# FUNCTIONAL CHARACTERIZATION OF A SMALL NONSTRUCTURAL PROTEIN OF THE *PARVOVIRUS* B19.

by

MANNIE MAN YEE FAN

B.Sc. (Honours), McGill University, 1998

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

Department of Biochemistry and Molecular Biology

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April 2001

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Biochemistry & Molecular Biology

The University of British Columbia
Vancouver, Canada

Date April 2001

DE-6 (2/88)

# ABSTRACT

The pathogenic human parvovirus B19 produces a class of small, 11kDa proteins whose function is as yet undetermined. In the absence of a continuous permissive cell line in which B19 can be readily propagated, *in vitro* studies have been used to assess the functional role of the 11kDa proteins.

In previous studies, the 11kDa proteins have not been shown to be targets of phosphorylation or binding partners for other viral proteins, nor have they demonstrated $Zn^{++}$-binding or transactivation activities. However, there is preliminary evidence that the 11kDa proteins are capable of interacting with the cellular growth factor receptor-binding protein 2 (Grb2) *in vitro*, and this thesis aims to investigate this interaction in greater detail.

In studies reported in this thesis, far western experiments confirm *in vitro* interactions between Grb2 and the 11kDa proteins. Moreover, a fusion construct of the 11kDa protein with glutathione S-transferase (GST) is able to specifically pull down endogenous Grb2 from HEK 293 cell lysates, providing further support for our hypothesis that the two proteins interact.

Grb2 is an important adaptor molecule in the MAPK signal transduction pathway leading to defined cellular responses. The protein is composed of a series of modular domains each specialized for a different function, including two SH3 domains that are capable of binding proline-rich sequences. Such sequences correspond to consensus "ligand" motifs found in binding partners for Grb2 such as son of sevenless (Sos), a guanine nucleotide exchange factor for the Ras small GTP-binding protein that is implicated in MAPK activation.

Sos-like proline-rich regions are also present in the 11kDa proteins. Further studies have focused on the requirement for these regions in the Grb2/11kDa interaction. Far western studies employing 11kDa proteins mutated at proline residues suggest reduced interactions with Grb2. Furthermore, GST-11 mutant constructs do not precipitate Grb2 from mammalian cell lysates as readily as does the wild-type. These results indicate possible involvement of the proline-rich

region in mediating interactions of the 11kDa proteins with Grb2, in an SH3-dependent manner.

The findings from this thesis strongly support an interaction of the 11kDa proteins with the host protein Grb2 to influence host cell environment during the viral replicative cycle.

# TABLE OF CONTENTS


Abstract ii

Table of Contents iv

List of Tables ix

List of Figures x

List of Abbreviations xii

Acknowledgments xix

**1. INTRODUCTION** 1

1.1. Taxonomy 1

1.1.1. Dependoviruses 1

1.1.2. Parvoviruses 3

1.1.3. Erythroviruses 3

1.2. The Erythrovirus B19 4

1.2.1. B19 Infection and Transient Aplastic Crisis 6

1.2.2. Persistent B19 Infection 6

1.2.3. B19 Infection *In Utero* 7

1.2.4. B19 Infection in the Immunocompetent Host 7

1.2.5. B19 and Other Disorders 11

1.3. B19 in Cell Culture 11

1.4. The B19 Genome 12

1.4.1. The B19 Nonstructural Proteins 15

1.4.2. The B19 Structural Proteins 15

1.4.3. The B19 11kDa Proteins 16

1.5. Review of Relevant Signal Transduction Concepts and Pathways 17

1.6. Review of Functions of Other Viral Nonstructural Proteins 19

1.6.1. Virus Entry 19

1.6.2. Nuclear Targeting and Replication of Viral Genome 19

1.6.3. Viral Gene Expression 21

1.6.4. Processing and Nuclear Export of mRNA Transcripts 22

1.6.4.1. Processing and Nuclear Export of Viral Transcripts 22
1.6.4.2. Processing and Nuclear Export of Host Transcripts 23
1.6.5. Processing of Viral Proteins 24
1.6.6. Host Cell Protein Trafficking and Degradation 24
1.6.7. Virion Packaging, Assembly, and Egress 24
1.6.8. Cell Cycle Arrest 25
1.6.9. Immune Evasion 25
1.6.9.1. Inhibition of Humoral Immune Responses 25
1.6.9.2. Inhibition of Interferon Responses 26
1.6.9.3. Modulation of Cytokine and Chemokine Responses 27
1.6.9.4. Modulation of Apoptosis 28
1.6.9.4.1. Inhibition of Apoptosis 28
1.6.9.4.2. Induction of Apoptosis 29
1.6.9.5. Modulation of Major Histocompatibility Complex (MHC) Function 30
1.7. Other Proline-Rich Viral Nonstructural Proteins 31
1.8. Previous Studies on the 11kDa Proteins and the Present Study 32

**2. MATERIALS AND METHODS** 34
2.1. Chemicals and Supplies 34
2.2. Bacterial Culture 35
2.2.1. Growth and Maintenance 35
2.2.2. Preparation of Competent Bacteria for Heat Shock Transformation 35
2.2.3. Heat Shock Transformation of *E. coli* 36
2.3. Tissue Culture 36
2.3.1. Growth and Maintenance 36
2.3.2. Transfection of Plasmid DNA into COS-7 Cells 37
2.3.3. Lysis of Tissue Culture Cells for GST Protein Pull Down and Immunoprecipitation Experiments 37
2.4. Molecular Cloning Techniques 38

2.4.1. Small-Scale Plasmid DNA Isolation 38
2.4.2. Large-Scale Plasmid DNA Isolation 39
2.4.3. DNA Quantitation and Purity Check 39
2.4.4. Plasmid DNA Digestions 39
2.4.5. Plasmids 40
2.4.5.1. pGEX2T 40
2.4.5.2. pGEX-11 40
2.4.5.3. pGEX-11 P42,45A/P42,45,46A/P42,46A/P43A/P43,45A /P43,45,46A/P45A/P45,46A 42
2.4.5.4. pGEX-11 P42A 42
2.4.5.5. pGEX-11 P46A 42
2.4.5.6. pGEX2T-tag 42
2.4.5.7. pGEX2T-tag-Grb2 42
2.4.5.8. pEGFP-C2 42
2.4.5.9. pEGFP-11 44
2.4.6. Oligonucleotides 44
2.4.6.1. 11kDPro2 44
2.4.6.2. 11COMPL 44
2.4.6.3. P42A1 44
2.4.6.4. P42A2 45
2.4.6.5. P46A1 45
2.4.6.6. P46A2 45
2.4.6.7. pGEX3' 45
2.4.6.8. 11Seq1 45
2.4.7. Polymerase Chain Reaction (PCR)-Based Site-Directed Mutagenesis 46
2.4.8. G-Ladder Screen for Mutant Plasmid DNA 46
2.4.9. Automated Sequencing of Plasmid DNA 48
2.5. Protein Techniques 48
2.5.1. GST/GST Fusion Protein Purification 48
2.5.2. Protein Quantitation 49

2.5.3. Antibodies 50
2.5.3.1. Primary Antibodies 50
2.5.3.1.1. Rabbit anti-B19 11kDa 50
2.5.3.1.2. Mouse anti-GFP 50
2.5.3.1.3. Rabbit anti-Grb2 50
2.5.3.1.4. Mouse anti-GST 50
2.5.3.1.5. Mouse anti-HA 50
2.5.3.1.6. Mouse anti-Sos1 51
2.5.3.2. Secondary Antibodies 51
2.5.3.2.1. HRP-Conjugated Goat anti-Mouse 51
2.5.3.2.2. Super X-Absorbed HRP-Conjugated Donkey anti-Rabbit 51
2.5.4. Western Blotting 51
2.5.5. Far Western Blotting 52
2.5.6. GST Protein Pull Down Assay 52
2.5.7. Immunoprecipitation 53

**3. RESULTS** 55
3.1. Expression of Glutathione S-Transferase (GST) and GST-Tagged Fusion Proteins 55
3.2. GST-HA-Grb2 Interacts with GST-11 but not GST on a Far Western Blot 55
3.3. GST-HA-Grb2, but not GST-HA, Interacts with GST-11 on a Far Western Blot 58
3.4. Anti-Hemagglutinin (HA) Antibody Recognizes Both GST-HA and GST-HA-Grb2 58
3.5. GST-11 but not GST Interacts with Cellular Grb2 in a GST Protein Pull Down Assay 61
3.6. Exogenously Added GST and GST-11 Do Not Appear to Interact with Cellular Grb2 in Co-Immunoprecipitation Studies 61
3.7. Addition of Exogenous GST and GST-11 Proteins Do Not Affect Grb2/Sos Interaction 65

3.8. Neither the Enhanced Green Fluorescent Protein (EGFP) Nor the EGFP-11kDa Fusion Protein (EGFP-11) Interact with Cellular Grb2 in Co-Immunoprecipitation Studies *In Vivo* 65

3.9. Levels of Sos Protein Co-Immunoprecipitated by Anti-Grb2 Antibody Are Comparable in COS-7 Cells Transiently Expressing EGFP or EGFP-11 69

3.10. The GST-HA-Grb2/GST-11 Interaction on a Far Western Blot Requires 11kDa Proline-Rich Region 69

3.11. The Grb2/GST-11 Interaction in a GST Protein Pull Down Assay Requires 11kDa Proline-Rich Region 73

**4. DISCUSSION** 75

4.1. Characterization of the B19 11kDa/Grb2 Interaction 75

4.1.1. Far Western Studies 75

4.1.2. Pull Down Assays 75

4.1.3. Immunoprecipitation Studies 76

4.2. Src Homology 3 (SH3)-Mediated Interactions and B19 11kDa Proteins 77

4.3. Effects of Proline-to-Alanine Mutations on B19 11kDa/Grb2 Interactions 78

4.3.1. Far Western Studies 78

4.3.2. Pull Down Assays 78

4.4. Implications of Present Findings on Possible Functions of B19 11kDa Proteins 79

4.5. Future Directions 79

4.6. Conclusions 80

**5. REFERENCES** 81

# LIST OF TABLES


| | | |
|---|---|---|
| Table 1.1. | Selected *Parvoviridae* and their target host species. | 2 |
| Table 1.2.5. | Other disorders associated with parvovirus B19 infection. | 11 |
| Table 2.4.5. | Plasmid vectors and the proteins they express. | 40 |

# LIST OF FIGURES


| | | |
|---|---|---|
| Figure 1.2.4.1. | Development of B19-specific IgM and IgG after experimental B19 inoculation of seronegative volunteers and a volunteer with trace amounts of IgG to B19. | 9 |
| Figure 1.2.4.2. | Time course of experimental inoculation of Parvovirus B19 in a seronegative host. | 10 |
| Figure 1.4. | Transcription and coding map of Parvovirus B19. | 14 |
| Figure 1.8. | Model of B19 11kDa protein-mediated disruption of the Grb2/Sos/Ras signaling pathway. | 33 |
| Figure 2.4.5.1. | pGEX2T plasmid map. | 41 |
| Figure 2.4.5.8. | pEGFP-C2 plasmid map. | 43 |
| Figure 3.1. | Coomassie-stained polyacrylamide gel of purified GST/GST-fusion proteins. | 56 |
| Figure 3.2. | Western blot and far western blot demonstrating interaction of GST-11 but not GST with GST-HA-Grb2. | 57 |
| Figure 3.3. | Western blot and far western blots demonstrating interaction of GST-HA-Grb2 but not GST-HA with GST-11. | 59 |
| Figure 3.4. | Western blots demonstrating ability of anti-HA antibody to recognize HA epitope in both GST-HA and GST-HA-Grb2. | 60 |
| Figure 3.5. | Western blots demonstrating interaction of exogenously added GST-11 but not GST with cellular Grb2 in the GST protein pull down assays. | 62 |
| Figure 3.6. | Western blots of anti-Grb2-immunoprecipitated samples in the presence of exogenously added GST or GST-11. | 63 |
| Figure 3.7. | Western blots of anti-Grb2-immunoprecipitated samples in the absence or presence of exogenously added GST or GST-11. | 66 |

| | | |
|---|---|---|
| Figure 3.8. | Western blots of anti-Grb2-immunoprecipitated samples in COS-7 cells transfected with pEGFP-C2 or pEGFP-11. | 67 |
| Figure 3.9. | Western blots of anti-Grb2-immunoprecipitated samples in COS-7 cells transfected with pEGFP-C2 or pEGFP-11. | 70 |
| Figure 3.10. | Far western blot and western blots demonstrating differential strength of interaction of GST-11 wt/mutant proteins with GST-HA-Grb2. | 71 |
| Figure 3.11. | Western blots demonstrating differential strength of interaction of exogenously added GST-11 wt/mutant proteins with cellular Grb2 in a GST protein pull down assay. | 74 |

# LIST OF ABBREVIATIONS

| | |
|---|---|
| A | adenine |
| $A_{260}$, $A_{280}$, or $A_{562}$ | absorbance at 260, 280, or 562 nm |
| aa | amino acid |
| AAV | adeno-associated virus |
| Ab | antibody |
| Ad | adenovirus |
| Ala | alanine |
| Amp | ampicillin |
| ASFV | African swine fever virus |
| ATCC | American Type Culture Collection |
| ATP | adenosine 5'-triphosphate |
| BCA | bicinchroninic acid |
| βME | β–mercaptoethanol |
| BPB | bromophenol blue |
| BPV | bovine papilloma virus |
| BSA | bovine serum albumin |
| C | cytosine |
| $CaCl_2$ | calcium chloride |
| cdc | cell division cycle mutant |
| cDNA | complementary DNA |
| $CH_3COOK$ | potassium acetate |
| Ck | chemokine |
| CkR | chemokine receptor |
| CMV | cytomegalovirus |
| CNS | central nervous system |
| $CO_2$ | carbon dioxide |
| CPSF | cleavage and polyadenylation specificity factor |
| CPV | canine parvovirus |
| crm | cytokine response modifier |

| | |
|---|---|
| CTL | cytotoxic T-lymphocyte |
| dATP | deoxy-ATP |
| ddGTP | dideoxy-GTP |
| DEAE | diethylaminoethyl |
| $dH_2O$ | distilled water |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| dNTPs | deoxy-NTPs |
| ds | double-stranded |
| EBNA | EBV nuclear antigen |
| EBV | Epstein-Barr virus |
| ECL | enhanced chemiluminescence |
| EDTA | ethylene-diamine tetra-acetic acid |
| EGF | epidermal growth factor |
| EGFP | enhanced green fluorescent protein |
| EGFP-11 | enhanced green fluorescent protein-11kDa fusion |
| EI | erythema infectiosum |
| ER | endoplasmic reticulum |
| ERK | extracellular signal-regulated kinase |
| FasL | Fas ligand |
| FBS | fetal bovine serum |
| FLICE | Fas-associated death domain-like ICE |
| FLIP | FLICE-inhibitory protein |
| FPV | feline parvovirus |
| FW | far western blot |
| g | gram |
| G | guanine |
| GDP | guanosine diphosphate |
| GEB | glutathione elution buffer |

| | |
|---|---|
| GEF | guanine nucleotide exchange factor |
| GFP | green fluorescent protein |
| gp | glycoprotein |
| Grb2 | growth factor receptor-binding protein 2 |
| GS or HS | goat or horse serum |
| GSH | glutathione |
| GST | glutathione S-transferase |
| GST-11 | glutathione S-transferase-11kDa fusion |
| GTP | guanosine 5'-triphosphate |
| HIV | human immunodeficiency virus |
| $H_2O$ | water |
| HA | hemagglutinin |
| HCOOH | formic acid |
| HCV | hepatitis C virus |
| HEK | human embryonal kidney |
| HEPES | (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) |
| HHV | human herpesvirus |
| hnRNP | heteronuclear ribonucleoprotein |
| HPV-2 | human parvovirus |
| HRP | horse radish peroxidase |
| HSV | herpes simplex virus |
| hSos | human Sos |
| HTLV | human T-lymphotropic virus |
| IAP | inhibitor of apoptosis protein |
| ICE | interleukin-1β-converting enzyme |
| ICP | infected cell protein |
| IE | immediate early |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |

| | |
|---|---|
| IMP | inflammation modulatory protein |
| IN | integrase |
| IP | immunoprecipitation |
| IPTG | isopropylthio-β-D-galactoside |
| ITR | inverted terminal repeat |
| Kan | kanamycin |
| kb | kilobase |
| kDa | kilodalton |
| KLH | keyhole limpet hemacyanin |
| KSHV | Kaposi's Sarcoma-associated herpesvirus |
| LB | Luria-Bertani |
| LMP | latent membrane protein |
| MA | matrix |
| MAC | membrane attack complex |
| MAPK | mitogen-activated protein kinase |
| MCS | multiple cloning site |
| MCV | molluscum contagiosum virus |
| MEK | MAPK/ERK kinase |
| mg | milligram |
| μg | microgram |
| $MgCl_2$ | magnesium chloride |
| $MgSO_4$ | magnesium sulfate |
| MHC | major histocompatibility complex |
| MHV | murine γ-herpesvirus |
| MIP | macrophage inflammatory protein |
| ml | milliliter |
| μl | microliter |
| mM | millimolar |
| mm | millimeter |
| mRNA | messenger RNA |

| | |
|---|---|
| mSos | murine Sos |
| mu | map unit |
| MVM | minute virus of mice |
| MW | molecular weight |
| NaCl | sodium chloride |
| NAK | nef-associated kinase |
| NaOH | sodium hydroxide |
| NES | nuclear export signal |
| ng | nanogram |
| NK | natural killer |
| NLS | nuclear localization signal |
| nm | nanometer |
| NS | nonstructural |
| nt | nucleotide |
| NTP | nucleoside triphosphate |
| OD | optical density |
| ORF | open reading frame |
| OS | oligoadenylate synthetase |
| P(number)A | proline-to-alanine mutation at specified amino acid position |
| p6 | promoter at map unit 6 |
| PABII | poly(A)-binding protein II |
| PAGE | polyacrylamide gel electrophoresis |
| PAK | p21-activated protein kinase |
| PAP | poly(A) polymerase |
| PBS | phosphate-buffered saline |
| PBS-T | PBS supplemented with 0.05% Tween-20 |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| PIC | pre-integration complex |
| PKR | dsRNA-dependent protein kinase |

| | |
|---|---|
| pmol | picomole |
| PMSF | phenylmethylsulfonyl fluoride |
| PNK | polynucleotide kinase |
| poly(A) | polyadenylated |
| PP-II | polyproline type II |
| PrCl | preclear |
| pRB | retinoblastoma protein |
| Pro | proline |
| PSB | protein sample buffer |
| PTMV | pig-tailed macaque virus |
| PVDF | polyvinylidene fluoride |
| RF | replicative form |
| RMV | rhesus macaque virus |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| RRE | rev-responsive element |
| $sdH_2O$ | sterilized distilled water |
| SDM | site-directed mutagenesis |
| SDS | sodium dodecylsulfate |
| SFV | shope fibroma virus |
| SH | Src homology |
| SMP | skim milk powder |
| snRNA | small nuclear RNA |
| Sos | son of sevenless |
| SPV | simian parvovirus |
| ss | single-stranded |
| STET | sucrose-Triton-EDTA-Tris |
| SV40 | simian virus 40 |
| T | thymine |
| TAC | transient aplastic crisis |

| | |
|---|---|
| TAE | tris-acetate-EDTA |
| TBE | tris-borate-EDTA |
| TNF | tumor necrosis factor |
| TNFR | TNF receptor |
| tris | tris[hydroxymethyl]aminomethane |
| U | unit |
| UTR | untranslated region |
| UV | ultraviolet |
| vEGF | viral EGF homolog |
| VETF | vaccinia virus early transcription factor |
| v-FLIP | viral FLIP homolog |
| VGF | vaccinia growth factor |
| Vif | virion infectivity protein factor |
| vIL | viral IL homolog |
| vMIP | viral MIP homolog |
| VP | viral protein (structural) |
| Vpr | viral protein R |
| Vpu | viral protein U |
| VSV | vesicular stomatitis virus |
| vTNFR | viral TNFR homolog |
| WB | western blot |
| wt | wild-type |
| $Zn^{++}$ | zinc ion |

# ACKNOWLEDGMENTS

I would like to express my gratitude to the people who have helped me through my studies and who have been providing support and encouragement through my family crisis. I am forever indebted to Dr. Caroline Astell, who gave me this opportunity to train, and to Dr. Cynthia Shippam-Brett, who taught me my first lessons in molecular biology at the bench. Special thanks go to members of the Astell lab, past and present, for being wonderful friends and for being there when I need you (to answer a question, to listen to my nonsensical babbles, and so on). Many thanks to members of my committee Dr. Roger Brownsey, Dr. Shoukat Dedhar, and Dr. Alice Mui, for their continual guidance and useful suggestions. Also thank you to all my other friends in the department--without you my life would have been that much less enjoyable...

I would also like to thank my family for the love and support they've shown. Last but not least, I'd like to thank the Almighty for placing me on this path and for giving me love, hope, and strength. I have not been your best pupil but I try. May I live to see the day my father will recover and be able to appreciate the contents of this thesis. Amen.

# INTRODUCTION

## 1.1. Taxonomy

Parvoviruses are small eukaryotic viruses (diameter ranges from 18-25nm) with linear, single-stranded DNA (ssDNA) genomes roughly 5kb long. These non-enveloped viruses have a T=1 icosahedral protein capsid coat which protects against inactivation by moderate pH changes, high temperatures, high salt concentrations and lipid solvent extraction. The members of the family *Parvoviridae* are classified according to sequence similarity, genome organization, host range, and requirement for co-infection by a helper virus (Pringle, 1993). The family consists of two subgroups, the *Densovirinae* and the *Parvovirinae*. While the former infects arthropods, members of *Parvovirinae* infect vertebrate organisms and can in turn be subdivided into three genera: the *Dependoviruses*, *Parvoviruses*, and *Erythroviruses* (reviewed in Berns, 1996; Young, 1996; Astell, 1999a; Astell, 1999b).

### 1.1.1. Dependoviruses

While all members of *Parvoviridae* require cellular functions associated with S phase of the cell cycle to replicate successfully, *Dependoviruses* such as adeno-associated virus (AAV) further require co-infection by a helper virus (adenovirus or herpesvirus) in order to establish a productive infection. The requirement appears to be more for an altered cellular environment than for specific functions associated with the helper virus, as it has been shown that treatment of cells by various chemicals or UV irradiation can replace the need for a helper virus (Yakobson *et al.*, 1987; Yakobson *et al.*, 1989). In the absence of a helper virus, infection by AAV results in site-specific integration of the AAV genome into the host genome (Kotin *et al.*, 1990). AAV replication is initiated upon subsequent infection by adenovirus or herpesvirus. (An exception is AAV5, a recently discovered member of the family that can replicate in the absence of a helper virus.)

Table 1.1. Selected *Parvoviridae* and their target host species.

- *Parvoviridae*
  - *Densovirinae* (invertebrate)
    - *Contravirus*
    - *Densovirus*
    - *Iteravirus*
  - *Parvovirinae* (vertebrate)
    - *Dependovirus*
      - Adeno-Associated Virus (most animals)
      - Avian Adeno-Associated Virus (bird)
      - Bovine Adeno-Associated Virus (cattle)
    - *Parvovirus*
      - Aleutian Mink Disease Virus (mink)
      - Bovine Parvovirus (cattle)
      - Canine Parvovirus (dog)
      - Feline Panleukopenia Virus (cat)
      - Goose Parvovirus (goose)
      - H1 Virus (rodent)
      - Kilham Rat Virus (rat)
      - LuIII (unknown)
      - Minute Virus of Canines (dog)
      - Minute Virus of Mice (mouse)
      - Mouse Parvovirus (mouse)
      - Hamster Parvovirus (hamster)
      - Mink Enteritis Virus (mink)
      - Porcine Parvovirus (pig)
    - *Erythrovirus*
      - B19, Human Parvovirus-2 (human)
      - V9 Parvovirus (human)
      - Pig-Tailed Macaque Virus (monkey)
      - Rhesus Macaque Virus (monkey)
      - Simian Parvovirus (monkey)
      - Chipmunk Parvovirus (chipmunk)

Infection by *Dependoviruses* produces no known pathological symptoms. Hence viruses such as AAV are attractive for use as vectors in gene therapy. AAV not only integrates site-specifically into the host genome; it does so in both dividing and senescent cells, without causing cellular transformation in the infected cell.

### 1.1.2. Parvoviruses

In contrast to the *Dependoviruses*, members of the *Parvoviruses* and *Erythroviruses* are capable of replicating autonomously, in the absence of any helper viruses. *Parvoviruses* infect a wide range of domestic animals as well as several murine species. Because successful replication of *Parvoviruses* requires progression of the host cell into the S phase, cells that are rapidly dividing are the most susceptible to infection. These include cells of the hematopoietic system, epithelial cells lining the gastrointestinal tract, as well as fetal or neonatal cells. Correspondingly, *Parvovirus* infection in newborn domestic animals and livestock are frequently lethal, leading to significant financial consequences.

### 1.1.3. Erythroviruses

While members of *Erythroviruses* share some common characteristics with the *Parvoviruses*, they differ from each other in genome organization and in specific host range. These viruses appear to require certain transcription factors or other conditions unique to progenitor cells of the erythroid lineage in mammals and therefore display a tropism towards red cell precursors in humans and other primate and rodent species.

Among the *Parvovirinae*, *Erythrovirus* is the most recently discovered genus. Currently, the genus is comprised of six members: simian parvovirus (SPV) (O'Sullivan *et al.*, 1994; Brown *et al.*, 1995a), rhesus macaque virus (RMV) (Green *et al.*, 2000), pig-tailed macaque virus (PTMV) (Green *et al.*, 2000), chipmunk parvovirus (Yoo *et al.*, 1999), V9 (Nguyen *et al.*, 1999), and B19 (also known as human parvovirus or HPV-2) (Cossart *et al.*, 1975).

## 1.2. The Erythrovirus B19

The B19 parvovirus was first discovered by Yvonne Cossart in a screen for hepatitis in blood donors (Cossart *et al.*, 1975; structure reported in Agbandje *et al.*, 1994) and is the only member of the *Parvoviridae* known to cause disease in humans (reviewed in Young, 1995; Rogers, 1999; molecular aspects reviewed in Astell *et al.*, 1997). Roughly 45% of children test seropositive for antibodies against the virus, and the antibody prevalence rises in an age-dependent manner up to about 70% in the adult population (Gay *et al.*, 1994), indicating infection during childhood. There are no known animal or insect vectors for B19, and viral transmission can take place through respiratory (foamites and aerosol droplets), oral-fecal, blood-borne, and transplacental routes.

The tropism of B19 parvovirus for human erythroid progenitor cells appears to be regulated at multiple levels. One determinant is the presence of the blood group P antigen, which has been identified as the cell surface receptor for B19 parvovirus (Brown *et al.*, 1993). The virus binds with high affinity and specificity to the carbohydrate moiety of the P antigen, also known as globoside or tetrahexose ceramide. Cells lacking the P antigen are resistant to B19 infection (Brown *et al.*, 1994a). While the P antigen is expressed in a variety of cell types including mature erythrocytes, erythroid progenitors, megakaryocytes and platelets, endothelial cells, and fibroblasts (von dem Borne *et al.*, 1986), only bone marrow cells expressing the erythrocyte P antigen are permissive to B19 infection (Kerr *et al.*, 1995). In non-permissive cells, it has been proposed that the block in replication may be mediated in part by an inhibition of transcript maturation (Liu *et al.*, 1992). Other studies suggest that the 3′ untranslated region (3′ UTR) of the mRNAs encoding B19 structural proteins may block their own translation, thereby inhibiting viral replication (Pallier *et al.*, 1997). Moreover, Brunstein and co-workers (Brunstein *et al.*, 2000) have identified a novel splicing pattern for B19 transcripts in semi-permissive cells which may lead to a translational block of structural protein expression. Their findings implicate differences in host cell splicing machinery as one determinant of B19 tropism. In other studies, the presence of as yet

uncharacterized transcription factors in erythroid progenitor cells has been implicated in the restriction of B19 replication to these cells. The B19 p6 promoter cannot function in non-erythroid cells (Kurpad *et al.*, 1999) but is able to direct erythroid cell-specific expression in infected cells when cloned into a recombinant AAV-2 virus (Wang *et al.*, 1995).

In permissive erythroid progenitor cells, entry of the virus is believed to be mediated by endocytosis via clathrin-coated pits, independent of cell cycle progression. B19 DNA is detected in the nucleus two days following infection (Morey *et al.*, 1993), and the virus proceeds to induce apoptosis in the infected cell (Moffatt *et al.*, 1998). The cessation of erythropoiesis leads to a deficiency in reticulocytosis and a decrease in red cell count (hematological consequences of B19 infection reviewed in Brown, 2000). Despite similar pathological alterations, clinical manifestations of B19 infection are highly variable and dependent on the hematological and immunological state of the infected host. In some cases, infection is asymptomatic or the symptoms may be sub-clinical. In the majority of cases, however, B19 infection is typically associated with mild pyrexia and flu-like illness within the first week, concurrent with viremia and a depression in red cell count.

This occurrence is usually transient and not problematic in healthy individuals. However, B19 infection becomes clinically significant and may even be life-threatening in certain groups of patients, namely in patients with an abnormality in red cell function or with a weakened immune system.

## 1.2.1. B19 Infection and Transient Aplastic Crisis

B19 infection can become life-threatening in patients with an underlying hematological disorder (eg. sickle cell anemia) (Pattison *et al.*, 1981; Rao *et al.*, 1992; Serjeant *et al.*, 1993), with a pre-existing hemolytic condition (eg. β-thalassemia, hereditary spherocytosis) (Kelleher *et al.*, 1983; Davidson *et al.*, 1984; Goss and Szer, 1997), or under erythroid stress (eg. iron deficiency, hemorrhage) (Kojima *et al.*, 1988; Kamoshita *et al.*, 1993). In these individuals, because they have a low red cell count or shortened red cell half-life to begin with, a cessation in red blood cell production resulting from B19 infection may lead to a life-threatening transient aplastic crisis (TAC) (Young *et al.*, 1984; Chorba *et al.*, 1986). While this hematological condition is ultimately self-limiting, the resulting anemia may be so severe as to not only cause fatigue but possibly precipitate congestive heart failure and/or cerebrovascular accidents as well. The anemic condition arising from TAC may be resolved by blood transfusion.

## 1.2.2. Persistent B19 Infection

In individuals with congenital or acquired immunodeficiencies (eg. from HIV infection, bone marrow or solid organ transplant procedure, chemotherapy treatment, etc.), B19 establishes a chronic infection due to the inability of the host to clear the virus, thus leading to chronic red cell aplasia (Neild *et al.*, 1986; Kurtzman *et al.*, 1988; Coulombel *et al.*, 1989; Frickhofen and Young, 1989; Graeve *et al.*, 1989; Weiland *et al.*, 1989; Frickhofen *et al.*, 1990; Bertoni *et al.*, 1995). Analysis of B19-specific antibodies in these individuals indicates a persistence of the IgM antibody and a lack of seroconversion of the IgG antibody from the anti-major capsid species to the anti-minor capsid species. Immunocompromised individuals with chronic hypoplastic anemia resulting from persistent B19 infection may be treated by immune globulin therapy (Kurtzman *et al.*, 1989).

