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Dr. Dennis E. Hruby

Vaccinia virus (VV), the prototype member of the orthopoxvirus family, is a large virus of complex morphology which contains a 191 Kbp double-stranded DNA genome whose expression is tightly regulated in a temporal fashion during viral replication. The regulation of gene expression can be exerted at various of levels, including transcriptional, translational, and post-translational points of control. In addition to transcriptional regulatory mechanisms, the occurrence of a variety of post-translational modifications in VV has been demonstrated. In an effort to better understand the role played by post-translational modifications during the viral replication cycle, we chose to focus on one specific modification event, ADP-ribosylation.

Experiments were designed to determine whether any VV proteins might be subject to ADP-ribosylation. The ability to metabolically label a subset of viral proteins by growth of the virus in the presence of [^{3}H]adenosine, in addition to the effects of the ADP-ribosylation inhibitor nicotinamide on viral core protein precursor processing and replication, provided evidence that this or some similar modification is an obligatory event during VV replication. Immunological reagents were used to identify several of the modified

proteins. Biochemical evidence obtained via labeling with various precursor compounds, boronate affinity chromatography, and reverse phase HPLC analysis confirmed that the proteins were modified by ADP-ribose or a closely related compound.

Additional ADP-ribosylation inhibitor studies provided further support for the initial finding that the viral proteins are subject to ADP-ribosylation or some related modification, and the evidence obtained from these experiments supports a model where this modification event might serve a function in either the proteolytic processing of the core protein precursors, or in localization of the mature core proteins to sites of VV replication within infected cells.

Evidence for the Modification of Vaccinia Virus Core Proteins by ADP-ribosylation

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CHAPTER I

INTRODUCTION

Because of the historical importance of variola virus, the causative agent of smallpox, and the use of the related orthopoxvirus, vaccinia virus, as a vaccine, the orthopoxviruses have been of interest to researchers since the earliest days of microbiology (Fenner *et al.*, 1989). Although the exact origin of vaccinia virus (VV) is unknown to this day (Buller and Palumbo, 1991), it appears to have been a distinct species for at least the last 50 to 100 years (Fenner, 1990), and at some point in time VV replaced (or possibly originated from) cowpoxvirus to become the virus used in vaccination against smallpox. Because VV virions were among the largest virus particles, and because they proved to be amenable to physical and chemical analyses even with the more rudimentary biophysical instrumentation available prior to 1950, VV provided a tool which allowed observations of fundamental importance to virology to be made (Fenner *et al.*, 1989). For example, VV was the first animal virus to be observed with a microscope, grown in tissue culture, titered, purified, chemically analyzed, and shown to contain enzymatic activity (Moss, 1990 b.).

As an experimental system, VV has many attributes which make it ideal for studying a variety of problems such as virus-host cell interactions, DNA replication, and the regulation of gene expression. The genome of VV is 191 kilobase-pairs (Kbp) in size and encodes approximately 196 different open reading frames whose expression is tightly regulated in a temporal fashion during viral replication (Wittek, 1982; Goebel *et al.*, 1990). Since approximately 90% of the sequences contained within the genome are unique, the virus is functionally haploid, making it an effective substrate for genetic manipulation (Condit and Motyczka, 1981; Bertholet *et al.*, 1986). In addition, the cytoplasmic site of VV transcription and replication necessitates that the virus contain or encode many, if not all, of the enzymes and regulatory factors essential for the replication and expression of its genetic information (Moss, 1985), and the packaging of an array of enzymes and structural proteins within the virus particles makes VV readily exploitable for biochemical investigations (Moss, 1990 b.).

In eukaryotic organisms, the regulation of gene expression can be exerted at a variety of different levels, including transcriptional, translational, and post-translational control mechanisms (Creighton, 1983), and this seems to be true for the regulation of VV gene expression as well. Transcription of VV open reading frames appears to occur by a cascade mechanism, with specific transcription factors activating the expression of certain genes at early (prior to DNA replication), intermediate, and late times during the infection cycle (Moss, 1990), as will be discussed in greater detail in the next section. In addition to transcriptional regulatory controls, a variety of post-translational modifications of VV proteins have been reported, including proteolytic processing, glycosylation, phosphorylation, acylation, and potentially ADP-ribosylation (reviewed by VanSlyke and Hruby, 1990), although the functional

role of most of these modifying groups during the VV replication cycle is still unclear at this time. The remaining portion of the introduction will be divided into an overview of the viral replication cycle with some emphasis on post-translational modifications in VV, and a brief summary of ADP-ribosylation and what is known about its functional role in various systems.

Vaccinia Virus Replication

The principal events in the poxvirus replication cycle include virus entry into the host cell, regulated gene expression, DNA replication, virion assembly, and dissemination of mature virus particles (Moss, 1990 b.), as is depicted schematically in Figure I.1 and described in more detail below.

Virus Entry

There are two forms of infectious VV virions, intracellular particles (INV), which obtain a single lipid envelope but remain associated with the cytoplasm of the host cell until cell lysis occurs, and extracellular virus (EEV), which gains a second lipid envelope by budding through the Golgi apparatus and subsequent release from the plasma membrane of the infected cell (Moss, 1990 b.; Hiller and Weber, 1985). Both forms of the virus fuse with the plasma membrane in a pH-independent manner, although the kinetics of EEV fusion are more rapid than for the INV virus (Doms *et al.*, 1990; Payne and Norrby, 1978). Entry of VV into cells can be blocked by monoclonal antibodies against at least five polypeptides in the virion membrane and by various polyclonal antisera (Buller and Palumbo, 1991), and attempts are currently being made to determine which of the five proteins are important for binding and which are important for penetration (Moss, 1990 b.). The mechanism of entry of poxviruses into cells differs from that of other viruses in that poxviruses do

