derived from many different parvoviruses and adenovirus infection studies, and while space does not allow a review of that literature, those include studies of structural variability and flexibility in the capsids that occur under various conditions, including at low pH, ion removal, after host cell, receptor, or antibody selection, ubiquitin modification, or after binding to sphingolipids or receptors [a small number of the relevant references are: (13, 16, 30, 34, 36, 38, 41, 47, 48, 56, 61, 65, 66)].

C1a2) The roles of structural variability and flexibility in viral proteins. Functional variation in structure is a key property of many virion proteins, with well recognized triggers for conformational change including low pH, ion binding or release, receptor binding, or protease cleavage, although other more subtle and less well understood changes are also likely to be common. The changes resulting often allow membrane penetration and/or fusion, or the complete or partial disassembly of the virion to expose internal components and to allow viral trafficking and genome release for replication. As examples, structural changes in the gp120 of HIV are induced upon CD4 binding, allowing binding of CCR5 or CXCR4 co-receptors that mediate infection (58). The binding of receptors to the picornavirus capsid can result in structural changes (7, 55), and antibodies binding to the flavivirus envelope protein can trap intermediate conformational forms of the protein (29, 39). In addition, variation in viral protein structures can result in antibodies binding to forms that differ from those seen by the receptors, allowing evasion of at least part of the antibody response [e.g. (10)]. Submolar cleavages, asymmetric structures such as portals, or incorporation of minor proteins into virions may be common in viral proteins but are often hard to define in detail and therefore not well understood. Here we seek to define the functional connections between the variation in the parvovirus structure, including submolar variation, and its functional significance for virus receptor and antibody binding and the infectious cycle.

C1a3) Asymmetry of functional sites in viral structures. Some of the flexible structures in parvoviral capsids are asymmetric, being present in only a small proportion of the capsid subunits. Examples include those resulting from cleavage of surface or internal loops of a small number of the VP1 and VP2, the inclusion of 5-6 VP1 molecules per capsid, or the packaging of one ssDNA molecule with its 5'-end protruding from the capsid (13, 15). These types of asymmetrically variant structures are likely present in the capsids of many different types of viruses, but can be difficult to detect without specific probes, and would not be seen after X-ray or cryo-EM analysis of viral capsids that involve symmetry averaging for the reconstructions.

The VP2 within the capsid can be cleaved at several positions. The cleavage of VP2 to VP3 occurs only in full capsids, and occurs between residues 15 and 24 depending on the specificity of the proteinase, and must involve exchange of cleaved and uncleaved N-termini through the fivefold axis pores of the capsid (15, 45). That VP2 to VP3 digestion is temperature dependent, most likely because the pores (and perhaps other parts of the capsid) have to expand to allow exchange of N-termini (15, 45). Divalent ions bound within the capsid are associated with changes in the flexibility of several surface loops, including those involved in binding to sialic acids (60).
Other asymmetric structures derive from the cleavages of small proportions (5-20%) of the proteins within the capsid. Some cleavages that would affect both antibody and TIR binding occur within surface loop 3, and those differ in susceptibility in different viruses due to differences of VP2 residues 297 and 300 and by other residues (45). Only 5-20% of the proteins become cleaved even after extensive digestion, suggesting there is pre-existing variability in the structure of that loop, or that a structural change occurs in the capsid after cleavage of some sites which prevents cleavage of further sites. Those cleavages are within the binding sites of the TIR and of many antibodies that bind the “B” antigenic site in the capsids (21), and those would show different binding properties to both ligands compared to the uncleaved structures. Another cleavage occurs at Lys 271 on the inside of the capsid, between two unbonded cysteines (Cys270 and 274), and in the vicinity of a small di-S bonded loop between Cys490 and 494. Although of unknown function, that cleavage is beneath the threefold region of the capsid and its greatest effect would likely be to alter the stability of the capsid or the DNA packaging.

C1b) Studies of capsid structures, and the role of capsid protein flexibility.

C1b1) Mutations that affect capsid flexibility. We have seen that capsid substitutions that affect important functions often cause only small changes in the viral structures determined by X-ray crystallography (2, 20, 40, 60, 64). However, those were often associated with changes in protein flexibility — of all 60 sites in the capsid, or within a subset of the capsid subunits. For example, substitutions within 3 of the 4 surface loops affect both TIR and antibody binding - in loop 1 residue Lys93 cross links to another loop in the VP2 and results in stabilization, while Asp93 or Asn93 do not; in loop 3 residues Gly299, Ala300 or Gly300 do not form H-bonds to other structures, while Glu299 or Asp300 would H-bond with Arg81; Asp323 forms a bond with Arg377, while Asn323 does not. Those viruses with single changes can be used in our studies of receptor and antibody binding, in addition to other variants that affect the structure (see below).

C1b2) Role of divalent ions controlling the parovirus capsid structure. Many different viruses bind ions within their structures, and in some cases those control “switches” between different structures. CPV and FPV capsids bind and release two or three divalent ions within each capsid subunit (giving 120 to 180 ions per capsid), and we have determined the crystal structures of capsids with varying numbers of ions bound, and showed that those stabilize two loops in the capsid (60). This may be a common mechanism in paroviruses, as in MVM it has recently been shown that ion binding in the capsid affects DNA packaging and release (13). We can remove or prevent ion binding by chelation, incubation at low pH, or by mutation of the residues involved in coordinating the ions. The 3 Ca^{2+} per capsid subunit in CPV (and the 2 Ca^{2+} in FPV) are coordinated by groups of surrounding Asp and His residues (Fig. 1). To test for effects of the ion binding on the viral functions, we would modify individual Asp or His residues to Asn or Ala, in order to change the charge and prevent the coordination of one or more of the ions. Those changes would be made in infectious clones of CPV or FPV, and the viruses tested for viability, as well as for structure and functions as described below.

C1b3) Examining capsids for submolar variation – protease cleavages and exposed structures.