## 1.2.3. B19 Infection *In Utero*

The B19 parvovirus is able to cross the placenta. Frequency of maternal infection with B19 is estimated at 1 in roughly every 400 pregnancies (Gay *et al.*, 1994), of which 9% of the cases result in fetal death (Gay *et al.*, 1994; Public Health Laboratory Service Working Party of Fifth Disease, 1990). Incidence of fetal loss is comparable in normal pregnancies and in pregnancies complicated by B19 infection during the first trimester, while the risk for spontaneous abortion increases substantially in cases of infection during the second trimester. Many more cells in the fetus are rapidly dividing and hence susceptible to B19 infection, and the severe anemia resulting from intrauterine infection with B19 could cause death of the fetus in a condition called non-immune hydrops fetalis (Brown *et al.*, 1984; Anand *et al.*, 1987; Anderson L.J., 1990; Wright *et al.*, 1996). The anemia could also cause hypoxia leading to severe developmental defects in the central nervous system (CNS) if the fetus survives to term (Torok, 1995). Surviving neonates would also be expected to develop immune tolerance to B19 capsid proteins as a result of exposure of the fetal immune system to the virus early in pregnancy.

## 1.2.4. B19 Infection in the Immunocompetent Host

Following infection, B19 propagates itself in infected erythroid progenitor cells leading to the transient depression in red cell count at the peak of viremia during the first week of infection. In the immunocompetent host who has not had a previous B19 infection, virus can be detected from days 5-6 post-infection onwards, reaching maximum levels at days 8-9 (serum titer can reach as high as $10^{11}$ viral particles per ml). Viremia is accompanied by non-specific flu-like symptoms such as headache, myalgia, and chills resulting from the action of various inflammatory cytokines. The host then launches an IgM-based immune response leading to the formation of immune complexes which are responsible for the secondary symptoms of B19 infection observed by about 15 days post-infection.

In children and adults alike, the neutralizing IgM response may coincide with the onset of a rubella-like rash, characterized by maculopapular cutaneous

eruptions on the cheeks, trunks, and extremities. This condition is known as "fifth disease" (as it is the 5th of 6 classical childhood exanthema) or erythema infectiosm (E.I.; Anderson M.J. *et al.*, 1983; Chorba *et al.*, 1986). E.I. symptoms typically resolve within a few days or could recur over a period of several weeks.

About half of B19-infected adults (especially women) may experience arthralgias and develop arthritis-like complications in the small joints of the hands and feet (Reid *et al.*, 1985; White *et al.*, 1985; Ueno *et al.*, 1993), which normally resolve within several weeks but have been documented to last as long as two years.

Both types of inflammatory responses are manifested secondary to immune complex formation, such that by the time the patients present to medical attention the period of viremia has usually passed. B19-specific IgG expression is also increased following viremia, at around 2 weeks following infection, and is essential for neutralization of the virus (Anderson M.J. *et al.*, 1985; Anderson L.J. *et al.*, 1986). The IgG antibody, which persists for life, is a convenient marker of past exposure to B19 and confers a strong and durable protective effect against recurrent infection by B19. The first wave of seroconversion usually occurs among school-age children; a second wave is observed among parents of school-age children if they had failed to seroconvert beforehand.

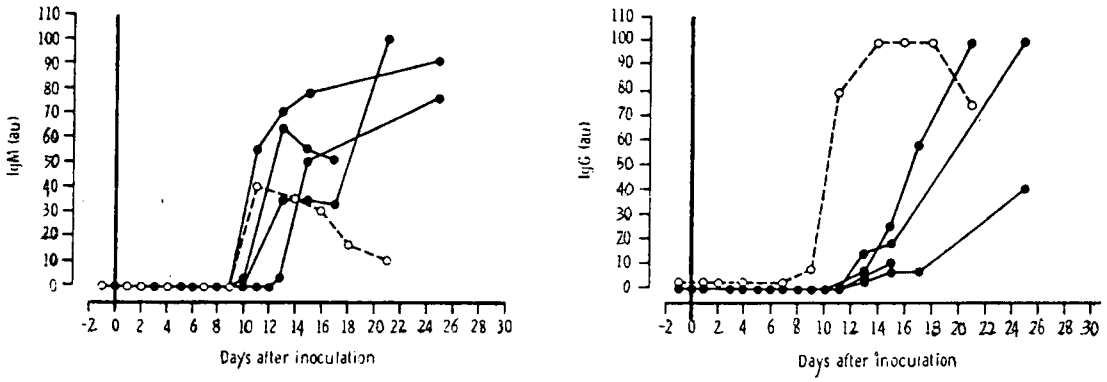


Figure 1.2.4.1. Development of B19-specific IgM (left) and IgG (right) after experimental B19 inoculation of seronegative volunteers (closed circles) and a volunteer with trace amounts of IgG to B19 (open circles). (Figure taken from Anderson M.J. *et al.*, 1985.)

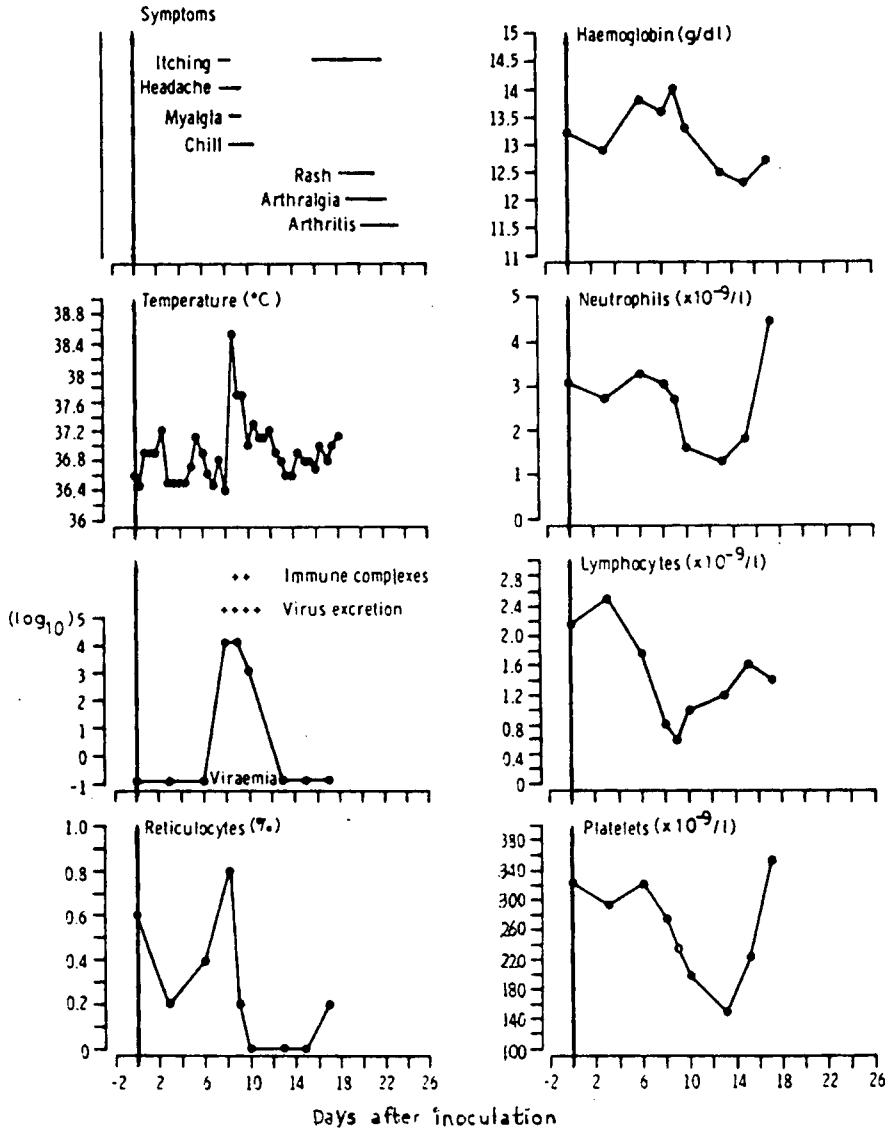


Figure 1.2.4.2. Time course of experimental inoculation of Parvovirus B19 in a seronegative host. (Figure taken from Anderson M.J. *et al.*, 1985.)

## 1.2.5. B19 and Other Disorders

A number of disorders not described above have also been associated with or attributed to B19 infection. These are summarized in Table 1.2.5 below.

Table 1.2.5. Other disorders associated with parvovirus B19 infection.

Cardiovascular manifestations
- Acute congestive heart failure
- Myocarditis
- Pericarditis

Cutaneous manifestations
- Peripheral edema
- Vascular purpura
- Vesicular lesions

Hematological disorders
- Aplastic anemia
- Autoimmune hemolytic anemia
- Chronic neutropenia
- Idiopathic thrombocytopenic purpura

Hepatobiliary tract disorders
- Acute hepatic sequestration
- Fulminant liver failure

Neurological diseases
- Coma
- Seizure
- Sensorineural abnormalities

Renal diseases
- Acute renal failure
- Nephrotic syndrome

Rheumatic diseases
- Juvenile rheumatoid arthritis
- Rheumatoid arthritis
- Vasculitis

## 1.3. B19 in Cell Culture

The species tropism of B19 parvovirus is retained in cell culture. B19 infects and replicates in burst forming erythroid progenitors (BFU-E) as well as in late erythroid progenitor cells (Mortimer *et al.*, 1983). When cultured in the presence of erythropoietin, B19 will also replicate in erythroid progenitor cells from human bone marrow (Ozawa *et al.*, 1986; Ozawa *et al.*, 1987b; Srivastava and Lu, 1988),

human fetal liver (Yaegashi *et al.*, 1989; Brown *et al.*, 1991; Westmoreland and Cohen, 1991), and normal peripheral blood (Serke *et al.*, 1991), as well as in cells from patients with chronic myelogenous leukemia (CML) or erythroleukemia (Takahashi *et al.*, 1989). Additionally, human megakaryoblastoid cells will also support low levels of B19 replication after being adapted to growth in the presence of erythropoietin (Shimomura *et al.*, 1992; Munshi *et al.*, 1993).

The B19-infected primary culture systems described thus far are not readily adaptable to laboratory studies, and to date no known continuous permissive cell line for propagating the virus has been identified. The use of transfected cell systems has aided in the elucidation of various molecular and cellular aspects of B19 replication and transcription. Viral transcripts and proteins are generated in HeLa cells transfected with plasmids containing part or almost all of the B19 genome (Doerig *et al.*, 1987; Ozawa *et al.*, 1988a; Doerig *et al.*,1990) and in COS-7 cells transfected with B19/SV40 hybrid vectors (Beard *et al.*, 1989). A full-length clone containing the entire B19 genome has also been generated (Deiss *et al.*, 1990), though it has not been shown to be infectious.

## 1.4. The B19 Genome

In contrast to other parvoviruses which package predominantly negative-sense ssDNA, B19 and AAV package their genome as both positive- and negative-sense ssDNA in separate particles in a one-to-one ratio. The 5112nt genome of B19 parvovirus is arranged as a single-stranded coding region flanked on either end by a palindromic inverted terminal repeat (ITR) sequence. The ITR consists of 383nt of which 365nt are folded into a duplex hairpin structure (Deiss *et al.*, 1990), providing a free 3'-hydroxyl group that is essential for initiation of DNA synthesis and replication of the viral genome (Astell, 1990) which take place in the nucleus.

The currently accepted model for parvoviral replication (reviewed in Berns, 1990; Berns, 1996) stipulates that the viral ssDNA genome monomer is first converted into a double-stranded DNA (dsDNA) monomer, referred to as the replicative form (RF). This conversion into the RF requires a functional host DNA

polymerase, while the viral nonstructural (NS) protein becomes essential in subsequent stages of the replicative cycle. The dsDNA RF monomer is converted into a dimer intermediate which is then resolved into two monomer RF molecules. From these RF monomers, viral progeny ssDNA strands (both positive- and negative-sense) are generated and packaged into capsids in the nucleus, and assembled virions are released from the infected cell upon lysis.

Unlike some insect densoviruses which encode proteins from opposite strands of the genome, B19 produces proteins from one strand only. The coding strand, by definition the plus strand, contains two large and one smaller open reading frames (ORFs). In contrast to other parvoviruses, B19 produces all of its transcripts from a single promoter located at map unit 6 (p6) (Blundell *et al.*, 1987; Doerig *et al.*, 1987). At least nine different transcripts are known to be generated from the three open reading frames (Figure 1.4), through the extensive use of alternate splicing and end processing mechanisms (Ozawa *et al.*, 1987a; Beard *et al.*, 1989; St. Amand *et al.*, 1991; Luo and Astell, 1993). All transcripts in permissive cell lines have a 56nt leader sequence and are 3'-end processed (polyadenylated) either in the middle or at the far right end of the genome. The open reading frame on the left gives rise to the nonstructural (NS) proteins, while the structural capsid proteins (viral proteins VP1 and VP2) are encoded by the open reading frame spanning the right half of the genome (Ozawa *et al.*, 1988b). ). Differential levels of transcript production are observed in non-permissive and permissive cells; VP transcripts predominate in B19-infected cells (Brown *et al.*, 1994b), while NS transcripts accumulate in HeLa cells transfected with a B19-based plasmid due to a functional block in transcription towards the middle of the genome(Liu *et al.*, 1992). In addition to the transcripts encoding the NS and VP proteins, B19 also produces two classes of abundant small mRNAs encoding proteins with molecular weights of 7.5kDa and 11kDa (St. Amand *et al.*, 1991; St. Amand and Astell, 1993; Luo and Astell, 1993).

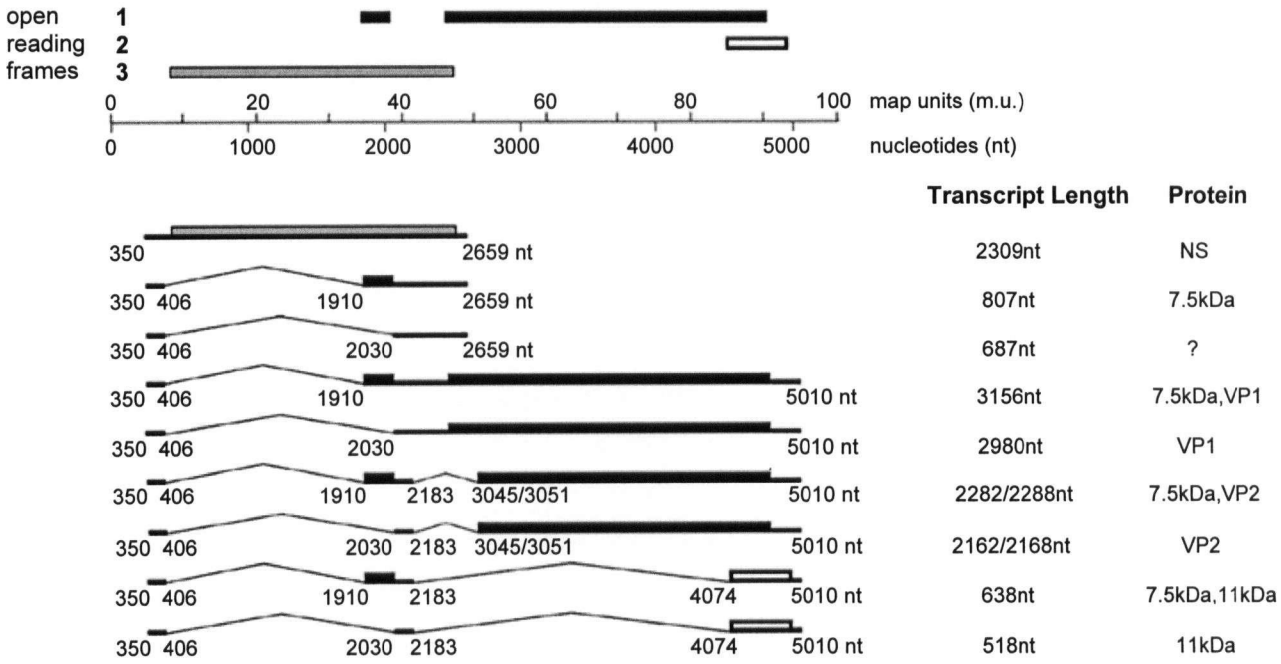


Figure 1.4. Transcription and coding map of Parvovirus B19.

## 1.4.1. The B19 Nonstructural Proteins

The full-length NS protein translated from the 2309nt transcript has a molecular weight (MW) of ~70kDa (Cotmore *et al.*, 1986; Ozawa and Young, 1987). There are, however, several lower molecular weight species, and it is not clear whether these represent degradation products or whether they arise from post-translational modifications of the full-length protein. The functions of NS proteins are highly conserved among the different members of *Parvoviridae*. In a manner analogous to the NS1 protein of the minute virus of mice (MVM, a prototype member of the genus *Parvovirus*) and the Rep proteins of AAV2, the NS protein of B19 erythrovirus effects transactivation of the p6 promoter (Doerig *et al.*, 1990) and is likely indispensable for initiation of viral replication through its associated helicase/ATPase and presumed site-specific endonuclease activities. Recent evidence also suggests that the B19 NS protein mediates cytotoxicity by induction of apoptosis in infected erythroid cells (Moffatt *et al.*, 1998).

## 1.4.2. The B19 Structural Proteins

Capsid proteins VP1 and VP2 are translated from overlapping open reading frames. They share an identical amino acid sequence except for 227 additional amino acid residues at the amino-terminus of VP1 which are absent from the VP2 sequence. The capsid coat of a native B19 particle is composed of 60 capsomers of VP1 and VP2, of which VP2 is the predominant species. The shorter VP2 protein (58kDa) comprises about 95% of the capsid coat and is termed the "major" structural protein, while the longer VP1 protein (83kDa) accounts for about 5% and is referred to as the "minor" capsid species. It has been shown by other investigators that VP2 protein produced from a baculovirus expression system in insect cells is capable of self-assembly into viral particles, with or without VP1, while VP1 does not self-assemble in the absence of VP2 (Kajigaya *et al.*, 1991). In addition to forming the capsid to house the viral genome, structural proteins may also be important in determination of host range and tissue specificity of parvoviruses. Recent evidence points to VP1 and VP2 as being at least partly

responsible for the tissue tropism of different strains of MVM (Spalholz & Tattersall, 1983) as well as for host range differences between canine and feline parvoviruses (CPV and FPV, respectively; Parrish, 1991). Immunodominance studies suggest that the major neutralization epitopes in the viral capsid reside in the unique amino-terminal portion of the VP1 protein (Saikawa *et al.*, 1993; Brown *et al.*, 1995b), which is believed to protrude from the surface of the viral particle (Rosenfeld *et al.*, 1992).

### 1.4.3. The B19 11kDa Proteins

The 11kDa proteins are produced from the third, smaller open reading frame in the B19 genome. Due to the use of alternate start codons, three related proteins of similar molecular weight are translated from both the 638nt and 518nt mRNAs (see Figure 1.4) (St. Amand *et al.*, 1991; St. Amand and Astell, 1993). A functional role for these small proteins in the B19 life cycle has yet to be identified. Other viruses have been shown to encode gene products which modulate the host immune response and evade the immune surveillance system (reviewed in Spriggs, 1994; McFadden, 1995; Spriggs, 1996; Barry and McFadden, 1997; Ploegh, 1998; Spriggs, 1999; Lalani and McFadden, 1999; Lalani *et al.*, 2000), to prevent premature apoptosis of the infected cell, or to induce apoptosis after a productive infection has been established (reviewed in Shen and Shenk, 1995; Teodoro and Branton, 1997; Roulston *et al.*, 1999).

The B19 11kDa proteins do not appear to be related in sequence to any other known protein, nor do other members of the *Parvoviridae* produce them, though a comparable ORF with three regions of homology exists in the SPV genome. Absence of 11kDa expression has not been shown to affect either viral capsid assembly (Cohen *et al.*, 1995) or expression of other viral proteins (St. Amand, 1992). The 11kDa proteins do not appear to have any region corresponding to transmembrane domains, DNA-binding domains, or $Zn^{++}$-binding domains, and immunoprecipitation experiments have not shown them to interact with other virally encoded proteins. A region has been identified as a putative transactivation motif, though previous experiments have failed to demonstrate any such activity

from the 11kDa proteins (W. Luo and C.R. Astell, unpublished results). Although several potential target phosphorylation sites for protein kinase C and casein kinase II have been identified, there is no evidence to suggest that the 11kDa proteins are phosphorylated *in vivo*. Analysis of the amino acid sequence of the 11kDa proteins indicates an unusually high proportion of proline residues; 14 out of 94 residues or 15%. The proline residues can be grouped into three regions which share similarity with certain sequences involved in mediating protein-protein interactions, thus leading to the speculation that the 11kDa proteins may be involved in binding to other proteins via their proline-rich regions.

## 1.5. Review of Relevant Signal Transduction Concepts and Pathways

In order for an organism to be viable, each cell must perform specific functions at specific times. Co-ordination of behavior in different cells must be exact and occurs through a concerted series of actions cued by various signals from outside or inside the cell. A large number of signals are transduced to the interior of a cell by a relatively small number of signaling molecules, and specificity of signaling is achieved through the assembly of selected signaling molecules at specific times and locations. Signals are propagated through a series of molecules, with each level or "node" subject to signaling divergence or convergence to or from other signaling networks. Proteins interact with each other through various means, including the use of specialized modular domains which were first identified as regions of high sequence homology in signaling molecules. Subsequently, these domains were found to belong to a growing assortment of non-catalytic folding protein modules whose primary role is to mediate moderate to high affinity binding with other proteins.

Prototypical protein binding domains include the Src homology (SH) domains, first identified within the signaling molecule and non-receptor protein tyrosine kinase Src. SH2 domains mediate inducible, specific high affinity binding to phosphotyrosine residues on target proteins, while SH3 domains are involved in

largely constitutive, moderate affinity binding to proline-rich sequences. The latter acts more as a general recruitment type of mechanism than as a molecular on/off switch.

One example of a signaling protein which contains these types of domains is the growth factor receptor binding protein (Grb2; Downward, 1994), with two SH3 domains flanking a single SH2 domain. This protein belongs to a class of "adaptor" molecules which possess no intrinsic enzymatic activity and are believed to function solely to bring one protein into close proximity with another protein so that the signal can be propagated.

In the case of the signaling network involving Grb2, a paradigm pathway involving receptor tyrosine kinases has been well characterized. A signal originating from outside the cell in the form of a growth factor (for example epidermal growth factor or platelet-derived growth factor) is transmitted to its cell surface receptor. The receptor typically traverses the plasma membrane and either possesses intrinsic tyrosine phosphorylation activity or is associated with a non-receptor tyrosine kinase which mediates self-phosphorylation on critical tyrosine residues on the cytoplasmic tail upon ligand stimulation. The phospho-tyrosine residues then serve as docking sites for SH2 domain-containing proteins such as Grb2, which then recruits another protein, son of sevenless (Sos), to the plasma membrane through SH3 interactions with proline-rich regions or "ligands" on Sos. Sos is a guanine nucleotide exchange factor (GEF) which, when brought into close vicinity to membrane-bound GTPase Ras, is able to exchange GDP for GTP on Ras to activate it.

Activated Ras is responsible for propagating the signal via a series of phosphorylation events through downstream kinase effectors Raf1, MAPK/ERK kinase (MEK), and mitogen-activated protein kinase (MAPK) (reviewed in Guan, 1994; Margolis and Skolnik, 1994; Garrington and Johnson, 1999). Phosphorylated MAPK is translocated from the cytoplasm to the nucleus, where it exerts its effects by phosphorylating target proteins on serine residues. A number of target proteins have been identified, including the transcription factor Elk-1 which controls expression of c-fos. C-fos is known to form the AP1 complex in conjunction with c-

jun, which in turn regulates expression of various genes involved in cell cycle progression and proliferation. In this way, a signal initiated outside the cell is transmitted with high fidelity to the interior of the cell and is capable of eliciting a highly specific response within the cell.

## 1.6. Review of Functions of Other Viral Nonstructural Proteins

In order to maximize chances of survival and propagation, viruses have evolved numerous mechanisms to manipulate the host environment for their own benefit, through interactions with viral or host cell components and alterations in host cell signaling events. At this point, it is useful to review the functions of other viral nonstructural proteins in order to better understand the possible functional roles that the B19 11kDa proteins may play in the viral replicative cycle.

### 1.6.1. Virus Entry

Viral entry into the host cell is typically mediated by interactions of viral capsid or envelope proteins with receptors on the host cell membrane. Recent findings point towards a role for nonstructural proteins in enhancing the process of intracellular delivery of virions into the infected cell. In the case of retrovirus human immunodeficiency virus type 1 (HIV-1), it has been well established that viral entry takes place by fusion, though increasing evidence suggests endocytosis may also play a role. This process is CD4- and chemokine receptor-dependent and appears to be enhanced by the HIV-1 accessory protein Nef, which is believed to carry out a number of other additional functions that contribute to increased viral infectivity (to be discussed further) (Schaeffer *et al.*, 2001).

### 1.6.2. Nuclear Targeting and Replication of Viral Genome

The *Rhabdoviridae* vesicular stomatitis virus (VSV) expresses a small, basic C′ protein which functions to enhance viral replication. The C′ protein increases production of full-length genomic RNAs by stabilizing the viral genome/transcriptase complex (Peluso *et al.*, 1996).

Poliovirus encodes a nonstructural polyprotein, 2BC, which is also involved in viral replication. Sites of genome replication have been localized to membrane vesicles whose formation is coupled to phospholipid synthesis (Guinea and Carrasco, 1990). The 2BC polyprotein enhances viral replication through the induction of membrane vesicle proliferation.

HIV-1 also expresses a variety of proteins (the so-called accessory proteins) which are involved in multiple aspects of nuclear targeting and replication of the genome. Up to 100 molecules of the 23kDa virion infectivity protein factor (Vif) are packaged into a single viral particle. These proteins co-localize with and are required for proper packaging of the nucleoprotein core structures (Liu *et al.*, 1995; Hoglund *et al.*, 1994). Vif is required for the synthesis of proviral DNA in infected cells (von Schwedler *et al.*, 1993) and has been suggested to be involved in nuclear targeting of the genome (Strebel and Bour, 1999) once the viral particle has been uncoated to release the genome in the form of a ribonucleoprotein complex known as the pre-integration complex (PIC). Another protein implicated in nuclear targeting of the PIC is the above-mentioned Nef protein, which has been shown to stimulate replication in quiescent CD4 lymphocytes (Spina *et al.*, 1994) and reverse transcription (Aiken and Trono, 1995; Schwartz *et al.*, 1995) in association with cellular serine/threonine kinases (Sawai *et al.*, 1994).

At least three other HIV-1-encoded proteins are involved in the actual translocation and import of the PIC into the nucleus of the infected cell. These include the viral protein R (Vpr) (reviewed in Bukrinsky and Adzhubei, 1999), which connects the PIC with cellular nuclear import machinery even in the absence of active cell cycling (Heinzinger *et al.*, 1994; Popov *et al.*, 1998; Vodicka *et al.*, 1998), thereby facilitating subsequent proviral DNA synthesis and integration into the host genome without the need for cell division. The integrase (IN) and matrix (MA) proteins both contain nuclear localization signals and are also involved in mediating nuclear import and translocation of the PIC (Bukrinsky *et al.*, 1993; von Schwedler *et al.*, 1994; Gallay *et al.*, 1997)

## 1.6.3. Viral Gene Expression

Viruses encode numerous gene products to regulate expression at the level of transcription (reviewed in Flint and Shenk, 1997). These include the HIV-1 Tat protein (reviewed in Kingsman and Kingsman, 1996), which transactivates cellular and viral genes (Mavankal *et al.*, 1996; Parada and Roeder, 1996), and the bovine papilloma virus type 1 (BPV-1) E2 protein (reviewed in Ham *et al.*, 1991; McBride *et al.*, 1991), which stimulates transcription through the binding of enhancers and transcription factors (Rank and Lambert, 1995; Grossel *et al.*, 1996). Another member of the *Papovaviridae* family, simian virus 40 (SV40), expresses the large T-antigen which both stimulates late viral gene transcription as well as autorepresses early transcription (Reed *et al.*, 1976; Casaz *et al.*, 1991; Gilinger and Alwine, 1993; Gruda *et al.*, 1993; Rice and Cole, 1993).

The small DNA viruses Ad2 and Ad5 from *Adenoviridae* also encodes several transcriptional regulators. The E1A protein (reviewed in Hagmeyer *et al.*, 1995; Pruzan and Flint, 1995; Shenk, 1996) binds a cellular co-factor p300 and stimulates E2F-dependent transcription by sequestering the cellular retinoblastoma protein (pRB). E1A has been shown to activate early viral transcription (Nevins, 1981) and to regulate transcription of genes by either RNA polymerase II or III. Ad2 and Ad5 also encode the IVa2 protein which binds the major late promoter to stimulate late viral gene transcription (Tribouley *et al.*, 1994; Lutz and Kedinger, 1996).

Examples of transcriptional regulators from large DNA viruses include the virus early transcription factor (VETF) from vaccinia virus of the *Poxviridae* family (reviewed in Moss *et al.*, 1991). VETF is a DNA-dependent ATPase which binds the early promoter and is essential for early gene transcription by the vaccinia virus RNA polymerase (Li and Broyles, 1993a; Li and Broyles, 1993b; Cassetti and Moss, 1996). Herpes simplex virus type 1 (HSV-1) of the *Herpesviridae* encodes the tegument viral protein VP16, also known as ICP25 or α-TIF (reviewed in O'Hare, 1993; Roizman and Sears, 1996), which activates immediate early (IE) gene transcription from the HSV-1 genome (Batterson and Roizman, 1983; Campbell *et al.*, 1984).

## 1.6.4. Processing and Nuclear Export of mRNA Transcripts

Following transcription of the cellular genes, nascent pre-mRNA species normally undergo a series of post-transcriptional processing steps to become mature mRNA ready for nucleocytoplasmic export as heteronuclear ribonucleoprotein (hnRNP) complexes. The export process is tightly regulated to prevent escape of any improperly processed or potentially deleterious transcripts, with one of the requirements being the complete removal of all introns within the transcript before it will be exported. This "spliceosome retention" hypothesis stipulates that the mRNA export machinery recognizes only fully spliced mRNAs, and that partially spliced or unspliced pre-mRNA species are retained in the nucleus.

### 1.6.4.1 Processing and Nuclear Export of Viral Transcripts

Viruses have evolved a number of strategies to ensure export of viral transcripts, be they fully spliced or not. One of the best characterized modes of viral transcript escape involves the HIV-1 accessory protein Rev (reviewed in Pollard and Malim, 1998). This 19kDa protein has both nuclear localization and export signals (NLS and NES, respectively) (Fischer *et al.*, 1995) and binds to a sequence known as the Rev-responsive element (RRE) on partially spliced or unspliced viral transcripts. When Rev is bound to the RRE, the NLS becomes masked and the NES associates with the nuclear export machinery to mediate nucleocytoplasmic transport of the viral transcript (Malim *et al.*, 1989; Cullen, 1995). This NES-dependent export is dependent on the cellular protein Crm1 (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997) and may also require the U1 small nuclear RNA (snRNA) from the splicing machinery (Lu *et al.*, 1993).

In a similar manner, the adenovirus 55kDa E1B and 34kDa E4 proteins form a complex to mediate export of late viral transcripts (Krug, 1993; Dobbelstein *et al.*, 1997), possibly by sequestration of nuclear export factors away from spliced host mRNAs to sites of viral replication. Other viral proteins involved in viral transcript processing or export include the influenza NS1 protein, which regulates transport of

spliced NS2 mRNA and its precursor NS1 mRNA (Alonso-Caplen *et al.*, 1992), and the 63kDa HSV-1 IE protein ICP27, which stabilizes the 3′ ends of late viral pre-mRNAs and enhances viral transcript export (Brown *et al.*, 1995; Phelan *et al.*, 1996; Sandri-Goldin, 1998).