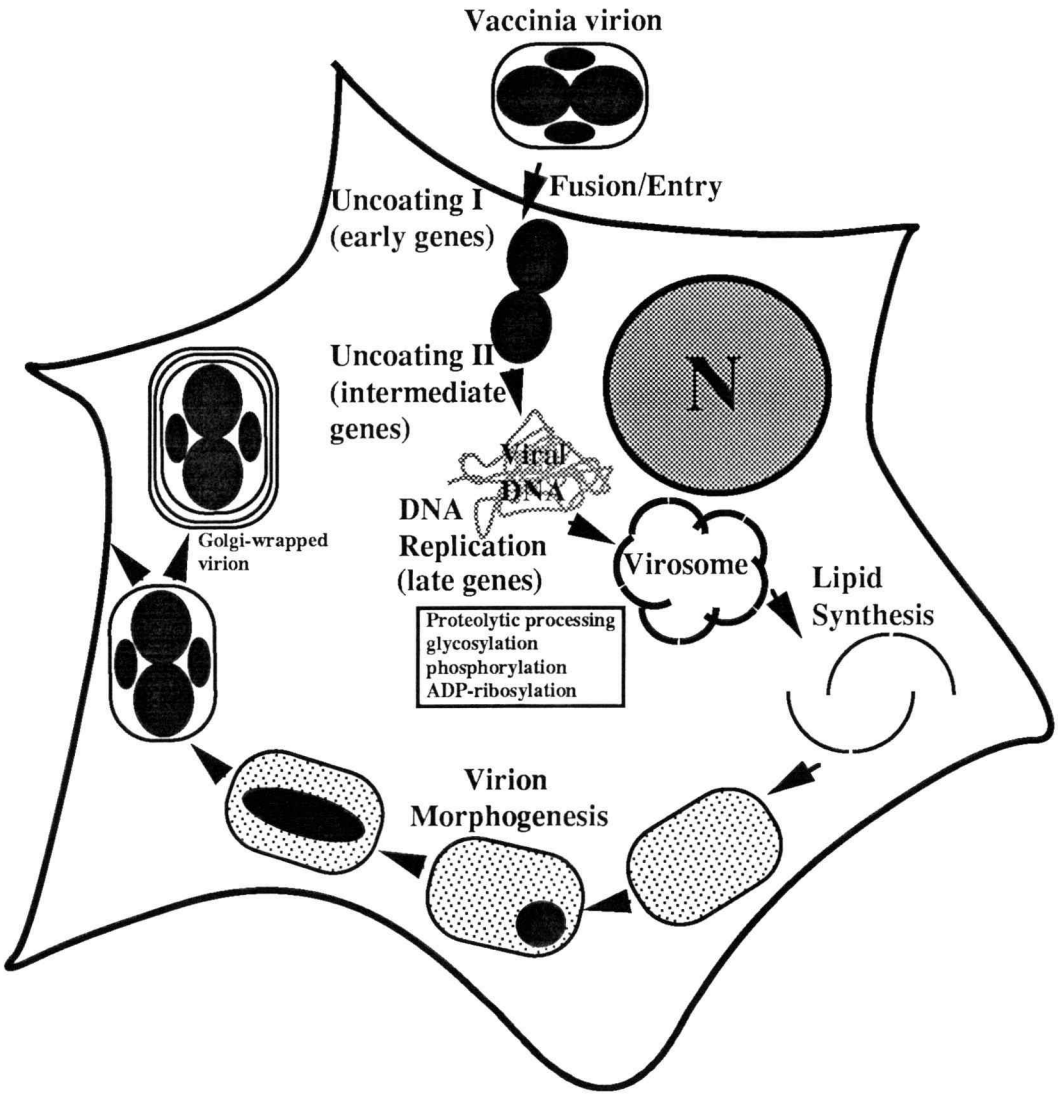


Figure I.1 *Vaccinia virus replication cycle.*

not possess one major ligand important for receptor-mediated binding (Buller and Palumbo, 1991).

Uncoating

Following entry into the cell, the VV membrane is degraded, with hydrolysis of some of the virion proteins and all of the viral phospholipid, and this process constitutes the first stage of uncoating (Dales, 1965; Fenner *et al.*, 1989). The second uncoating step occurs when the viral DNA is released from the core through distinct breaks in the core wall (Dales, 1965 b.). It appears that initial expression of selected VV genes within the core is required in order for uncoating to occur, and a 23 kDa "uncoating protein" with trypsin-like activity seems to be necessary for this process (Pedley and Cooper, 1987).

Early Gene Expression

Almost immediately after entering the cell, the VV early genes are transcribed by the viral early transcription factor and RNA polymerase, which are packaged in the core along with proteins such as the capping enzyme and poly(A) polymerase (reviewed by Moss, 1990), producing capped and polyadenylated transcripts (Wei and Moss, 1975; Kates and Beeson, 1970). VV early promoters extend only about 30 nucleotides from the RNA start site, and are very A:T-rich (see Moss, 1990). The majority of early VV transcripts are of discrete length (Cooper *et al.*, 1981; Wittek *et al.*, 1980), with termination of transcription occurring approximately 50 nucleotides downstream of the sequence TTTTT(N)T (Rohrman and Moss, 1985; Yuen and Moss, 1987). The early gene products constitute mainly enzymes involved in replication of the viral genome and possibly in synthesis of late mRNA transcripts, as well as proteins potentially involved in modification of the cytoskeleton, the

shutdown of host cell macromolecular synthesis, inhibition of interferon-induced antiviral effects, *etc.* (Buller and Palumbo, 1991).

DNA Replication

Poxvirus DNA replication occurs in the cytoplasm in discrete areas termed virus factories or virosomes (Cairns, 1960), independently of the host cell nucleus (Pennington and Follet, 1974). In the case of VV, DNA replication occurs between two and five hours after infection, producing about 10,000 copies of the genome through the formation and resolution of large concatamers of DNA (Moss, 1990 b.; Buller and Palumbo, 1991). Resolution yields genome length linear double-stranded DNA molecules with covalently linked, incompletely base-paired terminal hairpin loops (Moss, 1990 b.). While much about poxvirus DNA replication has yet to be elucidated, there is some evidence for a strand displacement mechanism where small DNA fragments are covalently linked to RNA primers, while other evidence suggests a self-priming mechanism of DNA synthesis (see Moss, 1990 b.). High levels of recombination occur during DNA replication in poxvirus-infected cells (Ball, 1987; Evans *et al.*, 1988; Spyropoulos *et al.*, 1988; Merchlinsky, 1989), and this characteristic has been useful in the laboratory for mapping viral mutants as well as for inserting foreign genes into the VV genome for expression (Moss, 1990 b.).

Late Gene Expression

As DNA replication begins, expression of the early genes ceases, and transcription of what appear to be two additional temporal classes of genes commences; the "intermediate" genes, which are transcribed as DNA replication begins, and the late genes, which are expressed after some delay (Moss, 1990 b.). Three of the intermediate gene products appear to be transcription factors which are required for the expression of the late genes

(Keck *et al.*, 1990). Analysis of late gene promoters has revealed a common TAAAT motif near the start of most late open reading frames (Moss, 1990, 1990 b.), and when the TTTTT(N)T termination sequence is present at the 3' end of late genes it is not recognized by the late transcription complex (Yuen and Moss, 1987; Moss, 1990 b.). Late mRNA transcripts do not possess defined 3' ends and are heterogeneous in length (Cooper *et al.*, 1981; Mahr and Roberts, 1984), capped (Boone and Moss, 1977), and possess both a 5' poly (A) leader (Bertholet *et al.*, 1987; Schwer *et al.*, 1987) and a 3' poly (A) tail (Moss, 1990 b.). Late gene products include structural proteins and enzymes which are destined to be packaged into virions (Buller and Palumbo, 1991).