C1b3a) Role of VP2 to VP3 cleavages in viral capsid structure and functions. This cleavage occurs only in full capsids, and in CPV and similar paroviruses involves removal of 15 to 24 residues (45). The N-termini of a proportion of the VP2 must fill the channels at the fivefold axes of symmetry, and that may prevent initiation of packaging the viral DNA at more than 1 site. VP2 N-terminal sequences also influence nuclear export of the full particles after production (42). Cleavage of the VP2 to VP3, along with Ca^{2+} removal, would favor DNA release (13). We would examine the effects of the N-terminal sequences on the structure and permeability of the capsids by digesting the capsids with trypsin (which cuts after residue 19) or with subtilisin (which cuts after residue 24) at 37°C, converting ~80% of the VP2 to VP3. After adding proteinase inhibitors the capsids would be isolated by gel chromatography and examined for changes in the particle stability by spectrofluorometry.
tested for altered DNA exposure properties, and for their ability to bind the TfR and infect cells, as detailed below.

**C1b3b) Antibody detection of the VP1 N-terminus, or of variable and internal capsid peptides.** We have antibodies that recognize capsid sequences that are normally buried but that become exposed under different conditions. Those include MAb against the VP1 N-terminal sequences, anti-peptide antibodies against sequences on the inside of the capsid (VP2 residues 258-270), against peptides surrounding the fivefold pore, and MABs that recognize the variable loop between residues 362-373 in the capsid, the structure of which is in part controlled by bound Ca$^{2+}$ (60, 70, 74). Those antibodies would be used to directly detect those peptides in capsids in ELISA, where the amount of binding would be proportional to the amount of peptide exposure. Another assay would use the antibodies to immuno precipitate the capsid - where the amount of VP2 recovered would represent the proportion of particles that had the specific peptide exposed. The latter assay would be particularly sensitive for peptides or structures that are present at low numbers or transiently revealed on the capsids.

**C1b3c) Cleavage of residues in surface loops - a control of variable TfR and antibody binding?** The role of surface cleavages would be examined using several approaches: comparing cleaved and uncleaved capsids, altering the known cleavage sites, or by preparing new cleaved forms of the capsids by introducing additional digestion sites. Cleavages in loop 3 are controlled by VP2 residues 297 and/or 300; when residue 297 is an Ala the protein is not cleaved, while the Ser form is cut in 10-20% of the capsid proteins (45). Residue 300 also controls cleavages, as the Ala form is cleaved, while the Asp or Gly forms are not (Fig. 2). CPV mutants with Asp300 or Glu299 also do not bind the canine TfR and are therefore altered in canine host range, and also differ in antigenic structure. From the atomic structure of the Asp300 mutant, it is seen that the charged side chain forms a H-bond with Arg87 to stabilize that loop (and Glu299 also likely forms a similar bond), and that also likely prevents virus interaction with the canine TfR (27, 40).

We would therefore prepare mutants of several positions within loop 3 to increase or reduce the flexibility and cleavages of that structure. Those would include changes of residues 297, 299, 300, and 301 which prevent or allow cleavage, or change inter-chain bonds, depending on the specific residue introduced. To increase cleavage susceptibility over the wt capsid level, we would also introduce basic sequences that would be susceptible to trypsin or to furins on the cell surface. Mutants would be tested for viability, for canine and canine TfR binding, and for antibody binding and neutralization with Fabs with different specificities.

**C1b3d) The cleavage of VP2 residue Lys271.** This cleavage occurs in ~10% of the VP2 at Lys271 on the inner face of the capsid, and may be due to an intercellular protease or possibly to a previously unrecognized autocatalysis. After cleavage the new N and C termini would likely separate and change the capsid structure in the vicinity of the packaged DNA, and the Cys270 and Cys274 may be able to form a new disulfide bond. To investigate any functions associated with that cleavage, we will change residue 271 from Lys to Ala (or Arg), and would test the mutant virus for the formation of the cleaved protein and for any changes in the viral relative infectivity and particle stability.

**C1b3e) Testing the functions of mutant capsids.** Capsids with different proportions of surface loop cleavages or Ca$^{2+}$ bound would be tested for affinity and levels of binding to purified TfR ectodomains, to TfRs expressed on cells, and for binding to antibodies with different specificities, using standard methods used in our laboratory (19, 21, 45, 46, 49). Capsids would be examined for the numbers of TfRs bound in biochemical assays of the complexes, or by using negative staining EM to estimate the number of TfR bound, which is possible due to the large size of the TfR relative to the capsid (22, 49). The binding of Mab-derived Fabs that we know attach over the positions that are cleaved would be examined and the relationship to TfR binding would be examined as described below (Section C2b1 and C3b).
C1b3f) DNA exposure in full particles. For full particles the exposure or release of DNA occurs during infection. To test for changes in the DNA exposure, wild type or mutant capsids would be examined, and capsids bound to wild type or mutant TIRs, or treated with proteases or with Ca\(^{2+}\) removed. The 3' end of the viral DNA can be exposed without particle disintegration, and we would detect that release by using it as a template for synthesis with the T7 DNA polymerase. Synthesis initiates on the 3' DNA hairpin, so that DNA synthesis is proportional to the amount of the 3' hairpin exposed (13, 74). The capsids would be incubated with T7 polymerase and dNTPs, and the amount of product determined by incorporation of \(^{32}\text{P}\)- or Cy5.5-labeled nucleotides which would indicate the degree of 3'-end exposure.

The 5'-end of the DNA is exposed on the outside of the full capsid, with the NS1 protein attached to that DNA in newly-made particles. We would detect the NS1-associated with the capsid using the gold labeled Fab of the CE10 antibody against NS1 protein, and examine in EM. We can also detect the 5' end of the DNA directly using an hnRNP A/B protein that recognizes a sequence close to the 5'-end of the viral DNA that is exposed on the outside of the capsid (68). That protein would be purified and gold labeled, then used to detect the DNA on the outside of the capsid. The protein binds close to the surface of the capsid, and should be tightly localized adjacent to the capsid. After initially screening the binding by negative staining, cryoEM analysis should allow the position of DNA exposure to be seen, most likely at or adjacent to a fivefold axis of the capsid.