### 1.6.4.2. Processing and Nuclear Export of Host Transcripts

A number of viral proteins mentioned above in the processing of viral transcripts also play a role in limiting host transcript processing or export. In addition to its positive effects in mediating viral transcript export, the HSV-1 ICP27 protein causes a decrease in host protein synthesis by blocking the splicing of host pre-mRNA species (Hardy and Sandri-Goldin, 1994). A variation of this strategy to block host transcript export is exemplified by the influenza NS1 protein, which binds the U6 snRNA to inhibit host pre-mRNA splicing (Fortes *et al.*, 1994; Qiu *et al.*, 1995). The NS1 protein also safeguards against host mRNA export a step further by selectively blocking the export of fully spliced mRNAs containing poly(A) tails that were generated by the cellular two-step cleavage/polyadenylation system, which most host transcripts employ. In this way, fully spliced host mRNAs are retained in the nucleus while export of viral transcripts (whose poly(A) tails are produced differently) is unaffected (Fortes *et al.*, 1994; Qiu and Krug, 1994).

The influenza NS1 protein also mediates its negative effects on cellular mRNA export by inhibiting the host cellular 3′-end processing machinery. NS1 launches a double attack to accomplish this: it binds to and blocks the function of the 30kDa subunit of the cleavage and polyadenylation specificity factor (CPSF) (Nemeroff *et al.*, 1998), which is required for 3′-end cleavage and subsequent polyadenylation of the transcript; it also binds to the poly(A)-binding protein II (PABII) (Chen *et al.*, 1999). Binding to PABII prevents the latter's interaction with poly(A) polymerase (PAP), thereby disrupting the processive elongation of poly(A) chains. It is also possible that NS1 may inhibit PABII's ability to directly shuttle cellular mRNAs into the cytoplasm (Chen and Krug, 2000).

## 1.6.5. Processing of Viral Proteins

Viruses also encode nonstructural proteins that function to process viral gene products. For example, the flavivirus hepatitis C virus (HCV) produces a 70kDa NS3 protein which is required for processing of the single HCV polyprotein. NS3 has an amino-terminal serine protease domain as well as a carboxy-terminal ATP-dependent RNA helicase domain. In association with its co-factor NS4A and several host cellular enzymes, NS3 mediates self cleavage from the polyprotein and then proceeds to cleave the rest of the peptide into distinct viral proteins (Tomei *et al.*, 1993; Bartenschlager *et al.*, 1995; Landro *et al.*, 1997).

## 1.6.6. Host Cell Protein Trafficking and Degradation

Some viral proteins function to alter host cell protein trafficking or degradation. HIV-1 alone encodes several of these, including gp160, viral protein U (Vpu), and Nef. The Env precursor gp160 protein retains CD4 in the endoplasmic reticulum (ER) to block its trafficking to the cell surface (Crise *et al.*, 1990; Jabbar and Nayak, 1990), and the ER-bound CD4 is then induced by Vpu to undergo degradation via the proteasomal pathway (Willey *et al.*, 1992; Jabbar, 1995). Nef also downregulates CD4 but does this through the enhancement of the rate of cell surface CD4 internalization into endosomes (Rhee and Marsh, 1994). The poliovirus 2BC polyprotein alters protein trafficking in a different manner; it induces membrane vesicle proliferation as discussed earlier and functions to block the exocytic pathway (Barco and Carrasco, 1995).

## 1.6.7. Virion Packaging, Assembly, and Egress

As mentioned above in the context of nuclear targeting of the viral genome, the HIV-1 Vif protein co-localizes with and is required for proper packaging of nucleoprotein core structures (Liu *et al.*, 1995). Additionally, the proper maturation, targeting and release of virions require the function of the 16kDa viral protein U (Vpu) (Subbramanian and Cohen, 1994; Jabbar, 1995; Schubert *et al.*, 1995a; Schubert

*et al.*, 1995b) and the co-ordinated expression and processing of the Gag, Pol, Env, Vif and Nef proteins (Strebel and Bour, 1999).

### 1.6.8. Cell Cycle Arrest

In addition to its role in facilitating translocation of the HIV-1 PIC into the nucleus, Vpr (reviewed in Bukrinsky and Adzhubei, 1999) also causes cell cycle arrest at the G2/M checkpoint via inhibition of Cdc2 kinase activity (He *et al.*, 1995; Jowett *et al.*, 1995; Re *et al.*, 1995; Bartz *et al.*, 1996).

### 1.6.9. Immune Evasion

In order to successfully establish a productive infection in the host, viruses need to escape surveillance or at least delay detection by the host immune system (reviewed in Alcami and Koszinowski, 2000) and avoid any associated anti-viral response. To accomplish this, different viruses have evolved widely ranging strategies to interfere with the host humoral immune responses, inhibit interferon responses (reviewed in Goodbourn *et al.*, 2000), regulate cytokine and chemokine function (reviewed in Barry and McFadden, 1997; Spriggs, 1999; Lalani and McFadden, 1999; Lalani *et al.*, 2000), modulate apoptosis signaling (reviewed in Roulston *et al.*, 1999), as well as regulate function of the major histocompatibility complex classes I and II molecules (reviewed in Ploegh, 1998).

#### 1.6.9.1. Inhibition of Humoral Immune Responses

The complement cascade forms part of the innate immune response and consists of a series of enzymatic events and formation of complexes involving ~20 proteins. The cascade is activated by the binding of a recognition component to an antibody bound to antigen and functions to mediate local inflammation and lysis of infected host cells by the membrane attack complex (MAC). The cowpox virus (member of the *Poxviridae* family) expresses an inflammation modulatory protein (IMP) which is believed to inhibit production of macrophage chemoattractant factors C3a and C5a to limit tissue inflammation/damage and to block MAC-

mediated lysis (Kotwal, 2000). The HSV-1 glycoprotein C (gC) also inhibits complement-mediated lysis of infected cells, by binding C3b, thereby blocking the stablizing effects of properdin on the C3b/Bb convertase complex (Harris *et al.*, 1990; Hung *et al.*, 1994). Several members of the *Coronaviridae* and the *Herpesviridae* families (including HSV-1, HSV-2, and murine cytomegalovirus (mCMV)) encode functional IgG Fc receptors. Sequestration of host antibodies thus prevents IgG Fc-dependent activation of the complement cascade (Alcami and Koszinowski, 2000).

### 1.6.9.2. Inhibition of Interferon Responses

Host interferon (IFN) expression is typically upregulated upon viral infection. Effects of the IFN response include (1) upregulation of the 2′-5′ oligoadenylate synthetase (2′-5′ OS) which activates RNase L to degrade viral mRNA, (2) activation of the double-stranded RNA (dsRNA)-dependent protein kinase (PKR) which phosphorylates and inactivates the translation factor eIF-2α to block translation of viral proteins, and (3) induction of apoptosis to prevent viral propagation (Samuel *et al.*, 1997; Clemens, 1997; Clemens and Elia, 1997; Diaz-Guerra *et al.*, 1997; Castelli *et al.*, 1997; Rivas *et al.*, 1998). Again, viruses have evolved numerous ways to counter host-mediated IFN responses (Goodbourn *et al.*, 2000).

The vaccinia virus from the *Poxviridae* family encodes a viral homolog of the IFN-α/β receptor, which prevents binding and activation of the cellular receptor and thereby blocks IFN function (Smith *et al.*, 1998). Other viral nonstructural proteins intercept the IFN response further downstream; the HCV proteins NS5A and E2, and the HIV Tat protein, bind to and inhibit PKR activity. The multifunctional influenza NS1 protein, on the other hand, binds the dsRNA directly to inhibit IFN-induced activation of PKR, so that protein synthesis continues unaffected (Lu *et al.*, 1995). NS1 binding to the dsRNA also inhibits activation of the 2′-5′ OS, hence viral mRNA does not become degraded by RNase L.

### 1.6.9.3. Modulation of Cytokine and Chemokine Responses

Cytokines are soluble proteins that mediate immune and inflammatory responses in cases of infection or injury, and chemokines in particular are chemoattractant cytokines that mediate chemotaxis and trafficking of leukocytes to the affected site. HIV-1 as well as many large DNA viruses have evolved a number of gene products derived from host counterparts to block or stimulate the function of various cytokine, cytokine receptors, chemokines (Ck's), or chemokine receptors (CkR's) in order to best serve the virus' needs for its survival and propagation.

The HIV-1 transcriptional activator Tat protein shares partial sequence similarity with a known cellular Ck and has been shown to function as a chemoattractant for monocytes (Albini *et al.*, 1998). Tat also causes changes in host cytokine expression (Brady *et al.*, 1995) by upregulating interleukin-4 receptor (IL-4R) expression in a human B cell line (Puri and Aggarwal, 1992) and by increasing IL-2 secretion from activated T cells (Westendorp *et al.*, 1994).

The *Herpesviridae* Epstein-Barr virus (EBV) expresses the latent membrane protein 1 (LMP1), which recruits components of the tumor necrosis factor (TNF) receptor (TNFR)-mediated signal transduction machinery to mimic cytokine responses beneficial to the virus, such as proliferation (Farrell, 1998). Towards the same end, EBV also expresses a viral IL-10 homolog (vIL-10) (Hsu *et al.*, 1990), which stimulates B cell growth and differentiation by inhibiting the synthesis of antiviral cytokines. The Kaposi's Sarcoma-associated herpesvirus (KSHV, also known as human herpesvirus 8 or HHV-8) encodes a viral homolog of the macrophage inflammatory protein-1α (vMIP-1α) from its K6 ORF (Moore *et al.*, 1996) which acts as a functional chemokine to inappropriately induce chemotaxis to favor viral survival. In contrast, the murine γ-herpesvirus 68 (MHV-68) secretes a Ck binding protein, vCkBP-III, which functions to sequester a broad range of Ck's (Parry *et al.*, 2000).

Members of the *Poxviridae* have also expended a great deal of effort, genetically speaking, to mimic and modulate the activities of various cellular cytokines, growth factors, and receptors. Vaccinia encodes a viral homolog of the

epidermal growth factor (vEGF) from its 19K ORF, known as the vaccinia growth factor (VGF), to inappropriately stimulate cell growth, while the molluscum contagiosum virus (MCV) produces from its MC148R ORF a viral MIP-1β homolog which lacks the amino-terminal domain required for activation of the CkR. The vMIP-1β is therefore believed to act as an inhibitor of chemotactic signaling by competing with cellular Ck's for binding to CkR and blocking subsequent leukocyte mobilization (Senkevich *et al.*, 1996).

Other poxvirus-encoded proteins are secreted and function as cytokine-binding proteins to inhibit cytokine signaling. Shope fibroma virus (SFV) and myxoma virus each encodes a T2 protein (Smith *et al.*, 1991; Upton *et al.*, 1991), and cowpox virus encodes a crmB protein (Hu *et al.*, 1994), which are secreted, soluble binding proteins for tumor necrosis factor (vTNFR). These soluble receptors act as a molecular sink to soak up TNF to prevent TNF-mediated cell lysis. In addition to M-T2, myxoma virus also produces M-T7, a secreted IFN-γ binding protein (vIFN-γR) which correspondingly inhibits IFN function (Nash *et al.*, 1999).

### 1.6.9.4. Modulation of Apoptosis

#### 1.6.9.4.1. Inhibition of Apoptosis

Anti-viral and anti-proliferative responses often involve induction of apoptosis to block viral replication and propagation, though viruses have developed ways to overcome these hindrances. The *Papovaviridae* SV40 large T-antigen not only transactivates viral gene expression but also binds and inactivates p53, an important mediator of the apoptotic response. Further downstream, expression of the anti-apoptotic factor Bcl-$X_L$ is increased in the presence of the Tax protein from human T-lymphotropic virus type 2 (HTLV-2) and is believed to mediate the viral protein's inhibitory effects on Fas-mediated apoptosis (Zehender *et al.*, 2001). The human CMV (hCMV) also encodes two immediate early proteins, IE-1 and IE-2, which have been shown to inhibit TNF-induced apoptosis (Hengel *et al.*, 1998).

At a level downstream of Fas and TNFR activation, caspase activity is also targeted by virally encoded proteins. Cowpox crmA is a serpin-like protein which inhibits initiator caspase (caspase 1/8) activity to protect against apoptosis mediated by cytotoxic T-lymphocytes (CTLs), TNF, or Fas (Ploegh, 1998; Alcami and Koszinowski, 2000), and KSHV produces a viral homolog of caspase 8/FLICE-inhibitory proteins (v-FLIP) that interfere with the recruitment and activation of caspase 8 to block TNF-induced apoptosis (Irmler *et al.*, 1997). African swine fever virus (ASFV), on the other hand, encodes a member of the inhibitor of apoptosis protein (IAP) family named A224L which interacts with the proteolytic fragment of caspase 3 to inhibit its activity. This inhibitory interaction protects the host cell against apoptosis induced by a variety of means including TNF-α, cycloheximide, and staurosporine in a transfected cell model (Nogal *et al.*, 2001).

### 1.6.9.4.2. Induction of Apoptosis

Occasionally, it may be to the viral pathogen's advantage to promote apoptosis, such as when cell lysis is desirable at the end of the replicative cycle for dissemination of viral progeny. Despite the wealth of knowledge on viral proteins that block apoptosis, those virally encoded proteins which stimulate the process are much less well characterized. Among the well-established examples are the adenovirus E1A protein which induces sensitivity to TNF (Duerksen-Hughes *et al.*, 1989) and upregulates p53 (Debbas and White, 1993; Lowe and Ruley, 1993), and the HIV-1 Tat protein which upregulates the FasL promoter to sensitize cells to Fas-mediated apoptosis (Westendorp *et al.*, 1995). And of course, the NS1 proteins from *Parvoviridae* members MVM and B19 have been shown to cause DNA strand breaks (Op de Beeck and Caillet-Fauquet, 1997), induce cell cycle arrest at the G2/M checkpoint (Op de Beeck *et al.*, 1995), and mediate apoptosis of the infected cell (Moffatt *et al.*, 1998).

### 1.6.9.5. Modulation of Major Histocompatibility Complex (MHC) Function

Major histocompatibility complex (MHC) molecules are important vehicles for antigen presentation and for host detection of foreign substance. Helper T cells express CD4 co-receptors for MHC class II product recognition (sampling endosomal compartments), while cytotoxic T-lymphocytes (CTLs) express CD8 co-receptors for MHC class I product recognition (sampling the proteasomal pathways). MHC products are typically formed from a pair of ER-associated heavy and light subunits stabilized by a short antigenic peptide derived from cleavage of proteins from the proteasomal or endosomal degradation pathways. The assembled products are then trafficked to the plasma membrane for cell surface presentation of the antigen.

Virtually every component of the MHC-mediated antigen presentation system is subject to viral counterattack, as are most elements of other immune response pathways. The EBV nuclear antigen 1 (EBNA-1) contains a glycine-alanine repeat domain that interferes with recognition of its own proteasomal cleavage site. By escaping proteolysis, EBNA-1 is able to avoid MHC class I presentation of its fragments and subsequent recognition by CTLs (Levitskaya *et al.*, 1997). Other proteins, such as ICP47 from HSV-1 and HSV-2, act to inhibit the peptide transporter protein TAP by competing with peptides for binding to the cytosolic surface of the TAP complex, thereby blocking transport of antigenic peptides to complete assembly of the MHC molecules (York *et al.*, 1994; Hill *et al.*, 1995; Tomazin *et al.*, 1996).

Assembled MHC molecules are by no means immune to viral countermeasures. For instance, the human CMV encodes a protein named US3 which binds and retains MHC class I molecules in the ER (Ahn *et al.*, 1996); HIV-1 Nef and KSHV K3 and K5 gene products also downregulate MHC class I products (Schwartz *et al.*, 1996; Ishido *et al.*, 2000; Coscoy and Ganem, 2000). HIV-1 Nef and EBV vIL-10 homolog also interfere with processing of MHC class II molecules, thus delaying recognition by T helper cells (Koppelman *et al.*, 1997).

Cells with downregulated display of MHC products are susceptible to killing by natural killer (NK) cells, as cells bearing surface MHC molecules normally send NK cells signals to prevent killing of themselves. The human CMV has come up with expression of a MHC class I homolog, UL18 (homologs also produced by murine mCMV and rat rCMV), to function as a decoy to fool NK cells and inhibit NK-mediated cell lysis (Farrell *et al.*, 1997).

## 1.7. Other Proline-Rich Viral Nonstructural Proteins

It is difficult to predict what the function of the 11kDa proteins may be, given the widely ranging roles that nonstructural proteins from other viruses play. In the characterization of unknown proteins, it has been instructive to look for similarities with known proteins in terms of sequence and structure in order to delineate function. In the case of the 11kDa proteins, there are very few clues upon which to base these types of comparisons, though other virally encoded, nonstructural proteins containing proline-rich regions or SH3 recognition sites have been catalogued. These include the aforementioned HCV NS5A protein, which has been shown to interact with the SH3 domain-containing protein Grb2 to disrupt mitogenic signaling in addition to its role in inhibition of IFN-induced PKR anti-viral activity (Tan *et al.*, 1999). In addition, the Nef protein from HIV-1 contains a highly conserved sequence with repeated proline residues in PxxP motifs which was shown to mediate binding to the SH3 domain in various Src-family non-receptor tyrosine kinases (Saksela *et al.*, 1995). This SH3 domain-binding function is also important in mediating interaction of Nef with a p21-activated protein kinase (PAK)-like Nef-associated kinase (NAK), which implicates the HIV-1 Nef protein in modulation of host cell signaling cascades that may be of importance in HIV-1 pathogenesis (Manninen *et al.*, 1998).

## 1.8. Previous Studies on the 11kDa Proteins and the Present Study

The 11kDa proteins, like the signaling protein Sos, contain proline-rich regions which conform to consensus SH3 recognition sites with the sequence xxxPPxPxx. Because such sequences have been identified in numerous signaling molecules and are known to mediate binding to SH3 domains on other proteins, we speculate that the 11kDa proteins, like Sos, may be involved in protein-protein interactions. To investigate this possibility, affinity chromatography experiments have been carried out to identify putative cellular interactors of the 11kDa proteins. An ~83kDa and a ~26kDa protein from an erythroleukemic cell lysate were shown to bind to an 11kDa protein fused carboxy-terminal to a glutathione S-transferase (GST) epitope tag but not to GST alone. Based on the assumption that the 11kDa proline-rich region may be mediating protein-protein interactions, a computer search was done to identify proteins with these molecular weights containing one or more SH3 domains. The adaptor protein Grb2 fits these criteria and was therefore tested for its ability to interact with the 11kDa proteins. Preliminary far western blotting studies suggested that a Grb2 fusion protein can interact specifically with the GST-11kDa fusion protein but not with GST, demonstrating interactions between 11kDa and Grb2, at least *in vitro* (Zagrodney, 1998).

The present study attempts to characterize the 11kDa/Grb2 interaction further, through the use of a mutagenic approach coupled with far western blotting studies and GST fusion protein pull down assays. Our working hypothesis is that the B19 11kDa proteins interact with Grb2, attenuating the normal cellular Grb2/Sos/Ras signaling pathway and affecting downstream signaling (see Figure 1.8).

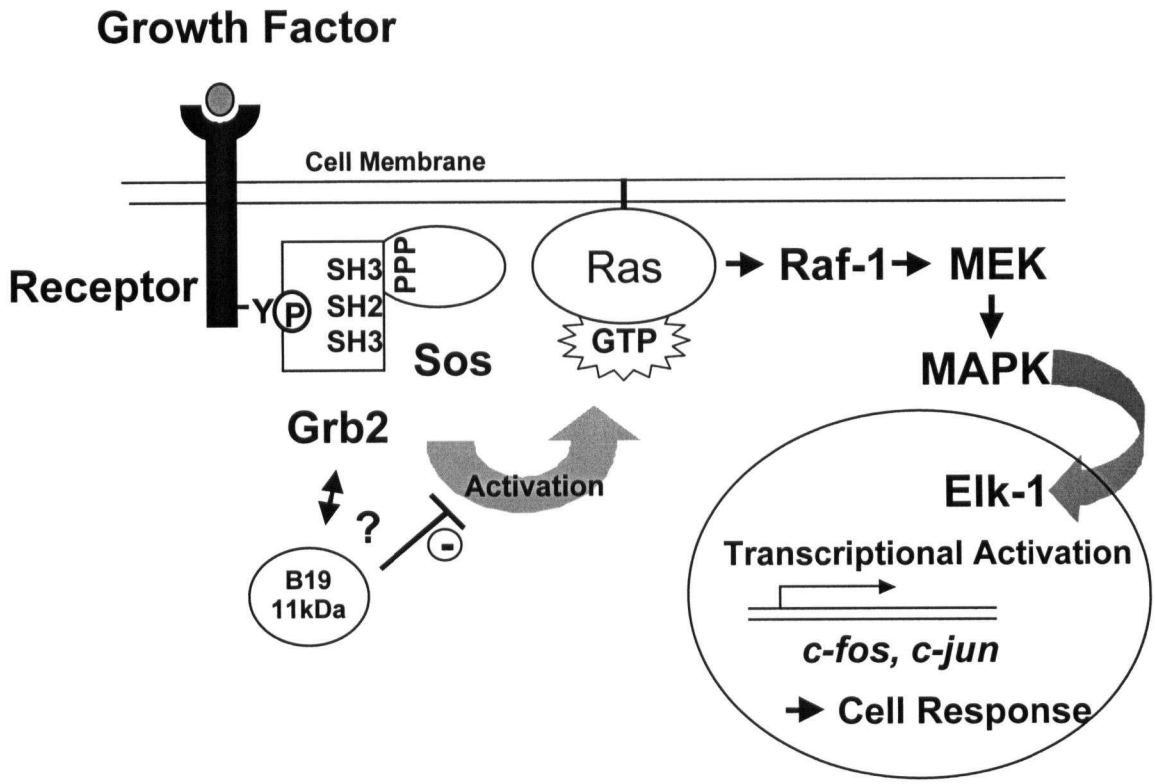


Figure 1.8. Model of B19 11kDa protein-mediated disruption of the Grb2/Sos/Ras signaling pathway.

# MATERIALS AND METHODS

## 2.1. Chemicals and Supplies

All chemicals used were purchased from Fisher Scientific Co., Sigma Chemical Co., Gibco BRL, or Roche Diagnostics (formerly Boehringer Mannheim) unless otherwise specified. Bacterial culture reagents were purchased from BDH Inc. or ABI. Tissue culture reagents were from Gibco BRL or Sigma Chemical Co. Cell Culture Reagents. Agarose and polyacrylamide gel supplies were from Gibco BRL or Bio-Rad Laboratories. DNA ladders and protein molecular weight standards were obtained from Gibco BRL and Bio-Rad Laboratories, respectively. DNA purification kits were obtained from Qiagen (QIAprep Spin Miniprep Kit) or Bio/Can Scientific (GeneClean II kit). Radiolabelled dATP was purchased from New England Nuclear (NEN) Dupont (now part of Perkin Elmer (PE) Applied Biosystems), ultrapure deoxyribonucleotide triphosphates (dNTP's) were from Amersham, and the BigDye Terminator Reaction Ready Mix for automated sequencing was supplied by PE Applied Biosystems. Restriction and deoxyribonucleic acid (DNA)-modifying enzymes were obtained from New England Biolabs (NEB), Gibco BRL, or Boehringer Mannheim unless otherwise noted.

Oligonucleotide primers were synthesized at the Nucleic Acid and Protein Synthesis (NAPS) unit at the University of British Columbia. Glutathione-Sepharose 4B and Protein A-sepharose CL-4B resins for protein purification, protein pull down assays and immunoprecipitation studies were supplied by Amersham Pharmacia Biotech. Polyvinylidene fluoride (PVDF) membranes and other apparatus for protein transfer were from Bio-Rad Laboratories (Immun-Blot PVDF) or Pall Corp. Gelman Laboratory (BioTrace PVDF).

Primary antibodies were from StressGen Biotechnologies, Boehringer Mannheim, or Becton Dickinson (BD) PharMingen/Transduction Laboratories unless otherwise noted. Secondary antibodies were purchased as part of the enhanced chemiluminescence (ECL) kit from Amersham Pharmacia Biotech or from

Jackson ImmunoResearch. Film for ECL detection was from Amersham Pharmacia Biotech.

## 2.2. Bacterial Culture

### 2.2.1. Growth and Maintenance

Routine cloning, plasmid propagation, and protein expression were carried out in DH10b *E. coli* [F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ⁻ *rps*L *nup*G] from Gibco BRL. Bacteria were routinely grown on Luria-Bertani (LB) agar plates (1% peptone, 0.5% yeast extract, 1% sodium chloride (NaCl), and 1.5% agar) or in LB media for plasmid isolation; for protein expression, bacteria were cultured in 2xYT media (16g peptone, 10g yeast extract, 5g NaCl per liter). Media or agar were supplemented with 100μg/ml ampicillin (Amp) or 30μg/ml kanamycin (Kan) when required for plasmid selection. Frozen stocks of bacterial cultures were routinely prepared with 25% glycerol and stored at -80°C.

### 2.2.2. Preparation of Competent Bacteria for Heat Shock Transformation

Competent DH10b *E. coli* bacteria were prepared from a 10ml overnight culture grown at 37°C in LB media which was used to inoculate 200ml LB media. The 200ml culture was then grown at 37°C with vigorous shaking until an optical density at 600nm ($OD_{600}$) of ~0.9 was reached. Bacteria were harvested by centrifugation at 5000rpm for 5 minutes at 0°C, and the supernatant was decanted. The pellet was resuspended in 50ml of ice-cold 100mM $MgCl_2$ and centrifuged again at 5000rpm for 5 minutes at 0°C. After the supernatant was removed, the cells were resuspended in 10ml ice-cold 100mM $CaCl_2$. A further 100ml of ice-cold 100mM $CaCl_2$ was added, and the solution was mixed and incubated for 1 hour on ice. The suspension was spun as before and the pellet was resuspended in ~12ml of ice-cold 85mM $CaCl_2$ solution containing 15% glycerol. The mixture was separated

into smaller aliquots and frozen in a dry ice/ethanol bath. Frozen stocks of competent bacteria were stored at -80°C for up to a year.

### 2.2.3. Heat Shock Transformation of *E. coli*

Plasmid DNA was routinely introduced into bacteria by heat shock transformation. 50µl of heat shock competent DH10b *E. coli* were mixed with 0.5µg or more of plasmid DNA or with 1µl of a ligation reaction. The mixture was incubated on ice for 30 minutes, heat shocked at 42°C for 1 minute, and replaced on ice for a further 2 minutes before being diluted with 950µl fresh LB media. After incubation for 1 hour at 37°C with vigorous shaking, the mixture was centrifuged for 5 minutes at 4000rpm. The supernatant was removed by aspiration, and the pellet was resuspended in 100µl of LB media and plated in entirety onto LB agar with appropriate antibiotic selection for overnight growth at 37°C.

## 2.3. Tissue Cell Culture

### 2.3.1. Growth and Maintenance

The human embryonal kidney (HEK) 293 cell line has been transformed with sheared human adenovirus type 5 (Ad 5) DNA and expresses the Ad 5 transforming gene (info from ATCC). COS-7 cells are simian CV-1 cells transformed with an origin-defective SV40 mutant virus which expresses the large T-antigen and therefore supports high copy number replication of plasmids containing the simian virus SV40 origin (info from ATCC). Both HEK 293 and COS-7 cells were cultured as monolayers in complete media consisting of low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10mM HEPES at pH 7.4, and 1x antibiotic-antimycotic (Gibco BRL; 100units penicillin, 100µg streptomycin, and 0.25µg amphotericin B per milliliter of media). Cells were propagated in 100mm culture dishes in a water-jacketed incubator at 37°C with 5% $CO_2$. Frozen stocks of cells were quickly thawed at 37°C and diluted 1:10 in fresh, complete media. Cells were routinely trypsinized and

passaged in a 1:10 dilution in fresh media when they have reached about 90% confluency, usually every 3 to 4 days. Frozen stocks of HEK 293 and COS-7 cells were routinely prepared as one milliliter aliquots of roughly 1-2 x $10^6$ cells in freezing media consisting of DMEM supplemented with 10% FBS and 15% dimethylsulfoxide (DMSO). Cells were frozen for several hours at -20°C and then overnight at -80°C before being transferred to liquid nitrogen for long term storage. Selected aliquots were thawed and checked for viability at three or more weeks after the initial date of freezing.

## 2.3.2. Transfection of Plasmid DNA into COS-7 Cells

COS-7 cells were transfected by a modified Diethylaminoethyl (DEAE)-Dextran procedure. Briefly, nearly confluent cells in 100mm culture dishes were passaged at a 1:6 dilution one day prior to transfection. Five micrograms of plasmid DNA purified by ethanol precipitation was dissolved in 100µl of filter-sterilized nanopure distilled water ($dH_2O$), mixed with 200µl of 2mg/ml DEAE-Dextran, and diluted to 3ml final volume with serum-free DMEM. Cells were washed twice with DMEM and then overlaid with the transfection solution. After 8hr of incubation at 37°C 5% $CO_2$, the transfection solution was removed and replaced with 3ml of phosphate-buffered saline (PBS) containing 10% DMSO. Cells were subjected to the DMSO shock for exactly 5 minutes and then washed twice with PBS before the addition of 10ml of complete media. Transfected cells were incubated at 37°C 5% $CO_2$ for 2-3 days before being analyzed for target protein expression.

## 2.3.3. Lysis of Tissue Culture Cells for GST Protein Pull Down and Immunoprecipitation Experiments

Nearly confluent cells were trypsinized, resuspended in complete media, and counted. The suspension was then centrifuged for 5 minutes at 1200rpm to pellet the cells. The supernatant was removed by aspiration and the pellet was washed once with PBS and centrifuged as above. After removal of the supernatant,

the washed pellet was resuspended in ice-cold hypotonic buffer (20mM Tris-Cl pH7.4) supplemented with protease inhibitors at 2μg/ml antipain, 0.5mM phenylmethylsulfonyl fluoride (PMSF), 1μg/ml leupeptin, and 4μg/ml aprotinin. Cells were resuspended at a concentration of roughly 3 x $10^6$ cells per milliliter of hypotonic buffer and incubated on ice for 15 minutes to allow for cell swelling. The cells were then transferred to a prechilled dounce homogenizer and lysed with 8 up and down strokes using a type-B pestle. Lysates were routinely prepared fresh and used within one hour.

## 2.4. Molecular Cloning Techniques

### 2.4.1. Small-Scale Plasmid DNA Isolation

Plasmid DNA was isolated according to a modified alkaline lysis protocol based on the method described in Le Gouill *et al.* (1994). The bacterial pellet from 1.5ml of an overnight culture of bacterial cells was resuspended in 100ul of Solution I (50mM glucose, 10mM ethylene-diamine tetra-acetic acid (EDTA), 25mM Tris-Cl, pH8.0). Then 200μl of Solution II (0.2N NaOH, 1% SDS; prepared fresh immediately before use) and 200μl of chloroform were added, mixed by vortexing, and the cells were lysed for 1 minute. 150μl of Solution III (29.4g $CH_3COOK$, 5ml HCOOH and $H_2O$ to 100ml final volume) was added and mixed by vortexing for 2-3 seconds. The mixture was then centrifuged at top speed for 2 minutes to pellet cell debris. The upper, aqueous phase was transferred to a fresh microfuge tube, and DNA was precipitated with 2 volumes of ice-cold 95% ethanol and pelleted by centrifugation. After the DNA pellet was washed once with 70% ethanol and recentrifuged, it was dissolved in 50ul of sterilized distilled $H_2O$ ($sdH_2O$) containing 0.05μg/μl of RNase A to remove contaminating RNA fragments. The DNA was then subjected to screening or verification by restriction endonuclease digestion, G-laddering, or automated sequencing, as required.