Various late VV proteins undergo post-translational modification events. It appears that a subset of at least five (Moss and Rosenblum, 1973), and possibly eleven (Pennington, 1974) or more of the late viral proteins are subject to proteolytic processing during virion morphogenesis. The processing of at least three of these proteins, the major core proteins 4A, 4B, and 25K, is an essential step in the formation of mature infectious virions (Katz and Moss, 1970; Moss and Rosenblum, 1973; Miner and Hruby, 1989; VanSlyke *et al.*, 1991). A number of virally-encoded or induced polypeptides are subject to glycosylation (Garon and Moss, 1971; Moss *et al.*, 1971), one of which is associated with the membrane of INV particles (Garon and Moss, 1971), and nine which are present in the outer envelope of EEV particles (Payne, 1979). All of these proteins can be labeled using radioactive glucosamine, suggesting that the modifying sugar residue consists of N-acetylglucosamine (Garon and Moss, 1971; Payne, 1979). Several VV proteins are also phosphorylated, as was determined by radiolabeling viral proteins in the presence of orthophosphate (Sarov and Joklik, 1972; Rosemond and Moss, 1973; VanSlyke and Hruby, 1990). It appears that several polypeptide species

of approximately 11 kDa are subject to phosphorylation, as are a number of higher molecular weight polypeptides, but the identities of most of these substrate proteins have yet to be determined (reviewed by VanSlyke and Hruby, 1990). A number of acylated VV proteins have also been described. The major outer envelope antigen of the EEV form of the virus, p37K, is modified by palmitic acid (Hiller and Weber, 1985), and although the function of the palmitate residue is unknown, p37K appears to be involved in the envelopment of VV by the Golgi apparatus and subsequent release from the plasma membrane of the infected cell (Payne, 1980; Schmutz *et al.*, 1991). A number of other virally induced or encoded proteins also appear to be palmitated, although this remains to be confirmed (Child and Hruby, unpublished data). In addition, at least two VV proteins of 25 and 35 kDa are subject to myristylation (Franke *et al.*, 1989). The 25 kDa polypeptide has been identified as the L1R gene product and shown to be modified on the N-terminal glycine residue (Franke *et al.*, 1990), but the role of the modified protein in the VV replication cycle has yet to be elucidated. In addition to these modification events, a subset of about eight viral core proteins, including the major core proteins 4A, 4B, and 25K, appear to be subject to ADP-ribosylation (Child *et al.*, 1988), and the remaining chapters of this thesis will provide evidence which supports this finding.

Virion Assembly and Dissemination

Virion assembly occurs entirely within the granular, electron dense virosomes in the cytoplasm, and begins with the formation of spicule-coated lipoprotein bilayers which are not believe to be associated with any cellular organelle (Moss, 1990 b.). Subsequently, these bilayers appear as circular envelopes surrounding a dense nucleoprotein mass embedded in a granular matrix (Moss, 1990 b.). During maturation, extensive biochemical and

morphological changes yield mature virions which contain a dense, bi-concave core and two lateral bodies of unknown function within the viral envelope (Dales and Pogo, 1981; Moss, 1990 b.). Proteolytic processing is thought to be integrally associated with assembly (Katz and Moss, 1970; Silver and Dales, 1982). As maturation is completed, the virions are transported to peripheral areas of the cytoplasm, where they may remain localized or become enveloped by a double membrane from the Golgi apparatus, transported to the plasma membrane, and released, retaining one of the Golgi-derived membranes (Buller and Palumbo, 1991).

ADP-Ribosylation

ADP-ribosylation refers to the post-translational modification of proteins by the covalent attachment of ADP-ribose, which can be bound to protein in either monomeric or homo-polymeric form (Figure I.2). The polymers generated by poly-ADP-ribosyl transferase can range up to 65 residues in length (Purnell *et al.*, 1980; Hacham and Ben-Ishai, 1990), and a helical conformation has been proposed for long poly-ADP-ribose chains which could allow the formation of higher order helices between ADP-ribose chains (Minaga and Kun, 1983 a,b) or even between the ADP-ribose polymer and DNA (Stockert, 1983).

Nicotinamide adenine dinucleotide (NAD^+) is the donor molecule for both mono- and poly-ADP-ribosyltransferases (Ferro and Oppenheimer, 1978; Moss *et al.*, 1979; Alvarez-Gonzalez *et al.*, 1988). There is much evidence that ADP-ribosylation reactions are ubiquitous; mono-ADP-ribosyl transferases are present in bacteria, protozoa, plants, animals, and viruses (such as some of the bacteriophage) (Althaus and Richter, 1987; Shall, 1989), while poly-ADP-riboyslyation reactions occur in all higher eukaryotes and some lower