C1b4) Dynamic properties of digested or mutant capsids. For the cleavage site variants and Ca\(^{2+}\) binding mutants, protease treatments and probing with a large panel of specific Mab will be used to examine for changes in the structure. Capsid stability can be measured by resistance to heating, either testing for effects on infectivity, or by changes in Trp fluorescence using spectrofluorometry, as we have described previously (45). The level of cleavage would be determined by SDS-PAGE analysis after protease treatments. To confirm the sites of cleavage seen, we would use micro-N-terminal sequencing or size analysis by mass spectrometry. We would particularly focus on cleavages generated during limited digestions, which would show the structures within the capsid that are changing in conformation.

C1b5) Receptor binding properties of the mutant or digested capsids. We would determine the binding affinity of wildtype or variant capsids to the cell receptors (19, 49). Plasmid expression efficiently displays the receptor on the surface of TIR-negative TRVb cells, and we can also readily purify TIR ectodomains after expression in insect cells from baculoviruses (49). The purified TIRs bind the capsids in vitro with similar relative affinities to those seen for the same receptors expressed on cells (49). To measure the affinity of virus binding to the wildtype TIR or mutants we would also use flow cytometry to measure binding and release of capsids to the receptors expressed on cells. In an alternative solid-phase binding assay, the His-tagged TIR ectodomain would be immobilized on lipid bilayers with Ni\(^{2+}\)-NTA, and fluorescently labeled capsids flowed over the receptor layer. Labeled viruses would be followed by fluorescent microscopy using TIR, and the on- and off-rates determined directly, allowing the affinity of binding to be determined directly (9). Additional studies are described in Section C2, below.

C1b6) Changes in capsid permeability. Changes in capsid permeability are associated with several structural changes in the virus, including cleavage of VP2 to VP3 in full capsids, the release of the VP1 N-termini from inside the capsid, and the release of the 3'-end of the viral DNA (see above). Some or all of these releases occur through pores at the 5-fold axes of symmetry. Capsid permeability can therefore be increased by mutations in the loops surrounding the pores at the fivefold axes of symmetry. We already have mutants of residues involved in the stabilizing the pore structure ("gatekeepers") including Asn187Ala, Asp168Ala and Thr170Ala, and those capsids would be examined for their permeability and the degree of exposure of the VP2 and VP1 N-termini or DNA under mild heating or other conditions. The permeability of empty capsids can be semi-quantitatively monitored using negative staining EM at neutral pH. Stains with differing properties include methylamine tungstate (NanoW), ammonium molybdate, uranyl acetate, Ni\(\text{SO}_4\), and sodium tungstate. NanoW does not enter intact empty particles at neutral pH, but can enter after different treatments, while Ni\(\text{SO}_4\) readily enters empty particles under most conditions (45) (Fig. 3). We would therefore examine the various mutant capsids under controlled conditions including heating, protease digestion, and receptor or antibody binding. For empty particles we would
particularly examine the penetration of NanoW, carefully examining the capsids by electron microscope and the proportion of penetrated particles determined.

**C1c) Outcomes, potential problems and difficulties, and alternative approaches.**

The goals of this first section are the characterization of any important variation, flexibility, and asymmetry in the parvovirus capsid structures, and effects on capsid permeability and the exposure of internal components important for infection. Structural variation clearly occurs in the capsid, and many of the variants or mutants we will be examining are already known to show differences in flexibility and/or protease cleavage susceptibility. The effects of structural changes on the capsid functions are harder to predict, but functions known to be altered include the affinity of receptor binding (in particular the host-specific binding to the glycosylated canine TfR), as well as sialic acid binding, antibody binding, and exposure of various peptides and of the viral DNA. Receptor binding will also be examined in detail in the experiments in Section C2, and the relationships between antibody and TfR binding will be examined in Section C3.

There are some potential challenges in these analyses of the capsids. First, we cannot predict the functional effects of some of the structural changes, and some effects may be relatively subtle. It is clear that the capsids act as finely regulated machines and perform their functions through controlled transitions, and do not (for example) simply fall apart, even to release the DNA, but likely expose internal structure or the DNA 3'-end only under controlled circumstances during infection. Asymmetry can be difficult to detect and quantify, but in this system we should be able to detect that using a variety of biochemical or other approaches. We know some of the capsid changes that control protease cleavages and can use that information to predict alternative sites, and have enough preliminary information to be able to prepare informative mutants for our functional analyses.

The divalent ion binding sites are well defined from the X-ray structures, as are natural mutations are known that prevent one ion binding and also increase the flexibility of the adjacent loop that controls sialic acid binding (60). Altering the binding sites of the other two ions is quite straightforward, although we do not know if capsids with mutations affecting the binding of those ions will be viable. However, we can also control ion binding by chelation with EDTA or EGTA, or by incubation at low pH, so there are several alternative approaches available. If mutant viruses are non-viable, we can test many functions by expression of capsids by plasmid transfection or by expression from baculoviruses (58).

**C2) Aim 2. To define the structural interactions between various parvovirus capsids and variants of the transferrin receptor or artificial receptors.** Hypothesis: That specific binding of capsids to the feline or canine TfRs is required for successful cell infection, and those interactions are controlled by viral structures varying in structure and flexibility.

**C2a) Background on receptor and capsid structures involved in binding.**

The FPV and CPV capsids bind the TfR on the surface of their host cells, and it appears that specific TfR-capsid interactions are required for infection, as other ligands that bind the virus at the cell surface and mediate uptake do not result in infection (26). The TfR is large butterfly shaped homodimer (11nm span), where each monomer is comprised of protease-like, apical and helical domains (Fig. 4). While FPV and CPV both bind the feline TfR, only CPV binds the canine TfR, an interaction that was critical for the host range shift of CPV (53). We have prepared a low resolution structure of the feline TfR:capsid structure determined by cryoEM which shows the footprint of the feline TfR on the capsids (22), and have also defined sites on the receptor that control interactions with the virus (19, 49, 50). The canine TfR has a distinct interaction with the CPV capsid compared to the feline TfR, as many viral changes that alter canine TfR binding do not change feline receptor binding (25, 51). Mutational studies show that the canopy that bears the furthest loops of the receptor domains is central to the interaction (25).