## 2.4.2. Large-Scale Plasmid DNA Isolation

Plasmid DNA was isolated according to an alkaline lysis protocol modified from the method described in Sambrook *et al.* (1989). Briefly, 200ml of LB media with appropriate antibiotic selection were inoculated with a single colony of bacteria carrying the plasmid DNA of interest for overnight growth at 37°C with vigorous shaking. Bacteria were harvested and lysed under alkaline conditions, and plasmid DNA was isolated and purified by polyethylene glycol (PEG) precipitation. The DNA was further purified by phenol/chloroform extraction before being sterilized by ethanol precipitation. The final DNA pellet was dissolved in sterilized distilled water ($sdH_2O$) and quantitated by spectrophotometric measurements at 260nm.

## 2.4.3. DNA Quantitation and Purity Check

Plasmid DNA and oligonucleotides were routinely quantitated by spectrophotometric measurements at 260nm in a Pharmacia Biotech Ultrospec 3000 UV/Visible spectrophotometer. Absorbance at 260nm ($A_{260}$) is related to DNA concentration as follows:

Concentration of plasmid DNA in μg/ml

$= (A_{260})(50\mu g/ml)$(dilution factor)

Concentration of oligonucleotide DNA in μg/ml

$= (A_{260})(33\mu g/ml)$(dilution factor)

The ratio of $A_{260}$ to $A_{280}$ gives an indication of the purity of the DNA; most pure DNA preparations generate ratios of between 1.8 and 2.0.

## 2.4.4. Plasmid DNA Digestions

All plasmid DNA digestions were done using restriction endonucleases, buffers, and protocols provided by NEB, Gibco BRL, or Boehringer Mannheim. In general, up to 1μg of plasmid DNA would be digested with restriction enzymes at a concentration of 4 units (U) of enzyme per microgram of DNA, in a final reaction volume of 20μl to 50μl and incubated at the specified temperature (usually 37°C) for

two or more hours. The samples were then resolved by agarose gel electrophoresis or purified for further use with a commercial DNA purification kit.

## 2.4.5. Plasmids

Table 2.4.5. Plasmid vectors and the proteins they express.

| PLASMID | PROTEIN |
|---|---|
| pGEX2T | GST |
| pGEX-11 wt/mutant | GST-11 wt/mutant |
| pGEX2T-HA | GST-HA |
| pGEX2T-HA-Grb2 | GST-HA-Grb2 |
| pEGFP-C2 | EGFP |
| pEGFP-11 | EGFP-11 |

2.4.5.1. **pGEX2T** (figure 2.4.6.1) directs expression in *E. coli* of glutathione S-transferase (GST), a 26kDa protein from *Schistosoma japonicum*, under the control of the inducible *tac* promoter. A multiple cloning site (MCS) following the GST-encoding sequence permits construction of a fusion protein, with the protein of interest fused carboxy-terminal to GST. Because GST binds strongly to glutathione, GST and GST fusion proteins can be readily purified by affinity chromatograhy on glutathione-sepharose resin (Pharmacia Biotech, 1997).

2.4.5.2. **pGEX-11** was constructed by Dr. Janet St. Amand by cloning the open reading frame (ORF) for the 11kDa protein from B19 parvovirus in frame into the *Bam* HI/*Eco* RI sites of pGEX2T. This vector directs the expression of a GST-11kDa fusion protein, GST-11.

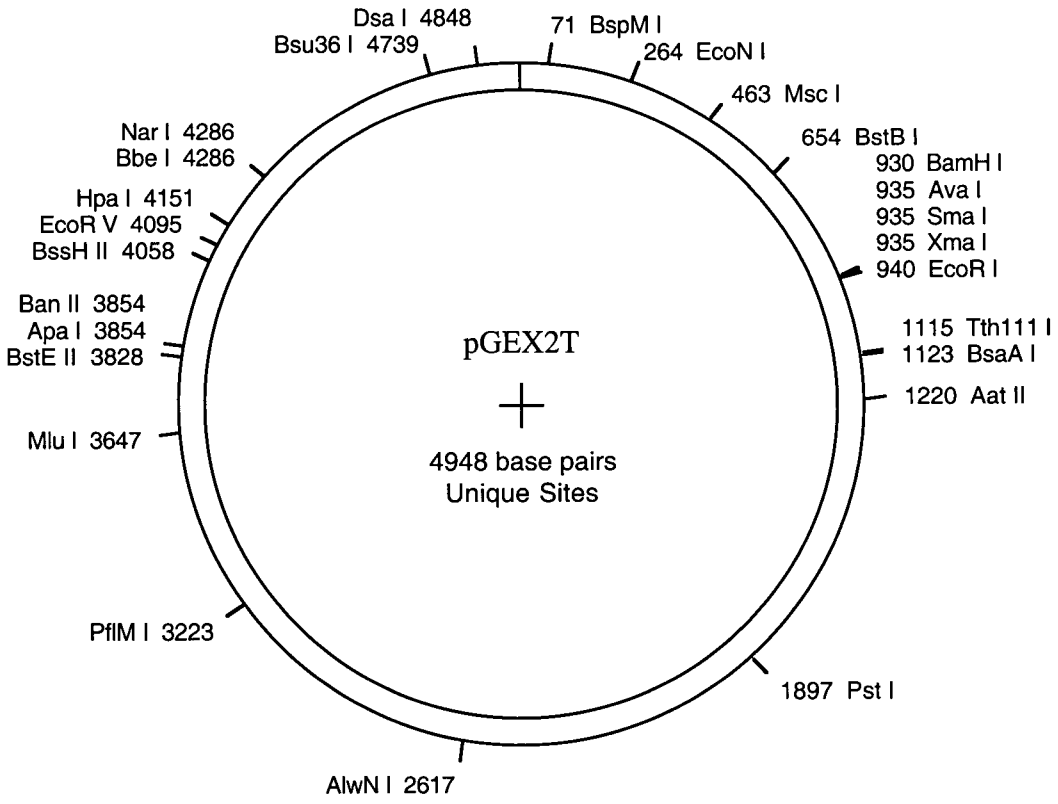


Figure 2.4.5.1. pGEX2T plasmid map.

2.4.5.3. **pGEX-11 P42,45A/P42,45,46A/P42,46A/P43A/P43,45A/P43,45,46A/P45A /P45,46A** were constructed from pGEX-11 by PCR-based site-directed mutagenesis using oligonucleotide primers 11kDPro2 and 11COMPL. The resulting GST-11 proteins have proline-to-alanine mutations at amino acid residues corresponding to the specified positions within the native 11kDa protein (i.e. P42,45A specifies a double amino acid substitution of Pro-42 and Pro-45 with Ala).

2.4.5.4. **pGEX-11 P42A** was constructed from pGEX-11 by PCR-based site-directed mutagenesis using oligonucleotide primers P42A1 and P42A2. The resulting GST-11 protein has a proline-to-alanine mutation at an amino acid residue corresponding to position 42 of the native 11kDa protein.

2.4.5.5. **pGEX-11 P46A** was constructed from pGEX-11 by PCR-based site-directed mutagenesis using oligonucleotide primers P46A1 and P46A2. The resulting GST-11 protein has a proline-to-alanine mutation at an amino acid residue corresponding to position 46 of the native 11kDa protein.

2.4.5.6. **pGEX2T-tag** was generously provided by Ken Harder and was constructed by insertion of a hemagglutinin (HA) epitope tag and polylinker into the *Bam* HI/*Eco* RI sites of pGEX2T. This vector encodes a GST-HA fusion protein.

2.4.5.7. **pGEX2T-tag-Grb2** was generously provided by Dr. John Schrader and was constructed by cloning the cDNA encoding the human growth factor receptor binding protein (Grb2) into the *Nhe* I/*Eco* RI sites of pGEX2T-tag. This vector directs the expression of a GST-HA-Grb2 fusion protein.

2.4.5.8. **pEGFP-C2** (figure 2.4.6.8) directs expression of a variant of wild-type green fluorescent protein (GFP). GFP is a protein from *Aequorea victoria* that fluoresces upon light excitation and is useful as a tag to localize proteins or monitor transgene expression in living cells. The present vector encodes a GFP variant referred to as enhanced GFP, or EGFP, under the control of a human cytomegalovirus (CMV)

immediate early promoter. EGFP has an excitation maximum at 488nm and an emission maximum at 507nm, and the protein has been optimized for brighter fluorescence and higher expression in mammalian cells. The vector contains an SV40 origin for high copy number replication in mammalian cells expressing the SV40 T-antigen (i.e. COS-7 cells). An MCS following the EGFP-encoding sequence permits construction of a fusion protein, with the protein of interest fused carboxy-terminal to EGFP.

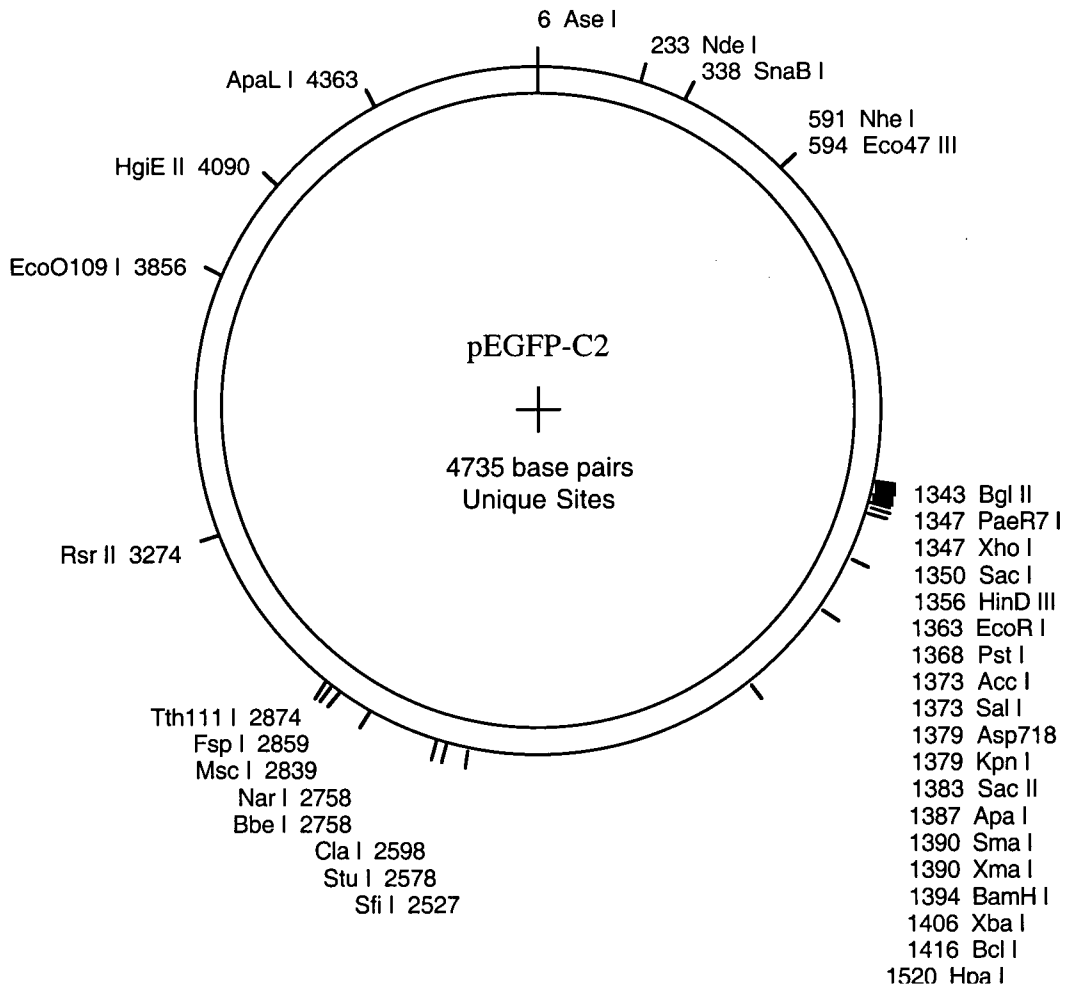


Figure 2.4.5.8. pEGFP-C2 plasmid map.

2.4.5.9. **pEGFP-11** was constructed by Dr. Hanson Chen by cloning the ORF for the B19 11kDa protein from pGFP-11 in frame into the *Bgl* II/*Eco* RI sites of pEGFP-C2. This vector encodes an EGFP-11kDa fusion protein, EGFP-11, which is presumed to have similar excitation and emission properties as does wild-type EGFP.

## 2.4.6. Oligonucleotides

Oligonucleotide primers were synthesized at the NAPS unit at UBC. Mutagenic oligonucleotides to be used in PCR-based site-directed mutagenesis experiments were first 5'-phosphorylated using T4 polynucleotide kinase (PNK) in the presence of the appropriate 1x reaction buffer containing 1mM ATP. Phosphorylation was carried out at 37°C for 30 minutes and terminated by heat inactivation at 70°C for 10 minutes.

2.4.6.1. **11kDPro2** is a mutagenic oligonucleotide with random C or G at 4 positions corresponding to the first nucleotide in triplet codons encoding Pro-42, 43, 45, or 46 in the native B19 11kDa protein, in the reverse complementary orientation.
5'-CAG GTA CAG TCT GGC/G TGC/G TAC TGC/G TGC/G GCG TTT AGT TAC-3'

2.4.6.2. **11COMPL** is a mutagenic oligonucleotide with random C or G at 4 positions corresponding to the first nucleotide in triplet codons encoding Pro-42, 43, 45, or 46 in the native B19 11kDa protein. 11COMPL is identical to 11kDPro2 in the reverse complementary orientation.
5'-GTA ACT AAA CGC C/GCA C/GCA GTA C/GCA C/GCC AGA CTG TAC CTG-3'

2.4.6.3. **P42A1** is a mutagenic oligonucleotide with a C-to-G mutation at the position corresponding to the first nucleotide in the triplet codon normally

encoding Pro-42 in the native B19 11kDa protein, leading to incorporation at that position with Ala.
5'-CGT AAC TAA ACG CGC ACC AGT ACC ACC-3'

2.4.6.4. **P42A2** is a mutagenic oligonucleotide with the reverse complementary sequence to P42A1.
5'-GGT GGT ACT GGT GCG CGT TTA GTT ACG-3'

2.4.6.5. **P46A1** is a mutagenic oligonucleotide with a C-to-G mutation at the position corresponding to the first nucleotide in the triplet codon normally encoding Pro-46 in the native B19 11kDa protein, leading to incorporation at that position with Ala.
5'-GCC CAC CAG TAC CAG CCA GAC TGT ACC TG-3'

2.4.6.6. **P46A2** is a mutagenic oligonucleotide with the reverse complementary sequence to P46A1.
5'-CAG GTA CAG TCT GGC TGG TAC TGG TGG GC-3'

2.4.6.7. **pGEX3'** anneals to the pGEX-11 (wild-type and mutant) vector ~200nt downstream of the targeted 11kDa proline-to-alanine mutation site and sequences along the reverse complementary strand.
5'-CCG GGA GCT GCA TGT GTC AGA GG-3'

2.4.6.8. **11Seq1** anneals within the 11kDa ORF ~70nt downstream of the targeted mutation site and sequences along the reverse complementary strand.
5'-GTT GTT CAT ATC TGG TTA AG-3'

## 2.4.7. Polymerase Chain Reaction (PCR)-Based Site-Directed Mutagenesis

The B19 11kDa protein contains three proline-rich regions conforming to SH3 ligand motifs. Site-directed mutagenesis was performed to substitute selected proline residues with alanines in the second of these regions to determine if this region is involved in interaction of the 11kDa protein with Grb2. The 25µl reaction mix consisted of 1x Cloned Pfu Buffer (Stratagene), 5ng of template pGEX-11 plasmid DNA, 62.5ng of each of two sequence-complementary 5'-phosphorylated mutagenic oligonucleotide primers, 0.2mM dNTP's, and 1.25 units (U) of Pfu Turbo DNA polymerase (Stratagene). After an initial denaturation step at 94°C, the reaction was cycled 16-18 times through denaturation for 1 minute at 94°C, primer annealing for 1 minute at 56°C, and nascent strand extension at 68°C for 11 minutes (approximately 2 minutes per kb of template multiplied by ~5.2kb of pGEX-11). The PCR products were treated with *Dpn* I which specifically digested methylated and hemimethylated DNA and was therefore used to select for newly-synthesized mutation-containing plasmid DNA. Samples with or without *Dpn* I treatment were resolved by 1.0% agarose gel electrophoresis in Tris-Acetate-EDTA (TAE) buffer and visualized by ethidium bromide staining. The *Dpn* I-digested samples were then transformed into competent *E. coli* bacteria with or without being treated with T4 DNA ligase. The ligase would repair the nicks within the nascent plasmid molecule, though the nicks, being staggered, would not be expected to interfere with circularization of the plasmid DNA or the subsequent transformation into bacteria.

## 2.4.8. G-Ladder Screen for Mutant Plasmid DNA

Site-directed mutagenesis using 11kDPro2 and 11COMPL as primers and pGEX-11 as template yielded products with variable sequence where either C or G could be incorporated in up to four different nucleotide positions, due to partial degeneracy of the primers at these positions. In order to screen for and identify mutant pGEX-11 plasmid DNA containing defined C-to-G mutations, a modified

procedure based on the Sanger dideoxy manual sequencing method (Sanger *et al.*, 1992) was employed. In this method, $\alpha$-$^{32}$P dATP from NEN Dupont was used as label in conjunction with the Reagent Kit for T7 Sequenase v2.0 DNA Polymerase from Amersham. The template typically consisted of 28µl of plasmid DNA from small-scale isolation and was denatured in 0.2M NaOH at 37°C for 30 minutes prior to ethanol precipitation. The washed and dried pellet of denatured DNA was annealed with 1pmol of either the pGEX3' or 11Seq1 oligonucleotide in 1x sequencing buffer. Instead of setting up four different reactions using a different dideoxyribonucleotide in each one, only dideoxyguanosine triphosphate (ddGTP) was employed in the sequencing reaction, hence the name "G-laddering" for this modified sequencing protocol. Radiolabeled dATP was incorporated into nascent DNA strands synthesized by T7 DNA polymerase. DNA fragments of different sizes were generated, depending on the position of incorporation of ddGTP, and these were resolved on a 6% polyacrylamide gel containing urea in Tris-Borate-EDTA (TBE) buffer. Gels were run under constant power at 32W (voltage ~2000V) for 1 or 3 hours using 11Seq1 or pGEX3' as the primer, respectively. Gels were then dried in a Bio-Rad gel dryer and exposed to Kodak or AGFA X-ray film overnight. The resulting banding pattern from a given sample would represent DNA fragments ending at all possible GTP positions. Because both primers employed here sequence along the reverse complementary strand from downstream of the 11kDa stop codon, the nucleotide C at the first position of codons specifying prolines would be read as the nucleotide G from the reverse complementary direction. Hence the sequence analysis of wild-type pGEX-11 should yield bands corresponding to DNA fragments ending with ddGTP at each of the four positions to be mutated. In the case where a C-to-G mutation has occurred, this would become a G-to-C mutation on the reverse complementary strand which would be manifested as a missing band in the G-ladder. In this way, pGEX-11 mutants (other than pGEX-11 P42A and pGEX-11 P46A) were identified and subjected to sequence verification by automated sequencing.

### 2.4.9. Automated Sequencing of Plasmid DNA

PE Applied Biosystems provides a comprehensive protocol for automated DNA sequence analysis which was followed with minor modifications. Briefly, double-stranded plasmid DNA template was isolated from a large or small scale preparation and was quantitated by $A_{260}$ measurements. 100-300ng of DNA was mixed with 3.2pmol of sequencing primer and 2µl of the "BigDye Terminator Reaction Ready" PCR mix (PE Applied Biosystems) on ice. Sterilized distilled $H_2O$ ($sdH_2O$) was added to a final reaction volume of 5µl prior to amplification in a Perkin Elmer GeneAmp PCR System 2400 instrument. Samples were denatured for 5 minutes at 95°C and then subjected to 25 cycles of 5 seconds at 95°C, 10 seconds at 55°C, and 4 minutes at 60°C. The DNA was then precipitated with 95% ethanol, washed with 70% ethanol, and dried in a Savant SpeedVac SC110 instrument. Samples were then submitted to Dr. Ivan Sadowski's laboratory here in the Department of Biochemistry and Molecular Biology at UBC for automated sequence analysis.

## 2.5. Protein Techniques

### 2.5.1. GST/GST Fusion Protein Purification

GST and GST fusion proteins were purified according to a protocol modified from the method described by Pharmacia, supplier of the pGEX2T plasmid. Briefly, 200ml of 2xYT media was inoculated with 5ml of overnight culture and incubated at 37°C with vigorous shaking until an $OD_{600}$ of ~0.5 was reached. Isopropylthio-β-D-galactoside (IPTG) was added to a final concentration of 0.2mM to induce expression of GST and GST fusion proteins from the *tac* promoter, and the culture was grown at 37°C for 3 more hours prior to being harvested by centrifugation at 7200rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet stored at -20°C until ready for protein purification. The pellet was first resuspended in 10ml of ice-cold PBS supplemented with protease inhibitors at 2µg/ml antipain, 0.5mM PMSF, 1µg/ml leupeptin, and 4µg/ml aprotinin. 1ml of 10mg/ml lysozyme

in STET buffer (8% sucrose, 0.5% Triton X-100, 50mM EDTA, 10mM Tris-Cl pH8.0) was added, and the solution was incubated at room temperature for 15 minutes with end-to-end rotation. Bacteria were lysed by sonication in 15ml Sarstedt screw-cap conical tubes on ice with two 30-second bursts at 40% intensity separated by a 1-minute break on ice in between bursts. Protein was solubilized by the addition of Triton X-100 to 1% final concentration and incubation for 30 minutes at 4°C with end-to-end rotation. After centrifugation at 9400rpm for 10 minutes at 4°C, the supernatant was treated with 2mM ATP/10mM $MgSO_4$/50mM Tris-Cl pH8 for 10 minutes at 37°C prior to incubation with PBS-equilibrated glutathione-sepharose 4B resin for 1 hour at 4°C with end-to-end rotation. The resin and all bound proteins were pelleted by centrifugation and washed three times with ice-cold PBS 5mM ATP supplemented with protease inhibitors. Bound proteins were eluted by resuspending the pellet in glutathione elution buffer (GEB, 10mM reduced glutathione (GSH) and 50mM Tris-Cl pH8.0) and incubating for 10 minutes at room temperature with end-to-end rotation. The resin was discarded after centrifugation and the supernatant was stored at -20°C as a resin-purified protein fraction.

## 2.5.2. Protein Quantitation

Purified protein preparations were routinely quantitated by the bicinchroninic acid (BCA) protein determination method using BCA reagents A and B from Pierce. Briefly, 100µl of 1:25 diluted protein samples were prepared with $sdH_2O$ alongside serial dilutions of bovine serum albumin (BSA) protein standards with known concentrations (ranging from 0-1.0mg/ml). The working reagent was prepared by mixing 1 part of reagent B with 50 parts of reagent A. 2ml of the working reagent is added to each 100µl sample and the resulting mixtures were incubated at 37°C for 30 minutes and then allowed to cool to room temperature. Absorbance at 562nm ($A_{562}$) was measured and protein concentrations were calculated based on a calibration curve relating $A_{562}$ to BSA protein content .

## 2.5.3. Antibodies

### 2.5.3.1 Primary Antibodies

2.5.3.1.1. Rabbit **anti-B19 11kDa** polyclonal antisera were prepared by Dr. Janet St. Amand (St. Amand, 1992) and raised against a 28-residue synthetic peptide conjugated to keyhole limpet hemacyanin (KLH). The peptide conjugate was used in complete Freund's adjuvant. The sequence of the synthetic peptide corresponds to amino acid residues 59-86 (PNTKDIDNVEFKYLTRYEQ HVIRMLRLC) of the B19 11kDa protein and does not contain any of the three putative SH3 ligand sequences in the 11kDa protein.

2.5.3.1.2. Mouse **anti-GFP** monoclonal antibody (StressGen) of the $IgG_2$ isotype recognizes the GFP GST fusion protein and will react with GFP, EGFP, as well as GFP/EGFP fusion proteins.

2.5.3.1.3. Rabbit **anti-Grb2** polyclonal antibody (StressGen) was raised against a 23-residue synthetic peptide with sequence corresponding to amino acid residues 195-217 (KGACHGQTGMFPRNYVTPVNRNV) of the human Grb2 protein. This peptide partially overlaps with the carboxy-terminal SH3 domain (residues 163-208) of Grb2 but does not contain the amino-terminal SH3 domain (residues 5-54) primarily responsible for the high affinity binding of Grb2 to Sos.

2.5.3.1.4. Mouse **anti-GST** monoclonal antibody (StressGen) of the $IgG_1$ isotype recognizes the purified GST protein and will react with GST and GST fusion proteins.

2.5.3.1.5. Mouse **anti-HA** monoclonal antibody (Boehringer Mannheim) of the $IgG_{2b\kappa}$ isotype recognizes a partial, 9-residue sequence (YPYDVPDYA) of the hemagglutinin epitope derived from the human influenza hemagglutinin protein.

2.5.3.1.6. Mouse **anti-Sos1** monoclonal antibody (BD PharMingen) of the $IgG_1$ isotype recognizes the amino-terminal 109 amino acid residues of the mSos1 protein. This peptide excludes the SH3 ligand that is known to bind Grb2. The antibody will cross-react with the human homologues hSos1 and hSos2 because of a high degree of amino acid identity among the proteins.

### 2.5.3.2 Secondary Antibodies

2.5.3.2.1. Horse radish peroxidase (HRP)-conjugated **Goat anti-mouse** antibody (Jackson ImmunoResearch Laboratories, catalog number 115-035-003) is used in conjunction with mouse anti-GFP, anti-GST, anti-HA, and anti-Sos1 primary antibodies in western and far western blotting experiments.

2.5.3.2.2. Super X-absorbed horse radish peroxidase (HRP)-conjugated **Donkey anti-rabbit** antibody (Jackson ImmunoResearch Laboratories, catalog number 711-035-152) is used in conjunction with rabbit anti-B19 11kDa and anti-Grb2 primary antibodies in western blotting experiments.

## 2.5.4. Western Blotting

Proteins resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to PVDF membranes for detection of specific proteins by western blotting. The transfer was carried out by semi-dry electrophoresis in Bjerrum & Schafer-Nielsen transfer buffer (39mM glycine, 20% methanol, 1.3mM SDS, 48mM Tris-Cl pH9.2) in a Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell apparatus run at 12V for 1 hour. Post-transfer membranes were soaked for ~1 minute in methanol before being equilibrated in PBS. Non-specific proteins were then blocked by incubating the membranes in blocking buffer, which consisted of either PBS 5% skim milk powder (PBS 5% SMP) or PBS 0.05% Tween-20 supplemented with 10% goat or horse serum (referred to as PBS-T/GS or PBS-T/HS, respectively), for 1 hour at room temperature with shaking. After quickly

rinsing 3x with PBS or PBS 0.05% Tween-20 (PBS-T), the membranes were incubated with primary antibody dissolved in blocking buffer at an appropriate dilution for 1 hour at room temperature with shaking. (Dilutions vary from 1:250 for anti-Sos1 antibody to 1:1000 for anti-GFP antibody to 1:5000 for anti-HA antibody to 1:10,000 for the other antibodies used in this study.) Membranes were then washed 3x 5 minutes each with PBS or PBS-T and then incubated with horse radish peroxidase (HRP)-conjugated secondary antibody dissolved in blocking buffer for 1 hour at room temperature with shaking. Membranes were washed 3x 5 minutes each with PBS or PBS-T, and proteins of interest were visualized by enhanced chemiluminescence (ECL) detection using a kit from Amersham Pharmacia Biotech according to manufacturer's instructions.

## 2.5.5. Far Western Blotting

The far western blotting procedure permits detection of interaction between a purified protein probe in solution and another protein immobilized on a PVDF membrane. The procedure is essentially identical to that of western blotting save for an additional step prior to incubation with the primary antibody solution. After the brief rinses of the membranes following blocking, the membranes were incubated for 1 hour at room temperature with shaking in a 0.5-1.0μg/ml protein probe solution prepared by dissolving the purified protein fraction in blocking buffer. The membranes were washed 3x 5 minutes each with PBS or PBS-T, and incubation with primary antibody and subsequent steps then proceeded as per western blotting. In this case, however, the primary antibody employed would be directed against the protein probe, and far western blotting results would indicate whether the protein probe has bound. The apparent molecular weight of the protein(s) which interacted with the protein probe could then be determined.

## 2.5.6. GST Protein Pull Down Assay

GST protein pull down assays were carried out according to a protocol derived from the procedure described in Byrne *et al.*, 1996. Briefly, HEK 293 cells

were resuspended at a concentration of roughly 3 x $10^6$ cells per milliliter of ice-cold hypotonic buffer (20mM Tris-Cl pH7.4) supplemented with protease inhibitors and lysed by dounce homogenization (see section 2.3.3). 500µl of lysate (~0.5-1mg protein) was incubated with 10µl bed volume of PBS-equilibrated glutathione-sepharose 4B resin, in the presence of 10µg of resin-purified GST or GST fusion protein. The samples were incubated for 2 hours at 4°C with end-to-end rotation. After centrifugation at 2000rpm for 5 minutes at 4°C to pellet the resin and associated proteins, the supernatant was discarded and the pellet was washed 3x with ice-cold PBS. The resin and bound proteins were finally resuspended in 2x protein sample buffer (2x PSB; 6.7% glycerol, 1.3% SDS, 3.3% β-mercaptoethanol (βME), 0.033% bromophenol blue (BPB), 41.7mM Tris-Cl pH6.8), boiled for 5 minutes, and resolved by SDS-PAGE. The presence of the Grb2 protein in the pull down samples was investigated by western blotting using an anti-Grb2 antibody.

## 2.5.7. Immunoprecipitation

The protocol used in immunoprecipitation studies was modified from the method described by Bonifacino *et al.* in *Current Protocols in Molecular Biology* (1999). Briefly, 15µl bed volume of equilibrated protein A-sepharose CL-4B resin was bound to 0.5-1.0µg rabbit anti-Grb2 polyclonal antibody in the presence of 0.1mg BSA in 10x bed volumes (150µl) of ice-cold hypotonic buffer (20mM Tris-Cl pH7.4) supplemented with protease inhibitors by incubation for 1 hour at 4°C with end-to-end rotation. The antibody-bound resin was then pelleted by centrifugation for 2-3 seconds at top speed at 4°C and washed twice with 10x bed volumes of hypotonic buffer. Parental HEK 293 cells or COS-7 cells transiently transfected with pEGFP-C2 or pEGFP-11 were grown to near confluency before being harvested for cell lysate. Cells were resuspended in ice-cold hypotonic buffer supplemented with protease inhibitors at roughly 1ml per 100mm culture dish's worth of cells and lysed by dounce homogenization (see section 2.3.3). 500µl of lysate (~0.5-1mg protein) was then incubated with anti-Grb2 antibody-bound resin, in the presence or absence of 5-10µg resin-purified GST or GST-11, for 2-3 hours at 4°C with end-to-

end rotation, with or without a prior preclearing step involving a 1-hour incubation of the lysate with protein A-sepharose resin alone. The resin was then washed twice with hypotonic buffer. Resins and associated proteins from both the preclearing and actual immunoprecipitation steps were resuspended in 2x PSB, boiled for 5 minutes, and resolved by SDS-PAGE. The presence of specific proteins in the immunoprecipitation samples was investigated by western blotting.