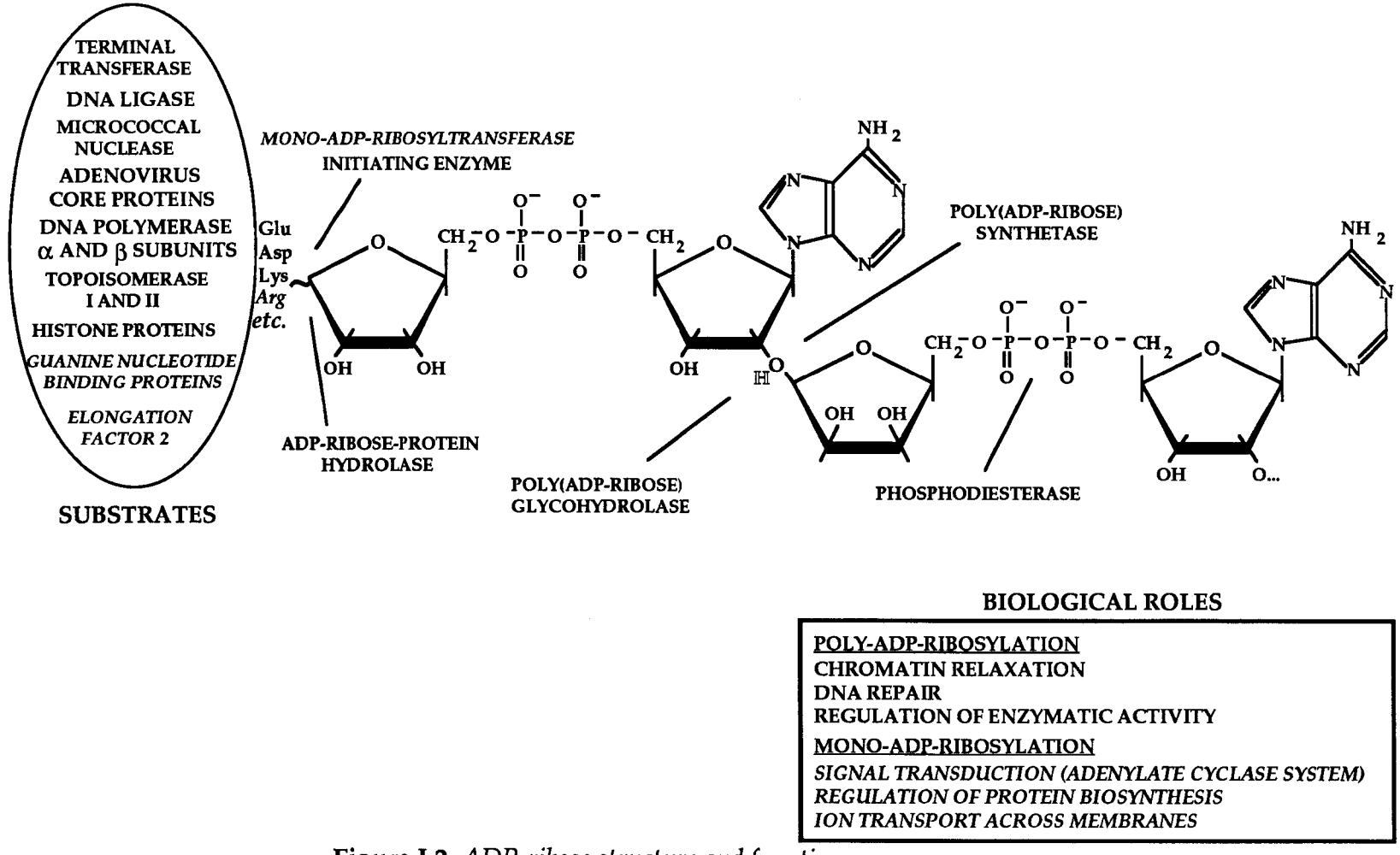


Figure I.2 *ADP-ribose structure and function.*

eukaryotes, although they have not been found in prokaryotic organisms (Purnell *et al.*, 1980; Althaus and Richter, 1987; Shall, 1989). The various ADP-ribosyltransferase enzymes utilize different amino acid residues as ADP-ribose acceptors, and there are at least three types of protein-ADP-ribose bonds which can be distinguished based on their stabilities under different incubation conditions (Purnell *et al.*, 1980; Althaus and Richter, 1987; Shall, 1989; Jacobson *et al.*, 1990). These linkages are comprised of carboxylate esters with aspartate or glutamate residues which demonstrate sensitivity to both alkaline pH and neutral hydroxlyamine, Schiff bases formed with lysine, arginine, or dipthamide (a modified histidine residue) whose N-glycosidic bonds are fairly resistant to neutral hydroxylamine (or very resistant in the case of dipthamide) but not to dilute alkali, extremely hydroxylamine-resistant but N-ethylmaleimide and $HgCl_2$ sensitive thioglycosidic bonds to cysteine residues (Aktories *et al.*, 1988), and possibly phosphodiesterase linkages with phosphoserine (Purnell *et al.*, 1980; Althaus and Richter, 1987; Jacobson *et al.*, 1990).

ADP-ribosylation reactions apparently regulate a wide variety of cellular functions. Poly-ADP-ribosylation is a nuclear event, and the physiological roles attributed to this modification include modulation of DNA repair and chromatin conformation (Gaal *et al.*, 1987; Shall, 1989), the regulation of cell cycle progression, and regulation of the activity of a number of enzymes involved in DNA replication including DNA polymerase, terminal deoxynucleotidyl transferase, DNA ligase, topoisomerase I and II, and poly-ADP-ribosyltransferase itself (Althaus and Richter, 1987). Most of what is known about the functional role of mono-ADP-ribosylation reactions comes from studies of the bacterial toxins which ADP-ribosylate specific amino acid residues in specific target proteins (Althaus and Richter, 1987; Jacobson *et al.*,

1990), but it appears that these proteins may be subject to modification by cellular enzymes as well, and there is evidence for the function of mono-ADP-ribosylation in the regulation of signal transduction, the "fine tuning" of protein synthesis by the modulation of elongation factor-2 activity, and in regulating the efflux of Ca^{2+} from mitochondria (Althaus and Richter, 1987). Further evidence that mono-ADP-ribosylation can act in regulating enzymatic activity came from the finding that the dinitrogen reductase protein in the photosynthetic bacterium *Rhodospirillum rubrum* is reversibly inactivated by ADP-ribosylation of a specific arginine residue (Ludden and Burris, 1978; Ludden *et al.*, 1982; Pope *et al.*, 1985 a, b).

Due to technical difficulties encountered in characterizing ADP-ribosylated proteins, these types of studies have proven to be challenging. Some of the problems encountered include the low steady-state levels of both mono- and poly-ADP-ribose within cells relative to other adenine-containing compounds which can interfere with analyses, the lack of effective, generally applicable *in vivo* labeling methods, the lability of protein-ADP-ribose bonds at neutral or alkaline pH which necessitates the careful control of experimental conditions in order to maintain the linkages, the apparently rapid turnover of ADP-ribose moieties (Purnell *et al.*, 1980; Jacobson and Jacobson, 1983; Aboul-Ela *et al.*, 1988), as well as the fact that different ADP-ribose acceptors are found depending on whether analyses are performed *in vitro*, in isolated nuclei, or in permeabilized or intact cells (Althaus and Richter, 1987). More sensitive methods for the detection and quantification of ADP-ribose residues have become available in recent years, however, and progress in this field has been made using improved *in vivo* labeling techniques, more sensitive radioimmunological or flurometric detection assays, improved protocols for purifying ADP-ribose away from

contaminants, and better *in vitro* assays for analyzing modified acceptor proteins as well as detecting novel ADP-ribosyltransferase activities (Purnell et al, 1980; Althaus and Richter, 1987).

Because the biological roles of ADP-ribosylation reactions are not yet well understood, and because no ADP-ribosylation-deficient cell lines exist for examining the function of the modification, inhibitors have also played a critical role in ADP-ribosylation studies (Althaus and Richter, 1987). Most of the known inhibitors are not selective for either mono- or poly-ADP-ribosylation, and the high extracellular drug concentrations ordinarily used in these inhibitor studies can lead to severe metabolic or even toxic side effects, so it is important to support the results of this type of study with experiments which do not rely on the use of inhibitors, or to at least use a number of chemically diverse compounds which would be unlikely to share common metabolic side effects (Althaus and Richter, 1987). The most commonly used inhibitor compounds to date have been the nicotinamides and the benzamides. Nicotinamide and its analogs, in addition to inhibiting ADP-ribosylation reactions, are now known to affect the synthesis of NAD^+. This may deplete cellular phosphoribosyl diphosphate pools, thus resulting in a decrease in nucleotide synthesis (Purnell and Whish, 1980). The benzamides, with 3-aminobenzamide being the most commonly used, were assumed to have no other significant metabolic effects other than inhibition of ADP-ribosylation, but recent studies have demonstrated that these compounds affect cell viability, glucose metabolism, and DNA synthesis (Milam and Cleaver, 1984). Both of these types of compounds effectively inhibit poly-ADP-ribosylation at fairly low concentrations (in most cases less than 1mM), but showed very low efficacy in inhibiting mono-ADP-ribosylation reactions; for example, 40 mM nicotinamide inhibited mono-ADP-ribosyltransferase

activity by only 32-39% (Smets *et al.*, 1990). Until recently, no inhibitors with specificity for mono-ADP-ribosyltransferases were known, but recent work indicates that the norepinephrine analog meta-iodobenzylguanidine appears to be a potent and specific mono-ADP-ribosylation inhibitor which exerts a number of effects in intact cells. These effects seem to be due to interference with endogenous mono-ADP-ribosyltransferase enzymes, including the inhibition of mitochondrial respiration (Loesberg *et al.*, 1990 a,b).

