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Delineation of the molecular events in virus interaction with the host cell following modification of the poliovirion.

Monica Celeste Frazier
University of Alabama at Birmingham

DELINEATION OF THE MOLECULAR EVENTS IN VIRUS INTERACTION WITH THE HOST CELL FOLLOWING MODIFICATION OF THE POLIOVIRION

by

MONICA CELESTE FRAZIER

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ABSTRACT OF DISSERTATION
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Name of Candidate Monica Celeste Frazier

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The binding of poliovirus to the poliovirus receptor (PVR) and A particle formation does not guarantee viral entry. Greater than 80% of all poliovirions that bind to PVR-bearing cells and undergo A particle formation fail to internalize and are subsequently sloughed off the cell surface. Consequently, the poliovirus infection process is highly abortive. A possible solution to such a problem is to enhance the adhesiveness of the virus. One way to do this is to use cell surface receptors called integrins. Integrins play a role in cell adhesion. The peptide most recognized by integrins is arginine, glycine, aspartic acid (RGD). To determine if we can facilitate interaction of A particles with the host cell, we reconstructed the infectious clone of poliovirus to include an RGD sequence. We substituted amino acids 95 to 102 of the viral protein 1 (VP1) capsid protein (BC loop) with amino acids RRGDLGSL, a consensus sequence from coxsackievirus A9 and foot and mouth disease virus, both known to use integrins as receptors for viral entry. Transfection of RNA derived from in vitro transcription of this clone resulted in production of virus that retained the RGD substitution in VP1 as determined by sequence analysis. The recombinant poliovirus showed greater binding to

integrin-positive and PVR-negative BHK cells than the wild-type virus. However, when using genome equivalents of RGD and wild-type viruses, the plaque-forming potential of the RGD virus was 9-fold less than wild-type virus. To characterize further the effect of the RGD mutation, we used a complementation system developed in this laboratory in which a defective RNA genome (replicon) was encapsidated by a poliovirus capsid protein (RGDP1) provided in *trans* by a recombinant vaccinia virus. Replicons encapsidated with RGDP1 also bound to baby hamster kidney cells like the RGD viruses, suggesting binding to integrins. In addition, data from replicons encapsidated with the RGD capsid suggested that these replicons were more infectious on a per genome basis when compared with replicons encapsidated with the wild-type capsid. Ultimately, this strategy might prove useful to expand the host range and infectivity of poliovirus replicons, which have potential uses in gene therapy and as recombinant vaccines.

DEDICATION

This dissertation is dedicated to my parents, Robert D. and Ida C. Frazier; my brother, Robert "Robbie" D. Frazier, Jr.; and my daughter, Celeste Marissa Frazier. With the exception of my daughter, they have been by my side since day one, and it is with great pride that I dedicate this dissertation to them. To my dad, I want to say thank you for being there for me. We wanted to celebrate our milestones (his retirement and my graduation) together; however, as fate would have it, you retired first. We may not have been able to celebrate together, but we do get to celebrate again. I thank my brother, for all his support, encouragement, and mechanical skills. Thanks to him, my car has never run or looked better! Seriously, we've always been the dynamic duo, wonder twins, sister/brother team, and I pray that we will always stay that way. I thank my mom, for everything, as well. I've called on her many times for help and she's always been there no matter what. There were many days when I thought that I could not go on, but she gave me the courage and strength to go on. Our relationship is a very special one, and I am proud to have a mother like her. All of the inspirational calls, encouraging words, and love she has given have meant so much to me. I do not think any words can express how much she means to me. I thank my daughter, so much for coming into my life. Her birth is a magnificent close to an important chapter in my life. She has truly been a wonderful and beautiful blessing to us all, and I would like her always to remember that I will always love her. In closing, since my coming to UAB to pursue this degree, there have been many times when I just wanted to give up, but none of them would let me. One by

one, they called in their own special way to say hang in there and don't give up. Well, I did hang in there and did not give up. Thanks to them, I can say that I made it through. Hence, I want to let them all know how much I love them for all they have done for me from the bottom of my heart. This is our dissertation and we have worked very hard for it. Congratulations on a job well done!

ACKNOWLEDGMENTS

As an undergraduate at Alabama State University (ASU), my goal in life had nothing to do with research. As a matter of fact, I did not know much about it. The only scientist I had heard of was Dr. George Washington Carver. Although I was very familiar with his work and thought it was fascinating, it just was not something I thought that I would someday end up doing. It was during a conversation with a fellow student that I was told of a program that paid money to students interested in research. Although I had always had this idea to find the cure for cancer, I still was not very interested in research; however, the money surely sounded good. Therefore, I tried it. Working in the laboratory was okay, but it just did not interest me. During the summers, the faculty at my university always encouraged their students to do summer internships in research. Therefore, I did. My first job was here at the University of Alabama at Birmingham (UAB) with Dr. Donald Miller. I came here as green as the grass outdoors. I had absolutely no skills in anything other than tissue culture and cell counting, but I learned so much in his laboratory and had such a wonderful time working with him that I returned to ASU with a new attitude towards research. Because of Dr. Miller, I took a second look at research, and for that I want to say thanks. The next summer, I went to the National Institutes of Health. I never thought that I would end up somewhere like this walking amongst some of the greatest scientists from all over the world. During my stay here, my mentor, Dr. William Murphy, showed me just how exciting research could be. He was so enthusiastic about research that I wanted to have that same feeling. My first publication came from his

laboratory and for that I want to say thanks. After graduating from ASU, I have had numerous other people who have greatly influenced me and stood by my decisions and me; two in particular are Drs. Thelma Ivery and Narendra Singh. Dr. Ivery, my chemistry professor at ASU, prepared me for what was to come in graduate school. I called her several times for advice and each time she was there to listen. I want her to know that I appreciate all that she has done for me. Dr. Narendra Singh, my former boss at Auburn University, and I have also had many wonderful talks. When I told him that I wanted to pursue my doctorate, he encouraged me to do so and told me to call on him if I needed him. I can boastfully say that he has kept his word. Anytime I have called on him, he has been there for me. It is not often that we get the opportunity to meet people like him and because of that I want to say thanks.

Since being at UAB, I have had the opportunity to meet a lot of interesting people and experience a lot of interesting things. Through it all, though, I made it. There are several people whom I would like to thank from this university. First, I want to thank my mentor, Dr. Casey Morrow, for allowing me into his laboratory. Through his laboratory, I have gained a vast amount of experience and laboratory skills in various areas of research. Next, I would like to thank my committee members, Drs. Candece Gladson, Jeff Engler, Ming Luo, and David Ansardi. They have all been very helpful and there for me when I needed them. I could not have asked for a better committee. Although I hate to point out any one person in particular for fear of offending others, I must say something about this person or I would truly be remiss. Words simply cannot express the gratitude and appreciation that I have for Dr. Gladson. I thank her so much for all that she has done for me. I would now like to thank all of those who have helped me to get the job done.

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Outside of UAB, there have been many people who have supported me not only through graduate school but also throughout various times in my life. Thanks go to my ASU family, sorors, UAB friends, dancing partners, and all other friends for supporting my efforts to obtain this degree and for all of the good times. I would also like to thank various members of my church family back home and here and my other mom, Mrs. Shirley Carter. Again, I would like to thank my parents, Robert and Ida Frazier; my

brother and sister-in-law, Robbie and Alison Frazier; my daughter, Celeste Frazier; my grandfather, Mr. Daniel Scott; and other family members, Midnight, Princess, and Angel, for being there for me when I needed them. Regrettably, some of my family are not here to celebrate this day with me, but I want to thank them, too, for being there. These family members include my grandmother, Mrs. Annie K. Menafee; my godmother, Ms. Ezella Gibson; my aunt, Mrs. Dorothy Council; and my four-legged brother, Spike. I cherish my relationship with my family and want them to know that had it not been for the love and support of them and my spiritual faith, I would have never made it. I would like to close my acknowledgments with one of several passages I have hanging in my room to help me get through the days and years I've spent here: "Have I not commanded thee? Be strong and of good courage; be not afraid, neither be thou dismayed: for the Lord thy God is with thee whithersoever thou goest" (Joshua 1:9).

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LIST OF ABBREVIATIONS

A	adenine
AUG	methionine codon
β-gal	beta-galactosidase
BHK	baby hamster kidney
BrdU	5-bromo-2'-deoxyuridine
C	cytosine
$CaPO_3$	calcium phosphate
CAR	coxsackievirus-adenovirus receptor
CAT	chloramphenicol acetyltransferase
CAV9	coxsackievirus A9
CBC	cap-binding complex
CNS	central nervous system
CPE	cytopathic effect
$3D^{pol}$	poliovirus RNA-dependent RNA polymerase
DI	defective-interfering
DMEM	Dulbecco's modified Eagle's medium
ds	double stranded
DTT	dithiothreitol
E, Glu	glutamic acid
ECMV	encephalomyocarditis virus

FCS	fetal calf serum
FMDV	foot-and-mouth disease virus
G, Gly	glycine
GFP	green fluorescent protein
H	heated
HIV-1	human immunodeficiency virus type 1
IRES	internal ribosomal entry site
K	lysine
L	leu
mM	millimolar
MOI	multiplicity of infection
N	asparagine, native
N-Ag	neutralization antigenic site
ntr	nontranslated region
ORF	open reading frame
P, Pro	proline
^{32}P	radioisotope phosphorus 32
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PND	principal neutralization domain
pT7-IC	poliovirus infectious clone plasmid
PVR	poliovirus receptor
RF	replicative form

RGD	arginine-glycine-aspartic acid
RI	replication intermediate
RIPA	radioimmunoprecipitation assay buffer
RLU	relative light units
RPA	ribonuclease protection assay
RT	reverse transcription
S, Ser	serine
^{35}S	radioisotope sulfur 35
SDS	sodium docecyl sulfate
T-Ag	T-antigen
TCA	trichloroacetic acid
TK^-	tyrosine kinase minus
TNF-α	tumor necrosis factor α
TUT	terminal uridylytransferase
VCAM	vascular cell adhesion molecule
VP	viral protein
VPg	virion protein genome
VVP1	vaccinia virus encoding poliovirus capsid P1
VVRGDP1	vaccinia virus encoding mutated poliovirus capsid RGD-P1
x *g*	times the force of gravity at sea level

INTRODUCTION

Poliovirus, a member of the family *Picornaviridae* and genus *Enterovirus*, is the RNA virus responsible for causing the devastating epidemic of poliomyelitis. Poliomyelitis, the potentially crippling disease caused by poliovirus, occurs in one of several forms (111). First, it can occur as abortive poliomyelitis, the most common form of the disease that causes only minor illness as characterized by fever, headache, sore throat, or nausea, to name a few. Second, it can occur as nonparalytic poliomyelitis, also known as aseptic meningitis. Finally, it can occur in its most well known form, paralytic poliomyelitis. However, only 1% of all natural infections by poliovirus result in the paralytic form of poliomyelitis (89, 111).

In 1909, Landsteiner and Popper discovered that poliomyelitis was caused by a virus (96), but the virus causing this disease was not named poliovirus until 1955 by vonMangus (204). The virus infects humans via a fecal to oral route. This mode of transmission can occur when excretions of humans are discharged into sewers, landfills, and rivers and lakes, thereby contaminating water supply.

In the late 19th and early 20th centuries, poliovirus caused devastating outbreaks of poliomyelitis. As a result, many people all over the world were crippled. However, after the development of two effective vaccines, poliomyelitis has been virtually eliminated. The first vaccine consisted of a killed or formaldehyde-inactivated poliovirus, as discovered by Salk in the 1950s (170, 171). The second vaccine consisted of a live attenuated poliovirus, as discovered by Sabin in the 1960s (169).

In 1988, the World Health Organization embarked on a worldwide effort to eradicate poliovirus (52). Since that time, the number of cases of poliomyelitis has dropped tremendously. The last case of polio in the western hemisphere is documented to have occurred in 1991 in Peru, whereas the last known case of polio in the eastern hemisphere is documented to have occurred in 1997 in Cambodia (52). However, in spite of these reported cases and other reports of objection to the eradication of poliovirus (53, 54), the World Health Organization hopes to achieve its goal by 2003 (52, 53).

Picornaviridae

The family *Picornaviridae* is not only one of the smallest RNA containing virus groups known but also one of the most important in human and agricultural pathogens. Moreover, the first animal virus discovered in 1898, foot-and-mouth disease virus (FMDV), is a member of this family (101). Currently, the family of RNA viruses is divided into five genera: rhinovirus (i.e., human rhinovirus and bovine rhinovirus), enterovirus (i.e., poliovirus, coxsackievirus, and echovirus), aphthovirus (i.e., FMDV), cardiovirus (i.e., encephalomyocarditis virus, Theiler's murine encephalomyelitis virus, and Mengo virus), and hepatovirus (i.e., human hepatitis A virus) (166, 206). Picornaviruses are small, icosahedral positive-strand viruses. Their icosahedral shells are comprised of 60 copies of four viral proteins (VP1, VP2, VP3, and VP4) (77). Each shell contains a single-stranded positive-sense RNA ranging from 7209 to 8450 bases in length (166). In spite of variations in length of the RNAs, their genomes are organized in similar fashion. They each have a small protein, virion protein genome (VPg), attached to the 5' terminus, followed by 5' nontranslated region (ntr) and a single polyprotein that consists

of the three precursor proteins P1, P2, and P3. Following the polyprotein is a 3' ntr and a poly(A)tail (62, 88, 166, 182). Aphthoviruses and cardioviruses deviate slightly from this genomic arrangement in that they carry a leader protein sequence located just between the 5' ntr and the polyprotein (138, 159). Protein synthesis of picornaviruses involves a series of cleavages, resulting in 11 protein products or 12 protein products in the case of aphthoviruses and cardioviruses that carry the additional leader sequence. Picornaviruses are very stable. They are acid labile in that they can survive at pH levels of 3 or lower, whereas rhinoviruses and aphthoviruses are labile at pH 6 or less (166). Although these viruses appear to be very stable and resistant to harsh conditions, studies show that the drug guanidine hydrochloride blocks the synthesis of many picornaviruses (31, 44, 91).

Poliovirus Genomic Organization

There are three serotypes of poliovirus. Each serotype has a prototypic strain. The prototypic strains are Mahoney for type 1, Lansing for type 2, and Leon for type 3 (111, 206). The genome length of each serotype varies. The genome lengths are 7441 nucleotides for type 1, 7440 nucleotides for type 2, and 7432 nucleotides for type 3 (196). The genome of poliovirus, as shown in Fig. 1, is organized like all other picornaviruses, with the exception of aphthoviruses and cardioviruses. The 5' end of poliovirus does not have the common 7-methylguanosine cap (126). Instead, the 5' end of poliovirus, like all other picornaviruses, is linked to a small protein (VPg) through a phosphodiester linkage to the phenolic (O^4) hydroxyl group of a tyrosine residue (5, 98, 126). Following the VPg protein is the 5' ntr of poliovirus. This ntr is approximately 740 bases in length (88, 152) and is conserved among the serotypes of poliovirus and other picornaviruses. Because

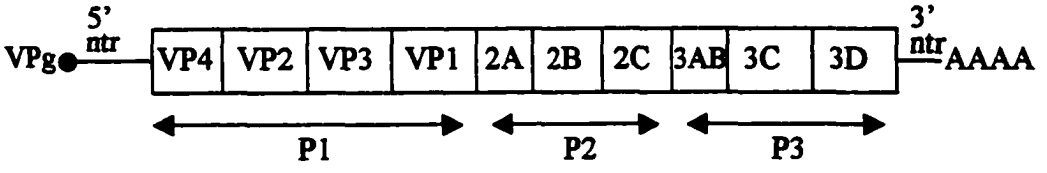


Fig. 1. Depiction of the poliovirus genome. The poliovirus genome has a small protein, VPg, linked to the 5' end as depicted by the black filled circle. Following VPg is the 5' ntr; the AUG start codon; and the P1, P2, and P3 proteins of poliovirus. P1, the structural protein, is comprised of VP4, VP2, VP3, and VP1. P2, a nonstructural protein involved in replication, consists of 2A, 2B, and 2C. P3, another nonstructural protein involved in replication, is made up by 3AB, 3C, and 3D. Following P3 is the 3' ntr, followed by the poly A tail.

this region tends to be highly conserved, it is thought that it must serve a critical viral function. Thus far, that function appears to be its involvement in cap-independent initiation of translation of viral RNA (135, 136, 198). Within the 5' ntr is the internal ribosomal entry site (IRES) (81, 82), or ribosome-landing pad (135), as it has also been called. The specific sequence that comprises the IRES has not been elucidated. However, it is contained within nucleotides 130 to 630 and has a cloverleaf-like structure, as supported through data gathered via site-directed mutagenesis and enzymatic analyses (7, 29, 69, 125). Through these studies, mutations within the IRES have been found to alter the efficiency of viral RNA translation (69).

Following the IRES is the methionine codon (AUG) or start codon that marks the beginning of translation of the long open reading frame (ORF) of poliovirus. This ORF produces a single translation product called the polyprotein. This polyprotein encodes the capsid proteins, the proteinases required for cleavage of the polyprotein, as well as the proteins required for RNA synthesis (111). As stated before, cleavage of the polyprotein gives rise to the 11 protein products found in poliovirus.

The 3' ntr is relatively short when compared with the 5' ntr and follows the polyprotein. This region is approximately 72 bases in length (62, 88, 166, 182) and has no known function at this time. However, studies suggest that this region may be important in poliovirus replication because of the production of temperature-sensitive mutants as a result of an 8-base insertion in this region (174).

Finally, there is a 3'-terminal poly(A) tail at the end of the poliovirus genome. The poly(A) tail averages 60 residues in length (2) and is genetically encoded because of transcription from the poly(U)found in the minus strands (51, 208, 209). Although there

is no known function of the poly(A) tail, what is acknowledged is that RNA molecules with short poly(A) tracts have lower specific infectivity (173, 186).

Poliovirus Life Cycle

Like most viruses, the life cycle of poliovirus begins with binding to the poliovirus receptor, PVR, as shown in Fig. 2. Upon binding, the poliovirion undergoes a conformational change that occurs from the native 160S particle to the 135S or A particle (16, 19, 36, 68). This conformational change results from the loss or release of the capsid protein VP4 and the protrusion of the N terminus of VP1 from the viral surface (64, 177, 178). The externalized N terminus is thought to form an amphipathic helix that allows the 135S particle to attach to the plasma membranes, forming a pore through which viral RNA passes into the cytoplasm of the cell (19, 64, 84). The message sense viral RNA is released into the cytoplasm after removal of the 5'-terminal VPg protein by a cellular enzyme, which cleaves the phosphodiester bond between the 5'-terminal phosphate of the viral RNA and the tyrosine in VPg (4, 126, 205). Following release into the cytoplasm, the RNA genome is translated by host ribosomes to generate viral proteins. As mentioned before, poliovirus RNA is not capped like other cellular mRNA; therefore, host cell ribosomes begin translation of polio VPs by binding to the IRES of poliovirus. The genome is translated as one long polyprotein, which is cleaved by viral proteases into the individual proteins necessary for replication and capsid production.

Replication of poliovirus begins with the transcription of plus-strand RNA to yield a complementary minus-strand RNA that has the VPg attached to the 5' end (12, 157, 166). This minus-strand serves as a template for the production of more positive-

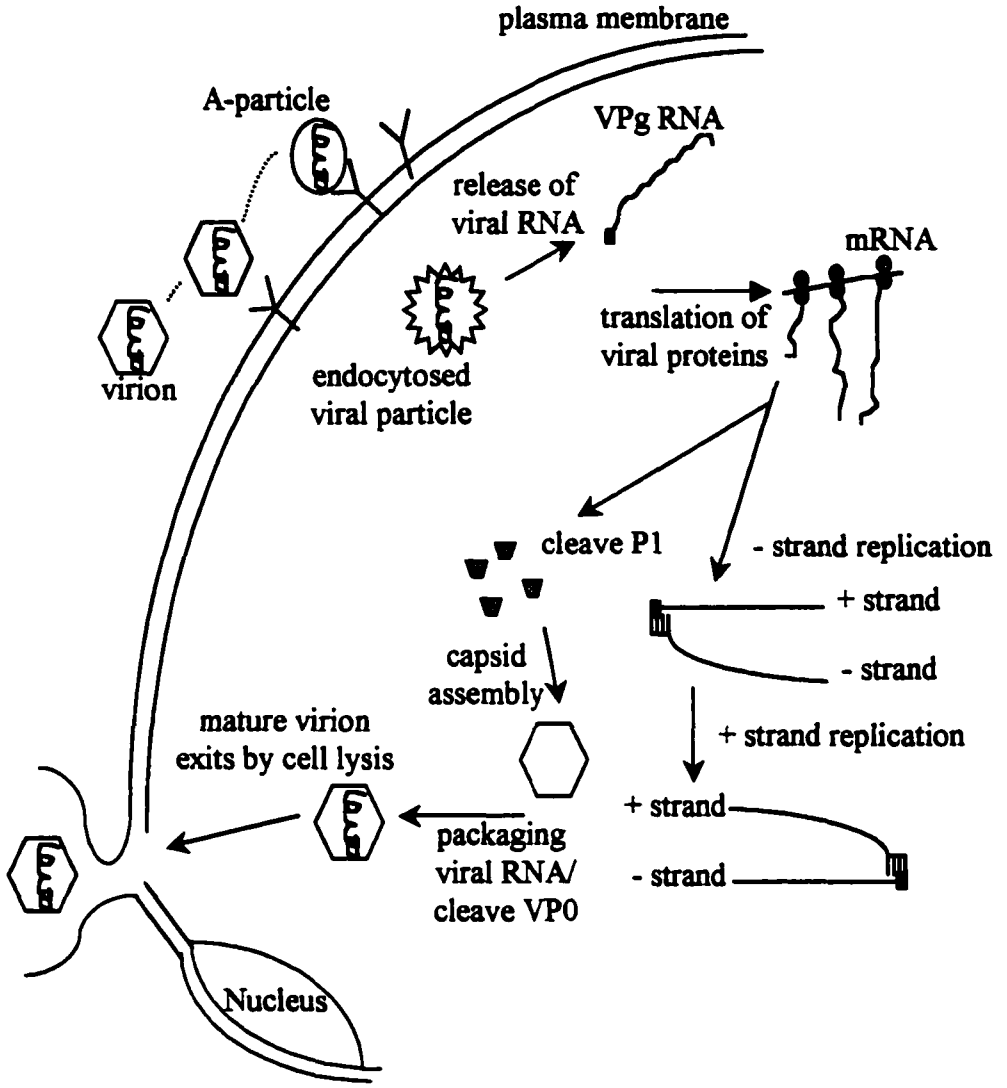


Fig. 2. Poliovirus life cycle. Poliovirus life cycle begins with the binding of virus to the poliovirus receptor, PVR (CD155). After binding to the receptor, the virus undergoes a conformational change to form the A particle. The virus is then thought to be endocytosed into the cytoplasm of the cell, where it uncoats and the viral RNA is released. The viral RNA is translated as a single polyprotein using host cell ribosomes and cleaved by virally encoded proteases. Once processed, the polymerase $3D^{pol}$ starts replication of the viral RNA. Newly synthesized polioviral RNA is then encapsidated, after which maturation cleavage occurs (VP0 – VP2 +VP4). Last, the mature virus is released from the cell by cell lysis.

strand RNA. The synthesis of plus- and minus-strand poliovirus RNA is uneven where the plus strand is produced in excess of approximately 10 times greater than the minus strand (12, 166). The plus-strand RNA produced can serve one of several roles. It can serve as the mRNA for synthesis of more viral proteins, as a template for the production of more minus-strand RNA, or as the viral RNA genome to be encapsidated for the production of new progeny virions (166). The only difference between the two RNAs is that RNAs that are to be used for the production of more viral proteins have no VPg linkage and those that are to be used for packaging into capsids to make progeny viruses are linked to the VPg protein (98, 126, 127). The process by which the RNA is packaged into the viral capsid is not clear. The two theories present are that the capsid forms a shell around the RNA or that the RNA is packaged into a previously formed empty capsid (80, 166). Although poliovirus is a lytic virus, there is some controversy as to how the newly formed viruses exit the cell. Some studies have shown the vectorial release of poliovirus from cells prior to cell lysis, whereas other studies show the induction of apoptosis of the host cell by poliovirus (194, 199).

Receptor binding and entry. The replicative success of any virus in terms of infectivity is dependent upon its recognition of a receptor and its subsequent internalization. For poliovirus, not all of the specific mechanisms for cell entry are well understood. However, studies on binding and uncoating have led to the following views on the initial stages of poliovirus cell entry. As stated before, upon viral recognition of its receptor, poliovirus binds to the PVR, which leads to the start of the uncoating process. The capsid residues found to be important for regulating binding to the PVR are located

in the canyon of the poliovirus capsid (43). PVR is thought to be the only element involved in uncoating. However, CD44H, an isoform of the lymphocyte homing receptor, has been shown to have some interaction with PVR, although it does not act as a receptor for poliovirus and is not required for replication (15, 30, 180). The conformational change of the virion that occurs as a consequence of binding involves loss of the internal capsid protein VP4 and the externalization of the amino terminus of the once internalized VP1 capsid protein (64, 177, 178). This conformational change leads to the formation of altered particles (A particles) that are approximately 4% larger than the virion (19). These A particles, also known as 135S particles for their sedimentation value, are sensitive to proteases and detergents and are no longer able to bind to susceptible cells (19, 64, 177). In addition, A particles are thought to be very important intermediates in the uncoating process. This importance is highlighted through several studies that show the reduction of poliovirus infectivity following inhibition of this conformational change, the potential to infect nonpermissive cells, and its affinity for liposomes (15, 19, 47, 63, 64). Once formed, the A particles can take one of two paths. They can be either internalized or sloughed off the cell surface (24, 166). Greater than 80% of viruses that bind PVR and form A particles are sloughed off, rendering the entry of poliovirus into cells an abortive process (166). For the remaining 20% that are destined for internalization, the path by which entry occurs is debated. One thought is that virions enter the cell through receptor-mediated endocytosis (64, 166). In this model, the virion is endocytosed into the cytoplasm to form clathrin-coated vesicles. Acidification of the vesicles leads to the release of VP4 and the externalization of the N terminus of VP1. The hydrophobic N terminus then attaches to liposomes, allowing release of viral RNA from the virion into

the cytoplasm (64, 166). The other view, however, involves a possible concerted effort between VP4 and VP1. VP4 is a myristylated 7-kDa capsid protein found on the inside of the virion that is known to play a role in the stabilization of the virus particle (37, 117, 118). Yet, there is some other evidence that VP4 may also be involved in later stages of cell entry (19, 118). This second model for poliovirus cell entry follows with binding of the virus to the receptor, which leads to the conformational change and formation of A particles. The capsid proteins VP4 and N terminus of VP1 are projected from the bottom of the canyon, which is the site of binding, as stated before. The N terminus of VP1, which is thought to form an amphipathic helix (19, 64, 84), and perhaps even the amino-terminal myristate of VP4 then come together and insert themselves into the cell membrane, forming a pore. A VP3 plug is removed, opening the pore, which allows the viral RNA to exit into the cytoplasm (165).