**Fig. 4.** The TfR structure, based on a model of the human receptor (domains are colored on one monomer – protease-like = red, apical = green, helical = yellow). Residues tested for effects on the binding of CPV are colored: no clear effect on virus binding dark blue, alteration of virus binding red or purple. Known Asn-linked glycosylation sites labeled in orange. A portion of the stalk domain is colored in cyan (left), and transferrin binding sites are brown (right). B) The position of residue Leu 221 in the apical domain.
of the feline and canine TFRs have defined receptor residues that control virus binding (19, 49, 50), and those show that one site is particularly critical for the interaction. When that site was changed to the other amino acids, a series of receptors with single changes were obtained that varied in affinity of binding, from no binding, low binding, to close to wild type (19). Those mutant TFRs are all available for the proposed studies.

These studies will provide fundamental information about the processes of receptor-virus interactions. In the case of TFR, the rodent TFR-1 is used for cell binding and infection by New World arenaviruses and by mouse mammary tumor virus, and those viruses also bind to the apical domain of the receptor, in some cases interacting with the same residues in the structure as CPV (1, 69). While the normal function of the apical domain is unknown, we have examined TFR sequences from many different large and small cats which likely have been infected by FPV for very long periods, and have identified a region in the apical domain with substitutions of residues 378 (Gln-Arg), 379 (Asn-Ile), and 380 (Trp-Ser) within the virus binding region, that is clearly under selection, perhaps by the virus. Those changes will therefore also be tested in our future studies to see whether they affect virus binding and infection.

C2b) How does capsid bind the TFR, and is that specific interaction important for infection of cells? We will further define the TFR-capsid interactions by analysis of the receptor and viral structures using a variety of approaches to alter the viral and TFR structures involved in the interaction, to explain how the functional contacts are controlled, and the effects on binding or infection of the structural variants being examined. In addition to studying specific mutants we will also prepare higher resolution cryoEM structures of various TFR-capsid complexes. The overall goals are to create a complete dynamic structural model of the interaction that explains the functions in binding and infection.

C2b1) Defining the functional capsid-TFR interactions. We have identified several residues in the receptor and capsid that control binding, and have determined the 27Å resolution asymmetric structure of the dimeric feline TFR ectodomain interacting with the CPV capsid (22)(Fig. 5). Here we will define the interactions between specific residues on the receptor and capsid in more detail, and will complement the mutational studies with higher resolution cryoEM structures of the complexes, so as to achieve a complete understanding of the interactions and their control of various viral functions.

C2b1a) Viral mutants to examine. Some cleavage site and flexibility mutants will be derived from Aim 1 by mutation of sites in the capsid structure. Other viruses will have altered affinities of TFR binding or TFR contacts. The CPV-2a natural variant is altered at VP2 residues 87, 101, 300 and 305 and shows reduced affinity for the feline TFR, but retains high infectivity (49). We have prepared intermediate mutants with single or double changes in the capsid (e.g. of VP2 residues Ile101Thr and Ala300Gly) that alter the affinity of receptor binding. VP2 residues 93 and 323 together control binding to the canine TFR but not the feline receptor (20).

C2b1b) Selection for viruses with altered binding properties by passaging on mutant receptors. To identify the capsid residues that interact with specific sites on the TFR, we will isolate mutant viruses by growth in CHO cells that are expressing TFR variants which show reduced virus binding and infection due to point mutations in the apical domain. We have prepared cell clones that express the altered affinity variants of the feline TFR (19). Those will be inoculated with CPV or FPV and then the virus passaged repeatedly to isolate variants that are better adapted. Once isolated those viruses will be sequenced to identify the candidate contacts between the receptor and virus that are selected for increased infection.

C2b1c) TFR variants with increased binding prepared by in vitro evolution and selection. To alter TFR binding we would use yeast surface display and selection to prepare receptor variants with altered capsid-binding properties, including those with higher affinities. Clones of the feline TFR ectodomain and of the apical domain alone have been prepared and those are displayed on the surface of yeast as fusions with the Aga2p mating agglutinin protein (4, 12). To select for altered binding variants of the protein, TFR sequences around the apical domain would be subjected to error-prone PCR, and then the mutant products transformed into yeast along with the expression plasmid to give incorporation by the homologous recombination (4). The mutant TFR yeast library would be selected by capsid binding, and fluorescence-based sorting used to enrich for yeast.
expressing receptors binding higher levels of virus compared to an HA epitope included in the expression vector - yeast showing higher virus binding relative to the levels of protein expression would be candidates for those with increased affinity. A BD-Biosciences FACSAria high speed flow cytometer/cell sorter at Cornell is available to the Parrish laboratory, which can sort up to 50,000 events per second, allowing us to enrich for even rare mutants with increased binding. Repeated selection and additional rounds of mutagenesis would be used if necessary to isolate the higher binding versions of the receptor. Tfr variants with altered binding would be re-introduced into the Tfr gene in an expression plasmid, transfected into TRVb cells, and then tested for binding affinity and for the ability to mediate infection. The relative binding and infectivity of each of the three major strains of virus would be compared to those seen for the wildtype Tfr, or to the lower affinity versions of the Tfr already produced in other studies (19, 50).

C2c) Use cryoEM and mutant mapping to obtain better structural models of the CPV-feline Tfr and canine Tfr interactions.

To directly visualize the Tfr-capsid interaction we would prepare higher resolution cryoEM reconstructions of Tfr-capsid complexes, by including more complexes and by using approaches that allow orientation of the receptor in the complex. We would examine complexes of the feline and canine Tfr ectodomains, as well as a canine Tfr mutant that has a single point mutation that removes a glycosylation site, which binds with higher affinity and which will likely still show the binding of the canine Tfr. Traditional cryoEM approaches can be difficult due to the dimeric and the aggregation-prone properties of the Tfr ectodomain, and we would avoid these problems using a number of approaches. Data would be collected at Purdue University, where Dr. Hafenstein worked for several years until 2009, so that she is familiar with the microscopes and systems required for these studies. A letter of support is included from Dr. Paul Chipman, who manages the EM facility at Purdue, indicating that Dr. Hafenstein would have access to the appropriate equipment for these studies.