The following chapters describe our efforts to determine whether certain of the VV core proteins might be subject to ADP-ribosylation, and, if so, what the biological relevance of this modification may be during the viral replication cycle.

CHAPTER II

INHIBITION OF VACCINIA VIRUS REPLICATION BY NICOTINAMIDE: EVIDENCE FOR ADP-RIBOSYLATION OF VIRAL PROTEINS

Stephanie J. Child, Christine A. Franke, and Dennis E. Hruby

SUMMARY

Replication of vaccinia virus (VV) in monolayers of BSC_{40} cells was inhibited 99.9% in the presence of 60mM nicotinamide (NIC), a competitive inhibitor of ADP-ribosylation reactions. Dot-blot hybridization analysis of infected cell extracts utilizing a VV DNA-specific probe indicated that the drug had only minimal effects on viral DNA synthesis. SDS:polyacrylamide gel electrophoresis of newly synthesized VV proteins pulse-labeled at early (2 h) or late (8 h) times post-infection revealed that although the full spectrum of expected viral polypeptides was evident, quantitative differences in the levels of expression of a distinct subset of viral proteins were observed in the presence of the drug. Velocity sedimentation of virus-infected cell lysates established that no mature particles were assembled in drug treated cells. Additional evidence suggesting that VV morphogenesis was abortive in the presence of NIC was obtained by pulse-chase labeling experiments which demonstrated that the two VV major late core polypeptide precursors P4A and P4B, whose proteolytic processing to 4A and 4B is intimately associated with viral assembly, were not cleaved in the presence of NIC. Interestingly, growth of VV in the presence of [^{3}H]adenosine resulted in the metabolic labeling of eight proteins that were associated with purified virions. These proteins co-migrated with proteins labeled with [^{3}H]adenosine that were present in extracts of VV-infected, but not uninfected, cells. These analyses also revealed that the [^{3}H]adenosine-labeling of a subset of cellular proteins (MW 18-20 kDa), possibly histones, was increased 4-fold by VV infection. The observed induction of either increased synthesis or hyper-modification of these 18-20 kDa proteins was inhibited by NIC. These results are discussed with respect to whether one or more VV polypeptides are subject to obligatory ADP-ribosylation modification reactions in order to attain their active

configuration, and if so, whether the enzymes catalyzing these reactions are specified by the virus or host cell.

INTRODUCTION

In many respects, vaccinia virus (VV) represents an ideal model eukaryotic system with which to investigate a variety of questions related to development, virus-host cell interactions, and gene regulation. The study of gene regulation in particular would appear to be most appropriate to the VV system since this virus apparently encodes more that 200 viral genes whose expression is tightly regulated in a temporal fashion during the viral replicative cycle (Wittek, 1982). As an experimental system, VV provides at least two significant advantages for this type of study. First, the 185 Kb genome contains approximately 90% unique sequences (Baroudy *et al.*, 1982). This renders it functionally haploid and makes it an effective substrate for both classical and directed genetic studies. Secondly, unlike other DNA-containing animal viruses (*e.g.*, adenovirus or herpesvirus), VV replicates within the cytoplasmic compartment of infected cells. Previous work has demonstrated that either physical removal of the host cell nucleus (Pennington and Follet, 1974), or functional inactivation of the nucleus by UV-irradiation or α-amanitin treatment (Hruby *et al.*, 1979) renders the cell incapable of supporting VV replication. Since the virus completes at least the bulk of its replicative cycle within the cytoplasm of infected cells, this would seem to necessitate that VV contain or encode many of the enzymes and regulatory factors necessary to replicate and express its own genetic information, since the cognate cellular enzymes are primarily located in the nuclear compartment. Thus, in theory, using VV as a model system, one should have ready access to viral genes encoding essential enzymatic and structural functions as well as the factors which regulate their expression.

In eukaryotic organisms, the regulation of gene expression can be exerted at a variety of levels including chromatin structure, gene copy number,

transcriptional initiation, RNA processing and transport, translation, post-translational modifications, and assembly of individual monomers into active macromolecular structures (Creighton, 1983). Since VV expresses its genetic program within the confines of a host cell, one would presume that many of these same mechanisms are employed by the virus. The only VV gene which has been examined in any detail with regard to these questions is the thymidine kinase gene (Hruby and Ball, 1981). As expected, evidence has been obtained to indicate that the expression of thymidine kinase activity in VV-infected cells is regulated at the level of transcription, translation, RNA turnover, and feedback inhibition (Hruby *et al.*,1983). Most of the current research efforts into the important elements of VV gene regulation are focused on the process of transcription (Cochran *et al.*, 1985; Weinrich and Hruby, 1986). It is likely that translation and post-translational modifications will prove to be equally important features of the overall regulatory schemes employed by VV.

Post-translational modification of VV polypeptides by proteolytic cleavage (Moss, 1985), glycosylation, and phosphorylation (Sarov and Joklik, 1972) has been reported. With regard to the possibility that VV proteins might be ADP-ribosylated, it was of interest to note the recent report by Déry *et al.* (1986) indicating that adenovirus core polypeptides are apparently ADP-ribosylated. This was shown both by direct labeling of viral proteins with [^{3}H]adenosine and by using the drug nicotinamide. Treatment of infected cells with nicotinamide (NIC), a competitive inhibitor of ADP-ribosylation reactions, depressed the yield of adenovirus particles by 9-fold and the production of infectious virions by 10^{-4}. It was therefore of interest to determine if this was an isolated phenomenon or if other animal viruses might also modify their proteins in this manner. The experiments reported in this paper demonstrate

an inhibitory effect of NIC on VV replication and gene expression and present evidence which suggests that one or more VV polypeptides are subject to obligatory ADP-ribosylation reactions.