Viral RNA translation and proteolytic cleavage. Polioviral proteins are synthesized by the translation of the genome's single polyprotein. The approximate time required for this translation is 10-15 min (165). As stated before, translation of the polyprotein is initiated through the binding of host cell proteins to the IRES of poliovirus just upstream of the AUG initiation codon. The host cell proteins thought to be involved in this process are La or p52 (188); poly (rC) binding protein 2 (28); and the polypyrimidine tract protein, p57 (72, 83). Following binding of host cell proteins to the IRES, translation of the polyprotein ensues. As in other picornaviruses, translation occurs solely in the cytoplasm of the cell with no nuclear products required (156). Also, it is during this translation that host protein synthesis is shut off. Whereas most cellular

mRNAs require the binding of the cap-binding complex (CBC) to the m^7cap for translation, poliovirus does not because it uses an IRES instead of an m^7cap. Therefore, to shut off host protein synthesis, it is implicated that the polioviral protein $2A^{pro}$ mediates cleavage of the protein p220, a large part of the CBC, which is needed for the initiation of host protein translation (22, 71, 94). It is through this cleavage of p220 that translation of capped mRNA molecules is prevented, thereby shutting off host protein synthesis. However, this is now controversial. Although there are some studies say purified $2A^{pro}$ does not directly cleave p220 but activates cellular proteases for this event (207), others show purified 2A proteins from two similar picornaviruses (rhinovirus 2 and coxsackievirus A) can cleave p220 directly (185). Ultimately, host protein synthesis is shut off, and viral protein synthesis from the polyprotein proceeds. As shown in Fig. 3, the polyprotein consists of 3 proteins, P1, P2, and P3. These proteins are produced through the cleavage of the polyprotein by virally encoded proteases. The first of these cleavages releases the structural protein P1 from the nonstructural proteins P2 and P3. This cotranslational cleavage occurs at the tyrosine-glycine junction of P1 and P2 and is mediated by $2A^{pro}$ (197). Subsequently, the nonstructural proteins P2 and P3 are cleaved by the viral protease $3C^{pro}$ at glutamine-glycine junctions (88, 93). The structural protein P1 is further cleaved into its individual proteins VP0, VP1, and VP3 by the viral protease 3CD also at glutamine-glycine junctions (88, 93, 97, 212). The 3CD polyprotein is then processed by $2A^{pro}$ at a tyrosine-glycine junction, resulting in the production of 3C' and 3D' (88, 93, 97).

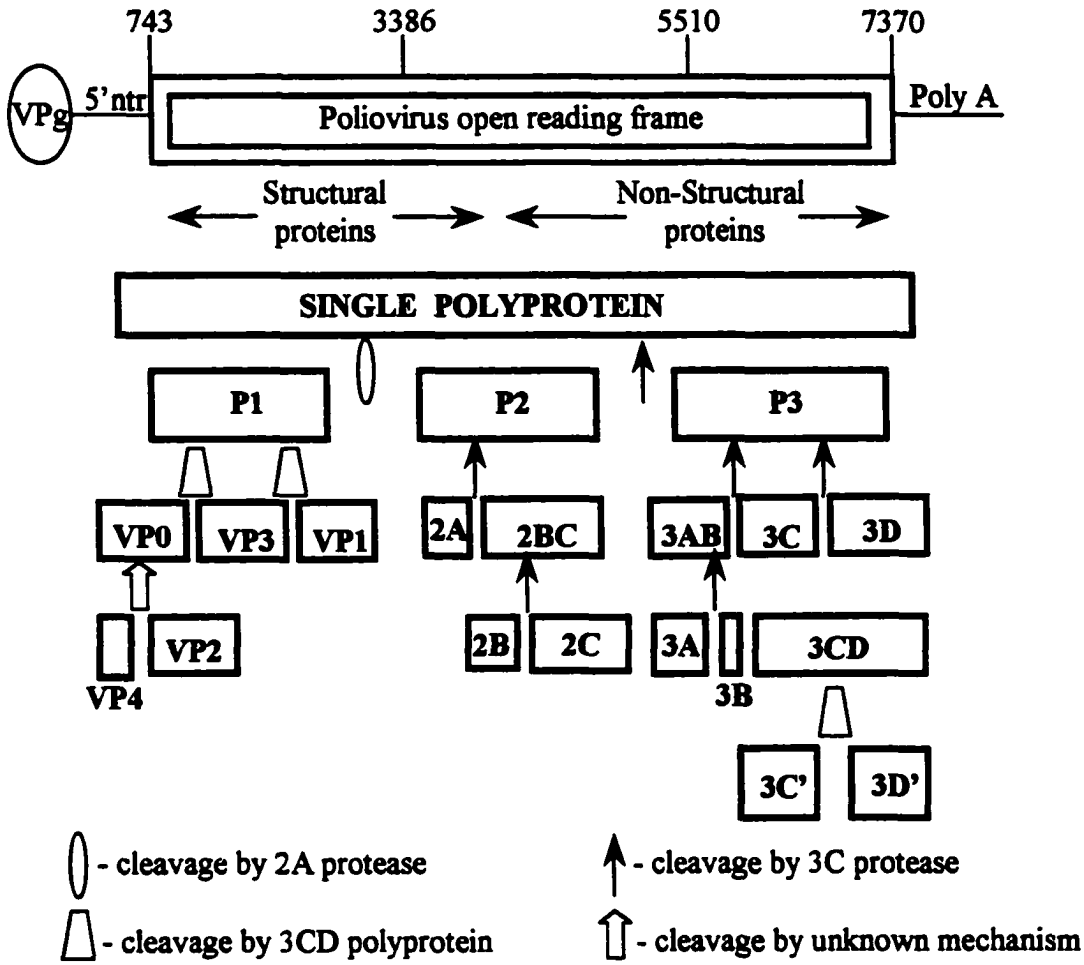


Fig. 3. Processing of the poliovirus polyprotein. Poliovirus is translated as a single polyprotein. This protein is proteolytically cleaved by virally encoded proteases to release the capsid protein P1 from the nonstructural proteins P2-P3. Following release of P1, P2 and P3 are then cleaved. The P1 or capsid is further cleaved by 3CD to form VP1, VP0, and VP3. The majority of the P2 and P3 proteins are cleaved by the protease 3C, with the exception of 3CD, which is cleaved into 3C' and 3D' by the protease 2A. The final cleavage of the capsid, maturation cleavage, is the cleavage of viral capsid protein VP0. This cleavage occurs by an unknown mechanism to produce VP4 and VP2.

Proteases. The virally encoded proteases play a very important role in the life cycle of poliovirus. In poliovirus, 3 proteases are encoded within the genome: $2A^{pro}$, $3C^{pro}$, and 3CD. The protease $2A^{pro}$ is a 16-kDa protein involved in the release of structural protein, P1, from the polyprotein, as well as in the shutoff of host protein synthesis (23, 71, 197). The release of the capsid precursor, P1, occurs by a cotranslational cleavage at a tyrosine-glycine bond (197). The second cleavage by $2A^{pro}$ also occurs at a tyrosine-glycine bond, but in *trans* with the viral protein 3CD (93, 97, 212). This cleavage results in the production of 3C' and 3D' proteins. The notion that $2A^{pro}$ is involved in the shutoff of host protein synthesis comes from a study in which a poliovirus whose $2A^{pro}$ was mutated with a single insertion proved unable to cleave p220 during infection and changed the host protein shutoff pattern (23). A few years later, a group demonstrated that $2A^{pro}$ alone could not cleave p220 but instead could activate other cellular proteases for this purpose (207). This latter study refuted the idea that a purified $2A^{pro}$ could directly cleave p220. However, studies of two very similar picornaviruses (rhinovirus 2 and coxsackievirus A) demonstrated that purified $2A^{pro}$ could indeed cleave p220 (185). In any case, studies do show that $2A^{pro}$ is not essential for either replication or encapsidation of poliovirus (11, 130).

The protease $3C^{pro}$ is a 43-kDa protein involved in cleavages relating to P2 and P3 (88, 93, 165). The cleavage of $3C^{pro}$ occurs in *cis* and in *trans* at glutamine-glycine bonds (88, 93). Although there are as many as 13 glutamine-glycine pairs in the poliovirus genome, only 8 are used by $3C^{pro}$, which suggests other determinants for site recognition (88, 114). When looking at sites cleaved by $3C^{pro}$, it was observed that $3C^{pro}$ had a

preference for either an alanine or valine residue four amino acids from the newly formed carboxy terminus after cleavage (114, 131).

The third protease involved in the poliovirus life cycle is 3CD. 3CD is a 72-kDa protein responsible for proteolytic processing of the P1 precursor at glutamine-glycine bonds (88, 93, 97, 212). Following cleavage of the P1 precursor, the formation of VP0, VP3, and VP1 results. Not only is 3CD involved in the proteolytic processing of capsid precursors, but it is also possibly involved in the synthesis of positive-strand RNA during RNA replication (6, 7).

Replication of Viral RNA. Replication of poliovirus RNA requires an RNA-dependent RNA polymerase called $3D^{pol}$. However, before replication can begin, translation must occur. All of the proteins required for replication are located in the P2 and P3 regions of the poliovirus genome. This includes $3D^{pol}$, which is derived from P3. Once $3D^{pol}$ is present, the first step in RNA synthesis is to copy the positive-strand RNA genome to make several minus strands (157). In order to make these minus-strand copies, $3D^{pol}$, a VPg donor, and host factor protein are needed (165, 181). The method by which initiation of minus-strand synthesis occurs is not clear. One theory is that VPg donates a VPg-pUpU primer that is elongated by $3D^{pol}$, using the positive-strand RNA genome as a template (134, 192, 193, 206). The second theory proposes that the host enzyme, terminal uridylytransferase (TUT) adds poly (U), forming a hairpin that can self-prime and be elongated by $3D^{pol}$. After elongation, VPg is added and cleaves the newly synthesized negative strand from the positive-strand template (210, 211). Once the negative strand has been synthesized, it is used as a template in the synthesis of more positive strands.

During replication, several intermediates are found. First, there is the replication intermediate (RI). The RI is a single-stranded molecule containing a full-length template with stretches of base pairing between nascent daughter strands (157, 158). Second, there is the replicative form (RF) intermediate. The RF is a double-stranded molecule that is sometimes found in a hairpin-like structure (in other words, a double-stranded molecule consisting of a covalently attached positive and minus strand [206]). Last, there is the single-stranded RNA intermediate. These RNAs are most abundant and are indistinguishable from the viral RNA. They have VPg covalently attached to the 5' end with a poly(A) tail at the 3' end (98, 157). These intermediates are found in replication complexes along with other viral proteins necessary for RNA replication, which are in association with smooth membranes (25, 26).

The other viral proteins necessary for replication are 2BC, 2B, and 2C. The precursor protein 2BC is thought to be responsible for providing the membranous structures necessary for RNA synthesis. This is done presumably by inducing membranes of the host cell to proliferate (26). The proteins 2B and 2C are both shown to be involved in RNA synthesis through mutational analyses of cDNA clones (23, 99). In addition, 2C has been shown to be sensitive to the drug guanidine, which results in loss of replication in poliovirus (17, 142, 143). As for P3, the proteins necessary for replication are the precursor 3AB, which is thought to initiate RNA synthesis (17, 132, 175), and 3B, better known as VPg, which has been discussed earlier as a major player in the early events surrounding RNA synthesis (134, 192, 193, 206).

Capsid assembly. Capsid assembly, or morphogenesis, is a very important event in the life cycle of poliovirus. Its importance is based on the fact that without capsid, no mature virion can be made. Due to its relatively simple structure and availability of subviral intermediates, which can be isolated from infected cells, poliovirus has been a very attractive model for studying morphogenesis. The poliovirus capsid consists of 60 copies each of four capsid proteins (VP1, VP2, VP3, and VP4) (77). The capsid has an icosahedral symmetry and is approximately 300 Å in diameter when RNA is packaged inside (18). As shown in Fig. 4, poliovirus morphogenesis begins with the initial cleavage of P1 from the P2-P3 precursor of the poliovirus genome by $2A^{pro}$ (197). After this initial cleavage, the P1 precursor or protomer is then cleaved by 3CD, producing VP0, VP3, and VP1 (73, 88, 93, 97, 212). The final processing step of the capsid, the cleavage of VP0 into VP2 and VP4, is called the maturation cleavage. This cleavage occurs late in assembly and is probably done in conjunction with encapsidation of viral RNA (73). Although the mechanism by which this cleavage occurs is thought to be autocatalytic (77), it has yet to be proven. However, studies have shown this cleavage to take place at an asparagine-serine bond (73, 75).

The assembly of these proteins is essentially a three-step process. Cleavage of P1 results in the formation of a 5S immature protomer that consists of one copy each of VP0, VP3, and VP1. Five 5S protomers then assemble into the 14S pentamer. Subsequently, 12 14S pentamers assemble to form the 75S empty capsid, which consists of 60 copies each of VP0, VP3, and VP1. The 75S empty capsid can then go on to become the 155S mature virion, which is composed of approximately 60 copies each of VP1, VP2, VP3, VP4, and one to two copies of VP0, the precursor of VP2 and VP4 (12, 18, 73). Although the

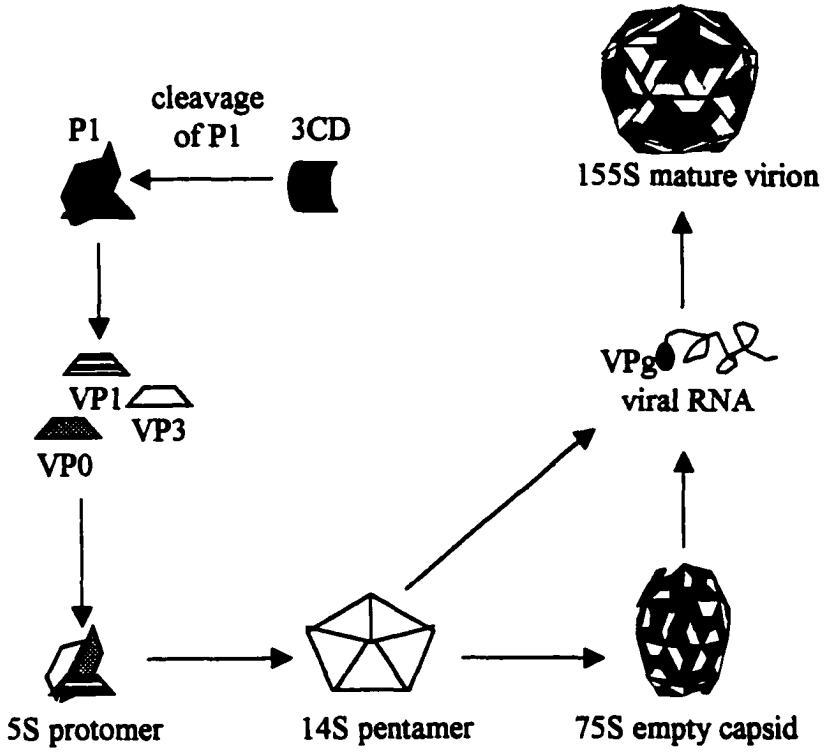


Fig. 4. Poliovirus capsid assembly. Capsid assembly begins with the cleavage of P1 by 3CD. 3CD cleaves P1 into the individual capsid proteins VP0, VP1, and VP3. These proteins assemble into the 5S protomer. Five 5S protomers come together to form the 14S pentamer. After the formation of the pentamer, 12 pentamers then assemble to form the 75S empty capsid. Viral RNA that has VPg covalently attached is then encapsidated, giving rise to the provirion, which undergoes a maturation cleavage. The maturation cleavage is the cleavage of VP0 into VP4 and VP2. Following maturation cleavage, the 155S mature virion is formed. Based on Ansardi et al., 1996.

stepwise assembly of the poliovirus capsid is generally accepted, there is controversy as to whether the pentamer is a direct precursor to the poliovirus virion (147, 165). A couple of pieces of evidence support the idea that 14S pentamers are immediate precursors to the virion. Pulse-chase experiments using poliovirus-infected cells demonstrate the ability of 14S pentamers to go on to form both empty capsids and virions (80). Lastly, experiments that inhibited replication of poliovirus-infected cells with the drug guanidine demonstrated the inhibition of virion formation and the accumulation of 14S pentamers (66). However, upon removal of this drug, the 14S pentamers were rapidly converted into poliovirions with no evidence of empty capsid formation, thereby providing further evidence for the proposed theory that the 14S pentamer is a direct precursor to the poliovirion.

Capsid intermediates. During morphogenesis, several intermediates evolve. These intermediates are the 5S protomer, the 14S pentamer, the 75S empty capsid, and the 125S provirion (147). The 5S protomer, generated from cleavage of the P1 precursor, consists of the capsid proteins VP0, VP3, and VP1 (165, 197). Their molecular weights are 37.4, 33, and 26 kDa, respectively (89). Although cleavage of P1 is completed by 3CD (88, 93, 97, 212), studies show that the protease $3C^{pro}$ can also cleave P1 but not as efficiently (92). Once P1 is cleaved into VP0, VP3, and VP1, it is proposed that the three proteins remain together as a 5S protomer (12). The next intermediate is the 14S pentamer. The 14S pentamer is comprised of five 5S protomers (147). It has been designated as one of the key intermediates in the assembly of picornaviral capsids (73). The reason for this recognition is that it can go on to form either 75S empty capsids or the 155S mature

virion (73, 147). Although the 14S pentamer is proposed to be the direct precursor to the virion, the 75S empty capsid has also been proposed to be the direct precursor to the virion (147). The 75S empty capsid, which consists of 12 pentamers, is probably the most contentious intermediate (147). It exists in two forms, native (N) or heated (H) (148, 165). The N-antigenic empty capsid is labile and has the same epitopes as the wild type or N virus (148). On the other hand, the H-antigenic empty capsid is more stable and has a totally different set of epitopes. The sedimentation coefficients of these empty capsids differ. The N-antigenic empty capsid has a sedimentation coefficient of 65-70S, whereas the H-antigenic empty capsid has a sedimentation coefficient of 80S (89, 148). The final intermediate is the 125S provirion. The provirion particle is the same as the empty capsid, except for the incorporation of the RNA genome (147). This particle is different from the mature virion in that VP0 has not been cleaved into VP2 and VP4 (12). The existence of this intermediate was first evident in sedimentation studies of poliovirus. A shoulder of 125S was observed on the 155S peak on sucrose density gradients (58, 59). It was also observed that these particles contained a substantial amount of VP0 compared to the 155S mature virion.

Encapsidation of viral RNA. The mechanism of packaging viral RNA into capsids, or encapsidation, is not well defined. One theory proposed by Jacobson and Baltimore is called the procapsid hypothesis (80). It is based on the packaging of RNA into a preformed empty capsid. There are two proposed models to explain the mechanisms behind this theory. First, the threading model suggests that RNA is threaded through a pore in the empty shell. Last, the transfiguration model suggests that the RNA

could wrap around the procapsid or empty capsid, prompting restructuring of the VP0, VP3, and VP1 subunits and thereby allowing internalization of the RNA. The second and most accepted hypothesis for encapsidation simply proposes the assembly of 14S pentamers around the viral RNA (66). It is also interesting to note that only positive-sense RNAs with a VPg covalently attached are packaged (98, 127). This leads to a question of whether this specificity of packaging is based on a packaging signal or sequence. However, one thought on this issue is that packaging of RNA is coupled to RNA replication (128). This is supported by several studies. One study shows that upon addition of low concentrations of guanidine hydrochloride, an agent that inhibits poliovirus replication, both RNA synthesis and formation of mature virions were arrested (80). Another study demonstrated that RNAs associated with active replication complexes were packaged into capsids, whereas those RNAs associated with guanidine-inhibited replication complexes were not (128). Another interesting note on the specifics of poliovirus encapsidation is its size constraints. Studies on defective-interfering (DI) genomes show that the smallest genome capable of encapsidation is between 80% and 87% of the 7.4-kb poliovirus genome (95, 104, 206). In terms of the longest genome capable of encapsidation, a genetically engineered dicistronic genome containing the IRES of encephalomyocarditis virus (EMCV), which was 108% of the length of the poliovirus genome, was encapsidated (115).

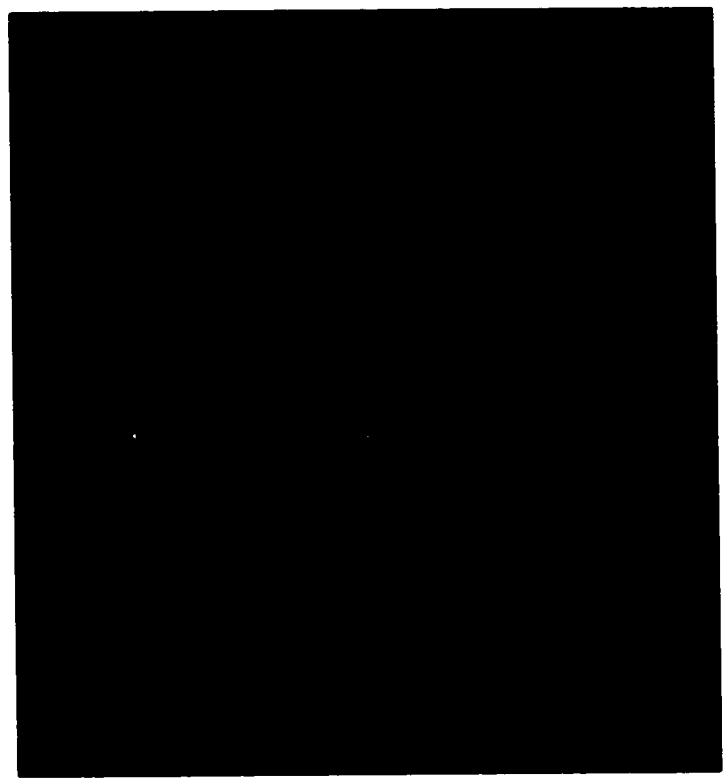
Poliovirus Capsid

The three-dimensional structure of poliovirus has been determined by X-ray crystallography (77). From that study, the poliovirion was again shown to be composed

of the viral capsid proteins VP1, VP2, VP3, and VP4 and a single positive-strand RNA genome (77). As stated before, only positive-strand RNA with VPg attached are packaged into capsids (98, 127). The capsid itself is approximately 5 nm thick and 30 nm in diameter (165). It is icosahedral in shape and has no lipid envelope. Its sedimentation coefficient is 156S and has a buoyant density of 1.34 g/cm^3. This low buoyant density is direct evidence of the impermeability of poliovirus to cesium (89). Poliovirus cesium-impermeable capsid prevents binding of heavy atoms to viral RNA (165). The capsid is also a very stable structure in that it is resistant to sodium dodecyl sulfate (SDS) up to 1% concentration and acidic conditions (89). The capsid is also resistant to 70% alcohol, 5% Lysol, ether, deoxycholate, and other detergents known to destroy other viruses (111). When the capsid is bound to the PVR, the sedimentation coefficient changes from 156S to 135S. The 135S particle differs from the native 156S particle in that it has lost VP4, is sensitive to proteases, can attach to liposomes, and has antigenic properties like heat-inactivated viruses (64).

Structure of the poliovirion. X-ray crystallographic structures have been resolved for several picornaviruses, including FMDV, HRV14, and Mengo virus (1, 105, 164). The three-dimensional structure of poliovirus, as determined by Hogle at 2.9 Å (77), shows a 180-subunit icosahedral shell with pseudo T = 3 symmetry as shown in Fig. 5. The shell has a pseudo T = 3 symmetry because the viral proteins VP1, VP2, and VP3, which make up the majority of the shell, have different sequences (12). Although these proteins have different sequences, the structural similarity is great. They all form what is called an eight-strand antiparallel β-barrel, or swiss roll (Fig. 6) (165). This shape allows

Fig. 5. Structure of the poliovirion. This figure is a stereo space-filling representation of the exterior capsid surface. VP1 is blue, VP2 is yellow, VP3 is red, and VP4 is green. Some notable features of the capsid are the fivefold peak formed by VP1, the saddle across the twofold axis, the threefold axis formed by alternating VP2 and VP3 proteins, and the icosahedral symmetry of the particle as indicated by the edges of the particle. Used with permission from Hogle et al., 1985.