# RESULTS

## 3.1. Expression of Glutathione S-Transferase (GST) and GST-Tagged Fusion Proteins

The proteins GST, GST-HA, GST-HA-Grb2, GST-11, GST-11 P42A, GST-11 P42,45A, GST-11 P42,45,46A, GST-11 P42,46A, GST-11 P43A, GST-11 P43,45A, GST-11 P43,45,46A, GST-11 P45A, GST-11 P45,46A, and GST-11 P46A were expressed in DH10b *E. coli* and purified on glutathione-agarose or glutathione-sepharose resin using a batch affinity procedure as described in the Materials and Methods section (Section 2.5.1). Selected fractions of purified proteins were resolved by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 3.1). GST appears to migrate at 27kDa, GST-HA at 32kDa, and GST-HA-Grb2 at 50kDa. Wild-type and mutant GST-11 proteins run as at least three distinct bands, at 37kDa (full-length), 32kDa, and 30kDa. The 32kDa and 30kDa species are likely products from proteolytic cleavage of the full-length GST-11 protein at the carboxy-terminal.

## 3.2. GST-HA-Grb2 Interacts with GST-11 but not GST on a Far Western Blot

Resin-purified GST, GST-11 and GST-HA-Grb2 fusion proteins described in section 3.1 were employed in far western studies to confirm preliminary observations that GST-11 interacts with GST-HA-Grb2 *in vitro* (Zagrodney, 1998) (Figure 3.2). Purified fractions of GST and GST-11 were resolved by SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membrane, and probed with GST-HA-Grb2. The membrane was then subjected to detection with an anti-hemagglutinin (HA) antibody. Results suggest that GST-HA-Grb2 binds to membrane-bound GST-11 but not GST, confirming specificity of the interaction of the Grb2 fusion protein with the 11kDa moiety of GST-11. The lower molecular weight species of GST-11 also fail to bind GST-HA-Grb2. This observation is consistent with our earlier hypothesis (see Figure 3.1) that the lower molecular

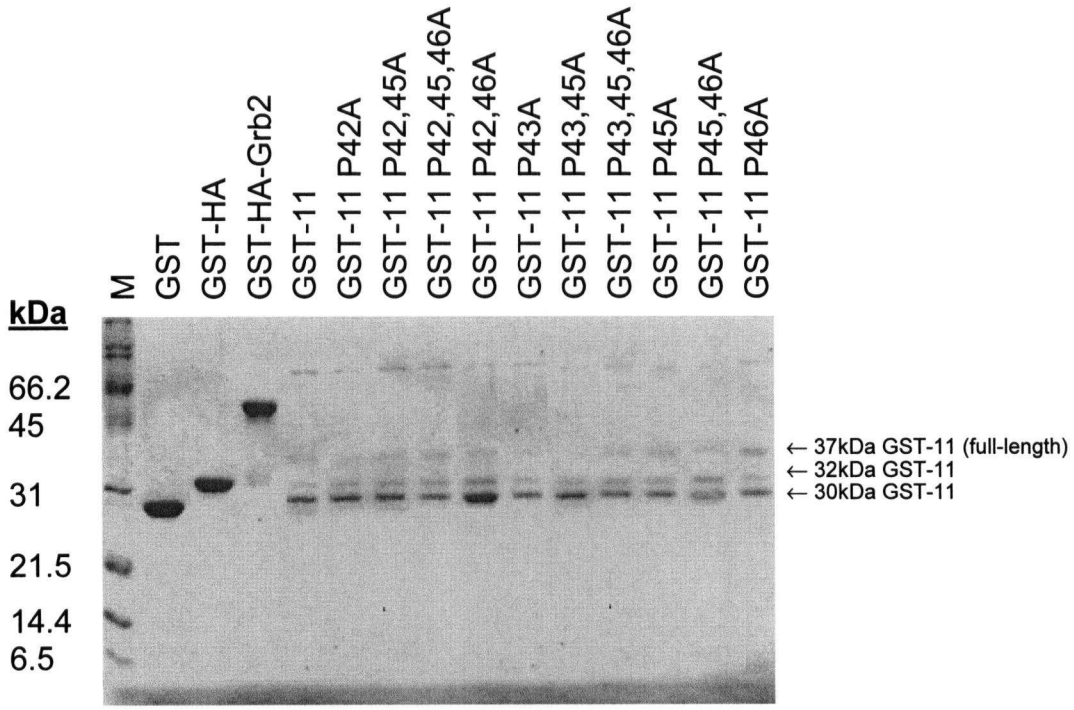


Figure 3.1. Coomassie-stained polyacrylamide gel of purified GST/GST-fusion proteins. Bacterially expressed and resin-purified fractions of GST and various GST-fusion proteins were resolved by 12.5% SDS-PAGE and visualized by staining with Coomassie Brilliant Blue. The apparent migration of GST-11 wild-type or mutant proteins is indicated.

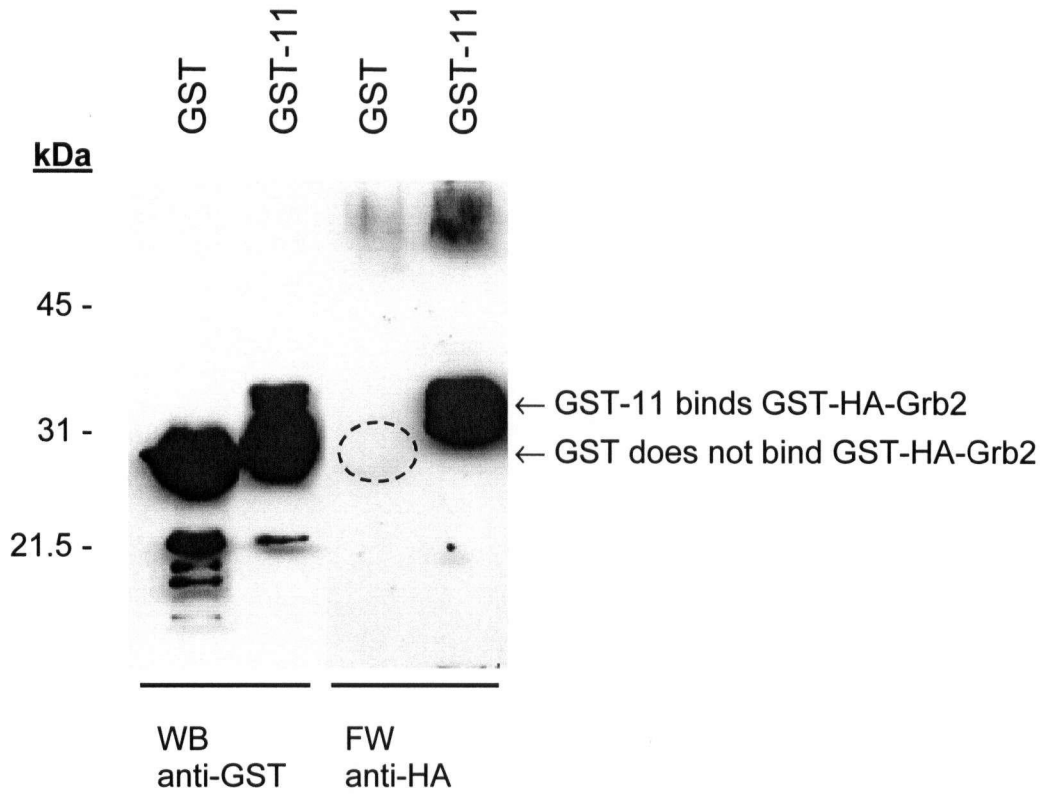


Figure 3.2. Western blot and far western blot demonstrating interaction of GST-11 but not GST with GST-HA-Grb2. Resin-purified protein preparations of GST and GST-11 were resolved by 12.5% SDS-PAGE, transferred to PVDF membrane, and subjected to either western blotting with an anti-GST antibody at 1:10,000 dilution (left) or far western blotting (right). For far western blotting, the membrane was first incubated with a protein probe consisting of resin-purified GST-HA-Grb2 diluted in blocking buffer to a final concentration of 1.0μg/ml. Any bound GST-HA-Grb2 was subsequently detected using an anti-HA antibody at 1:5000 dilution as per western blotting.

weight species represent degradation products of GST-11 lacking part or all of the 11kDa portion.

## 3.3. GST-HA-Grb2, but not GST-HA, Interacts with GST-11 on a Far Western Blot

Resin-purified GST, GST-11, GST-HA and GST-HA-Grb2 fusion proteins described in section 3.1 were employed in far western studies to confirm specificity of the interaction with Grb2 (Figure 3.3). Purified fractions of GST and GST-11 were immobilized on PVDF membrane and probed with either GST-HA or GST-HA-Grb2. Results demonstrate that GST-HA-Grb2 but not GST-HA binds to membrane-bound GST-11, suggesting that specificity of the interaction with GST-11 resides in the Grb2 portion of the GST-HA-Grb2 fusion protein.

## 3.4. Anti-Hemagglutinin (HA) Antibody Recognizes Both GST-HA and GST-HA-Grb2

In order to rule out the possibility that the lack of signal observed in the far western using GST-HA was due to the inability of the anti-HA antibody to detect GST-HA as compared with GST-HA-Grb2, both proteins were resolved by SDS-PAGE and subjected to western blotting using the anti-HA antibody (Figure 3.4). From figure 3.4, it can been seen that GST-HA and GST-HA-Grb2 are both detected with similar sensitivity by either the anti-HA antibody or the anti-GST antibody. The bands at ~31kDa in the GST-HA-Grb2 lanes likely represent degradation products of the protein. The higher molecular weight species detected by the anti-HA antibody in the GST-HA and GST-HA-Grb2 lanes may be dimerization products due to incomplete denaturation of proteins in the samples.

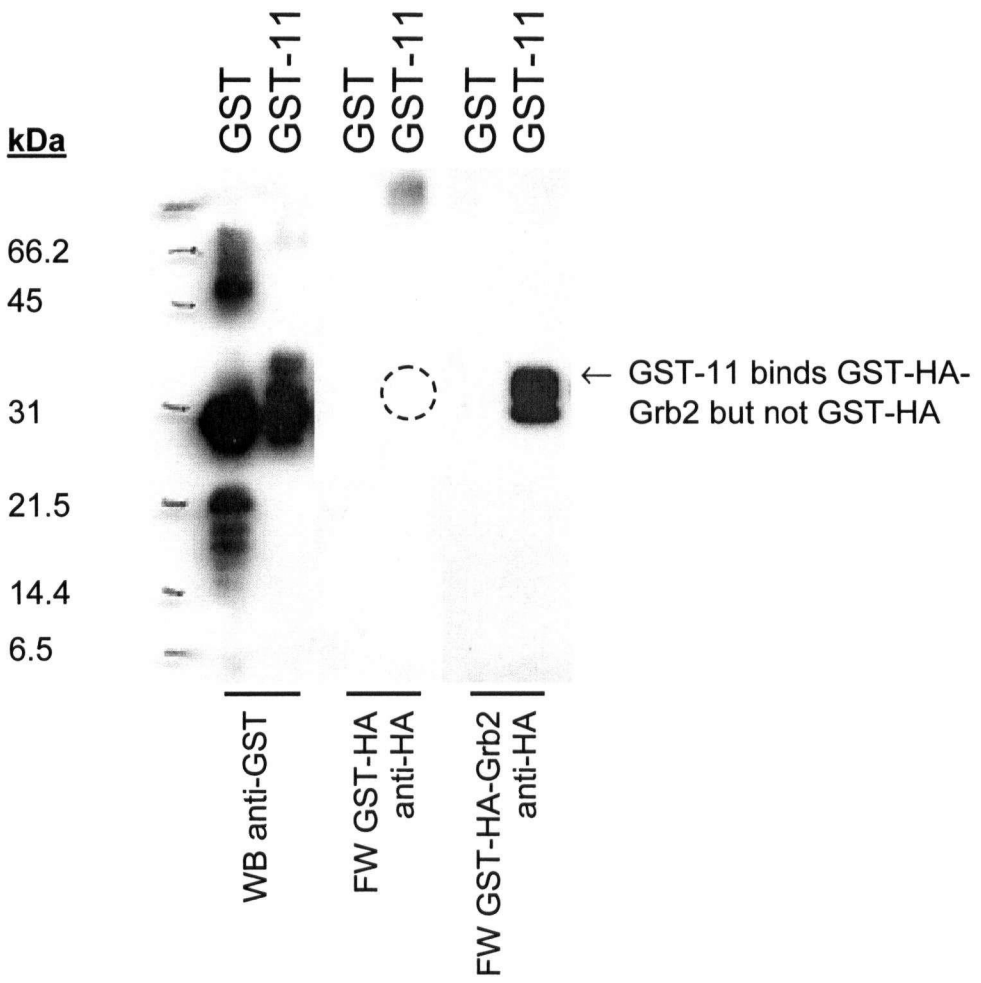


Figure 3.3. Western blot and far western blots demonstrating interaction of GST-HA-Grb2 but not GST-HA with GST-11. Resin-purified protein preparations of GST and GST-11 were resolved by 12.5% SDS-PAGE, transferred to PVDF membrane, and subjected to either western blotting with an anti-GST antibody at 1:10,000 dilution (left) or far western blotting (center and right). For far western blotting, the membranes were first incubated with a protein probe consisting of either resin-purified GST-HA (center) or resin-purified GST-HA-Grb2 (right) diluted in blocking buffer to a final concentration of 1.0μg/ml. Any bound GST-HA or GST-HA-Grb2 was subsequently detected using an anti-HA antibody at 1:5000 dilution using the western blotting protocol (Sections 2.5.4 and 2.5.5).

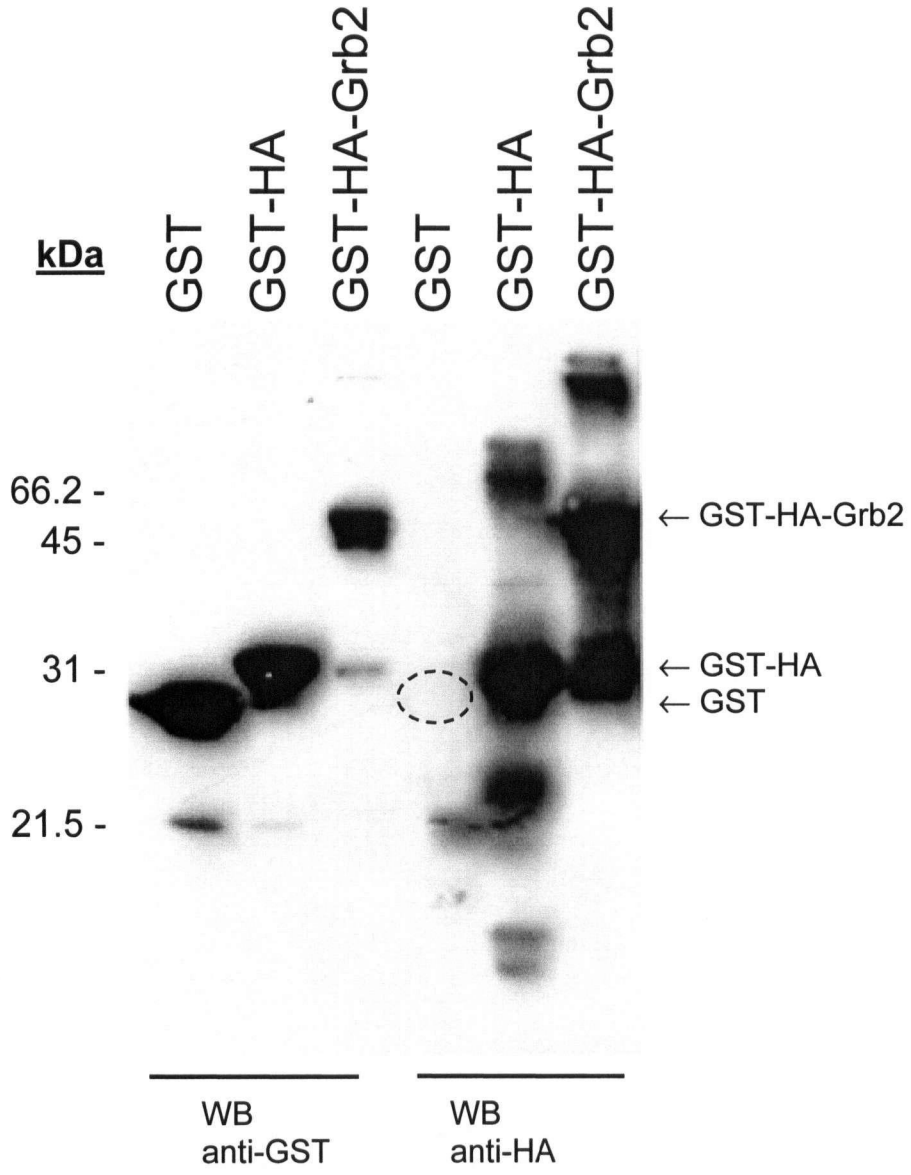


Figure 3.4. Western blots demonstrating ability of anti-HA antibody to recognize HA epitope in both GST-HA and GST-HA-Grb2. Resin-purified protein preparations of GST, GST-HA and GST-HA-Grb2 were resolved by 12.5% SDS-PAGE, transferred to PVDF membrane, and subjected to western blotting with either an anti-GST antibody at 1:10,000 dilution (left) or an anti-HA antibody at 1:5000 dilution (right).

## 3.5. GST-11 but not GST Interacts with Cellular Grb2 in a GST Protein Pull Down Assay

Resin-purified GST and GST-11 fusion proteins described in section 3.1 were employed in GST fusion protein pull down assays to investigate the ability of GST-11 to co-precipitate endogenous Grb2 from mammalian cells. This experiment assesses the interaction of soluble GST and GST-11 with cellular Grb2. 10μg of GST or GST-11 were incubated with an aliquot of the dounce-homogenized lysate from about $1.5 \times 10^6$ human embryonal kidney (HEK) 293 cells (~0.5-1mg protein) in the presence of glutathione (GSH)-sepharose resin. GST, GST-11 and any associated proteins were pelleted by centrifugation and analyzed by SDS-PAGE (Figure 3.5). Figure 3.5 shows that cellular Grb2 is effectively co-precipitated by GST-11 but not by GST, further providing support for an interaction between Grb2 and the 11kDa protein.

## 3.6. Exogenously Added GST and GST-11 Do Not Appear to Interact with Cellular Grb2 in Co-Immunoprecipitation Studies

10μg of resin-purified GST or GST-11 fusion proteins described in section 3.1 were added exogenously to a dounce-homogenized HEK 293 cell lysate (~0.5-1mg protein) in an immunoprecipitation experiment using an antibody directed against Grb2. Grb2 and any associated proteins were pelleted by centrifugation and analyzed by SDS-PAGE (Figure 3.6). In the absence of preclearing (lanes labeled "IP w/o PrCl"), GST-11 was co-immunoprecipitated with Grb2 (Figure 3.6B). However, this interaction was non-specific as GST was also co-immunoprecipitated in a parallel control experiment using GST in place of GST-11 (Figure 3.6C). Moreover, once the samples have been precleared, neither GST nor GST-11 were observed to co-immunoprecipitate with Grb2 under otherwise identical conditions (Figures 3.6B and C) even though Grb2 was clearly present in precleared samples (Figure 3.6A). Bands in the 50-55kDa range in the IP lanes (Figure 3.6B) likely

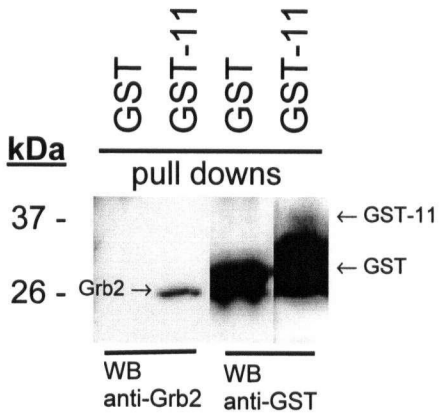


Figure 3.5. Western blots demonstrating interaction of exogenously added GST-11 but not GST with cellular Grb2 in the GST protein pull down assays. HEK 293 cell lysates were incubated with resin-purified GST or GST-11 in the presence of glutathione-sepharose resin. Any interacting proteins were co-precipitated upon centrifugation. Proteins from pull down samples were resolved by 12.5% SDS-PAGE, transferred to PVDF membrane, and subjected to western blotting with either an anti-Grb2 antibody (left) or an anti-GST antibody (right) at 1:10,000 dilution.

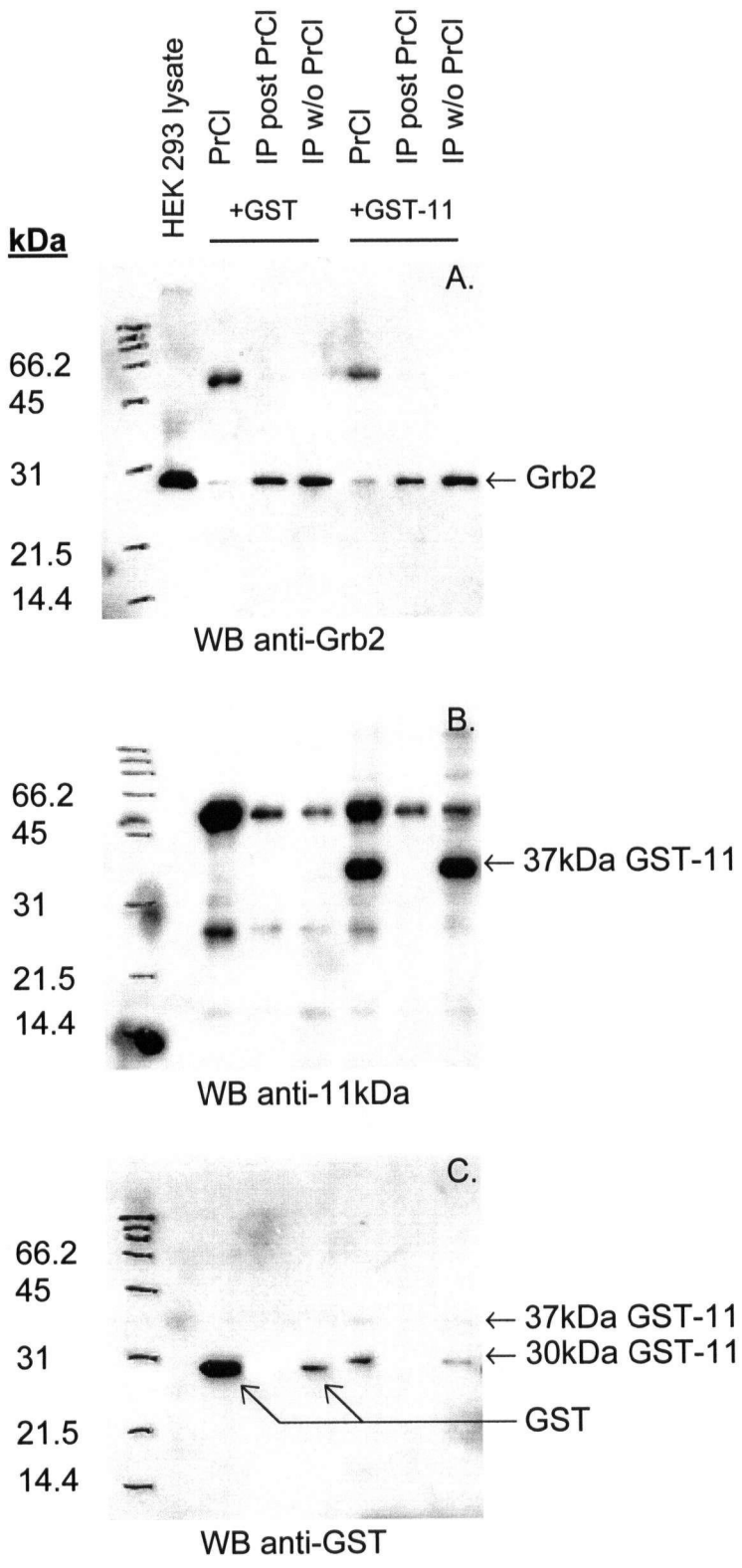


Figure 3.6. [Please see next page for figure legend.]

Figure 3.6. Western blots of anti-Grb2-immunoprecipitated samples in the presence of exogenously added GST or GST-11. HEK 293 cell lysates with added resin-purified GST or GST-11 were immunoprecipitated with an anti-Grb2 antibody in the presence of protein A-sepharose resin, with (post PrCl) or without (w/o PrCl) a prior preclearing step (using protein A-sepharose beads alone). Any interacting proteins were co-immunoprecipitated upon centrifugation. Proteins from immunoprecipitation samples were resolved by 12.5% SDS-PAGE, transferred to PVDF membrane, and subjected to western blotting with either an anti-Grb2 antibody (A), an anti-11kDa antibody (B), or an anti-GST antibody (C) at 1:10,000 dilution. Bands in the PrCl lanes correspond to proteins that bound nonspecifically to protein A-sepharose in the absence of antibody.

represent IgG heavy chains of the anti-Grb2 antibody. However, it is unclear what the other bands of a similar molecular weight in the PrCl lanes are (Figure 3.6A).

## 3.7. Addition of Exogenous GST and GST-11 Proteins Do Not Affect Grb2/Sos Interaction

If the 11kDa proteins affect the interaction of Grb2 with Sos as proposed in our hypothesis (Section 1.8), then one might expect the amount of Sos bound to Grb2 would be reduced in the presence of the 11kDa proteins. Hence immunoprecipitation samples from the experiment described in section 3.6 were subjected to western blot analysis for the Grb2-binding protein Sos. Briefly, proteins precipitated with an anti-Grb2 antibody from dounce-homogenized HEK 293 cell lysates in the absence or presence of 10μg of resin-purified GST or GST-11 fusion proteins were resolved by SDS-PAGE and probed with an antibody directed against Sos (Figure 3.7). The levels of Sos protein co-precipitated with Grb2 appear unchanged in the presence of exogenously added GST or GST-11.

## 3.8. Neither the Enhanced Green Fluorescent Protein (EGFP) Nor the EGFP-11kDa Fusion Protein (EGFP-11) Interact with Cellular Grb2 in Co-Immunoprecipitation Studies *In Vivo*

African green monkey kidney COS-7 cells transiently expressing either EGFP or EGFP-11 were dounce-homogenized and subjected to immunoprecipitation experiments using the anti-Grb2 antibody. Grb2 and any associated proteins were pelleted by centrifugation and analyzed by SDS-PAGE (Figure 3.8). In the absence of preclearing, EGFP-11 was co-immunoprecipitated with Grb2, but this interaction was non-specific as EGFP was also co-immunoprecipitated in a parallel control experiment. Different efficiencies of transfection were observed for each of the pEGFP-C2 and pEGFP-11-transfected cells, which necessitated the dilution of the EGFP-expressing cell lysate twenty-five fold with non-transfected COS-7 cell lysate in order to obtain roughly equivalent concentrations of EGFP and EGFP-11 in these

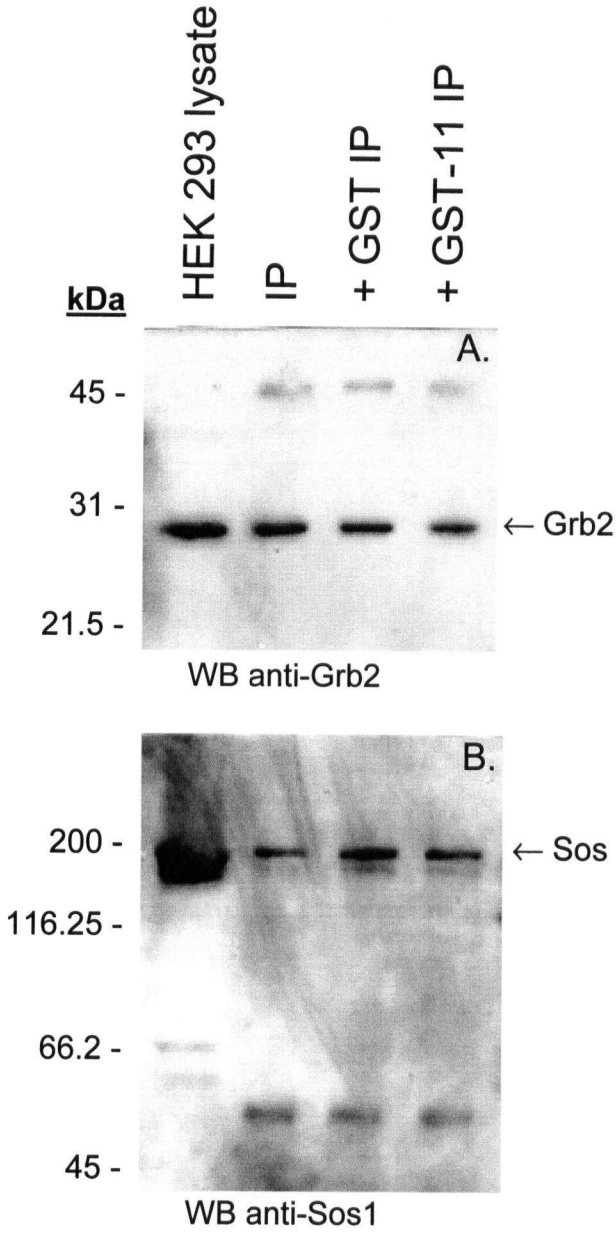


Figure 3.7. Western blots of anti-Grb2-immunoprecipitated samples in the absence or presence of exogenously added GST or GST-11. HEK 293 cell lysates with or without added resin-purified GST or GST-11 were immunoprecipitated with an anti-Grb2 antibody in the presence of protein A-sepharose resin. Any interacting proteins were co-immunoprecipitated upon centrifugation. Proteins from immunoprecipitation samples were resolved by 12.5% (A) or 7.5% (B) SDS-PAGE, transferred to PVDF membrane, and subjected to western blotting with either an anti-Grb2 antibody at 1:10,000 dilution (A) or an anti-Sos1 antibody at 1:250 dilution (B).