MATERIALS AND METHODS

Cells and virus

BSC_{40} cells, a clonal line of African Green Monkey kidney cells, were maintained in monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS, GIBCO Laboratories), 2 mM L-glutamine, and 50 μg/ml gentamycin sulfate.

VV (strain WR) was used to infect monolayer cultures of BSC_{40} cells. Crude virus stocks were prepared by harvesting infected cells into 1 ml of phosphate buffered saline, then lysing the cells by three cycles of freeze-thawing to release intracellular virus. The titer of the crude stocks was determined by plaque assay on confluent monolayers of BSC_{40} cells (Hruby *et al.*,1979). Partially purified virus stocks were prepared as previously described (Hruby *et al.*, 1979).

Nicotinamide (Sigma Chemical Company) was added at 60-80 mM concentrations to monolayer cultures at 0 hours post infection (T_0) unless otherwise specified.

Extraction and dot blot analysis of VV DNA

Duplicate dishes (100 mm) of BSC_{40} cells were infected with partially purified VV at a multiplicity of infection (MOI) of 1 PFU per cell. NIC (60 mM) was added to one dish in each set at T_0 during infection. Infected monolayers were incubated at 37°C in MEM plus 5% FCS. Infected cells were harvested at 0, 2, and 8 hours post infection (hpi) by loosening the cells with a rubber policeman and collecting the cells by centrifugation at 2,000 rpm for 5 min at 4°C in an International Equipment Co. clinical centrifuge. The cells were then resuspended in 1 ml of phosphate buffered saline, and lysed by three cycles of freeze-thawing. Lysates were treated with 0.25% trypsin (GIBCO Laboratories) at 37°C for 30 min. Cellular and viral DNA were

collected on nitrocellulose by filtration with a microsample manifold (Schleicher and Schuell, Inc.). Binding of viral DNA was carried out by blotting the nitrocellulose filter for 3 min on Whatman 3MM paper saturated with 0.5M NaOH, with this step repeated two more times, then blotted three times for 3 min with 1M Tris.Cl (pH 7.6)-0.15M NaCl for 15 min, and baked in a vacuum oven at 80°C for 2 h. The nitrocellulose-bound DNA was hybridized with a cloned [^{32}P]-labeled (nick-translated) VV *Hind*III D DNA fragment. Hybridization and wash conditions were as previously described (Villarreal and Hruby, 1986). The filter was then placed at -70°C and exposed to Kodak XAR-5 X-ray film.

Polypeptide analysis

Duplicate dishes (100 mm) of BSC_{40} cells were either infected with VV (25 PFU per cell) or mock infected, and incubated at 37°C in MEM plus 5% FCS. Pulse-labeling of viral and cellular polypeptides was carried out at 0, 2, and 8 hpi by incubating sets of infected and mock infected BSC_{40} cells (with 60 mM NIC present in one dish in each set) with MEM (minus cold methionine) containing 10 μCi/ml [^{35}S]L-methionine (New England Nuclear; 1,125 Ci/mmol) for 30 min at 37°C.

After labeling, the cells were loosened with a rubber policeman, pelleted by centrifugation at 2,000 rpm for 3 min at 4°C in a Beckman TJ-6 centrifuge, resuspended in 100 μL of 1 mM Tris.Cl (pH 9.0), and lysates stored at -20°C until subsequent gel analysis. Pulse-chase labeling of VV-infected and mock infected BSC_{40} cells (plus or minus 60 mM NIC) was done as described above, with the exception that the cells were pulse-labeled for 30 min at 8 hpi, followed by a 4 h chase period in which cells were incubated in MEM plus 5% FCS plus 10 mM (100X) unlabeled methionine. Lysates were prepared and stored as described above. Continuous labeling of viral and host polypeptides

(plus or minus 80 mM NIC) with radioactive adenosine was carried out by adding MEM plus 5% FCS containing 10 μCi/ml [^{3}H]adenosine (Amersham; 40 Ci/mmol) at 1 hpi, and incubating the infected and mock-infected cells at 37°C for an additional 18 h to allow a single cycle of virus maturation. Lysates were harvested as described above.

All labeled lysates were analyzed by electrophoresis on 12 or 15% SDS:PAGE gels (Studier, 1973). Gels with [^{3}H]adenosine were processed for fluorography, dried, and placed at -70°C while exposed to Kodak XAR-5 X-ray film. Gels with [^{35}S]L-methionine were dried and exposed to Kodak XAR-5 X-ray film at room temperature.

Densitometric scanning

Proteins which were subject to apparent hyper-ADP-ribosylation (18-20,000 MW) were subjected to electrophoresis on a 15% polyacrylamide gel, autoradiographed, and the autoradiograph scanned on a Biomed densitometric scanner (kindly provided by the laboratory of Dr. Kensal VanHolde) to give measurements of relative protein band density.

Velocity sedimentation of viral particles

Confluent BSC_{40} monolayers were infected with 5 PFU of VV per cell and incubated at 37°C in MEM plus 5% FCS. At 1 hpi, 1 μCi/ml [^{3}H]thymidine (New England Nuclear; 80 Ci/mmol) was added to two dishes of infected cells (one plus 60 mM NIC, one minus NIC), and 10 μCi/ml [^{3}H]adenosine added to one dish. The infected cells were incubated at 37°C for 24 h, then loosened with a rubber policeman and pelleted by centrifugation at 2,000 rpm for 5 min at 4°C in a Beckman TJ-6 centrifuge. The infected cells were then lysed by swelling in 10 mM Tris.Cl (pH 9.0) followed by Dounce homogenization, and the cell nuclei pelleted by centrifugation. Samples were layered on 36% (w/v, in 10 mM Tris.Cl, pH 9.0) sucrose cushions and virus particles pelleted in a

SW41 rotor at 18,000 rpm for 80 min at 4°C in a Beckman model L8-70M ultracentrifuge. Virus pellets were then Duall homogenized, layered on 25-40% (w/v) sucrose gradients, and centrifuged at 13,500 rpm for 40 min at 4°C. Fractions (0.5 ml) were collected from the bottom of the gradients, and 50 μL aliquots of each fraction were analyzed for radioactivity by liquid scintillation counting. Peak fractions of the [^{3}H]thymidine and [^{3}H]adenosine-labeled virus were also analyzed by polyacrylamide gel electrophoresis on a 12% SDS:PAGE gel, and the results visualized by autoradiography as described above.

RESULTS

Effect of nicotinamide on VV replication

The effect of nicotinamide (NIC) on VV replication was determined by infecting monolayers of BSC_{40} cells with VV in the presence of the concentrations of drug indicated in Figure II. 1 and assaying the yield of progeny virions after 24 h of incubation at 37°C. As is evident, increasing NIC concentrations elicited a concomitant reduction in the replication of VV. A 60 mM concentration of NIC was sufficient to suppress viral replication by greater than 99%, and this concentration of the drug was used for subsequent experiments unless otherwise indicated. To determine the effect of addition of NIC at different times prior to and after infection, a time of addition experiment was also carried out (data not shown), and the results of this experiment indicated that addition of NIC at 0 hours post infection (hpi) was as effective as pre-incubation of cells in the presence of drug, while addition at time points after infection decreased the ability of the drug to suppress viral replication. In addition, microscopic observation of virus-infected and uninfected cells incubated in the presence of NIC indicated no observable cytopathology caused by this drug treatment regimen.

