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Analysis of Cell-Induced Reovirus Conformational Change and Identification of a Putative Second Reovirus Receptor

by

John L. Fernandes

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Analysis of Cell-Induced Reovirus Conformational Change and Identification of a Putative Second Reovirus Receptor" submitted by John L. Fernandes in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

Conformational changes in reovirus capsid proteins were detected upon virus attachment to cells. A conformational change was additionally detected in the reovirus attachment protein, σ1, and mapped to the N-terminus of this protein by deletion mutagenesis. Conformational changes in all reovirus proteins were found to be reversible and dependent upon virus (or σ1) interaction with sialic acid - the minimal determinant necessary for virus attachment. Subsequent investigation revealed a protein from L cell membrane that specifically bound reovirus with inreased affinity upon virus alteration by sialic acid. This protein was identified as Annexin VI. Binding of reovirus to a low-affinity receptor, followed by a conformational change and binding to a high affinity receptor may be key steps in the programmed disassembly and entry of reovirus to susceptible cells.

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List of Abbreviations

AVI	Annexin VI
ATP	Adenosine triphosphate
BME	β-mercaptoethanol
BSA	Bovine serum albumin
cDNA	Cloned (Recombinant) DNA
CT	Chymotrypsin
ddH_2O	double-distilled water
DMEM	Dulbecco's minimal essential medium
DMSO	Dimethylsulfoxide
dsRNA	doube-stranded RNA
DMEM	Dulbecco's Minimal Essential Media
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr Virus
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol -bis-(β-Aminoethyl ether) N,N,N',N'-tetraacetic acid
FCS	Fetal Calf Serum
G5	Antibody specific for the C-terminus of Reovirus Protein σ1
HA	Hemagglutinin
HBsAg	Hepatitis B Virus Small Antigen
HBV	Hepatits B Virus
HEPES	N-2-(Hydroxymethyl)piperazine-N-2-ethanesulphonic acid
HIV	Human Immunodeficiency Virus
IM	Intramuscular

JMEM	Joklick's minimal essential medium
kDa	Kilodaltons
mA	Milliampere
MC2	Annexin VI-specific antibody
MOI	Multiplicity of infection
mRNA	Messenger RNA
PBS	Phosphate buffered saline, pH 7.4
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PFU	Plaque-forming units
PKC	Protein Kinase C
PMSF	phenylmethlysulfonylfloride
PPO	2,5 diphenyloxazole
RIP	Radioimmunoprecipitation
R3	Reovirus serotype 3
RNA	Ribonucleic Acid
SC	Subcutaneous
SDS	Sodium Dodecylsulphate
SDS-PAGE	Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis
TEMED	N,N,N',N'-tetramethylethyldiamine
TRIS	Tris(Hydroxymethyl)Aminomethane Hydrochloride
Trp	Trypsin
VAP	Viral attachment protein
VP	Virus protein

MAN'S MIND STRETCHED TO A NEW IDEA

NEVER GOES BACK TO ITS ORIGINAL DIMENSIONS

-Oliver Wendell Homes

Introduction

Overview

To productively infect their host cells, viruses must succeed in three major goals: they must transfer their genome and accessory proteins across the plasma membrane into the host cell cytosol, they must target these components to the nucleus of the host cell, and they must be able to release their genome and accessory proteins from their packaged, condensed transport configuration. For enveloped viruses, these processes are rather well understood to use refined methods of membrane fusion and uncoating (Helenius, 1982). Some of these viruses penetrate the host cell directly through the membrane, while others enter from endosomes (Helenius et al., 1980). For non-enveloped viruses, the early events in the replication cycle are much less clear; while recent progress has been made in identifying cell surface receptors and characterizing virus-receptor interactions, the mechanisms for penetration, intracellular targeting, and uncoating remain largely unknown. Understanding of virus-receptor interaction and the disassembly process of virions at the molecular level is rapidly gaining vast amounts of interest. The study of these virus-cell interactions is crucial to the development of cures for viral disease and methods for directing virus tissue tropism in gene and cancer therapy. Recent advances have been made in rational drug design, following an understanding of the molecular details of virus-receptor interactions. This recent success has been an inspiration of many investigators to pursue an understanding of viral receptors in recent years (Crowell and Lonberg-Holm, 1986).

In the introduction I will discuss recent advances that have been made in the understanding of virus-cell receptor interactions. I will further discuss the effect of virus-receptor interaction on the virus, in terms of cell-induced virus

conformational changes. This will be followed by a discussion of reovirus structure and life cycle. The structure of the reovirus attachment protein, σ1 will be discussed, as well as the current state of knowledge of the early stages of reovirus infection. Additionally, I will introduce a relatively new class of proteins, the annexins, that are appearing to play an important role in virus-receptor interactions.

1 Virus-Cell Receptor Interactions

The tissue tropism and host range of most viruses is determined by the presence of specific receptors on the surfaces of susceptible cells. It is probable that viruses have evolved to recognize these specific constituents of the surfaces of host cells that, in fact, serve some other cellular function. Although it is generally held that poliovirus could only infect cells of primate origin, production of poliovirus could be achieved in the cells of other species when poliovirus ribonucleic acid (RNA) was transfected directly into these cells, bypassing the receptor recognition process (Holland et al., 1959). Additionally, an "infection" ensued when viruses were injected directly into virus-resistant cells (Wilson et al., 1977). This observation indicated that the block to virus infection of the cell happened at the attachment stage, and not at the uncoating or replication stage. Since then, it has been very well established that virus host range is limited by a lack of appropriate cellular receptors. Indeed, viruses were shown to bind only to cells bearing appropriate virus receptors and not to cells that lack virus receptors (Holland, 1961, 1964; Kunin, 1962). Further investigations have revealed that viruses possess cell attachment proteins that

bind specifically to cell receptors. The following sections will detail some of these observations.

2 Virus Attachment Proteins

Viruses recognize cells through use of a single virus attachment protein (VAP) or a combination of proteins. These proteins are often located towards the outermost regions of the virus, where the greatest probability exists of these proteins to be the first virus proteins to interact with the cell surface. Because of their relatively high degree of exposure on virus surfaces, VAPs are often the preferred antigens for the production of neutralizing antibodies and tend to be major targets for the host's immune system.

2.1 Influenza Virus

The attachment proteins of many enveloped viruses have been identified and characterized. The VAPs often appear to be glycoproteins that function in cell attachment and susbsequent membrane fusion. Of particular interest is the attachment protein of the influenza virus. Cell attachment and membrane fusion of this virus is enabled by the trimeric, virus-envelope glycoprotein, hemagglutinin (HA). This protein specifically recognizes sialic acid, which is a normal component of cell membrane proteins and lipids (Carr and Kim, 1993). Each HA monomer is synthesized as a protein precursor, referred to as HA0. This polypeptide is proteolyzed to form a pair of disulfide-bonded peptides, denoted as HA1 and HA2 (Lazarowitz et al., 1971; Skehel, and Waterfield, 1975). Although native HA binds sialic acid, it is dormant in the virus for

fusogenic activity (Carr and Kim, 1993). The interaction of HA with sialic acid has been well described (Sauter et al., 1992) and this interaction is separate from the membrane fusion activity of HA. The central region of the HA2 polypeptide folds into a helical hairpin structure; a short α-helix is connected to a long α-helix by an extended loop region. This long α-helix interacts with the corresponding long α-helices from two other HA2 polypeptides to form an interwound rope of three α-helices, called a three-stranded α-helical coiled coil (Carr and Kim, 1993). The three shorter α-helices are displayed on the outside of the coiled coil. The sialic acid-binding domains of the HA1 subunits assemble on top of the fibrous stem, which is formed primarily by the three-stranded HA2 coiled coil. HA2 is the transmembrane subunit, which spans the envelope membrane once. The amino terminus of HA2 contains a hydrophobic sequence of approximately 25 amino acids, is known to be necessary for membrane fusion (Daniels et al., 1985).

2.2 Poliovirus

Of the non-enveloped viruses, relatively few have been successfully investigated as to the relative natures of their attachment proteins. However, x-ray crystallography has revealed interesting details of the attachment proteins for poliovirus (Hogel et al., 1985) and rhinovirus (Rossman et al., 1985) which are members of the picornavirus family. These viruses are among the smallest RNA-containing animal viruses with an external diameter of approximately 300 A. Their protein shells possess icosahedral symmetry and are made up of 4 different proteins. The three larger structural virus proteins (VP1, VP2, and VP3) form the exterior of the viral capsid, while VP4 is at the interface between the

capsid and the RNA (Smith et al., 1986). A 25 A deep "canyon" is present on the viral surface. Each canyon encircles one of the 12 vertices of the virus and has been identified as the site of receptor binding (Rossman et al. 1985). VP1 is the major structural contributor to the walls and floor of the canyon, and possesses the relatively conserved sequences of the known picornaviruses (Rossman et al. 1985).

2.3 Adenovirus

Adenoviruses are exceptionally well characterized in terms of structure and replication. These viruses are important as pathogens and transforming viruses (Grunhaus and Horwitz, 1992). Recently, they have received special interest as one of the most effective viral vehicles for gene therapy (Cotten et al., 1993; Jaffe et al., 1992; Ragot, et., al., 1993). The adenoviruses consist of an icosahedral particle with a double-stranded genomic DNA and 11-15 structural proteins (Horwitz, 1990). The capsid consists of 12 vertices and 20 facets and at least 6 different polypeptides (Horwitz, 1990). Surrounding each vertex is a penton base, which binds a protruding trimeric fibre protein. The adenoviruses are unique in their use of a two-step attachment process to host cells. The first interaction occurs via the fibre protein, which interacts with high affinity with cell surface receptors. Following this interaction, the penton base recognizes a specific cell surface structure, which leads to the internalization and dismantling of the virus (Greber et al., 1993).

3 Virus Conformational Changes

Viruses must remain stable under many adverse conditions that permeate the extracellular environment, yet must be capable of releasing their genomes under favorable intracellular conditions. It is unsurprising, therefore, that the release of these viral genomes to the cell cytosol happens following the initiation of a programmed disassembly cascade, and that this initiation would be reflected in conformational changes in the virus capsid soon after attachment to host cells.

3.1 Influenza Virus

Of the enveloped viruses, the changes that occur in the influenza virus attachment protein, hemagglutinin (HA), following internalization of the virus to the acidic environment of the endosome are particularly well characterized. Influenza virus infection begins with the binding of the virus to sialic acid on the cell surface via the homotrimeric virus attachment protein, HA. Following this interaction, the virus is internalized by receptor-mediated endocytosis into an endosome (Wiley, and Skehel, 1987). Recent findings lead to a model of the mechanism of the conformational change, where the coiled-coil state of the native HA molecule extends, relocating the amino-terminal hydrophobic fusion peptide 100 A closer to the target membrane (Carr and Kim, 1993). A kinetically controlled mechanism for the HA conformational change has been postulated, in which the native state is "metastable", and that although it is stably folded, it contains the potential to fold into an even more thermodynamically stable state (Carr and Kim, 1993). The acidic environment of the endosome additionally

causes structural changes in the native influenza virions, which are characterized by increased or decreased binding of monoclonal antibodies against four major antigenic regions (epitopes) of HA (Webster et al., 1983; Yewdell et al., 1983). These observations are suggestive of an endosome-induced disassembly cascade for the influenza virus (Bachi et al., 1985).

3.2 Picornavirus

Aside from the picornaviruses and most recently, the adenoviruses, virtually nothing is known about the programmed disassembly of the naked animal viruses during the early stages of virus infection.

The structure of picornavirus is drastically altered in the early stages of infection of susceptible cells (Crowell and Philipson, 1971; Lonberg-Holm et al., 1975; Lonberg-Holm et al., 1972). The change in structure likely results from attachment to the plasma membrane, since it is similarly observed after incubation of virions with membrane preparations from cells bearing the appropriate receptor (De Sena and Mandell, 1976; Guttman and Baltimore, 1977). Picornavirus particles eluted from the cell surface after attachment are characterized by an altered protease susceptibility and antigenic profile, lower sedimentation rate, and the loss of viral protein 4 (VP4) (Crowell and Philipson, 1971; Fricks and Hogle, 1990; Lonberg-Holm et al., 1975; Lonberg-Holm, 1972). The native structure of the picornavirus delegates VP4 to the interior of the virion, below each of the vertices and lacking any exposure at the virion surface (Hogel et al., 1985; Rossman et al., 1985). The absence of VP4 from eluted, prebound particles implies a major structural rearrangement and release of VP4 from the interior of the virion. Since VP4 is myristylated at its N-terminus (Chow

et al., 1987), it is conceivable that it may function in anchoring the virion to the cell membrane and retainment on the cell membrane following elution. It was additionally shown that picornaviruses, altered by attachment to cells, expose the lipophillic amino terminus of viral protein 1 (VP1), suggesting its role in an interaction between cell altered virions and cell membranes (Fricks and Hogel, 1990).

3.3 Adenovirus

For cell entry, adenoviruses attach with high affinity to cell surface receptors via the fibre protein (Defer et al., 1990). While the identity of the first adenovirus receptor remains unknown, the vitronectin-binding integrins have recently been identified as the second adenovirus receptors (Wickham et al., 1993). The integrins mediate virus internalization through attachment to the penton base protein, and cell surface-bound viruses are internalized into endosomes via receptor-mediated endocytosis (Wickham et al., 1993). The acidic pH of the endosome apparently triggers the penetration of the internalized virions into the cytosol (Wickham et al., 1993). Recent studies have shown that incoming adenovirus type 2 particles undergo a stepwise disassembly program necessary to allow progress of the virus in the entry pathway and release of the genome into the nucleus (Greber et al., 1993). The fibers are released, the penton base structures dissociated, the proteins connecting the DNA to the inside surface of the capsid are degraded or shed, and the capsid-stabilizing minor proteins eliminated. The uncoating process starts immediately upon endocytic uptake with the loss of fibres and ends with the uptake of dissociated hexon proteins and DNA into the nucleus (Wickham et al., 1993).

4 Virus Receptors

Regardless of their replication strategy, viruses require cell surface attachment sites. Cellular receptors for a variety of viruses have been identified. In fact, cellular receptors for viruses such as human immunodeficiency virus (HIV), vaccinia virus and rabies virus have been shown to be molecules of considerable functional significance (Table 1). Members of the immunoglobulin super family such as CD4, intercellular adhesion molecule type 1 (ICAM-1), and closely related molecules have been identified as viral receptors (Mendelsohn et al., 1989; White and Littman, 1989).

Of the non-enveloped viruses, only the reovirus and polyoma viruses have been shown to interact with their attachment proteins with sialic acid on the cell surface. The polyomaviruses are also non-enveloped viruses that are icosahedrally symmetrical. The outer shell of the virion contains 360 copies of VP1, arranged in pentamers which serve as virus receptor binding proteins (Rayment et al., 1982). Polyomavirus attaches to the surface of susceptible cells by stereospecific recognition of oligosaccharides terminating in (α2,3)-linked sialic acid (Fried et al. 1981 and Cahan et al. 1983). Studies of pathogenicity show that the specificity of viral binding to such oligosaccharides is an important determination of the virus' ability to establish a disseminated infection and to induce tumors in the natural host.

Table 1 Cell Surface Molecules Serving as Virus Receptors

Virus	Putative Receptor	References
Vaccinia	EGF Receptor	Blomquist et al., 1984; Eppstein et al., 1985.
Sendai	Gangliosides	Holmgren et al., 1980.
Epstein-Barr	CR2 glycoprotein/C3d receptor	Frade et al., 1985.
Lactic Dehydrogenase	Ia and Fc receptors	Inada and Mims, 1985.
Rabies	Acetylcholine receptors	Lentz et al., 1982.
Rhinovirus	ICAM-1 adhesion molecules	Greve et al., 1989; Staunton et al., 1989.
HIV (in T cells)	CD4	Dalgleish et al., 1984; Klatzman et al., 1984.
HIV (in brain)	Galactosyl Ceramide	Bhat et al., 1991.
HBV	IL-6	Neurath et al., 1992.
Friend spleen forming virus	EPO receptor	Li et al., 1990; Yoshimura et al., 1990.
Shope fibroma virus	TNF ?	Smith et al., 1991.
Murine type C ecotropic retrovirus	?W1 cDNA clone	Albritton et al., 1989.
HTLV-1	IL-2 receptor β p75	Kohtz et al., 1989

Adapted from Sauve et al., 1993

5 Annexin Proteins in Virus-Cell Interactions.

Recent literature has reported a new class of proteins, termed "annexins", that appear to serve as virus cell-receptor proteins in addition to their normal cell functions. Although there is little evidence to define precisely the normal cell functions of these proteins, research in this field is progressing at an impressive rate. The current state of knowledge about the structure and function of these proteins will be discussed, particularly with regard to their function in virus binding and internalization.

6 Annexin Proteins as Virus Receptors.

Recently, it was shown that human cytomegalovirus is able to bind to human umbelical vein endothelial cells in a specific, saturable, and calcium-dependent manner (Wright et al., 1994). Affinity adsorption of detergent-prepared lysates of surface-radiolabeled endothelial cells to virions resulted in the identification of a cell-derived protein of 36 kDa (Wright et al., 1994). Protein sequencing of peptides obtained by cyanogen bromide cleavage demonstrated that this protein was identical to human annexin II (Wright et al., 1994). Purified annexin II was subsequently shown to bind directly to virions, providing strong evidence that annexin II is the cell surface receptor for human cytomegalovirus (Wright et al., 1994).

Hertogs et al. (1993) recently demonstrated that a 34 kDa human liver plasma membrane protein specifically bound to the smallest of the three proteins found in the membrane of hepatitis B virus, called hepatitis B virus small antigen (HBsAg). Partial amino acid sequence analysis has determined that this protein

is annexin V (Hertogs et al., 1993). Furthermore, native human liver annexin V was shown to inhibit binding of HBsAg to intact human hepatocytes and shows specificity for binding to HBsAg (Hertogs et al., 1993). This binding was shown to be inhibited by human liver plasma membrane proteins, recombinant annexin V, or annexin V antibodies (Hertogs et al., 1993). Despite a 90% sequence homology, rat liver annexin V did not bind to HBsAg, and did not significantly inhibit binding of HBsAg to intact hepatocytes (Hertogs et al., 1993). This observation suggests that species specificity may be directed by certain, discrete regions of the annexin V protein. Interestingly, cross-linking of HBsAg with radiolabeled annexin V resulted in a specific additional protein complex on PAGE with an apparent molecular weight of 90 kDa, corresponding to a complex of annexin V and HBsAg with a ratio of 2:1 or 1:2 (Hertogs et al., 1993). Collectively, these findings suggest that annexin V may be a receptor for hepatitis B virus infection.

In normal human B lymphocytes, the receptor for the Epstein-Barr Virus (EBV) is the CR2 receptor, which is a membrane glycoprotein normally involved in human B lymphocyte activation (Barel et al., 1981). It was demonstrated that in normal human B lymphocytes, the CR2 receptor is bound by annexin VI. However, if the B lymphocytes are transformed, the annexin VI on the CR2 receptor is replaced with p53, an anti-oncoprotein (Barel et al., 1991). Because the CR2 receptor is involved in signal transduction, it is possible that annexin VI functions also in signal transduction as well as viral infection, and may direct such events as a result of its ability to bind calcium ions (Barel et al., 1991).

7 Annexin Structure and Function

The annexin proteins bind to acidic phospholipids in a calcium-dependent manner (Powell and Glenney, 1987). The annexin family is distinguished by the presence in each of its members of the "annexin fold", a 17 amino acid consensus sequence (Geisow et al., 1986). The annexin fold occurs within a conserved 70 amino acid sequence and is repeated four times in all the annexins, with the exception of annexin VI, where it is repeated eight times (Sudhof et al., 1988; Burgoyne and Geisow, 1989).

7.1 Members of the Annexin Family

Currently, there are nine members of the annexin family derived from vertebrate tissue and determined by sequence analysis (Huang et al., 1968; Wallner et al., 1986; Weber et al., 1987; Crompton et al., 1988; Kaplan et al., 1988; Pepinsky et al., 1988; Burns et al., 1989; Hauptman et al., 1989; Towle and Treadwell, 1992). Three annexins have also been identified in invertebrates (Johnston et al., 1990; Schlaepfer et al., 1992), which brings the total of presently identified annexins to twelve. Evidence of the ubiquitous distribution of the annexins results from the finding that those annexins originally found in invertebrates have also been found in lower organisms such as slime mould (Doring et al., 1991; Gerke et al., 1991; Greenwood and Tsang, 1991) and sponge (Robitzki et al., 1990). Throughout the development of the annexins as a family of proteins, many different laboratories have chosen many different names for these proteins. A list of the most common recent nomenclature has therefore been provided in Table 2.

Table 2 Nomenclature of the Annexins

Annexin	Other Name	References
I	Lipocortin I	Huang et al., 1987; Tamaki et al., 1987; Sakata et al., 1988; Sato et al., 1988
	p35	De et al., 1986.
	Calpactin II	Glenney et al., 1987
	Chromobindin 6	Creutz et al., 1987
II	Lipocortin II	Huang et al., 1986
	Calpactin I	Saris et al., 1986; Kristensen et al., 1986
	p36	Huang et al., 1986
	Chromobindin 8	Creutz et al., 1987
	PAP-IV	Tait et al., 1988
III	Lipocortin III	Pepinski et al., 1988
	PAP-III	Tait et al., 1988
	35 α Calcimedin	Kaetzel et al., 1989
IV	Endonexin	Geisow et al., 1986
	Protein II	Weber et al., 1987
	Lipocortin IV	Pepinski et al., 1988
	PAP-II	Tait et al., 1988
	PP4-X	Grundman et al., 1988
	35 β Calcimedin	Kaetzel et al., 1989
V	PAP-I	Funakoshi et al., 1987
	Lipocortin V	Pepinski et al., 1988
	Endonexin II	Kaplan et al., 1988
	PP4	Grundmann et al., 1988
	35 γ Calcimedin	Kaetzel et al., 1989
VI	p68	Crompton et al., 1988; Moss et al., 1988
	67K calelectrin	Sudhof et al., 1988
	Lipocortin VI	Pepinsky et al., 1988
	Chromobindin 20	Creutz et al., 1987
	67K Calcimedin	Smith and Dedman, 1986
	Synhibin	Creutz et al., 1987
VII	Synexin	Burns et al., 1989
VIII		Hauptman et al., 1989
IX		Johnston et al., 1990
X		Johnston et al., 1990
XI		Towle and Treadwell, 1992
XII		Schlaepfer et al., 1992

Adapted from Smith and Dedman (1990)

7.2 Common Domains in the Annexins

Sequence analysis and limited proteolysis studies have revealed that all of the annexins are comprised of two major domains (Geisow, 1986; Kristensen et al., 1986; Huang et al., 1986; Saris et al., 1986' Glenney and Tack, 1985; Wever and Johnsson, 1986). These domains are illustrated in Figure 1. The N-terminal domain is particularly sensitive to limited proteolytic digestion, and when this short N-terminus or tail is removed, the core protein remains, which consists of the C-terminus. In terms of homology between the N- and C-terminal regions within the annexin family, there is little homology within tail peptides, but a great deal of homology between the C-terminal regions of these proteins. Although annexins I and II show an overall homology of 25%, their N-termini display a 35% homology while their C-termini display a homology of 50% (Kristensen et al., 1986). The significance of the C-terminal homology is important from a functional perspective, in that this core protein displays both of the characteristic properties of this family of proteins: their ability to bind to calcium and their ability to specifically bind to phospholipids (Schlaepfer and Haigler, 1987; Glenney, 1986). The 17 amino acid annexin fold has been postulated to have a loop-helix secondary structure which is involved in calcium binding (Geisow et al., 1986), although an additional distinct, conserved calcium binding domain has been recently reported to be present in annexins I and V (Jost et al. 1994).

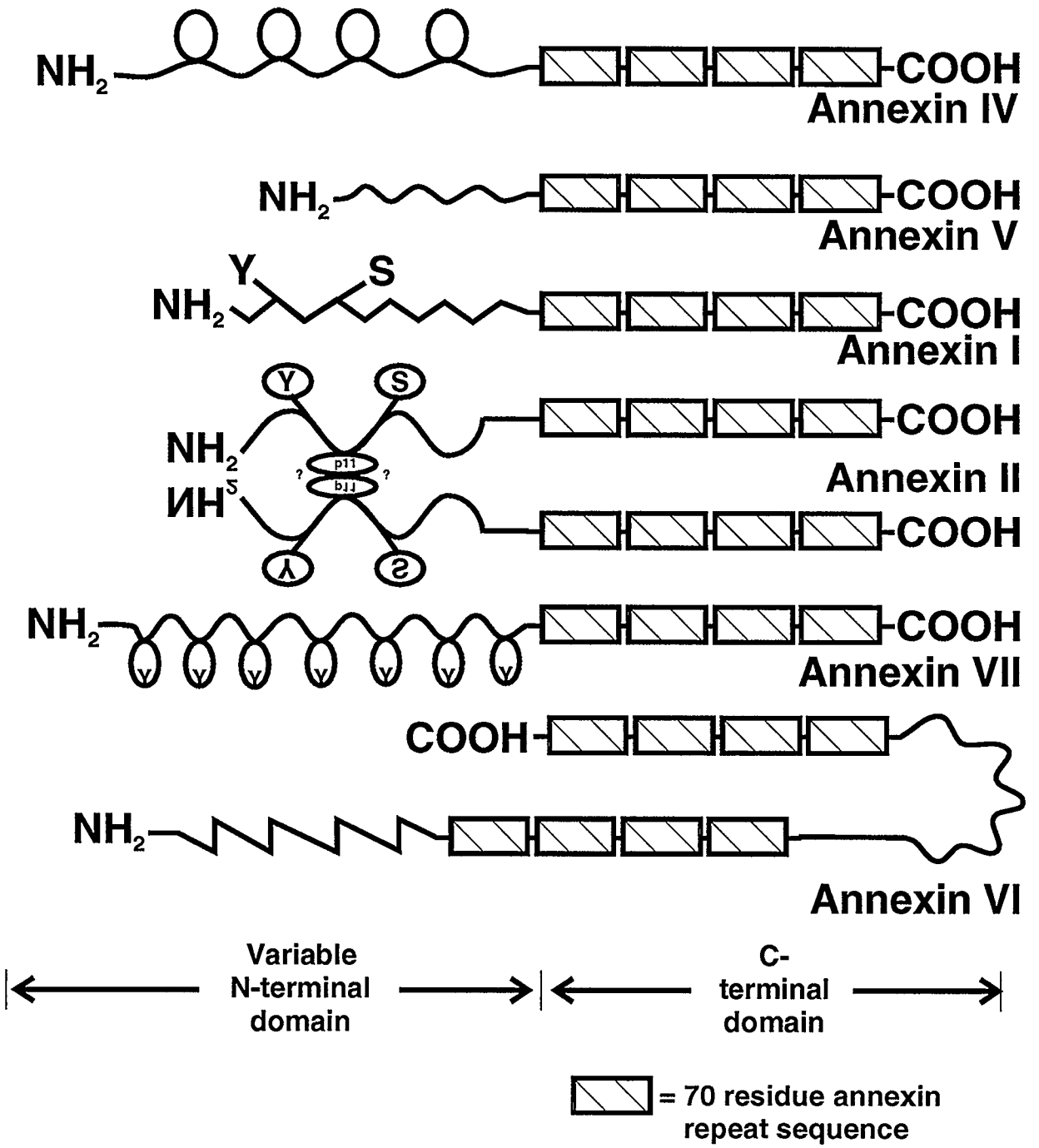


Figure 1 Schematic Showing the Common C- and N-terminal domains of Several Annexins.