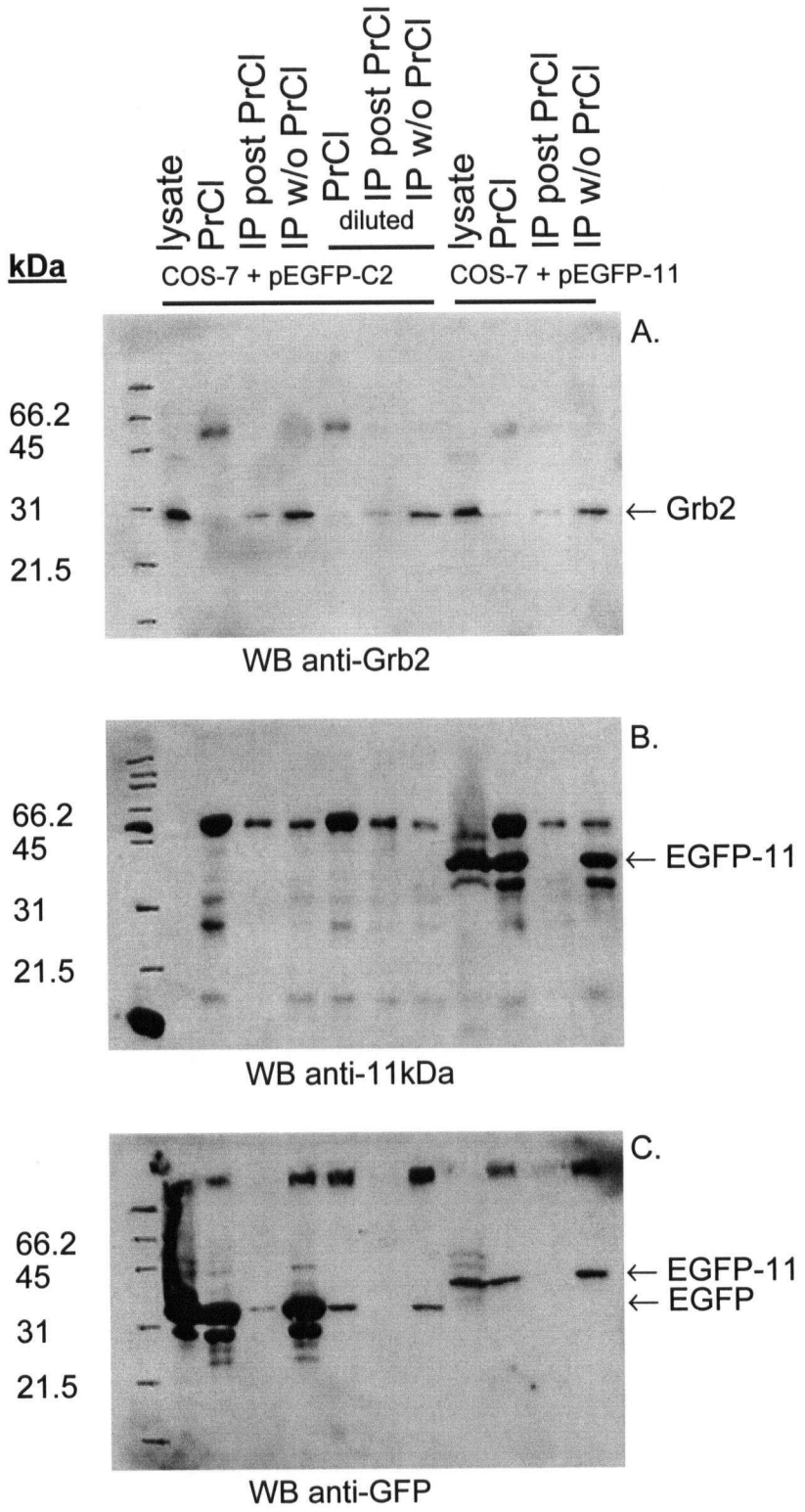


Figure 3.8. [Please see next page for figure legend.]

Figure 3.8. Western blots of anti-Grb2-immunoprecipitated samples in COS-7 cells transfected with pEGFP-C2 or pEGFP-11. Undiluted (EGFP and EGFP-11-expressing) or 25-fold diluted (EGFP-expressing) cell lysates were immunoprecipitated with an anti-Grb2 antibody in the presence of protein A-sepharose, with (post PrCl) or without (w/o PrCl) a prior preclearing step (using protein A-sepharose beads alone). Any interacting proteins were co-immunoprecipitated upon centrifugation. Proteins from immunoprecipitation samples were resolved by 12.5% SDS-PAGE, transferred to PVDF membrane, and subjected to western blotting with either an anti-Grb2 antibody at 1:10,000 dilution (A), an anti-11kDa antibody at 1:10,000 dilution (B), or an anti-GFP antibody at 1:1000 dilution (C). Bands in the PrCl lanes correspond to proteins that bound nonspecifically to protein A-sepharose in the absence of antibody.

studies. Once the samples have been precleared and transgene expression normalized against each other by dilution of the pEGFP-C2 lysate, neither EGFP nor EGFP-11 were observed to co-immunoprecipitate with Grb2 under otherwise identical conditions.

## 3.9. Levels of Sos Protein Co-Immunoprecipitated by Anti-Grb2 Antibody Are Comparable in COS-7 Cells Transiently Expressing EGFP or EGFP-11

Immunoprecipitation samples from the experiment described in section 3.8 were subjected to western blot analysis for the Grb2-binding protein Sos. Briefly, proteins precipitated with an anti-Grb2 antibody from dounce-homogenized COS-7 cells previously transfected with constructs expressing either EGFP or EGFP-11 were resolved by SDS-PAGE and probed with an antibody directed against Sos (Figure 3.9). The levels of Sos protein co-precipitated with Grb2 are comparable in COS-7 cells transfected with either construct.

## 3.10. The GST-HA-Grb2/GST-11 Interaction on a Far Western Blot Requires 11kDa Proline-Rich Region

Up to this point, it has been assumed that the interaction between Grb2 and the 11kDa proteins observed in far western blots and GST pull down experiments involved interactions of an SH3 domain (in Grb2) with the putative SH3 ligand sequences in the 11kDa proteins. To further investigate the nature of these interactions, resin-purified GST, GST-11 wild-type (wt) and mutant proteins described in section 3.1 were employed in far western studies (Figure 3.10). Purified fractions of GST and GST-11 wt/mutant proteins were resolved by SDS-PAGE, transferred to PVDF membrane, and probed with GST-HA-Grb2. The membrane was then subjected to detection with an anti-hemagglutinin (HA) antibody. Results indicate that GST-HA-Grb2 binds with reduced affinity to membrane-bound GST-11 proteins mutated in certain proline residues, as

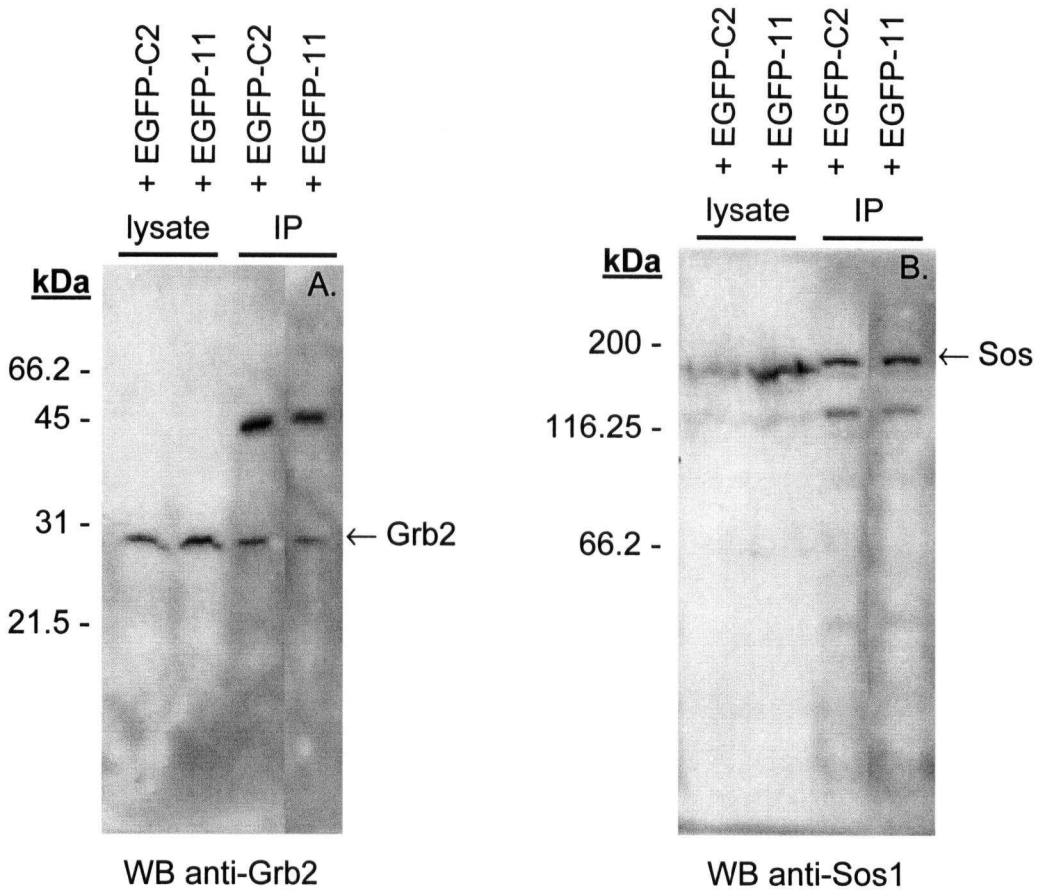


Figure 3.9. Western blots of anti-Grb2-immunoprecipitated samples from COS-7 cells transfected with pEGFP-C2 or pEGFP-11. Undiluted cell lysates were immunoprecipitated with an anti-Grb2 antibody in the presence of protein A-sepharose. Any interacting proteins were co-immunoprecipitated upon centrifugation. Proteins from immunoprecipitation samples were resolved by 12.5% (A) or 7.5% (B) SDS-PAGE, transferred to PVDF membrane, and subjected to western blotting with either an anti-Grb2 antibody at 1:10,000 dilution (A) or an anti-Sos1 antibody at 1:250 dilution (B).

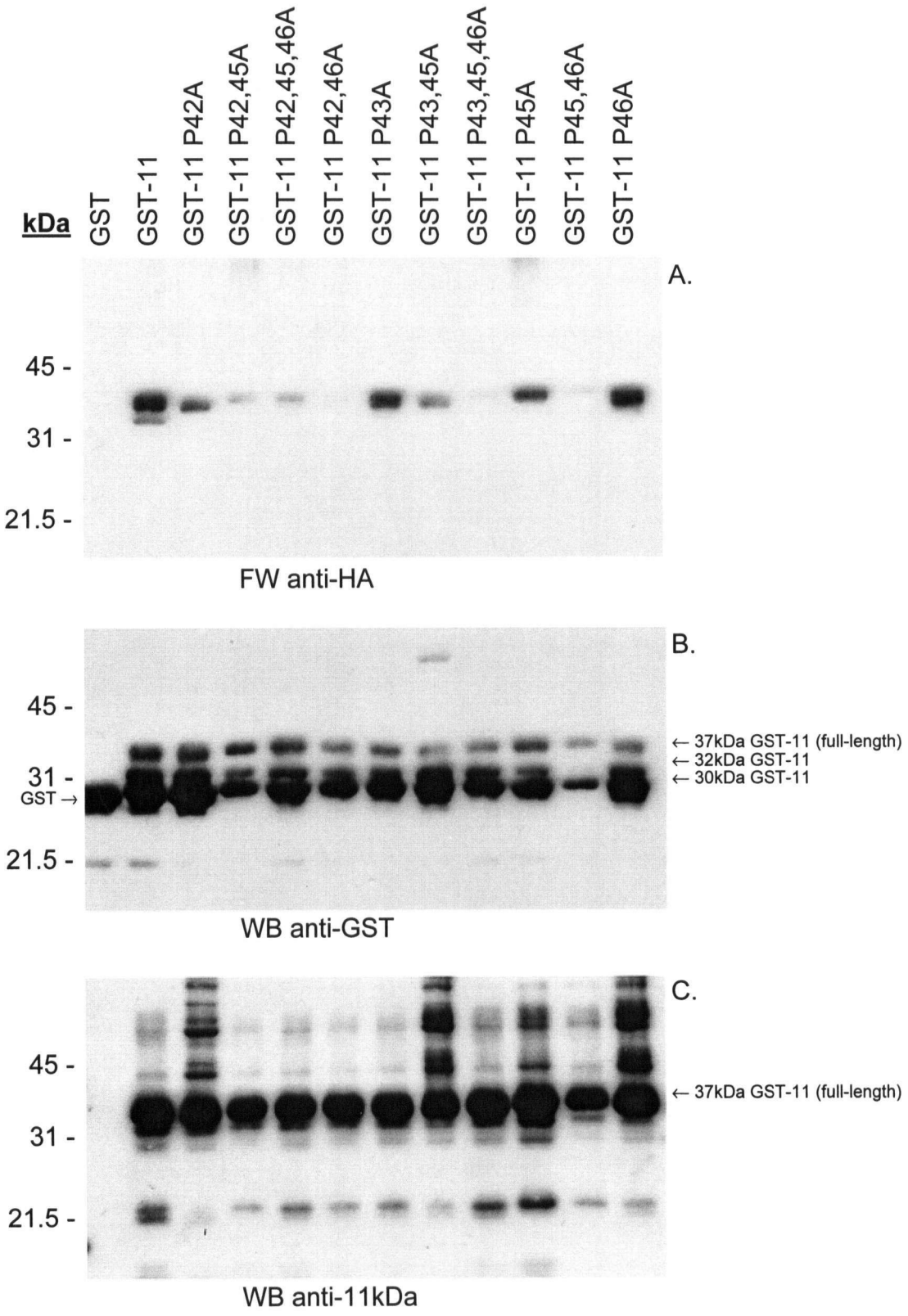


Figure 3.10. [Please see next page for figure legend.]

Figure 3.10. Far western blot and western blots demonstrating differential strength of interaction of GST-11 wt/mutant proteins with GST-HA-Grb2. Resin-purified protein preparations of GST, GST-11, and GST-11 mutants were resolved by 12.5% SDS-PAGE, transferred to PVDF membrane, and subjected to either far western blotting (A) or western blotting (B and C). For far western blotting, the membrane was first incubated with a protein probe consisting of resin-purified GST-HA-Grb2 diluted in blocking buffer to a final concentration of 0.5μg/ml. Any bound GST-HA-Grb2 was subsequently detected using an anti-HA antibody at 1:5000 dilution. For western blotting, the membranes were detected with either an anti-GST antibody (B) or an anti-11kDa antibody (C) at 1:10,000 dilution.

compared with wt GST-11. While the binding of Grb2 to GST-11 is not greatly affected by single mutations at proline residues 43 or 46, multiple mutations in the GST-11 protein appear to significantly reduce the interaction. In particular, a marked decrease in interaction with GST-HA-Grb2 is observed in GST-11 proteins mutated at proline positions 42 and 46; 43, 45 and 46; and 45 and 46. These results strongly suggest this proline-rich region is involved in mediating interactions of the 11kDa protein with Grb2 in far western experiments.

## 3.11. The Grb2/GST-11 Interaction in a GST Protein Pull Down Assay Requires 11kDa Proline-Rich Region

The effect of mutating specific proline residues on Grb2/11kDa interactions as observed in GST protein pull down experiments was also examined. Resin-purified GST, GST-11 wild-type (wt) and GST-11 mutant proteins described in section 3.1 were compared for their ability to co-precipitate endogenous Grb2 from mammalian cells. 10μg of GST, GST-11 or GST-11 mutant were incubated with a dounce-homogenized lysate from about $1.5 \times 10^6$ human embryonal kidney (HEK) 293 cells in the presence of glutathione (GSH) sepharose resin. GST, GST-11 (wt/mutant) and any associated proteins were pelleted by centrifugation and analyzed by SDS-PAGE (Figure 3.11). Results again suggest that Grb2 binds with reduced affinity to GST-11 proteins mutated in certain proline residues, as compared with wt GST-11. Single mutations at proline positions 43 or 46 do not appear to interfere with the ability of GST-11 to co-precipitate Grb2. These results parallel those from the far western blotting experiments (Section 3.10) and provide further support that the proline-rich region is involved in mediating interactions of the 11kDa protein with Grb2.

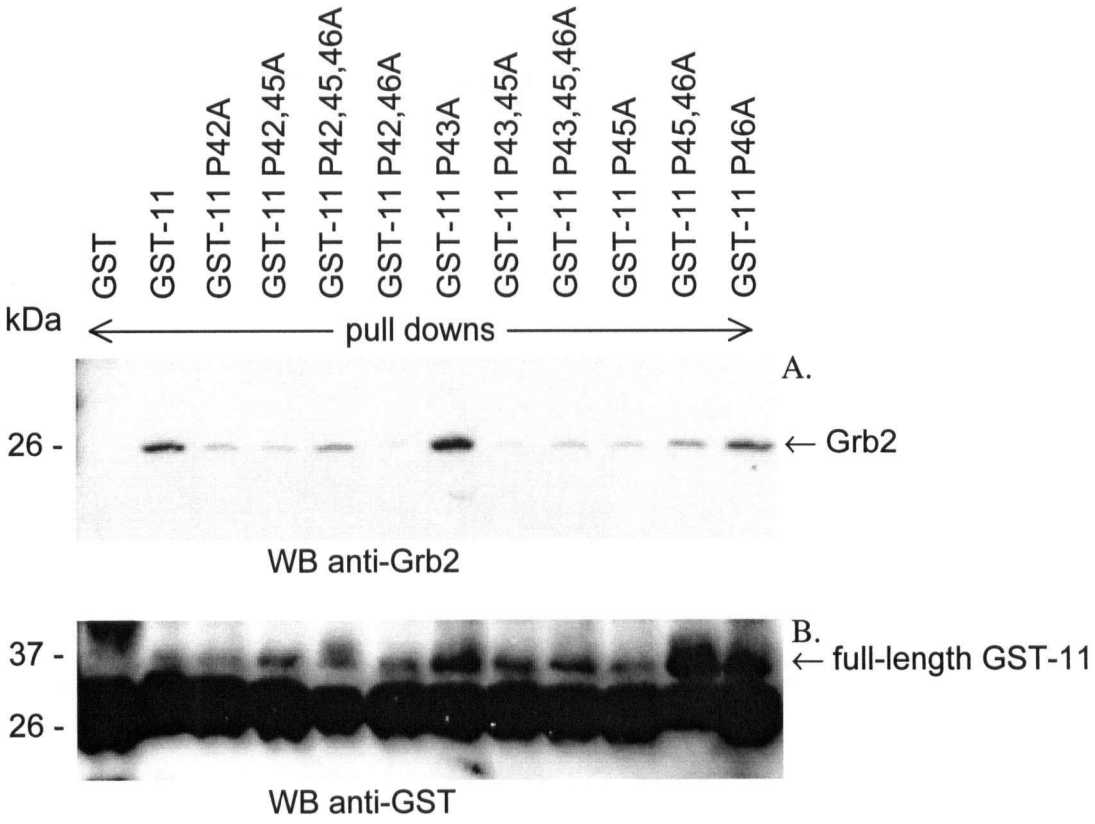


Figure 3.11. Western blots demonstrating differential strength of interaction of exogenously added GST-11 wt/mutant proteins with cellular Grb2 in a GST protein pull down assay. HEK 293 cell lysates were incubated with resin-purified GST, GST-11, or GST-11 mutants in the presence of glutathione-sepharose resin. Any interacting proteins were co-precipitated upon centrifugation. Proteins from pull down samples were resolved by 12.5% SDS-PAGE, transferred to PVDF membrane, and subjected to western blotting with either an anti-Grb2 antibody (A) or an anti-GST antibody (B) at 1:10,000 dilution.

# DISCUSSION

In this study, further evidence is provided in support of the hypothesis that the small, unique 11kDa proteins produced by parvovirus B19 may play a role in the viral life cycle by interfering with the host environment through its interactions with cellular signaling molecules such as the growth factor receptor-binding protein 2 (Grb2).

## 4.1. Characterization of the B19 11kDa/Grb2 Interaction

### 4.1.1. Far Western Studies

The initial observation that the 11kDa protein in the form of a GST-11kDa fusion protein (GST-11) interacted with GST-HA-Grb2 in a far western experiment provides the basis for the investigation carried out in this study. This interaction has since been confirmed. Membrane-immobilized GST-11 interacts with GST-HA-Grb2 in solution, and this interaction is specific to the 11kDa portion of the protein as GST alone fails to bind GST-HA-Grb2 in the far western. Similarly, while an antibody directed against the hemagglutinin (HA) epitope readily detects GST-HA-Grb2 which has bound to immobilized GST-11, no signal is observed if GST-HA-Grb2 is replaced with GST-HA in the far western protocol. This demonstrates that specificity of the interaction resides in the Grb2 portion of the fusion protein as predicted.

### 4.1.2. Pull Down Assays

A GST fusion protein pull down assay was carried out to investigate the ability of GST-11 to interact with endogenous, cellular Grb2 in mammalian cells. A human embryonal kidney (HEK) cell line, 293, was used in these studies. Results from the assay indicate that Grb2 is readily co-precipitated with GST-11 but not GST, once again confirming the specific binding of 11kDa to Grb2.

### 4.1.3. Immunoprecipitation Studies

Numerous attempts were made to characterize this interaction further in an *in vivo* cell model, but all immunoprecipitation experiments to date have failed to yield conclusive results.

While pilot studies suggested an interaction in a COS-7 cell model between the transfected EGFP-11kDa fusion protein (EGFP-11) and cellular Grb2, later experiments have shown this binding to be nonspecific and removable by an earlier preclearing step. In these studies, efficiency of transfection was consistently low despite various attempts at optimization by varying transfection conditions. To compound the difficulties, transfection of pEGFP-11 (expressing the EGFP-11 protein, ~2% fluorescing cells) typically yielded 10- to 20-fold fewer fluorescing cells than pEGFP-C2 (expressing the EGFP protein, ~20-50% fluorescing cells), suggesting a significant decrease in transfection efficiency for pEGFP-11. The reduced number of fluorescing cells may also be due to misfolding of a sub-population of expressed EGFP-11, which may affect the excitation/emission properties of the fusion protein. However, this second possibility is not supported by data from western blotting experiments which indicate a 10- to 20-fold increase in EGFP expression as compared with EGFP-11 expression from a comparable number of cells, consistent with observed differences in the apparent levels of fluorescence between pEGFP-C2 and pEGFP-11-transfected cells. These findings may indicate a reduced half life of the fusion protein and leave one to question if over-expression of the 11kDa fusion protein is toxic in these cells.

A different model system was investigated in another attempt to demonstrate the Grb2/11kDa interactions *in vivo*. Untransfected HEK 293 cell lysates were subjected to immunoprecipitation in the presence of bacterially expressed and affinity purified GST or GST-11 protein. Similar observations of nonspecific binding were noted. While we were able to show the Grb2/11kDa interaction in both far western and pull down experiments, we have not been successful in using co-immunoprecipitation to demonstrate an interaction between endogenous Grb2 and the 11kDa protein expressed as either an EGFP or GST fusion

construct. This suggests that while the binding between the two proteins is strong enough to be detected by far western and pull down approaches, it may not be stable enough to allow co-immunoprecipitation of the two proteins under the conditions tested.

## 4.2. Src Homology 3 (SH3)-Mediated Interactions and B19 11kDa Proteins

The binding of Grb2 to 11kDa is thought to perturb the normal function of the Grb2 protein in binding Sos. Interactions of Grb2 with the guanine nucleotide exchange factor Sos involves the binding of proline-rich motifs (known as Src homology 3 or SH3 ligands) in Sos with the cognate domains (SH3 domains) in Grb2. Sos contains multiple such SH3 recognition sites, consisting of proline-rich PxxP motifs which adopt a polyproline type II (PP-II) helix conformation (Ren *et al.*, 1993; Yu *et al.*, 1994). The B19 11kDa proteins are also rich in similar proline-rich sequences, and in fact, one of these motifs (RPPVPPR) strongly resembles a known SH3 binding site on Sos with the sequence PPPVPPR. It therefore seems likely that the binding of Grb2 to 11kDa may be mediated by the same type of SH3 domain/ligand interactions as those responsible for Grb2/Sos interactions during normal host signaling.

To test the requirement for specific proline residues within the afore-mentioned SH3 ligand-like sequence of the 11kDa in mediating interactions with Grb2, PCR-based site-directed mutagenesis was carried out to substitute selected proline residues with alanine residues. The resulting mutant proteins were expressed as GST-fusion proteins and subjected to far western studies to test their ability to bind the GST-HA-Grb2 fusion protein *in vitro*.

## 4.3. Effects of Proline-to-Alanine Mutations on B19 11kDa/Grb2 Interactions

### 4.3.1. Far Western Studies

Ten mutants were derived from GST-11 where one or more proline residues were selectively replaced with alanine residues. These were tested alongside GST and GST-11 in far western experiments to determine the requirement of the 11kDa/Grb2 interaction for the proline-rich region in question. Singly mutated GST-11 proteins appeared to retain the ability to interact with GST-HA-Grb2, while a reduced interaction was observed for several of the double and triple mutants. The interaction was markedly decreased for GST-11 proteins mutated at proline positions 42 and 46; 43, 45 and 46; and 45 and 46. These results suggest that while no single proline residue is critical for SH3 binding, multiple prolines together contribute to the interaction.

### 4.3.2. Pull Down Assays

The GST-11 mutants were also subjected to pull down assays to further investigate the ability of various GST-11 mutant constructs to pull down endogenous Grb2 from mammalian cell lysates. Results obtained here generally mirror those from the far western studies, namely that most of the GST-11 mutant proteins displayed reduced interaction with Grb2, as compared with wild-type GST-11. Single mutations at proline positions 43 or 46 did not appear to interfere with the ability of GST-11 to co-precipitate Grb2, and indeed, no single-proline mutation studied here was capable of completely abrogating the interaction. Nevertheless, these results, which mimic the results of the far western studies (Section 4.3.1), provide strong support for the involvement of the proline-rich region of the B19 11kDa proteins as a conformationally defined unit in mediating interactions with Grb2.

## 4.4. Implications of Present Findings on Possible Functions of B19 11kDa Proteins

The process of elucidating the function of the B19 11kDa proteins is ongoing. These proteins do not seem to be involved in structural aspects of virion production as their expression does not appear to affect viral capsid formation (Cohen *et al.*, 1995). The ability of the 11kDa proteins to interact with cellular Grb2, however, suggests a possible mechanism of the B19 parvovirus to manipulate the host cell environment by modulating signaling pathways. The 11kDa proteins contain multiple proline-rich regions analogous to SH3 recognition sites on the signaling molecule Sos, which may facilitate binding to Grb2 in a similar manner. As such, it is possible that interaction of 11kDa with Grb2 may affect downstream signaling events including those stemming from mitogenic stimuli (Figure 1.8). Examples of virally encoded nonstructural proteins which interact with SH3 domain proteins and/or disrupt mitogenic signaling include the hepatitis C virus NS5A protein (Tan *et al.*, 1999) and the human immunodeficiency virus type 1 Nef protein (Saksela *et al.*, 1995; Manninen *et al.*, 1998), both of which are implicated in modulation of host signaling cascades involved in virulence and pathogenesis. The B19 11kDa proteins may function in a similar way to enhance viral replication and propagation.

## 4.5. Future Directions

Far western and GST pull down experiments indicate an interaction between the B19 11kDa proteins and Grb2, and studies using proline-to-alanine mutants of the 11kDa proteins implicate a role for the proline-rich region in binding the SH3 domain of Grb2. The inability to observe this interaction in co-immunoprecipitation studies suggests that these interactions may not be sufficiently stable to be detected by this approach. If the interaction is relatively weak and not stable to the multiple washing steps, it may be useful to include a cross-linking step prior to washing.

Having established the involvement of at least one of the three proline-rich sequences of the 11kDa proteins in binding to Grb2 in far western and pull down

assays, it will be instructive to mutate the remaining two regions individually or in combination to examine the requirement for, and possible synergistic or compensatory effects from, these proline-rich regions in the Grb2/11kDa interaction.

Some additional problems to be addressed include the need to obtain higher levels of transfection efficiency and possibly the selection of stable cell lines with inducible expression, as we suspect the 11kDa proteins may be toxic in cells. It is also important to scale up the purification of cellular proteins shown to interact with the 11kDa proteins in affinity chromatography experiments (Zagrodney, 1998) and to use mass spectrometry analysis to determine the identities of the ~83kDa and ~26kDa putatively interacting proteins.

## 4.6. Conclusions

This study was undertaken in an attempt to further elucidate the functional role of the small 11kDa proteins produced by B19 parvovirus. Preliminary findings pointing to an interaction between the 11kDa proteins and cellular Grb2 have been confirmed, and the nature of this interaction has been determined to require a proline-rich region within the 11kDa proteins. Progress has been made to further our understanding of the function of these proteins, though much remains to be learned from this class of small proteins so unique to the parvovirus B19.

# REFERENCES

Agbandje, M.; Kajigaya, S.; McKenna, R.; Young, N.S. and Rossmann, M.G. (1994). "The Structure of Human Parvovirus B19 at 8A Resolution". *Virology*. 203(1): 106-115.

Ahn, K.; Angulo, A.; Ghazal, P.; Peterson, P.A.; Yang, Y. and Fruh, K. (1996). "Human Cytomegalovirus Inhibits Antigen Presentation by a Sequential Multistep Process". *Proceedings of the National Academy of Sciences of the U.S.A.* 93(20): 10990-10995.

Aiken, C. and Trono, D. (1995). "Nef Stimulates Human Immunodeficiency Virus Type 1 Proviral DNA Synthesis"" *Journal of Virology*. 69: 5048-5056.

Albini, A.; Ferrini, S.; Benelli, R.; Sforzini, S.; Giunciuglio, D.; Aluigi, M.G.; Proudfoot, A.E.; Alouani, S.; Wells, T.N.; Mariani, G.; Rabin, R.L.; Farber, J.M. and Noonan, D.M. (1998). "HIV-1 Tat Protein Mimicry of Chemokines". *Proceedings of the National Academy of Sciences of the U.S.A.* 95(22): 13153-13158.

Alcami, A. and Koszinowski, U.H. (2000). "Viral Mechanisms of Immune Evasion". *Immunology Today*. 21(9): 447-455.

Alonso-Caplen, F.V.; Nemeroff, M.E.; Qiu, Y. and Krug, R.M. (1992). "Nucleocytoplasmic Transport: The Influenza Virus NS1 Protein Regulates the Transport of Spliced NS2 mRNA and Its Precursor NS1 mRNA". *Genes and Development*. 6(2): 255-267.

Anand, A.; Gray, E.S.; Brown, T.; Clewley, J.P. and Cohen, B.J. (1987). "Human Parvovirus Infection in Pregnancy and Hydrops Fetalis". *New England Journal of Medicine*. 316(4): 183-186.

Anderson, L.J. (1990). "Human Parvovirus B19". *Pediatric Annals*. 19(9): 509-510, 512-513.

Anderson, L.J. and Hurwitz, E.S. (1988). "Human Parvovirus B19 and Pregnancy". *Clinics in Perinatology*. 15: 273-286.

Anderson, L.J.; Tsou, C.; Parker, R.A. ; Chorba, T.L. ; Wulff, H. ; Tattersall, P. and Mortimer, P.P. (1986). "Detection of Antibodies and Antigens of Human Parvovirus B19 by Enzyme-Linked Immunosorbent Assay". *Journal of Clinical Microbiology*. 24(4): 522-526.

Anderson, M.J.; Higgins, P.G.; Davis, L.R.; Willman, J.S.; Jones, S.E.; Kidd, I.M.; Pattison, J.R. and Tyrrell, D.A. (1985). "Experimental Parvoviral Infection in Humans". *Journal of Infectious Diseases*. 152(2): 257-265.

Anderson, M.J.; Jones, S.E.; Fisher-Hoch, S.P.; Lewis, E.; Hall, S.M.; Bartlett, C.L.; Cohen, B.J.; Mortimer, P.P. and Pereira, M.S. (1983). "Human Parvovirus, the Cause of Erythema Infectiosum (Fifth Disease)?" *Lancet*. 1: 1378. (letter)

Astell, C.R. (1990). "Terminal Hairpins of Parvovirus Genomes and Their Role in DNA Replication". In: Tijssen, P. ed. *Handbook of Parvoviruses*. Boca Raton: CRC Press, Inc. Vol. 1 pp. 59-79.