In one approach we would purify the capsid-Tfr complex in a Sepasore CL2B column to isolate monomeric complexes (22), and after concentrating those would be frozen and examined by cryoEM. An EM defocus level of 4-6 microns is necessary for high resolution reconstructions, but at those defocus levels there can be insufficient contrast to easily determine the position of the Tfr bound to the capsid. We would therefore seek to increase the Tfr density by saturating the Tfr with iron-loaded transferrin, which would both double the mass of the Tfr and add Fe3+ ions to the complex, allowing better visualization.

In a second approach we would prepare liposomes containing up to 10% (w/w) DOGS-NTA which would be nickel loaded, incubated with soluble 6-His Tfr ectodomain, and then with purified virus, and frozen for cryoEM (5, 8). A similar approach would be to use "monolayer purification" (32), where a lipid monolayer containing 2 to 20% Ni-NTA lipids would be used to bind the Tfr ectodomain, followed by incubation with capsids. That monolayer could be picked up on an EM grid and examined by negative staining, or frozen for cryoEM. The latter method has been used to visualize Tfr-transferrin complexes and ribosomes (32), giving sufficient resolution to define their structures. An additional advantage of these methods is that they would result in the virus-binding apical domain of the Tfr orientated away from the lipid surface, and the density of the Tfr-capsid complex can be adjusted by varying the amount of the Ni-NTA lipid in the membrane. Using proper controls for non-specific capsid binding, this would allow us to ensure that each particle had one or more receptors located between the capsid and the membrane layer.

C2c1) CryoEM reconstruction methods, asymmetric structures. The methods to be used here for cryoEM reconstructions are now well established, including those that do not rely on the standard icosahedral averaging methods during reconstruction. To visualize the feline Tfr interacting asymmetrically with CPV, we used a reconstruction procedure that was developed at Purdue (22, 35). The program involved a combination of the programs SPI/DER (17) and a modified version of XMIPP (52), and utilized the particle orientations found in an icosahedral reconstruction of the CPV-feline Tfr complex. A spherical mask is defined that corresponded to one icosahedral asymmetric unit, then the area of each of the 60 possible projections of the mask is examined to determine the best correlation between the density of the image and the projected density of the mask, given the angles that define the virus orientation for each specific difference image. These orientations are used to compute an asymmetric reconstruction. The procedure is iterated using the resultant map to re-select, from the 60 orientational possibilities, the preferred angles that define the Tfr position relative to the orientation of the virus. In our previous studies, convergence of the particle orientations was reached after about six cycles. To obtain three-dimensional reconstructions of individual virus symmetry features, Briggs et al. describe a method of cryoEM reconstruction allowing for the classification and reconstruction of individual features of icosahedral virus particle (6). This allows examination of features that
deviate from perfect icosahedral symmetry, such as a unique vertex or incorporation of proteins with less than full occupancy.

C2d) Can alternative receptor binding ligands in the CPV capsids mediate infection?

An open question is still whether the TFR binding is required for cell infection because of some structural specificity, or whether other receptors would also work. This would be examined directly by using alternative receptors to bind the capsid to cells, and examining the effects on cell entry and infection. In studies of other paroviruses and AAVs the insertion or replacement of peptides or protein domains into the capsid structures has allowed the selection of variants with altered receptor binding and tropisms (11, 44). We will examine CPV variants with a variety of insertions into surface loops that have been shown to allow peptide addition (57). An initial candidate would be the insertion of the integrin-binding peptide Arg-Gly-Asp into the 4 surface loops, some of which would be expected to affect the binding to the TFR, while others would be outside the TFR binding site, so that they may be able to be grown on normal cells, and tested for infectivity on cells expressing other receptors (such as the αvβ5 integrin). This has been shown to allow some transduction of CPV vectors in previous studies (43), and also to work for AAV vectors (18). Peptides that bind the human TFR have been reported (37, 72), and we would insert those sequences into the CPV capsids in the surface loops. Capsids would be prepared after transfection and tested for their ability to bind the feline, canine or human TFRs when those were expressed on TRVb cells, and to mediate infection.

We would also use our experience with antibody engineering to prepare bi-specific antibodies that contain the binding domains of non-neutralizing anti-capsid Mab as well as those of Mab that we have prepared against the feline TFR (3, 50). Alternatively the anti-TFR scFv itself would be inserted into the capsid structure as an additional domain, either into one of the surface loops as has been shown to work for various sized peptides, or added to the C- or N-terminus of VP2 as has been reported for AAV vectors (73).

Each mutant or sequence-inserted capsid would be tested for assembly after transfection, and then for viability in feline or canine cells. Assembling viruses that do not infect and replicate independently would be prepared by transfection and the virions purified and concentrated before being used for testing. Binding of the capsids to cells expressing various receptors (or no receptors as controls) would be tested using our standard methods of flow cytometry or fluorescence microscopy (19, 24, 26). The TFR binding peptide- or scFv-expressing capsids, or those tested with the bi-specific antibody linker would be inoculated onto cells expressing mutant versions of the feline TFR that do not bind capsids [e.g. Ile221Ser (50)]. Infection would be measured by staining for virus capsids in cells, or the NS1 protein.

C2e) Expected outcomes, possible problems and solutions and alternative approaches.

The studies to be conducted here extend from our previous work which has largely been published, and we expect that most will be straightforward and lead to clear results. The preparation of mutant and chimeric capsids containing peptides would be straightforward, as would be the bi-specific antibody linker. The results are difficult to predict, as it does appear from previous results that the CPV capsid is not as permissive for use of alternative ligands as is AAV. However, by trying several different approaches we believe that we can answer the basic question about the structural dependence on TFR binding for infection.

We do not currently have a way to directly connect capsid mutations with specific structures of the TFR, and so we will select for viral variants using TFRs with known changes in their structure, and also to prepare higher resolution TFR:capsid complex structures. While it is not certain that the viral and TFR changes selected will necessarily be in direct contact, that is a reasonable possibility, and the residues identified can be used in docking and orientation studies of the TFR-capsid complexes. The higher resolution TFR-capsid structures will involve refinements of methods that we have already developed, as well as preparation of purified complexes, or of immobilized receptors on lipid layers. These would increase the quality of the results by reducing the background of the images, and where the TFR was immobilized on a monolayer or liposome, its orientation relative to the capsid would be constrained to one or a small number of positions in the images collected. We can also use tomography with a tilting stage to collect more images of each complex, to obtain additional structural information.