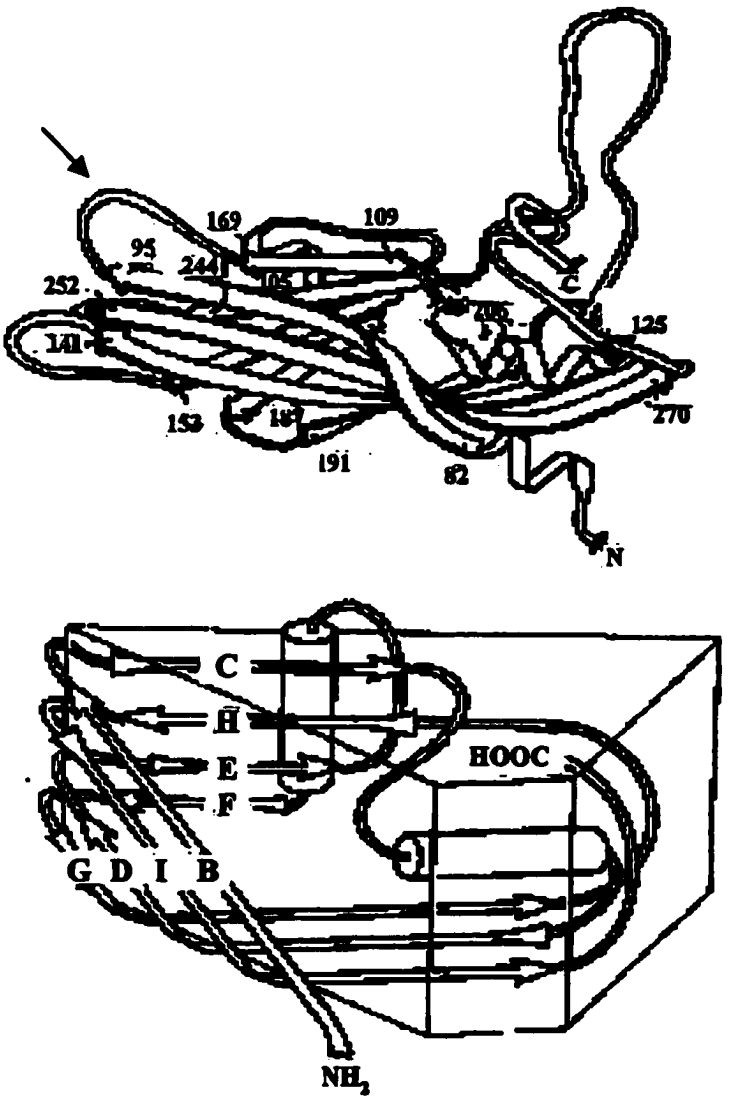


Fig. 6. Schematic representation of the poliovirus capsid proteins. The top figure is a ribbon diagram of VP1. The BC loop, identified with an arrow, is formed from the β-strands B and C starting at amino acid 95 and ending at amino acid 105, as shown in this ribbon figure. The bottom figure is a schematic of the β-barrel structure of the capsid proteins. It depicts the two four-stranded β-sheets arranged in antiparallel fashion. The sheets are depicted as arrows and the loops connect them. Coils are depicted as tubes. The PROW, which is made up of the BC, HI, DE, and EF loops, is shown on the left. The carboxyterminus and aminoterminus of the protein is given for clarity. Used with permission from Hogle et al., 1985.

each subunit to form compact triangular wedge-like structures. These wedge-like structures are the basic core structures that form the virion shell, with five VP1 subunits making up the fivefold axes and with alternating VP2 and VP3 subunits making up the three-fold or quasi-sixfold axes (19, 73). Crystallographic studies also revealed that VP1, VP2, and VP3 were exposed on the surface of the virion, whereas VP4 lies buried inside in close proximity to the RNA genome (12, 73, 165). The interactions of the wedge-like structures, VP4, and the amino-terminal extensions of VP1, VP2, and VP3 help stabilize the virion (19). In addition to sequence difference, these proteins also differ in size and in arrangement of the loops that connect the strands of the β-barrels (73). In terms of size, VP1, VP2, and VP3 are about 250 amino acids in length; however, VP4 is much smaller (165). It is about 70 amino acids in length and is thought of as an amino-terminal extension of VP2 because of its position inside the virion (12, 73). The β-barrel structure (Fig. 6) is created by two four-stranded β-sheets that are arranged in antiparallel fashion enclosing a hydrophobic pocket (12, 73). The β-sheets are alphabetically labeled from B to I as they occur from the amino to carboxy termini of the protein (12). The loops that connect the strands of the β-barrels, β-sheets, decorate the surface of the virion and serve as antigenic determinants of the virus (73, 165). These loops are labeled according to the strands they connect. With the exception of loop GH, the other loops, BC, HI, DE, and EF, form what is referred to as the "PROW" of the wedge-like structure (165). There are several other prominent features of the virus structure. The canyon, which is the putative binding site, surrounds the peak formed at the fivefold axis by VP1 (73, 77, 163). Last, there are three protrusions found at the five-, three-, and twofold axes, respectively. The first protrusion, at the fivefold axis, involves the loops connecting the top three strand

pairs of the narrow side of the VP1 β-barrel. The second protrusion, at the twofold axis, involves the EF loop of VP2 and the GH loop of VP1. Finally, the third protrusion, at the threefold axis, involves the top two strands of the narrow side of VP2 and VP3 β-barrels (129).

Neutralization antigenic sites. The viral capsid proteins VP1, VP2, and VP3 all form an eight-stranded, antiparallel β-barrel structure, as stated before. Each of these proteins comes together to form the shell or capsid, which surrounds the viral RNA. The surface of the virion is decorated by the loops that link the eight antiparallel β-strands of each capsid protein (VP1, VP2, and VP3). These loops, as previously stated, serve as the antigenic determinants of poliovirus. Although the antigenic structure of poliovirus is the same for all three serotypes, the antigenicity of each serotype is different so that neutralizing antibodies of one serotype has no effect against the other two (120, 122). Poliovirus has three neutralization antigenic sites (N-Ag) to which these neutralizing antibodies can bind (120, 122, 129): N-AgI, N-AgII, and N-AgIII (120, 122). For all of the amino acids discussed within the three major antigenic sites of poliovirus, a four-digit nomenclature is used (120). The first digit of each number represents the capsid protein from which it is located (i.e., VP1, VP2, or VP3). The remaining three numbers represent the actual position of the amino acid within the protein. N-AgI, unlike the other two, is a continuous sequence of amino acids located on a well-exposed loop linking the β-strands B and C, called the BC loop of VP1 (50, 77, 119). It includes amino acids 1097 to 1101 of the BC loop and also amino acids 1142, 1144, and 1147 of the DE loop of VP1 (120) and is located at the apex of the viral particle (122). The second loop, N-AgII, is a

discontinuous stretch of three amino acid segments. These segments consist of amino acids 1221 to 1226, which make up the GH loop of VP1; amino acids 2164 to 2170 of the EF loop of VP2; and amino acid 2270, which is located at the C terminus of VP2 (129). The last loop, N-AgIII, is also discontinuous but is composed of two different sites. As a result, N-AgIII is designated as N-AgIIIA and N-AgIIIB. N-AgIIIA is composed of amino acids 3058 to 3060 of the β-turn preceding the β-B strand of VP3 as well as amino acids 3071 to 3073 of the VP3 β-B strand itself (120). N-AgIIIB is comprised of amino acids 2072 of the BC loop of VP2 and amino acids 3076 to 3079 of the BC loop of VP3 (120). Both N-AgIIIA and N-AgIIIB make up loops that are prominent features near the three-fold axis of the virion surface (129).

PVR

The initiation of most viral infections begins with the binding of a virus to a receptor. The receptor for poliovirus is also called CD155 (67, 112). The gene encoding the PVR has been mapped to human chromosome 19 (113). Two clones of PVR (H20A and H20B) have been cloned and sequenced and found to be approximately 3.3 kb in length, differing only in the carboxy terminus (112). The amino acid sequences derived from the nucleic acid sequences of the PVR cDNA clones have determined PVR to be a member of the immunoglobulin superfamily (112, 150). This distinction was proven when it was shown that the structure of PVR is characteristic of members of the immunoglobulin superfamily. The structure of PVR consists of an amino-terminal signal sequence, three extracellular immunoglobulin-like domains, a transmembrane domain, and a cytoplasmic tail (112). Studies done on the PVR a year later revealed four other

clones in addition to H20A and H20B (90). These clones were named PVRα, PVRβ, PVRγ, and PVRδ. PVRα and PVRδ were discovered to be the same as H20A and H20B; however, PVRβ and PVRγ were found to be missing the transmembrane domain found in the other two PVR clones (90). These studies showed that PVR protein could be produced in both a transmembrane and a secreted form after alternative splicing of the transmembrane form (90). Also, two other genes related to PVR (PRR1 and PRR2) have been identified, although their ability to function as a PVR is not yet known (55, 103, 150). The portion of the PVR thought to be involved in binding to the virus is believed to be found in the first immunoglobulin-like domain 1 (D1) of PVR (150). Mutational studies show that the main sites in D1 that touch the viral surface are the C-C' loop, the D-E loop, and the G strand of PVR (70, 116, 150). However, of the three sites, the C-C' loop is believed to be a major player in binding; the D-E loop is thought to be support for the C-C' loop (150). Mutations in strand G were found to inhibit binding to serotypes 1 and 2 of poliovirus but not type 3 (70); therefore, its importance in binding is dependent upon the serotype involved.

DI Particles

Many interactions between viruses can affect the phenotypic expression of the wild type virus, thereby covering up the true viral genotype. These interactions include phenotypic mixing, viral defectiveness, integration, viral persistence, and interference (38). There are several types of interference, but the one most important in the study of poliovirus is homologous interference. Homologous interference occurs within the cell between homologous viruses or some closely related viruses (38). One form of

homologous interference occurs in viruses that have been serially passaged at high multiplicities of infection (MOIs). Following these passages, the number of viral particles remains constant; however, the number of infectious particles drops. Consequently, the interference of growth of the infectious population seems apparent. Analysis of these interfering viruses show that a large portion of them have deletions, thereby giving rise to a large number of DI viral particles (38). Poliovirus, among other viruses, is known to have DI particles within its population. These were first described in 1971 (42). After passaging poliovirus at a high MOI (>200 PFU/cell), a population of viruses appeared with slower sedimentation properties in sucrose density gradients. These viruses also had a lower buoyant density of 1.31-1.325 g/cm^3, compared with the 1.34 g/cm^3 buoyant density of wild type polio (42). Not unlike other viruses, the poliovirus DI particles also exhibited the characteristic interference of wild type poliovirus production in mixed infections, thereby resulting in an enrichment of the poliovirus DI population over wild type poliovirus (40,41). As stated before, a characteristic of interfering particles is that they generally have large deletions within their genomes. For poliovirus DI particles, those deletions were found to occur within the 5' region of the RNA genome (42, 104). As a result of sequencing several DI particles from poliovirus type 1 Sabin, it was found that these entities contained deletions within the P1 capsid region while maintaining the translational reading frame for P2 and P3 of the RNA genome (95). These deletions within P1 were localized to nucleotides 1226 to 2705, which cover a large portion of the VP2 and VP3 genes (95). This finding along with others demonstrated that sequences at the 5' end of the P1 capsid were not essential for RNA replication (12, 35, 85).

Using Poliovirus as an Expression Vector

Poliovirus replicons. Previous genetic studies of poliovirus have shown several segments of the genome to be either important or not essential for many viral functions such as replication and encapsidation (10, 86, 198). Studies on the DI particles have shown that large portions of the 5' end of P1 can be deleted and have no effect on replication (42, 85, 95). These findings prompted studies on the use of poliovirus as an expression vector. Early studies in the use of poliovirus as an expression vector were done by constructing "minireplicons," which contained in-frame substitutions of human immunodeficiency virus type 1 (HIV-1) *gag, pol,* and *env* segments for portions of P1 (35). Although these "minireplicons" showed that the poliovirus replication machinery was capable of replication through expression of the *gag, pol* and *env* proteins, they were unable to be encapsidated, probably because of the inability to compete with wild type poliovirus for capsids (35). More studies involving the replacement of foreign genes into portions of P1 have since evolved. In a study by Percy et al (1992), the reporter gene chloramphenicol acetyltransferase (CAT) was substituted for nucleotides 756 to 1805 and shown to replicate and encapsidate by superinfection of a helper virus (137). In a second study, the rotavirus capsid gene VP7 was inserted into either the 5' or 3' end of P1 (109). Like others, this study not only demonstrated replication but also proved that one could insert genes that result in an increase in the size of the poliovirus genome. Until this time, studies had shown that large segments of P1 could be removed and replication would still ensue. However, studies by Andino et al. (1994) proved that the entire P1 region could be deleted without disrupting replication (8). In this study, the entire P1 gene from nucleotides 756 to 3371 was removed and replaced with the firefly luciferase gene.

Whereas this study proved that deletion of the entire P1 allowed replication, it did not mention whether encapsidation also occurred (8). Nevertheless, this study did prove, along with that of Mattion et al. (1994), that foreign genes could be inserted into the P1 region, including those that resulted in an increase in genome size, without blocking RNA replication (8, 109).

Poliovirus replicon encapsidation. The finding that the entire P1 gene was dispensable and could be replaced with a foreign gene without disrupting replication was a major discovery in the development of poliovirus as an expression vector. In addition, the discovery that poliovirus cDNA was infectious was also a major contribution to allowing molecular genetic manipulations of the genome, which would allow better use of this system (151, 176). However, a major obstacle towards fulfilling the potential use of poliovirus expression vector systems was finding out how to encapsidate these mutated genomes since they did not compete well for capsids in mixed infections with wild type polio. This obstacle has been successfully overcome in this laboratory. Previous studies from this laboratory have shown the encapsidation of replicon and DI genomes through the use of recombinant vaccinia viruses that express the poliovirus capsid P1 (VVP1) (14, 144). These replicons expressed foreign genes that replaced either the entire P1 region or all but VP4 of P1 (35, 145). In the replicon shown in Fig. 7, the foreign gene, which has $2A^{pro}$ cleavage sites on both ends, is placed between VP4 and 2A of the poliovirus genome. The purpose of the $2A^{pro}$ cleavage sites, which flank the ends of the foreign gene, is to allow release of the foreign protein following translation. The method by which these replicons are encapsidated is shown in Fig. 8. In brief, cells permissive to

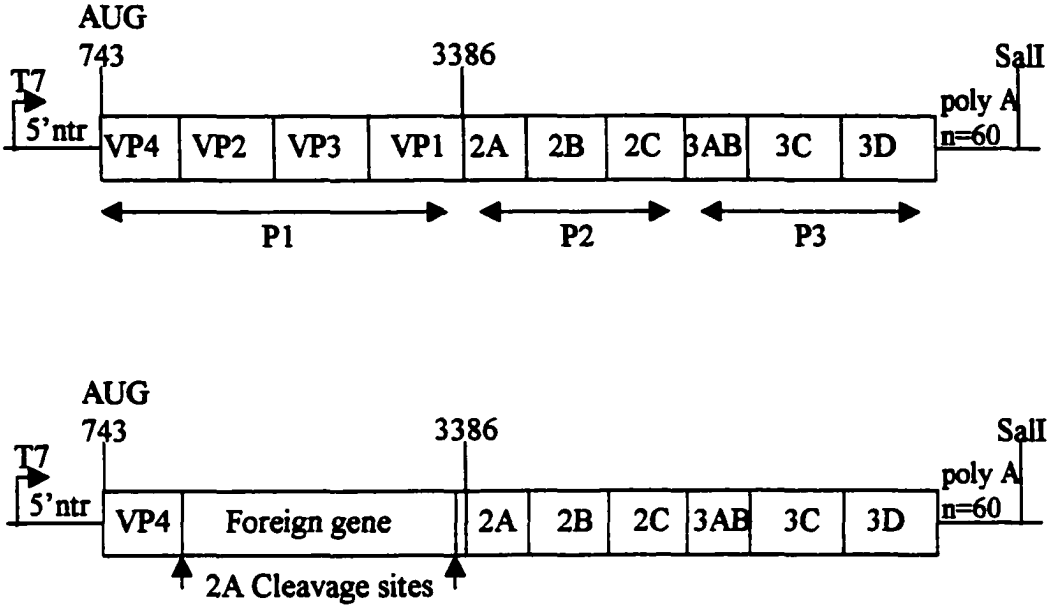


Fig. 7. Replicon genome. The top figure is a depiction of the poliovirus cDNA from which the replicon is generated. It has unique restriction sites on the 5'and 3' ends and a T7 promoter, which allows the use of T7 RNA polymerase for the generation of large quantities of RNA in vitro. The genome of the replicon, as shown in the lower figure, consists of a foreign gene inserted between VP4 and 2A of the poliovirus genome. It also has 2A cleavage sites flanking the ends of the foreign gene to allow for easy release. Based on Porter et al., 1995.

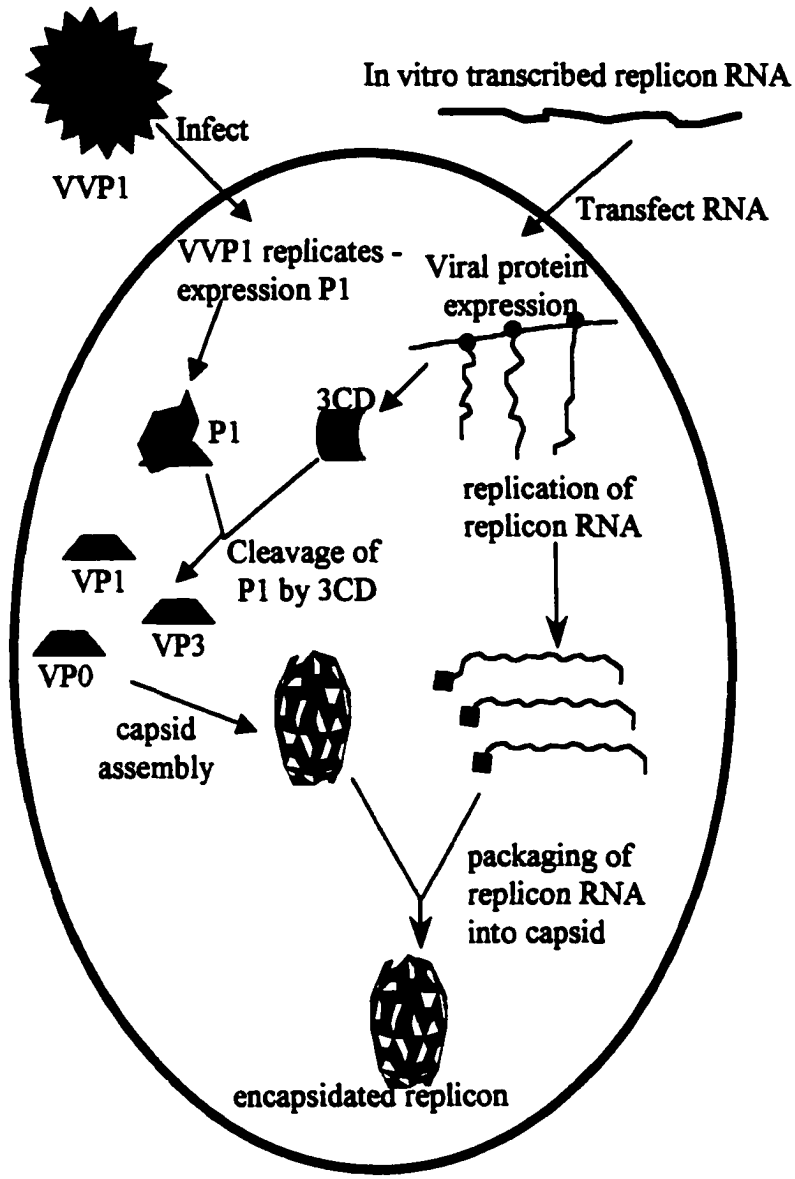


Fig. 8. Encapsidation of poliovirus using a recombinant vaccinia virus. Vaccinia virus encoding the poliovirus P1, VV-P1, is used to infect Hela cells. Two hours after infection, in vitro transcribed replicon RNA is transfected into the cells. Following transfection, the RNA is translated and replicon proteins are produced. The P1 protein, as produced by the vaccinia virus, is cleaved by 3CD. In addition, the replicon RNA undergoes replication within the cytoplasm of the cell. The capsids produced are then assembled and the replicon RNA encapsidated, giving rise to encapsidated replicons. Based on Ansardi et al., 1996.

poliovirus infection such as HeLa cells are infected with VVP1. Two hours after infection, the replicon RNA, which has been in vitro transcribed from an infectious clone of poliovirus cDNA, is transfected into the cell. Following transfection of the viral RNA, viral proteins such as $3CD^{pro}$ are produced. This $3CD^{pro}$ then proteolytically processes in *trans* the capsid P1 that has been produced by the recombinant vaccinia virus VVP1. The individual capsid proteins VP1, VP3, and VP0 then assemble to form the capsid intermediates needed to encapsidate the RNA genome. In the meantime, the RNA that was transfected into the cell replicates within the cell, making multiple copies of it, which are later encapsidated by the capsids derived from VVP1. These encapsidated replicons can then be serially passaged by infecting cells with replicons that have been previously infected with VVP1 to generate higher titer stocks of the replicon. By using this strategy, encapsidation of replicons expressing several foreign proteins such as HIV-1 *gag* and *pol* (144), firefly luciferase (146), green fluorescent protein (GFP), and *E. coli* β-galactosidase (β-gal) (Bledsoe and Morrow, unpublished data) has been accomplished. The β-gal replicon is the largest gene to date (3 kb) that has been successfully encapsidated and serially passaged to generate large stocks (Bledsoe and Morrow, unpublished data); however, it is important to note that after approximately 17 passages, deletions of the β-gal gene were discovered (Bledsoe and Morrow, unpublished data). Studies using the luciferase replicon, which expresses enzymatically active firefly luciferase, showed that the amount of luciferase produced directly correlated with the infectious dose of replicon used in the infection (146). Another important replicon developed in this laboratory is a replicon that expresses murine tumor necrosis factor α (TNF-α). For the first time, this laboratory showed that the biologically active molecule,

murine TNF-α, could be safely delivered to the central nervous system (CNS) by poliovirus, causing inflection of CNS function but no pathogenesis in the CNS (27). Studies with this replicon established profound evidence for the safe use and potential of poliovirus replicons as expression vectors.

Mutagenesis of Poliovirus

Great strides have been made in the genetic study of poliovirus through the construction of full-length cDNA clones that were found to be infectious in mammalian cells (151, 152). However, prior to the evolution of molecular cloning, the poliovirus mutants needed for genetic studies were derived from viral stocks by selection with or without prior chemical treatment of the inoculate (206). One of the most popular and important mutants of poliovirus derived in this manner was the attenuated virus that is presently used in the oral vaccine (169). Today, mutants of poliovirus are selected by one of the following ways: (i) viral selection via drug resistance or antibody recognition, (ii) random mutagenesis through chemical treatment or enzymatic alteration, (iii) semi-random mutagenesis through restriction enzyme cleavage sites, (iv) site-directed mutagenesis, or (v) swapping or exchange of poliovirus gene segments through recombination or with restriction sites within the cDNA (206). Consequently, most of the methods used today depend heavily on the use of cDNA clones. However, the first cDNA clones developed had some restrictions. Although these clones could produce virus following transfection into mammalian cells, the plasmids used were deficient in transcriptional control of the viral cDNA and the infectivity of these clones were low (10 PFU/μg of DNA) (151, 206). This limitation then led to the development of clones that

contained promoters, SV40 DNA origins of replication, and use of COS cells that expressed a T-antigen (T-Ag), which promotes replication of plasmids with SV40 origins of replication (176). These modifications increased infectivity to that of 10^3 PFU/μg of DNA (176, 206). To use cDNAs better and to ensure recovery of virus, the use of phage T7 RNA polymerase allowed researchers to acquire infinite quantities of infectious viral RNA that could be transfected into cells, yielding an infectivity of $>10^5$ PFU/μg (206).

Site-directed mutagenesis. The discovery that the cDNA of poliovirus was infectious was very important in the genetic analysis of poliovirus. The production of specific mutations in poliovirus was also very important in determining specific sequences or genes necessary for certain viral functions. One very powerful method used to make these mutations was oligonucleotide-mediated, site-directed mutagenesis. This method has been applied to studies involving cleavage sites of the polyprotein, to specific sites within the noncoding regions of the poliovirus genome, and in the introduction of foreign sequences into specific regions of the capsid (10, 11, 50, 106, 206). The mutagenesis of infectious poliovirus cDNA by this method is straightforward. Using PCR, the mutation is introduced into the cDNA by a mutagenic primer. This cDNA, which contains the mutation, is then either transfected as DNA or transcribed into RNA and then transfected into cells. Following transfection of the DNA or RNA, the phenotype of the progeny virus can be studied. However, if the mutation is lethal, no virus is produced. One precaution in using this method is that the phenotype seen must be a direct result of the mutation; therefore, the mutated cDNA and progeny virus must be sequenced to ensure that this is indeed the case.

Mutagenesis of the poliovirus BC loop. The advances that have been made in poliovirus pathogenesis and receptor recognition can be attributed to three important discoveries. The discoveries are the determination of the three-dimensional structure of poliovirus (77); the identification of its receptor, PVR (112); and the discovery of the infectious potential of its cDNA (151). Although studies via the genetic manipulation of poliovirus cDNA have occurred throughout its genome, one area that has received a great bit of attention is the capsid. As stated before, the capsid is composed of 60 protomers, each containing the capsid proteins VP1, VP2, VP3, and VP4 (77). The surface has a prominent peak at the fivefold axis, a small protrusion at the threefold axis, a deep depression called the canyon surrounding the fivefold axis, and a hydrocarbon binding pocket underneath the canyon floor (77). Also, the surface is decorated with loops that connect the eight antiparallel β-strands of the viral proteins. These loops form the neutralization antigenic sites on the virions' surface.

As stated above, the capsid has been a large focus in the genetic manipulation of poliovirus. However, more specifically, the BC loop of N-AgI is a region of the capsid that has received the most attention. This site has been found to be important in many aspects of poliovirus pathogenesis. In terms of binding, it was thought that the BC loop was in direct contact with the receptor. However, through mutational analysis, it was discovered that the EF loop and not the BC loop of N-AgI made contact with the receptor (100). Still, it is thought that the BC loop may play a role in conformation or flexibility of the virion, thereby having an influence in receptor binding (100). Other studies have shown that amino acids 95, 97, 99, 102, and 104 of the BC loop may be directly responsible for determining conformation of the virion (106).

In addition to its role in virion conformation, the BC loop was also found to be a determinant of host range (119, 121). Normally, poliovirus type 1/Mahoney (P1/Mahoney) is avirulent in mice. However, two studies have devised mechanisms to overcome this host restriction. Murray et al. (1988) found that upon the exchange of only six amino acids (95, 97, 99 to 102) of P1/Mahoney with the N-AgI of poliovirus type 2/Lansing (P2/Lansing), P1/Mahoney could become very neurovirulent in mice (121). Not only could the host restriction of P1/Mahoney be overcome by substituting the VP1 BC loop of P1/Mahoney with P2/Lansing (106, 119, 121), but it could also be overcome by intragenic suppressors. Studies by Moss and Racaniello (119) found that by substituting a glycine (Gly) for a glutamic acid (Glu) at amino acid 40 of VP1 and a serine (Ser) for a proline (Pro) at amino acid 54 of VP1, P1/Mahoney was more virulent in normal mice than wild type P1/Mahoney, which is avirulent in normal mice.

Finally, the BC loop has been used to express foreign antigenic regions as a way to elicit neutralizing antibodies. One such use of the BC loop in this capacity is through the expression of various HIV antigenic domains. One study removed amino acids 94 to 102 of P1/Mahoney VP1 and replaced them with 18 amino acids of the transmembrane glycoprotein gp41 of HIV-1 (57). This chimera produced viable virus that elicited neutralizing antibodies to HIV-1 (57). A second study, using the same method, replaced N-AgI of P1/Mahoney with the principal neutralization domain (PND) of HIV-1 strains LAI and RF and generated a significant but weak HIV-neutralizing response (50). In conclusion, all of these studies show the flexibility of the virions' surface and the adaptability of the BC loop to foreign sequences.

Integrins

Cell-cell and cell-extracellular matrix interactions are mediated by a superfamily of cell adhesion molecules called integrins. These interactions are important in cell growth and differentiation, tumor cell growth and metastasis, apoptosis, wound healing, inflammation, hemostasis, and cell migration (45, 49). All of these observed effects arose due to the adhesive and signaling properties of integrins. Integrins can be found on virtually all cell types, especially those that have an adhesive capacity (183). They are composed of one α and one β subunit that are noncovalently linked (Fig. 9). To date, approximately 16 α and 8 β subunits have been identified (49). These subunits can combine to form at least 20 receptors. Although the three-dimensional structure of integrins is not known, the primary structures of several integrins have been derived (56, 76, 78, 153). As stated earlier, integrins are composed of one α and one β subunit. Most α subunits consist of a heavy chain and a light chain, which are linked by a disulfide bond. They are derived from the same gene but are proteolytically cleaved during maturation of the receptor (183). The light chain of the α subunit contains a transmembrane region and a cytoplasmic tail. The α subunit also has a couple of notable features. The most notable feature of this subunit is the seven genetic repeats found at the amino terminus of the molecule (61). Studies demonstrated that in all integrins, 3 or 4 of the 7 repeats are highly homologous to calcium binding loops of the EF-hand protein (168, 191, 200). These calcium or cation binding sites found in integrins are major players in the binding of ligands to the receptor (183). Another segment of the α-subunit that is thought to influence ligand binding is the I or insertion domain. This domain is a 200-residue insertion that is found in place of the first calcium/cation site (46).