The relatively invariable C-terminus contains the 4 annexin repeats (8 in annexin VI). The N-terminal is highly variable from one annexin to another. Y and S represent tyrosine and serine residues, respectively. (Adapted from Creutz, 1992)

7.3 Annexin V Structure

For annexin V, high resolution studies (Huber et al., 1990; Huber et al., 1990; Huber et al., 1992) have provided insights into two possible conformations of this protein which, because of the sequence similarities between the four seventy amino acid repeats of the annexins, may provide information on the core structures of the other annexins.

In their earlier study of annexin V, Huber et al. (1990) concluded that the polypeptide chain of this molecule is folded into four domains which coincide with the 70 amino acid repeats of the molecule (see Figure 2). The N-terminal tail, which consists of residues 5-16, runs across the surface of domain I across to domain IV where it binds and helps to anchor domain I to domain IV. Domain I is comprised of residues 17-88; domain II, residues 89-159; the domain II to domain III connector is residues 160-167; domain III, residues 168-246; domain IV residues 247-317. The short links between domains I and II and also III and IV are described by Huber et al. (1990) as "sharp kinks between the C- and N-terminal α-helices of contiguous domains" and in a helical cylinder plot of annexin V, they propose that a potential ion channel is formed by four of the constituent helices which are coated with charged residues, although they consider that slight side chain conformational changes would be necessary for ease of ion movement. Subsequent studies by Huber et al. (1990) discuss the possible resistance of this channel to ion flow in terms of voltage-dependent gating. It has already been shown that annexin V displays a voltage-dependence for its open state to occur (Rojas et al., 1990). It is therefore speculated that because annexin V is electrically dipolar (modules II and III have

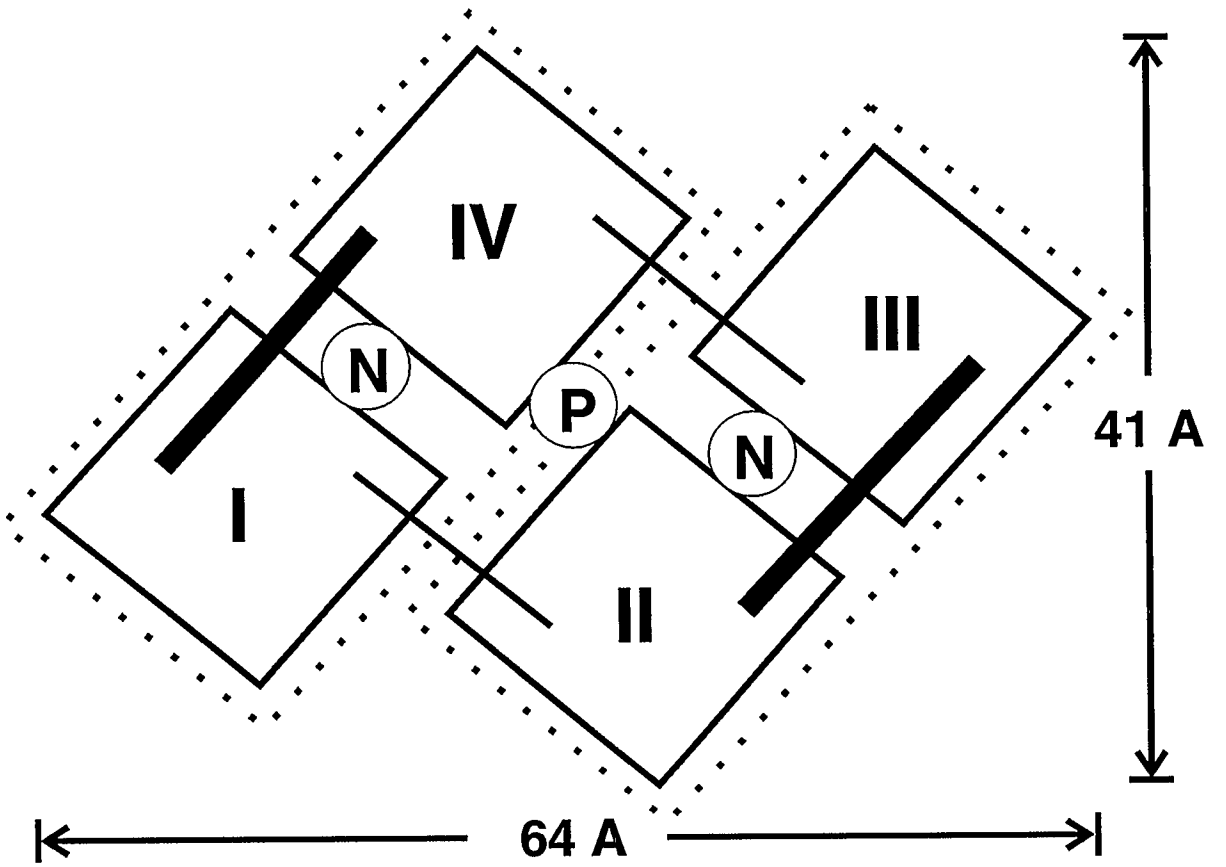


Figure 2 Model of Annexin V Domain Arrangement Predicted from X-ray Crystallographic Data.

Domains I and IV and II and III are considered to interact tightly and form modules (shown with broken lines). These modules interact loosly with each other (bold lines). The areas around **N** are nonpolar, while the inter-module area around **P** is polar and could confer ion-channel activity to the molecule.

(adapted from Huber, et. al., 1990)

an internal positive charge and modules I and IV have an internal negative charge) an electric field might act to alter their orientation with respect to each other, and thus change the diameter of the core of the molecule. The change would result in less resistance to ion flux through the center of annexin V.

8 Annexin VI

Previously, it was noted that structural information gained from the study of annexin V molecular organization may have relevance to the structural organization of other annexins, especially considering that the conservation of amino acid sequence is generally higher than 40% between members of this family. In the case of annexin VI, this protein incorporates eight repeats of the annexin consensus sequence (Moss et al., 1988) which have been shown by sequence alignment to be a duplication of the four annexin consensus domains common to the rest of the family (Crompton et al., 1988). When these two-dimensional aspects of the similarities between the structures of annexin V and annexin VI were considered in conjunction with the observations of Newman et al. (1989), who determined the dimensions of annexin VI through electron microscopy to be 35 Å X 100 Å, Huber et al., speculated that annexin VI may fold to form a molecule of similar organization to annexin V, but with two or more added domains. The model for one possible planar organization of annexin VI derived by Driesen et al. (1992) is shown in Figure 3.

Annexin VI is unique within the annexin family of proteins, in the sense that all other members of the family contain four conserved annexin repeats and have molecular weights ranging between 32-50 kDa , whereas annexin VI

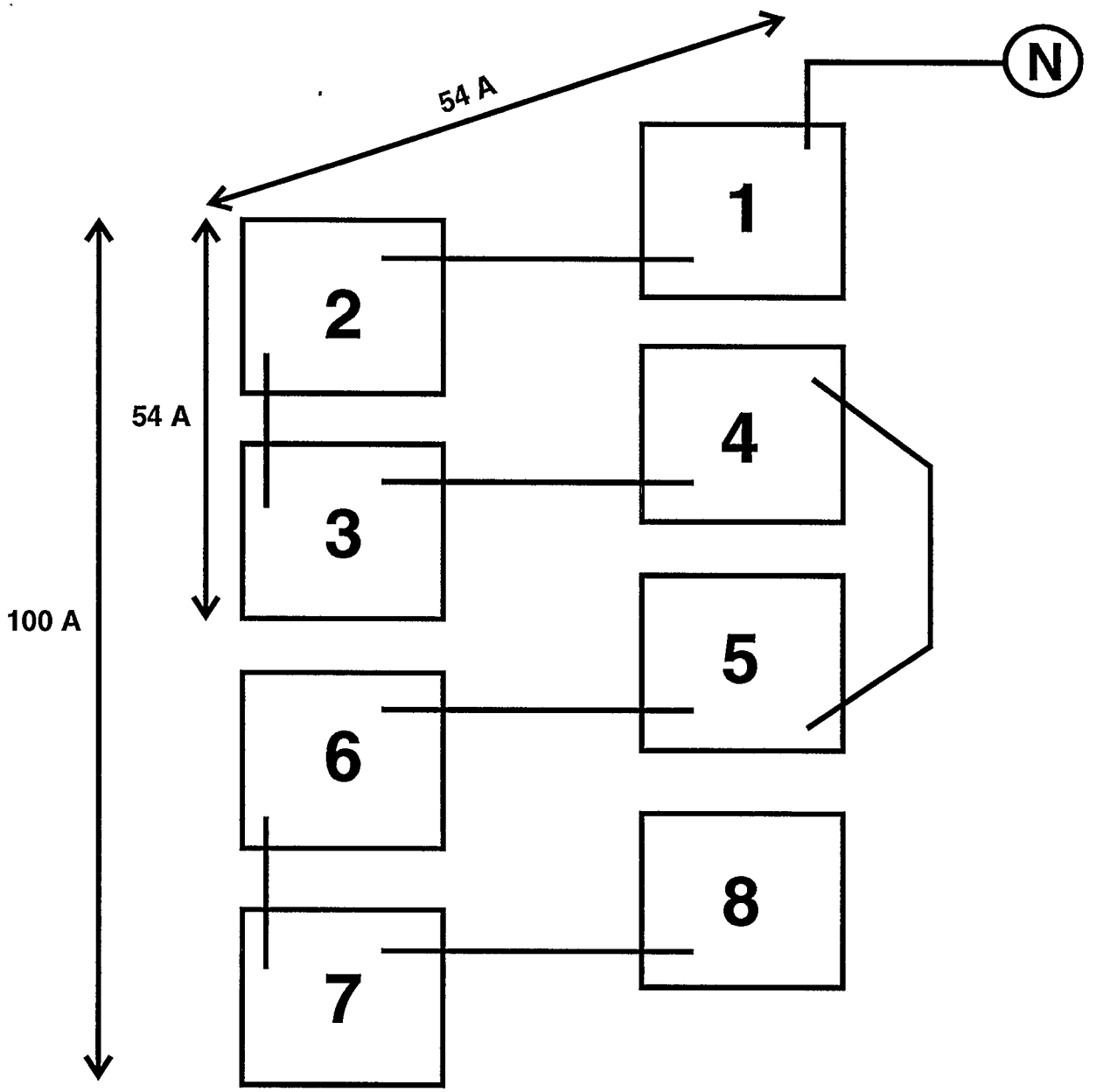


Figure 3 Diagram of a Possible Planar Arrangment of the 8 Domains of Annexin VI.

1-8 represent the eight domains of the C-terminus of annexin VI. The putative a-helix in the peptide linking domains 4 and 5 is shown in an arbitrary position. The N-terminus is labelled N.

(adapted from Driesen, et. al., 1992)

has a molecular weight of 68 000 kDa and contains eight repeats (Sudhof et al., 1988; Crompton et al., 1988). This protein is often observed on SDS-PAGE as a 68 kDa doublet that arises as a consequence of alternate splicing (Moss and Crumpton, 1990).

8.1 Tissue and Intracellular Distribution of Annexin VI

Most current evidence suggests that annexin VI is present only as an intracellular protein. An early anti-annexin VI antibody study identified annexin VI in lymphocytes and a number of cell lines, finding by subfractionation that 90% of it was bound to the plasma membrane and other intracellular membranes, whereas 10% was associated with the cytosol (Crumpton et al., 1984). Recently, however, the heterogenous expression of certain annexins (I-VI) were detected on the external cell surfaces of certain non-metastatic and metastatic murine, rat and human cell lines (Yeatman et al., 1993). Annexin VI was found to exhibit a relatively strong degree of cell surface expression on the cell lines investigated (Yeatman et al., 1993). In a recent comprehensive screening of non-pathological human tissue, it was noted that annexin was expressed in hormone-secreting cells such as adrenal cortex, testicular Leydig cells, and pancreatic islet cells and some secretory epithelia such as salivary gland ductal epithelia, sweat glands and their ducts, and epithelia of non-lactating breast (Clark et al., 1991). Recently, a study of the localization of annexin VI in ventricular, skeletal and smooth muscle cells revealed that annexin VI is a sarcolemma-associated calcium binding protein that is expressed in both striated myocytes and intestinal smooth muscle cells (Iida et al., 1992).

8.2 Annexin VI in Calcium Binding

Annexins do not contain EF-hand structures found in the troponin-C/calmodulin/S-100 superfamily or demonstrate sequence similarity with other calcium-regulated proteins, such as protein kinase C and synaptophysin (Sudhof et al., 1988).

Although annexin VI possesses more of the previously mentioned 17-residue calcium binding domains than any other annexin, this protein has been shown to bind calcium with a stoichiometry of approximately 1:1 and a K_D of 1.2 μM, indicating the presence of one calcium binding site per annexin VI molecule (Owens and Crumpton, 1984). Hazarika et al. (1991) first showed that annexin VI is associated with calcium-sequestering organelles, and suggested that annexin VI may play a regulatory role in the calcium-release/uptake cycle in the sarcoplasmic reticulum of muscle cells as well as in non-muscle organelles, a key process in stimulus-response systems. Annexin VI has further been reported by Diaz-Munoz et al. (1990) to dramatically alter the gating properties of the ryanodine-sensitive calcium-release channel from sarcoplasmic reticulum.

It has been demonstrated that annexin VI as well as annexins IV and VII undergo a calcium-dependent self-association on membrane surfaces (Zaks and Creutz, 1991). The self-association event was observed to occur through a positively cooperative manner and more dramatic in membranes containing phosphatidylserine and phosphatidylcholine rather than in membranes containing phosphatidylserine and phosphatidylethanolamine (Zaks and Creutz, 1991). Annexin VI has been shown to bind to lipid membranes as a trimer to form a hexagonal lattice, resulting in observable "ring-like structures" of about 45 A in diameter (Newman et al. 1989).

8.3 Annexin VI Binding of Lipid Membranes

Biochemically, annexins V and VI as well as protein kinase C exhibit a unique cooperative membrane binding that produces a highly sequential interaction of proteins on the same membrane particle (Bazzi and Nelsestuen, 1991). The association of annexins V and VI with membranes is very responsive to the nature of the membrane so that these annexins constitute very sensitive biological probes of membrane structure (Bazzi and Nelsestuen, 1992).

Biological membranes display asymmetrical organization of phospholipids (Devaux, 1991). Most acidic phospholipids are found in intracellular membranes where they are embedded in a matrix composed largely of phosphatidylethanolamine (PE). On the exterior leaflet of the plasma membrane, phosphatidylcholine (PC) is the major phospholipid. For many purposes, PE and PC may appear similar since they are both neutral, zwitterionic phospholipids that may function as a dispersion medium for more bioactive molecules. The association of annexin VI with membranes containing PC has been observed to induce extensive clustering of acidic phospholipid components (Bazzi and Nelsestuen, 1991a). Consequently, protein binding appeared to involve lateral motion of phospholipids in the membrane. It was suggested that association of annexin VI with membranes involves multiple "calcium bridges" between protein and phospholipids (Bazzi and Nelsestuen, 1991). Like annexin VI, protein kinase C (PKC) binds very little calcium by itself. However, PKC in its complex with membrane lipids was observed to bind a large number (eight to nine) of calcium ions. It was further observed that annexin VI binds a similar number of calcium ions (eight to nine) when bound to phospholipids in a calcium-dependent manner (Bazzi and Nelsestuen, 1991).

8.4 Phosphorylation of Annexin VI

Many proteins are functionally regulated by post-translational modifications and the identification of these modifications frequently provides insights into protein function. Annexins I and II are both substrates for tyrosine kinases and it has been shown *in vitro* that tyrosine phosphorylation of these two annexins has opposing effects on their calcium requirements for phospholipid binding (Moss et al., 1992). Annexin VI was reported to be phosphorylated at low levels on serine and threonine in cells in a growth-dependent manner. Most of the phosphate (approximately 80% of the incorporated phosphate), however, was found to be incorporated into an unidentified complex that has high mobility on thin-layer electrophoresis. It was found to be improbable that this phosphate group is incorporated as a phosphoamino acid, and its identity remains unknown (Moss et al., 1992). Although annexin VI has a tyrosine residue (position 10) and a serine residue (position 13) (Crompton et al., 1988), phosphorylation of these residues has yet to be observed. It is interesting to note, however, that the pattern of phosphorylation observed for annexin VI in cells shares similarities with that described previously for the retinoblastoma gene product, p105RB (Buchkovich et al., 1989). Neither protein is phosphorylated in quiescent cells, whereas both are phosphorylated during the latter stages of the cell cycle and are dephosphorylated as cells exit log-phase growth and enter G_0 (Moss et al., 1992). Expression, synthesis and turnover of both annexin VI and p105-RB appear to be relatively constant throughout the cell cycle (Moss et al., 1992).

8.5 Function of Annexin VI

The function of annexin VI has not been defined, but there are three principal schools of thought. The first proposes that it has a structural role in the cortical submembrane skeleton of the cell. Thus annexin VI, through its association with non-ionic detergent-insoluble fraction of lymphocyte plasma membrane (Davies et al., 1984), and by analogy with annexins I (Glenney et al., 1987) and II (Gerke and Weber, 1984; Glenney, 1986), may interact with elements of the cytoskeleton, especially actin. The second holds that it has a role in membrane/membrane fusion. Evidence for this is derived from studies with Annexin VI, which induces aggregation of secretory vesicle/granule membranes in a calcium-dependent manner (Geisow and Burgoyne, 1987). Thirdly, it is thought that annexin VI may resemble annexin II and have a similar capacity to inhibit phospholipase A2 (Flower et al., 1984), although there is some uncertainty as to whether this represents true inhibition of the protein.

Lin et al. (1992) reported that annexin VI is required for budding of coated pits from purified plasma membranes. It was postulated that annexin VI works, either directly or indirectly, to alter enzymatically crucial lipids in the cell membrane, leading to detachment of the exocytotic stalk (Lin et al., 1992). It is possible that the coordinate binding and trimerization of annexin VI might constrict the stalk sufficiently to force the opposing membranes together and cause fusion. Alternatively, annexin VI may have an intrinsic mechanical activity that squeezes the membranes like a "purse string" to sever the stalk (Lin et al., 1992). The activity displayed by annexin VI in vitro suggested that it is required for coated vesicle formation within the cell. None of the other annexins were observed to have budding activity, which may indicate that annexin VI is

specifically designed for endocytosis/exocytosis reactions (Lin et al., 1992). Smyth et al. (1994), however, recently reported that annexin VI is not an essential component of the endocytic pathway, and that in A431 (human epidermal carcinoma) cells, annexin VI fails to exert any influence on internalization and recycling of the transferrin receptor.

9 Reovirus

The Reoviridae are a family of non-enveloped, icosahedral, double-stranded (ds) ribonucleic acid (RNA) genomes. They have been isolated from plants, insects, fish, birds and snakes (Joklik, 1983; Ahne et al., 1987), and are implicated in a number of human and animal diseases. These include infantile diarrhea (rotavirus), Colorado tick fever, and blue tongue. The best characterized viruses in this family are the human reoviruses (Respiratory Enteric Orphan Viruses), which were first isolated from humans in the 1950's (Robbin's et al., 1951; Stanley et al., 1953; Stanley, 1961). The three distinct serotypes have been extensively studied (Joklik, 1981; Joklik, 1983; Joklik, 1985), and this has resulted in a vast foundation of knowledge resulting in an appealing system to study in the context of virus-receptor interactions.

9.1 Reovirus Molecular Structure

Each human reovirus virion is icosahedral in shape, with a molecular weight of 1.3×10^8, 15% of which is RNA (Rhim et al., 1961; Harvey et al., 1981). The viral protein components are arranged into an outer capsid (approximately 72 nm) and an inner capsid (approximately 56 nm), with the inner

"core" enclosing the ten individual gene segments of dsRNA that make up the viral genome (McCrae and Joklik, 1978; Mustoe et al., 1978). The dsRNA genome is separated into three size classes, L, M, and S (Shatkin et al., 1968; Bellamy and Joklik, 1967). There are three segments in the L class (M.W. about 2.8 X 10^6), three in the M class (M.W. about 1.4 X 10^6) and four in the S class (M.W. about 0.7 X 10^6). Each segment of the dsRNA genome codes for at least one protein and is assigned to a size class based on its relationship to a given dsRNA segment (Mustoe et al., 1978; McCrae and Joklik, 1978). The L class codes for lambda (λ) proteins, M class for mu (μ) proteins and S class for sigma (σ) proteins. Eight of the proteins coded for are incorporated into mature virions, the other two along with p14 are nonstructural (Zweering et al., 1971). The core structure is composed primarily of λ1, λ2, and σ2, with minor quantities of λ3, μ1 and μ2 (Joklik, 1981). The core proteins are responsible for an RNA transcriptase activity (Shatkin and Sipe, 1968), a guanyltransferase activity that "caps" mRNA and has been associated with the λ2 protein (Cleveland et al., 1986; Furuichi and Shatkin, 1976), and two methylase activities.

The nonstructural proteins, σNS and μNS found in large quantities in infected cells (Joklik, 1981), appear to function in reovirus morphogenesis. The σNS protein has been observed to bind ssRNA (Gomatos et al., 1980; Gomatos et al., 1981; Richardson and Furuichi, 1985). Recent reports suggest that μNS serves to anchor the structures involved in genome synthesis and assembly to the cell matrix. The outer capsid is composed of σ1, σ3 and μ1C (a cleavage product of μ1C) (Lee et al., 1981a). Protein σ3 has a strong affinity for μ1C (Lee et al., 1981a), binds tightly to dsRNA (Huismans and Joklik, 1982), and is thought to be responsible for inhibition of host protein and RNA synthesis (Fields, 1982). Protein μ1C is in part responsible for virulence and tissue

tropism (Hardy et al., 1982). Together, the σ3 and μ1C proteins make up discrete subunits or capsomeres, which in turn form the outer icosahedral shape of the virion. "Spikes" of the λ2 pentamers which originate at the twelve vertices of the core icosahedron from a tunnel through the outer capsid, which allows mRNA transcripts to move from the core to the outer cellular environment (Ralph et al., 1980; Lee et al., 1981b), where the tips of the λ2 pentamers are exposed. Capping these λ2 pentamers are molecules of the σ1 protein, the most type-specific of all the reovirus proteins (Lee et al., 1981b; Gaillard and Joklik, 1982; Cashdollar et al., 1985). The σ1protein is the reovirus cell attachment protein and exists as a homo-oligomer (Lee et al., 1981).

9.2 Structure and Function of the S1 Gene Product σ1

The σ1 protein has been shown to be a homotrimer (Strong et al., 1991) and have a fibre-like structure with a globular head extending outwards from the λ2 protrusions (Furlong et al., 1988). It is clear that the most divergent of the reovirus genes is S1, which codes for the σ1 protein. Nucleotide sequencing has shown a 28% homology between serotypes 1 and 2 with only 5% and 9% homology, respectively with the serotype 3 gene (Cashdollar et al., 1985). The sequences translate with serotypes 1 and 2 having 41% of their amino-acid residues shared with serotype 3 sharing no more than 20% of its amino acids with either serotype 1 or 2, resulting in a hydrophobicity profile distinct from the other two. The conclusions from the sequence analysis are confirmed by the observation that antibodies against the serotype 3 protein σ1 are absolutely specific, whereas those against serotypes 1 and 2 σ1 proteins are partly cross-reactive (Gaillard and Joklik, 1980).

Despite large differences in the sequences of the σ1 protein certain functional attributes are maintained. It has been observed that all three serotypes of reovirus compete for the same receptor on L cells, suggesting a conserved attachment site (Lee et al., 1981b). Additionally, the production of genetic reassortants of reovirus with exchanges S1 genes suggests a structural conservation in the region of σ1 attachment to λ2 (Joklik, 1985). The best characterized σ1 protein is that belonging to reovirus, serotype 3.

The S1 gene of reovirus serotype 3 is 1416 nucleotides in length and the first reading frame has a coding capacity of 455 amino acids, sufficient to account for the 42 kDa of its σ1 protein (Nagata et, al., 1984). Theoretical analysis of the S1 sequence predicts an amino terminal portion containing an α-helical coiled coil structure and a globular C-terminal region of indeterminant form (Bassel-Duby et al., 1985). The coiled coil amino terminal region is thought to represent the portion of σ1 that anchors the protein to the λ2 pentamers, with the globular C-terminal region containing the cell binding domain (Bassel-Duby et al., 1985). Additionally, in a more elaborate analysis where the functional capacities of expressed σ1 deletion mutants were examined, the cell binding domains were mapped to the C-terminal region of the σ1 protein (Nagata et al., 1987). It is also clear that within the C-terminal region of the σ1 protein, the erythrocyte and cell binding domains are unique. This has been demonstrated by epitope mapping studies with monoclonal antibodies (Burstin et al., 1982; Spriggs et al., 1983), as well as the observation that when in-frame deletion mutants of the σ1 protein which divide the protein into four segments (5'- I - II - III - IV -3') are produced, segments III and IV contain the host cell binding domain , but segment II must also be present to allow recognition of the erythrocyte receptor (Nagata et al., 1987).

In addition to its role in virus-cell interactions, the s1 protein also plays a major role in other host-virus interactions. The σ1 protein is responsible for evoking a number of host immune responses which include: the elicitation of neutralizing antibodies (Weiner and Fields, 1977), the development of delayed hypersensitivity (Weiner et al., 1980) the generation of cytotoxic (Finberg et al., 1979) and suppressor T cells (Fontana and Weiner, 1980), and in the specific case reovirus serotype 1, the induction of autoimmunity (Onodera et al., 1981; Haspel et al., 1983) and immunosuppression (Garzelli et al., 1985). The σ1 protein may also mediate binding of virions to microtubules (Babiss et al., 1979), and separates tissue tropism from virulence (Weiner et al., 1977).

9.3 Tissue Tropism and Virus-Cell Interaction

As the viral attachment protein and most genetically divergent of the reovirus proteins, the σ1 protein is responsible for inducing the greatest number of host organism immune responses, by being the most immunodominant of the reovirus outer capsid proteins (Gaulton et al., 1986), and clearly regulates the pattern of viral cell and tissue tropism (Fields and Green , 1982; Sharpe and Fields, 1985). Injection of reovirus, serotype 3 into the brains of newborn mice resulted in the development of an acute, often fatal encephalitis, brought about by the specific infection of neuronal, but not ependymal cells (Margolis et al., 1971). In contrast, serotype 1 was only able to infect ependymal cells, leading to a non-fatal ependymitis (Kilham and Margolis, 1970). The construction of reassortants between reovirus serotypes 1 and 3 resulted in the determination that the S1 gene was responsible for the differential tropism previously observed (Weiner et al., 1977). S1 exchanged reassortants were also used to

demonstrate that antigenic variants of reovirus, selected in the presence of monoclonal anti-σ1 antibodies, demonstrated alterations in neurovirulence, despite a conserved ability to infect L cells and spread normally from the intestine (Spriggs et al., 1983; Spriggs and Fields, 1982; Kaye et al., 1986). However, the loss of neurovirulence of one reassortant was traced to the M2 gene (encoding the major outer capsid proteins, μ1C). This was found to be due to the reduced ability of the mutant to grow in neuronal tissues, and not as a result of altered tissue specificity (Hardy et al., 1982) Therefore, it is clear that other viral components play a role in reovirus tissue tropism and pathogenesis in addition to the S1 gene and the σ1 protein.