Astell, C.R. (1999a). "Parvoviruses: General Features". In: Granoff, A. and Webster, R.G. eds. *Encyclopedia of Virology*. 2nd Edition. Academic Press. pp. 1151-1155.

Astell, C.R. (1999b). "Parvoviruses: Molecular Biology". In: Granoff, A. and Webster, R.G. eds. *Encyclopedia of Virology*. 2nd Edition. Academic Press. pp. 1155-1159.

Astell, C.R.; Luo, W.; Brunstein, J. and St. Amand, J. (1997). "B19 Parvovirus: Biochemical and Molecular Features". In: Anderson, L.J. and Young, N.S. eds. "Human Parvovirus B19". In: Parks, W.P. *Monographs in Virology*. Basel: Karger. Vol. 20 pp. 16-41.

Barco, A. and Carrasco, L. (1995). "A Human Virus Protein, Poliovirus Protein 2BC, Induces Membrane Proliferation and Blocks the Exocytic Pathway in the Yeast Saccharomyces Cerevisiae". *EMBO Journal*. 14(14): 3349-3364.

Barry, M. and McFadden, G. (1997). "Virus Encoded Cytokines and Cytokine Receptors". *Parasitology*. 115: S89-S100.

Bartenschlager, R.; Lohmann, V.; Wilkinson, T. and Koch, J.O. (1995). "Complex Formation Between the NS3 Serine-Type Proteinase of the Hepatitis C Virus and NS4A and Its Importance for Polyprotein Maturation". *Journal of Virology*. 69(12): 7519-7528.

Bartz, S.R.; Rogel, M.E. and Emerman, M. (1996). "Human Immunodeficiency Virus Type 1 Cell Cycle Control: Vpr is Cytostatic and Mediates G2 Accumulation by a Mechanism Which Differs From DNA Damage Checkpoint Control". *Journal of Virology*. 70: 2324-2331.

Batterson, W. and Roizman, B. (1983). "Characterization of the Herpes Simplex Virus Virion-Associated Factor Responsible for Induction of Alpha Genes". *Journal of Virology*. 46: 371-377.

Beard, C.; St. Amand, J. and Astell, C.R. (1989). "Transient Expression of B19 Parvovirus Gene Products in COS-7 Cells Transfected with B10-SV40 Hybrid Vectors". *Virology*. 172: 659-664.

Berns, K.I. (1990). "Parvovirus Replication". *Microbiology Reviews*. 54: 316-329.

Berns, K.I. (1996). "Parvoviridae: The Viruses and Their Replication". In: Fields, B.N.; Knipe, D.M.; Howley, P.M. et al. eds. *Virology*. 3rd Edition. Philadelphia: Lippincott-Raven. pp. 2173-2197.

Bertoni, E.; Rosati, A.; Zanazzi, M. ; Azzi, A. ; Zakrzewfka, K. ; Guidi, S. and Salvadori, M. (1995). "Severe Aplastic Anaemia due to B19 Parvovirus Infection in Renal Transplant Recipient". *Nephrology, Dialysis, Transplantation*. 10(8): 1462-1463.

Blundell, M.; Beard, C. and Astell, C.R. (1987). "In Vitro Identification of a B19 Parvovirus Promoter". *Virology*. 157: 534-538.

Bonifacino, J.S.; Dell'Angelica, E.C. and Springer, T.A. (1999). "Immunoprecipitation". In: Ausubel, F.M.; Brent, R.; Kingston, R.E.; Moore, D.D.; Seidman, J.G.; Smith, J.A. and Struhl, K. eds. *Current Protocols in Molecular Biology*. John Wiley & Sons, Inc. pp. 10.16.1-10.16.29.

Brady, H.M.; Abraham, D.J.; Pennington, D.J.; Miles, C.G.; Jenkins, S. and Dzierzak, E.A. (1995). "Altered Cytokine Expression in T Lymphocytes from Human Immunodeficiency Virus Tat Transgenic Mice". *Journal of Virology*. 69(12): 7622-7629.

Brown, C.R.; Nakamura, M.S.; Mosca, J.D.; Hayward, G.S.; Straus, S.E. and Perera, L.P. (1995). "Herpes Simplex Virus Trans-Regulatory Protein ICP27 Stabilizes and Binds to 3' Ends of Labile mRNA". *Journal of Virology*. 69(11): 7187-7195.

Brown, K.E. (2000). "Haematological Consequences of Parvovirus B19 Infection". *Bailliere's Best Practice in Clinical Haematology*. 13(2): 245-259.

Brown, K.E.; Anderson, S.M. & Young, N.S. (1993). "Erythrocyte P Antigen: Cellular Receptor for B19 Parvovirus". *Science*. 262: 114-117.

Brown, K.E.; Green, S.W.; O'Sullivan, M.G. and Young, N.S. (1995a). "Cloning and Sequencing of the Simian Parvovirus Genome". *Virology*. 210(2): 314-322.

Brown, K.E.; Green, S.W.; Young, N.S.; Anderson, S.; Momoeda, M.; Kawase, M.; Kajigaya, S. and Young, N.S. (1995b). "Peptides Derived from the Unique Region of B19 Parvovirus Minor Capsid Proteins Elicit Neutralizing Antibodies in Rabbits". *Virology*. 206: 626-632.

Brown, K.E.; Hibbs, J.R.; Gallinella, G.; Anderson, S.M.; Lehman, E.D.; McCarthy, P. and Young, N.S. (1994a). "Resistance to Parvovirus B19 Infection due to Lack of Virus Receptor (Erythrocyte P Antigen)". *New England Journal of Medicine*. 330(17): 1192-1196.

Brown, K.E.; Mori, J.; Cohen, B.J. and Field, A.M. (1991). "In Vitro Propagation of Parvovirus B19 in Primary Foetal Liver Culture". *Journal of General Virology.* 72: 741-745.

Brown, K.E.; Young, N.S. and Liu, J.M. (1994b). "Molecular, Cellular and Clinical Aspects of Parvovirus B19 Infection". *Critical Reviews In Oncology/Hematology.* 16: 1-31.

Brown, T.; Anand, A.; Ritchie, L.D.; Clewley, J.P. and Reid, T.M. (1984). "Intrauterine Parvovirus Infection Associated with Hydrops Fetalis". *Lancet.* 2: 1033-1034. (letter)

Brunstein, J.; Soderlund-Venermo, M. and Hedman, K. (2000). "Identification of a Novel RNA Splicing Pattern as a Basis of Restricted Cell Tropism of Erythrovirus B19". Virology. 274(2): 284-291.

Bukrinsky, M.; Haggerty, S.; Dempsey, M.P.; Sharova, N.; Adzhubel, A.; Spitz, L.; Lewis, P.; Goldfarb, D.; Emerman, M. and Stevenson,M. (1993). "A Nuclear Localization Signal within HIV-1 Matrix Protein that Governs Infection of Non-Dividing Cells". *Nature.* 365: 666-669.

Bukrinsky, M. and Adzhubei, A. (1999). "Viral Protein R of HIV-1". *Reviews in Medical Virology.* 9(1): 39-49.

Byrne, J.L.; Paterson, H.F. and Marshall, C.J. (1996). "p21Ras Activation by the Guanine Nucleotide Exchange Factor Sos, Requires the Sos/Grb2 Interaction and a Second Ligand-Dependent Signal Involving the Sos N-Terminus". *Oncogene.* 13(10): 2055-2065.

Campbell, M.E.; Palfreyman, J.W. and Preston, C.M. (1984). "Identification of Herpes Simplex Virus DNA Sequences Which Encode a Trans-Acting Polypeptide Responsible for Stimulation of Immediate Early Transcription". *Journal of Molecular Biology.* 180: 1-19.

Casaz, P.; Sundseth, R. and Hansen, U. (1991). "Trans-Activation of the Simian Virus 40 Late Promoter by Large T Antigen Requires Binding Sites for the Cellular Transcription Factor TEF-1". *Journal of Virology.* 65: 6535-6543.

Cassetti, M.A. and Moss, B. (1996). "Interaction of the 82kDa Subunit of the Vaccinia Virus Early Transcription Factor Heterodimer with the Promoter Core Sequence Directs Downstream Binding of the 70kDa Subunit". *Proceedings of the National Academy of Sciences of the U.S.A.* 93: 7540-7545.

Castelli, J.C.; Hassel, B.A.; Wood, K.A.; Li, X.L.; Amemiya, K.; Dalakas, M.C.; Torrence, P.F. and Youle, R.J. (1997). "A Study of the Interferon Antiviral

Mechanism: Apoptosis Activation by the 2-5A System". *Journal of Experimental Medicine*. 186: 967-972.

Chen, Z.; Li, Y. and Krug, R.M. (1999). "Influenza A Virus NS1 Protein Targets Poly(A)-Binding Protein II of the Cellular 3'-End Processing Machinery". *EMBO Journal*. 18(8): 2273-2283.

Chen, Z. and Krug, R.M. (2000). "Selective Nuclear Export of Viral mRNAs in Influenza-Virus-Infected Cells". *Trends in Microbiology*. 8(8): 376-383.

Chorba, T.L.; Coccia, P.; Holman, R.C.; Tattersall, P.; Anderson, L.J.; Sudman, J.; Young, N.S.; Kurczynski, E.; Saarinen, U.M.; Moir, R.; Lawrence, D.N.; Jason, J.M. and Evatt, B. (1986). "The Role of Parvovirus B19 in Aplastic Crisis and Erythema Infectiosum (Fifth Disease)". *Journal of Infectious Diseases*. 154(3): 383-393.

Clemens, M.J. (1997). "PKR: A Protein Kinase Regulated by Double-Stranded RNA". *The International Journal of Biochemistry and Cell Biology*. 29(7): 945-949.

Clemens, M.J. and Elia, A. (1997). "The Double-Stranded RNA-Dependent Protein Kinase PKR: Structure and Function". *Journal of Interferon and Cytokine Research*. 17: 503-524.

Cohen, B.J.; Field, A.M.; Mori, J.; Brown, K.E.; Clewley, J.P.; St. Amand, J. and Astell, C.R. (1995). "Morphology and Antigenicity of Recombinant B19 Parvovirus Capsids Expressed in Transfected COS-7 Cells". *Journal of General Virology*. 76: 1233-1237.

Coscoy, L. and Ganem, D. (2000). "Kaposi's Sarcoma-Associated Herpesvirus Encodes Two Proteins that Block Cell Surface Display of MHC Class I Chains by Enhancing Their Endocytosis". *Proceedings of the National Academy of Sciences of the U.S.A.* 97: 8051-8056.

Coulombel, L.; Morinet, F.; Mielot, F. et al. (1989). "Parvovirus Infection, Leukaemia, and Immunodeficiency". *Lancet*. 1: 101-102.

Cossart, Y.E.; Cant, B.; Field, A.M. and Widdows, D. (1975). "Parvovirus-Like Particles in Human Sera". *Lancet*. 1: 72-73.

Cotmore, S.F.; McKie, V.C.; Anderson, L.J.; Astell, C.R. and Tattersall, P. (1986). "Identification of the Major Structural and Nonstructural Proteins Encoded by Human Parvovirus B19 and Mapping of Their Genes by Procaryotic Expression of Isolated Genomic Fragments". *Journal of Virology*. 60: 548-557.

Crise, B.; Buonocore, L. and Rose, J.K. (1990). "CD4 is Retained in the Endoplasmic Reticulum by the Human Immunodeficiency Virus Type 1 Glycoprotein Precursor". *Journal of Virology*. 64: 5585-5593.

Cullen, B.R. (1995). "Regulation of HIV Gene Expression". *AIDS*. 9: S19-S32.

Davidson, R.J.; Brown, T. and Wiseman, D. (1984). "Human Parvovirus Infection and Aplastic Crisis in Hereditary Spherocytosis". *Journal of Infection*. 9: 298-300.

Debbas, M. and White, E. (1993). "Wild-Type p53 Mediates Apoptosis by E1A, Which is Inhibited by E1B". *Genes and Development*. 7: 546-554.

Deiss, V.; Tratschin, J.D.; Weitz, M. and Seigl, G. (1990). "Cloning of the Human Parvovirus B19 Genome and Structural Analysis of Its Palindromic Termini". *Virology*. 175: 247-254.

Diaz-Guerra, M.; Rivas, C. and Esteban, M. (1997). "Activation of the IFN-Inducible Enzyme RNase L Causes Apoptosis of Animal Cells". *Virology*. 236: 354-363.

Dobbelstein, M.; Roth, J.; Kimberly, W.T.; Levine, A.J. and Shenk, T. (1997). "Nuclear Export of the E1B 55-kDa and E4 34-kDa Adenoviral Oncoproteins Mediated by a Rev-Like Signal Sequence". *EMBO Journal*. 16(14): 4276-4284.

Doerig, C.; Beard, P. and Hirt, B. (1987). "A Transcriptional Promoter of the Human Parvovirus B19 is Active In Vitro and In Vivo". *Virology*. 157: 539-542.

Doerig, C.; Hirt, B.; Antonietti, J. and Beard, P. (1990). "Nonstructural Protein of Parvovirus B19 and Minute Virus of Mice Controls Transcription". *Journal of Virology*. 64: 387-396.

Downward, J. (1994). "The Grb2/Sem-5 Adaptor Protein". *FEBS Letters*. 338: 113-117.

Duerksen-Hughes, P.; Wold, W.S. and Gooding, L.R. (1989). "Adenovirus E1A Renders Infected Cells Sensitive to Cytolysis by Tumor Necrosis Factor". *Journal of Immunology*. 143: 4193-4200.

Farrell, H.E.; Vally, H.; Lynch, D.M.; Fleming, P.; Shellam, G.R.; Scalzo, A.A. and Davis-Poynter, N.J. (1997). "Inhibition of Natural Killer Cells by a Cytomegalovirus MHC Class I Homologue In Vivo". *Nature*. 386: 510-514.

Farrell, P.J. (1998). "Signal Transduction From the Epstein-Barr Virus LMP-1 Transforming Protein". *Trends in Microbiology*. 6: 175-177.

Fischer, U.; Huber, J.; Boelens, W.C.; Mattaj, I.W. and Luhrmann, R. (1995). "The HIV-1 Rev Activation Domain is a Nuclear Export Signal that Accesses and Export Pathway Used by Specific Cellular RNAs". *Cell*. 82: 475-483.

Flint, J. and Shenk, T. (1997). "Viral Transactivating Proteins". *Annual Review of Genetics*. 31: 177-212.

Fornerod, M.; Ohno, M.; Yoshida, M. and Mattaj, I.W. (1997). "Crm1 is an Export Receptor for Leucine-Rich Nuclear Export Signals". *Cell*. 90: 1051-1060.

Fortes, P.; Beloso, A. and Ortin, J. (1994). "Influenza Virus NS1 Protein Inhibits Pre-mRNA Splicing and Blocks mRNA Nucleocytoplasmic Transport". *EMBO Journal*. 13(3): 704-712.

Franciosi, R.A. and Tattersall, P. (1988). "Fetal Infection with Human Parvovirus B19". *Human Pathology*. 19(4): 489-491.

Frickhofen, N.; Abkowitz, J.L.; Safford, M.; Berry, J.M.; Antunez-de-Mayolo, J.; Astrow, A.; Cohen, R.; Halperin, I.; King, L.; Mintzer, D.; Cohen, B. and Young, N.S. (1990). "Persistent B19 Parvovirus Infection in Patients Infected with Human Immunodeficiency Virus Type 1 (HIV-1): a Treatable Cause of Anemia in AIDS". *Annals of Internal Medicine*. 113(12): 926-933.

Frickhofen, N. and Young, N.S. (1989). "Persistent Parvovirus B19 Infections in Humans". *Microbial Pathogenesis*. 7: 319-327.

Fukuda, M.; Asano, S.; Nakamura, T.; Adachi, M.; Yoshida, M.; Yanagida, M. and Nishida, E. (1997). "Crm1 is Responsible for Intracellular Transport Mediated by the Nuclear Export Signal". *Nature*. 390: 308-311.

Gallay, P.; Hope, T.; Chin, D. and Trono, D. (1997). "HIV-1 Infection of Non-Dividing Cells Through the Recognition of Integrase by the Importin/Karyopherin Pathway". *Proceedings of the National Academy of Sciences of the U.S.A.* 94: 9825-9830.

Garrington, T.P. and Johnson, G.L. (1999). "Organization and Regulation of Mitogen-Activated Protein Kinase Signaling Pathways". *Current Opinion in Cell Biology*. 11: 211-218.

Gay, N.J.; Hesketh, L.M.; Cohen, B.J.; Rush, M.; Bates, C.; Morgan-Capner, P. and Miller, E. (1994). "Age Specific Antibody Prevalence to Parvovirus B19: How Many Women are Infected in Pregnancy?" *Communicable Disease Report. CDR Review*. 4(9): R104-R107.

Gilinger, G. and Alwine, J.C. (1993). "Transcriptional Activation by Simian Virus 40 Large T Antigen: Requirement for Simple Promoter Structures Containing Either TATA or Initiator Elements With Variable Upstream Factor Binding Sites". *Journal of Virology*. 67: 6682-6688.

Goodbourn, S.; Didcock, L. and Randall, R.E. (2000). "Interferons: Cell Signalling, Immune Modulation, Antiviral Response and Virus Countermeasures". *Journal of General Virology*. 81: 2341-2364.

Goss, G.A. and Szer, J. (1997). "Pancytopenia Following Infection with Human Parvovirus B19 as a Presenting Feature of Hereditary Spherocytosis in Two Siblings". *Australian And New Zealand Journal of Medicine*. 27: 86-87.

Graeve, J.L.A.; de Alarcon, P.A. & Naides, S.J. (1989). "Parvovirus B19 Infection in Patients Receiving Cancer Chemotherapy: the Expanding Spectrum of Disease". *American Journal of Pediatric Hematology/Oncology*. 11: 441-444.

Green, S.W.; Malkovska, I.; O'Sullivan, M.G. and Brown, K.E. (2000). "Rhesus and Pig-Tailed Macaque Parvoviruses: Identification of Two New Members of the Erythrovirus Genus in Monkeys". Virology. 269(1): 105-112.

Grossel, M.J.; Barsoum, J.; Prakash, S.S. and Androphy, E.F. (1996). "The BPV-1 E2 DNA-Contact Helix Cysteine is Required for Transcriptional Activation but Not for Replication in Mammalian Cells". *Virology*. 217: 301-310.

Gruda, M.C.; Zabolotny, J.M.; Xiao, J.H.; Davidson, I. and Alwine, J.C. (1993). "Transcriptional Activation by SV40 Large T Antigen: Interactions with Multiple Components of the Transcription Complex". *Molecular and Cellular Biology*. 13(2): 961-969.

Guan, K.L. (1994). "The Mitogen Activated Protein Kinase Signal Transduction Pathway: From the Cell Surface to the Nucleus". *Cellular Signalling*. 6(6): 581-589.

Guinea, R. and Carrasco, L. (1990). "Phospholipid Biosynthesis and Poliovirus Genome Replication, Two Copled Phenomena". *EMBO Journal*. 9: 2011-2016.

Hagmeyer, B.; Angel, P. and van Dam, H. (1995). "Modulation of AP-1/ATF Transcription Factor Activity by the Adenovirus-E1A Oncogene Products". BioEssays. 17: 621-629.

Ham, J.; Dostatni, N.; Gauthier, J.M. and Yaniv, M. (1991). "The Papilloma Virus E2 Protein: A Factor with Many Talents". *Trends in Biochemical Sciences*. 16(11): 440-444.

Hardy, W.R. and Sandri-Goldin, R.M. (1994). "Herpes Simplex Virus Inhibits Host Cell Splicing, and Regulatory Protein ICP27 is Required for This Effect". *Journal of Virology*. 68(12): 7790-7799.

Harris, S.L.; Frank, I.; Yee, A.; Cohen, G.H.; Eisenberg, R.J. and Friedman, H.M. (1990). "Glycoprotein C of Herpes Simplex Virus Type 1 Prevents Complement-

Mediated Cell Lysis and Virus Neutralization". *Journal of Infectious Diseases.* 162: 331-337.

He, J.; Choe, S.; Walker, R.; Di Marzio, P.; Morgan, D.O. and Landau, N.R. (1995). "Human Immunodeficiency Virus Type 1 Viral Protein R (Vpr) Arrests Cells in the G2 Phase of the Cell Cycle by Inhibiting P34 Cdc2 Activity". *Journal of Virology.* 69: 6705-6711.

Heinzinger, N.K.; Bukrinsky, M.I.; Haggerty, S.A.; Ragland, A.M. and Emerman, M. (1994). "The Vpr Protein of Human Immunodeficiency Virus Type 1 Influences Nuclear Localization of Viral Nucleic Acids in Nondividing Host Cells". *Proceedings of the National Academy of Sciences of the U.S.A.* 91(15): 7311-7315.

Hengel, H.; Brune, W. and Koszinowski, U.H. (1998). "Immune Evasion by Cytomegalovirus: Survival Strategies of a Highly Adapted Opportunist". *Trends in Microbiology.* 6(5): 190-197.

Hill, A.; Juovic, P.; York, I.; Russ, G.; Bennink, J.; Yewdell, J.; Ploegh, H. and Johnson, D. (1995). "Herpes Simplex Virus Turns Off the TAP to Evade Host Immunity". *Nature.* 375: 411-415.

Hoglund, S.; Ohagen, A.; Lawrence, K. and Gabuzda, D. (1994). "Role of Vif During Packing of the Core of HIV-1". *Virology.* 201: 349-355.

Hsu, D.H.; de Waal Malefyt, R.; Fiorentino, D.F.; Dang, M.N.; Vieira, P.; de Vries, J.; Spits, H.; Mosmann, T.R. and Moore, K.W. (1990). "Expression of Interleukin-10 Activity by Epstein-Barr Virus Protein BCRF1". *Science.* 250: 830-832.

Hu, F.; Smith, C.A. and Pickup, D. (1994). "Cowpox Virus Contains Two Copies of an Early Gene Encoding a Soluble Secreted Form of the Type II TNF Receptor". *Virology.* 204: 343-356.

Hung, S.L.; Peng, C.; Kostavasili, I.; Friedman, H.M.; Lambris, J.D.; Eisenberg, R.J. and Cohen, G.H. (1994). "The Interaction of Glycoprotein C of Herpes Simplex Virus Types 1 and 2 with the Alternative Complement Pathway". *Virology.* 203: 299-312.

Irmler, M.; Thome, M.; Hahne, M.; Schneider, P.; Hofmann, K.; Steiner, V.; Bodmer, J.L.; Schroter, M.; Burns, K.; Mattmann, C.; Rimoldi, D.; French, L.E. and Tschopp, J. (1997). "Inhibition of Death Receptor Signals by Cellular FLIP". *Nature.* 388: 190-195.

Ishido, S.; Wang, C.; Lee, B.S.; Cohen, G.B. and Jung, J.U. (2000). "Downregulation of Major Histocompatibility Complex Class I Molecules by Kaposi's Sarcoma-Associated Herpesvirus K3 and K5 Proteins". *Journal of Virology.* 74(11): 5300-5309.

Jabbar, M.A. and Nayak, D.P. (1990). "Intracellular Interaction of Human Immunodeficiency Virus Type 1 (ARV-2) Envelope Glycoprotein gp160 with CD4 Blocks the Movement and Maturation of CD4 to the Plasma Membrane". *Journal of Virology*. 64: 6297-6304.

Jabbar, M.A. (1995). "The Human Immunodeficiency Virus Type 1 Vpu Protein: Roles in Virus Release and CD4 Downregulation". *Current Topics in Microbiology and Immunology*. 193: 107-120.

Jowett, J.B.M.; Planelles, V.; Poon, B.; Shah, N.P.; Chen, M.L. and Chen, I.S.Y. (1995). "The Human Immunodeficiency Virus Type 1 Vpr Gene Arrests Infected T Cells in the G2 + M Phase of the Cell Cycle". *Journal of Virology*. 69: 6304-6313.

Kajigaya, S.; Fujii, H.; Field, A.; Rosenfeld, S.; Anderson, L.J.; Shimada, T. and Young, N.S. (1991). "Self-Assembled B19 Parvovirus Capsids Produced in a Baculovirus System are Antigenically and Immunologically Similar to Native Virions". *Proceedings of the National Academy of Sciences of the U.S.A.* 88: 4646-4650.

Kamoshita, M.; Ohtani, K.; Hasegawa, Y.; Satoh, Y.; Kozima, H.; Shibuya, A.; Ninomiya, H.; Nagasawa, T.; Matsunaga, Y. and Abe, T. (1993). "Human Parvovirus B19-Induced Aplastic Crisis in an Elderly Man with Intestinal Hemorrhage". *Japanese Journal of Clinical Hematology (Rinsho Ketsueki)*. 34(4): 493-495

Kay, B.K.; Williamson, M.P. and Sudol, M. (2000). "The Importance of Being Proline: The Interaction of Proline-Rich Motifs in Signaling Proteins with Their Cognate Domains". *FASEB Journal*. 14: 231-241.

Kelleher, J.; Naomi, M.; Luban, L.; Mortimer, P. and Kamimura, T. (1983). "Human Serum Parvovirus: A Specific Cause of Aplastic Crisis in Children with Hereditary Spherocytosis". *Journal of Pediatrics*. 102: 720-722.

Kerr, J.R.; McQuaid, S. and Coyle, P.V. (1995). "Expression of P Antigen in Parvovirus B19-Infected Bone Marrow". *New England Journal of Medicine*. 332: 128.

Kingsman, S.M. and Kingsman, A.J. (1996). "The Regulation of Human Immunodeficiency Virus Type 1 Gene Expression". *European Journal of Biochemistry*. 240: 491-507.

Kojima, S.; Matsuyama, K. & Ishii, E. (1988). "High Serum Iron in Human Parvovirus-Induced Aplastic Crisis in Iron Deficiency Anemia". *Acta Haematologica*. 80: 171-172.

Koppelman, B.; Neefjes, J.J.; de Vries, J.E. and de Waal Malefyt, R. (1997). "Interleukin-10 Down-Regulates MHC Class II Alphabeta Peptide Complexes at the Plasma Membrane of Monocytes by Affecting Arrival and Recycling". *Immunity*. 7(6): 861-871.

Kotin, R.M.; Siniscalco, M.; Samulski, R.J.; Zhu, X.D.; Hunter, L.; Laughlin, C.A.; McLaughlin, S.; Muzyczka, N.; Rocchi, M. and Berns, K.I. (1990). "Site-Specific Integration by Adeno-Associated Virus". *Proceedings of the National Academy of Sciences of the U.S.A.* 87(6): 2211-2215.

Kotwal, G.J. (2000). "Poxviral Mimicry of Complement and Chemokine System Components: What's the End Game?" *Immunology Today.* 21: 242-248.

Krug, R.M. (1993). "The Regulation of Export of mRNA from Nucleus to Cytoplasm". *Current Opinion in Cell Biology.* 5(6): 944-949.

Kurpad, C.; Mukherjee, P.; Wang, X.S.; Ponnazhagen, S.; Li, L.; Yoder, M.C. and Srivastava, A. (1999). "Adeno-Associated Virus 2-Mediated Transduction and Erythroid Lineage-Restricted Expression from Parvovirus B19p6 Promoter in Primary Human Hematopoietic Progenitor Cells". *Journal of Hematotherapy and Stem Cell Research.* 8: 585-592.

Kurtzman, G.J.; Cohen, B.; Meyers, P. ; Amunullah, A. and Young, N.S. (1988). "Persistent B19 Parvovirus Infection as a Cause of Severe Chronic Anaemia in Children with Acute Lymphocytic Leukaemia". *Lancet.* 2: 1159-1162.

Kurtzman, G.; Frickhofen, N.; Kimball, J.; Jenkins, D.W.; Nienhuis, A.W. and Young, N.S. (1989). "Pure Red-Cell Aplasia of 10 Years' Duration due to Persistent Parvovirus B19 Infection and Its Cure with Immunoglobulin Therapy". *New England Journal of Medicine.* 321: 519-523.

Lalani, A.S.; Barrett, J.W. and McFadden, G. (2000). "Modulating Chemokines: More Lessons from Viruses". *Immunology Today.* 21(2): 100-106.

Lalani, A.S. and McFadden, G. (1999). "Evasion and Exploitation of Chemokines by Viruses". *Cytokine and Growth Factor Reviews.* 10: 219-233.

Landro, J.A.; Raybuck, S.A.; Luong, Y.P.C.; O'Malley, E.T.; Harbeson, S.L.; Morgenstern, K.A.; Rao, G. and Livingston, D.J. (1997). "Mechanistic Role of an NS4A Peptide Cofactor with the Truncated NS3 Protease of Hepatitis C Virus: Elucidation of the NS4A Stimulatory Effect Via Kinetic Analysis and Inibitor Mapping". *Biochemistry.* 36(31): 9340-9348.

Le Gouill, C.; Parent, J-L.; Rola-Pleszczynski, M. and Stankova, J. (1994). "Analysis of Recombinant Plasmids by a Modified Alkaline Lysis Method". *Analytical Biochemistry.* 219: 164.

Levitskaya, J.; Sharipo, A.; Leonchiks, A.; Ciechanover, A. and Masucci, M.G. "Inhibition of Ubiquitin/Proteasome-Dependent Protein Degradation by the Gly-

Ala Repeat Domain of the Epstein-Barr Virus Nuclear Antigen 1". *Proceedings of the National Academy of Sciences of the U.S.A.* 94(23): 12616-12621.

Li, J. and Broyles, S.S. (1993a). "Recruitment of Vaccinia Virus RNA Polymerase to an Early Gene Promoter by the Viral Early Transcription Factor". *Journal of Biological Chemistry.* 268(4): 2773-2780.

Li, J. and Broyles, S.S. (1993b). "The DNA-Dependent ATPase Activity of Vaccinia Virus Early Gene Transcription Factor is Essential for Its Transcription Activation Function". *Journal of Biological Chemistry.* 268(27): 20016-20021.

Liu, H.; Wu, X.; Newman, M.; Shaw, G.M.; Hahn, B.H. and Kappes, J.C. (1995). "The Vif Protein of Human and Simian Immunodeficiency Viruses is Packaged into Virions and Associates with Viral Core Structures". *Journal of Virology.* 69: 7630-7638.

Liu, J.M.; Green, S.W.; Shimada, T. and Young, N.S. (1992). "A Block in Full-Length Transcript Maturation in Cells Nonpermissive for B19 Parvovirus". *Journal of Virology.* 66(8): 4686-4692.

Lowe, S.W. and Ruley, H.E. (1993). "Stabilization of the p53 Tumor Suppressor is Induced by Adenovirus 5 E1A and Accompanies Apoptosis". *Genes and Development.* 7: 535-545.

Lu, S.; Heimer, J.; Rekosh, D. and Hammarskjold, M.L. (1993). "U1 Small Nuclear RNA Plays a Direct Role in the Formation of a Rev-Regulated Human Immunodeficiency Virus Env mRNA that Remains Unspliced". *Proceedings of the National Academy of Sciences of the U.S.A.* 87: 7598-7602.

Lu, Y.; Wambach, M.; Katze, M.G. and Krug, R.M. (1995). "Binding of the Influenza Virus NS1 Protein to Double-Stranded RNA Inhibits the Activation of the Protein Kinase that Phosphorylates the eIF-2 Translation Initiation Factor". *Virology.* 214(1): 222-228.

Luo, W. and Astell, C.R. (1993). "A Novel Protein Encoded by Small RNAs of Parvovirus B19". *Virology.* 195: 448-455.

Lutz, P. and Kedinger, C. (1996). "Properties of the Adenovirus IVa2 Gene Product, an Effector of Late-Phase-Dependent Activation of the Major Late Promoter". *Journal of Virology.* 70: 1396-1405.