Since NIC effectively inhibited VV replication, it was of interest to determine at which level of replication this inhibition is exerted. The effect of NIC on VV DNA replication was first examined. Cytoplasmic extracts were prepared and analyzed as described in the Materials and Methods section. Since viral DNA replication does not begin until 1.5 hpi in BSC_{40} cells (Hruby *et al.*, 1980), little or no viral DNA was detectable at 0 h and 2 hpi (Figure II. 2.A) either in the presence or absence of drug. At 8 hpi, VV DNA was evident both in the presence and absence of NIC, although DNA synthesis in the presence of the drug seemed to be somewhat reduced. These results suggest

Figure II. 1. *Inhibition of VV replication by nicotinamide.* BSC_{40} cell monolayers were infected at 5 PFU per cell with VV. The indicated concentrations of drug were added at T_0. After 24 h of infection, progeny virus was harvested and titrated by plaque assay.

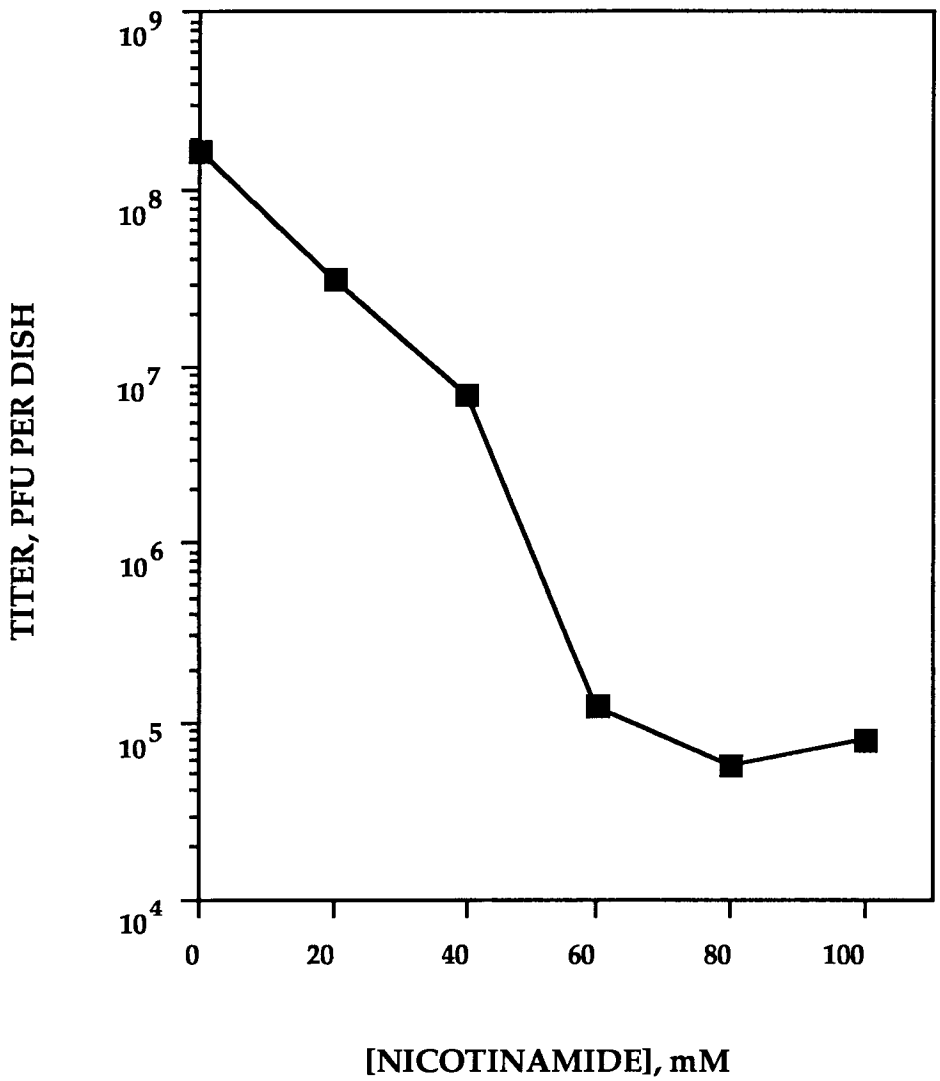

TITER, PFU PER DISH
10^9
10^8
10^7
10^6
10^5
10^4
0
20
40
60
80
100
[NICOTINAMIDE], mM

Figure II. 1

Figure II. 2. *Effect of nicotinamide on VV DNA and protein synthesis.* A. BSC_{40} cell monolayers were infected at 1 PFU per cell in the presence (+) or absence (-) of 60 mM NIC, and the cells harvested at the indicated times. The lysates were then subjected to dot blot filtration and hybridized with a VV DNA-specific probe. B. Gel electrophoresis of radiolabeled viral polypeptides. Cellular and viral polypeptides from mock infected (MI) and VV-infected (WT) BSC_{40} cells in the presence (+) or absence (-) of 60 mM NIC were pulse-labeled for 30 min by the addition of [^{35}S]methionine at the indicated times post infection, and the lysates subjected to electrophoresis on a 12% SDS:PAGE gel. The two major VV core polypeptide precursors P4A and P4B are indicated on the right. The molecular weights (x 10^3) of marker proteins are indicated on the left.