The gross observations of reovirus tissue tropism and pathogenesis in model systems has generated a great deal of interest in the observation of reovirus-cellular interactions at the molecular level. Early studies on reovirus receptors suggested, that as least on erythrocytes, a major determinant in reovirus recognition of target cells was the presence of particular carbohydrates.

9.4 The Reovirus Receptor

Details of the specific interaction between σ1 and the receptors on host cells are largely unknown, but it is clear that sugar moieties on the receptors are important recognition signals (Armstrong et al., 1984; Gentsch and Pacitti, 1985; Pacitti and Gentsch, 1987), specifically the α-anomeric form of sialic acid (Paul et al., 1989). Because of this, glycophorin was identified as the reovirus receptor on human erythrocytes; this interaction being presumably due to the relatively high content of the α-anomeric form of sialic acid on glycophorin (Paul and Lee, 1987). The demonstration that the α-anomeric form of sialic acid is the

minimal determinant recognized by reovirus is compatible with the finding that the virus binds to multiple sialoglycoproteins on the host cell surface. However, the identities of these proteins remain unknown. A cell-surface receptor for reovirus serotype 3 was previously isolated by using anti-idiotypic anti-receptor antibodies. The receptor was found to be a glycoprotein of 67 kDa and a pI of 5.9 (Co et al., 1985). Other evidence, however, has shown that the epidermal growth factor receptor (EGFR) is recognized by reovirus (Tang et al., 1993). Additionally, the epidermal growth factor receptor (EGFR) on host cells has been shown to confer reovirus infection efficiency (Strong et al., 1993).

The identity of the reovirus receptor has yet to be determined. Because all cell types and tissues contain, on their surfaces, the α-anomeric form of sialic acid - yet not all cell types and tissues are infectable by reovirus, it is probable that there exists another receptor determinant. The nature of this other reovirus receptor will be discussed in the context of this thesis.

10 The Research Project

The main goals of this work were to detect and characterize changes in the structure of reovirus as a result of interaction with host cells. These changes were predicted to occur during the earliest stage of reovirus infection (during attachment) and be manifested as conformational changes in the reovirus capsid proteins. We also attempted to characterize the minimal determinant necessary for these confomational changes to occur in reovirus. Further to these investigations, we established the nature of the cell-induced reovirus conformational changes in terms of effects on the virus. Because conformational changes in reovirus likely result from the interaction of reovirus with its cellular

receptor, we attempted to purify this receptor to determine its identity, and to characterize its interaction with reovirus.

Elucidation of the initial virus-cell interactions would allow us to follow the next steps in virus-cell interactions, such as elucidation of second messages within the cell or internalization events. Advancement of knowledge in the reovirus system would also be of value for the sake of comparison to other viral systems, since it appears that despite there being gross dissimilarities among viral systems, certain critical elements in virus-cell interactions appear to be fundamental to diverse virus groups.

Methods and Materials

1 Cell Culture Procedures

1.1 Cultivation of L Cells

The L-929 mouse fibroblast cell line was used for the propagation of reovirus, σ1 binding studies and production of receptor-containing membrane fractions. Unless otherwise indicated, mouse fibroblast L-929 cells were used in all experiments. Cells were grown in Joklik's modification (for suspension cultures) of Eagle's minimal essential medium (JMEM, Gibco Laboratories, Burlington, Ontario) supplemented with 5% fetal calf serum (FCS) (Gibco) containing penicillin G (75 units/mL) and streptomycin (50μg/mL). A 20 litre package was dissolved in 20 litres of double-distilled water along with 60 g of sodium bicarbonate, and the medium then sterilized by filtration. The L cells were maintained as monolayers in 5% carbon dioxide at 37 ^{o}C, and split when confluent using trypsin-EDTA (Gibco, 0.05% trypsin (1:250), and 5mM EDTA) according to established procedures (Jakob and Paston, 1979; Bird and Forrester, 1981). Suspension cultures were grown in flat-bottomed Florence flasks, incubated in 37^{o}C water baths, with the L cells being maintained in suspension with magnetic stir bars. The cell density was maintained between 0.5 - 1.0 X 10^{6} cells/mL, with the maximum volume being 4 litres of cells in a 6 litre flask (McCrae, 1985).

1.2 Propagation of Virus

The virus strain used throughout these studies was reovirus serotype 3 (Dearing Strain). Plaque plugs were obtained from a virus titration of L-cells, utilizing the agar overlay method (Bellamy et al., 1967; Barret and Inglis, 1985) and were stored in 1 mL aliquots of JMEM + 5% fetal calf serum (FCS) at -70^{o}C. An inoculum was started by freeze-thawing a plaque plug three times, alternating between a dry ice/ethanol bath. This was used to infect a 75 cm^{2} L cell monolayer (McCrae, 1985), which was used to infect a spinner culture of L-929 cells for virus growth and purification.

Four litres of L-929 cells were grown to approximately 1 X 10^{6} cells/mL in JMEM with 5% fetal calf serum (FCS) (Gibco). Two 75 cm^{2} flasks of 24 hour-infected L cells were subjected to alternate freeze-thawing in a -70^{o}C freezer and a 37^{o}C water bath. The 4 litres of L-929 cells were gently pelleted at 80 X g at 4^{o}C for 10 minutes and immediately resuspended in 200 mL of JMEM containing 5% FCS in a sterile 500 mL flat-bottomed Florence flask, containing a magnetic stir bar. The contents of the freeze-thawed 75 cm^{2} (reovirus-infected L-929 cells) flasks were decanted into the Florence flask , and the mixture was allowed to stir gently in a 35^{o}C water bath for approximately 2 hours.

Following incubation, the contents of the 500 mL Florence flask were decanted into a sterile 6 litre flat-bottomed Florence flask, containing a magnetic stir bar. The solution volume was adjusted to 3.5 litres with fresh JMEM/5% FCS and incubated at 32^{o}C for approximately 36 hours. At 36 hours, an additional 500 mL of JMEM/5% FCS were added, bringing the total volume up to 4 litres. Following another 24-48 hour incubation, when approximately 60% cell lysis was

observed (visualized microscopically), virus was harvested according to the following protocol.

1.3 Harvesting of Virus from Infected Cells

Infected L cells (at about 60% cell lysis) were pelleted by centrifugation at 2000 X g for 20 minutes 4°C. The supernatant was discarded and the pellet resuspended in 20 mL of homogenization buffer (10mM TRIS, pH 7.5; 250mM NaCl). The resulting L-cell solution was then sonicated continuously on ice at the microtip output limit (80% duty cycle) for 3 minutes.

Following sonication, a 10% (W/V) sodium deoxycholate solution (in virus homogenization buffer) was added to a final concentration of 0.1%, and the solution incubated on ice for 30 minutes. Approximately 2/3 of a volume (approximately 15 mL) of freon (pre-chilled for 30 minutes at -20°C) were then added, followed by sonication of the solution at the microtip output limit (70% duty cycle) until a stable, homogeneous solution of relative viscosity was achieved. The solution was immediately centrifuged in a 50 mL conical centrifuge tube at approximately 500 X g for 5 minutes at 4°C. The resulting supernatant was decanted, the pellet discarded, and a 2/3 volume of freon (pre-chilled for 30 minutes at -20°C) was added to the supernatant. The solution was sonicated immediately at the microtip output limit (65% duty cycle) until a relatively homogeneous, viscous and stable solution was achieved. The resulting solution was centrifuged for 5 minutes at 4°C in a 50 mL conical centrifuge tube, and the resulting supernatant decanted (the pellet was discarded). This supernatant was then loaded onto 1.2 g/mL - 1.4 g/mL CsCl gradients (prepared by dissolving 27g or 54g, respectively, of CsCl into 100 mL

(total volume) of ddH_2O, containing 2 mL of 1M TRIS, pH 7.4), so that the supernatant volume did not exceed the gradient volume by more than 1/3. The gradients were ultracentrifuged at 26 000 rpm for 2 hours at 4°C in a JS 28.1 rotor (Beckman). The resulting top component (reovirus shells) and lower component (reovirus particles) were separately collected by aspiration and dialyzed against phosphate buffered saline (PBS) (130mM NaCl; 2mM KCl; 1.5mM KH_2PO_4; 8.1mM $NaHPO_4$), pH 7.4 and stored at 4°C until needed. Concentration (in mg/mL) of virus was determined by multiplying the dilution factor (usually 100) by its optical density at 280nm (ultraviolet) and dividing by 5.42). plaque-forming units (PFU)/mL of virus was determined using the agar overlay method. Virus titres of 10^{10}-10^{11} (PFUs)/mg virus were routinely obtained.

1.4 Plaque Titration of Reovirus

6 well, 30 mm tissue culture plates were seeded with L cells and allowed to grow to 70% confluence in JMEM/5% FCS. Dilutions of virus were prepared, ranging from 10^{-5} to 10^{-10} in PBS. The growth medium was removed from each plate of L-cells by aspiration, and 100 μL of each dilution of virus were added to duplicate plates. The plates were incubated for 1 hour at 37°C with intermittent rocking to ensure that the whole L cell surface was exposed to virus. Agar overlay medium was prepared by adding 3.3 mL of FCS to 30 mL of 2X JMEM, followed by incubation at 45°C in a water bath. To this solution, 30 mL of 2% bacto-agar (pre-equilibrated to 45°C) was added and the final solution left in a water bath at 45°C.

After the one hour incubation period, the plates of L cells (infected with reovirus) were each treated with 7 mL of agar overlay medium and incubated at 37^{o}C overnight in 5% Carbon dioxide (tissue culture incubator). The next day, 0.5 mL of neutral red (0.1 % (W/V)) were added to each plate, followed by rocking to coat the surface of the agar evenly. The plates were then incubated for 6-16 hours in the tissue culture incubator before visualization of plaques was achieved. Plaques were collected with a sterile glass pasteur pipette and resuspended in 1 mL of JMEM/5% FCS and stored at -70^{o}C until required.

Titre of the virus was calculated by determining the average number of plaques on duplicate plates, followed by the division of this average number by the dilution factor of the virus used to infect the respective plates. The resulting number was multiplied by ten, and taken as the number of plaque forming units per mL of virus (PFU/mL). This was repeated for at least 5 different dilutions of virus and the mean of each PFU/mL was taken to be the actual PFU/mL of virus.

1.5 Preparation of a Reovirus Column

1.0 g of CNBr-activated sepharose 4b (Sigma) were allowed to reswell in 1mM HCl in a volume of 200 mL, followed by washing with coupling buffer (0.1M $NaHCO_3$, pH 8.3; 500mM NaCl). 5 mL of reovirus, serotype 3 (approximately 4 mg/mL) were dialyzed against coupling buffer and then added to the reswelled sepharose in a 10 mL plastic sterile conical centrifuge tube. The tube was then incubated overnight in the dark, while gently tipping end-over-end at 4^{o}C for 16-24 hours.

The sepharose was then transferred to a buffer containing a blocking agent (0.2M glycine, pH 8.0) and incubated in a similar tipping end-over-end

manner at 4°C for 16 to 24 hours. The sepharose was then washed with coupling buffer followed by acetate buffer (0.1M, pH 4.0; 500mM NaCl) followed by coupling buffer, followed by three more washes of coupling buffer. The sepharose was assayed for reovirus content by SDS-PAGE and stored at 4°C in PBS (containing 0.02% sodium azide).

1.6 L Cell Binding Assay

The L cell binding assay was essentially the same as previously described (Lee *et al.,* 1981). Confluent L-929 cell monolayers in 60 mm plates (Falcon) were preincubated with cold phosphate buffered saline (PBS) containing 10 mg/ml bovine serum albumin (BSA) for 20 minutes at 4°C. After rinsing once with cold PBS, 200 µL of one protein translation reaction (diluted to a 200 µL final volume in PBS of ^{35}S-methionine labelled protein) was added to the plates. The plates were incubated at 4°C with intermittent rocking for 60 minutes, after which the supernatant was removed and the monolayers were washed five times with cold PBS. The cells were lysed with 200 µl of lysis buffer (PBS containing 1% NP-40, 0.5% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) and the nuclei were pelleted for 2 minutes in a microfuge at 5000 x g. Protein sample buffer was added to the supernatant and the bound protein was analyzed by SDS-PAGE and autoradiography of the ^{35}S-methionine labelled proteins.

1.7 Storage of Cells for Tissue Culture

Cells for tissue culture were maintained in storage by pelleting at least 10 X 10^6 cells at no more than 200 X g, followed by resuspension in 1-2 mL of storage media (10% tissue culture grade DMSO, 40% Dulbecco's minimal essential medium (DMEM), and 50% FCS). The cells were then transferred to cryogenic vials which were then sealed and wrapped together in insulation (fiberglass and styrofoam), followed by incubation overnight at -70^{o}C. The next day, the frozen cryogenic vials were transferred to liquid nitrogen for long term storage. Cells were resuspended by thawing them with the addition of 1 mL of JMEM/5% FCS at room temperature, with constant resuspension, followed by incubation in a 75 cm^2 tissue culture flask (containing 10 mL of JMEM/5% FCS in the tissue culture incubator (37^{o}C, 5% CO_2).

1.8 Lysis of Cells

Cells were lysed in cell lysis buffer (20 mM TRIS-Cl, pH 7.6; 150 mM NaCl; 1 mM EGTA (Ethyleneglycol -bis-(β-Aminoethyl ether) N,N,N',N'-tetraacetic acid); 1% Triton X-100; 1mM PMSF) on ice for 5 minutes. Cells were collected by scraping with a rubber policeman and pipette aspiration, followed by microcentrifugation at low speed to pellet the nuclei. Supernatants were transferred to another tube and either used immediately for experimentation or electrophoresis, or stored at -70^{o}C until required.

1.9 Isolation of Cell Membranes

Cells were grown (L-cells from a 4 L spinner culture or A431 cells in lasagna dishes) and pelleted by centrifugation at 2000 X g at 4°C for 15 minutes. The cell pellets were resuspended in resuspension buffer (20mM TRIS, pH 7.4; 5mM $MgCl_2$; 2mM PMSF) and homogenized by hand, using a glass homogenizer and no more than 30-40 strokes. The nuclei were removed by centrifugation at 500 X g for 3 minutes, followed by decanting of the supernatant and discarding of the pellet. The resultant supernatant was centrifuged at 33 000 rpm in a Beckman ultracentrifuge, using an SW55 rotor (Beckman). The pellet was resuspended in 2.5 mL of PBS and layered onto a 2.5 mL 35% (W/V) sucrose (made up in PBS) cushion in a 5 mL centrifuge tube and centrifuged at 16 000 rpm for one hour at 4°C. The resultant interphase band was collected by aspiration and diluted to 5 or 10 mL in PBS. This solution was then centrifuged (using 5 mL tubes in an SW55 rotor (Beckman)) at 33 000 rpm for 30 minutes at 4°C. The resulting pellet was resuspended in PBS or membrane buffer (20mM HEPES, pH 7.4; 150mM NaCl; 2mM PMSF) and stored at -70°C until required.

1.10 Binding of Reovirus or σ1 Protein to L cells and Plasma Membrane

Confluent L cell monolayers in 96 well tissue culture plates (Falcon) were preincubated with cold PBS containing 5 mg/mL of BSA for 15 minutes at 4°C. The PBS was removed by aspiration and [^{35}S]methionine labeled reovirus or aliquots of translation mixtures of full-length σ1 or dII were added to the wells and incubated at 4°C for 60 minutes, after which the supernatants were

removed and the monolayers washed 4 times with cold PBS, followed by the addition of 100µL of 1 X protein sample buffer (PSB) and boiling for 5 minutes prior to SDS-PAGE.

1.11 Fixing of Cells to Plates with Glutaraldehyde

Confluent monolayers of cells were washed twice with an excess of room temperature (RT) PBS, and then covered with no more than 2mm of PBS containing 0.25% glutaraldehyde (V/V). The plates were covered and incubated at room temperature for twenty minutes. The fixed monolayers were washed with excesses of 4°C PBS four times and used immediately or covered with PBS containing 0.02% sodium azide (W/V) and stored covered at 4°C until required.

1.12 Preparation of [^{35}S]methionine Labeled Reovirus

Reovirus was prepared exactly as described in section 1.3, with the addition that approximately 33µCi/mL of [^{35}S]methionine was included in the JMEM+5% FCS after dilution of the reovirus-adsorbed (infected) cells to 3.5 L. If less [^{35}S]methionine-reovirus was required, the whole procedure was scaled down to a 2 L preparation.

2 Routine Procedures

2.1 *In Vitro* Transcription (σ1 and dII in Conformational Change Studies)

For the conformational change studies, transcription of DNA templates to RNA was performed according to the following protocol.

50μL transcription reactions were made up in 1 X reaction buffer (40mM TRIS-HCl (pH 7.5); 6mM $MgCl_2$; 2mM spermidine; 10mM NaCl; [Promega]), containing 1mM ATP, CTP and UTP; 0.1mM GTP; 0.5mM cap analog, 6mM dithiothreitol; 10 mg/mL bovine serum albumin; 1.0 U/μL RNA guard (Promega); 5 μg of linearized template DNA; and 15U of SP6 RNA polymerase per μg of DNA. The reactions were incubated for 30 minutes at 37°C, at which time GTP was added to a final concentration of 1.0 mM and incubated for an additional 45 minutes. The DNA templates were removed by digestion with the addition of 15μg of RNase-free DNase I (Betheseda Research Laboratories) and 1.0 U/μL of RNAguard (Promega), followed by a 15 minute incubation at 37°C. The reactions were then diluted with 10mM TRIS (pH7.5) and 1mM EDTA, and extracted with phenol, phenol-chloroform, and then chloroform. The RNA was precipitated in 95% ethanol, containing 2.5 M ammonium acetate at -70°C for at least 4 hours. The pellets were washed, resuspended in sterile water, and stored at -70°C in small aliquots until required. The resulting mRNA was >95% full length (determined by agarose gel analysis), and yields were approximately 10-15 μg per reaction.

2.2 *In Vitro* Transcription (Recombinant Annexin VI and σ1)

Transcription of DNA templates to RNA was accomplished through use of a commercially available kit from Ambion (Ambion SP6 transcription kit). Use of this kit resulted in high yields of very high quality RNA.

Briefly, to a 1.5 mL microfuge tube, the following reagents were added in order: 6μL of RNase-free ddH_2O, 2μL of 10X transcription buffer (Ambion), 2μL of ATP solution, (Ambion), 2μL of CTP solution (Ambion), 2μL of GTP solution (Ambion), 2μL of UTP solution (Ambion), 2μL of template DNA (0.5 mg/mL), and 2μL of enzyme mix (Ambion).

The reaction mixture was incubated at 37°C for six hours. Following incubation, 1μL of RNase-free DNase I (2U/μL) (Ambion) were added, followed by a brief pulse-microcentrifugation, followed by incubation at 37°C for 15 minutes. The reaction was stopped by the addition of 30μL of RNase-free ddH_2O and 25μL of Lithium Chloride Precipitation Solution (Ambion) and mixed thoroughly. The reaction was chilled for 60 minutes at -20°C and then centrifuged at maximum speed in a microcentrifuge to pellet the RNA. The supernatant was gently removed by aspiration and the pellet washed once with 70% ethanol and then re-centrifuged. The final RNA pellet was resuspended in ddH_2O to approximately 100ng/μL and assayed for quality by agarose gel electrophoresis and translation in rabbit reticulocyte lysate (see Methods and Materials section 2.3).

2.3 *In Vitro* Translation

Capped or uncapped mRNAs were translated *in vitro* in rabbit reticulocyte lysates (RRLs) according to the manufacturer's specifications (Promega). The 25μL translation reactions contained 50-100 μg of RNA (2 μL of approximately 50 μg/μL mRNA), 2μL (20μCi) of [^{35}S]methionine (1100 Ci/mMol (New England Nuclear)), 2 μL of amino acids minus methionine (1mM each) (Promega), 18 μL of RRL, and 1 μL of ddH_2O.

The reaction mixtures were incubated at 37^{o}C for one hour and then diluted to 200μL with 4^{o}C PBS, pH 7.4 and used immediately for experimentation or stored until needed at -70^{o}C.

2.4 Radioimmunoprecipitation

Samples to be immunoprecipitated were diluted to appropriate concentrations according to the relative experiments and mixed with an equal volume of antibody (antibody concentrations determined by titration), diluted in PBS to appropriate working concentrations of antibody and incubated at room temperature for 60 minutes. Fixed Staphylococcus aureus (10% suspension (W/V) in NET buffer (50 mM TRIS-Cl, pH 7.5; 150 mM NaCl; 1 mM EDTA, pH 8.0; 0.05 (V/V) NP-40; 0.02% (W/V) sodium azide) that had been presorbed with BSA (5 mg/mL) was then added to the solution in equal volume and incubated with gentle agitation every 10 minutes for 30 minutes. Immunoprecipitates were washed three times with sarcosyl wash buffer (50 mM phosphate buffer; pH 7.4; 0.1 M KCl; 0.5% Sarcosyl (W/V); 0.02% sodium azide (W/V)) and intermittent pelleting of the Staphylococcus aureus in a microcentrifuge at maximum speed

(12 000 rpm) for 10 seconds. Staphylococcus aureus pellets were resuspended by vortexing until no visible pellet remained (20 seconds). After the final wash, pellets were resuspended in 1 X protein sample buffer (PSB), followed by boiling for 5 minutes prior to SDS-PAGE.

2.5 Radioiodination of Proteins

50 μg of target protein was dissolved in 100 μL of fresh 1x PBS, pH 7.4. Separately, 2 iodo-beads (Pierce) were placed in a 1.5ml eppendorf tube and washed twice with 1ml of PBS. The beads were then blotted on 3M whatman paper to dry, and placed in a fresh 1.5mL eppendorf tube. The protein solution was added to the beads and work was transferred into a fume hood. 5μL of ^{125}I (approximately 0.5 mCi) was added to the protein/beads. The tube was capped and placed in a lead pig at room temperature. The reaction time was determined empirically and was generally between 30 - 60 seconds. The reaction was stopped after the appropriate amount of time, with the addition of 100 μL of 1M KI in PBS. The reaction mix (200μL now) was added to 800 μL of PBS and this mixture was deposited onto a 10DG desalting column (Biorad) previously equilibrated with 2 column volumes of PBS. 1mL fractions were collected by alternately adding 1mL of PBS to the column, then collecting 1mL of eluant until 10 fractions were collected in total. Typically, the iodinated protein product eluted in fraction 4 or 5 as determined via gamma counter analysis (LKB-Wallac). 5μL samples from each fraction were placed in 0.5mL eppendorf tubes, which in turn were placed into carrier tubes and analyzed on the gamma counter.

3 Antisera

Rabbit antisera to total reovirus proteins obtained by intramuscular (IM) and subcutaneous (SC) injection into female New Zealand white rabbits as previously described. Rabbit antisera to σ1 was prepared as described by Yeung et al. (1987). Rabbit antiserum (purified immunoglobulin G (IgG) fraction) to annexin VI (MC2 antibody) was obtained as a generous gift from Dr. Stephen E. Moss (University of London, U.K.).

4 Electrophoresis and Gel Processing

4.1 Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis of Proteins (SDS-PAGE)

A discontinuous SDS-polyacrylamide slab gel as described by Laemmli (1970) was used for all SDS-PAGE procedures. The stacking gel was composed of: 3.4% acrylamide (W/W); 0.09% (W/W) N,N'-methylene-bisacrylamide; 2.8% (V/V) glycerol; 145mM TRIS-Cl, pH 6.8; 0.1% SDS. 1 mg/mL ammonium persulfate (APS) and 0.05% (V/V) N,N,N',N'-tetramethylethyldiamine (TEMED). The resolving gel was composed of: 10% acrylamide:bisacrylamide (37.5:1 ratio); 380 mM TRIS-Cl (pH 8.8); 0.05% SDS; 0.7 mg/mL APS, and 0.04% (V/V) TEMED. The gel size utilized was 13.5 cm (height) X 17.5 cm (width) X 0.3 cm (thickness), and electrophoresis was

performed at a constant current of 35 milliamps (mA) for 2-4 hours. Running buffer composition was: 25 mM TRIS-base, pH 8.45; 0.19 M glycine; 5% (V/V) glycerol; and 0.1% SDS. Samples were incubated in protein sample buffer (final concentration: 50 mM TRIS, pH 6.8; 1% SDS; 2% β-mercaptoethanol; 10% glycerol; and 0.01% bromophenol blue) for 30 minutes at either 4°C or 37°C or boiled immediately for 5 minutes prior to SDS-PAGE, which was performed at either room temperature at 35mA for 2.5 hours or at 4°C (for temperature-sensitive experiments) at 25mA for 4 hours, until the tracking dye had reached the desired distance along the gel.

4.2 Processing of SDS-PAGE Gels

Gels of experiments that incorporated [^{35}S]methionine as a label were developed by fixing with gel fix (ddH2O:Methanol:Acetic Acid in a 30:6:4 ratio) for at least 20 minutes, followed by a 5 minute wash in DMSO (Dimethylsulfoxide), followed by rocking in DMSO for 30 minutes, followed by rocking the gel for 45 minutes in 20% PPO (2,5-diphenyloxazole (W/V) in DMSO), followed by washing through constantly running tap water for at least 30 minutes. The gels were then dried onto 3mm filter paper (Whatman) for 90 minutes at 77°C on a slab drier (Biorad, model 583).and exposed at -70°C to Kodak XAR-5 film.

Gels of experiments that incorporated ^{125}Iodine as a label were developed by fixing with gel fix for 30 minutes, followed by washing under a continuous flow of tap water for 5 minutes, followed by drying for 90 minutes at

77°C on a slab drier (Biorad, model 583).and exposed at -70°C to Kodak XAR-5 film.

4.3 Coomassie Staining

Gels electrophoresed to completion were placed into a coomassie staining solution (1 .0 % (w/v) coomasie brilliant blue R-250 (Biorad), 10% (V/V) methanol, 15% (V/V) acetic acid in ddH_2O), rocking gently at room temperature for at least 4 hours. The unincorporated stain was removed from the gel with successive changes of destaining solution (10% (V/V) methanol, 15% (V/V) acetic acid in ddH_2O) until protein bands could be visualized. The gels were then incubated while rocking at room temperature in ddH_2O overnight to remove the background stain. Glycerol was then added to a final concentration of no more than 0.5%, followed by further incubation for 60 minutes, and the gels preserved between sheets of acetate (Biorad) or photographed immediately.