We have several versions of the feline and canine TFRs that bind with lower affinities than the wildtype due to single changes in their sequence (19). The yeast expression technology has been used for many studies expressing cell surface glycoproteins [e.g. (28, 33)], and the method is being used in the Parrish laboratory for antibody engineering (see also Section C3). The TFR is a complex protein structure, and it may not fold correctly or bind virus efficiently in yeast, and there may be differences in glycosylation. Despite these difficulties if these studies worked they would give us valuable data, and so we see this as a "high risk but high
payoff experiment, but not essential for the studies - even if the yeast expression does not result in an improved receptor, we have many other TIR mutants and capsids with variable properties that will give us most of the information we are seeking about the roles of the receptor:capsid interactions in virus infection.

**C3) Aim 3. Use antibodies to probe the capsid structure, and also to determine how binding to overlapping sites leads to neutralization in some cases, but not others.** Hypothesis: That antibodies can be used to detect variant structures in the viral capsid, and that the specific position and orientation of binding controls the likelihood of competition with the TIR, and neutralization of infection.

**C3a) Background.**

The properties of viral antigens and the effects of antibody binding vary, with some viruses being efficiently neutralized, while others show varying degrees of escape from antibody binding or inactivation (23, 54). The parvovirus capsid is a potent multivalent antigen which rapidly stimulates strong antibody responses, and each virion can bind up to 60 antibody molecules (21). We have prepared many different antibodies that recognize the CPV capsid, and have recently mapped the binding footprints of 8 separate antibody Fabs on the virion surface structure using cryoEM (21). Those antibodies covered a total of ~70% of the capsid surface, and could be divided into two major clusters of overlapping binding sites (termed “A” and “B”) (21, 63). Several of the antibody footprints clearly overlapped the binding footprint of the feline TIR (Fig. 6). Those antibodies are very specific for particular structures in the capsids, and therefore can be used to probe for changes (21, 70). While 7 of 8 antibodies bound with similar affinities and overlapped the TIR binding site on the capsids, 4 showed little neutralization of the virus when tested as Fabs, while two showed efficient neutralization (46). Some neutralizing and non-neutralizing Fabs represent pairs with essentially the same footprint, indicating that there can be distinct interactions between TIR and antibody binding that give different effects on viral infection. One model derived from the cryoEM structures is that the angle of attachment is important in neutralization, and that Fabs binding at an oblique angle may block TIR binding to both the capsid subunit where the Fab bound, as well as to an adjacent two-fold related asymmetric unit (21)(Fig. 7). Competition between the Fabs and the feline TIR was much more efficient for the highly neutralizing Fabs suggesting that those had a different mechanism of blocking receptor attachment (46). While other possibilities such as differences in the affinity of binding of the neutralizing and non-neutralizing Fabs seem unlikely due to the similar affinities of most of the antibodies examined, we will also be able to test that possibility directly in the studies proposed by selecting for increased affinity variants (see below).

**C3b) Antibody and TIR binding – uneven Fab binding and examining for unoccupied sites.**

The poor neutralization by several antibody Fabs indicates that even in the presence of excess Fab (when all 60 capsid sites appear occupied by cryoEM), there are still sites on the capsids available for receptor binding. As described above, cleaved VP2 surface loops would likely form such open sites, as they are unlikely to allow antibody attachment, but those may still allow (or perhaps even favor) TIR binding. Such a mechanism would explain why Fabs that bind at oblique angles could sterically block TIR access to an adjoining cleaved site on the capsid which did not directly bind that antibody. Here we would use the capsids with either varying numbers of cleavages or different levels of cleavage susceptibility to examine for effects on antibody and TIR binding.

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**Fig. 6.** Binding footprints on one asymmetric unit of the CPV capsid, showing a B-site antibody (blue), an A-site antibody (red), and of the feline TIR (black). Residues 297 and 300 control protease cleavages.

**Fig. 7.** The cryoEM structures of the complexes between the Fab of Mab F (neutralizing) and Mab8 (largely non-neutralizing as Fab), showing the different orientations of those Fabs, which otherwise bind with similar footprints on the capsid.
C3b1a) TIR binding to Fab-occupied capsids. To test this model, we would examine for such open sites on the capsids using viruses with alterations in capsid cleavages (derived in Section C1 studies). Mutant or wildtype capsids would be digested with proteinases to cleave varying proportions of the surface loop around VP2 residue 300. After purification, those capsids would be incubated with excess Fab, and then with purified TIR labeled with 5 nm colloidal gold. After washing, the complexes would be examined in the EM either unstained or with light negative staining, looking for capsid-associated gold particles, compared to control capsids, or those incubated with labeled TIR in the presence of non-specific Fabs. A prediction of our hypothesis is that most Fabs would allow some TIR binding, but that binding would be blocked by the highly neutralizing Fabs of antibodies E and F.

C3b1b) Are there open sites on Fab-saturated capsids that can be detected with antibodies of different specificities? Open sites on capsids incubated with excess Fab would also be assayed by leaving the Fab with the capsids in large excess, and incubating with gold labeled Fabs of antibodies binding to different regions of the viral asymmetric unit. For example, cleavages around VP2 residue 300 would likely prevent the B-site antibodies from binding, while most A-site specific Fabs would still bind to those digested capsid subunits. As some A-site antibodies (e.g. shown in red in Fig. 6) clearly overlap the binding footprints of some B-site Fabs (as shown in blue in Fig. 6) (21), they could be used as specific probes for the open sites not occupied by the B-site Fabs. For these assays controls are critical to distinguish possible artifacts. One would be provided by the comparison of the antibodies with different effects on receptor binding. The possibility that the second antibody binds due to the natural exchange due to low affinity of the different antibodies would be examined by swapping the two antibodies in the study (gold labeling the first antibody and adding excess second antibody), or by using antibody variants with increased affinities selected as described below.