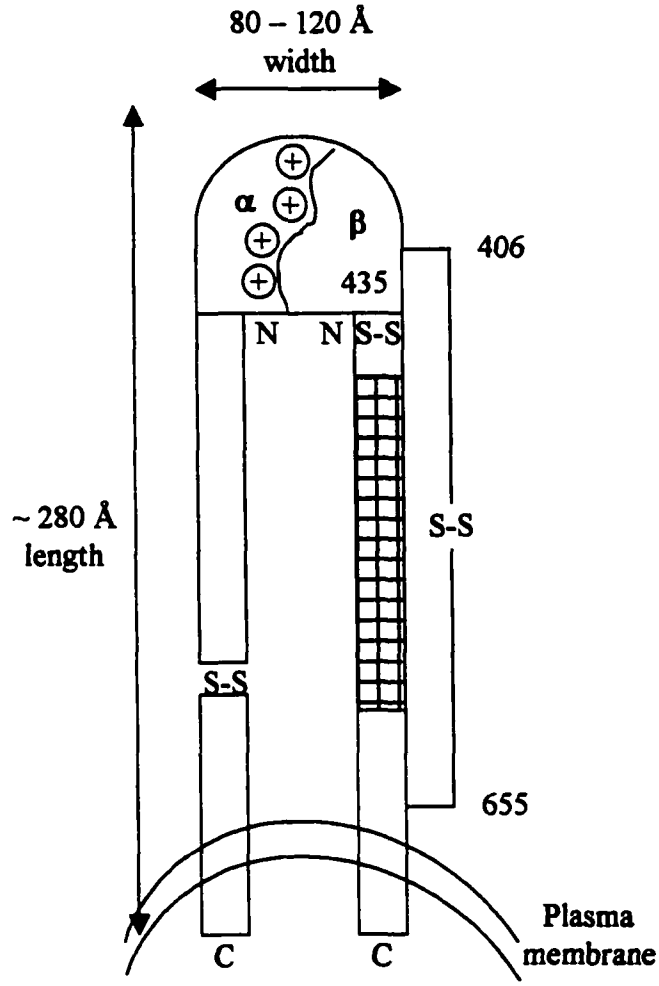


Fig. 9. Structure of integrins. Integrins consists of one α and one β subunit. The α subunit, as depicted on the left, consists of a heavy chain and a light chain connected by a disulfide bond. The light chain consists of a transmembrane and cytoplasmic tail. It has 7 genetic repeats, of which 3 or 4 are homologous to the calcium binding loops of the EF-hand protein. The repeats are depicted as the symbol + enclosed in a circle. The β subunit on the right has a cysteine-rich core, depicted as a grid box, and two important disulfide bonds between residues 5 and 435, and 406 and 655. It also has a transmembrane region and a cytoplasmic tail. The integrin has a globular head of about 80-120Å and a length of about 280Å. Based on Smith, 1994.

The β-subunit is a highly folded subunit that consists of 56 cysteine residues (183). Based on the β_3-subunit, it is thought that all other β-subunits have extensive internal disulfide bonding located between residues 5 and 435 in the amino-terminal domain (32). A cysteine-rich core is found on the carboxy-terminal side of residue 435, as well as a disulfide bond between residues 406 and 655 (Fig. 9). All β-subunits have transmembrane domains and cytoplasmic tails like the α-subunits. As stated before, the three-dimensional structure of integrins is not available; however, through electron microscopy, the structure of integrin $\alpha_5\beta_1$ has been elucidated (124). Through the examination of $\alpha_5\beta_1$, the integrin was found to contain an 80- to 120- Å globular head with long extended tails giving rise to a molecule approximately 280 Å in length (124). Based on this finding, all other integrins are speculated to have the same shape. The extracellular domains of integrins play a role in the divalent cation-dependent association of molecules within the extracellular matrix and other integral plasma membrane receptors. On the other hand, the intercellular domains interact with the components of the actin cytoskeleton, as well as protein kinases and calcium binding proteins (49).

Integrins, ligands, and (RGD). Integrins are adhesive molecules that bind to many different ligands. Some of the ligands used by integrins are fibronectin, vitronectin, osteopontin, collagens, thrombospondin, fibrinogen, and von Willebrand factor (183). Each of these ligands can be found in association with extracellular matrix molecules, blood-borne proteins, immunoglobin superfamily proteins, bacterial membrane proteins, and snake venom proteins (183). As a result, the effect of integrins can be felt in various

tissues and organisms (i.e., T and B lymphocytes, platelets, monocytes, neurons, and tumor cells) (183).

Integrins are adhesive molecules or receptors that bind to ligands. Like any other receptor, in order for binding to take place, the receptor must first recognize its binding site or ligand. The recognition site of integrins involves specific peptide sequences that are found in the ligands. Table 1 lists the minimal sequences necessary for integrin binding (183).

Table 1. Integrins, ligands, and peptides

Integrin	*Ligand*	*Peptide*
αvβ5	Vn	RGD
α2β1	collagen	DGEA
α3β1	Fn, laminin, collagen	RGD
α4β1	Fn, VCAM	RGD, EILDV
αvβ1	Vn, Fn	RGD
αIIIBβ3	Fg, Fn, Vn, vW	RGD, KQAGDV
αvβ3	Fg, Fn, Vn, vW	RGD
α4β7	Fn, VCAM	EILDV

The abbreviations used are as follows: Fn, fibronectin; Vn, vitronectin; VCAM, vascular cell adhesion molecule; Fg, fibrinogen; vW, von Willebrand factor. Based on Smith, 1994.

As shown in Table 1, there is one peptide sequence that is recognized by a large number of integrins. That peptide sequence is the (RGD) sequence. This peptide sequence was

first identified through studies involving fibronectin. In 1981, Pierschbacher et al. discovered an 11-kDa fragment from fibronectin that retained the ability to allow cell adhesion (140). Further studies by this group dissected the 11-kDa fragment to find that a small synthetic peptide containing an RGD fragment could simulate the binding capacity of fibronectin, which allowed cell adhesion (141). Following this discovery, several other receptors have been isolated using affinity chromatography. Affinity columns containing synthetic RGD peptides have isolated the fibronectin receptor ($\alpha_5\beta_1$), the vitronectin receptor ($\alpha_v\beta_3$) and the platelet integrin GPIIb-IIIa receptor ($\alpha_{IIb}\beta_3$) (149, 183). The integrin $\alpha_v\beta_3$, originally called the vitronectin receptor but now known to bind other adhesive proteins, plays a role in cell adhesion, bone resorption, and tumor metastasis (3, 48, 179). Some studies have shown that by preventing $\alpha_v\beta_3$ from binding to its ligand, apoptosis of endothelial cells during angiogenesis, the formation of new blood vessels, can occur (133, 168). In terms of affinity for the RGD peptide, $\alpha_v\beta_3$ has the highest affinity with a K_d in the nanomolar range (183). The major fibronectin receptor, $\alpha_5\beta_1$, has a moderate affinity for the RGD peptide (183). This is because $\alpha_5\beta_1$ requires both RGD and synergy sites for maximal binding (3). The integrin, $\alpha_5\beta_1$, plays a role in cell adhesion, cell migration, cytoskeleton assembly, and assembly of the fibronectin extracellular matrix (3). The platelet integrin, $\alpha_{IIb}\beta_3$, is a lot like $\alpha_v\beta_3$ in that it has the capacity to bind several ligands, as shown in Table 1. It also has a high affinity for RGD like the $\alpha_v\beta_3$ integrin (183). As stated before, $\alpha_{IIb}\beta_3$ has the ability to bind several different ligands. One of these ligands is fibrinogen. Studies have shown the RGD sequence to play an important role in the binding of fibrinogen to platelets (149); therefore, in terms of therapeutics, it has been proposed that peptides containing RGD

could possibly inhibit the binding of fibrinogen to platelets, thereby preventing platelet aggregation (149, 168).

Integrin/ligand binding and divalent cations. Divalent cations play a very important role in the binding of integrins to ligands. As stated before, the α subunit contains 7 genetic repeats (61). Three or 4 of these genetic repeats have sequence homology to the calcium binding loops of the EF-hand proteins, which have a helix-loop-helix motif (61, 65, 191). Studies have shown that a divalent ion resides within this loop (191). Direct evidence of the effect of divalent ions on ligand binding first came from studies on the $\alpha_{IIb}\beta_3$ integrin. Studies of this integrin found that, although only low levels of Ca^{2+} at approximately -10^{-6}M were needed to sustain association of the α and β subunits, higher levels of approximately 10^{-4}M were needed for ligand binding (60, 139). Studies have also shown the cation Mn^{2+} to affect the function or binding of integrins to ligands (65, 183, 184). The integrin $\alpha_v\beta_3$ has also been studied extensively. Studies on this integrin have shown the association rate of this receptor to its ligand to be drastically affected by divalent cations. The integrin $\alpha_v\beta_3$ is unable to bind fibrinogen in the presence of Ca^{2+} but can bind fibrinogen in the presence of Mn^{2+} (183). Ion binding to integrins has been demonstrated to occur at two sites. These sites are the low affinity and high affinity sites. There are three to four low affinity sites that have a requirement to be filled with Ca^{2+}, Mg^{2+}, or Mn^{2+} ions in order for ligand recognition to occur (65, 184). The high affinity site maintains subunit association (65, 184). Through these studies and others, the type of divalent cations found in the cellular surroundings has been demonstrated to have a significant impact on the function of integrins.

Integrins as viral receptors. Integrins have been implicated in having an active role in several biological responses such as wound healing, inflammation, apoptosis, and tumor metastasis. They have also been shown to act as signaling molecules (49, 167). More recently, integrins have been found to play the role of viral receptor. Many organisms contain RGD sequences that allow them to bind integrins. Mutational analyses of these RGD sequences show disruption in binding, virulence, and stability of these organisms (190, 201). However, not all organisms that contain RGD sequences bind to integrins or use them as receptors for entry into cells. Lately, several viruses have been found to exploit the use of integrins as receptors for cellular entry. Viruses that have been shown to use integrins in their life cycle include coxsackievirus A9 (CAV9), echovirus 1 and 22, FMDV, and rotavirus SA11 (20, 22, 74, 123, 162, 187). Other viruses such as adenovirus use integrins as a secondary viral receptor, which is required for internalization (21, 34, 195). Of all the viruses listed, FMDV was the first virus suspected of using integrins for cell attachment (102). The RGD sequence of FMDV that allows attachment to integrins has been localized to a highly exposed loop called the GH loop (1, 107, 202), which is found in the capsid protein VP1 of FMDV (107, 202). In addition to the RGD sequence, studies by Mateu et al. (1996) have shown that two leucine residues located at positions +1 and +4 downstream of the RGD and a more minor leucine residue located at the +2 position are critical for recognition of the viral ligand (108). Although, through mutational analysis, the RGD sequence in FMDV was found to be important in binding, no integrin had been identified as the cellular receptor. However, in 1995, Berinstein et al. identified integrin $\alpha_v\beta_3$ as the receptor for FMDV (22). More recently, a second integrin, $\alpha_5\beta_1$, has been described as a receptor for FMDV (79); however,

previous studies were unable to show this integrin to act as a viral receptor for FMDV (22). The integrin that acts as a cellular receptor for CAV9 is $\alpha_v\beta_3$ (20, 162). Prior to the identification of this integrin, several studies had implicated the importance of the RGD motif found in the VP1 of CAV9 in viral entry (33). In addition, studies revealed the ability of CAV9 to bypass entry into cells via RGD, thereby showing the flexibility and adaptability of CAV9 (160, 161). SA11 rotavirus is the latest virus to demonstrate the use of integrins as receptors (74). It is also the only one to date that uses not only RGD but also a second recognition peptide, DGEA. The integrins found to act as viral receptors for SA11 are $\alpha_2\beta_1$ and $\alpha_4\beta_1$ (74). The integrin $\alpha_2\beta_1$ is a receptor for the ligand collagen and recognizes the peptide sequence DGEA, whereas the integrin $\alpha_4\beta_1$ recognizes the RGD peptide and has fibronectin and vascular cell adhesion molecule (VCAM) as its ligands (183). Finally, adenovirus utilizes the integrin $\alpha_v\beta_5$ or $\alpha_v\beta_3$ and the coxsackievirus-adenovirus receptor (CAR) for entry into permissible cells (21, 34, 195). The binding of integrin, $\alpha_v\beta_5$, to adenovirus types 2 and 12 was resolved by cryo-EM (34). In addition, the RGD peptide used for binding was found to be located on the adenovirus penton base protein, which is highly flexible (189). For adenovirus, infection begins with the binding of virus to CAR. Following binding to CAR, the virus is internalized into clathrin-coated vesicles mediated by the association of the integrin $\alpha_v\beta_5$ or $\alpha_v\beta_3$ with the penton base protein (21, 34). Consequently, in the case of adenovirus, integrins are only responsible for internalization and not for binding and internalization of the virus.

Despite all of this knowledge, there is no explanation as to why or how these viruses have evolved to use integrins as viral receptors. However, it would be of interest to determine if other viruses could be manipulated such that integrins could be used as

viral receptors. Having the capability to alter viral-host interactions would have great implications in changing possible routes of infection, the inherent infectivity of a virus, and virulence of a virus.

Poliovirus is a virus that is known to have the capability of accepting foreign sequences without disruption of its ability to replicate, encapsidate, and infect host cells. Knowing this, it was of interest to determine if poliovirus could use integrins as a second mode of entry. Its normal mode of entry involves binding of the virus to the PVR; alteration of the viral capsid; and, last, internalization into the host cell. However, if an integrin was used instead, that could cause an alteration of the viral capsid that would lead to more efficient viral entry. If so, it would be plausible to that a change in viral-host interaction could occur, thereby changing the virus's host range. To date, poliovirus is only able to infect a select group of organs found in humans and primates; however, the PVR is found in other tissues. Also, poliovirus has a very abortive infectious process in that ≤20% of viruses that bind the PVR actually get internalized. Through the use of integrins as a receptor, it would be interesting to note what effect, if any, this change would have on poliovirus infectivity. These questions are important in that if host range and infectivity could be enhanced or altered by this change, this would lead to a proposal for the use of poliovirus vectors as efficient vehicles for the targeting of various cell types.

Using the adhesive properties of integrins, this project characterized the use of an RGD peptide to ascertain the effects of poliovirus infectivity and replicon infection. Prior to this study, no virus known to lack interaction with integrins had been altered to allow integrin binding. In terms of replicons, no studies have been done in an attempt to change

the inherent infectivity of the replicons. Because poliovirus has a highly abortive infection process due to the large amount of viral sloughing, it is reasonable to suggest the use of adhesive molecules to prevent this sloughing effect, which may influence the infectious potential. That is, the number of replicons that enter cells might increase, allowing the infection of cells with replicon to be more efficient.

Article 1 describes the production and characterization of a chimeric poliovirus that contains an RGD motif in the capsid of poliovirus. An RGD consensus derived from the RGD sequences found in other picornaviruses was inserted into the BC loop of VP1 via an infectious cDNA clone of poliovirus. Following transfection of the in vitro transcribed RNA, virus was produced and shown to retain the original RGD sequence. In addition, all of the viral proteins necessary for replication, translation, and encapsidation were produced like wild type poliovirus. We also demonstrated that this virus was lytic and could bind to cells expressing the appropriate integrins by plaque assay. However, the RGD mutation resulted in fewer viruses being produced in comparison to wild type.

In the study described in article 2, we used the replicon encapsidation method to produce replicons encapsidated with capsids containing RGD. The RGD capsids were shown to be capable of proteolytic processing by the viral protease $3CD^{pro}$. Knowing that the capsid could be proteolytically processed, an attempt to encapsidate two replicon genomes with this RGD capsid was pursued. The genomes used were ones that encoded firefly luciferase and GFP. Both replicons demonstrated the ability to be encapsidated and the ability to be serially passaged to generate stocks of the replicons. Unlike the wild type luciferase and GFP replicons, the titers of RGD replicons were not as high as the wild type replicons. On the other hand, the RGD replicons did demonstrate the ability to

bind integrin-expressing cells. In addition, data generated using equal genomes of RGD replicons and wild type replicons suggested a possible increase in infectivity for the replicons encapsidated with capsids containing RGD.

INTEGRIN BINDING CAPACITY CONFERRED TO A RECOMBINANT POLIOVIRUS ENCODING AN RGD AMINO ACID MOTIF IN THE BC LOOP

by

MONICA C. FRAZIER AND CASEY D. MORROW

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ABSTRACT

Poliovirus undergoes a conformational change upon interaction with the cellular receptor used for virus entry. Most of the altered A particles do not enter and are shed from the cell. Recent studies suggest these particles are infectious if entry into the cell is facilitated. To explore this possibility further, we have cloned an amino acid sequence containing an arginine-glycine-aspartic acid (RGD) motif known to interact with molecules on the cell surface called integrins, into the BC loop of the capsid of poliovirus type 1 Mahoney cDNA. The progeny virus (RGD poliovirus) was shown to replicate in HeLa cells and to retain the RGD sequence after several passages. The virus produced plaques smaller than wild type poliovirus. Analysis of protein expression revealed that the RGD viruses produced all proteins necessary for replication and encapsidation, albeit at a slightly slower rate than the wild type poliovirus. Binding assays were used to demonstrate the interaction of RGD poliovirus with poliovirus receptor (PVR)-negative/integrin-positive baby hamster kidney cells (BHK-21) unlike wild type poliovirus. Cyclical binding of the RGD poliovirus to BHK cells, followed by amplification in HeLa cells, resulted in the enrichment of viruses that retained the RGD mutation but had a single amino acid change in sequence flanking the RGD motif. The results of these studies established the feasibility of alteration of the cellular binding properties of poliovirus through manipulation of the capsid amino acid sequences.

INTRODUCTION

The poliovirus life cycle begins with binding of the virus to poliovirus receptor (PVR or CD155) (20). Upon binding, a conformational change occurs in the capsid in

which VP4 protein is lost and the amino terminus of VP1 protrudes from the surface, allowing binding to liposomes (11) and resulting in an altered particle (A particle). This A particle is endocytosed into the cytoplasm where, following release of the genome RNA, translation/replication takes place. The binding of poliovirus to the PVR does not, however, guarantee entry into the cell. A substantial portion of bound polioviruses are released or eluted from the cell as A particles (25). As a result, the particles are no longer able to attach to susceptible cells (3, 11, 27). This sloughing of viral particles from the cell surface contributes, in part, to the high particle to PFU ratio for poliovirus.

Many studies have reported mutated capsid residues that alter recognition of poliovirus by PVR or that affect the host range and neurovirulence of polio (7, 16, 21, 22). One region of capsid found to be involved in this is the neutralization antigenic site 1 (N-Ag1). A substantial part of N-Ag1 is composed of a loop that connects the β-strands of Ь and C of VP1; this loop is called the BC loop of VP1. This loop is highly exposed on the surface of polio near the fivefold axis of symmetry (13). Previous studies show that this BC loop can be substituted with various sequences from different proviruses, resulting in chimeric viruses of dual antigenicity (9, 21). Additional studies have reported polioviruses, which contain immunogenic epitopes from other pathogens substituted in BC loop (9, 10).

To date, no studies designed to alter binding of the virus to cells via modulation of the BC loop have been reported. To test the feasibility of altering the binding properties of the virus, we mutated the cDNA in order for the VP1-BC loop to encode an amino acid motif containing the amino acids, arginine-glycine-aspartic acid (RGD), which should facilitate interaction with cell surface adhesion molecules, integrins. We used an RGD

motif, which is most recognized by integrins and found in the capsid proteins of other picornaviruses (19). The gene encoding RRGDLGSL was inserted into the VP1 BC loop of poliovirus type 1. Experiments were performed to compare the cell binding and infectivity of this virus (RGD poliovirus) with the wild type. The results of these experiments establish that it is possible to alter the interaction of poliovirus with the host cell by changing the amino acids in the BC loop.

MATERIALS AND METHODS

Chemicals and Enzymes

All chemicals were purchased from Sigma unless otherwise specified. The restriction enzymes, cell culture media, antibiotic/antimycotic, and DH5α competent cells were purchased from Gibco/BRL. The fetal calf serum (FCS) was purchased from Biocell Laboratories. The [^{35}S]Translabel (methionine-cysteine) and methionine- and cysteine-free media for metabolic labeling were purchased from ICN Biomedical. The ^{35}S-dATP used for DNA sequencing was purchased from Amersham. The synthetic DNA primers used for PCR were obtained from Oligos, Etc., and Gibco/BRL. The SeaKem Low melting agarose was purchased from ECM Laboratories. The peptides used for the peptide binding experiment were purchased from Research Genetics; the three peptides used were as follows: RGE 5'-GRGESP-3', RGD 5'-GRGDSP-3', and vRGD 5'-RRGDLG-3'.

Cells and Viruses

BHK-21 and HeLa H1 cells were purchased form American Type Culture Collection. BHK-21 and HeLa H1 cells were maintained in monolayers culture in

complete media that consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FCS and 1% antibiotic/antimycotic. The antisera to poliovirus RNA-dependent RNA polymerase ($3D^{pol}$) and poliovirus have been previously described (1, 15).

Mutagenesis of the Poliovirus BC Loop

The RGD peptide, RRGDLGSL, replaced the wild type amino acid PASTTNKD within the BC loop of poliovirus by site-directed mutagenesis by PCR (26). The three synthetic primers used were as follows: primer A, 5'-CAT-GTC-GAC-AAA-GTG-AGA-CTC-ATT-ATC-3', which covers nucleotides 640 to 658 and encodes a unique Sal I restriction site; primer B, 5'-ACC-GTG-GAT-AAC-AGA-CGA-GGT-GAT-CTA-GGG-AGT-TTA-AAA-TTA-TTT-GCA-GTG-TGG-AAG-3', which is the mutagenic primer that covers nucleotides 2750 to 2806; and primer C, 5'-GGG-CCC-GGG-CTA-ATA-TGT-GGT-CAG-ATC-3', which covers nucleotides 3371 to 3385 and encodes a unique SmaI restriction site. To modify the BC loop of poliovirus, a poliovirus infectious clone plasmid (T7-IC) cDNA, which contains the full-length poliovirus infectious clone, was used as the starting plasmid for cloning the entire P1 region (6). In the first round of PCR, the mutagenic primer B and primer C were used to generate a megaprimer of 635 bp, which encoded the RGD mutation. In the second and final round of PCR, primer A, the megaprimer DNA segment generated from the first round of PCR and the T7-IC cDNA plasmid, was used. This final PCR generated a 2.64-kb RGDP1 segment comprising the full-length P1 region containing the RGD mutation flanked by SalI and SmaI restriction

sites. The 2.64-kb segment designated RGDP1 was used for the generation of a recombinant poliovirus.

Construction of a Recombinant Poliovirus

To generate a recombinant poliovirus, the infectious clone of poliovirus, pT7-IC, was used (Fig. 1). Both the vector pT7-IC and the 2.64-kb RGDP1 segment generated from site-directed mutagenesis by PCR are digested with SalI and SmaI enzymes. Following digestion, the PCR product is ligated into pT7-IC plasmid overnight at 20°C and transformed into *Escherichia coli* DH5α. The colonies were selected on ampicillin-containing LB plates after an overnight incubation at 37°C. The plasmids isolated were screened for the described insert by digests with SalI and SmaI restriction enzymes and sequenced for the desired mutation by dideoxy sequencing of double-stranded DNA. The plasmid generated was called pT7-IC-RGDP1 and used to produce the recombinant poliovirus, RGD poliovirus.

Generation of a Recombinant Poliovirus

To generate the recombinant poliovirus, RGD poliovirus, the pT7-IC-RGDP1 plasmid was restricted with SalI to linearize. In vitro transcription using the T7 DNA-dependent RNA polymerase was used under standard conditions to generate RNA transcripts. The RNA was processed as previously described and transfected into cells using lipofectin (Gibco/BRL) according to manufacturer's instruction.

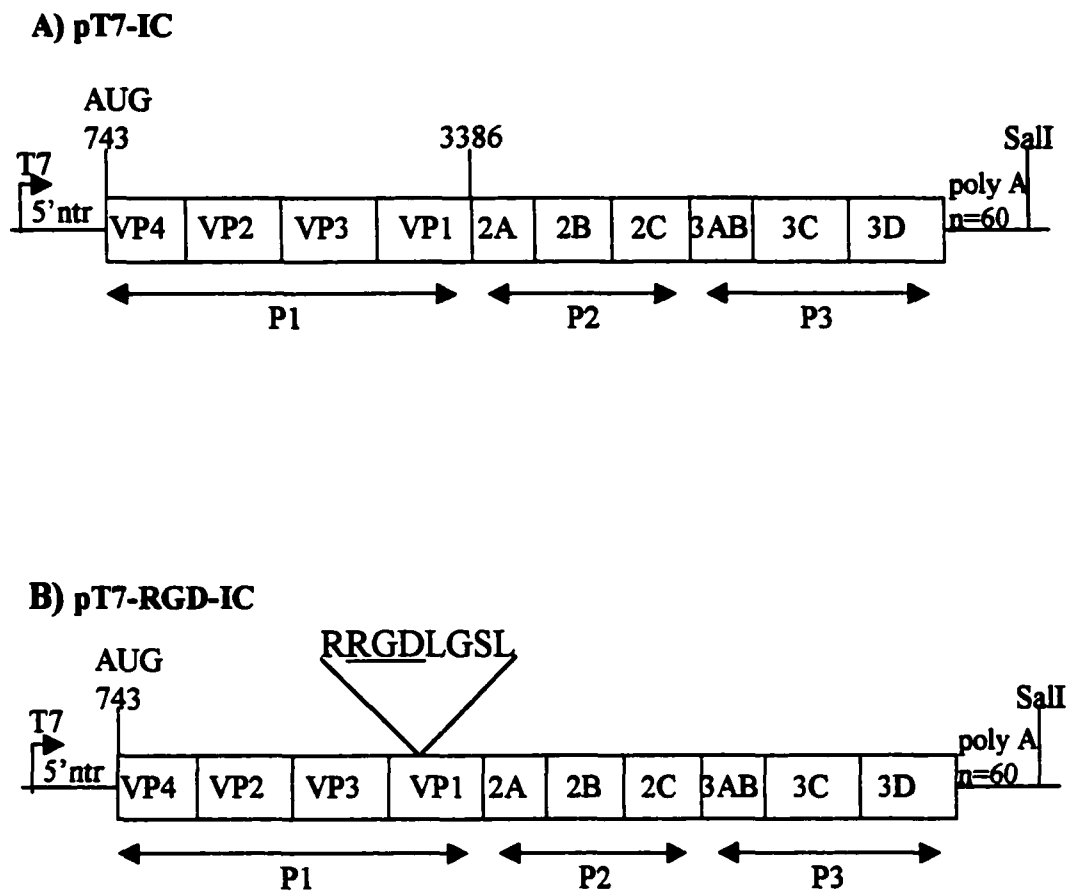


Fig. 1. Depiction of the plasmids pT7-IC and pT7-RGD-IC. The plasmids are cDNA constructs of P1/Mahoney genome. (A) The plasmid contains the three regions of the poliovirus genome, P1, P2, and P3. The P1 region consists of VP4, VP2, VP3, and VP1. The P2 region consists of 2A, 2B, and 2C; the P3 regions consists of 3AB, 3C, and 3D. At the 5' end is a T7 promoter followed by the 5' ntr. The 3' end consists of a poly (A) tail and a unique SalI restriction site. (B) This plasmid is the same as (A) with the exception of the substituted RGD sequence, RRGDLGSL, for the amino acids PASTTNKD at positions 95 to102 of VP1.