4.4 Agarose Gel electrophoresis

1% agarose gels were made by adding 1 g of dry agarose to 100 mL of 1 X TRIS-acetate buffer (TAE) (50 X solution made by 242 g TRIS base into 57.1 mL glacial acetic acid and 100 mL of 0.5 M EDTA, pH 8.0), and heating the heterogeneous solution in a microwave oven until all the agarose had dissolved. 5µL of 10 mg/mL ethidium bromide were then added to the dissolved agarose, and the resulting solution mixed and poured into an agarose gel mould which was allowed to cool at room temperature until the gel was set. Agarose gels were run in 1 X TAE buffer at a constant voltage of 120 volts until the desired

degree of band separation was achieved. Nucleic acid bands were visualized directly by placing the agarose gels on top of an ultraviolet light source, and viewed through protective eyeware.

5 Cloning and Bacteriology Protocols

5.1 Propagation and Storage of Bacterial Cells and Plasmids

A variety of E. coli host strains were used in this work, including, HB101 and XL1blue as general work horse strains. The JM110 strain was utilized in instances where dam-, dcm- markers were required. Master stocks of the host strains, or host strains containing plasmid, were picked from single colonies and grown in LB broth, at 37°C for 16 to 24 hours, with shaking. All culture medium used for growing host cells with plasmid contained 100 µg/mL of ampicillin, since all plasmids used carried a gene for ampicillin resistance. 0.5 mL aliquots were mixed with an equal volume of 100% glycerol in 2mL cryotubes, and were then quick frozen by immersion in liquid nitrogen and stored at -70°C. Master stocks were also maintained as needed by streaking onto LB agar plates, incubating at 37°C for 16-24 hours and storing at 4°C for up to one month. Plasmids maintained in E. coli include; pG4T3s1,pG4T3dII and PG4AVI. All parental plasmids were derivatives of PGEM-4Z (Promega).

5.2 Large Scale Preparation of Plasmid DNA

Two methods of preparing large scale quantities of plasmid DNA were utilized, Triton-lysate plasmid isolation and Magic Maxi-Preps (Promega). For the triton-lysate preparation, plasmid DNA was prepared from 1L cultures inoculated with the desired host strain containing plasmid. The innoculum was obtained either from an isolated colony on an LB agar plate, or a loop full of frozen master stock was used to inoculate 5mL of LB broth in a culture tube. This was shaken at 37°C for 6 to 8 hours and used to inocculate 1L of LB broth. The 1L was shaken at 37°C for 16 to 24 hours. Cells were harvested by centrifugation at 3500 rpm in a J.S 4.2 rotor (2000 x g) for 20 minutes at 4°C. The supernatant was removed and the pellet rinsed with a 4°C solution of 10mM TRIS-HCl, 1mM EDTA (TE), pH 7.5. The pellet was resuspended in 4.5 ml of ice cold sucrose buffer (50 mM TRIS-HCl pH 8.0, 1 mM EDTA, 25% sucrose (W/V)) and transferred to a clean / sterile 36 mL high speed polypropylene centrifuge tube. Solutions were kept on ice from then on. After a 10 minute incubation, 1 mL of a freshly prepared 10 mg/mL lysozyme was added and mixed gently. This was incubated a further 10 minutes, and then 1.8 mL of cold 0.25 M EDTA pH 8.0 was added and gently mixed and the solution was incubated 10 minutes. After incubation, 13 mL of ice cold triton-lytic buffer (50 mM TRIS-HCl pH 8.0, 60 mM EDTA, 2% Triton-X100) was slowly added while mixing gently on ice. This was allowed to incubate 20 minutes before a 30 minute centrifugation was performed, at 16k rpm / 4°C in a JA-20 rotor. After centrifugation the supernatant was decanted into a disposable 50 ml conical centrifuge tube. To this, 1 g of CsCl was added per mL of supernatant, as well as, 300 µL of 10 mg/mL ethidium bromide. This was mixed until everything was dissolved. Subsequently, this

mixture was decanted into Beckman polyallomer quick heat seal tubes. These were centrifuged in a TI 70 rotor at 60k rpm for 16 hours at 22°C. DNA bands were illuminated under ultraviolet light, harvested by aspiration into test tubes, and the ethidium bromide extracted using water-saturated n-butanol as follows. Approximately one volume of water saturated n-butanol was mixed with the DNA solution, allowed to separate and the n-butanol phase removed by careful aspiration from the top down to the meniscus. This was repeated until the DNA solution was completely clear upon visual inspection (usually three or four times). If necessary, the volume was decreased by sequential additions of 0.6 volume sec-butanol, with mixing and subsequent removal of the butanol phase as described above. The DNA solution was then dialyzed against TE, pH 8.0, with at least 4 changes of buffer, allowing a minimum of 2 hours between buffer changes. The DNA solution was then decanted into 15 mL polypropylene tubes (Falcon) and was precipitated by the addition of 3 volumes of -20°C 95% EtOH and incubation at -20°C overnight. Following centrifugation the pellets were washed twice with 70% EtOH and once with 95% EtOH. The pellet was dried and resuspended in ddH_2O. DNA concentration was estimated by standard A_{260} measurements (Sambrook *et al.,* 1989). DNA was stored at -70°C until required.

The alternative method of large scale DNA preparation was via the Magic Maxiprep DNA purification System (Promega). The procedure followed is outlined in Promega Technical Bulletin no. 139 with minor modifications. Briefly, 500 ml of culture was centrifuged, and the pellet was resuspended in 10 ml of cell resuspension buffer (Promega) and transferred to a 36 mL high speed centrifugation tube. Once thoroughly resuspended, 10 mL of cell lysis solution (Promega) was added with gentle mixing and was allowed to stand until the solution became clear, or to a maximum of 5 minutes. 10 mL of neutralization

solution (Promega) was then added with immediate mixing by inversion. Following centrifugation (17,000 x g / 30 minutes), the supernatant was transferred to a fresh tube and mixed with 0.8 volumes of isopropanol. Following centrifugation the pellet was resuspended in 2 mL of TE, and mixed with 10 mL of Magic Maxiprep DNA Purification Resin (Promega). The DNA/resin mix was added to a Magic Maxicolumn (Promega) and drawn into the column by applying a vacuum to the end of the column. The DNA/resin mix was washed twice with 12.5 mL of Column Wash Solution (Promega) and rinsed with 20 mL of 80% EtOH and dried by continuing to draw a vacuum for 10 minutes. 1.5 mL of preheated (76^{o}C) ddH_2O was then applied to the maxicolumn and the DNA eluted by centrifugation in a swinging bucket rotor (1,300 x g) for 5 minutes. The DNA was quantitated as described above.

5.3 Small Scale Preparation of Plasmid DNA

Small quantities of plasmid DNA, suitable for analytical purposes, was prepared according to two methods - the lysozyme-boiling method and the Magic Minipreps DNA Purification System (Promega). The lysozyme-boiling method will be described first.

Following centrifugation of a 5 mL overnight culture (4,000 x g / 10 minutes) the cells were resuspended in 200 µL of STET buffer (8% (W/V) Sucrose; 5% (V/V) Triton X-100; 50mM EDTA; 50mM TRIS, pH 8.0) and transferred to a 1.5 mL microfuge tube. 50µL of 10 mg/mL lysozyme was added and the solution was incubated for 1 minute and then boiled for 45 seconds. After pelleting in a microfuge (10 -15 minutes / 4°C), 10 µL of preboiled RNase A

in ddH_2O (10 mg / mL) was added to the supernatant followed by incubation at 65°C for 15 minutes. The mixture was then extracted once with phenol /chloroform (containing 4% isoamyl alcohol) and twice with chloroform only. The upper aqueous phase was then precipitated by adding 0.5 volumes of 7.5 M ammonium acetate and three volumes of 95% EtOH, with overnight incubation at -20°C. After pelleting the DNA at 4°C for 20 minutes in a microfuge, the pellet was washed twice with 70% EtOH, and once with 95% EtOH and allowed to air dry. The pellet was resuspended in 50 µL of ddH_2O and quantitated by standard A_{260} measurements (Sambrook *et al.,* 1989). DNA was stored at -70°C.

The alternative method of small scale DNA preparation was via the Magic Miniprep DNA purification System (Promega). The procedure followed is outlined in Promega Technical Bulletin no. 117 with minor modifications. Briefly, 5 mL of culture was centrifuged, and the pellet was resuspended in 200 µL of cell resuspension buffer (Promega) and transferred to a 1.5 mL microcentrifuge tube. 200 µL of cell lysis solution was then added with gentle mixing and was allowed to stand until the solution became clear, or to a maximum of 5 minutes. 200 µL of neutralization solution (Promega) was then added with immediate mixing by inversion. Following microcentrifugation at high speed, the supernatant was transferred to a fresh 1.5 mL tube and mixed with 1 mL of Magic Miniprep DNA Purification Resin (Promega). The DNA/resin mix was added to a 3 mL luer lock disposable syringe (Becton Dickinson) and pushed into a Magic Minicolumn (Promega) with the syringe plunger. The DNA/resin mix was washed with 2 mL of Column Wash Solution (Promega). The minicolumn was then transferred to a 1.5 mL microcentrifuge tube and spun in a microfuge at top speed for 30 seconds to dry the resin. The minicolumn was then transferred to a fresh microfuge tube and 50 µL of preheated (70°C) ddH_2O was then applied to the

minicolumn and incubated for 1 minute. The DNA was eluted by microcentrifugation at top speed for 30 seconds. The DNA was quantitated as above.

5.4 Restriction Endonuclease Digestion

All restriction endonuclease digestions were performed following manufacturer's recommendations for the supplied buffers. For complete digestion of plasmid DNA, 3 units of enzyme per μg of DNA was used. Plasmid from small scale cultures was resuspended in a total of 50 μL, and 5 to 10 μL was used for analysis by restriction enzyme digestion and gel electrophoresis. Plasmid prepared from large scale cultures was resuspended to a concentration of approximately 0.5 μg / μL and approximately 20 to 30 μg of DNA was used for digestion if fragments were to be isolated and purified from agarose gels. Digestion mixtures were incubated at 37°C for 30 minutes to 1 hour, and 1 μL of the mixture analyzed by agarose gel electrophoresis. Procedures requiring digestion with two different enzymes in different buffers were performed by either of two methods. If the two buffers required were similar, the second buffer was obtained by adding the additional buffer components to the mixture. In addition the first enzyme would be heat inactivated by incubation at 65°C for 15 minutes, to limit star activity. If the two buffers required could not be obtained via simple addition of components, the digestion mixture was cleaned using the Magic DNA Clean-Up System (Promega). The procedure for clean up was followed as outlined in Promega Technical Bulletin no. 141. The DNA mixture was combined with 1 mL of Magic DNA Clean-Up Resin (Promega), and added to a 3 mL luer lock disposable syringe (Becton Dickinson). This mixture was then pushed into a

Magic Minicolumn (Promega) with the syringe plunger. The DNA/resin mix was washed with 2 mL of 80% isopropanol, and then the minicolumn was transferred to a 1.5 mL microcentrifuge tube and spun in a microfuge at top speed for 30 seconds to dry the resin. The minicolumn was then transferred to a fresh microfuge tube and 50 µL of preheated (70°C) ddH_2O was then applied to the minicolumn and incubated for 1 minute. The DNA was then eluted by microcentrifugation at top speed for 30 seconds. The DNA was then used for subsequent restriction endonuclease digestion.

5.5 Agarose Gel Purification of Restriction Endonuclease Fragments

Restriction endonuclease fragments were separated on a 0.7 % agarose gel containing 0.25 µg of ethidium bromide / mL of gel in TAE buffer. The gel was removed from the apparatus and the fragment of interest was identified by visual comparison with a concurrently run 1 kbp DNA ladder (BRL) using ultraviolet light illumination. The band of interest was cut out by trimming away as much agarose as possible that did not contain DNA. This DNA block, was then cut up into fine fragments with a clean, sterile scalpel and transferred to a microcentrifuge tube containing 1 mL of Magic DNA Clean-Up Resin (Promega). After this point the methods for cleaning the DNA are as above for the use of the Magic DNA Clean-Up System (Promega). Interestingly, the resin would dissolve the agarose and alleviated the need for expensive low melting agarose. The purified DNA was then run on an agarose preparatory gel to ensure integrity and quantitated using standard A_{260} measurements (Sambrook *et al.,* 1989). The purified fragments were subsequently used in ligation reactions.

5.6 T4 DNA Ligase Reactions

Ligations were performed in a 10 µL volume using 1.0 to 2.0 units of T4 DNA ligase and previously prepared 5x ligation buffer (50 mM TRIS-HCl pH 7.5; 50 mM $MgCl_2$; 50 mM DTT; 5 mM ATP; filter sterilized and stored at -20°C). Usually the molar ratio of vector : insert was 1:2 or 1:3, with a total DNA concentration of 100 to 300 ng per reaction. Ligation mixtures were incubated in a thermocycler at 13°C overnight (12 to 16 hours). The ligation mixture was then diluted to 45 µL with distilled water and 15 µL of the diluted reaction mixture was used to transform 100 µL of competent cells.

5.7 Preparation of Competent Cells

A culture tube containing 5mL of LB broth was inoculated with the desired bacterial host and shaken at 37°C for 16 to 20 hours. 1mL of this culture was used to inocculate 50 mL of fresh LB broth which was then shaken at 37°C for ~2.5 hours (until OD_{600} = 0.4). The flask was then placed on ice for 10 minutes; thereafter the culture was transferred to a clean, sterile 50 mL conical tube (prechilled) and the cells were harvested by centrifugation (2500 x g) at 4°C for 15 minutes. The supernatant was discarded and the cells resuspended in 10 mL of ice cold $CaCl^2$ buffer (50mM $CaCl_2$; 10 mM TRIS-HCl pH 8.0). The cells were incubated 15 minutes on ice, and then centrifuged (2500 x g) at 4°C for 15 minutes. The cells were resuspended in 2 mL of $CaCl_2$ buffer and aliquoted in 200 µL aliquots into sterile 1.5 mL microfuge tubes. The cells were stored at 4°C and were used for transformation after a 24 hour waiting period, but before 48 hours.

5.8 Transformation of E. coli

100 µL of competent cells were placed into a prechilled 15 mL Falcon 2059 polypropylene tube and 1.7 µL of 1:10 dilution of (stock 14.4 M) β-mercaptoethanol was added, giving a final concentration of 25 mM. The cells were swirled gently and allowed to sit on ice for 10 minutes with occasional swirling. 50 to 100 ng of DNA was added to the cells with swirling. In addition, 11 µl of 10x TCM (100mM TRIS-HCl pH 7.4; 100 mM $CaCl_2$; 100 mM $MgCl_2$) was added with swirling. The mixture was allowed to incubate on ice for 30 minutes, before a heat pulse in a 42°C water bath was performed for 45 seconds.

Following the heat pulse, the cells were placed on ice for 2 minutes and then 0.5 mL of preheated (42°C) LB broth was added. This was followed by incubation with shaking for 1hr at 37°C. 200uL of cells were then plated on LB agar plates (containing either 100 µg / mL of ampicillin). The plates were incubated overnight (16-20 hours) in a 37°C incubator and individual colonies were picked for subsequent procedures.

Results

Part I Conformational Change in Reovirus

6 Introduction

The initial interaction between an invading virus and the target cell involves both the cell attachment protein on the virus and the virus receptor on the host cell plasma membrane. Of all the reovirus proteins, σ1 alone possesses intrinsic cell binding capabilities, and as such, is responsible for the specific high affinity attachment of virions to susceptible cells (Lee et al., 1981; Armstrong et al., 1984) and thus plays a pivotal role in viral infectivity and tissue tropism (Sharpe and Fields, 1985). In agreement with predictions from sequence analysis, biochemical and biophysical studies have provided unequivocal evidence that the globular head and the fibrous tail represent the C- and N-terminal portions, respectively, of σ1 (Strong et al., 1991). It was further shown that σ1 is a trimer and that the N- and C-terminal halves each harbors an independently active trimerization domain (Leone et al., 1992). The two halves are also functionally independent since the C-terminal globular head can bind to cell receptors even when it is separated from the N-terminal fibrous tail (Yeung et al., 1989; Duncan et al., 1991) and since the N-terminal one-quarter of σ1 possesses intrinsic virion-anchoring function (Mah et al., 1990; Leone, et., al., 1991). However, as revealed in the following investigation, there is communication between the two termini upon σ1 binding to cell receptors.

While significant revelations have been made on the structure-function relationships of σ1, the nature of the reovirus receptor is less well-defined. It was suggested earlier that a 67K protein which shares structural similarities with

the ß-adrenergic receptor is the reovirus receptor (Co et al., 1985a; Co et al., 1985b). However, subsequent studies on human epidermoid carcinoma A431 cells and mouse L fibroblasts have cast serious doubts on this claim (Choi and Lee, 1988; Sawutz et al., 1987). Indeed, evidence that sialic acid is the major signal for reovirus recognition (Paul et al., 1989; Gentsch and Pacitti, 1985; Gentsch and Pacitti, 1987) suggests that reovirus binds to multiple sialoglycoproteins, rather than to a single homogeneous species, on the cell surface. Results from ligand-blot analyses concur with this notion (Choi et al., 1990). There is now little doubt that the initial interaction between reovirus and the host cell involves the binding of the σ1 globular head to cell surface sialic acids. A pertinent question would then be whether this interaction alone could trigger conformational changes in σ1 and in other viral capsid proteins.

In the following study the protease pepsin was used to demonstrate that the main body of the reovirus undergoes conformational change upon cell binding. Structural changes in σ1 were also detected when it alone bound to cells. These changes were observed to be reversible upon detachment of the virion, or σ1, from cells. It was further demonstrated that the binding of σ1 to the α-anomeric form of sialic acid is sufficient to trigger this reversible conformational change.

6.1 Cell Binding Induces Conformational Change in Reovirus

Since a conformational change in reovirus upon cell attachment would likely involve a reorganization of capsid proteins, protease digestion experiments were carried out in an attempt to detect this change. Various proteases were used, and it was determined that the best results were obtained with pepsin.

^{35}S-labeled reovirus was allowed to bind to an L cell monolayer at 4^{o}C for an hour after which pepsin was added and the cells were incubated further for 15 minutes at 37^{o}C. For the control sample, pepsin was added to the cell monolayer at the same time as reovirus. The results (figure 4A) show that reovirus was totally degraded when added to the monolayer at the same time as pepsin. However, cell-bound reovirus was protected from degradation by the protease. Interestingly, the σ3 protein of reovirus was not protected from pepsin degradation to the extent of the other structural proteins.

To rule out the possibility that enhanced resistance to pepsin was due to virus internalization, however unlikely, during incubation at 4^{o}C, similar experiments were carried out on purified plasma membranes of L cells. Again, with the exception of protein σ3, other capsid proteins of membrane-bound reovirus were protected from pepsin degradation (figure 4B). Essentially the same results were obtained using glutaraldehyde-fixed cells instead of live cells (figure 5). It would appear, therefore, that the binding of the virion to the cell surface is sufficient to trigger conformational changes in the viral capsid proteins. The fact that protein σ3 of cell- and membrane-bound virions remained relatively susceptible to pepsin suggests that the observed effects are not due to the masking of bound virions by cell surface structures in their vicinity, rendering them less accessible to pepsin.

6.2 Cell-Induced Conformational Change in Reovirus is Reversible

It has previously been shown for picornaviruses that upon temperature-upshift (from 20^{o}C to 37^{o}C), a significant fraction of the bound virus is released

Figure 4 Pepsin Digestion of Prebound, Cell-bound and Membrane-bound Reovirus.

(A) Pepsin digestion of prebound virus was carried out by adding pepsin (+) or buffer (-) to L cell monolayers (in 24-well plates) at the same time as ^{35}S-reovirus, followed by incubation at 37^{o}C for 15 minutes For cell-bound virus, pepsin digestion was carried out on monolayers to which reovirus had been allowed to bind (see Materials and Methods). Protein sample buffer was then added to the wells, and the cell lysates were harvested, boiled, and analyzed by SDS-PAGE.

(B) Same as (A) except immobilized L cell membranes were used instead of cell monolayers.

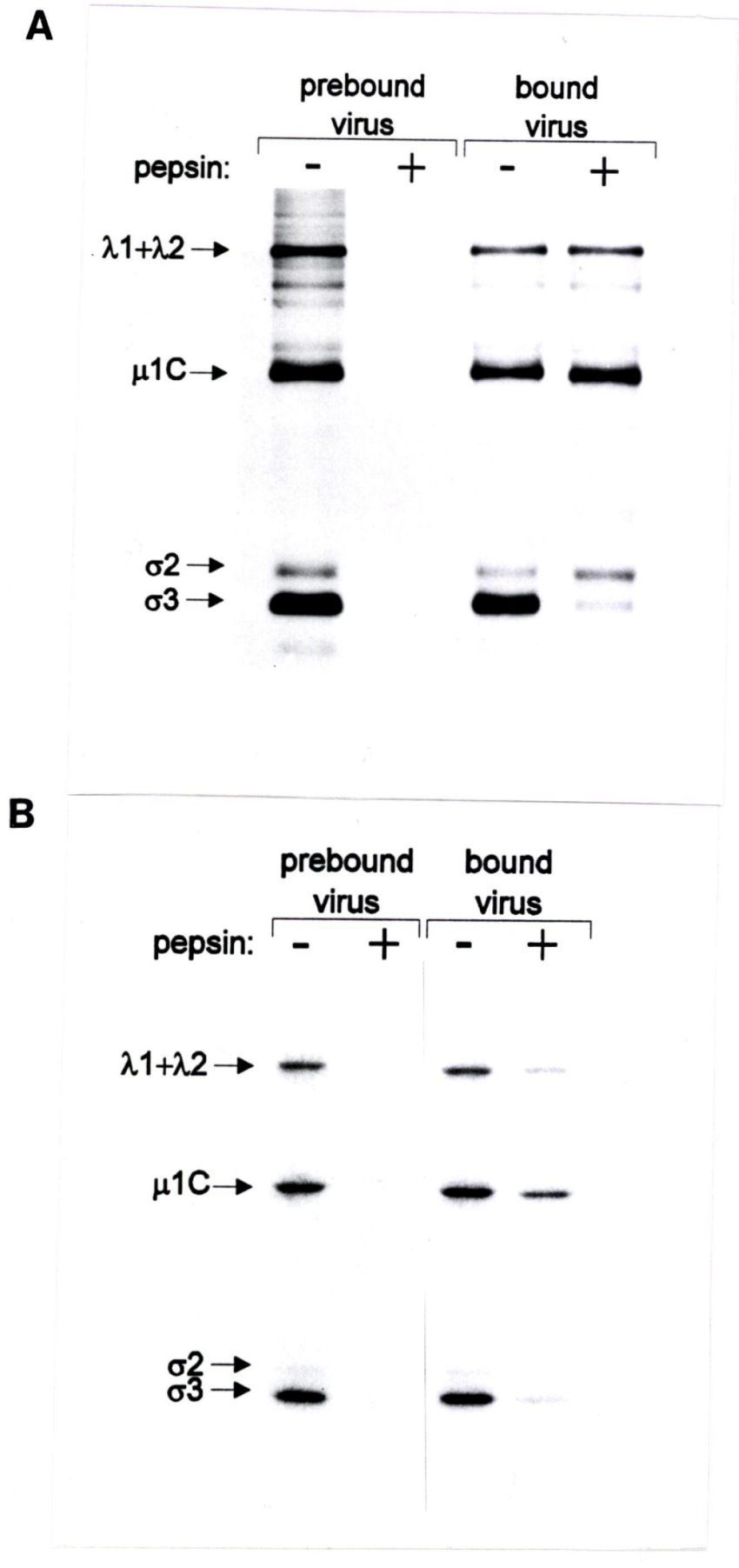
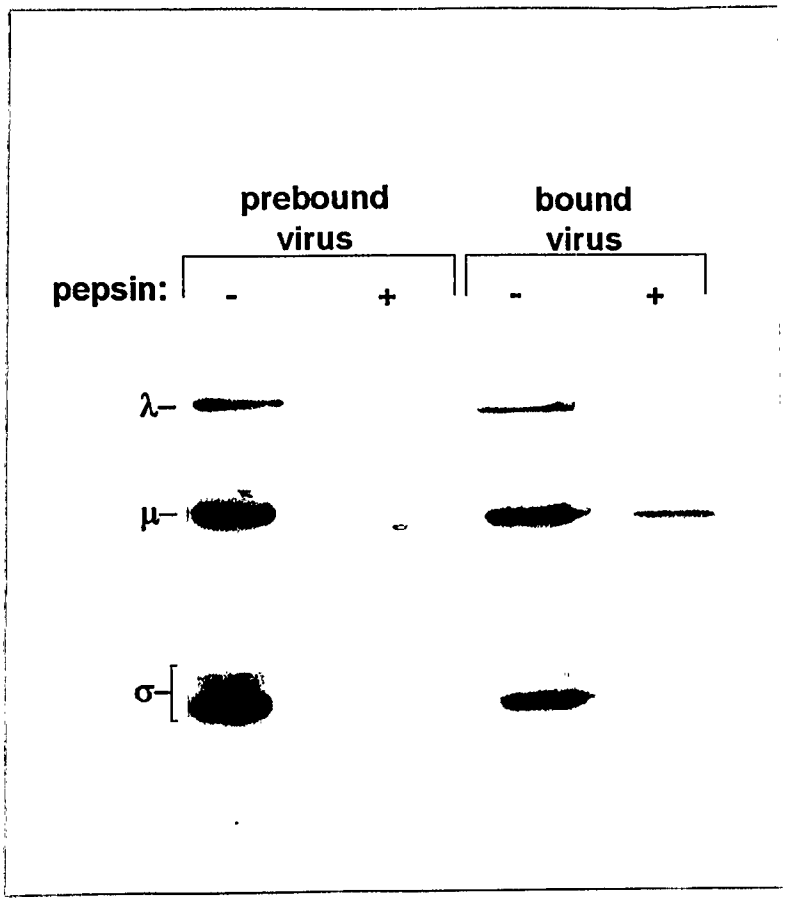

A
prebound virus
bound virus
pepsin: - + - +
λ1+λ2
μ1C
σ2
σ3
B
prebound virus
bound virus
pepsin: - + - +
λ1+λ2
μ1C
σ2
σ3

Figure 5: Pepsin Digestion of Prebound and Fixed Cell-bound Reovirus

Pepsin digestion of prebound virus was carried out by adding pepsin (+) or buffer (-) to glutaraldehyde-fixed monolayers (in 24 well plates) at the same time as ^{35}S-reovirus, followed by incubation at 37^{o}C for 15 minutes. For cell-bound reovirus, pepsin digestion was carried out on fixed monolayers to which reovirus had been allowed to bind (see Methods and Materials). Protein sample buffer was then added to the wells, and the cell lysates were harvested, boiled, and analyzed by SDS-PAGE.


prebound virus
bound virus
pepsin:
-
+
-
+
λ–
μ–
σ–

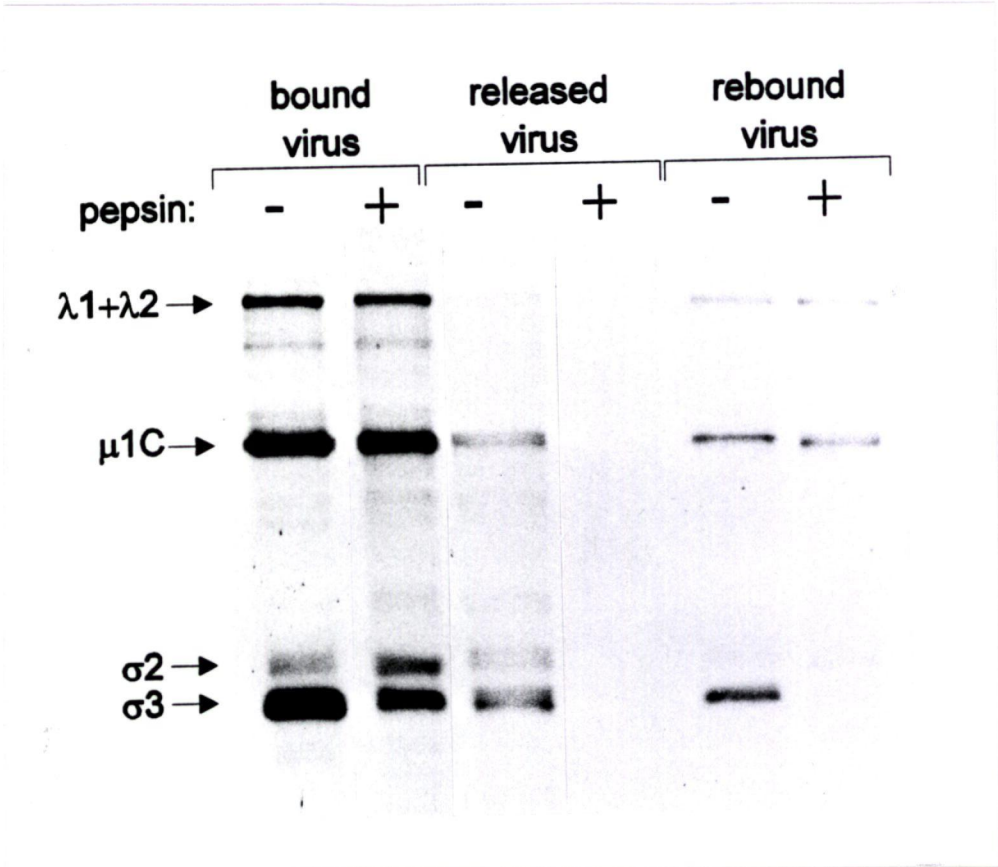
from the cells. The released viruses are structurally and antigenically altered and no longer able to bind to cells. To see whether reovirus, like picornaviruses, is also irreversibly altered after cell binding, reovirus was allowed to attach to cells at 4°C for 1 hour after which the cells were washed with cold (4°C) medium, followed by incubation at 37°C. A significant portion (approximately 70-80%) of bound virus became detached upon temperature-upshift, again confirming the extracellular nature of these viruses. The released virus particles were then tested for their susceptibility to pepsin and ability to rebind to cell receptors. Figure 6 clearly shows that whereas cell-bound virions were relatively resistant to pepsin digestion, the released virions reverted back to the pepsin-sensitive state and retained the ability to bind to cells at 4°C. The rebound virions again became relatively pepsin-resistant. This experiment demonstrates that unlike picornaviruses, reovirus undergoes a reversible conformational change upon binding to cells.