C3b1c) Detecting low levels of open sites by precipitating capsids with biotinylated antibodies. An additional assay for open sites would be to use biotinylated Fabs or TIR, and to incubate those with the Fab:capsid complexes in the presence of excess unlabeled Fab. We would then precipitate the biotinylated protein in the complex with streptavidin beads, or would precipitate the TIR with an anti-TIR monoclonal antibody we have prepared (50). The amount of capsid precipitated would be determined by Western blotting. This would show the proportion of capsids with sites that were accessible to the probe in question, even if present at low levels or transiently.

C3c) Is the affinity of the antibodies important for the TIR competition and neutralization? An alternative mechanism would be that neutralization is related to the specific affinity of the antibody, with increased affinity allowing a more efficient blocking of viral functions - such as receptor binding or capsid trafficking and specific structural changes in the endosome. We could test this by preparing antibody domains with higher and lower affinities and testing their abilities to neutralize the virus as scFv or Fabs. We have cloned the light and heavy variable chains of several antibodies and expressed those domains in yeast, as described for TIR (Section C2b1c). We would initially concentrate on neutralizing:non-neutralizing pairs of antibodies that bind to essentially the same sites, but that differ in neutralization - such as antibodies F and 8 (21). Initial studies show that the initial set of 4 antibodies that we have expressed are produced in good amounts on the surface of yeast cells, and that they show functional capsid binding when expressed (e.g. Fig 8). Those antibody V\textsubscript{H} and V\textsubscript{L} domains would therefore be readily mutated by error prone PCR and then introduced back into the yeast to allow recombination, producing a library that can be selected for altered (generally higher) affinity forms (9). We have already produced such a library for the clone of antibody 8 in yeast, and started to select for higher affinity versions. Antibodies selected would be expressed as either scFvs, or linked to the heavy and light chain constant regions to produce Fabs (71), and tested for binding affinity and for

![Fig. 8](image)

Fig. 8. The functional antibody scFv expression on the surface of yeast, where expression was detected with an anti-Fab antibody staining, and the binding activity detected using Alexa-fluor labeled capsids. Three examples are shown for of the antibodies to be examined here.
neutralization using standard methods (46). The affinities of binding can be directly measured for the antibody expressed on the yeast surface using flow cytometry to estimate on- and off-rates (9, 67).

An additional variant on this protein engineering approach would involve determining the virus-specific contacts and specificity determinants of the antibodies. We have already solved the X-ray crystal structure of the Mab 14, which specifically binds CPV but not FPV (21). Other specific antibodies do not recognize certain natural or antibody-selected antigenic variants (escape mutants) of the capsid (63). We would therefore use the same yeast expression library and mutagenesis to select antibody mutants that recognize these variant capsids, and thereby determine the antibody structural determinants of site-specific recognition. This would allow better models of the antibody-capsid contacts to be obtained.

C3c1) Functional testing of the mutated antibodies. To determine the effects of the different changes in the capsids and antibodies on the process of infection and the relationship to neutralization, we would examine the effects of the purified antibodies or antibody domains (as scFv or Fab) on the viral functions. We would purify the wild type and mutant forms of the capsids and antibodies. Antibodies would be tested for their ability to compete with Tfr in binding assays, and for their ability to neutralize standard virus preparations. Wildtype or mutant capsids would also be used, and treated with proteases to cleave varying proportions of the VP2 in some studies. Those would then be examined for their ability to bind the Tfr in solid phase or on cells, for uptake into the normal pathways of cell entry, or to be neutralized, using our well established methods (24, 46).

C3d) Expected outcomes, potential problems and their solutions or alternative approaches.

These studies will use existing well-characterized antibodies and engineered variable domains to answer important questions about the mechanisms of antibody attachment, recognition of specific capsid structures, and their interactions with receptor binding leading to neutralization. We already have the antibody-capsid complex structures of a representative set of 8 antibodies (21), and have several of the antibodies expressed in either bacteria or yeast and have confirmed that those are expressed and bind the viral capsids (e.g. Fig. 8). We are therefore well positioned to carry out the studies proposed.

While the lack of binding by some of the antibodies to sites with cleaved loops has not been formally shown, that is highly likely given the properties of the known escape mutations and binding sites. The preparation of the gold-labeled Fab should be quite straightforward, while the Tfr labeling may be more challenging. However, the Tfr ectodomain dimer, as expressed, displays two 6-His tags on the underside of the receptor, and that should be readily labeled by Ni²⁺-gold conjugates. The selection for antibody domains with altered affinities should be straightforward given that we already know that the most interesting antibody pairs are expressed in a functional form on the surface of yeast, and our close collaborator, Dr. Moonsoo Jin, has used all of the methods proposed for preparing and screening mutant libraries for ligands with altered affinities (see letter of collaboration).

C4) Overall Summary and Conclusions.

These studies will integrate our understanding of the structures of viral capsids with a more detailed knowledge of the binding properties and functions of host cell receptors and antibodies. The results would be correlated with biochemical and structural analyses of the capsid flexibility, variation, and/or asymmetry. These are central questions that apply to any non-enveloped animal virus, and have parallels to the structural changes and interactions seen for many enveloped virus glycoproteins, and so the results will clarify some of the underlying rules about how viruses interact with their host ligands and infect cells.

The work builds on a solid intellectual and methodological foundation resulting from our previous studies, and we have most of the materials and background information required. For each of the projects we combine well established methods with new approaches, and have alternative approaches for each of the experiments where the technology is novel or untested. Studies already underway would be continued in the first phase of the funding period, while studies requiring the development of reagents or information from previous studies will be done later in the project.

TIMELINE

This project would take 5 years to complete. The sequence of studies will initiate in years 1 and 2 with the preparation of the capsid mutants and their testing, along with development of the new methods for sample preparation for cryoEM, and collection of the cryoEM data for analysis. Analysis of the role of cleaved and stabilized capsids would initiate with the currently available mutants, and continue through years 3 and 4. Mutant forms of the Tfr and antibodies would be prepared in the first years, and tested in later years up to year 5. The preparation of capsids with altered receptor binding sites (peptides or domains), and selected on mutant receptors, would occur during years 3 to 5.
VERTEBRATE ANIMALS:

Parrish, Colin R., Cornell University

The animal care facilities and programs of the Baker Institute meet the requirements of the law and NIH regulations. The College of Veterinary Medicine at Cornell University, of which the Baker Institute is a part, is accredited by the American Association of Laboratory Animal Care. The animals are subjected to regular veterinary care on a routine basis. An Animal Care Protocol has been approved for these studies: Protocol #1988-0139, approved 10/5/09 for a three-year period.