DNA Sequencing

The pT7-IC-RGDP1 plasmid and recombinant RGD poliovirus sequences were confirmed by dideoxy sequencing of the double-stranded DNA using the Sequenase™ 4.0 kit (U.S. Biochemical) according to manufacturer's directions. The radioactive label used was [^{35}S]dATP purchased from Amersham. The sequence of VP1 was done using automated sequencing. The primer used consisted of sequences just upstream of the mutational site. The primer used was 5'-CAAATCCACTAGTCC-3'.

Analysis of Viral Proteins

The analysis of proteins was done similarly as previously published (1). To analyze the expression of proteins, monolayers of HeLa H1 cells were infected with either RGD poliovirus or wild type poliovirus at 20 PFU/cell for 3.5 h at 37°C. The medium was removed and washed 1X with DMEM -methionine, - cysteine. Cells were starved with this same media for 30 min following starvation; the cells were labeled with [^{35}S]Translabel (methionine-cysteine) for 2 h. After labeling, the cells were lysed and proteins immunoprecipitated using a radioimmunoprecipitation assay buffer (RIPA) (150 mM NaCl, 10 mM Tris pH 7.8, 1% Triton X-100, 1% sodium deoxycholate, and 0.2% sodium dodecyl sulfate [SDS]). Cellular debris was cleared after centrifugation at 14,000 x *g* for 10 min at 4°C and supernatants were allowed to incubate overnight at 4°C with antipoliovirus antiserum, αP1. Protein A sepharose beads were added to the supernatants and allowed to incubate at room temperature for 1 h. Following several washes with RIPA buffer, the bound proteins were stripped from the beads by heating samples at 95°C for 5 min in gel sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 20% glycerol, 0.05%

Bromophenol Blue, and 0.7 M β-mercaptoethanol). All samples for these experiments were run on 14% SDS- polyacrylamide gel electrophoresis (PAGE) gels for the detection of P1 precursor and P1 proccessivity.

Analysis of Virus Binding

Monolayers of baby hamster kidney (BHK) cells (integrin +/PVR -) were adsorbed with wild type poliovirus and RGD poliovirus for 30 min at 37°C. The viruses were removed, and cells were washed three times with 1X phosphate-buffered saline (PBS), pH 7.0. The cells were lysed in 0.5 ml of incomplete media (FCS-) by freezing and thawing. After spinning at 14,000 x *g* for 20 min, the supernatants were placed onto monolayers of HeLa H1 cells and allowed to infect for 2 h at 37°C. Following the 2-h infection, the medium is removed and an agar overlay (50% Basal Media, 33% Sea-Kem agarose, and 16% FCS) is applied. After 3 days of incubation at 37°C, the cells are fixed using 5% trichloroacetic acid (TCA), the overlays are removed and the cells are stained with Coomasie Blue (50% methanol, 25% acetic acid, 0.01% w/v Brilliant Blue) to count plaques.

To ascertain the specificity of binding, peptides were used in the binding assays, which are known to interact with integrins. Prior to adsorption onto monolayers of BHK cells, wild type poliovirus and RGD poliovirus are mixed with 50 mM of each of the three peptides (RGE-GRGESP, RGD-GRGDSP, and vRGD-RRGDSL). The amount of viruses bound to BHK cells was then determined as described above via plaque assay.

Ribonuclease Protection Assay (RPA)

Viral RNA was isolated using Tri-Reagent according to manufacturer's instruction. The antisense RNA probe for detection of viral RNA was created using an in vitro transcription kit by Ambion according to manufacturers instruction. For detection of poliovirus specific plus-strand RNA, a plasmid comprising nucleotides 671 to 1174 of poliovirus was linearized with BamHI and used as a DNA template for making the 530-base antisense probe (6). The RPA was performed using an Ambion RPA kit according to manufacturer's instruction. The protected RNA fragment was electrophoresed using a 5% polyacrylamide, 8 M urea gel. The RNA fragment of 503 bases was quantitated by scanning the gel with a PhosphorImager (Molecular Dynamics).

RT-PCR and PCR

Reverse transcription (RT)-PCR was done following manufacturer's instruction (Gibco/BRL Superscript RT-PCR kit). Amplification of the cDNA was done by PCR. PCR fragments were analyzed by agarose gel electrophoresis using a 1% agarose gel. PCR fragments were quantitated using a densitometer.

RESULTS

Construction of a Poliovirus That Contains an RGD Motif Substituted in the BC loop

Several groups have reported on changes within the major neutralization antigenic loops of the poliovirus to produce chimeric viruses (2, 12). However, there have been no reports that examine whether these changes influence the binding of poliovirus to cells. To

study whether amino acids inserted in the BC loop region affect binding of poliovirus, an RGD motif was inserted within the capsid of poliovirus type 1 Mahoney. Integrins, which mediate cell-cell attachment and can act as viral receptors for several picornaviruses, are mostly recognized by RGD binding motifs (4, 14, 28). If the RGD motif is expressed on the surface of poliovirus, it could allow cell adhesion of poliovirus to the cell surface via integrins. For these studies, a consensus RGD peptide, RRGDLGSL, was derived from RGD sequences found in other picornaviruses (5) and substituted for amino acids, PASTTNKD, located at position 95 to 102 of VP1 (BC loop) in poliovirus type 1 Mahoney (26). This region was chosen because previous studies show that this loop can be substituted with various sequences from different viruses and because amino acids within this loop that are critical to viral viability have been defined (9, 21). The plasmid pT7-IC (Fig. 1A), which contains the complete poliovirus infectious cDNA, is used to clone the RGD motif into the P1 region (6). In the first PCR cycle, a 635-bp fragment covering nucleotides 2750 to 3385 of P1 and containing the RGD mutation is produced. In the final round of PCR, this 635-bp fragment is used to produce the entire 2.64-kb RGD-P1 capsid. This RGD-P1 capsid was then cloned into the plasmid pCR-BLUNT (Invitrogen), digested with NheI and SnaB1 to isolate RGD-P1, and then cloned into pT7-IC to generate pT7-RGD-IC (Fig.1B). The mutation in pT7-RGD-IC was confirmed by DNA sequence analysis (data not shown).

Once the capsid mutation was confirmed, the mutant virus was analyzed for infectivity. The plasmid pT7-RGD-IC, which contains promoter sequences for T7 RNA polymerase positioned 5' to the complete poliovirus cDNA, was transcribed and transfected into HeLa H1 cells using Lipofectin. Following transfection of the RNA, the

HeLa H1 cells were examined to determine whether a cytopathic effect (CPE) was apparent. A plaque assay revealed the RGD-poliovirus plaques were generally smaller in size than the wild type poliovirus (Fig. 2).

To determine if the mutation was stable in cell culture, the virus was passaged several times in HeLa cells. The genome region corresponding to VP1 was amplified by RT-PCR. A sequence analysis of the VP1 region confirmed the presence of the nucleotides encoding the RGD amino acid sequence. To characterize further the RGD poliovirus, analysis of polioviral proteins 3CD and P1 was done using immunoprecipitation. HeLa H1 cells were infected with lysates from the transfection, followed by metabolic labeling at 2 h after infection. The radiolabeled proteins were immunoprecipitated with anti-3CD and anti-P1 antibodies. No expression of 3CD or P1 was seen in mock-infected cells. However, an abundance of 3CD and processed capsids was seen in the RGD virus-infected cells (Fig. 3). A time course of viral protein production was done. RGD poliovirus and wild type poliovirus at 1 x 10^6 PFU were allowed to infect HeLa H1 cells at 0, 1, 2, 4, and 6 h, followed by a 15-min chase with [^{35}S]Translabel (methionine-cysteine). The cell lysates were analyzed by 10% SDS-PAGE, followed by autoradiography to visualize viral proteins. A short (1-h) lag in viral protein synthesis of the RGD virus was seen in comparison to wild type. By 6 h after infection, all proteins necessary for replication and encapsidation were evident for both viruses (Fig. 4).

RGD Displayed on the Viral Capsid Is Functional

In order to determine whether the RGD encoded within the RGD virus capsid was functional, a cell line that expressed no PVR but did express integrins, BHK-21, was used

Fig. 2. Analysis of wild-type and RGD poliovirus plaques on HeLa H1 cells. Cells were infected with wild-type and RGD poliovirus for 2 h. After infection, an agarose overlay, as described in Materials and Methods, was placed over the cells and allowed to incubate for 3 days at 37°C. The cells were then fixed in 5% TCA, overlays removed, and cells stained with Coomasie Blue as described in Materials and Methods.

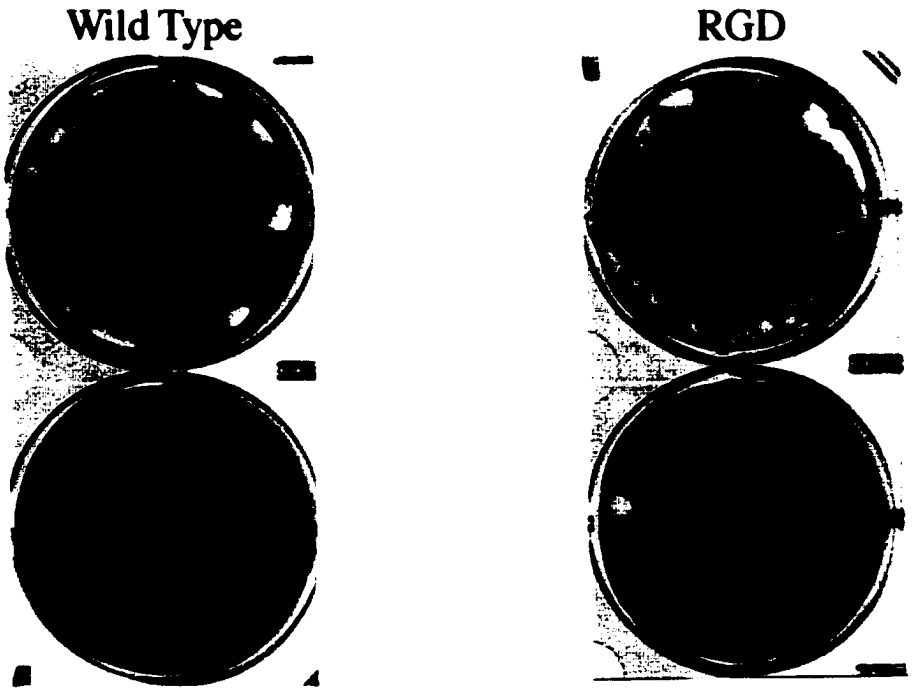


Fig. 3. Analysis of protein expression from HeLa H1 cells infected with RGD and wild-type poliovirus. Cells were infected with equal amounts of both RGD and wild-type poliovirus, as quantitated through plaque assay, for 3.5 h. Following infection, cells were metabolically labeled 2 h. After labeling the cell lysates were incubated with antibodies to $3D^{pol}$ and poliovirus. The immunoreactive proteins were analyzed on SDS-PAGE gel. (A) Results are shown for mock-infected cells (lane 1) and for cells infected with wild-type poliovirus (lane 2) and RGD poliovirus (lane 3). (B) Results are shown for mock-infected cells (lane 1) and for cells infected with wild-type poliovirus (lane 2) and RGD poliovirus (lane 3). Molecular weight markers and position of relevant proteins are indicated.

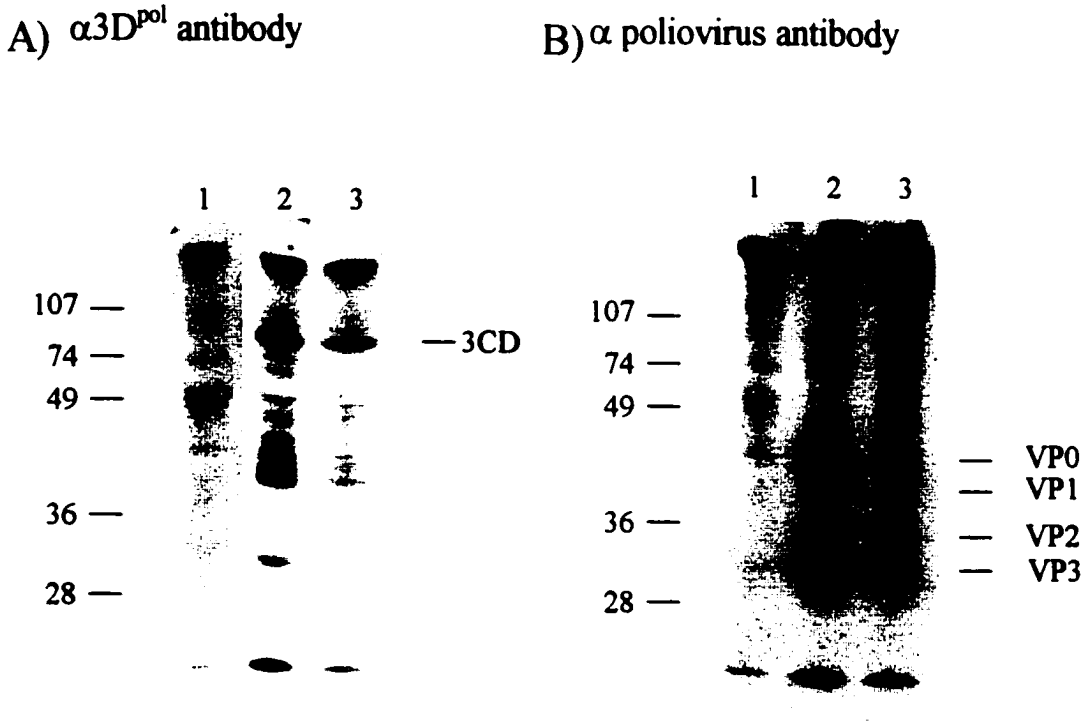
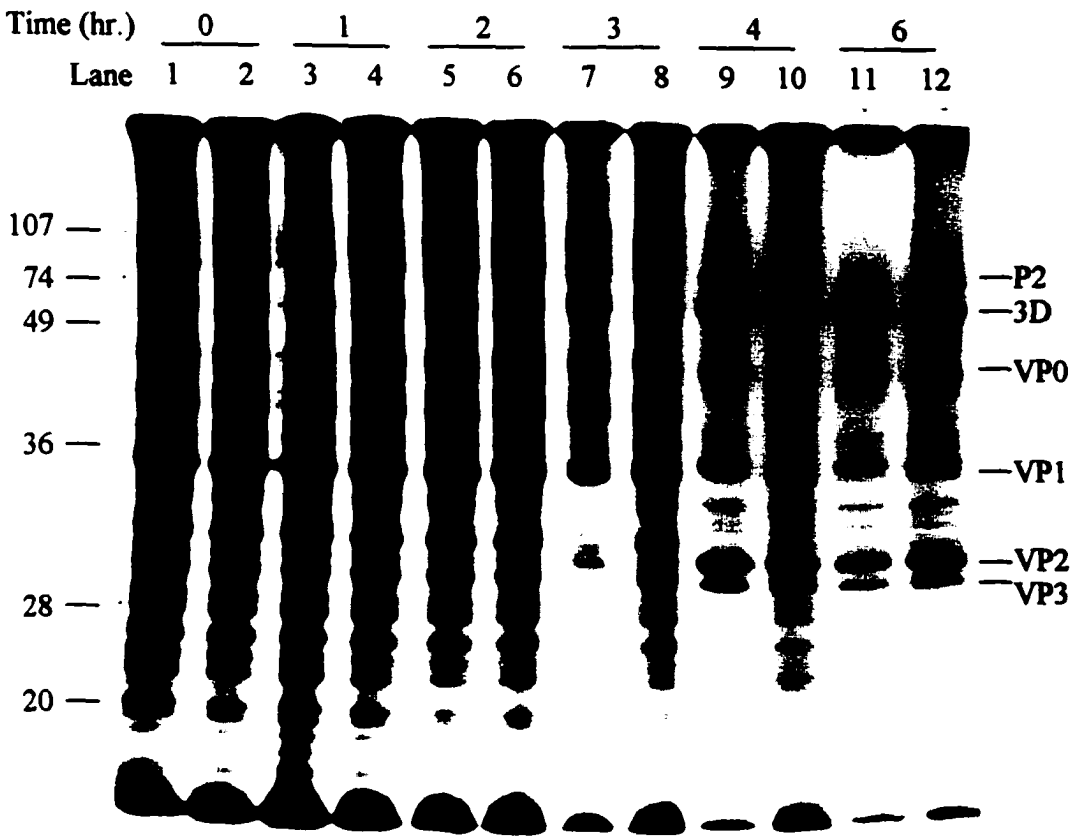


Fig. 4. Analysis of viral protein expression from RGD and wild-type poliovirus in HeLa H1 cells. HeLa H1 cells were infected with equal amounts of both RGD and wild-type poliovirus, as quantitated through plaque assay, for 3.5 h. Following infection, cells were metabolically labeled for 0, 1, 2, 3, 4, and 6 h. After labeling the cell lysates were analyzed on a 14% SDS-PAGE gel. Results are shown for wild-type poliovirus (lanes 1, 3, 5, 7, 9, and 11) and RGD poliovirus (lanes 2, 4, 6, 8, 10, and 12). Molecular weight markers and position of relevant proteins are indicated.



for binding studies. These cells are commonly used in cell binding (RGD to integrin) assays on foot-and-mouth disease virus (FMDV) and coxsackievirus, two picornaviruses with strains known to use integrins via an RGD sequence expressed within the capsid as viral receptors (4, 23, 24). Similar amounts of RGD and wild type poliovirus (1×10^6 PFU) were adsorbed onto BHK cells for various times and at various temperatures. In the first experiments, both viruses were adsorbed onto BHK cells at 37°C and 4°C for 2 h. Following adsorption, the cells were washed and lysed to separate cells from virus once bound to them. These viruses were then titered. The difference in titer of RGD virus versus wild type virus that bound to BHK cells at 37°C was greater than the titer of those that bound at 4°C. At 4°C, the difference in binding between RGD virus and wild type virus is only 1.3-fold (data not shown). Both wild type and RGD virus were adsorbed onto BHK cells for various times at 37°C. These data demonstrate that the recovery of virus bound to BHK cells is the same from 30 min to 2 h (data not shown). The subsequent binding experiments were done at 37°C for 30 min as shown in Fig. 5; under these conditions there was a 10-fold difference in binding between the RGD poliovirus and wild type poliovirus. To characterize further the binding of the viruses to BHK cells, increasing amounts of both viruses were adsorbed onto BHK cells and the amounts bound were determined by plaque assay. These results show that as the amount of wild type virus increases, the amount of virus recovered plateaus. However, as the titer of RGD virus increases, so does the amount of virus recovered (data not shown).

To demonstrate the specificity of binding of RGD virus to BHK cells, a peptide inhibitor assay was performed using RGE, RGD, and a vRGD peptide that was analogous to the one placed in the BC loop of the virus. All peptides at a 50-mM concentration were

$$\frac{\text{RGD virus}}{\text{wt virus}} = \frac{\text{2000 pfu}}{\text{200 pfu}} = \text{10 fold difference in binding}$$

Fig. 5. BHK binding analysis of wild-type and RGD poliovirus. Wild-type and RGD virus was adsorbed onto BHK cells (integrin positive/PVR negative) for 30 min at 37°C. After adsorption, cells were washed with PBS and lysed to release virus bound to BHK cells. Lysates are used in a plaque assay. Graph depicts the titer of virus bound to BHK cells. Values are given in PFU.

premixed with the RGD and wild type polioviruses and adsorbed onto BHK cells for 30 min at 37°C. As before, the cells were washed and virus bound to cells was quantitated by plaque assay. As shown in Fig. 6, when the RGD virus was mixed with the RGD peptide and the vRGD peptide, the amount of virus recovered from BHK cells was diminished. However, when the same amount of RGE peptide was mixed with the RGD virus, the amount of virus recovered from BHK cells was similar to the virus put in as determined by plaque assay. The peptides used had no effect on the background binding by wild type poliovirus.

Enhanced Infectious Potential of RGD Poliovirus

The fact that the RGD viruses show enhanced binding to BHK cells suggests that the RGD motif retains integrin binding capacity. If this is the case, we would expect that the RGD virus might have a greater infectious potential because the A particles or virus would be able to interact with PVR cells (e.g., HeLa cells). To address this question, we wanted to compare equal amounts of RGD viral RNA genome to wild type poliovirus for infection. To do this, HeLa H1 cells are infected with both wild type poliovirus type 1 Mahoney and RGD poliovirus and are grown at 37°C. Following an overnight incubation, the RNAs of both are isolated using Tri-Reagent followed by RPA. To detect poliovirus-specific plus-strand RNA in the RPA, an antisense 503-base riboprobe comprising nucleotides 671 to 1174 of the poliovirus genome was made (6). The riboprobe, used at saturation, was hybridized to the RNAs of both viruses at various dilutions. The protected RNA fragments were run out on a polyacrylamide gel and phosphorimaged. After phosphorimaging the gel, the results, as shown through a graph of pixel values given from

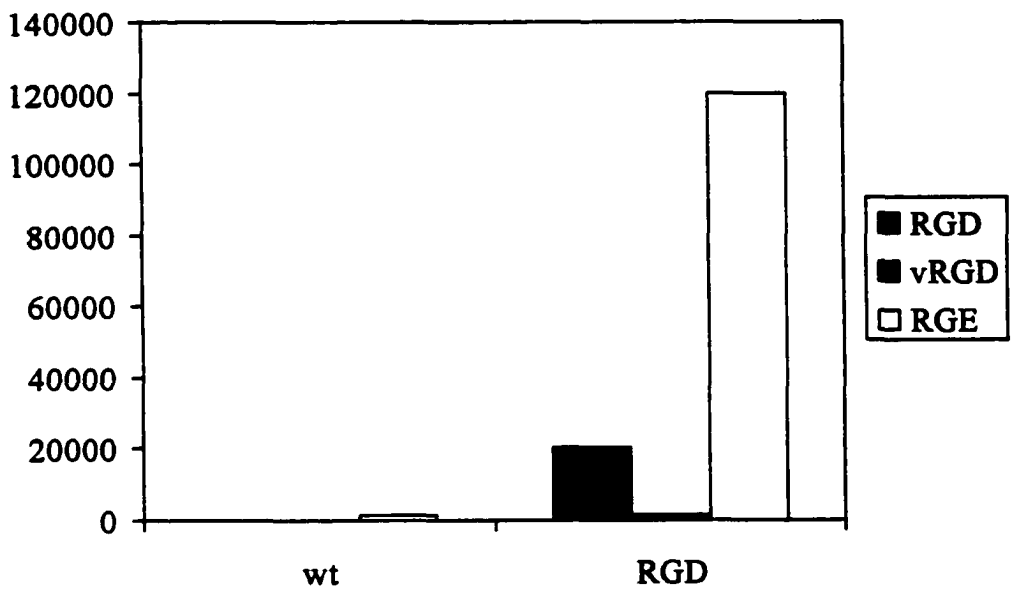


Fig. 6. Graph of competitive peptide assay. Wild-type and RGD poliovirus at equal amounts was mixed with 50 mM of RGE, RGD, and vRGD peptides and placed onto BHK cells for 30 min at 37°C. Following adsorption, the cells were washed with PBS and lysed to remove virus bound to BHK cells. The lysates are used in a plaque assay. Graph depicts titer of virus bound to BHK cells. Values are given in PFU.

the PhosphorImager (Molecular Dynamics), show the ratio of RNA between the stocks of RGD and wild type virus to be 1:5 respectively; that is, the stocks of poliovirus wild type contained 5 times more genome than the RGD virus. Using equal amounts of viral RNA genomes, HeLa H1 cells were infected and viral titer calculated using a plaque assay. The results of the assay show that the RGD virus produced nine-fold less PFU than the wild type poliovirus, which undoubtedly contributed to the small plaque phenotype (Fig. 7).

Enrichment of BHK Binding Viruses

The lower production of virus (as measured by PFU) from the RGD virus in comparison to wild type suggests that the mutation in the BC loop did affect some aspect of the viral replication cycle. If this is the case, it might be possible through selection to derive a virus with improved binding while still maintaining infectivity (7). To see if a virus could mutate to accommodate the RGD peptide better, the RGD virus was bound to BHK cells to derive an enriched population of viruses. This procedure was repeated for up to five consecutive bindings on BHK cells. On the final pass, the viruses were grown up on HeLa H1 cells for further analysis. Following RT-PCR of the capsid region, the product was cloned into pCI and transformed into *E. coli* and colonies were grown up and sequenced. From sequence analysis of the VP1 region, the RGD virus retained the nucleotides encoding the RGD sequence originally placed in the virus. However, a change from a leucine to a serine at amino acid position 102 of the BC loop was found (Fig. 8). Most probably, the amino acid change contributed to the stabilization of the RGD motif within the BC loop.

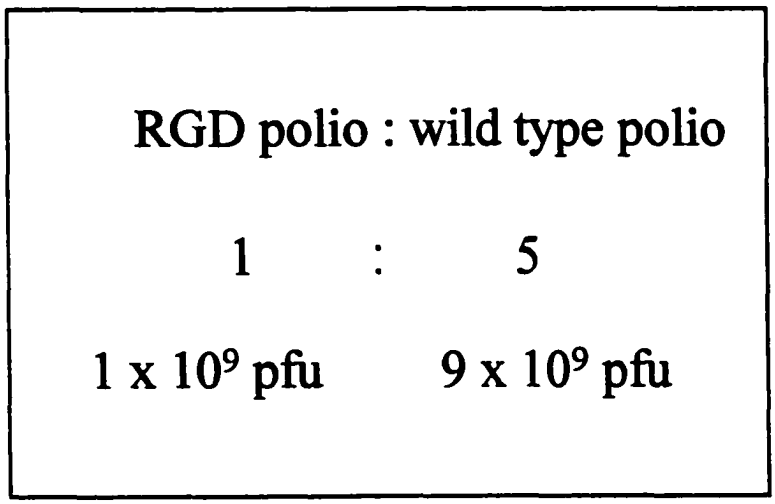


Fig. 7. Plaque assay of wild-type and RGD poliovirus. Wild-type and RGD poliovirus genomes were equilibrated via RPA. RPA revealed a 1:5 ratio between genomes (e.g., 1 genome equivalent of RGD poliovirus equals 5 genome equivalents of wild-type poliovirus). Equal genome amounts of wild-type poliovirus and RGD poliovirus were subjected to a plaque assay. Values given are in PFU.