6.3 Cell Binding Induces Conformational Change in σ1

The observation that receptor binding induces a conformational change in the main body of the virion implies that a signal is relayed from the viral attachment protein, σ1, to the other capsid proteins. It would therefore be of interest to see whether receptor binding at the C-terminal globular head of σ1 could cause a conformational change at the N-terminal fibrous tail which is the region anchored to the virion. To this end, we used the previously characterized protease chymotrypsin which removes 24 amino acids from the N-terminal end of σ1, yielding a functional 46 KDa polypeptide. *In vitro* synthesized σ1 was applied to L cells and binding was carried out at 4°C. Subsequent digestion

Figure 6: Pepsin digestion of cell-bound and released reovirus.

^{35}S-reovirus was allowed to bind to L cell monolayers and analyzed for sensitivity to pepsin degradation (bound virus) as in Fig. 4A. Following binding to cells at 4^{o}C, unbound virus was washed away and the monolayers were re incubated in PBS (pH 7.2) for 1 hour at 37^{o}C. Supernatants that contained eluted virus (released virus) were then collected, cleared by microcentrifugation, and added to new L cell monolayers at the same time as pepsin (+) or buffer (-). Alternatively, the released virus was allowed to bind to new L cell monolayers for 1 hour at 4^{o}C (rebound virus) and subsequently treated with pepsin (+) or buffer (-). All samples were boiled in protein sample buffer prior to analysis by SDS-PAGE.


bound virus
released virus
rebound virus
pepsin: - + - + - +
λ1+λ2
μ1C
σ2
σ3

with chymotrypsin resulted in the total cleavage of unbound σ1 (to either the 46 kDa protein or smaller fragments), whereas a significant amount of cell-bound σ1 remained intact (figure 7). These results suggest, but do not prove, that binding of σ1 to cell receptors induces a conformational change at the N-terminal end of the protein.

6.4 Cell Binding Enhances Stability of the N-terminal α-Helical Coiled Coil

It is important to complement the above chymotrypsin studies with another method of measuring receptor-induced structural alterations in σ1, in particular the N-terminus of σ1. Since the N-terminal heptad repeats confer stability of the σ1 trimer via the formation of a three-stranded α-helical coiled coil, any receptor-induced changes in σ1 stability would be indicative of structural alterations in σ1, or more specifically, the N-terminal fibrous tail of σ1. The clearest results were obtained with a σ1 deletion mutant (designated dII) lacking part of the α-helical coiled coil region (residues 123-223). This mutant nonetheless forms functional trimers (i.e., capable of binding to cells) that are less stable than full-length σ1 trimers (see below). When subjected to SDS-PAGE at 4°C after preincubation (also at 4°C) in protein sample buffer, the dII protein migrated as a trimer (figure 8). However, if the preincubation was carried out at 37°C, it migrated as a monomer. This is distinct from full-length σ1 which retains its trimeric state under the same conditions.

The stability of cell-bound dII trimers was then assessed. The results (figure 8) clearly show that dII manifested enhanced stability upon cell-binding, migrating as a trimer after preincubation in SDS at 37°C. Moreover, after

Figure 7: Chymotrypsin digestion of prebound and cell-bound protein σ1.

Full-length σ1 was synthesized *in vitro* and applied to L cell monolayers. Chymotrypsin was added to the cells either at the same time as σ1 (prebound) or 1 hour later (bound). Digestion was carried out at 4°C for 15 minutes Protein sample buffer was then added to the cells either prior to washing (for prebound σ1) or after washing (for bound σ1). Lysates were harvested, boiled, and analyzed by SDS-PAGE.

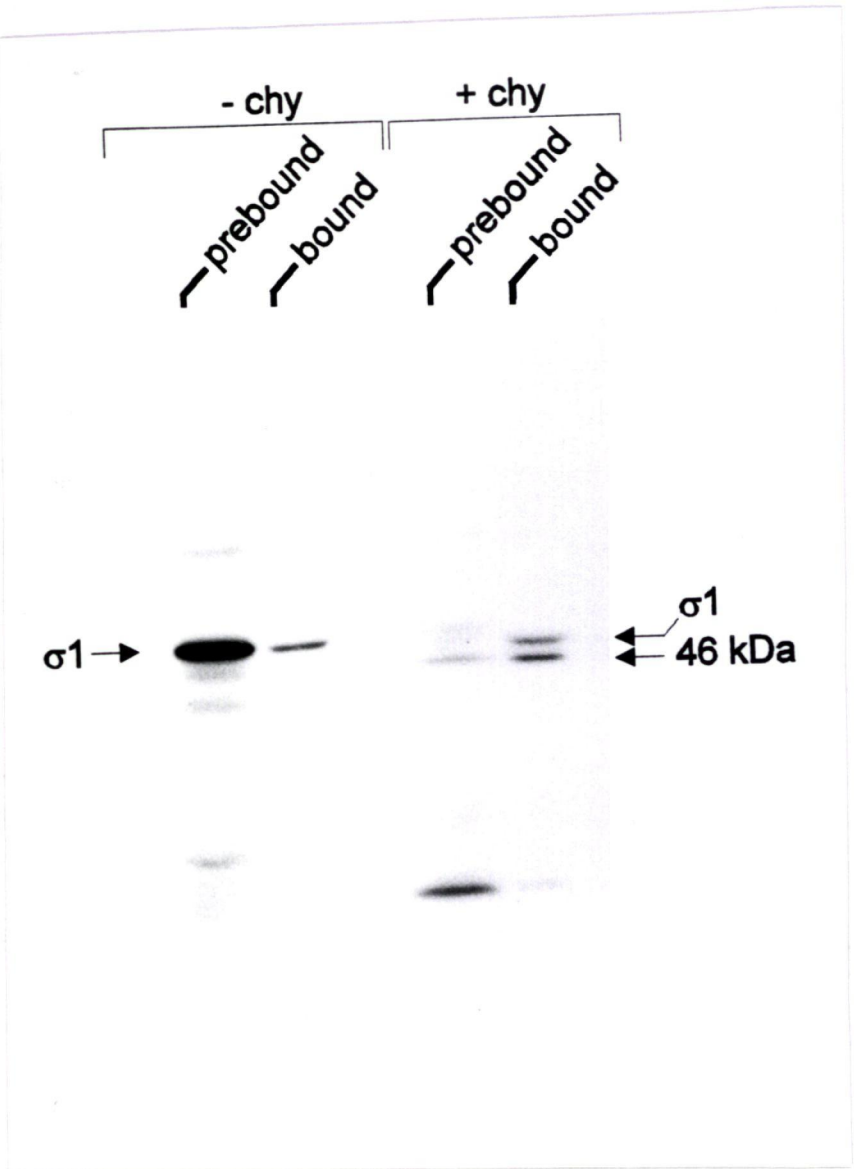
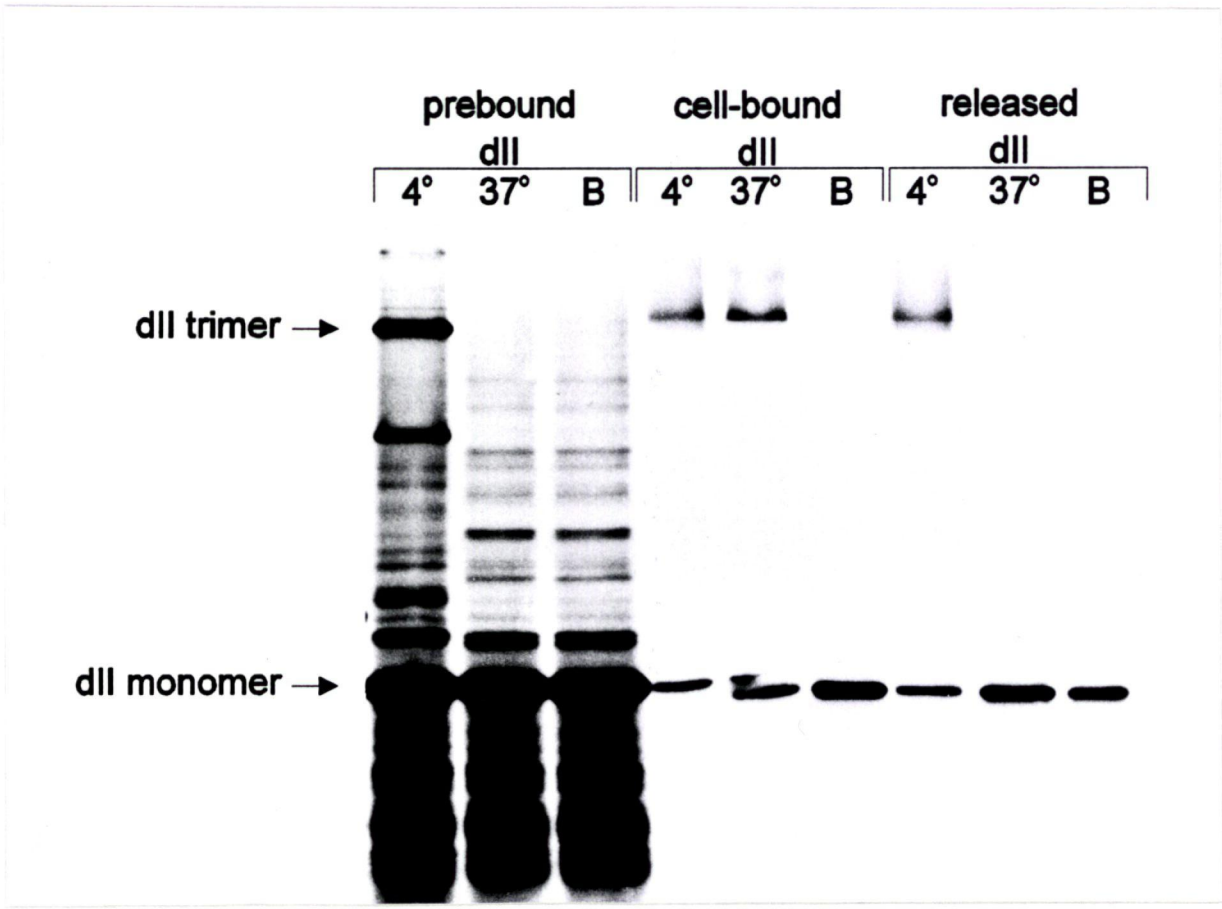

- chy
+ chy
prebound
bound
prebound
bound
σ1
σ1
46 kDa

Figure 8 Comparison of Thermostability between prebound, cell-bound, and released protein dII.

Protein dII (lacking part of the N-terminal portion of σ1) was synthesized *in vitro* (prebound) and incubated in protein sample buffer at 4°C, 37°C, or boiled (B). Alternatively, dII was allowed to bind to L cell monolayers as described in Methods and Materials. The monolayers were then washed, and were either harvested in protein sample buffer and incubated at the temperatures indicated (cell-bound) or reincubated in PBS for 1 hour at 37°C. The supernatants from the latter procedure (containing released dII) were then incubated in protein sample buffer at the temperatures indicated. All samples were subjected to SDS-PAGE carried out at 4°C.


prebound
dll
cell-bound
dll
released
dll
4°
37°
B
4°
37°
B
4°
37°
B
dll trimer
dll monomer

spontaneous detachment from cells at 37°C, the dII trimer reverted back to its original, unstable state. Although it is safe to assume that conformational changes most likely also occur in the C-terminal globular head upon cell-binding, analysis of the pre- and post-bound C-terminal tryptic fragment of σ1 did not reveal any detectable difference in stability between these two forms (figure 9). These results therefore confirm that the enhanced stability of the dII trimer is due to structural changes at the N-terminal α-helical region of σ1.

6.5 Sialic Acid-Binding Induces Conformational Change in σ1

In view of the overwhelming evidence that α-sialic acid is the first recognition signal on the cell surface for reovirus binding, we examined the ability of sialic acid alone to induce conformational change in σ1, again using the dII mutant protein as probe. To maintain the α-anomeric form of sialic acid in solution, a sialo-BSA conjugate (Paul et al., 1989) was used instead of pure sialic acid. The dII protein was incubated with either BSA (control) or sialo-BSA at 4°C, followed by incubation in SDS-containing protein sample buffer at either 4°C or 37°C. The samples were then subjected to SDS-PAGE. The results (figure 10) show that dII trimers incubated with the BSA control were stable in SDS at 4°C, but became unstable at 37°C. However, in the presence of sialo-BSA, a moderate, yet highly reproducible, enhancement of dII trimer stability at 37°C (in SDS) was consistently observed. Thus, the effects of σ1 binding to sialic acid alone and to cells are similar since both lead to enhanced stability of the σ1 N-terminal α-helical coiled coil. Like cell-bound dII trimers, the sialo-BSA-bound dII protein reverted back to its unstable configuration when the incubation temperature for binding of the dII/sialo-BSA mixture was raised from

Figure 9: Thermal Stability of the C-terminal Trimer of dII

Protein dII (lacking part of the N-terminal portion of σ1) was synthesized *in vitro* (prebound) and cleaved with insoluble chymotrypsin in PBS, according to titration of the chymotrypsin with dII protein. The cleaved product was allowed to bind to L cell monolayers as described in Methods and Materials. The monolayers were then washed, and harvested in protein sample buffer and incubated at the temperatures indicated for 30 minutes or boiled (B) for 5 minutes. Unbound dII was incubated in protein sample buffer at the indicated temperatures for 30 minutes. All samples were subjected to SDS-PAGE carried out at 4°C.

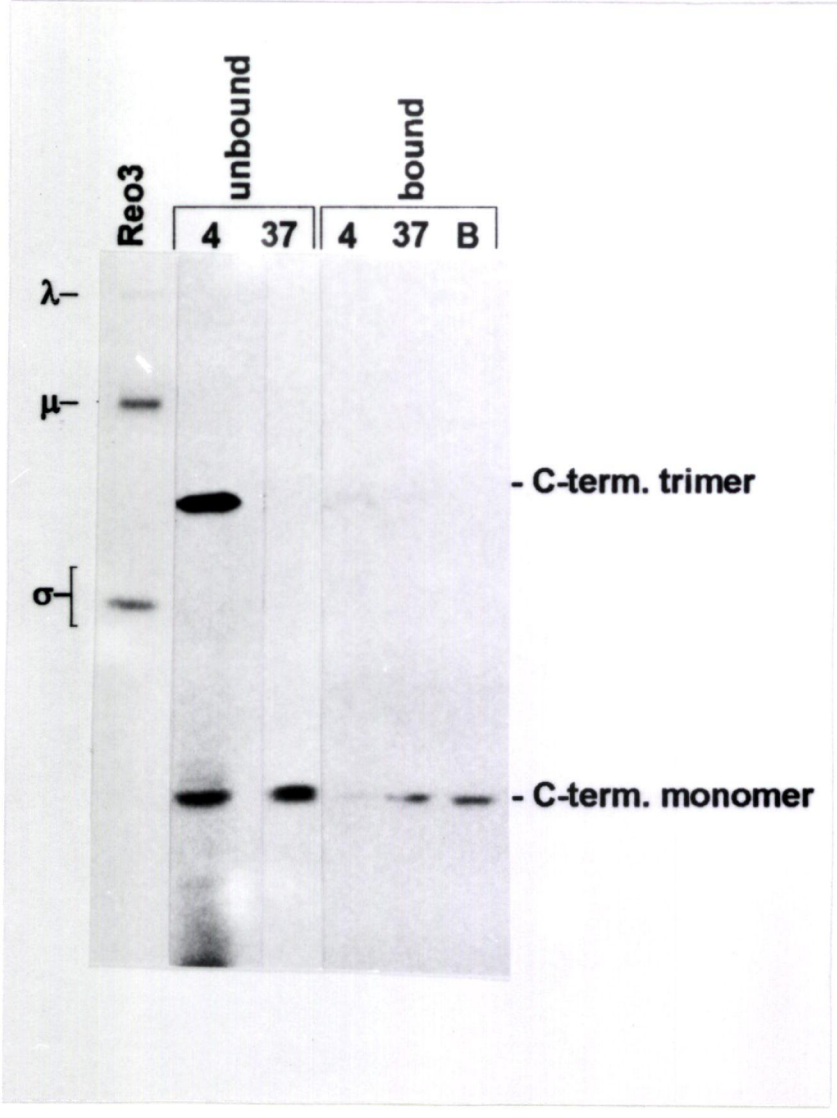
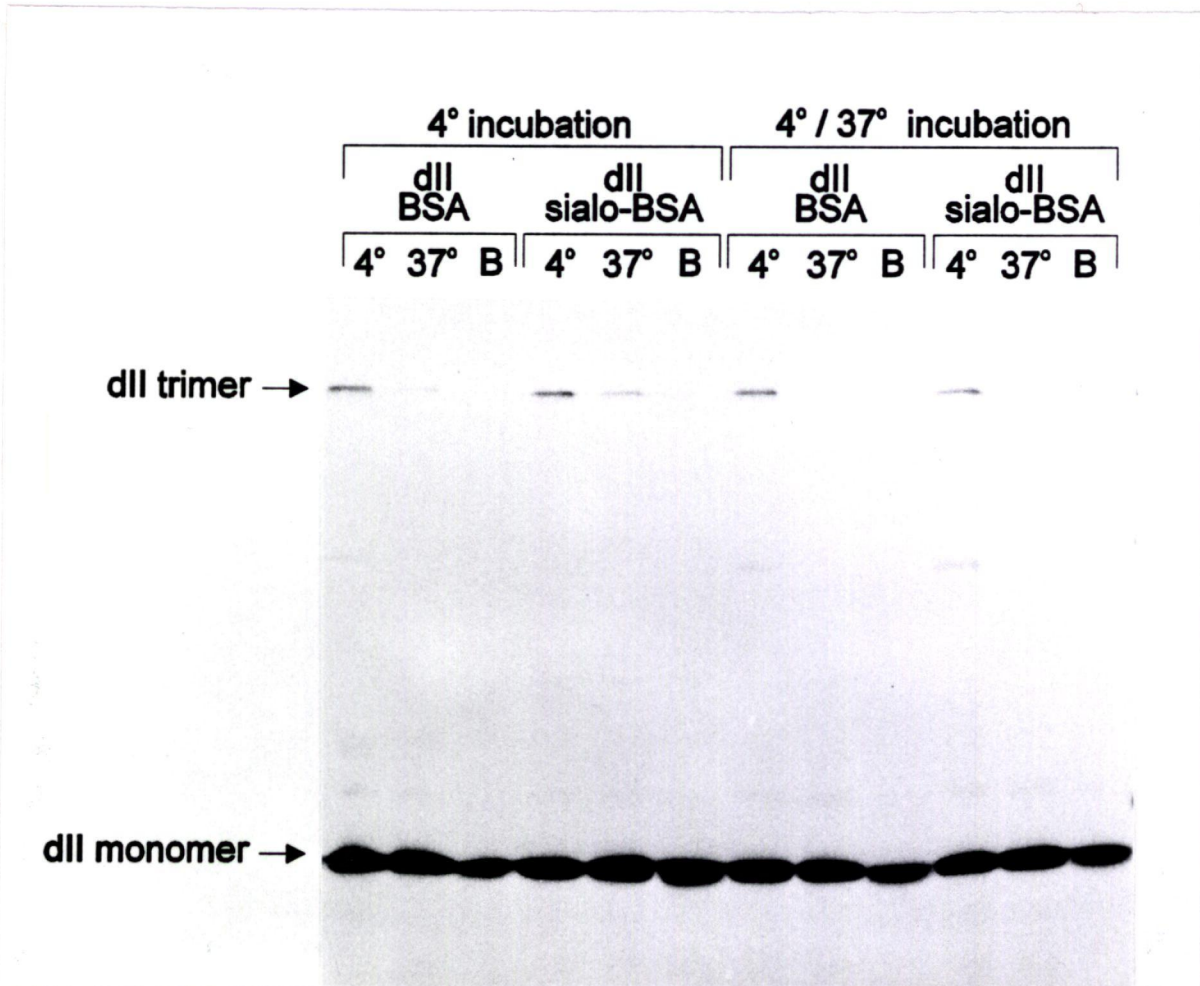

Reo3
unbound
bound
4
37
4
37
B
λ–
μ–
σ–
- C-term. trimer
- C-term. monomer

Figure 10. Thermostability of protein dII in the presence and absence of sialic acid.

Protein dII synthesized *in vitro* was incubated with either bovine serum albumin (BSA) or a sialo-BSA conjugate (both adjusted to pH 7.4) for 1 hr at 4°C. Protein sample buffer was then added and the samples were incubated at 4°C, 37°C, or boiled (B). Alternatively, after the initial incubation at 4°C, the samples were shifted to 37°C and incubated for an additional hour prior to the addition of protein sample buffer and incubation at the indicated temperatures. Samples were then subjected to SDS-PAGE carried out at 4°C.


4° incubation
4° / 37° incubation
dll BSA
dll sialo-BSA
dll BSA
dll sialo-BSA
4° 37° B
4° 37° B
4° 37° B
4° 37° B
dll trimer
dll monomer

4°C to 37°C. Based on similar observations made on cell-bound virions, σ1, and dII described above, it seems reasonable to deduce that this conformation reversion was due to the detachment of a significant portion of dII from sialo-BSA at 37°C. These experiments therefore illustrate that sialic acid-binding alone is accountable for the reversible conformational change in σ1. Unfortunately, attempts to demonstrate conformational changes in whole virions using sialo-BSA were not feasible due to the large amount of the conjugate that would be required.

6.6 Discussion

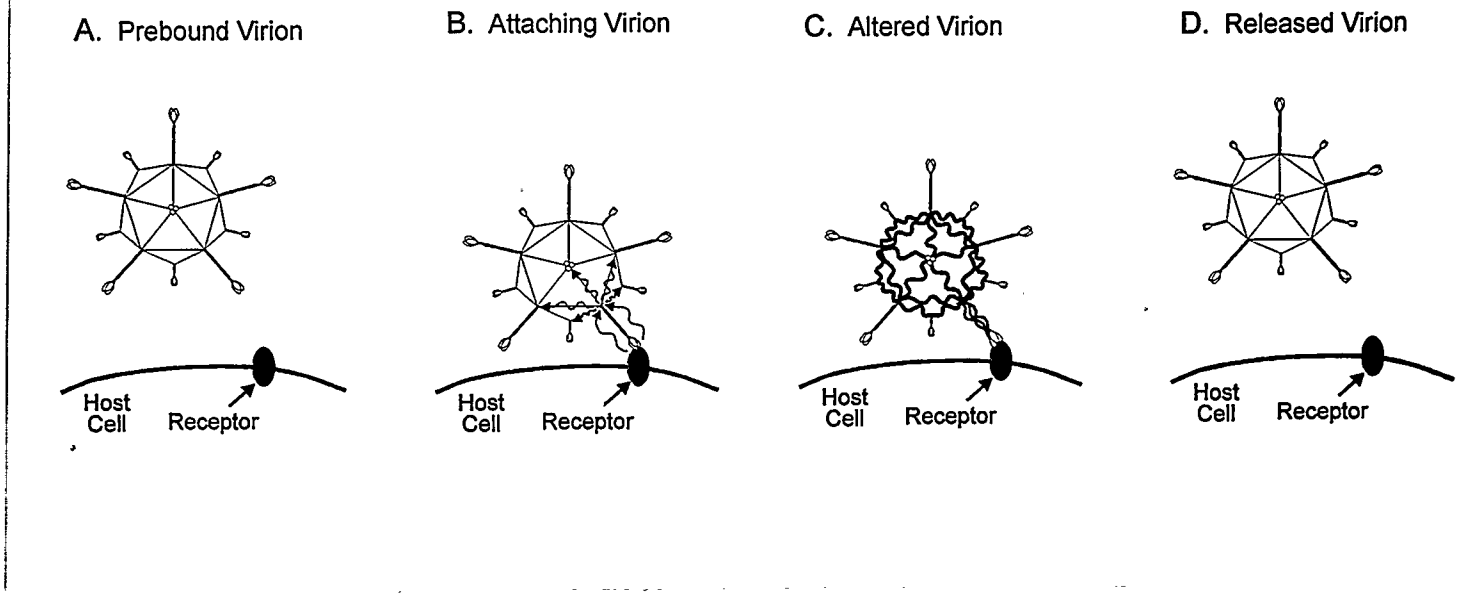
The mechanism of viral entry has been an area of intense research of late. In the case of enveloped viruses, it is widely believed that attachment of the virus to the host cell receptor leads to the exposure of hydrophobic domains on the viral attachment or fusion protein which in turn mediates the fusion of the viral envelope with the host cell plasma or endosomal membrane (for review see Bentz, 1993 and Helenius, 1992). How nonenveloped viruses cross the cell membrane is less clear. However, it has long been established that attachment of picornavirus to cells is accompanied by drastic conformational changes of the virion, resulting in alterations in antigenicity, increased protease sensitivity, loss of the internal capsid protein VP4 and of the ability to reattach to cells (Crowell and Philipson, 1971; DeSena and Mandell, 1977; Fenwick and Wall, 1973; Lonberg-Holm and Korant, 1972). Of particular interest is the finding that the N-terminus of the poliovirus VP1 protein, which is entirely internal in the native

virion, becomes externalized upon attachment to cells and confers the ability to attach to liposomes (Fricks and Hogel, 1990). Since the putative receptor binding sites on picornavirus reside in deep depressions on the viral capsid (Hogle et al., 1985; Luo et al., 1987; Rossman et al., 1983), it can easily be seen how receptor binding could result in the perturbation of the overall structural integrity of the virus. On the other hand, there is little, if any, information on receptor-induced conformational changes in other nonenveloped viruses such as adenovirus and reovirus that use extended, icosahedrally-arranged fibers as attachment proteins. This, coupled with the fact that the reovirus cell attachment protein, σ1, has been well-characterized, prompted the present studies.