1. Description of animal use; species and numbers of animals to be used. Rabbits would be used to prepare polyclonal sera against peptides and expressed proteins. Mice of the BALB/c strain would be used for hybridoma production for preparing structure-specific monoclonal antibodies.

   Rabbits: Maximum number of animals used each year 1-5: 2  Total: 10
   Mice: Maximum number of animals used each year 1-5: 10 Total: 50

2. Justification for use of animals. Rabbits would be required for preparing polyclonal sera against synthetic peptides and expressed proteins. Rabbits are the standard species for preparation of such sera. All rabbit handling and immunizations would be conducted by the Department of Laboratory Animal Medicine at Cornell on an at-cost basis, or from commercial vendors.

Mice of the BALB/c strain would be used for hybridoma production as those are compatible with the myeloma cells to be used for fusions. Mice are the standard and best characterized species for preparing specific monoclonal antibodies. MAbs will be produced in tissue culture; no production of ascitic fluids in mice will be carried out.

3. Veterinary Care and services of the Center for Research Animal Resources are available if any other veterinary care is required, and they are available on-call 24 hours per day. Any animals suffering from a disease would be examined and treated by a veterinarian. If the condition is untreatable the animal would be euthanized.

4. Discomfort, Distress and Injury. The experiments in this research will not cause undue discomfort or distress to the animals being used. However, when needed, veterinary care will be provided; if the condition is untreatable the animal would be euthanized by approved methods.

5. Euthanasia. Euthanasia will be performed by methods specified and approved by the AVMA Panel on Euthanasia.

   Mice: Euthanized by CO₂ anesthesia.
   Rabbits: Barbital overdose - I.V. administration.
BIBLIOGRAPHY AND REFERENCES CITED:


References Cited


References Cited
61. Sonntag, F., S. Bleker, B. Leuchs, R. Fischer, and J. A. Kleinschmidt. 2006. Adeno-associated virus type 2 capsids with externalized VP1/VP2 trafficking domains are generated prior to passage through the cytoplasm and are maintained until uncoating occurs in the nucleus. Journal of virology 80:11040-11054.

References Cited
CONSORTIUM / CONTRACTUAL ARRANGEMENTS:

A consortium arrangement is proposed with Pennsylvania State University-Hershey Medical Center, as described in the Research Plan. The appropriate programmatic and administrative personnel of each institution involved in this grant application are aware of the NIH consortium grant policy and are prepared to establish the necessary inter-institutional agreement consistent with that policy.
Dr. Colin R. Parrish  
Baker Institute for Animal Health  
College of Veterinary Medicine  
Cornell University  
Ithaca, NY 14853

Dear Colin,

This is to confirm my interest and ability to assist with your studies that involve the engineering and selection of altered versions of Tfr ectodomain or antibody domains with increased or decreased affinities. One approach that you have proposed to use includes yeast expression and display using Aga2p fusions and selection of altered versions with high affinities after error-prone PCR. I have had considerable experience with these methods in my studies of integrins and antibodies. I have also been developing methods for expression of the mouse Tfr ectodomain in the system for my own studies of blood-brain barrier dynamics, and for the development of isolated ICAM-1 domains for rhinovirus binding studies.

The tools for the assays are available in both our laboratories and can be readily adapted to the expression of the different antibody domains or the canine and feline Tfrs. The post-doctoral fellows in our laboratories have been working on these projects for the past several months, and are making good progress, having prepared the candidate proteins in yeast expression systems. We are able to use either the fluorescence-based cell sorter or magnetic sorting of the yeast using either labeled parvoviruses or antibodies as the detection methods. I also have a Biacore 2000 analyzer in my laboratory which can be used for the assays of the binding affinities of the purified components.

I look forward to continuing collaborations, and expect that these studies will increase our understanding of the processes of virus binding to cells for infection, as well as antibody binding and neutralizations.

Best Regards,

Moonsoo Jin, Sc.D.  
Assistant Professor of Biomedical Engineering  
Cornell University
Dear Susan and Colin,

This letter is written in support of your application for an NIH grant studying the structures and functions of the parovirus capsids. During the last few years, I have worked closely and collaborated on several projects with Dr. Hafenstein while she was at Purdue. These efforts have resulted in the publication of “Asymmetric binding of transferrin receptor to parovirus capsids” in the Proceedings of the Nation Academy of Sciences and “The Interaction of Decay-accelerating Factor with Coxsackievirus B3” in the Journal of Virology. Dr. Hafenstein was first author on both of these publications.

The Structural Biology Group of Purdue’s Biology Department has recently moved into their new building. The building is also the new home of the Biological Electron Microscopy Facility with three electron microscopes capable of performing high-resolution, cryo-electron microscopy of macromolecules such as viruses. This includes a recently acquired FEI Titan Krios instrument, a state of the art transmission electron microscope capable of achieving atomic resolution. This instrument is the most advanced electron microscope currently available. It has accessories to permit examination of frozen hydrated samples, a Gatan Tridiem energy filter to improve electron tomograms and an automatic loading device, eliminating the need for a technician to be present during each sample change. With automated image acquisition, optimized performance throughout the 80 to 300kV range and a Gatan 4k x 4k CCD camera, this instrument is highly versatile and designed for high throughput studies.

I am very enthusiastic about your project and would be happy to assist in the use of the microscopes for cryoEM, as well as in the data processing. I look forward to continuing to aid your research.

Best Regards,

[Signature]

Paul Chipman,
Director, Electron Microscopy Facility
RESOURCE SHARING PLAN:

Sharing Model Organisms: We do not anticipate the development of any model organisms from this study. Should any be developed, they will be made available to other NIH funded researchers via applicable Cornell University Material Transfer Agreements and/or licensing agreements through the Cornell Center for Technology, Enterprise, and Commercialization.