Amino Acid	92	93	94	95	96	97	98	99	100	101	102	103	104	105
Wild-Type	V	D	N	R	R	G	D	L	G	S	L	K	L	F
	gtg	gat	aac	aga	cga	ggt	gat	cta	ggg	agt	tta	aaa	tta	ttt
RGD	*	*	*	*	*	*	*	*	*	*	tca	*	*	*
	*	*	*	*	*	*	*	*	*	*	S	*	*	*

Fig. 8. Sequence data of enriched RGD poliovirus on BHK cells. RGD polioviruses were passed on BHK cells 5 times. After five passages, the viruses were grown up on HeLa H1 cells and the P1 region of several clones amplified by RT-PCR, cloned into pCI, and sequenced. The sequence shown is representative of six clones.

DISCUSSION

In this article, we described the construction of a poliovirus that has the RGD peptide RRGDLGLS cloned into the BC loop of the virus capsid. We present results that the substitution of the amino acids in the BC loop with the RGD binding motif was not lethal. The resultant virus, RGD poliovirus, was shown to be infectious; however, it grew to a lower titer than the wild type virus and produced smaller plaques. We observed a slight delay in protein expression following infection. Most important, we demonstrated that the RGD binding motif was functional on the surface of this virus as evidenced by a greater binding capacity than the wild type for BHK cells, which are integrin positive/PVR negative. We selected a mutant RGD poliovirus which contains a leucine to serine change at position 102 of the BC loop following sequential binding to BHK-21 cells, followed by amplification in HeLa cells.

Previous studies have described the construction and characterization of polioviruses, which have been modified within the BC loop-antigen site 1 (9, 10, 17, 18, 22). Most of these studies focused on deriving chimeras between type 1 and type 2 by substitution of amino acids corresponding to the neutralization site of type 2 into the BC loop (8, 17, 18). Additional studies have been reported where new amino acids have been inserted into this neutralization loop for the purpose of generating polioviruses, which can elicit immune responses to variety of different pathogens (9, 10). Consistent with the results obtained in this study, the substitution of the amino acids in the antigenic site resulted in viruses that had different growth characteristics from the wild type. All the recombinant viruses obtained grew to slightly less titers than the wild type virus and produced smaller plaques at several different temperatures examined. From the results of

these studies, it was concluded that mutations within this region could affect steps in the virus life cycle involved in uncoating, genome release, and later steps in the life cycle such as assembly. The results are consistent with the results presented in the study, which found the mutant RGD poliovirus produced ninefold less virus than wild type. Since we did not observe a drastic reduction in viral protein synthesis for the RGD poliovirus compared to wild type, we interpret these results to mean that the substitution in the BC loop affected steps in the assembly/encapsidation of the virus.

The results of our studies are unique because for the first time, polioviruses have been described in which the BC loop has been substituted with an RGD motif that could facilitate interaction with target cells. Previous studies have established that mutations within this region that substitute amino acids corresponding to the type 2 Lansing strain, resulted in a virus that was neurovirulent in mice (18). These type 1/type 2 chimeras were able to induce paralysis following intracerebral injections into normal mice. Based on these studies, the investigators concluded that mutations within this region had the capacity to alter the host range of poliovirus. Even with these changes, though, the type 1/type 2 chimeras did not possess the capacity to infect murine cells in vitro. Consistent with these results, we found that the substitution of the RGD binding motif into this region did not confer the capacity of poliovirus to infect murine (or other rodent) cell lines. The RGD was functional, though, as evidence by the capacity to enhance binding to BHK cells that were competed by peptides specific for the RGD sequence. Thus, the initial elements of the poliovirus infection have not been altered with these mutant viruses. Most probably, the virus needs to interact with the poliovirus receptor to induce the initial events in uncoating. The additional RGD sequence might facilitate interaction with the cell prior to

interaction with receptor or could promote interaction following the conversion of the virion to the 135S particle that occurs following interaction with receptor. The enhanced infectivity, then, that we observed on a per genome basis for RGD poliovirus supports the idea that the addition of the RGD sequence has provided some benefit for the infectious process of poliovirus. Additional studies will be required to delineate the exact mechanism of how the RGD/integrin interaction facilitates infection.

ACKNOWLEDGMENTS

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ENHANCED INFECTIVITY OF POLIOVIRUS REPLICONS ENCODING AN ARGININE-GLYCINE-ASPARTIC ACID (RGD) AMINO ACID MOTIF IN THE BC LOOP: AN APPROACH TO TARGETING OF GENE DELIVERY BY REPLICONS

by

MONICA C. FRAZIER AND CASEY D. MORROW

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ABSTRACT

Following interaction of poliovirus with the cellular receptor, the virus undergoes a conformational change. Many of these altered A particles are not internalized and sloughed off from the cell. To enhance the binding of viral particles to cells, we engineered a sequence encoding arginine-glycine-aspartic acid (RGD), known to interact with cell surface molecules called integrins, into the BC loop of the capsid of poliovirus type 1. The capsid was cloned into a recombinant vaccinia virus vector for further study, (vaccinia virus encoding mutated poliovirus capsid RGD-P1) [VVRGDP1]. Immunoprecipitation analysis using immunoprecipitation with an antibody to poliovirus capsids revealed VVRGDP1 produced a full-length capsid capable of being processed by viral protease 3CD into individual capsids. VVRGDP1 was used to encapsidate poliovirus RNA replicons encoding luciferase or green fluorescent protein (GFP). The replicons encapsidated by VVRGDP1 were capable of binding integrins expressed on the surface of cells. The encapsidated RGD replicons were more infectious compared to replicons encapsidated with the wild type capsid, as determined by luciferase assays and direct observation of infected cells for GFP. The results of our studies demonstrate the ability to influence infectivity through changes within the BC loop and point to the possibility of developing a cellular targeting approach for replicon gene delivery.

INTRODUCTION

Poliovirus is a positive-strand RNA virus with an icosahedral virion structure. The virion structure is known from X-ray crystallographic studies (19). Poliovirions are composed of 60 copies of the virus capsids, viral proten (VP)0, VP3 and VP1. The

capsids are initially expressed as a precursor protein, P1, which is proteolytically cleaved by a virally encoded enzyme (3CD), to yield the capsid proteins VP0, VP1, and VP3 (18). The cleaved proteins (5S) assemble to form a 14S pentamer and, finally, a 75S empty capsid (3, 18). It is still not clear whether the RNA genome is encapsidated by insertion into the empty capsid or, more likely, the RNA genome interacts with the 14S pentamer as a step in the encapsidation. The formation of a mature virion occurs after cleavage of VP0 to VP2 and VP4 (7, 18).

The availability of an infectious cDNA clone of poliovirus has allowed the analysis of the effect of l mutation within various regions of the capsid P1 protein on capsid assembly, encapsidation, and viral release (1, 21). Previous studies from this laboratory have demonstrated the expression of the P1 precursor protein and the 3CD polyprotein by using recombinant vaccinia viruses (4, 6). Coinfection of cells with a vaccinia virus encoding poliovirus capsid P1 (VVP1) and the vaccinia that expresses the poliovirus 3CD polyprotein (VVP3) resulted in the processing of the P1 precursor into VP0, VP1, and VP3 proteins which assemble to form 75S empty capsids (4). Other studies in this laboratory have used VVP1 to encapsidate replicons, which are genomes of poliovirus that contain foreign genes in place of the P1 capsid sequence (2, 12, 24, 25). These studies have shown the ability to encapsidate various foreign genes substituted for the entire P1 capsid, in *trans*, using the recombinant vaccinia virus VVP1 (25, 26).

A feature of poliovirus infection is that after interaction with the cellular poliovirus receptor (PVR; CD155), the virus undergoes an irreversible conformational change. These altered A particles have lost the VP4 capsid protein and now protrude the amino terminus of VP1 (16, 31, 32). Greater than 80% of these A particles are not

internalized into the cell and subsequently shed from the cell surface (29). Recent studies have shown that A particles are infectious if internalization in the cell is facilitated (14). In support of this result, we described the characterization of a recombinant poliovirus, which encodes an amino acid sequence in a virion protein known to facilitate interaction with cellular molecules called integrins (Frazier and Morrow, to be submitted). This virus encoded an RGD amino acid motif substituted for the amino acids with the BC loop of the poliovirion. This virus appeared to have greater infectious potential than the wild type virus. However, following selection on cells expressing integrins as a result of virus growth, we found a mutation within the amino acid of the BC loop of the recovered virus.

To characterize further the effects of the substitution of an RGD amino acid motif in the BC loop, we have used a complementation system previously described by this laboratory (5, 24). In this system, the capsid gene (P1) is expressed from a recombinant vaccinia virus. Foreign genes are substituted for the capsid gene in the viral genome (referred to as replicon). The infectivity of replicons can be analyzed because of the absence of reversion of the mutation since vaccinia virus has a proofreading mechanism. In this study, a vaccinia virus was engineered to express this RGD-VP1. The new capsid was then analyzed for its ability to be processed by the viral protease 3CD, to form mature capsid particles, and to encapsidate viral RNAs. The infectivity of the new arginine-glycine-aspartic acid (RGD) encapsidated replicons was compared to those replicons encapsidated with the wild type poliovirus capsid. The results of these studies establish that replicons encapsidated in the RGD capsid were more infectious than identical replicons encapsidated in wild type capsids. The results of this study are

discussed with respect to early steps in infection and the development of methods for targeting replicons to different cells.

MATERIALS AND METHODS

Chemicals and Enzymes

All chemicals were purchased from Sigma unless otherwise specified. The restriction enzymes, cell culture media, antibiotic/antimycotic, and DH5α-competent cells were purchased from Gibco/BRL. The fetal calf serum (FCS) was purchased from Biocell Laboratories. The [^{35}S]Translabel (methionine-cysteine) and methionine- and cysteine-free media for metabolic labeling were purchased from ICN Biomedical. The ^{32}P-UTP used for generating radiolabeled RNA probes and the ^{35}S-dATP used for DNA sequencing were purchased from Amersham. The synthetic DNA primers used for PCR were obtained from Oligos, Etc. and Gibco/BRL. The SeaKem Low melting agarose was purchased from ECM laboratories.

Cells and Viruses

Baby hamster kidney (BHK)-21, thymidine kinase minus(TK)$^-$143$_B$ mouse L and HeLa H1 cells were purchased form American Type Culture Collection. BHK-21 and HeLa H1 cells were maintained in monolayers culture in complete media that consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FCS and 1% antibiotic/antimycotic. TK$^-$143$_B$ mouse L cells were maintained in monolayers culture in F12 media (Gibco/BRL) with the same supplements as DMEM complete media. The wild type vaccinia viruses used for production of the recombinant vaccinia virus

encoding mutated poliovirus capsid RGD-P1 (VVRGDP1) have been previously described (4). The recombinant vaccinia viruses encoding poliovirus capsids 1 and 3, VVP1 and VVP3, used for encapsidation of poliovirus replicons and for experiments that investigated expression and processing of the poliovirus P1 capsid protein have been previously described (2, 4). The antiserum to poliovirus RNA-dependent RNA polymerase ($3D^{pol}$)and poliovirus has also been previously described (1, 20).

Mutagenesis of the Poliovirus P1 Gene

The RGD peptide, RRGDLGSL, replaced the wild type amino acid PASTTNKD within the BC loop of poliovirus by site-directed mutagenesis by PCR of the P1 genes (30). The three synthetic primers used were as follows: primer A 5'-CAT-GTC-GAC-AAA-GTG-AGA-CTC-ATT-ATC-3', which covers nucleotides 640 to 658 and encodes a unique SalI restriction site; primer B 5'-ACC-GTG-GAT-AAC-AGA-CGA-GGT-GAT-CTA-GGG-AGT-TTA-AAA-TTA-TTT-GCA-GTG-TGG-AAG-3', which is the mutagenic primer that covers nucleotides 2750 to 2806; and primer C 5'-GGG-CCC-GGG-CTA-ATA-TGT-GGT-CAG-ATC-3', which covers nucleotides 3371 to 3385 and encodes a unique SmaI restriction site. To modify the BC loop of poliovirus, a T7-IC cDNA, which contains the full-length poliovirus infectious clone, was used as the starting plasmid for cloning the entire P1 region (12). In the first round of PCR, the mutagenic primer B and primer C were used to generate a megaprimer of 635 bp, which encoded the RGD mutation. In the second and final round of PCR, primer A, the megaprimer DNA segment generated from the first round of PCR, and the T7-IC cDNA starting plasmid were used. This final PCR generated a 2.64-kb RGDP1 segment comprising the full-

length P1 region containing the RGD mutation flanked by SalI and SmaI restriction sites. The 2.64-kb segment designated RGDP1 was used for the generation of a recombinant vaccinia virus.

Construction of a Recombinant Vaccinia Virus Vector

To generate a recombinant vaccinia virus, the vaccinia virus recombination vector pSC65 was used (Fig. 1). Both the vector pSC65 and the 2.64-kb RGDP1 segment generated from site-directed mutagenesis by PCR are digested with SalI and SmaI enzymes. Following digestion, the PCR product is ligated into pSC65 plasmid overnight at 20°C and transformed into *Escherichia coli* DH5α. The colonies were selected on ampicillin containing LB plates after an overnight incubation at 37°C. The plasmids isolated were screened for the described insert by restriction digests with SalI and SmaI enzymes and sequenced for the desired mutation by dideoxy sequencing of double-stranded DNA. The plasmid generated was called pSC65-RGDP1 and used to produce the recombinant vaccinia virus, VVRGDP1.

Generation and Selection of a Recombinant Vaccinia Virus

To generate the recombinant vaccinia virus, VVRGDP1, the pSC65-RGDP1 plasmids were transfected into wild type vaccinia virus-infected HeLa H1 cells by the calcium phosphate ($CaPO_3$) method using a kit purchased from Stratagene and following manufacturer's directions. Selection of the recombinant vaccinia viruses was by resistance to the drug 5-bromo-2'-deoxyuridine (BrdU) and by a blue plaque phenotype as previously described (2) on TK$^-$143B cells overlaid with 2.5% SeaKem agar

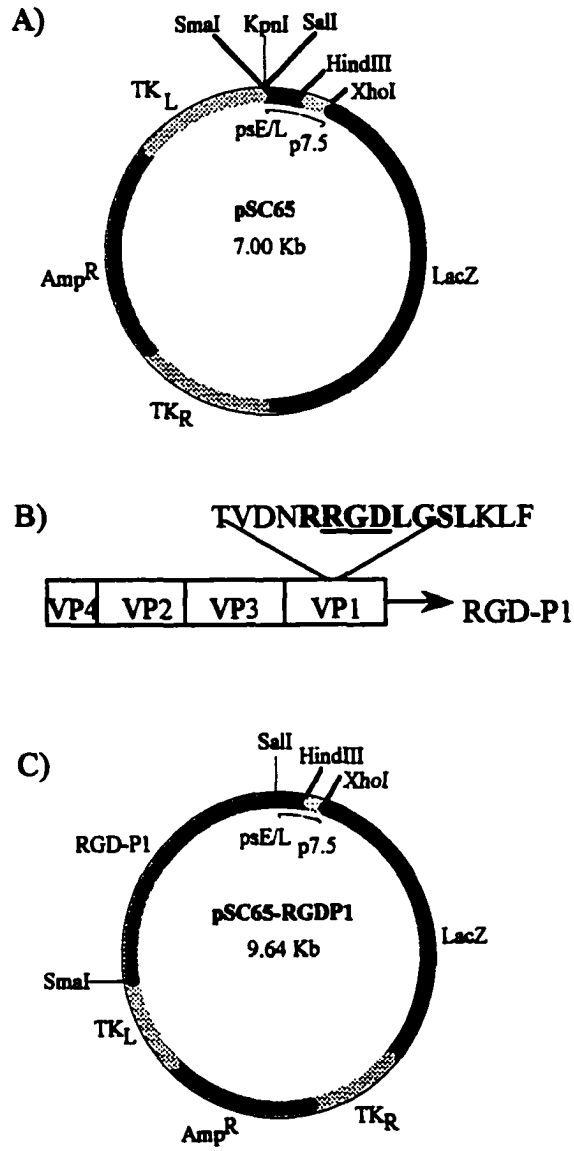

Fig. 1. Schematic of recombinant vaccinia virus vector pSC65 and of RGDP1 sequence. (A) The vaccinia virus vector, pSC65, is composed of ampicillin resistance, thymidine kinase, and LacZ genes. It also contains the vaccinia early/late promoter and a multiple cloning site. (B) Depiction of the RGDP1 protein. Letters in bold represent the mutated sequence; all others are of wild-type sequence. (C) Depiction of the pSC65-RGDP1 plasmid used to generate the vaccinia virus VVRGDP1. The RGDP1 sequence, which has unique SmaI and SalI sites flanking the sequence, was cloned into the SmaI/SalI restriction sites of the pSC65 plasmid.

supplemented with 5% FCS and xylene galactosidase. The putative recombinant vaccinia viruses were then plaque purified on TK$^-$143$_B$ cells overlaid with the same agar mixture as above several times before being cultured and grown up to large amounts for the final experiments. In addition, the first 5 passages of the viruses in culture were done under selection in BrdU media, after which the viruses were switched completely over to growth in complete media. The VVRGDP1 viruses were analyzed for P1 expression by metabolic radiolabelling and immunoprecipitation with an antipoliovirus antiserum.

DNA Sequencing

The pSC65-RGDP1 plasmid and recombinant vaccinia virus, VVRGDP1, sequences were confirmed by dideoxy sequencing of the double-stranded DNA using the Sequenase™ 4.0 kit (U.S. Biochemical) according to manufacturer's directions. The radioactive label used was [^{35}S]dATP, purchased from Amersham.

Analysis of P1 Expression Following Infection

The analysis of RGDP1 was done similarly as previously published (1). To analyze the expression of P1, monolayers of HeLa H1 cells were infected with either VVRGDP1 or VVP1 at 20 PFUs/cell for 3.5 h at 37°C. The medium was removed and washed 1X with DMEM -methionine, - cysteine. Cells were starved with this same medium for 30 min. Following starvation, the cells were labeled with [^{35}S]Translabel (methionine-cysteine) for 2 h. After labeling, the cells were lysed and proteins immunoprecipitated using a radioimmunoprecipitation assay buffer (RIPA) (150 mM NaCl, 10 mM Tris, pH 7.8, 1% Triton X-100, 1% sodium deoxycholate and 0.2% sodium

dodecyl sulfate [SDS]). Cellular debris was cleared after centrifugation at 14,000 x *g* for 10 min at 4°C and supernatants were allowed to incubate overnight at 4°C with antipoliovirus antiserum, αP1. Protein A sepharose beads were added to the supernatants and allowed to incubate at room temperature for 1 h. Following several washes with RIPA buffer, the bound proteins were stripped from the beads by heating samples at 95°C for 5 min in gel sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 20% glycerol, 0.05% Bromophenol Blue, and 0.7 M β-mercaptoethanol). To determine proccessivity of the capsid by the viral protease 3CD, a vaccinia virus, VVP3, expressing the viral protease 3CD was used. All of the steps for detection of P1 were the same for detection of proccessivity except infection. Unlike before, monolayers of HeLa H1 cells were coinfected with VVP3 and VVRGDP1 or VVP3 and VVP1 at 20 PFU/cell for 3.5 h at 37°C. The cells were washed, starved, labeled, and immunoprecipitated as previously described. All samples for both experiments were run on 14% SDS-polyacrylamide gel electrophoresis (PAGE) gels for the detection of P1 precursor and P1 proccessivity.

Encapsidation of Poliovirus Replicons

Procedures for the encapsidation of replicons have been described previously (5). Briefly, HeLa H1 cells were infected with 20 PFU/cell of VVRGDP1 for 2 h. The cells were then transfected with either in vitro transcribed T7-lucIC RNA or T7-GFPIC RNA using DEAE-dextran method (12). After an overnight infection, the cells and medium were harvested and cell lysates spun for 20 min at 14,000 x *g*. The lysates were then treated with RNase A at a final concentration of 20 μg/ml for 15 min at 37°C. Following RNase A treatment, the samples are diluted with wash buffer (30 mM Tris-HCl, pH 8.0,

and 0.1 M NaCl) and overlaid on a 0.5 ml sucrose cushion (30% sucrose, 30 mM Tris-HCl, pH 8.0, and 1 M NaCl) in SW55 tubes. The samples were spun at 45,000 rpm for 1.5 h at 4°C. Following centrifugation, the pellet was resuspended in complete medium. This was designated pass 1. For serial passage of encapsidated replicons for the generation of large stocks, the following steps were taken. HeLa H1 cells were infected with VVRGDP1 at 20 PFU/cell for 2 h. Next, pass 1 of encapsidated replicons was added. After an overnight infection, the culture was harvested by freezing and thawing cells three times and spinning out cell debris for 20 min at 14,000 x *g* at 4°C. The supernatants were then reused or frozen at –70°C for another pass.

Luciferase Assay

HeLa H1 cells are infected with encapsidated luciferase replicons for 6 h at 37°C. Following infection, the cells are washed in phosphate-buffered saline (PBS), pH 7.0, three times; scraped; and pelleted in PBS at 14,000 x *g* for 2 min. Using a luciferase assay kit (Promega) and following manufacturer's instructions, the luciferase activity of the replicon-infected cells was measured after lysing and addition of luciferase substrate via a luminometer as described previously (26).

Visualization of GFP-Expressing Cells

HeLa H1 cells are infected with encapsidated GFP replicons for 6 h at 37°C. Following infection, the cells were analyzed using a Leica TCS NT confocal microscope.

Replicon Binding Assays

Monolayers of BHK cells (integrin +/PVR -) are adsorbed with wild type luciferase replicons and RGD luciferase replicons for 30 min at 37°C. The replicons were removed and placed onto monolayers of HeLa H1 cells and allowed to infect for 6 h at 37°C. As a control, wild type luciferase and RGD luciferase replicons were placed directly onto HeLa H1 cells (BHK untreated) and allowed to infect for 6 h at 37°C. Following infection, the cells were subjected to a luciferase assay as explained previously.

Ribonuclease Protection Assay (RPA)

Viral RNA was isolated using Tri-Reagent according to manufacturer's instruction. The antisense RNA probe for detection of viral RNA was created using an in vitro transcription kit by Ambion according to manufacturer's instruction. For detection of poliovirus-specific plus-strand RNA, a plasmid (pLuc5) complementary to the luciferase gene encoded within the replicon was linearized with EcoRI and used as a DNA template for making the antisense probe (22). The RPA was performed using an Ambion RPA kit according to manufacturer's instruction. The protected RNA fragment was electrophoresed using a 5% polyacrylamide, 8 M urea gel. The RNA fragment of 503 bases was quantitated by scanning the gel with a PhosphorImager (Molecular Dynamics).

Reverse Transcription (RT)-PCR and PCR

RT-PCR was done following manufacturer's instruction. The kit used was the Gibco/BRL Superscript RT-PCR kit. Amplification of the cDNA was done by PCR. PCR

fragments were analyzed by agarose gel electrophoresis using a 1% agarose gel. PCR fragments were quantitated using a densitometer.

RESULTS

Construction of a P1 Encoding an RGD Motif

The neutralization antigenic site 1 (N-AgI) of poliovirus type 1 is located on a highly exposed loop of the capsid that connects the B and C antiparallel strands of VP1, called the BC loop. Previous studies have shown that this BC loop can be substituted with various amino acid sequences (15, 23). Mutational analyses of this loop have also defined amino acids within the wild type sequence of the BC loop that are critical to virus viability and stability. Viruses can use integrins as a receptor to enter cells (8, 27, 28). Coxsackievirus and foot-and-mouth disease virus (FMDV), members of the picornaviridae family, have RGD sequences on exposed loops within the VP1 region of their capsids. The RGD encoded by these picornaviruses have the following consensus sequence, RGD (L/M) XXL (where x denotes any residue) (11). The amino acid sequence, RRGDLGSL, was chosen to be inserted into the BC loop (VP1) at amino acid position 95 to 102 of poliovirus type 1 Mahoney.

Using site-directed mutagenesis by PCR (30), the wild type amino acids, PASTTNKD, within the BC loop of poliovirus were replaced with the RGD peptide, RRGDLGSL. In the first round of PCR, a 635-bp fragment covering nucleotides 2750 to 3385 of P1 was generated using the mutagenic primer B, which introduced the RGD mutation, primer C, as described in Materials and Methods, and a T7-IC cDNA, which encodes the entire poliovirus genome. In the second round of PCR, the 2.64-kb RGDP1

segment was generated using the 635-bp fragment (megaprimer) from the first round of PCR, T7-IC cDNA, and primer A as described in the Materials and Methods. Following the second PCR, the pSC65-RGDP1 clone was created by digesting the 2.64-kb RGDP1 PCR product and the vaccinia recombination vector, pSC65 with SmaI and SalI (Fig. 1). Following digestion, the RGDP1 PCR product was ligated into pSC65 to create the plasmid pSC65-RGDP1. This plasmid was sequenced and found to contain the full-length P1 with the RGD mutation, RRGDLGSL, substituted for PASTTNKD within the BC loop.

Construction and Characterization of a Vaccinia Virus That Encodes RGD

To produce a recombinant vaccinia virus that would express the RGDP1 sequence, HeLa H1 cells infected with wild type vaccinia virus and transfected with the pSC65-RGDP1 plasmid produced vaccinia viruses that were subjected to several rounds of plaque purification. Following plaque purification, the recombinant vaccinia viruses, VVRGDP1, were amplified by PCR and the region corresponding to the mutation was sequenced. Analysis of the sequence confirmed the RGD mutation within P1. The vaccinia viruses were analyzed for expression of RGDP1 by metabolic radiolabeling of HeLa H1 cells and immunoprecipitation with an antipoliovirus antiserum (αP1). The immunoprecipitated samples were run out on an SDS-PAGE gel; the autoradiograph showed a band about 97 kDa, a size that corresponded to the capsid P1 in the VVP1 samples. The levels of expression were comparable to those found in VVP1 (Fig. 2).