The results indicate that upon attachment to cellular receptors, reovirus capsid proteins undergo a structural rearrangement which is detectable in the main body of the virion as well as in protein σ1. Since the initial recognition between reovirus and cell receptors involves σ1-sialic acid interaction, it is important to demonstrate that sialic acid alone can induce a similar conformational change in σ1. This was indeed found to be the case. The binding of σ1 to cell receptors or sialic acid leads to detectable conformational changes at the N-terminal fibrous stalk of the protein. Collectively, these results suggest that receptor (sialic acid) binding leads to a temporal progression of structural alteration in the virion, starting from the C-terminal receptor binding domain of σ1, down the shaft of the protein towards the N-terminal virion-anchoring domain, and being subsequently relayed to the rest of the virion (figure 11). Based on what is known about other virus systems, it seems logical to conjecture that these conformational changes are necessary for subsequent steps in reovirus entry. Our results are also compatible with the recent

Figure 11 Model of reovirus interaction with the cell surface

Binding of virion (A) via protein σ1 to a sialic acid-bearing moiety on the cell surface (first step interaction) induces a conformational change which is initiated in the C-terminal receptor-binding domain of σ1, progresses to the N-terminus of the protein, and subsequently spreads to other capsid proteins (B and C). The structurally-altered virion (C) is then ready for the second step interaction which likely involves additional host cell factors and leads to subsequent viral entry (not depicted). However, if conditions are unfavorable for this interaction, the virus may elute from the cell surface, reassume its original prebound structure (D), and be ready to attach again.


A. Prebound Virion
B. Attaching Virion
C. Altered Virion
D. Released Virion
Host Cell
Receptor
Host Cell
Receptor
Host Cell
Receptor
Host Cell
Receptor

demonstration, by cryoelectron microscopy and image reconstruction, that reovirions and *in vitro*-generated intermediate subviral particles and cores (the latter two being accepted as true intermediates in reovirus infection) display significant differences in supramolecular structure and protein conformation that are related to the early steps of reovirus infection (Dryden et al., 1993). Of the two major outer capsid proteins (μ1C and σ3), the μ1C protein, but not σ3 protein, becomes particularly resistant to pepsin upon receptor binding. Thus, receptor-mediated conformational change in μ1C is likely important for reovirus penetration. It is interesting to note in this regard that results from recent genetic (Lucia-Jandris et al., 1993) and biochemical (Nibert et al., 1991; Nibert and Fields, 1992) studies suggest that proteolytic fragments of the μ1 protein (from which μ1C is derived) play a role in interacting with cell membranes. It is therefore not inconceivable that receptor binding causes μ1C to adopt a conformation more amenable to membrane association.

The observation that the receptor-induced conformational changes are reversible upon release of the reovirus or of σ1 from the receptor is interesting and compatible with the concept that the binding that leads to reovirus entry is a two-step process involving distinct viral and cellular components, and with the second step being a consequence of the first. Unlike picornaviruses, which recognize very specific cell surface structures (members of the immunoglobulin super gene family), reovirus uses the ubiquitous sialic acid as its first recognition signal. This initial interaction leads to conformational changes in the virion that are most likely a prerequisite for the second interaction. Under conditions which do not favor this latter step (for example, due to inaccessibility of the altered virion to the pertinent cell component involved), the virus may detach from the sialic acid, revert back to its original conformation, and be ready to attach again

(figure 11). This process is repeated until conditions which favor the second interaction are met, and viral entry ensues as a consequence (Fernandes et al., 1994). It can easily be seen how such a "ping-pong" binding mechanism which rejuvenates inconsequential encounters could enhance the probability of productive infection.

In view of the morphological similarities between adenovirus and reovirus, a comparison between these two viruses in terms of attachment and entry is inevitable. Recent evidence suggests that indeed, adenovirus entry also involves a two-step mechanism, with the first step being the binding of the icosahedrally-distributed fibers to cellular receptors yet to be identified (Philipson et al., 1968; Defer et al., 1990), and the second step involving the interaction between the penton base (connected to the N-terminus of the fiber) and cell membrane integrins, which then leads to virus internalization (Wickham et al., 1993). It is not known at present whether the adenovirus fiber and other capsid proteins undergo conformational changes upon virus attachment. However, based on our present findings on reovirus, it is conceivable that this may well be the case, with the end result being a drastic enhancement of the second step interaction. Conversely for reovirus, an interaction similar to the one between the adenovirus penton and integrins likely occurs and is being probed. These predictions, if confirmed, will provide general and unifying concepts pertaining to entry strategies employed by nonenveloped, fiber-bearing viruses, analogous to those already established for enveloped viruses.

Part II The Second Reovirus Receptor

7 Introduction

It is well known that reovirus recognizes sialic acid on cell surfaces (Armstrong et al., 1984; Gentsch and Pacitti, 1985; Pacitti and Gentsch, 1987; Paul and Lee, 1987; Paul and Lee, 1989), and that the α-anomeric form of sialic acid is the minimal determinant necessary for L-cell binding to cell surfaces (Paul et al., 1988). In light of the finding that reovirus undergoes a reversible, sialic acid-dependent conformational change upon L-cell binding, it seems reasonable to hypothesize the existence of a second reovirus cell surface receptor. Precedent for the phenomenon of a dual receptor binding mechanism in an unenveloped animal virus has been discussed (See Introduction, chapters 2-4 and Discussion in Results, chapter 6.6).

The identification of a specific reovirus receptor (other than sialic acid) was attempted previously on various cell types. No ubiquitously present proteinaceous receptor, however, has been identified for reovirus. Reovirus recognition of sialic acid-bearing proteins of 54 (Verdin et al., 1989) and 67 kDa (Co et al., 1985) on both endothelial and murine thyoma (R1.1) cells, respectively, has been reported. However, it has also been reported that reovirus recognizes multiple proteins on mouse fibroblast (L-929) cell surfaces (Choi et al., 1990). This multiple recognition, however, was likely the result of reovirus recognizing sialic acid, which is a component of many cell surface glycoproteins.

Previous work by others in our lab resulted in observations of a protein of 67 kDa that was conjectured to be the reovirus receptor protein. This protein

was isolated from purified and solublized L-929 cell membranes that had been passed through a wheat germ affinity (WGA) column, which selected for sialic acid-bearing proteins. This protein was later found to be capable of inhibiting reovirus binding to L-929 cells (Paul, R., Choi, A., Taylor, P., and Lee, P. *Unpublished Data*).

Further work in our lab showed that a 68 kDa protein eluted from a reovirus (reovirus immobilized onto sepharose 4b) column only under extreme elution conditions when purified, solublilized human epidermal carcinoma (A431) cell membrane had been passed through the column. Partial amino acid degradation analysis, as well as specific antibody recognition led us to postulate the identity of this protein as annexin VI, a protein belonging to a relatively new family of proteins (see Introduction, chapters 5-8). The following section describes our efforts to both purify this protein to near homogeneity from L-929 cells and to identify this protein, which we hypothesize to function as the second reovirus receptor.

7.1 Isolation of a 68 kDa Protein from Mouse L-929 Cell Fibroblasts

Cell plasma membranes were purified from approximately 12 litres of L929 cell membrane (approximately 9.6 X 10^{10} cells) as described in the methods and materials, with the exception that 4 litres of L-929 cells were allowed to reach 2 X 10^{6} cells/mL before the membranes were harvested. Purified plasma membranes were solublized on ice for 10 minutes in membrane solublization buffer (20mM HEPES, pH 7.4; 150mM NaCl; 1% (V/V) Triton X-100). After pelleting the insoluble membrane fraction at 12 000 rpm for 1 minute in a microcentrifuge, the supernatant containing the solublized membrane

fraction was retained, and the pellet discarded. The solublized membrane was diluted with load buffer (20mM HEPES, pH 7.4; 150mM NaCl) to make the final Triton X-100 concentration 0.1% (V/V). All subsequent steps were carried out at 4°C. A 2 mL reovirus column (see methods and materials) was prewashed with 20 mL of load buffer. The solublized membrane was loaded onto the column at a rate of 10 mL/hour and recirculated onto the column for 4 hours. The membrane load was collected and the wash and elution processes initiated.

The column was washed with 10 bed volumes of low salt wash buffer (20mM TRIS, pH 7.4; 150mM NaCl), followed by 10 bed volumes of high salt wash buffer (20mM TRIS, pH 7.4; 150mM NaCl; 0.037% (V/V) Triton X-100). 1 mL fractions were collected in sterile polypropylene microfuge tubes on an LKB 2112 REDIRAC fraction collector. The column was then treated with 65 bed volumes of elution buffer (100mM glycine, pH 2.6; 100mM NaCl; 0.037% (V/V) Triton X-100). 1 mL fractions were collected as described above, with the exception that approximately 30μL of 1.5 M TRIS, pH 7.4 was added to each fraction collection tube (prior to collection) to buffer each of the approximately 130 fractions to neutrality. 100 μL of each fraction was boiled with 25 μL of 5X protein sample buffer (final concentration: 50 mM TRIS, pH 6.8; 1% SDS; 2% β-mercaptoethanol; 10% glycerol; and 0.01% bromophenol blue) for 5 minutes before being subjected to SDS-PAGE (followed by Coomassie staining - see Methods and Materials, chapters 4.1 and 4.3) for analysis.

The results (figure 12) indicated that a protein band of approximately 68 kDa eluted from the reovirus column between fractions 53 and 64. The protein band appeared to be relatively homogeneous and a relatively minor component of the solublized cell plasma membrane preparation (figure 13). No observable

Figure 12: Low pH Elution Fractions from a Reovirus Column

Solublized L-929 cell membrane was loaded onto a 2 mL reovirus column as described. The column was washed with 10 bed volumes of low salt wash buffer, followed by 10 bed volumes of high salt wash buffer (see results). The remaining protein was eluted with elution buffer (glycine, pH 2.6) and 1 mL fractions were collected as described. Each fraction was boiled in SDS-containing protein sample buffer and analyzed by SDS-PAGE, followed by Coomassie staining. A 68 kDa protein was observed to elute between fractions 53 and 64, representing 26.5 and 32 bed volumes , respecively, elution buffer.

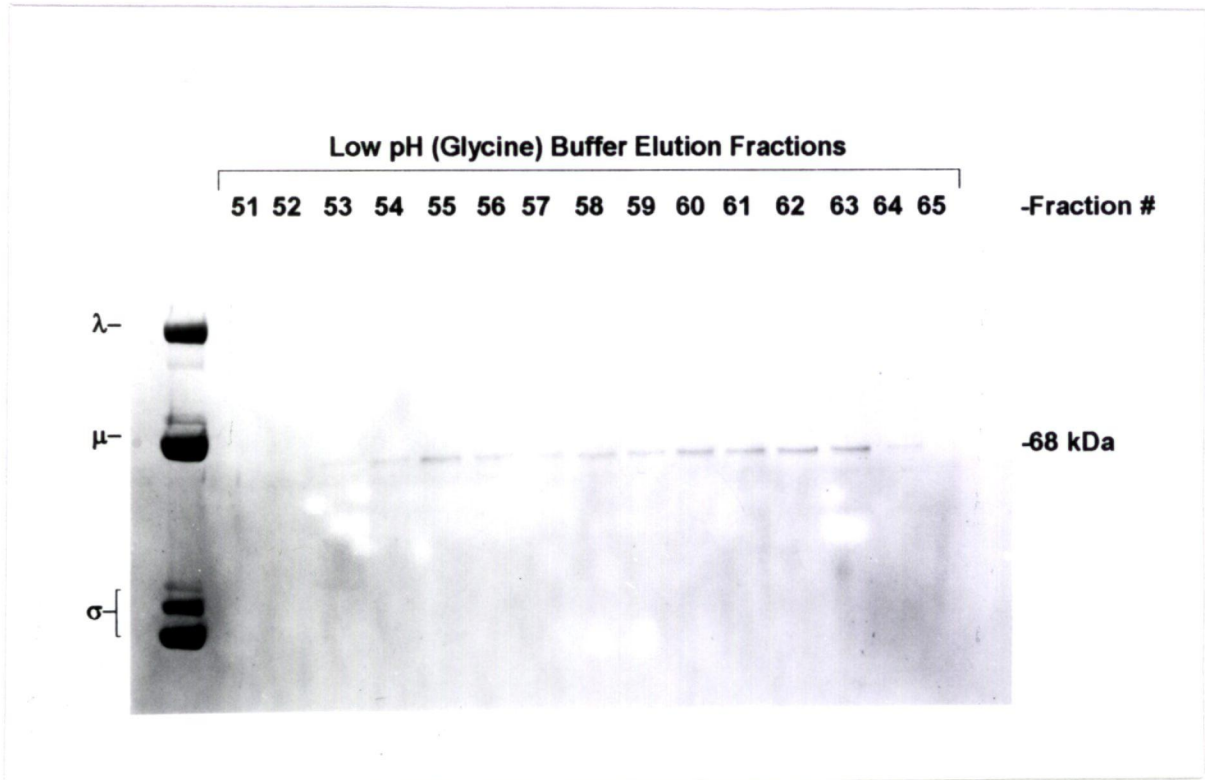
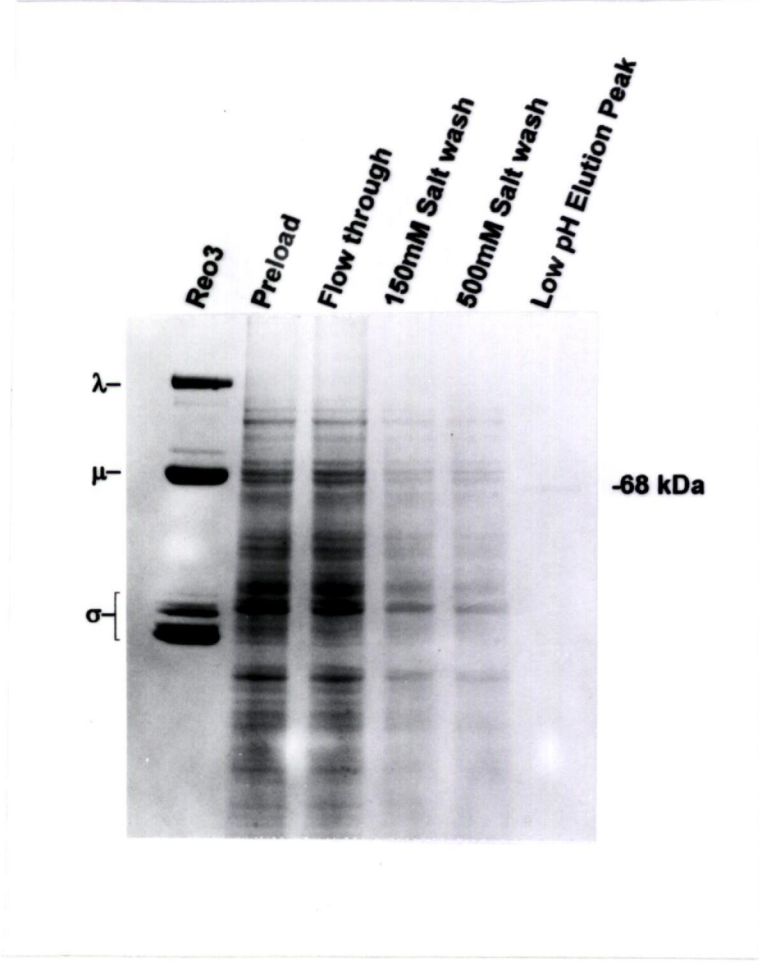

Low pH (Glycine) Buffer Elution Fractions
51 52 53 54 55 56 57 58 59 60 61 62 63 64 65
-Fraction #
λ–
μ–
σ–
-68 kDa

Figure 13: Peak Fractions from the Reovirus Column Washes and Elution

Solublized L-929 cell membrane was loaded onto a 2 mL reovirus column as described. The column was washed with 10 bed volumes of low salt wash buffer, followed by 10 bed volumes of high salt wash buffer (see results). The remaining protein was eluted with elution buffer (glycine, pH 2.6) and 1 mL fractions were collected as described. Each fraction (including the preload, flow-through and wash fractions) was boiled in SDS-containing protein sample buffer and subjected to SDS-PAGE. Peak fractions were identified by Coomassie staining and samples of each were subjected to SDS-PAGE, followed by Coomassie staining for analysis.


Reo3
Preload
Flow through
150mM Salt wash
500mM Salt wash
Low pH Elution Peak
λ
μ
σ
~68 kDa

protein was eluted when low salt elution buffer, high salt elution buffer, or elution buffer were run through the reovirus column and samples collected for analysis (data not shown). Additionally, this protein appeared to elute from the reovirus column relatively late in the elution process, indicating a relatively strong interaction with the column-immobilized reovirus.

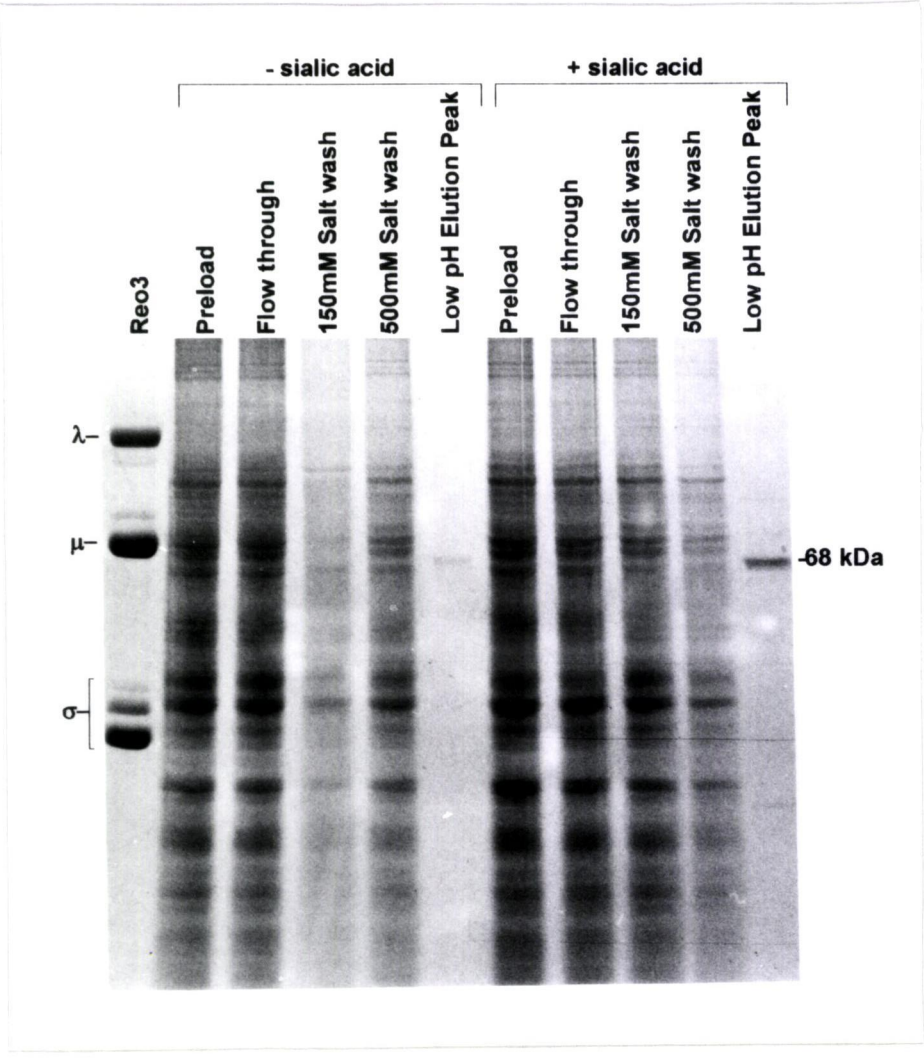
7.2 Sialic Acid Enhanced Recovery of the 68 kDa Protein from a Reovirus Column

Because the minimal determinant for binding and conformational change in reovirus is sialic acid, it was postulated that sialic acid-altered reovirus could bind to its second receptor (ie. the 68 kDa protein) with greater affinity. We surmised that the treatment of the column-immobilized reovirus with sialic acid could increase the relative amount of p68 bound to (and therefore eluted from) the column. To test this hypothesis, the same reovirus receptor isolation was performed as described previously with the exception that 0.1 M sialic acid (N-acetyl Neuraminic Acid) (final concentration) was included in the membrane preparation prior to loading on the reovirus column.

The results (figure 14) show that the inclusion of sialic acid in the column load drastically increased the amount of p68 eluted from the reovirus column. Again, this protein appeared to be relatively homogeneous. Samples from the membrane load, the flow-through, as well as peak fractions from the low salt and high salt washes indicate that p68 (from the sialic acid-enhanced recovery) is a relatively minor membrane protein, and has a seemingly high affinity for reovirus.

Figure 14: Sialic Acid-Enhanced Recovery of p68 from a Reovirus Column

Solublized L-929 cell membrane was loaded onto a 2 mL reovirus column as described, with the exception that 100mM of sialic acid in 100mM TRIS, pH 7.4 (+ sialic acid) or 100mM TRIS, pH 7.4 (- sialic acid) were included in the solublized membrane preparation prior to loading onto the reovirus column. The column was washed with 10 bed volumes of low salt wash buffer, followed by 10 bed volumes of high salt wash buffer (see results). The remaining protein was eluted with elution buffer (glycine, pH 2.6) and 1 mL fractions were collected as described. Each fraction (including the preload, flow-through and wash fractions) was boiled in SDS-containing protein sample buffer and subjected to SDS-PAGE. Peak fractions were identified by Coomassie staining and samples of each were subjected to SDS-PAGE, followed by Coomassie staining for analysis.


- sialic acid
+ sialic acid
Reo3
Preload
Flow through
150mM Salt wash
500mM Salt wash
Low pH Elution Peak
Preload
Flow through
150mM Salt wash
500mM Salt wash
Low pH Elution Peak
λ
μ
σ
~68 kDa

7.3 Sialic Acid Enhanced Binding of Reovirus to p68

Although the previous experiment infers that sialic acid-altered reovirus has increased affinity for p68, questions regarding this interaction remained. To eliminate questions regarding the functional viability of sepharose-immobilized reovirus, a more direct experiment was performed *in-vitro*.

25 µL of reovirus (4 mg/mL) in PBS was incubated with 100 µL of ^{125}I-labeled p68 (from a reovirus column purification) for 1 hour at 37°C in the presence or absence of 0.1M sialic acid (N-acetyl Neuraminic acid, final concentration). Solutions were buffered to neutrality by the inclusion of 100mM TRIS, pH 7.4 (final concentration). The solutions were then immunoprecipitated (see methods and materials, chapter 2.4) with an anti-reovirus antibody, run on an SDS-PAGE and analyzed by autoradiography.

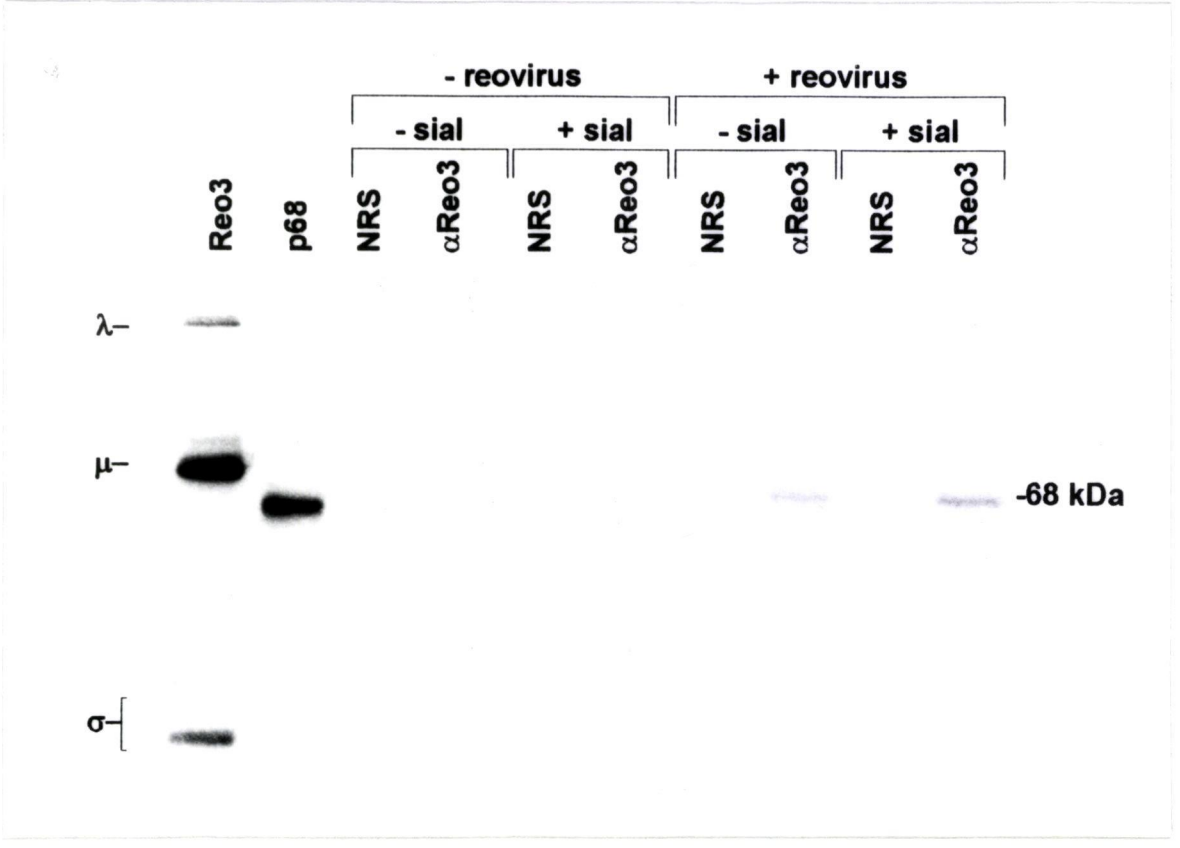
The results (figure 15) indicated that reovirus interacted with p68 *in vitro*, in both the presence and absence of sialic acid. Sialic acid ,however, was observed to increase the interaction of reovirus by two to three times. Immunoprecipitation of reovirus by normal rabbit serum did not result in any signal, indicating that the interaction between reovirus and p68 is relatively specific.