Malim, M.H.; Hauber, J.; Le S-Y; Maizel, J.V. and Cullen, B.R. (1989). "The HIV-1 Rev Trans-Activator Acts Through a Structured Target Sequence to Activate Nuclear Export of Unspliced Viral mRNA". *Nature.* 338: 254-257.

Manninen, A.; Hiipakka, M.; Vihinen, M.; Lu, W.; Mayer, B.J. and Saksela, K. (1998). "SH3-Domain Binding Function of HIV-1 Nef is Required for Association with a PAK-Related Kinase". *Virology*. 250: 273-282.

Margolis, B. and Skolnik, E.Y. (1994). "Activation of Ras by Receptor Tyrosine Kinases". *Journal of the American Society of Nephrology*. 5(6): 1288-1299.

Mavankal, G.; Ignatius-Ou, S.H.; Oliver, H.; Sigman, D. and Gaynor, R.B. (1996). "Human Immunodeficiency Type 1 and 2 Tat Proteins Specifically Interact with RNA Polymerase II". *Proceedings of the National Academy of Sciences of the U.S.A.* 93: 2089-2094.

Mayer, B.J. and Gupta, R. (1998). "Functions of SH2 and SH3 Domains". *Current Topics in Microbiology and Immunology*. 228: 1-22.

McBride, A.A.; Romanczuk, H. and Howley, P.M. (1991). "The Papillomavirus E2 Regulatory Proteins". *Journal of Biological Chemistry*. 266: 18411-18414.

McFadden, G. ed. (1995). *Viroceptors, Virokines, and Related Immune Modulators Encoded by DNA Viruses*. Austin, Texas: R.G. Landes Company.

Moffatt, S.; Yaegashi, N.; Tada, K.; Tanaka, N. and Sugamura, K. (1998). "Human Parvovirus B19 Nonstructural (NS1) Protein Induces Apoptosis in Erythroid Lineage Cells". *Journal of Virology*. 72: 3018-3028.

Moore, P.S.; Boshoff, C.; Weiss, R.A. and Chang, Y. (1996). "Molecular Mimicry of Human Cytokine and Cytokine Response Pathway Genes in KSHV". *Science*. 274: 1739-1744.

Morey, A.L.; Ferguson, D.J.P.; Leslie, K.O.; Taatjes, D.J. and Fleming, K.A. (1993). "Intracellular Localization of Parvovirus B19 Nucleic Acid at the Ultrastructural Level by In Situ Hybridization with Digoxigenin-Labelled Probes". *Histochemical Journal*. 25: 421-429.

Mortimer, P.P.; Humphries, R.K.; Moore, J.G.; Purcell, R.H. and Young, N.S. (1983). "A Human Parvovirus-Like Virus Inhibits Haematopoietic Colony Formation In Vitro". *Nature*. 302: 426-429.

Moss, B.; Ahn, B-Y; Amegadzie, B.; Gershon, P.D. and Keck, J.G. (1991). "Cytoplasmic Transcription System Encoded by Vaccinia Virus". *Journal of Biological Chemistry*. 266: 1355-1358.

Munshi, N.C.; Zhou, S.; Woody, M.J.; Morgan, D.A. and Srivastava, A. (1993). "Successful Replication of Parvovirus B19 in the Human Megakaryocytic Leukemia Cell Line MB-02". *Journal of Virology*. 67: 562-566.

Nash, P. ; Barrett, J.; Cao, J.X.; Hota-Mitchell, S.; Lalani, A.S.; Everett, H.; Xu, X.M.; Robichaud, J.; Hnatiuk, S.; Ainslie, C. ; Seet, B.T. and McFadden, G. (1999). "Immunomodulation by Viruses: The Myxoma Virus Story". *Immunological Reviews*. 168: 103-120.

Neild, G.; Anderson, M.; Hawes, S. and Colvin, B.T. (1986). "Parvovirus Infection after Renal Transplant". *Lancet*. 2: 1226-1227. (letter)

Nemeroff, M.E.; Barabino, S.M.L.; Li, Y.; Keller, W. and Krug, R.M. (1998). "Influenza Virus NS1 Protein Interacts with the 30kD Subunit of Cleavage and Specificity Factor and Inhibits 3' End Formation of Cellular Pre-mRNAs". *Molecular Cell*. 1(7): 991-1000.

Nevins, J.R. (1981). "Mechanism of Activation of Early Viral Transcription by the Adenovirus E1A Gene Product". *Cell*. 26: 213-220.

Nguyen, Q.T.; Sifer, C.; Schneider, V.; Allaume, X.; Servant, A.; Bernaudin, F.; Augusted, V. and Garbarg-Chenon, A. (1999). "Novel Human Erythrovirus Associated with Transient Aplastic Anemia". Journal of Clinical Microbiology. 37(8): 2483-2487.

Nogal, M.L.; de Buitrago, G.G.; Rodriguez, C.; Cubelos, B.; Carrascosa, A.L.; Salas, M.L. and Revilla, Y. (2001). "African Swine Fever Virus IAP Homologue Inhibits Caspase Activation and Promotes Cell Survival in Mammalian Cells". *Journal of Virology*. 75(6): 2535-2543.

O'Hare, P. (1993). "The Virion Transactivator of Herpes Simplex Virus". *Seminars in Virology*. 4(3): 145-155.

Op de Beeck, A.; Anouja, F.; Mousset, S.; Rommelaere, J. and Caillet-Fauquet, P. (1995). "The Nonstructural Proteins of the Autonomous Parvovirus Minute Virus of Mice Interfere with the Cell Cycle, Inducing Accumulation in G2". *Cell Growth and Differentiation*. 6: 781-787.

Op de Beeck, A. and Caillet-Fauquet, P. (1997). "The NS1 Protein of the Autonomous Parvovirus Miute Virus of Mice Blocks Cellular DNA Replication: A Consequence of Lesions to the Chromatin?" *Journal of Virology*. 71: 5323-5329.

O'Sullivan, M.G.; Anderson, D.C.; Fikes, J.D.; Bain, F.T.; Carlson, C.S.; Green, S.W.; Young, N.S. and Brown, K.E. (1994). "Identification of a Novel Simian Parvovirus from Cynomolgus Monkey with Severe Anemia: A Paradigm of Human B19 Parvovirus Infection". *Journal of Clinical Investigation*. 93: 1571-1576.

Ozawa, K.; Ayub, J.; Hao, Y.S.; Kurtzman, G.; Shimada, T. and Young, N. (1987a). "Novel Transcription Map for the B19 (Human) Pathogenic Parvovirus". *Journal of Virology*. 61: 2395-2406.

Ozawa, K.; Ayub, J.; Kajigaya, S.; Shimada, T. and Young, N. (1988a). "The Gene Encoding the Nonstructural Protein of B19 (Human) Parvovirus May be Lethal in Transfected Cells". *Journal of Virology*. 62: 2884-2889.

Ozawa, K.; Ayub, J. and Young, N. (1988b). "Functional Mapping of the Genome of the B19 (Human) Parvovirus by In Vitro Translation After Negative Hybrid Selection". *Journal of Virology*. 62: 2508-2511.

Ozawa, K.; Kurtzman, G. and Young, N. (1986). "Replication of the B19 Parvovirus in Human Bone Marrow Cell Cultures". *Science*. 233: 883-886.

Ozawa, K.; Kurtzman, G. and Young, N. (1987b). "Productive Infection by B19 Parvovirus of Human Erythroid Bone Marrow Cells In Vitro". *Blood*. 70: 384-391.

Ozawa, K. and Young, N. (1987). "Characterization of Capsid and Noncapsid Proteins of B19 Parvovirus Propagated in Human Erythroid Bone Marrow Cell Cultures". *Journal of Virology*. 61: 2627-2630.

Pallier, C.; Greco, A.; le Junter, J.; Saib, A.; Vassius, I. and Morinet, F. (1997). "The 3' Untranslated Region of the B19 Parvovirus Capsid Protein mRNAs Inhibits Its Own mRNA Translation in Nonpermissive Cells". *Journal of Virology*. 71: 9482-9489.

Parada, C.A. and Roeder, R.G. (1996). "Enhanced Processivity of RNA Polymerase II Triggered by Tat-Induced Phosphorylation of its Carboxy-Terminal Domain". *Nature*. 384: 375-378.

Parrish, C.R. (1991). "Mapping Specific Functions in the Capsid Structure of Canine Parvovirus and Feline Panleukopenia Virus Using Infectious Plasmid Clones". *Virology*. 183: 195-205.

Parry, B.C.; Simas, J.P.; Smith, V.P.; Stewart, C.A.; Minson, A.C.; Efstathiou, S. and Alcami, A. (2000). "A Broad Spectrum Secreted Chemokine Binding Protein Encoded by a Herpesvirus". *Journal of Experimental Medicine*. 191(3): 573-578.

Pattison, J.R.; Jones, S.E.; Hodgson, J.; Davis, L.R.; White, J.M.; Stroud, C.E. and Murtaza, L. (1981). "Parvovirus Infections and Hypoplastic Crisis in Sickle-Cell Anaemia". *Lancet*. 1: 664-665. (letter)

Peluso, R.W.; Richardson, J.C.; Talon, J. and Lock, M. (1996). "Identification of a Set of Proteins (C' and C) Encoded by the Bicistronic P Gene of the Indiana Serotype of Vesicular Stomatitis Virus and Analysis of Their Effect on Transcription by the Viral RNA Polymerase". *Virology*. 218(2): 335-342.

Phelan, A.; Dunlop, J. and Clements, J.B. (1996). "Herpes Simplex Virus Type 1 Protein IE63 Affects the Nuclear Export of Virus Intron-Containing Transcripts". *Journal of Virology*. 70(8): 5255-5265.

Ploegh, H.L. (1998). "Viral Strategies of Immune Evasion". *Science*. 280: 248-253.

Pollard, V.W. and Malim, M.H. (1998). "The HIV-1 Rev Protein". *Annual Review of Microbiology*. 52: 491-532.

Popov, S.; Rexach, M.; Ratner, L.; Blobel, G. and Bukrinsky, M. (1998). "Viral Protein R Regulates Docking of the HIV-1 Preintegration Complex to the Nuclear Pore Complex". *Journal of Biological Chemistry*. 273: 13347-13352.

Pringle, C.R. (1993). "Virus Taxonomy Update". *Archives of Virology*. 133: 491-495.

Pruzan, R. and Flint, J. (1995). "Transcription of Adenovirus RNA Polymerase III Genes". *Current Topics in Microbiology and Immunology*. 199(Pt.1): 201-226.

Puri, R.K. and Aggarwal, B.B. (1992). "Human Immunodeficiency Virus Type 1 Tat Up-Regulates Interleukin-4 Receptors on a Human B Lymphoblastoid Cell Line". *Cancer Research*. 52: 3787-3790.

Qiu, Y. and Krug, R.M. (1994). "The Influenza Virus NS1 Protein is a Poly(A)-Binding Protein that Inhibits Nuclear Export of mRNAs Containing Poly(A)"" *Journal of Virology*. 68: 2425-2432.

Qiu, Y.; Nemeroff, M. and Krug, R.M. (1995). "The Influenza Virus NS1 Protein Binds to a Specific Region in Human U6 snRNA and Inhibits U6-U2 and U6-U4 snRNA Interactions During Splicing". *RNA*. 1: 304-316.

Rank, N.M. and Lambert, P.F. (1995). "Bovine Papilloma-Virus Type 1 E2 Transcriptional Reulators Directly Bind Two Cellular Transcription Factors, TFIID and TFIIB". *Journal of Virology*. 69: 6323-6334.

Rao, S.P.; Miller, S.T. and Cohen, B.J. (1992). "Transient Aplastic Crisis in Patients with Sickle Cell Disease: B19 Parvovirus Studies During a 7-Year Period". *American Journal of Diseases of Children*. 146: 1328-1330.

Re, F.; Braaten, D.; Franke, E.K. and Luban, J. (1995). "Human Immunodeficiency Virus Type 1 Vpr Arrests the Cell Cycle in G2 by Inhibiting the Activation of P34 Cdc2-Cyclin B". *Journal of Virology*. 69: 6859-6864.

Reed, S.I.; Stark, G.R. and Alwine, J.C. (1976). "Autoregulation of Simian Virus 40 Gene A by T Antigen". *Proceedings of the National Academy of Sciences of the U.S.A.* 73(9): 3083-3087.

Reid, D.M.; Reid, T.M.; Brown, T.; Rennie, J.A. and Eastmond, C.J. (1985). "Human Parvovirus-Associated Arthritis: A Clinical and Laboratory Description". *Lancet*. 1: 422-425.

Ren, R., Mayer, B.J.; Cicchetti, P. and Baltimore, D. (1993). "Identification of a Ten-Amino Acid Proline-Rich SH3 Binding Site". *Science*. 259: 1157-1161.

Rhee, S.S. and Marsh, J.W. (1994). "Human Immunodeficiency Virus Type 1 Nef-Induced Down-Modulation of CD4 is Due to Rapid Internalization and Degradation of Surface CD4". *Journal of Virology*. 68: 5156-5163.

Rice, P.W. and Cole, C.N. (1993). "Efficient Transcriptional Activation of Many Simple Modular Promoters by Simian Virus 40 Large T Antigen". *Journal of Virology*. 67: 6689-6697.

Rivas, C.; Gil, J.; Melkova, Z.; Esteban, M. and Diaz-Guerra, M. (1998). "Vaccinia Virus E3L Protein is an Inhibitor of the Interferon (IFN)-Induced 2-5A Synthetase Enzyme". *Virology*. 243: 406-414.

Rogers, B.B. (1999). "Parvovirus B19: Twenty-Five Years in Perspective". *Pediatric and Developmental Pathology*. 2: 296-315.

Roizman, B. and Sears, A.E. (1996). "Herpes Simplex Viruses and Their Replication". In: Fields, B.N.; Knipe, D.M.; Howley, P.M. et al. eds. *Virology*. 3rd Edition. Philadelphia: Lippincott-Raven. pp. 2231-95.

Rosenfeld, S.J.; Yoshimoto, K.; Kajigaya, S.; Anderson, S.; Young, N.S.; Field, A.; Warrener, P.; Bansal, G. and Collett, M.S. [published erratum appears in J. Clin. Invest. (1992). 90(6): 2609.] (1992). "Unique Region of the Minor Capsid Protein of Human Parvovirus B19 is Exposed on the Virion Surface". *Journal of Clinical Investigation*. 89(6): 2023-2029.

Roulston, A.; Marcellus, R.C. and Branton, P.E. (1999). "Viruses and Apoptosis". *Annual Review of Microbiology*. 53: 577-628.

Saikawa, T.; Anderson, S.; Momoeda, M.; Kajigaya, S. and Young, N.S. (1993). "Neutralizing Linear Epitopes of B19 Parvovirus Cluster in the VP1 Unique and VP1-VP2 Junction Regions". *Journal of Virology*. 67(6): 3004-3009.

Saksela, K.; Cheng, G. and Baltimore, D. (1995). "Proline-Rich (PxxP) Motifs in HIV-1 Nef Bind to SH3 Domains of a Subset of Src Kinases and Are Required for the Enhanced Growth of Nef+ Viruses but not for Down-Regulation of CD4". *EMBO Journal*. 14: 484-491.

Sambrook, J.; Fritsch, E.F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. 2nd Edition. New York: Cold Spring Harbor Laboratory Press. pp. 1.38-1.41.

Samuel, C.E.; Kuhen, K.L.; George, C.X.; Ortega, L.G.; Rende-Fournier, R. and Tanaka, H. (1997). "The PKR Protein Kinase: an Interferon-Inducible Regulator of Cell Growth and Differentiation". *International Journal of Hematology*. 65(3): 227-237.

Sandri-Goldin, R.M. (1998). "ICP27 Mediates HSV RNA Export by Shuttling Through a Leucine-Rich Nuclear Export Signal and Binding Viral Intronless RNAs Through an RGG Motif". *Genes and Development*. 6: 868-879.

Sanger, F.; Nicklen, S. and Coulson, A.R. (1977). "DNA Sequencing with Chain-Terminating Inhibitors". *Proceedings of the National Academy of Sciences of the U.S.A.* 74(12): 5463-5467.

Sawai, E.T.; Baur, A.S.; Struble, B.M.; Peterlin, B.M.; Levy, J.A. and Cheng, M.C. (1994). "Human Immunodeficiency Virus Type 1 Nef Associates with a Cellular Serine Kinase in T Lymphocytes". *Proceedings of the National Academy of Sciences of the U.S.A.* 91: 1539-1543.

Schaeffer, E.; Geleziunas, R. and Greene, W.C. (2001). "Human Immunodeficiency Virus Type 1 Nef Functions at the Level of Virus Entry by Enhancing Cytoplasmic Delivery of Virions". *Journal of Virology*. 75(6): 2993-3000.

Schubert, U.; Clouse, K.A. and Strebel, K. (1995a). "Augmentation of Virus Secretion by the Human Immunodeficiency Virus Type 1 Vpu Protein is Cell Type Independent and Occurs in Cultured Human Primary Macrophages and Lymphocytes". *Journal of Virology*. 69(12): 7699-7711.

Schubert, U.; Henklein, P.; Bour, S.; Ferrer-Montiel, A.F.; Oblatt-Montal, M.; Strebel, K. and Montal, M. (1995b). "Identification of an Ion Channel Activity of the Vpu Transmembrane Domain and Its Involvement in the Regulation of Virus Release from HIV-1-Infected Cells". *FEBS Letters*. 398(1): 12-18.

Schwartz, O.; Marechal, V.; Danos, O. and Heard, J.M. (1995). "Human Immunodificiency Virus Type 1 Nef Increases the Efficiency of Reverse Transcription in the Infected Cell". *Journal of Virology*. 69(7): 4053-4059.

Schwartz, O.; Marechal, V.; Le Gall, S.; Lemonnier, F. and Heard, J.M. (1996). "Endocytosis of Major Histocompatibility Complex Class I Molecules is Induced by the HIV-1 Nef Protein". *Nature Medicine*. 2(3): 338-342.

Senkevich, T.F.; Bugert, J.J.; Sisler, J.R.; Koonin, E.V.; Darai, G. and Moss, B. (1996). "Genome Sequence of a Human Tumorigenic Poxvirus: Prediction of Specific Host Response-Evasion Genes". *Science*. 273: 813-816.

Serjeant, G.R.; Serjeant, B.E.; Thomas, P.W.; Anderson, M.J.; Patou, G. and Pattison, J.R. (1993). "Human Parvovirus Infection in Homozygous Sickle Cell Disease". *Lancet*. 341: 1237-1240.

Serke, S.; Schwarz, T.F.; Baurmann, H.; Kirsch, A.; Hottentrager, B.; Von Brun, A.; Roggendorf, M.; Huhn, D. and Deinhardt, F. (1991). "Productive Infection of In Vitro Generated Haemopoietic Progenitor Cells from Normal Human Adult Peripheral Blood with Parvovirus B19: Studies by Morphology, Immunocytochemistry, Flow-Cytommetry and DNA Hybridization". *Journal of Hematology*. 79: 6-13.

Shen, Y. and Shenk, T. (1995). "Viruses and Apoptosis". *Current Opinion in Genetics and Development*. 5: 105-111.

Shenk, T. (1996). "Adenoviridae and Their Replication". In: Fields, B.N.; Knipe, D.M.; Howley, P.M. et al. eds. *Virology*. 3rd Edition. Philadelphia: Lippincott-Raven. pp. 2111-2148.

Shimomura, S.; Komatsu, N.; Frickhofen, N.; Anderson, S.; Kajigaya, S. and Young, N.S. (1992). "First Continuous Propagation of B19 Parvovirus in a Cell Line". *Blood*. 79: 18-24.

Smith, C.A.; Davis, T.; Wignall, J.M.; Din, W.S.; Farrah, T.; Upton, C.; McFadden, G. and Goodwin, R.G. (1991). "T2 Open Reading Frame From the Shope Fibroma Virus Encodes a Soluble Form of the TNF Receptor". *Biochemical and Biophysical Research Communications*. 176: 335-342.

Smith, G.L.; Symons, J.A. and Alcami, A. (1998). "Poxviruses: Interfering with Interferon". *Seminars In Virology*. 8(5): 409-418.

Spalholz, B.A. & Tattersall, P. (1983). "Interaction of Minute Virus of Mice with Differentiated Cells: Strain-Dependent Target Cell Specificity is Mediated by Intracellular Factors". *Journal of Virology*. 46: 937-943.

Spina, C.A.; Kwoh, T.J.; Chowers, M.Y.; Guatelli, J.C. and Richman, D.D. (1994). "The Importance of Nef in the Induction of HIV-1 Replication from Primary Quiescent CD4 Lymphocytes". *Journal of Experimental Medicine*. 179(1): 115-123.

Spriggs, M.K. (1994). "Cytokine and Cytokine Receptor Genes 'Captured' by Viruses". *Current Opinion in Immunology*. 6: 526-529.

Spriggs, M.K. (1996). "One Step Ahead of the Game: Viral Immunomodulatory Molecules". *Annual Review of Immunology*. 14: 101-131.

Spriggs, M.K. (1999). "Virus-Encoded Modulators of Cytokines and Growth Factors". *Cytokine and Growth Factor Reviews.* 10: 1-4.

Srivastava, A. and Lu, L. (1988). "Replication of B19 Parvovirus in Highly Enriched Hematopoietic Progenitor Cells from Normal Human Bone Marrow". *Journal of Virology.* 62: 3059-3063.

St. Amand, J.L. (1992). *Identification and Characterization of Small RNAs and Proteins Expressed by the Human Parvovirus B19.* Ph.D. Thesis.

St. Amand, J. and Astell, C.R. (1993). "Identification and Characterization of a Family of 11kDa Proteins Encoded by the Human Parvovirus B19". *Virology.* 192: 121-131.

St. Amand, J.; Beard, C.; Humphries, K. and Astell, C.R. (1991). "Analysis of Splice Junctions and In Vitro and In Vivo Translation Potential of the Small, Abundant B19 Parvovirus RNAs". *Virology.* 183: 133-142.

Strebel, K. and Bour, S. (1999). "Molecular Interactions of HIV with Host Factors". *AIDS.* 13 (Suppl.A): S13-S24.

Subbramanian, R.A. and Cohen, E.A. (1994). "Molecular Biology of the Human Immunodeficiency Virus Accessory Proteins". *Journal of Virology.* 68(11): 6831-6835.

Takahashi, T.; Ozawa, K.; Mitani, K.; Miyazono, K.; Asano, S. and Takaku, F. (1989). "B19 Parvovirus Replicates in Erythroid Leukemic Cells In Vitro". *Journal of Infectious Diseases.* 160: 548-549.

Tan, S-L, Nakao, H.; He, Y; Vijaysri, S.; Neddermann, P.; Jacobs, B.L.; Mayer, B.J. and Katze, M.G. (1999). "NS5A, a Nonstructural Protein of Hepatitis C Virus, Binds Growth Factor Receptor-Bound Protein 2 Adaptor Protein in a Src Homology 3 Domain/Ligand-Dependent Manner and Perturbs Mitogenic Signaling". *Proceedings of the National Academy of Sciences of the U.S.A.* 96(10): 5533-5538.

Teodoro, J.G. and Branton, P.E (1997). "Regulation of Apoptosis by Viral Gene Products". *Journal of Virology.* 71: 1739-1746.

Tomazin, R. ; Hill, A.B.; Jugovic, P.; York, I.; van Endert, P.; Ploegh, H.L.; Andrews, D.W. and Johnson, D.C. (1996). "Stable Binding of the Herpes Simplex Virus ICP47 Protein to the Peptide Binding Site of TAP". *EMBO Journal.* 15(13): 3256-3266.

Tomei, L.; Failla, C.; Santolini, E.; De Francesco, R. and La Monica, N. (1993). "NS3 is a Serine Protease Required for Processing of Hepatitis C Virus Polyprotein". *Journal of Virology.* 67(7): 4017-4026.

Torok, T.J. (1995). "Human Parvovirus B19". In: Remington, J.S. and Klein, J.O. eds. *Infectious Diseases of the Fetus and Newborn Infant.* 4th Edition. Philadelphia: W.B. Saunders. pp. 668-702.

Tribouley, C.; Lutz, P.; Staub, A. and Kedinger, C. (1994). "The Product of the Adenovirus Intermediate Gene IVa2 is a Transcriptional Activator of the Major Late Promoter". *Journal of Virology.* 68: 4450-4457.

Ueno, Y.; Umadome, H.; Shimodera, M.; Kishimoto, I.; Ikegaya, K. and Yamauchi, T. (1993). "Human Parvovirus B19 and Arthritis". *Lancet.* 341: 1280. (letter)

Upton, C.; Macen, J.L.; Schreiber, M. and McFadden, G. (1991). "Myxoma Virus Expresses a Secreted Protein With Homology to the Tumor Necrosis Factor Receptor Gene Family that Contributes to Viral Virulence". *Virology.* 184: 370-382.

Vodicka, M.A.; Koepp, D.M.; Silver, P.A. and Emerman, M. (1998). "HIV-1 Vpr Interacts with the Nuclear Transport Pathway to Promote Macrophage Infection". *Genes and Development.* 12: 175-185.

von dem Borne, A.E.G.K.; Bos, M.J.E.; Joustra-Maas, N.; Tromp, J.F.; van Wijngaarden-du Bois, R. and Tetteroo, P.A.T. (1986). "A Murine Monoclonal IgM Antibody Specific for Blood Group P Antigen (Globoside)". *British Journal of Haematology.* 63: 35-46.

von Schwedler, U.; Kornbluth, R.S. and Trono, D. (1994). "The Nuclear Localization Signal of the Matrix Protein of Human Immunodeficiency Virus Type 1 Allows the Establishment of Infection in Macrophages and Quiescent T Lymphocytes". *Proceedings of the National Academy of Sciences of the U.S.A.* 91(15): 6992-6996.

von Schwedler, U.; Song, J.; Aiken, C. and Trono, D. (1993). "Vif is Crucial for Human Immunodeficiency Virus Type 1 Proviral DNA Synthesis in Infected Cells". *Journal of Virology.* 67(8): 4945-4955.

Wang, X.S.; Yoder, M.C.; Zhou, S.Z. and Srivastava, A. (1995). "Parvovirus B19 Promoter at Map Unit 6 Confers Autonomous Replication Competence and Erythroid Specificity to Adeno-Associated Virus 2 in Primary Human Hematopoietic Progenitor Cells". *Proceedings of the National Academy of Sciences of the U.S.A.* 92: 12416-12420.

Weiland, H.T.; Salimans, M.M.M.; Fibbe, W.E.; Kluin, P.M. and Cohen, B.J. (1989). "Prolonged Parvovirus B19 Infection with Severe Anemia in a Bone Marrow Transplant Recipient". *British Journal of Haematology.* 71: 300.

Westendorp, M.O.; Frank, R.; Ochsenbauer, C.; Stricker, K.; Dhein, J.; Walczak, H.; Debatin, K.M. and Krammer, P.H. (1995). "Sensitization of T Cells to CD95-Mediated Apoptosis by HIV-1 Tat and gp120". *Nature*. 375: 497-500.

Westendorp, M.O.; Li-Weber, M.; Frank, R.W. and Krammer, P.H. (1994). "Human Immunodeficiency Type 1 Tat Upregulates Interleukin-2 Secretion in Activated T Cells". *Journal of Virology*. 68: 4177-4185.

Westmoreland, D. and Cohen, B. (1991). "Human Parvovirus B19 Infected Fetal Liver as a Source of Antigen for a Radioimmunoassay for B19 Specific IgM in Clinical Samples". *Journal of Medical Virology*. 33: 1-5.

White, D.G.; Woolf, A.D.; Mortimer, P.P.; Cohen, B.J.; Blake, D.R. and Bacon, P.A. (1985). "Human Parvovirus Arthropathy". *Lancet*. 1: 419-421.

Willey, R.L.; Maldarelli, F.; Martin, M.A. and Strebel, K. (1992). "Human Immunodeficiency Virus Type 1 Vpu Protein Induces Rapid Degradation of CD4". *Journal of Virology*. 66: 7193-7200.

Wright, C.; Hinchliffe, S.A. and Taylor, C. (1996). "Fetal Pathology in Intrauterine Death due to Parvovirus B19 Infection". *British Journal of Obstetrics and Gynaecology*. 103: 133-136.

Yaegashi, N.; Shiraishi, H.; Takeshita, T.; Nakamura, M.; Yajima, A. and Sugamura, K. (1989). "Propagation of Human Parvovirus B19 in Primary Culture of Erythroid Lineage Cells Derived from Fetal Liver". *Journal of Virology*. 63: 2422-2426.

Yakobson, B.; Hrynko, T.A.; Peak, M.J. and Winocour, E. (1989). "Replication of Adeno-Associated Virus in Cells Irradiated with UV Light at 254nm". Journal of Virology. 63(3): 1023-1030.

Yakobson, B.; Koch, T. and Winocour, E. (1987). "Replication of Adeno-Associated Virus in Synchronized Cells Without the Addition of a Helper Virus". Journal of Virology. 61(4): 972-981.

Yoo, B.C.; Lee, D.H.; Park, S. M.; Park, J.W.; Kim, C.Y.; Lee, H-S.; Seo, J.S.; Park, K.J. and Ryu, W-S. (1999). "A Novel Parvovirus Isolated from Manchurian Chipmunks". *Virology*. 253(2): 250-258.

York, I.A.; Roop, C.; Andrews, D.W.; Riddell, S.R.; Graham, F.L. and Johnson, D.C. (1994). "A Cytosolic Herpes Simplex Virus Protein Inhibits Antigen Presentation to CD8+ T Lymphocytes". *Cell*. 77(4): 525-535.

Young, N.S. (1995). "B19 Parvovirus". *Bailliere's Clinical Haematology*. 8(1): 25-56.

Young, N.S. (1996). "Parvoviruses". In: Fields, B.N.; Knipe, D.M.; Howley, P.M. et al. eds. *Virology*. 3rd Edition. Philadelphia: Lippincott-Raven. pp. 2199-2220.

Young, N.S.; Mortimer, P.P.; Moore, J.G. and Humphries, R.K. (1984). "Characterization of a Virus that Causes Transient Aplastic Crisis". *Journal of Clinical Investigation*. 73: 224-230.

Yu, H.; Chen, J.K.; Feng, S.; Dalgarno, D.C.; Brauer, A.W. and Schreiber, S.L. (1994). "Structural Basis for the Binding of Proline-Rich Peptides to SH3 Domains". *Cell*. 76: 933-945.

Zagrodney, D.B. (1998). *An Investigation into the Function of Two Small Nonstructural Proteins of the Parvovirus B19*. M.Sc. Thesis.

Zehender, G.; Varchetta, S.; de Maddalena, C.; Colasante, C.; Riva, A.; Meroni, L.; Moroni, M. and Galli, M. (2001). "Resistance to Fas-Mediated Apoptosis of Human T-Cell Lines Expressing Human T-Lymphotropic Virus Type-2 (HTLV-2) Tax Protein". *Virology*. 281(1): 43-50.

Author-less References

"ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit, with AmpliTaq DNA Polymerase, FS" Protocol. (1998). The Perkin-Elmer Corporation.

"GST Gene Fusion System" Protocol. 3rd Edition. Revision 1. (1997). Pharmacia Biotech.

"Public Health Laboratory Service Working Party on Fifth Disease. Prospective Study of Human Parvovirus (B19) Infection in Pregnancy". British Medical Journal. (1990). 300(6733): 1166-1170.