To establish if the mutant RGDP1 was capable of proteolytic processing by the viral protease 3CD to produce VP0, VP1, and VP3, VVRGDP1 was coinfected with

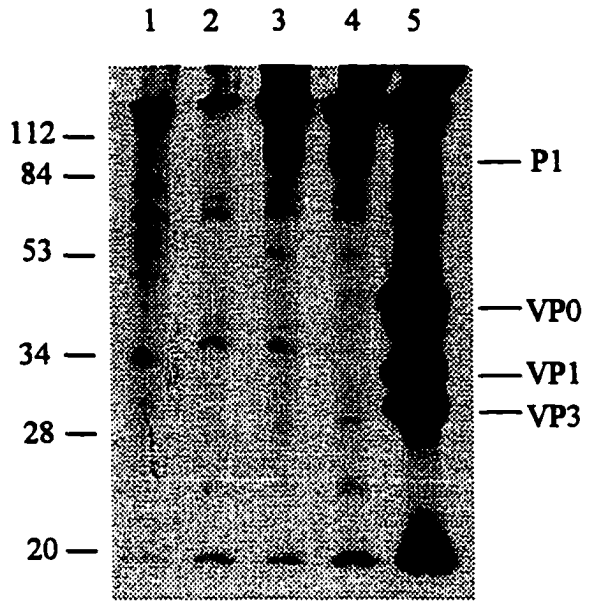

Fig. 2. Autoradiograph of P1 expression. HeLa H1 cells were infected with wild-type vaccinia viruses, VVP1 and VV-RGDP1 and wild-type polio for 5 h at 37°C. The cells were then labeled with ^{35}S methionine for 2 h at 37°C and lysed. The cell lysates were immunoprecipitated with an antipoliovirus antibody overnight at 4°C and run on an SDS-PAGE gel. Results are shown for mock-infected cells (lane 1), wild-type vaccinia virus (lane 2), VVP1 (lane 3), VVRGDP1 (lane 4), and wild-type poliovirus (lane 5). Molecular weight markers (kDa) and relevant proteins are indicated.

VVP3, which expresses the viral protease 3CD (20). HeLa H1 cells infected with VVP1 or VVRGDP1 at 20 PFU/cell were coinfected with VVP3 at 20 PFU/cell for 3 h. Following infection, cells were starved and labeled with [^{35}S]Translabel (methionine-cysteine) for 1 h. The cell lysates were then analyzed for the presence of VP0, VP1, and VP3 by immunoprecipitation with the αP1 antibody. The mutant RGDP1 precursors were processed by the viral protease 3CD (Fig. 3).

Encapsidation of Replicons Using VVRGDP1

Previous studies from this laboratory have demonstrated the ability to trans-encapsidate poliovirus replicons using VVP1 to provide the P1 protein (5, 26). To determine if RGD could encapsidate, replicons encoding firefly luciferase were used. The poliovirus replicon was made using the plasmid, pT7-lucIC, which has the entire P1 region removed and replaced with a luciferase gene. HeLa H1 cells were infected with VVRGDP1 at 20 PFU/cell. Following a 2-h infection, luciferase replicon RNA from in vitro transcription of the T7-lucIC was transfected into the vaccinia-infected cells using a DEAE-dextran method (12). The replicons produced following transfection were purified and serially passed with VVRGDP1 (Fig. 4) to generate large stocks of RGD luciferase replicons. Various amounts of the VVRGDP1-encapsidated luciferase replicons (RGDluc) were used to infect HeLa H1 cells for 6 h at 37°C. After incubation, the cells were lysed, luciferase substrate was added, and lysates were measured for luciferase activity using a luminometer. Infection of cells with the RGD luciferase replicon resulted in significant levels of luciferase activity demonstrating encapsidation (Fig. 5).

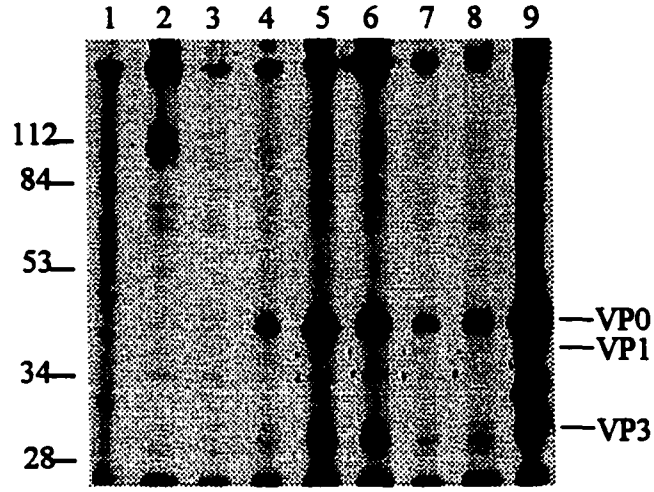

Fig. 3. Autoradiograph of P1 processing by the poliovirus protease 3CD. VVP1, VVP3, VV-RGDP1, and wild-type poliovirus were adsorbed onto HeLa H1 cells for 2 h at 37°C. Following adsorption, infection was allowed to continue for 4 h. The cells were then labeled with ^{35}S methionine for 2 h at 37°C and lysed. The cell lysates were immunoprecipitated with an anti-P1 antibody overnight at 4°C and run on an SDS-PAGE gel. Results are shown for mock-infected cells (lane 1), VVP1 (lane 2), VVP3 (lane 3), VVP1/VVP3 (lane 4), VVRGDP1/VVP3 (lanes 5-8), and wild-type poliovirus (lane 9). Molecular weight markers (kDa) and relevant proteins are indicated.

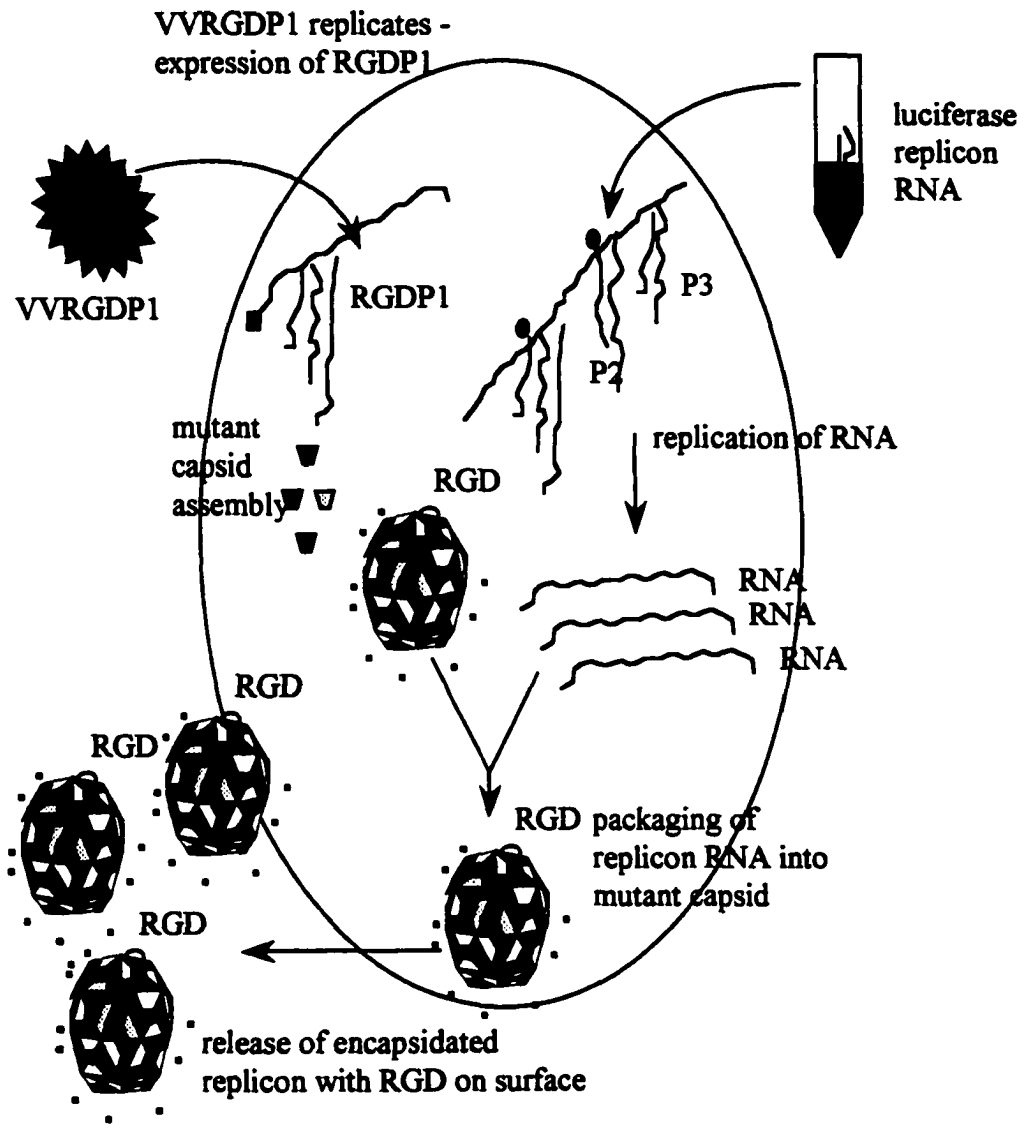

Fig. 4. Encapsidation of RGD luciferase replicons using VVRGDP1. HeLa H1 cells were infected with VVRGDP1 for 2 h at 37°C. After infection, the in vitro transcribed luciferase replicon RNA was transfected into the cells. Following transfection, the RNA was translated and replicon proteins were produced. The RGDP1 protein, as produced by the vaccinia virus, was cleaved by 3CD. The capsids assembled and the luciferase replicon RNA was encapsidated. The RGD luciferase replicons were serially passaged to generate large stocks.

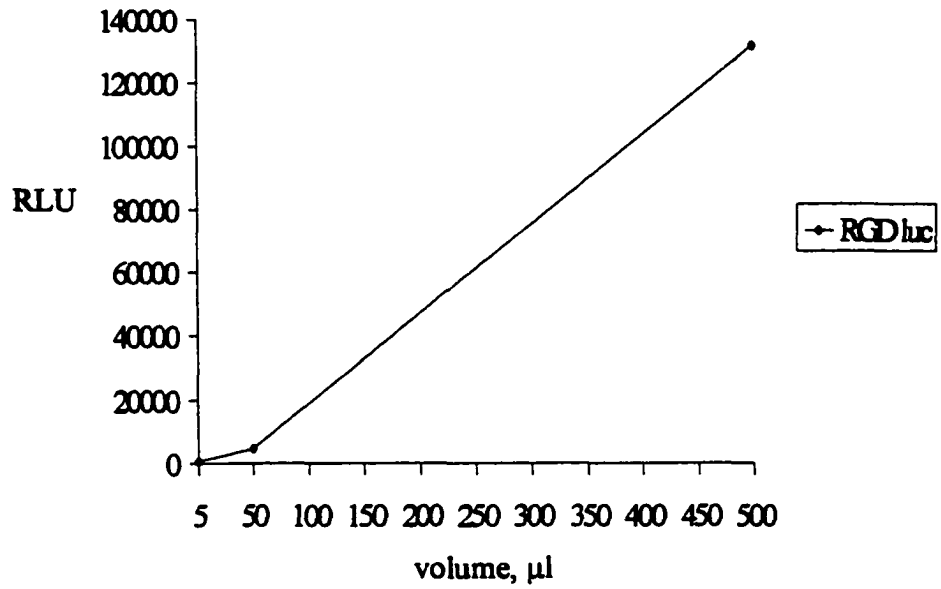

Fig. 5. Luciferase assay of encapsidated RGD luciferase replicon. HeLa H1 cells were infected with various amounts of replicon for 5 h at 37°C. Following infection, the cells were lysed and the lysates used to measure luciferase activity. Activity is given in relative light units (RLU).

Interaction of Replicons With Integrin Positive Cells

It was important to establish whether the RGD was expressed on the surface of the capsid, could bind to integrins, and could influence the infectivity of the replicon. For these experiments, BHK cells were used since they are known to express integrins capable of binding to RGD motifs and possess no receptor for poliovirus. The poliovirus capsid P1, which does not express an RGD motif, should not bind or allow infection of BHK cells. To test binding to BHK cells, luciferase replicons encapsidated with VVP1 (wild type luciferase) and VVRGDP1 (RGD luciferase) were adsorbed onto BHK cells (BHK treated) at 37°C for 30 min in DMEM minus FCS. Following incubation, the supernatants or media from the BHK cells were then placed onto HeLa H1 cells and allowed to infect for 6 h at 37°C. Simultaneously, the same amount of each replicon that was placed on BHK cells was also placed directly onto HeLa H1 cells (untreated) and allowed to infect for 6 h, like the BHK-treated samples. Following infection, a luciferase assay was performed. The results of these assays showed no binding of wild type luciferase replicons to BHK cells (Fig. 6). However, there was about a 75% decrease in luciferase activity for RGD luciferase replicons that were bound to BHK cells in comparison to the untreated RGD luciferase replicon samples.

Because of the low number of poliovirus particles that actually enter cells for a productive infection, it was important to see if the infectious potential of the virus had changed as a result of the RGD peptide now expressed on the capsid's surface. To explore this, the genomes of both wild type luciferase and RGD luciferase were equilibrated via an RPA. Using a pLuc5 plasmid (22) that contained the 5' end of the luciferase genome as a riboprobe for the detection of the amount of luciferase RNA

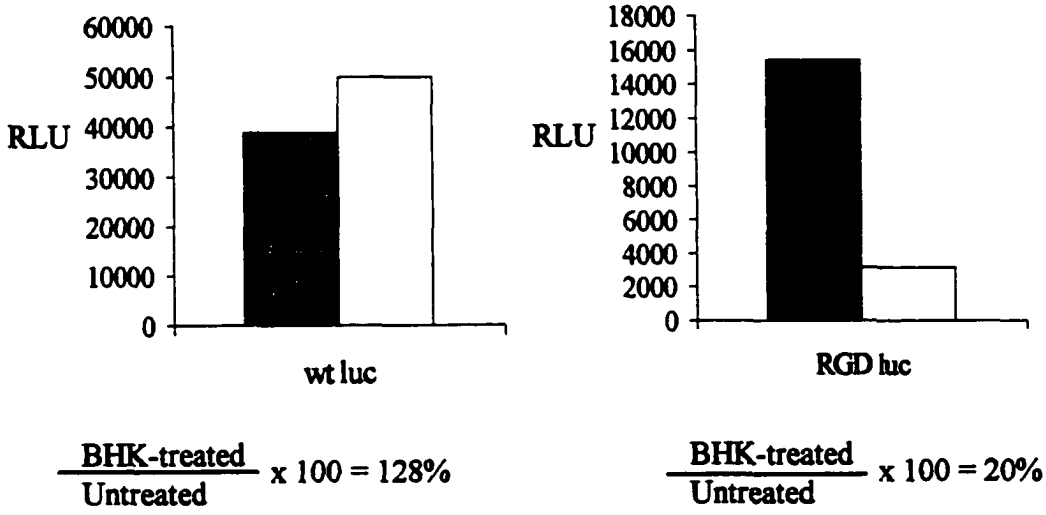

Fig. 6. BHK binding analysis of wild-type and RGD luciferase replicons. Wild-type and RGD luciferase replicons were adsorbed onto BHK cells for 30 min at 37°C. After adsorption, replicons were removed and placed onto HeLa H1 cells for 6 h at 37°C (BHK treated). Wild-type and RGD luciferase replicons were placed onto Hela H1 cells for 6 h at 37°C (untreated). Lysates were then used in a luciferase assay. Graph depicts relative light units (RLU) of virus not bound to BHK cells.

expressed in each replicon, the amount of luciferase RNA in each replicon could be equilibrated. In doing so, genome equal amounts of wild type luciferase and RGD luciferase could be studied. The RPA results demonstrated that there was 5 times more of the luciferase genome in replicons encapsidated by the wild type P1 than in those encapsidated with RGDP1 (data not shown). In other words, the amount of RGD luciferase replicon used was 5 times more than the wild type luciferase. Another luciferase assay was done using genome equivalents of the two replicons. The luciferase assay showed the RGD luciferase replicons to elicit higher relative light units (RLU) than wild type luciferase replicons (Fig. 7). These data suggest that as a result of RGD expression on the capsid, the infectious potential of the replicon has been increased. To characterize further the effects of the RGD motif on infection potential, we utilized a replicon that encodes GFP. Upon infection of cells with this replicon, the cells were green upon examination under UV fluorescence. Both VVP1 and VVRGDP1 were used to encapsidate a replicon encoding green fluorescent protein (GFP). Analysis of normalized encapsidated replicon stocks revealed that the replicons encapsidated with VV-P1 contained more genome equivalents (as determined by RPA) than the encapsidated replicons using VVRGDP1 (data not shown). Thus, it appears that the RGD amino acid displayed on the surface of the encapsidated replicons facilitated the infection. The same experiment was repeated using a GFP replicon. The results from this replicon were the same as those found in the luciferase assay (Fig. 8).

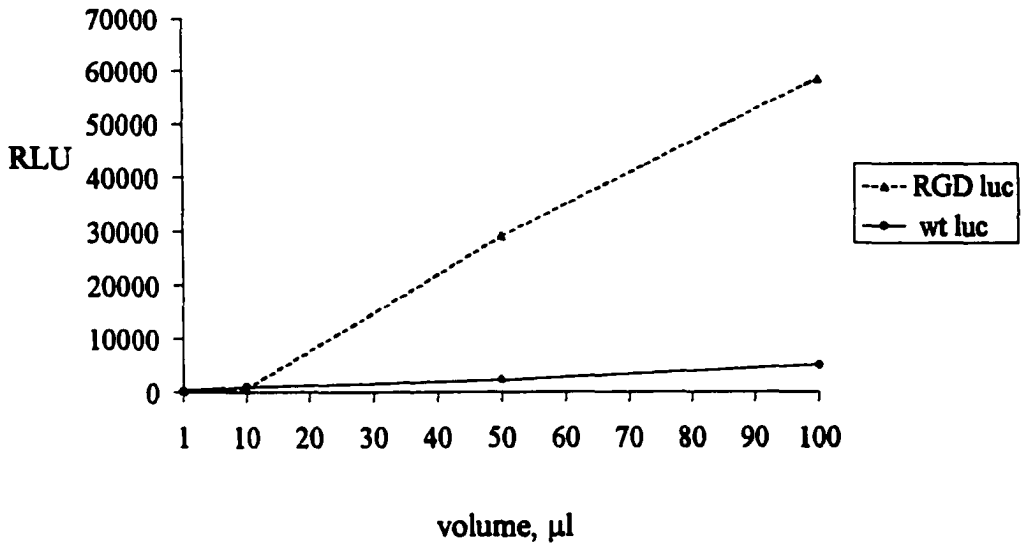

Fig. 7. Luciferase assay of wild-type and RGD luciferase replicons. Wild-type and RGD luciferase replicon genomes were equilibrated via RPA. Equal genomes of wild-type and RGD luciferase replicons were subjected to a luciferase assay. Numbers shown are in relative light units (RLU).

Fig. 8. Picture of wild-type and RGD GFP replicons. Using approximately equal genomes, HeLa H1 cells were infected with wild-type and RGD GFP replicons for 6 h at 37°C. After infection, cells were analyzed for infection using confocal microscopy and 20X objective. Top panel is wild-type GFP and bottom panel is RGD GFP.

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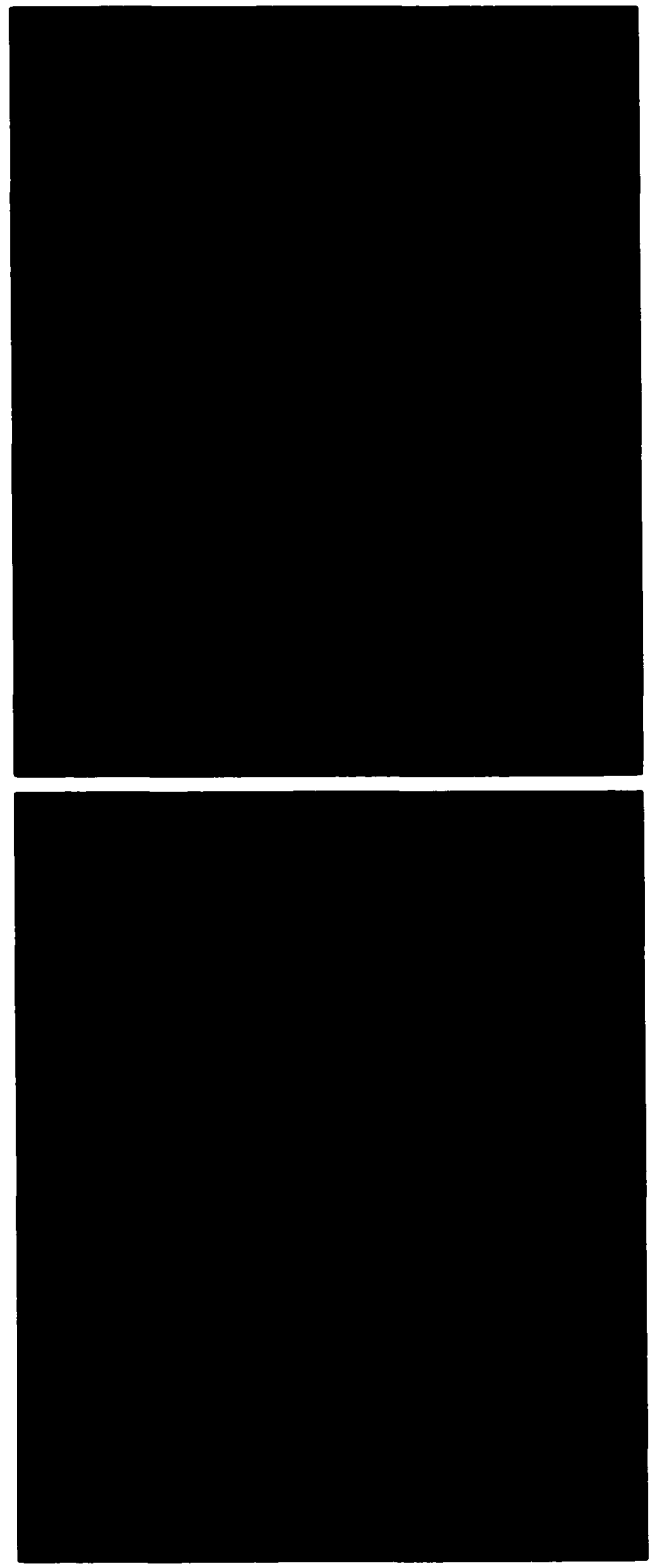

DISCUSSION

In this article, we described the construction and characterization of poliovirus capsids that contain an RGD motif within the BC loop of the poliovirus capsid. The entire P1 region was cloned into a recombinant vaccinia virus expression vector to produce VVRGDP1. Expression of the RGDP1 capsid protein from this virus was characterized with respect to processing by viral protease 3CD, encapsidation of viral RNA, and propagation of a replicon. The proteins derived from the vaccinia virus were shown to be full-length P1 proteins that were capable of being cleaved by 3CD. The capsid proteins were functional as evidenced by encapsidating poliovirus replicons encoding luciferase or GFP. Comparison of equal amounts of the replicon encapsidated with RGDP1 mutation revealed a greater infectivity when compared with replicons encapsidated with the wild type P1.

In a previous study, we described the construction and characterization of a recombinant poliovirus, which contained the RGD amino acid motif substituted for amino acids within the BC loop (Frazier and Morrow, unpublished data). We demonstrated that a viable virus was obtained which encoded the RGD amino acid motif in the BC loop. However, this virus exhibited defects in growth as manifested by a small plaque phenotype, although the virus had enhanced binding to integrin-positive BHK-21 cells. The results of the current study extended these studies by showing that replicons encapsidated with a P1 encoding the RGD amino acid motif were more effectively able to infect target cells. Replicons encapsidated with P1 encoding the RGD amino acid motif were more infectious as evidenced by the fact that fewer genome equivalents were required for infection of target cells. It is not clear, though, whether this enhanced

infectivity is the result of the replicon's interacting with the target cell prior to conversion to an A particle or converted A particles are now infectious as a result of the RGD amino acid motif on the BC loop. It appears that this enhanced infectivity can compensate for some of the effects that the insertion of the RGD amino acid motif in the BC loop has on the assembly/encapsidation of the replicon. We noted that for the most part, lower overall titers of the replicon were obtained following serial passage in the presence of VVRGDP1. Consistent with our previous study (Frazier and Morrow, unpublished data), the lower amounts could be a result of a defect in the encapsidation/assembly of the RGD containing P1 precursors and subviral particles. Interestingly, previous studies have established that mutations within this region can affect the capacity of the virus to grow at different temperatures (13). Indeed, we found that a greater encapsidation was obtained using VVRGDP1 if encapsidation was at 33°C rather than 37°C (unpublished). The results of this study collectively suggest that mutations within the BC loop can positively influence the infectivity of the replicon while simultaneously negatively affecting the encapsidation/assembly of the replicon.

The results of our study have impact on the development and use of replicons as a gene delivery vector. In recent studies, we have described experiments which point to the potential of poliovirus-based replicons for gene delivery into the central nervous system (CNS) (9, 10). Replicons encoding foreign genes were shown to express recombinant proteins for a limited time in motor neurons (10). No expression was noted in other surrounding cells of the CNS such as astrocytes and or oligodendrocytes. The exact reason replicons would not infect these cells is not clear. Recent studies have suggested that the level of PVR expression in the CNS in different cells is extremely low (17). The

promoter for the PVR has been shown to be active only at an early stage in development and is virtually undetectable in the adult mouse. It is possible, then, that neurons express sufficient receptor to allow infection, whereas astrocytes/oligodendrocytes do not express sufficient amounts of receptor to allow virus to initiate an infection. With this in mind, it would be interesting to use replicons, which encode the RGD amino acid motif in the BC loop, to determine whether they have enhanced infectious potential within the CNS. If, as we speculate, the RGD sequence facilitates either the initial interaction or the recovery of A particles, we would suspect that the expression of recombinant proteins within the CNS using replicons encapsidated with VVRGDP1 would be different from that with the same replicon encapsidated by VVP1.

Finally, the results of our studies have implications for the future design of replicons with amino acids substituted in the BC loop that would allow targeting to different cell types. These cell types will need to express the PVR in order to mediate infection. That is, we were unable to eliminate the need for the PVR by substitution of the BC loop with the RGD amino acid motif. We could, though, conceivably generate replicons which would have an enhanced binding to particular cell types mediated via interaction with a specific receptor. Thus, with a low-level PVR expression, the targeted replicons could infect a particular cell type. Additional experiments will be needed to explore this possibility.

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DISCUSSION AND CONCLUSIONS

The focus of the studies described in this dissertation has been to define the molecular events surrounding virus-host cell interactions. Article 1 examined the binding properties of poliovirus following modification of its capsid to include an RGD motif. To determine whether changes in binding had taken place, the binding of modified poliovirus was compared to wild-type poliovirus by using cell lines that do not encode the PVR. Article 2 examined the infectivity of replicons encapsidated with capsids containing an RGD motif. The infectivity of these replicons was compared to replicons encapsidated with a wild-type poliovirus capsid. The results of the first article determined the capability of inserting an RGD motif within the capsid of poliovirus and viral production. It also determined changes in binding and the production of proteins necessary for replication and capsid assembly. The second article analyzed the potential to increase the infectivity of replicons through integrin association via an RGD motif.