7.4 Preliminary Identification of p68 by an Annexin VI-Specific Antibody

As previously mentioned, preliminary data led us to speculate that the identity of p68 was annexin VI. Both proteins have been isolated from cell plasma membranes, and migrate on SDS-PAGE at 68 kDa. Preliminary evidence from antibody recognition studies, as well as partial amino acid

Figure 15: Sialic Acid-Enhanced Recognition of p68 by Reovirus

^{35}S-Reovirus (+reovirus) or buffer (-reovirus) was incubated with ^{125}I-labelled p68 in the presence (+sial) or absence (-sial) of 100mM sialic acid at 37^{o}C for 30 minutes. Samples were then immunoprecipitated (see Methods and Materials) with normal rabbit serum (NRS) or anti-total reovirus serum (αReo3). Samples were boiled in protein sample buffer for 10 minutes and subjected to SDS-PAGE, followed by processing and autoradiography (see Methods and Materials).


- reovirus
+ reovirus
- sial
+ sial
- sial
+ sial
Reo3
p68
NRS
αReo3
NRS
αReo3
NRS
αReo3
NRS
αReo3
λ–
μ–
σ–
-68 kDa

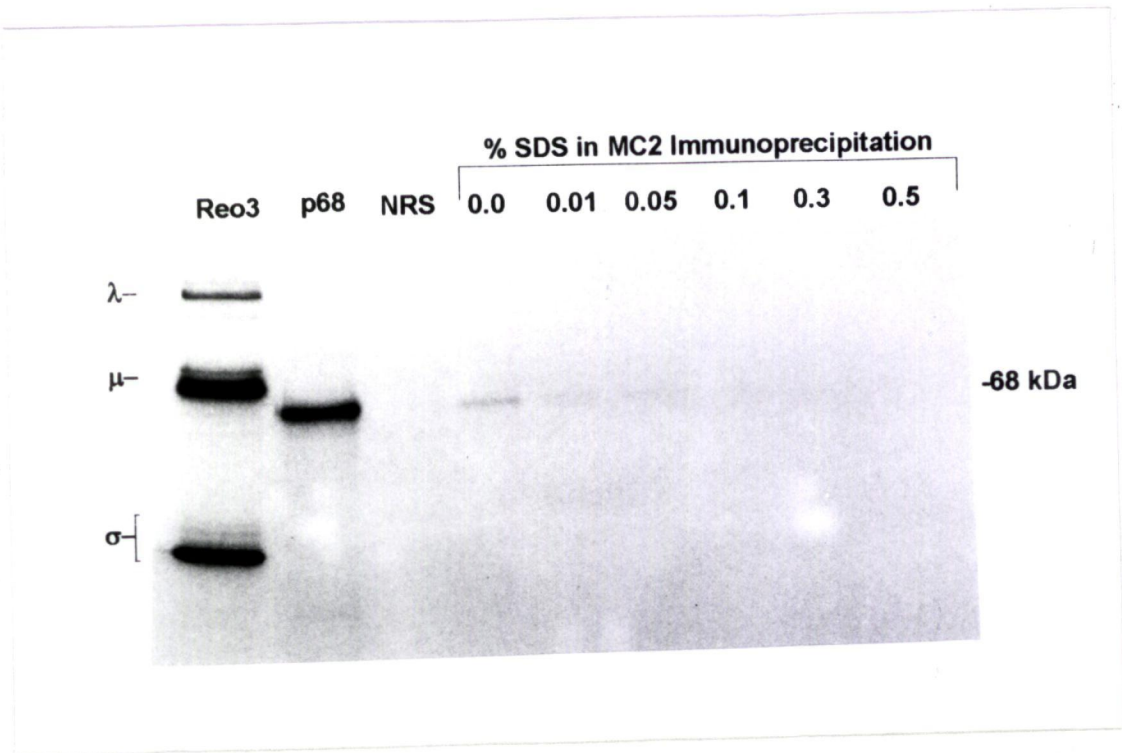
hydrolysis of a 68 kDa reovirus column-purified A431 cell membrane protein indicated several similarities between this protein and annexin VI. For these reasons, we subjected the L-929 cell membrane, reovirus column purified p68 protein to immunoprecipitation with an antibody specific for annexin VI (a generous gift from Dr. Stephen Moss, University of London, U.K.). Normally, this antibody is capable of immunoprecipitating annexin VI only in the presence of at least 0.3% (W/V) SDS, presumably because the antibody is directed against a partially denatured form of annexin VI. The results (figure 16) show that the annexin VI antibody (MC2) immunoprecipitated the p68 protein. The results further indicate that the presence of various amounts of SDS inhibited the ability of MC2 to immunoprecipitate the reovirus column-purified p68. This observation is probably due to the fact that the p68 protein was eluted from the reovirus column under relatively strong denaturing conditions (pH 2.6), and was at least partially denatured as a result of this treatment.

7.5 Aggregation of p68 and Annexin VI by Calcium

Although there are few, if any, defining functional features of annexin VI, one feature has been accepted by virtually all investigators in the annexin field. Annexin VI as well as all of the other annexin proteins (see introduction, chapters 5-8) are able to bind calcium with high affinity and be strongly aggregated by calcium ions. We therefore attempted to observe this phenomenon in both purified annexin VI as well as the reovirus column-purified p68 protein.

Figure 16: Immunoprecipitation of p68 by an Anti-Annexin VI Antibody

^{125}I-labelled annexin VI was immunoprecipitated (see Methods and Materials) with normal rabbit serum (NRS) or an anti-annexin VI antibody (MC2) in the presence of various concentrations of SDS (W/V) as indicated. Samples were boiled in protein sample buffer for 5 minutes and subjected to SDS-PAGE, followed by processing and autoradiography (see Methods and Materials).


% SDS in MC2 Immunoprecipitation
Reo3
p68
NRS
0.0
0.01
0.05
0.1
0.3
0.5
λ–
μ–
σ–
-68 kDa

Purified annexin VI from bovine lung was obtained (a generous gift from Dr. D. Waisman, Department of Medical Biochemistry, University of Calgary) and subjected to incubation (alongside with iodinated p68) with 10mM $CaCl_2$ for 10 minutes at 37°C. Samples were vortexed and then boiled in SDS and β-mercaptoethanol-containing protein sample buffer for 10 minutes, prior to analysis on SDS-PAGE, subjected to coomassie staining and autoradiography.

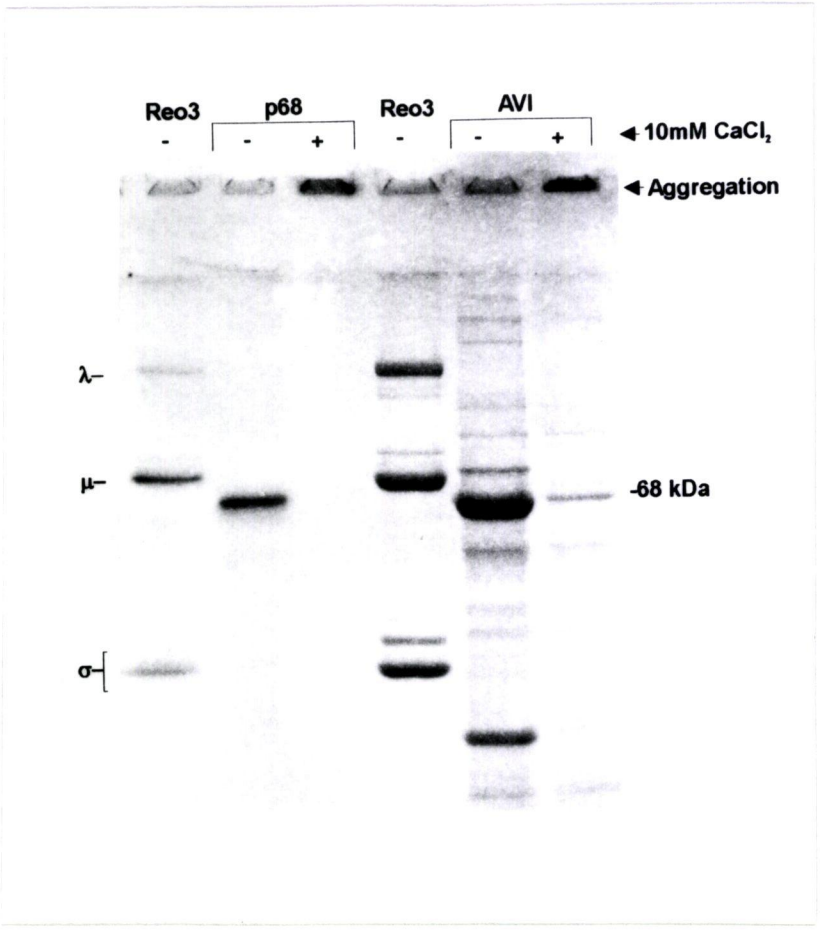
The results (figure 17) indicate that in the presence of 10mM $CaCl_2$, both purified bovine annexin VI and p68 are strongly aggregated, and are retained as aggregates after boiling in protein sample buffer, as that these samples did not even penetrate the gel matrix. Other work in our lab has indicated that reovirus column-purified p68 from A431 cell membrane is also a calcium binding protein (unpublished data). The calcium binding activity for this protein, however, was manifested as an SDS-PAGE protein band shift, rather than simple aggregation of the protein.

7.6 Sub-cloning of Human Recombinant Annexin VI

Because we hypothesize the identity of L-929 cell membrane derived p68 to be annexin VI, we attempted to repeat the previously described experiment (section 7.3) in which reovirus was found to recognize p68 in a sialic acid-enhanced manner. Additionally, because we had an in vitro expression system for reovirus protein σ1, we decided to perform a σ1/annexin VI cotranslation experiment to determine if there were any detectable interaction between annexin VI and σ1. To do this experiment, we obtained the cDNA for human recombinant annexin VI (a generous gift from Dr. S. Moss, University of London, U.K.) and subcloned this gene into an expression vector

Figure 17: Aggregation of p68 and Annexin VI by Calcium

I-^{125}-labeled p68 or Annexin VI (AVI) were incubated with 10mM $CaCl_2$ (+) or buffer (-) for 10 minutes at 37^{o}C. Samples were boiled in protein sample buffer for 5 minutes and subjected to SDS-PAGE, followed by processing and autoradiography for the radioactive samples, or coomassie staining for the non-radioactive samples (see Methods and Materials).


Reo3
p68
Reo3
AVI
-
-
+
-
-
+
10mM CaCl2
Aggregation
λ–
μ–
σ–
-68 kDa

for *in vitro* transcription and translation. Briefly, human recombinant cDNA for annexin VI was supplied in a bluescript KS vector (Stratagene), and flanked on both the 5' and 3' ends by Pst1 sites. The both the annexin VI-containing bluescript vector and a PGEM-4Z expression vector (Promega) were digested to completion with Pst1 (see Methods and Materials, chapter 5). The relevant agarose gel-purified fragments were ligated, and the resulting vectors transformed into competent E.coli (see Methods and Materials, chapter 5) for amplification and screening for the SP6-translatable clone of annexin VI in the PGEM-4Z vector. Digestion of small scale plasmid preparations (see Methods and Materials, chapter 5) with BamH1, EcoR1, Pst1 and Ssp1 revealed a bacterial colony containing an SP6-translatable clone of human recombinant annexin VI in the PGEM-4Z vector (figure 18) . The bacterial colony was grown into a large scale plasmid preparation (see Methods and Materials, chapter 5) and RNA was produced *in vitro* from a HindIII-linearized plasmid preparation (see Methods and Materials, chapter 5).

7.7 Interaction Between Recombinant Annexin VI and Reovirus is Enhanced by Sialic Acid.

Because we observed an interaction between p68 and reovirus, we elected to see if a similar observation could be made of reovirus and recombinant human annexin VI. To this end, [^{35}S]methionine-labeled human annexin VI was translated *in vitro* as previously described (chapter 2), and one fourth of a reaction (50 μL) was incubated with 25 μL of 4 mg/mL of reovirus for one hour at 37^{o}C in the presence or absence of 0.1M sialic acid (N-acetyl

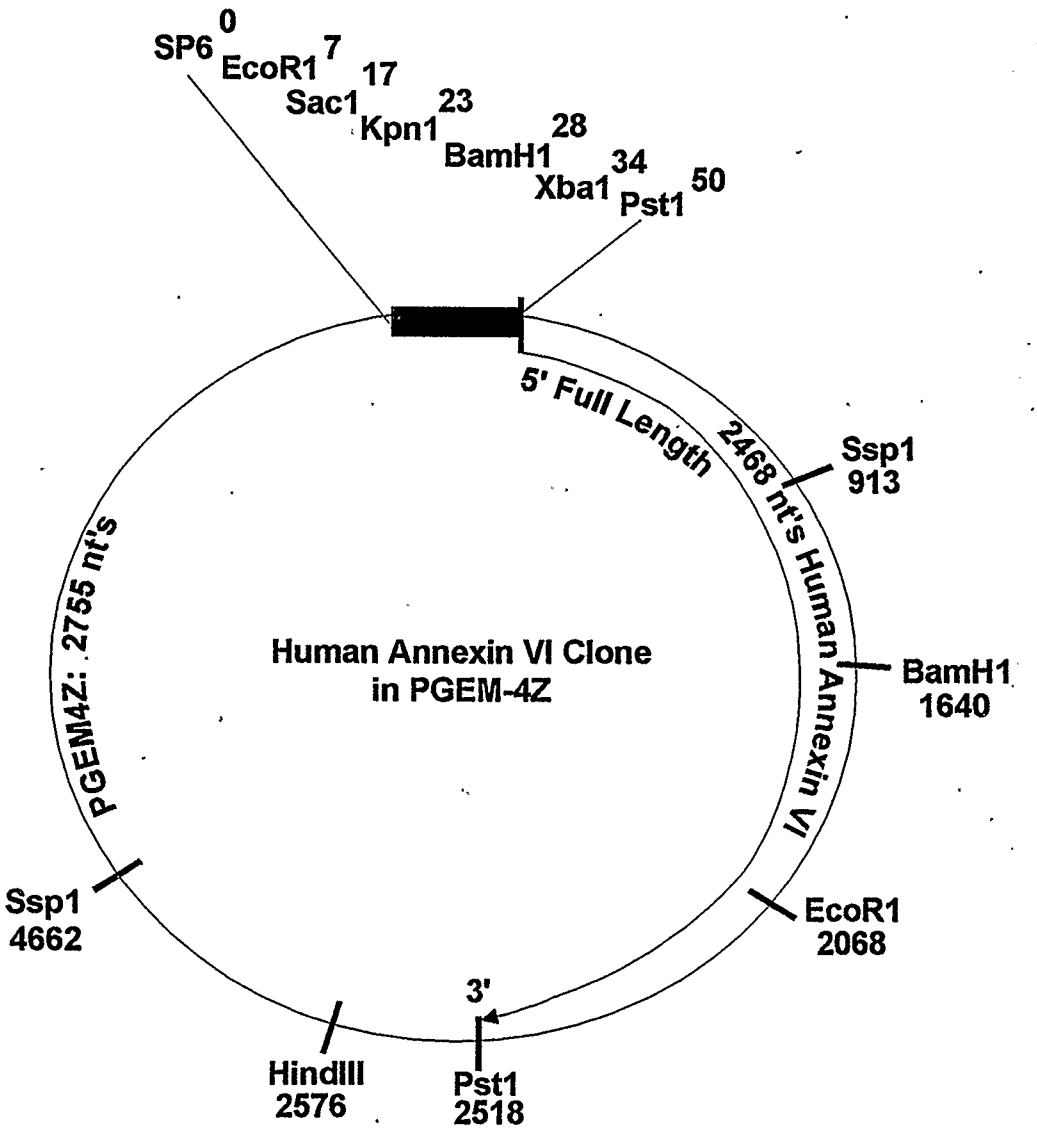


Figure 18: Map of the Human Annexin VI Clone in PGEM-4Z

Neuraminic acid, final concentration). Solutions were buffered to neutrality by the inclusion of 100mM TRIS, pH 7.4 (final concentration). The solutions were then immunoprecipitated (see Methods and Materials, chapter 2.4) with normal rabbit serum, an anti-annexin VI antibody, and an anti-reovirus antibody. Samples were boiled for 10 minutes in SDS-containing protein sample buffer and subjected to SDS-PAGE , processed and analyzed by autoradiography (see Methods and Materials).

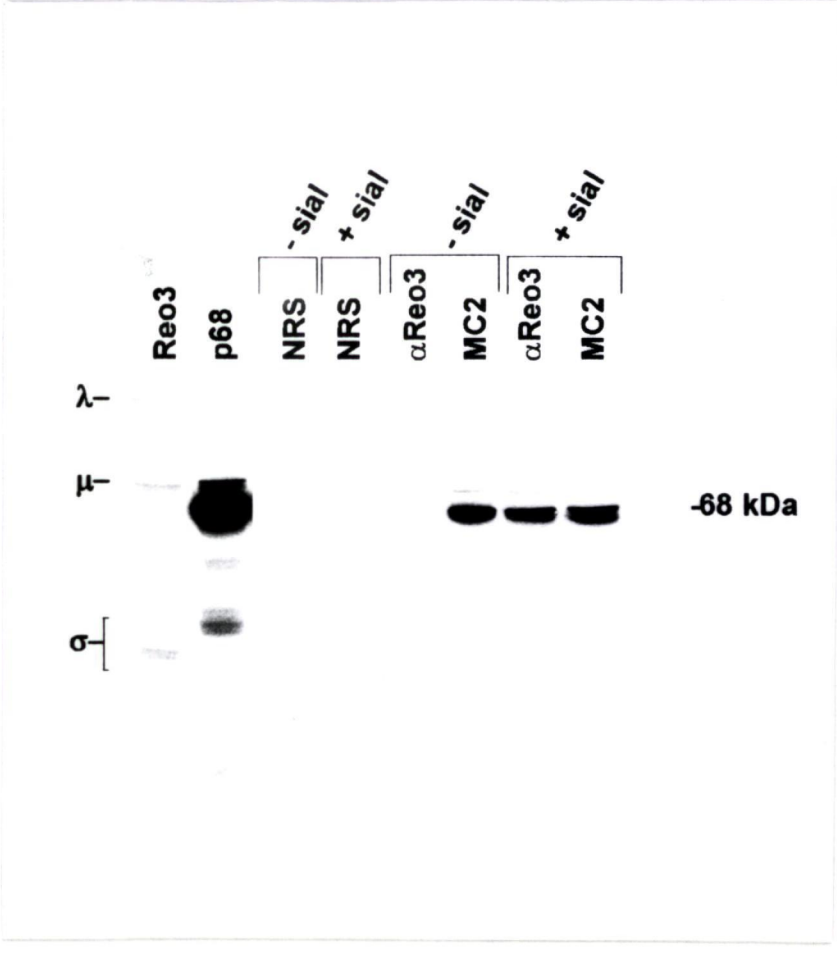
The results (figure 19) indicated that reovirus interacted with recombinant human annexin VI *in vitro*, in both the presence and absence of sialic acid. Sialic acid ,however, was observed to drastically increase the interaction of reovirus and annexin VI. Immunoprecipitation of reovirus by normal rabbit serum did not result in any signal, indicating that the interaction between reovirus and recombinant human annexin VI is relatively specific.

7.8 Cotranslation of Recombinant Human Annexin VI and Reovirus Protein σ1

Because the reovirus attachment protein is σ1, we postulated that association of the virus with annexin VI could occur through σ1. Because association between reovirus and p68 as well as annexin VI appeared to be relatively strong, we predicted that an association between protein σ1 and reovirus would exhibit similar strength. To this end, we performed a cotranslation of [^{35}S]methionine-labeled proteins σ1 and annexin VI, and subjected this cotranslation to incubation in protein sample buffer at various temperatures to determine whether there was an association between these two proteins. Normally, after incubation in protein sample buffer at 4^{o}C, protein σ1

Figure 19: Sialic Acid-Enhanced Recognition of Annexin VI by Reovirus

Reovirus was incubated with *in-vitro* translated [^{35}S]-methionine-labelled annexin VI (p68) in the presence of 100mM sialic acid (+sial) or buffer (-sial) at 37^{o}C for 30 minutes. Samples were then immunoprecipitated (see Methods and Materials) with normal rabbit serum (NRS) or anti-total reovirus serum (αReo3) or anti-annexin VI (MC2). Samples were boiled in protein sample buffer for 10 minutes and subjected to SDS-PAGE, followed by processing and autoradiography (see Methods and Materials).


Reo3
p68
NRS
NRS
αReo3
MC2
αReo3
MC2
- sial
+ sial
- sial
+ sial
λ–
μ–
σ–
-68 kDa

migrates as a compact form. At 37°C incubation in protein sample buffer, however, σ1 migrates as a less compact "hydra", which represents the partial denaturation of this protein at its C-terminus (the cell binding domain).

The results (figure 20) show that there was no difference in the compact to hydra shift of σ1 when translated alone or with annexin VI. Annexin VI migrated the same at all temperatures, whether or not it was translated with σ1. This lack of observable interaction suggests, but does not prove that reovirus interaction with annexin VI occurs though a reovirus protein other than σ1.

7.9 Lack of Interaction between Recombinant Proteins σ1 and Annexin VI

Because we were able to observe an interaction between reovirus and both p68 and recombinant human annexin VI through use of radioimmunoprecipitation, we attempted a similar experiment, using recombinant σ1 in place of reovirus. Briefly, human recombinant annexin VI and recombinant reovirus protein σ1 were separately translated *in vitro* (see Methods and Materials, chapter 2). Equal amounts of each translation mixture were then added to the same 1.5 mL microfuge tube and the resulting mixture was incubated at 37°C for 1 hour. The mixture was then immunoprecipitated with an anti-total reovirus antibody, an antibody specific for the C-terminus of σ1 (G5), and an antibody specific for the N-terminus of σ1.

The results (figure 21) indicate that although each of the σ1 antibodies, as well as the anti-total reovirus antibody, recognized σ1, none of these immunoprecipitations resulted in a signal from annexin VI, indicating a lack of detectable association of annexin VI with protein σ1.

Figure 20: *In Vitro* Cotranslation of Annexin VI and σ1

σ1 and annexin VI were either translated alone (σ1 and AVI, respectively), or cotranslated (Cotrans.) for one hour at 37°C (see Methods and Materials). Samples were then incubated in protein sample buffer at the temperatures indicated for 30 minutes or boiled (B) for 10 minutes. The samples were subjected to SDS-PAGE, processed, and analyzed by autoradiography.

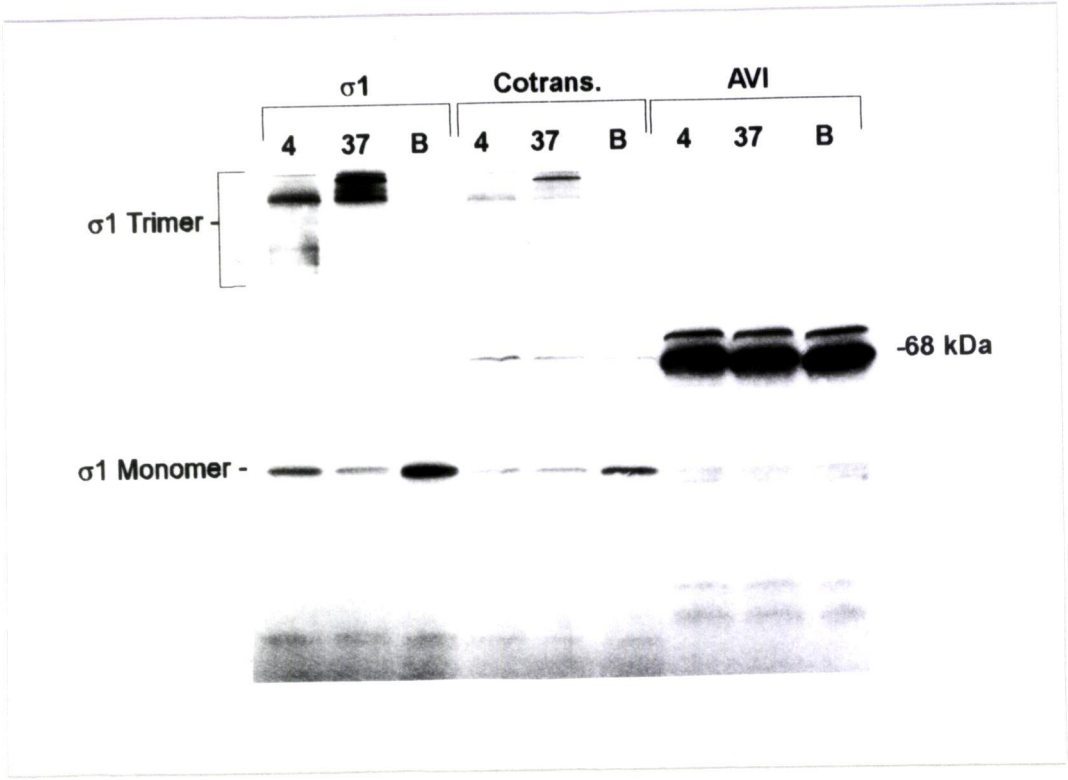
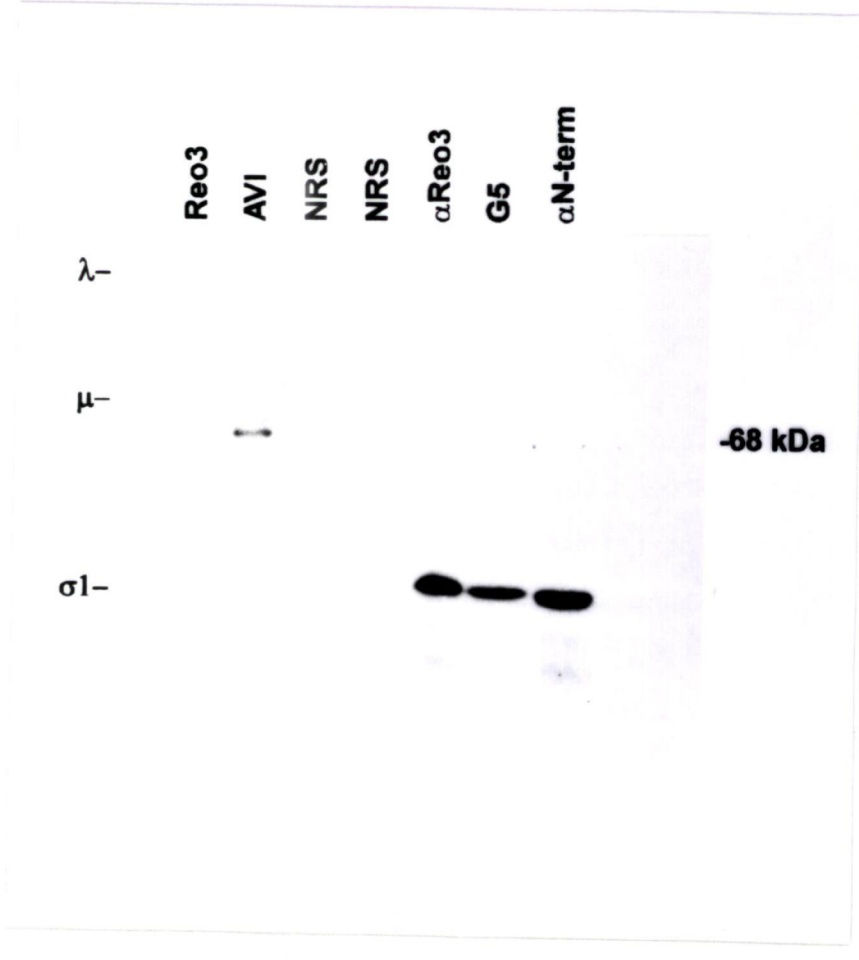

σ1
Cotrans.
AVI
4
37
B
4
37
B
4
37
B
σ1 Trimer
-68 kDa
σ1 Monomer

Figure 21: Lack of Interaction Between Annexin VI and σ1

Annexin VI and σ1 were serarately translated in vitro, mixed and incubated at 37°C for one hour. The mixture was then immunoprecipitated (see Methods and Materials) with normal rabbit serum (NRS), an anti-total reovirus antibody (αReo3), an anti-C-terminus σ1 antibody (G5) and an anti-N-terminus σ1 antibody (αN-term). The samples were then boiled in SDS-containing protein sample buffer for 10 minutes and subjected to SDS-PAGE, processed, and analyzed by autoradiography.