Article 1: Integrin Binding Capacity Conferred to a Recombinant Poliovirus Encoding an RGD Amino Acid Motif in the BC loop

The life cycle of poliovirus has been well characterized. It begins with the binding of poliovirus to its receptor, PVR, after which a conformational change (A particle formation) occurs and infection ensues. Of all the virions that bind to the PVR, greater than 80% are sloughed off the cell surface and are no longer able to attach to susceptible cells (165). This viral sloughing leads to not only a high particle to PFU ratio but also an

abortive infection process. To overcome this problem, it was thought that if a peptide could be displayed on the surface of the virus, which would allow interaction with an adhesive molecule, the infectivity of poliovirus might increase. This idea came about from a theory that if A particles, which are normally sloughed off the cell, could remain associated with the cell, then infection could ensue (47). On the other hand, these particles by some accounts have been rendered noninfectious if not taken in by the receptor (165). The goals of the experiments described in this study were to alter the binding of poliovirus such that it could remain more tightly associated with the PVR, allowing internalization via the PVR, and to determine whether using integrins in place of or with PVR could alter internalization of poliovirus. To accomplish this task, a poliovirus that encoded an RGD peptide on the capsid surface was made. This RGD peptide would allow binding to adhesive molecules called integrins. We then used this virus to analyze binding, replication, and protein synthesis in host and nonhost cells.

Mutagenesis of the poliovirus BC loop. The finding that full-length cDNA clones of poliovirus were infectious has caused an explosion in genetic studies surrounding poliovirus (151, 152). Studies involving the mutagenesis of the BC loop demonstrated that substitutions of amino acids 94 to 102 in this region did not affect the production of viable viruses (50, 106, 119). Therefore, for these studies, the amino acids PASTTNKD of P1/Mahoney BC loop, located at positions 95 to 102, were substituted for the consensus RGD peptide RRGDLGSL. This consensus sequence was derived from RGD sequences found in other picornaviruses, the family of viruses in which poliovirus is also a member (33). Using site-directed mutagenesis by PCR (172), the RGD consensus

peptide was cloned into the BC loop of the infectious cDNA clone of P1/Mahoney. Transfection of RNA from the in vitro transcribed plasmid into HeLa cells resulted in the production of viable virus. This finding confirmed previous studies, which showed viral production following substitution of amino acids within this region (50, 106, 119). RNA viruses, like poliovirus, encodes RNA-dependent RNA polymerases. As a result, these viruses have a high rate of mutation during replication. The mutation rate of polioviruses is estimated to be in the range of 10^{-5} to 10^{-4} (206). In working with viruses of this nature, it is important to consider the fact that substitution of a foreign sequence following several passages of an RNA virus like poliovirus may result in the removal of this sequence and acquisition of a reversion back to a wild-type sequence. Therefore, for these studies, stability is important. Sequence analysis of the chimeric RGD polioviruses showed stable expression of the RGD mutation following several passages on Hela cells. In addition, the chimeric viruses exhibited a small plaque phenotype with the exception of a few large plaque revertants. The small plaque phenotype, usually indicative of a mutant virus, is a phenotypic characteristic found in poliovirus mutants (106, 119).

Virus host cell interactions. Studies on the chimeric RGD virus had shown it to be able to infect cells and to produce stable, viable progeny viruses that retained the RGD peptide placed within the BC loop. For some picornaviruses, such as foot and mouth disease virus, echovirus, and coxsackievirus, these RGD sequences play an important role in their life cycles. These RGD sequences allow integrins to act as viral receptors (20, 22, 123, 162, 187). This is to say that the integrins not only allow binding, but also internalization of these viruses. In the case of FMDV, the virus binds to the integrin $\alpha_v\beta_3$

and is internalized by endocytosis into the cell much like poliovirus is by the PVR. The only difference is, whereas poliovirus is recognized by PVR structurally at the canyon, FMDV is recognized by integrins through recognition of the RGD sequence. For each of the viruses named above, the RGD sequence was found on highly exposed loops within the capsid protein VP1 (1, 33, 107, 202). Therefore, the RGD sequence for the chimeric poliovirus was also placed on a highly exposed loop in VP1, the BC loop. Some viruses that use cell surface integrins as receptors can also use nonintegrin receptors. Viruses known to have the capability of using different cell surface molecules as receptors are coxsackievirus A9, echovirus 9, and rotavirus SA11, two of which are members of the picornaviridae family (74, 161, 213). Because other viruses, as well as picornaviruses, had the ability to use other cell surface molecules to infect host cells, it was of interest to see if the RGD poliovirus could also bypass entry into host cells by the PVR. But, before that could be shown, we first had to prove binding to integrin positive, receptor negative cells. Baby hamster kidney (BHK) cells, which are known to have integrins expressed on the cell surface but do not express PVR, are used to test viral RGD binding via integrins (22, 203). Because BHK cells do express integrins, but do not possess the poliovirus receptor, entry should theoretically not occur if internalization of poliovirus requires the presence of the PVR. However, if the internalization of poliovirus does not require the PVR, entry would occur. To test binding of RGD to integrin positive cells, a couple of assays were done. First, a binding assay was done to calculate the amount of both RGD and wild-type poliovirus recovered from the BHK cells following binding. This assay demonstrated a 10-fold difference in binding to BHK cells, which suggested that the RGD was functional and capable of binding to BHK cells. This difference in binding

could have been influence by a couple of factors. These factors include temperature and flexibility of the BC loop. At low temperatures, binding is impaired because of a loss in membrane fluidity (24, 43). Therefore, as the temperature increases, the membrane becomes more fluid and binding increases. The second factor possibly involved in binding is the BC loop. The BC loop has been suspected of playing a role in virion flexibility, thereby playing a role in receptor binding (100). A loss of flexibility in the BC loop due to low temperatures can cause steric restraints on the ability of the viral BC loop to bind integrins. This phenomenon has been seen in studies involving viruses that had mutations within several regions of the capsid including the BC loop in VP1. These mutants exhibited reduced binding to Hela cells at 4°C but not at 37°C (43). This effect was also speculated to be as a result of the loss of membrane fluidity and multivalent interactions between virus and receptor. Lastly, the specificity of binding to BHK cells via the RGD sequence was examined by a competitive peptide assay to inhibit binding to these cells. Integrins recognize specific ligands via specific peptide sequences. One of the peptide sequences recognized by several integrins is RGD. Although other peptide ligands are recognized by integrins, most all of these peptides have an aspartic acid residue encoded within them that is essential for function (110). It was thought that the acidic side chain of aspartic acid was the factor essential for receptor binding; however, substitution of a glutamic acid, which also has an acidic side chain for aspartic acid, did not usually allow binding. Therefore, it was hypothesized that the longer side chain of aspartic acid allowed for better penetration into the binding site making aspartic acid and not glutamic acid suitable for ligand recognition. Hence, for these studies, unlike the RGD peptide, the RGE peptide will not inhibit binding of viruses encoding RGD to the

integrins expressed on BHK cells due to this structural difference. Consequently, the specific RGD and virally specific RGD peptides demonstrated inhibition of RGD virus binding to BHK cells, whereas the RGE peptide did not. As expected, inhibition of the chimeric virus was greater with the virally specific RGD peptide than with the specific RGD peptide. These data, in addition to the previous data, demonstrated the functionality of the RGD expressed and its ability to bind integrin-positive, PVR-negative BHK cells. It also suggests the expression of RGD on the surface of the virion.

The BC loop has been found to be a determinant of host range (119, 122). Studies on the BC loop by several groups have shown that the host restriction of P1/Mahoney in normal mice could be overcome through mutating the BC loop (106, 119, 122). Furthermore, mutations within the BC loop could also allow P1/Mahoney to use mutant PVRs (43, 100). Also, as previously stated, some viruses have shown the ability to use not only their natural receptors but also other cell surface molecules to gain entry into host cells.

Following A particle formation, poliovirus is thought to penetrate and uncoat either via receptor-mediated endocytosis or via release of viral RNA through a pore formed by the capsid proteins VP1 and VP4. For the chimeric RGD poliovirus, the method of entry could be different. The RGD expressed within the capsid of poliovirus could facilitate A particle entry in several ways. First, the RGD could allow better interaction with liposomes. In receptor-mediated endocytosis, the virion is endocytosed to form clathrin-coated vesicles. Acidification of these vesicles results in the externalization of VP1, which attaches to liposomes, thereby allowing release of viral RNA into the cytoplasm. Because the RGD peptide is located within VP1, this mutation may cause the

virion to become more sensitive to the acidification process, which may allow a more efficient externalization of VP1 and/or attachment to liposomes, thereby resulting in RNA release. Second, the RGD mutation could affect the structure of VP1, allowing it to form a larger pore for viral RNA release. Finally, the RGD could allow interaction with a second co-receptor, as in the case of adenovirus, which together with PVR would allow a more efficient poliovirus entry.

As a result of the findings stated earlier, it was of interest to see if the RGD poliovirus could not only overcome host restriction but also use a different method of entry. To test this theory, chimeric RGD virus was placed onto BHK cells and observed over several days for CPE. After 10 days of incubation at 37°C, no CPE was observed. To rule out the possibility of a persistent infection, a radiolabeling of BHK cells 17 h after infection on BHK cells was done. Following immunoprecipitation of protein with an antibody to $3D^{pol}$, no expression of the viral protein 3CD was observed. To prove that virus was indeed present, the same lysates from the 17 h BHK-infected cells were radiolabeled on HeLa cells and immunoprecipitated with the same antibody. These results revealed expression of the viral protein 3CD. Together, these studies provided evidence which suggested that the RGD virus could only bind BHK cells and not enter or infect BHK cells. Therefore, the RGD mutation made in the BC loop of P1/Mahoney did not allow use of a different receptor for viral entry and did not allow replication in BHK cells.

Studies by Liao and Racaniello and Colston and Racaniello illustrated that after several passages on mutant receptor-expressing cell lines, P1/Mahoney viruses had produced mutations within the BC loop to permit use of mutant receptor-expressing cell

lines (43, 100). The studies by Colston et al. demonstrated that mutation P1095S/T, which is within the region of the BC loop mutated for the studies described in this dissertation, greatly affected the ability of the P1/Mahoney capsid to accommodate mutant receptors (100). Interestingly, this expansion in receptor recognition is thought to be as a result of an introduction of a hydrogen bond between residues 1095 and 1102, altering the BC and adjacent EF loop. This EF loop forms part of an interface that is important in controlling assembly and disassembly of poliovirus (43). It is interesting to note that for these studies, following the P1095R substitution, the mutation L1102S arose. Although no sequential data in the Colston et al. studies stated a change at 1102, it is reasonable to think that a change could have occurred in those studies as it did in the studies reported in this dissertation. However, in concert with the Liao and Racaniello and Colston and Racaniello studies, this study demonstrated the adaptability of the BC loop following selection (43, 100). It was therefore of interest to determine then if the BC loop of the RGD virus could also mutate after several passages on BHK cells as a way to select for those viruses that better bound to BHK cells. To ascertain whether this could happen with the RGD virus, this virus was passaged on BHK cells six times and examined for changes within the BC loop by sequence analysis. The results of this experiment revealed the mutation L1102S. Therefore, the L1102S mutation, which is a change from a nonpolar amino acid to an uncharged, polar amino acid, may have also resulted in a small change in the BC loop structure, as suggested in the Colston et al. studies, by allowing the capsid to become more flexible (100). This increase in flexibility may allow recognition of a wider range of receptors; or alternatively, this change could have simply changed the interactions between amino acids that comprise the N-AgI,

allowing the loop to become more exposed on the cell surface for better binding via RGD, the latter thought being the most probable of the two. Because this change only involves one amino acid, no drastic or substantial change is proposed but only speculated because this change may have no effect on the structure at all. Studies to characterize the effects of this change on binding, host range, and structure are needed.

Molecular events after binding. Analysis of the chimeric RGD virus has shown that this virus has the capability to bind integrin-expressing BHK cells but not enter them. The virus continued to demonstrate a dependency on the PVR for viral entry. The initial transfection of viral RNA also demonstrated the ability of virus to replicate and release progeny virions. However, the smaller plaque phenotype detected for this virus indicated a possible defect in RNA release or some other process after binding and entry. Therefore, it was of interest to characterize further the molecular events following binding and entry of the RGD virus into the host cell. Mutational studies on poliovirus VP1 has shown it to be important in capsid assembly, encapsidation, and RNA release (9, 86). In terms of assembly, Ansardi et al. illustrated that the mutants VP1-R129K and VP1-R129Q had an inability to form empty capsids and virions at 33° C and 37°C (9). In addition, the individual capsid proteins produced and needed for capsid assembly were rapidly degraded in cells, thereby causing a defect in capsid assembly (9). In another study, two other mutants, VP-101 and VP-102, were shown to cause defects in encapsidation and viral RNA release (86). The mutant VP-101 had a deletion of amino acids 8 and 9 in VP1 of P1/Mahoney and the mutant VP-102 had a deletion of amino acids 1 through 4 in VP1 of P1/Mahoney. Both mutants displayed a very small plaque

phenotype and defects in RNA release, a probable cause for the very small plaque phenotype; however, the VP1-102 mutant also demonstrated a defect in RNA packaging or encapsidation. This diminished ability to encapsidate was measured by the relative abundance of subviral particles (86). Through these studies, the importance of VP1 in capsid assembly, encapsidation, and RNA release was shown; therefore, to characterize further this mutation in VP1, studies on protein expression and plaque formation were done. To determine whether all the proteins necessary for replication and encapsidation were being produced, pulse-chase experiments were done. The results of these experiments revealed the production of 3CD, P1, and all other proteins necessary for replication and encapsidation of the genome. However, the RGD virus demonstrated a slight lag in protein production when compared with the wild-type. At 3 h, the lag in protein production was apparent; however, by 6 h, this lag was no longer apparent. This lag in production may have resulted from a change in the 2° structure of the RNA as a result of the RGD mutation. Therefore, the polymerase $3D^{pol}$ was not able to synthesize RNA as efficiently. On the other hand, the virus may have mutated at the cleavage sites, causing a delay in cleavage by the viral proteases. However, sequence analysis of VP1 showed retention of the cleavage sites that flank it; therefore, the latter theory is not plausible. To investigate the possibility of a defect in assembly/encapsidation, the viral genomes of RGD and wild-type polioviruses were equilibrated via an RPA and used in a plaque assay. When the plaque forming potential of RGD and wild-type polioviruses using equal genome amounts was compared, a nine-fold difference was found. The RGD virus was much less efficient in plaque formation than wild-type virus. This result indicated a possible defect in virion release or lag in assembly/encapsidation.

To summarize the work presented thus far, the RGD mutation has been shown to be stable and not to interfere in virus production. Through the use of BHK cells and peptide, the ability of RGD to bind integrins is highly suggestive, although not proven. However, the RGD virus is capable of replicating and producing infectious viral particles, although there appears to be a lag in protein production compared with wild-type poliovirus. This lag in protein production may be an important link to the decrease seen in the plaque forming potential of RGD poliovirus compared with wild-type poliovirus, indicating a possible defect in capsid assembly/encapsidation. This lag may be the rate-limiting step in production of capsid proteins needed for capsid assembly.

Alternatively, the decrease in plaques could be a result of virion release. To speculate that this mutation may be the cause for this lag in protein production or virion release is just a suggestive notion. Unfortunately, studies involving mutations within the VP1 BC loop of poliovirus have focused on the effect of this loop on conformation and flexibility of the virion and its role as a determinant of host range (100, 106, 119, 121). This is the first study that suggests the possible role of the BC loop in capsid assembly/encapsidation and virion release.

Article 2: Enhanced Infectivity of Poliovirus Replicons Encoding an RGD Amino Acid Motif in the BC loop: an Approach to Targeting of Gene Delivery by Replicons

Replicons are polioviruses whose capsid (P1) has been replaced with a foreign gene. Because the genomes of replicons contain no capsid, one must be provided. By using the system developed by this laboratory, the capsid is provided in *trans* through another virus, vaccinia virus. The encapsidation of replicons by this method has been

shown many times by others in this laboratory (14, 144); therefore, this system of encapsidating replicon genomes by a providing a vaccinia virus that produces a poliovirus capsid in *trans* has been long established. The study of the effects of mutations within the capsid is effective in this system because of the absence of reversion of the mutation. The inherent proofreading property of vaccinia virus, for the most part, eliminates concerns for loss of the mutation. In addition, this system also allows the characterization of a mutation in a single round of infection.

The goal of this article was to determine if infectivity would increase due to the association of an RGD peptide with adhesive molecules called integrins. To investigate infectivity, a replicon was used. The replicon was to be encapsidated by a capsid that contained an RGD motif within the BC loop. Through the protein expressed by this replicon, infectivity would be analyzed. The RGD sequence that is to be used is identical to the one used in the viral studies of the first article. Unlike the construction of the RGD poliovirus, once the mutated P1 region generated from site-directed mutagenesis by PCR was generated, it was used to create a recombinant vaccinia virus for capsid expression.

Characterization of RGDP1. The mutant RGDP1 capsids expressed by the recombinant vaccinia viruses were characterized for the capacity to be processed by 3CD and for the encapsidation of replicons. In order for a capsid to encapsidate a viral genome properly, it must first be able to be proteolytically processed by the protease 3CD to form the capsid proteins VP0, VP1, and VP3, required for assembly. Previous studies have shown that by coinfecting cells with a vaccinia virus that expresses the wild-type poliovirus capsid P1, VVP1, and with a vaccinia virus that expresses the 3CD protease,

VVP3, processing of the P1 precursor could occur (9, 11, 13). Therefore, to determine the capability of RGDP1 to be proteolytically processed by 3CD, the above method, as described by Ansardi et al., was used (9, 11, 13). The results from this method illustrated expression of the RGDP1 precursor and production of VP0, VP1, and VP3 via cleavage of the RGDP1 precursor by the protease 3CD.

Because processing of RGDP1 had been established, it was important to prove that the capsid proteins VP0, VP1, and VP3 of RGDP1 could assemble in order to encapsidate viral genomes. To prove that the RGDP1 capsid could encapsidate a viral genome, attempts were made to encapsidate a replicon expressing firefly luciferase and GFP. The method of encapsidating replicon RNA by providing a capsid in *trans* had been long established in this laboratory. Likewise, VVRGDP1 was used in the same manner. Encapsidation of these replicons were measured in several ways: immunoprecipitation, luciferase assay, and microscopy. Results from immunoprecipitation illustrated a prominent 3CD band approximately 76 kDa in size, indicative of replication. Results of the luciferase assay revealed an increase in luciferase activity as passages increased. Infection of HeLa H1 cells with the GFP replicon produced green fluorescent cells as seen under a microscope. These assays proved not only replication but also the production of encapsidated luciferase and GFP replicons.

Binding of RGD replicons to integrins. To determine whether the RGD expressed within the capsid of the replicon was functional or capable of binding to integrin-expressing cells, a luciferase replicon was used. Like the studies used to implicate binding of RGD virus to integrins, similar studies were used to test binding of RGD

luciferase replicon to integrins. Because replicons only undergo one round of infection, they do not produce plaques. Therefore, the plaque assays used in the first study could not be used as a determinant of binding to BHK cells. To determine binding of replicons to BHK cells, a luciferase assay was used. The results of the binding assay showed an 80% loss in luciferase activity for RGD luciferase replicons pre adsorbed onto BHK cells, in comparison to RGD luciferase replicons not treated on BHK cells. The wild-type luciferase replicon showed no difference in luciferase activity with or without treatment on BHK cells. The overall result of the RGD binding assay suggested binding of RGD luciferase replicons to integrins expressed on the surface of BHK cells. The data also suggested that the RGD motif was expressed not within the interior of the capsid but on the surface of the replicon.

Infectious potential of RGD expressing replicons. The infectious process of poliovirus is highly abortive. This is due to viral sloughing following binding of poliovirus to the PVR. These particles, which are sloughed off the cell surface, are rendered noninfectious because they are no longer able to bind susceptible cells (19, 64, 177). Therefore, the binding of poliovirus to PVR does not guarantee entry into the host cell. As a matter of fact, the number of virions that actually enter the cell is ≤20% (165). In an attempt to increase the number of virions that enter the cell after binding, an RGD was placed on a highly exposed loop in order to allow binding to adhesive molecules, which would allow poliovirions to remain more tightly associated to the cell, thereby improving the chances of entry into the cell. To test this hypothesis, RGD and wild-type luciferase and GFP replicons would be used. Previous studies by this laboratory have

shown that the proteins produced by replicons directly correlate with the infectious dose of replicon used in infection (146). Using equivalent amounts of luciferase replicon genomes, a luciferase assay was performed. The results of the luciferase assay demonstrated a significant difference in luciferase activity. The RGD luciferase expressed a great deal more activity than wild-type luciferase. Although the same amount of genome was used from each replicon, the RGD replicon had greater activity, thereby suggesting that more RGD replicon entered cells than wild-type replicon. As a result, these data strongly suggest that the particle to PFU ratio was lowered, resulting in a more infectious replicon. This study was repeated using the GFP replicon. These data also demonstrated more GFP activity in RGD replicons than in wild-type GFP replicons. Like the luciferase assay, the GFP assay also suggests a lowering of the particle to PFU ratio, which suggests that the RGD replicon is more infectious.

Stability and propagation of the RGD replicon. During the preparation of large stocks of the RGD luciferase replicon and purification of these replicons, several observations were made. During serial passages of the replicon for the generation of large stocks, it was noticed that the RGD replicon did not produce the large amounts of encapsidated replicons that the wild-type did under the same growth conditions. One reason for this difference could be that the number of 5S capsids present for encapsidation of wild-type replicons was much greater than those present for encapsidation of the RGD replicon. This was unlikely because when we used equal PFU of VVP1 and VVRGDP1, P1 expression appeared to be of equal amounts and the amount of processed proteins was the same. Another reason may be in the release of RNA into

the cell. This is also unlikely in that the luciferase and GFP assay experiments show that when equal genomes were used, the RGD replicon seemed to enter cells more efficiently than the wild-type replicon. A final reason could be that the process of assembly/encapsidation has been slowed. Studies that involved decreasing the actual temperature for passaging of replicons from 37°C to 33°C resulted in a dramatic increase in the amount of replicon produced at similar numbers of passages. Therefore, the actual assembly/encapsidation of replicon seems the most plausible defect that would cause this difference in replicons produced. This is supported not only by the virus data generated in this dissertation but also by previous studies that show mutations in the VP1 region of the capsid can cause defects in encapsidation (9, 87).

Summary and Future Directions

The studies in this dissertation have demonstrated the ability to change virus-host cell interactions through modification of the viral capsid. We have shown the ability to modify the BC loop in such a manner that the RGD mutation introduced is stably expressed and can bind integrin-expressing BHK cells. In studies investigating the effects of the RGD motif on the infectious cycle of poliovirus, the data suggest that while there appears to be no defect in viral entry, there may be a defect in virion release or capsid assembly/encapsidation.

The studies described in this dissertation have laid the foundation from which many more projects can be built. Studies from this work have shown that after several passages on BHK cells, the BC loop of the RGD virus produced the mutation L1102S. This newly formed capsid could be characterized in both the viral and replicon systems

for changes in binding, infectious potential, capsid assembly, and encapsidation and then could be compared with the results described in this dissertation. In terms of binding, one would expect the L1102S virus to be a better binder to integrins or integrin-expressing cells. If the above theory is correct, then it is possible that the L1102S virus could be more infectious than the RGD and wild-type polioviruses described in this dissertation. This would be possible if, indeed, the A particles that are sloughed off are infectious and could be rescued by integrins expressed on the cell surface. Also, the capsid for this virus may be less stable than the other two viruses mentioned. This may be a direct result of the L1102S mutation, which may have come about due to a change in the EF loop, which is known to be an important factor in capsid stability. Also, these viruses (wild-type poliovirus, RGD poliovirus, and L1102S RGD poliovirus) could be structurally characterized for the effects that the RGD mutation may have on the structure of the BC loop and the capsid as a whole. Previous studies have determined not only the three-dimensional structure of poliovirus but also the structural transitions of poliovirus through crystallography to better understand receptor binding and RNA entry/exit into cells (19, 77). For that reason, the same methods could be used to characterize the viruses named above. However, both the RGD and L1102S viruses would be suspected of having less stable capsids due to the results seen in the studies of this dissertation and those of Colston and Racaniello and Liao and Racaniello (43, 100). As for the interaction between RGD and integrins, one would expect the L1102S virus to have better interactions with integrins due to the manner in which it was derived. As a result of the L1102S mutation, it is not unreasonable to believe that this interaction has been optimized.

Last, the pathogenesis of these viruses could be analyzed. The pathogenesis of poliovirus has been well characterized (89, 111, 155). It is known that poliovirus is transmitted via a fecal-to-oral route and that only 1% of natural infections result in paralysis (89, 111). Since the development of transgenic mice that express the PVR, studies involving poliovirus pathogenesis have increased immensely (154). Preliminary studies on the pathogenesis of viruses containing an RGD motif show them to infect only neurons and to cause paralysis, as does wild-type poliovirus. They also display a similar inflammatory response following inoculation in the CNS, including that of neuronal destruction. Finally, the modes of infection are the same for both viruses, with the exception that the RGD virus also appears to be able to infect orally as shown through the recovery of viruses from the CNS. This may be possible because of the involvement of integrins in cell migration. Viruses can become dislodged from their original location, and the RGD expressed on the viral surface rebind to integrin receptors expressed at a distant location. However, studies could also show the RGD virus to be more pathogenic than wild-type poliovirus in that the BC neutralization loop is no longer recognizable by antibodies currently in use.

Integrins are found on a wide array of cell types. Integrins that recognize the RGD motif are found in tissues and cells directly involved in the natural route of infection of poliovirus in humans. These tissues and cells are lymph nodes, tonsils, and colonic epithelial cells (214). Second, mice are known to express integrin in their tissues, as well. As a result, the possibility of integrins mediating migration of the RGD virus from the point of ingestion through the orthopharynx, tonsils, lymph nodes, and eventually to the blood, which leads to viremia prior to infection of the CNS, is not totally inconceivable.

Therefore, based on these unpublished findings, similar studies could be done to characterize the pathogenesis of the L1102S RGD poliovirus. In addition, these studies could be extended to include the replicons. This L1102S mutation could be expressed in a recombinant vaccinia virus to produce a replicon (L1102S RGD replicon) with this capsid. In terms of binding to integrins, this L1102S RGD replicon would be expected to demonstrate greater binding to BHK cells than the RGD replicons. This replicon, when comparing equal genome amounts, would also be expected to be even more infectious than the RGD replicons. The effect of these L1102S RGD replicons on gene delivery to the CNS could also be studied and compared with replicons previously studied in the CNS. One would expect a larger number of genes to be delivered to the CNS by the L1102S RGD replicons when compared to the wild-type replicons, thereby making the poliovirus replicon a more attractive vector for targeted gene delivery.

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I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that she may be recommended for the degree of Doctor of Philosophy.

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Casey D. Morrow	**Chair**	
David C. Ansardi		
Jeffrey A. Engler		
Ming Luo		
Candece L. Gladson		

Director of Graduate Program

Dean, UAB Graduate School

Date 5/3/01