Reo3
AVI
NRS
NRS
αReo3
G5
αN-term
λ–
μ–
σ1–
-68 kDa

7.10 Amino Acid Composition Analysis Reveals Similarity Between p68 and Annexin VI.

In order to convincingly demonstrate the identity of p68, we attempted to purify enough of this protein for amino-terminal sequence analysis. To purify more of this protein, approximately 54 litres of L-929 cells (approximately 4.86 X 10^{11} cells) were grown in spinner cultures (final growing cell density was allowed to reach 2 X 10^6 cells/mL) and the membranes harvested as previously described (see Methods and Materials, chapter 1). The protein was collected from elution through a 2 mL reovirus column as previously described. The resulting elution fractions were subjected to SDS-PAGE and analyzed by coomassie staining (see Methods and Materials, chapter 4). The identified fractions were vacuum-dried to a final volume of 0.5 mL and dialyzed against 5% (V/V) acetic acid to remove the Triton X-100. The dialyzed protein was then lyophilized and stored as a powder at -70^{o}C until required. An aliquot was resuspended in PBS for quantitation at 280 nm (UV), and it was determined that approximately 15 μg of p68 had been recovered from the reovirus column.

Because previous efforts to sequence the 68 kDa protein from A431 cell membrane resulted in the determination of an N-terminal blockage of this protein, and because annexin VI is blocked from sequencing at its N-terminus, we attempted to sequence fragments of p68. Fragments of approximately 10 μg of p68 were prepared by digestion with an excess of cyanogen bromide (CNBr) in the presence of 0.1N HCl. The resulting fragments were vacuum dried and sent for sequencing. Unfortunately, it was determined that there were not enough protein fragments to obtain any meaningful sequence. We therefore sent 5 more μg of the p68 protein for amino acid composition analysis

(Performed by Dr. Don McKay, Dept. of Medical Biochemistry, University of Calgary).

The results (table 4) indicated measurable amounts of each amino acid. These results were compared to a computer simulation of amino acid composition analysis of murine annexin VI, tabulated (table 4) and rendered for graphical comparison (figure 22). The graphical comparison of the relative amino acid contents of both murine p68 and annexin VI were virtually identical, indicating a very strong probability that murine p68 is actually murine annexin VI.

7.11 Reovirus is Cross-linked to the Cell Cytoskeleton

Because reovirus attaches to L-929 cell surfaces (and undergoes a conformational change) in a totally reversible manner, it seems that infection of the cell would require a further interaction with reovirus at the cell surface. It seems likely that this further interaction would be relatively irreversible and may involve the second reovirus receptor. Additionally, evidence has suggested that annexin VI binds to F-actin in a calcium-dependent manner (see Introduction). We therefore postulated that at least some of the reovirus-cell receptor interactions are irreversible, and may involve a relatively irreversible interaction on the surface of susceptible cells. To gain support for this hypothesis, an experiment was performed to determine the nature of reovirus association on the cell suface.

Briefly, reovirus was allowed to bind to a confluent monolayer of L-929 cells as described in the Methods and Materials (chapter 1.10) for 1 hour at 4^{o}C (to prevent virus internalization). The monolayers were then washed three times with pre-chilled (4^{o}C) PBS, and the monolayers then covered with PBS

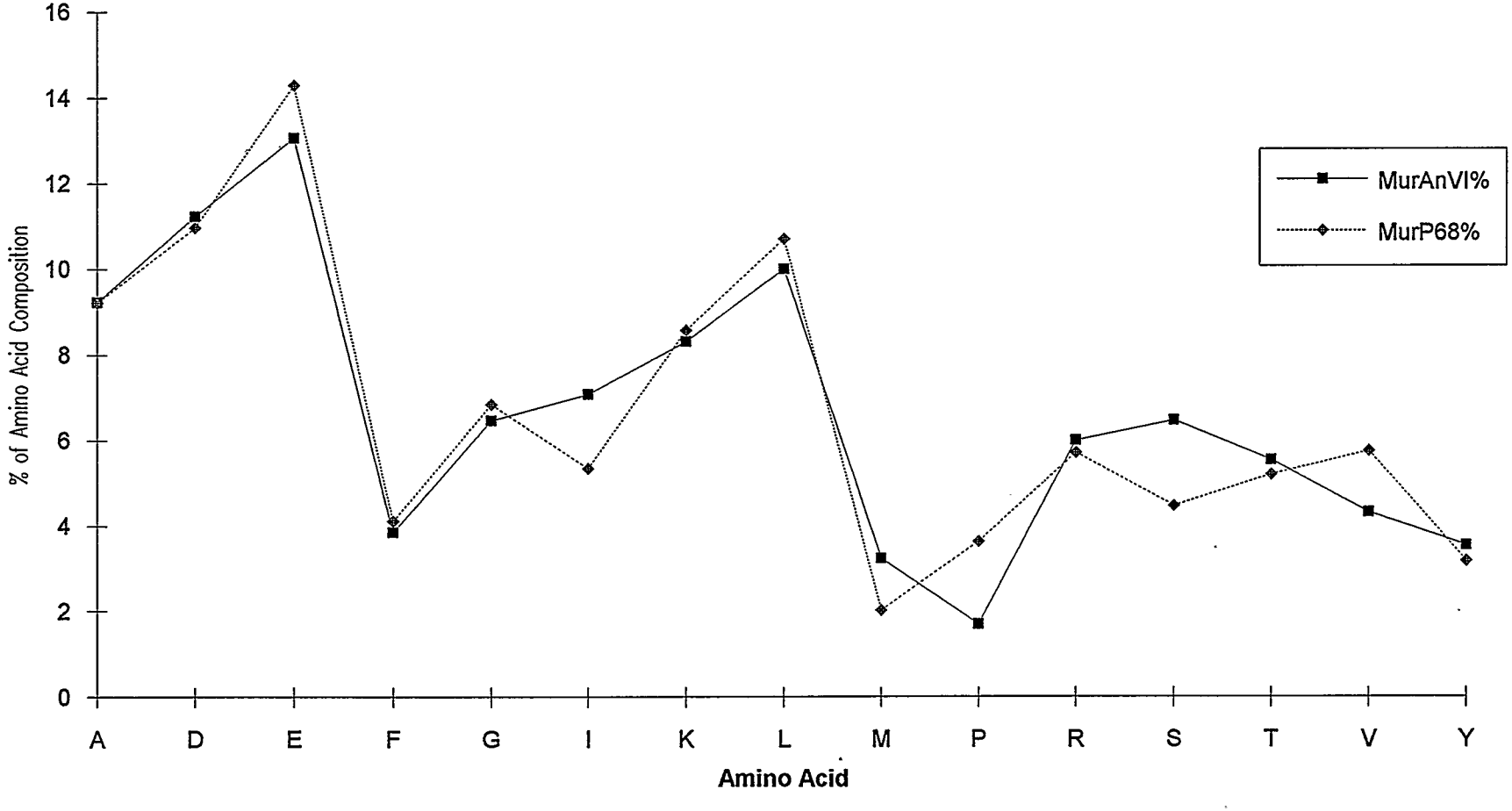
Table 3: Amino Acid Compositions and % Compositions of Murine Annexin VI and p68

Amino Acid	Annexin VI	p68	% Annexin VI	%p68
A	60	43	9	9
D	73	51	11	11
E	85	67	13	14
F	25	19	4	4
G	42	32	6	7
I	46	25	7	5
K	54	40	8	9
L	65	50	10	11
M	21	9	3	2
P	11	17	2	4
R	39	27	6	6
S	42	21	6	4
T	36	24	6	5
V	28	27	4	6
Y	23	15	4	3

Figure 22: Comparison of Amino Acid Compositions of Murine Annexin VI and p68

p68 was purified from L-929 cell membranes as described, and submitted for amino acid composition analysis (performed by Dr. D. McKay, Protein Sequencing Facility, University of Calgary). The data was compared to the murine annexin VI amino acid composition derived from the Genbank database library. Cysteine and tryptophan were not included, due to degradation by the analysis procedure. Gln and Glu are summarized as Glu (E), and Asn and Asp are summaraized as Asp (D). The histidine value was also not included, due to conflict with a ninhydrin-detectable contamination.

Percent Composition Comparison of Murine p68 with Murine Annexin VI



containing 1% (V/V) Triton X-100. The monolayers were incubated on ice for 10 minutes, washed gently with three changes of pre-chilled PBS, and harvested with SDS-containing protein sample buffer. Samples were boiled, subjected to SDS-PAGE, and coomassie stained for analysis (see chapter 4).

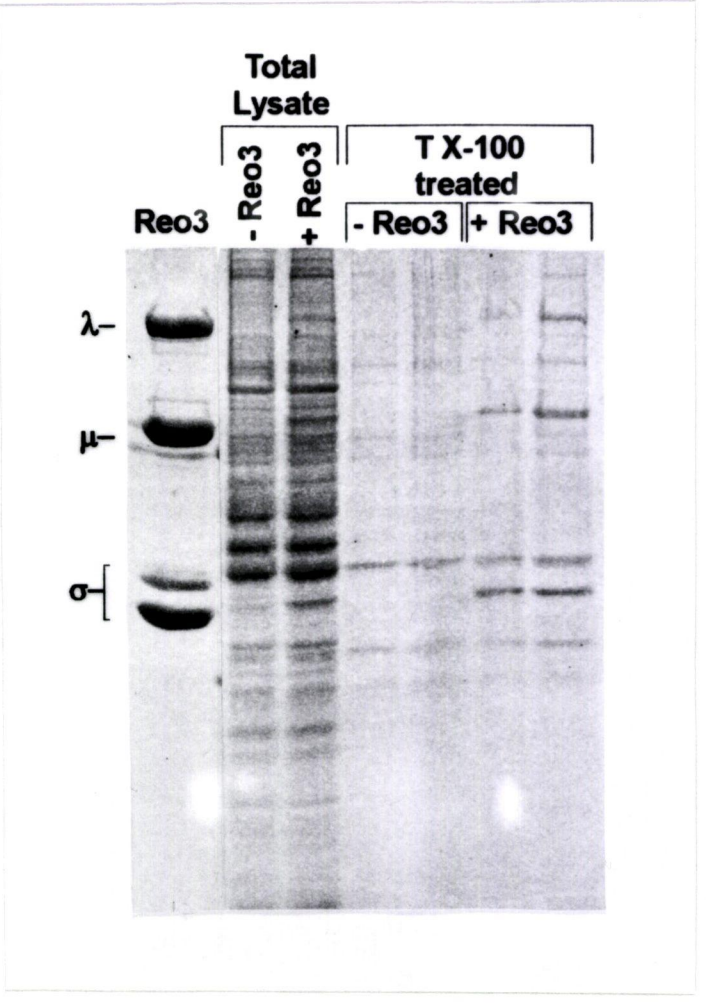
The results (figure 23) show that reovirus remains associated with the cell monolayer after treatment with Triton X-100. Because cell plasma membrane lipids are solublized by Triton X-100, the remaining proteins observed after this treatment were those proteins belonging to, or are associated with the cell cytoskeleton. It is clear from this data, therefore, that reovirus becomes cross-linked to the cell cytoskeleton sometime after binding, but before internalization. It is probable that this interaction represents the irreversible, but productive reovirus attachment to the cell surface, associating with the cell cytoskeleton possibly through annexin VI.

7.12 Discussion

While it is clear that reovirus binds to multiple cell surface proteins, it is not known whether all of these interactions lead to a productive viral infection. Although the 68 kDa proteins seems to be the major protein recognized by reovirus in solublized L-929 cell membrane preparations, its role in the reovirus infection process remains in question. The similarities in $CaCl_2$ aggregation, annexin VI antibody recognition, sialic acid-enhanced reovirus binding and amino acid composition analysis strongly suggest, but do not prove that these proteins are identical.

Figure 23: Reovirus Becomes Cross-Linked to the Cell Cytoskeleton

Reovirus (+Reo3) or buffer (-Reo3) was exposed to an L-929 cell monolayer for binding at 4^{o}C (see Methods and Materials) and the monolayers washed as described and then treated with buffer containing Triton X-100 (TX-100 treated) or harvested in SDS-containing protein sample buffer (Total Lysate). The Triton X-100 treated monolayers were washed with buffer as described and then harvested with SDS-containing protein sample buffer. Samples were boiled for 10 minutes, subjected to SDS-PAGE, and Coomassie stained for analysis (see Methods and Materials).


Total
Lysate
T X-100
treated
Reo3
- Reo3
+ Reo3
- Reo3
+ Reo3
λ
μ
σ

The association between p68 and reovirus is likely very strong, as that approximately 25 bed volumes of a very low pH buffer were required to elute this protein from the reovirus column. It is possible that the annexin VI-like 68 kDa protein reported here is coupled to sialic acid-bearing receptors on the cytoplasmic membrane, while retaining its affinity for reovirus. Although there are no reports on the glycosylation state of annexin VI in current literature, the possiblity of this protein being a sialoglycoprotein (and therefore a sialic acid-bearing reovirus receptor) has not yet been ruled out. The unusually strong interaction of reovirus and p68, however, seemingly precludes the relatively weak ionic interaction common between a protein and a sugar moiety. An interaction between a virus and an annexin VI-coupled receptor protein has been reported for the Epstein-Barr virus. It was found that the EBV receptor (CR2) was associated with p53 in transformed B lymphocytes, but is associated with annexin VI in normal B lymphocytes (Barel et al., 1991).

Because annexin VI has been shown to be functional in the budding of clathrin coated pits (in the presence of ATP and calcium), it is possible that this protein may function similarly in reovirus internalization. Further, annexins V and VII have recently been shown to form calcium channels, while annexin VI has been found to function in the regulation of a cacium-release channel in the sarcoplasmic reticulum (Rojas et al., 1990; Huber et al., 1990; Hazarika et al., 1991; Diaz-Munoz et al., 1990). Annexin VI has also been shown to trimerize and bind to the inside surface of cell plasma membrane to form flat "pore-like" lattices, that concentrate acidic phospholipids at their centers (Newman et al., 1989). The localized concentration of acidic phospholipids by annexin VI could lead to localized alterations in membrane structure that may be taken advantage of by cell surface-bound reovirus. This is especially intriguing

in light of the recent demonstration that the μ1C reovirus capsid protein interacts with cell plasma membranes.

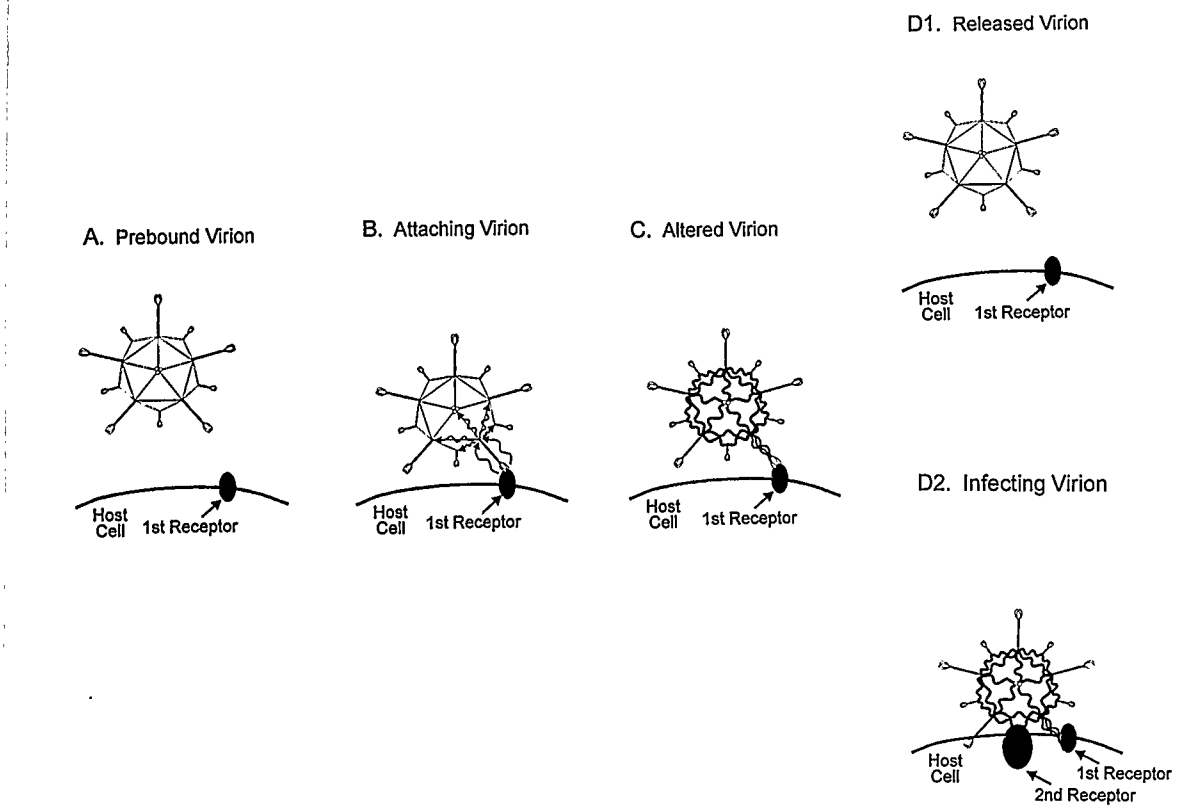
The observations that the second reovirus receptor is probably annexin VI, and that reovirus becomes cross-linked to the cell cytoskeleton soon after cell attachment leads us to hypothesize on a function for annexin VI in reovirus infection (figure 24). Annexin VI has been shown to bind F-actin in a calcium-dependent manner (Davies et al., 1984; Glenney et al., 1987; Glenney 1986; Gerke and Weber, 1984), and may serve to link reovirus to the cell cytoskeleton prior to the process of internalization.

Preliminary experiments have resulted in observations that reovirus causes a drastic increase in intracellular calcium concentration within seconds of cell attachment (Fernandes and Lee, Unpublished Data). This phenomenon may lead to an annexin VI-calcium interaction, which may cause annexin-VI to trimerize and associate with the cell plasma membrane as well as the cell cytoskeleton (Newman et al., 1989). This possibility is the focus of ongoing research in our lab.

Identification of annexin VI as the second reovirus receptor has led to intriguing insights as to the mechanism of reovirus internalization. Elucidation of the initial virus-cell interactions allow us to follow the next steps in virus-cell interactions, such as the elucidation of calcium-mediated second messages within the cell that may lead to internalization and shut-off of host protein synthesis. Advancement of this knowledge in the reovirus system is of potentially enormous value to the understanding of other virus internalization mechanisms, since it appears that despite gross dissimilarities among viral systems, certain critical elements such as the functions of the annexin proteins

Figure 24 Model of Reovirus Interaction with its Two Cell Surface Receptors

Binding of virion (A) via protein σ1 to a sialic acid-bearing moiety on the cell surface (first step interaction) induces a conformational change which is initiated in the C-terminal receptor-binding domain of σ1, progresses to the N-terminus of the protein, and subsequently spreads to other capsid proteins (B and C). The structurally-altered virion (C) is then ready for the second step interaction which likely involves annexin VI (second step interaction) (D2) and a reovirus protein other than σ1 and leads to subsequent viral entry (not depicted). However, if conditions are unfavorable for this interaction, the virus may elute from the cell surface, reassume its original prebound structure (D1), and be ready to attach again.


A. Prebound Virion
Host Cell
1st Receptor
B. Attaching Virion
Host Cell
1st Receptor
C. Altered Virion
Host Cell
1st Receptor
D1. Released Virion
Host Cell
1st Receptor
D2. Infecting Virion
Host Cell
1st Receptor
2nd Receptor

(see introduction, chapter 5) in virus-cell interactions, appear to be fundamental to a variety of virus families.

Discussion and Future Prospects

Significant progress has been made in the understanding of reovirus-cell receptor interactions. It has been previously shown that the first reovirus receptor is sialic acid. As a result of this interaction, we have observed reversible alterations in reovirus structure, and have additionally identified a second receptor protein.

The existence of a two-receptor mechanism of virus attachment may serve several possible functions. First, the reovirus does not "waste" itself by irreversible attachment to non-permissive cells. Second, reovirus becomes localized to the cell surface , which effectively concentrates it for possible co-localization with its second receptor. Third, it is possible that reovirus is structurally more sensitive to environmental damage in its sialic acid-altered conformation. This conformation may be described as the "entry conformation" which, by nature of its reversibility, renders the reovirus only temporarily vulnerable.

The use of sialic acid by reovirus for its first receptor seems logical, as this carbohydrate moiety is a normal component of cell surfaces (Carr and Kim, 1993). It is difficult to imagine, therefore, that sialic acid alone defines reovirus tissue tropism and cell specificity. The use of annexin VI as a second reovirus receptor also seems logical, as that this protein exhibits extraordinary evolutionary conservation (Moss et al., 1988). The use of annexin VI as a possible determinant of cell specificity and tissue tropism by reovirus is supported by recent observations. It was found that Human Hepatitis B virus (which does not infect rat liver) small antigen (HBsAg) binds strongly to human liver-derived annexin V, and poorly to rat liver-derived annexin V (Hertogs et al., 1993). Annexin V is thus potentially capable of directing species specificity,

despite the rather high degree of evolutionary conservation of this protein (>90%).

Despite our recent findings, much work is still required to gain an intimate understanding of reovirus-cell receptor interactions. It is possible, for example, that the reovirus dual receptor phenomenon occurs on the same protein, as that it has not yet been demonstrated whether annexin VI is sialylated. It must also be shown whether annexin VI is capable of defining tissue tropism and cell specificity. This question may be addressed by the transfection of an annexin VI-deficient cell line with annexin VI cDNA, in an attempt to enhance reovirus infection efficiency in the transformed cells. This cell line has been acquired by our laboratory, and these studies will commence shortly. Further, deletion mutagenesis and point mutation may allow us to precisely define the regions of reovirus interaction on the annexin VI molecule, allowing us to "fine tune" our knowledge of these specific regions of interaction.

Conclusive identification of annexin VI as a second reovirus receptor must still be made by a combination of protein p68 peptide fragment sequencing (as that the p68 protein is blocked from sequencing at its N-terminus (unpublished observation)) and peptide mapping. Because reovirus possibly interacts with annexin VI through a protein other than σ1, the identity of this other reovirus protein remains to be demonstrated. It is probable that one or a combination of the other reovirus outer capsid proteins (σ3, μ1C and λ2) are responsible for this interaction. A likely candidate is protein μ1C. A mutant of this protein has long been known to lead to altered reovirus neurovirulence (Hrdy et al., 1982). This protein has also been shown to be myristoylated (Nibert et al., 1991) and is therefore capable of interacting in a hydrophobic medium, such as the cell plasma or endosomal membranes (Lucia-Jandris et al., 1993). μ1C has also

been shown to punch small anion-selective channels through planar lipid bilayers (Tosteson et al., 1993). Finally, conformational changes have been observed in protein μ1C (Dryden et al., 1993), that could lead to exposure of its myristyl group for insertion into lipid membranes.

The best method of investigation as to the intricacies of reovirus/annexin VI interaction would be through X-ray crystallography of three-dimensional reovirus/annexin VI crystals. Another method of determining which reovirus protein interacts with annexin VI would be to probe a blot of reovirus proteins with ^{125}I-labeled annexin VI, to see whether the μ1C band is recognized by the protein. Immunoprecipitation of annexin VI from a mixture of annexin VI and soluble reovirus proteins may also specify the identity of the annexin VI-binding protein of reovirus. Additionally, a temperature-sensitive, entry-deficient mutant of reovirus has recently been acquired by our laboratory. This mutant contains a point mutation which alters only one amino acid in the μ1C protein. It will be interesting, therefore, to immunoprecipitate this reovirus from a mixture of mutant reovirus and annexin VI, that had been incubated at permissive and non-permissive temperatures, to see whether annexin VI co-precipitates with the reovirus mutant at only the permissive temperature.

Future research will also address the potential of calcium for a role in the reovirus infection process. Preliminary observations have suggested that a calcium spike is effected within the cell only seconds after reovirus exposure (Unpublished observations). Because annexin VI is a calcium-dependent phospholipid-binding protein, it is possible that a mechanism exists whereby reovirus causes localization of its second receptor to the cell membrane. Because calcium is such a strong second messenger for signal transduction inside the cell, it is possible that phenomena, such as phosphorylation

cascades, may result from reovirus cell attachment. These phosphorylation cascades may effect various reovirus infection processes, such as shutoff of host protein synthesis, cytoskeletal reorganization, and viral entry. Isolation of endosomes containing reovirus and, presumably, its receptor or receptor complex may reveal alternative protein phosphorylation patterns and shed light on the mechanisms of reovirus internalization.

Intimate understanding of reovirus-cell receptor interactions and mechanisms of internalization has many potential benefits. Features of the reovirus internalization mechanism may be common to other virus systems, potentiating the development of rational antiviral drug designs. Further, understanding the precise mechanisms of reovirus attachment and entry may potentiate the development of new strategies and vectors for tissue targeting in gene therapy. Because viruses are infectious, it is possible that knowledge gained from the reovirus system could be applied to the development of new viruses that specifically target and penetrate solid tumors.

As described, the potentials for application of an intimate understanding of the reovirus attachment and internalization mechanisms are enormous. Thus far, we have gained only a glimpse of previously unimagined horizons in our attempts to understand the interactions of only a few molecules in the vastness of nature.

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