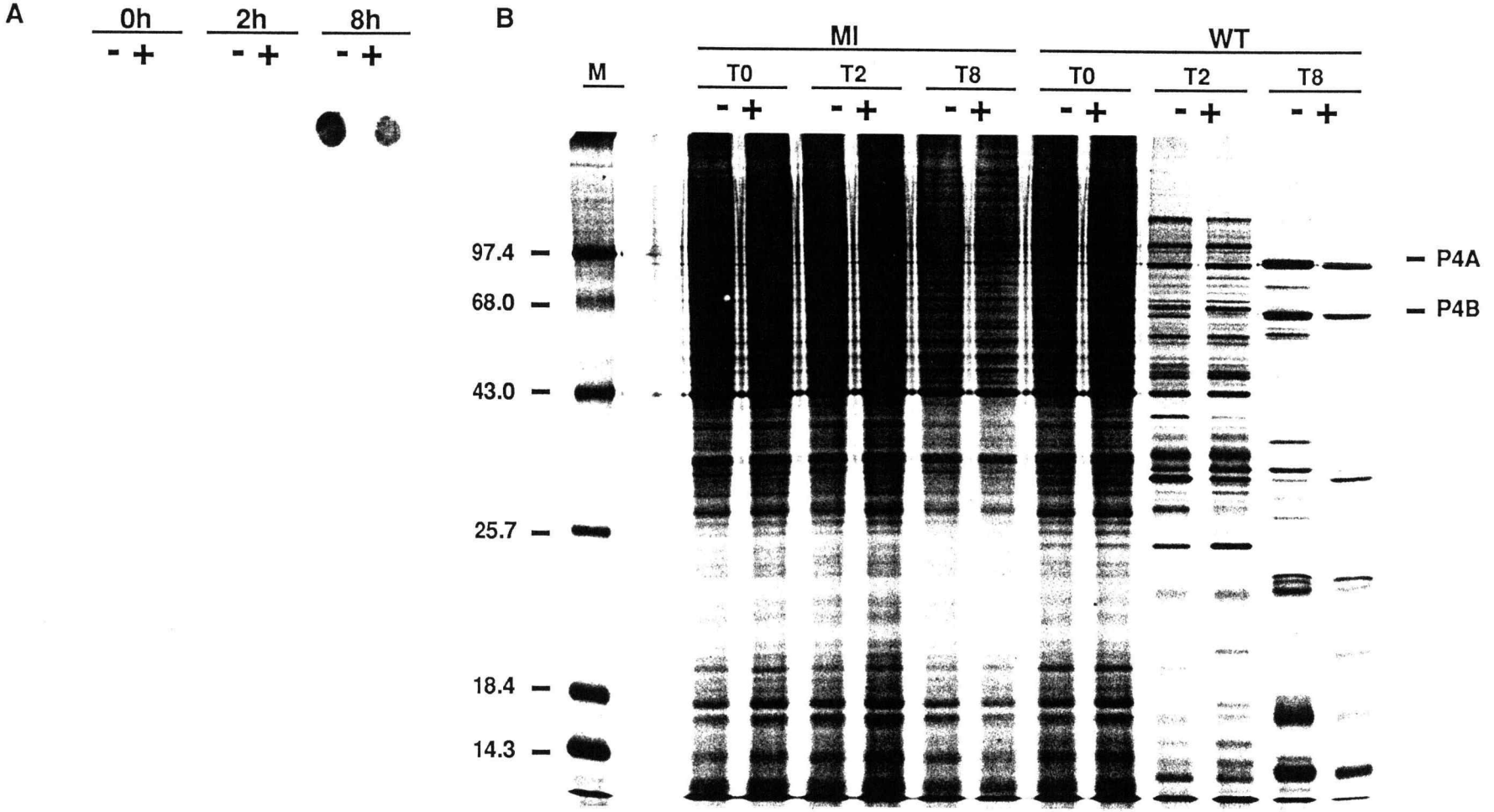


Figure II. 2

that both the early polypeptides, which are those expressed prior to DNA replication (Moss, 1985), and DNA are synthesized in the presence of NIC.

To further characterize what effects, if any, NIC treatment might cause at the level of protein synthesis, cellular and viral polypeptides were pulse-labeled with [^{35}S]L-methionine and analyzed by SDS:polyacrylamide gel electrophoresis as indicated in Figure II. 2.B. NIC does not appear to affect host cell protein synthesis at any of the times examined. At 0 hpi in VV-infected cells, the virus has not yet shut down host cell macromolecular synthesis, and again no effects from the drug are visible. Both early (T_2) and late (T_8) polypeptides are detectable for VV infections both in the presence and absence of NIC, although some quantitative differences in protein bands are evident in comparing the plus and minus drug lanes for both T_2 and T_8. For example, at 2 hpi, 25 kDa, 30 kDa, and 68 kDa proteins appear to be elevated in the presence of NIC, while 26 kDa, 29 kDa, and 40 kDa proteins seem to be reduced. Likewise, at 8 hpi, 20 kDa and 28 kDa polypeptides appear to be elevated, while those at approximately 24 kDa, 30 kDa, 65 kDa, and 94 kDa are reduced (Note that the latter two proteins have the same size as the major virion protein precursors P4A and P4B). Quantitative analysis of VV late protein synthesis in the presence or absence of NIC indicated that the drug caused approximately a 50% inhibition of incorporation of radioactive metabolites into viral proteins (data not shown). Thus, it appears that while VV early and late proteins are synthesized in the presence of NIC, some quantitative differences in protein levels do occur.

Since both viral DNA and proteins were synthesized in the presence of NIC, albeit in a somewhat altered fashion, it was of interest to determine whether viral particles were assembled. Cells were infected with VV in the presence or absence of NIC, and incubated in the presence of [^{3}H]thymidine to

label viral DNA. Virus particles were then purified and analyzed by velocity sedimentation (Figure II. 3). A peak representing labeled viral particles was present in extracts from cells infected in the absence of drug. Few, if any, viral particles were present in the extracts from drug-treated cells. The peak virus fractions (minus NIC) were analyzed by plaque titration and found to contain infectious viral particles, while the titers of the same fractions from extracts of cells infected in the presence of NIC were reduced by at least 3 logs. These results indicate that while VV early polypeptides, DNA, and late polypeptides are synthesized in the presence of NIC, infectious viral particles are not formed. Thus it was of interest to determine how NIC acts to prevent infectious particle formation.

The biological effects of NIC on VV infection are similar to those previously seen with the drugs α-amanitin or rifampicin, namely, VV early polypeptides, DNA, and late polypeptides are synthesized in the presence of these drugs, but as with NIC, mature infectious particles are not formed. Both drugs act, either directly or indirectly, to inhibit viral replication. It has also been shown that both rifampicin (Moss *et al.*, 1969) and α-amanitin (Villareal *et al.*, 1984) prevent the cleavage of the two major VV core precursor polypeptides, P4A and P4B, into the mature core proteins 4A and 4B. Since NIC also acts to prevent the formation of infectious virus particles, it was of interest to examine whether NIC would also inhibit these VV-specific proteolytic cleavages. Figure II. 4 shows the results of pulse-chase analysis of VV-infected cells incubated plus or minus NIC. Examination of the pulse and chase lanes minus NIC reveals that the core precursor proteins P4A and P4B are indeed cleaved into the mature core proteins 4A and 4B, while in the presence of the drug processing of the precursor polypeptides into their mature forms does not occur. Hence it would seem that NIC does act in a

Figure II. 3. *Velocity sedimentation of viral particles.* BSC_{40} cells were infected with VV in the presence (Δ) or absence (■) of 60 mM NIC, and viral DNA labeled for 24 h with [^{3}H]thymidine. Labeled virus particles were analyzed by velocity sedimentation and titers of the peak fractions plus (▲) and minus (❑) NIC determined by plaque titration.

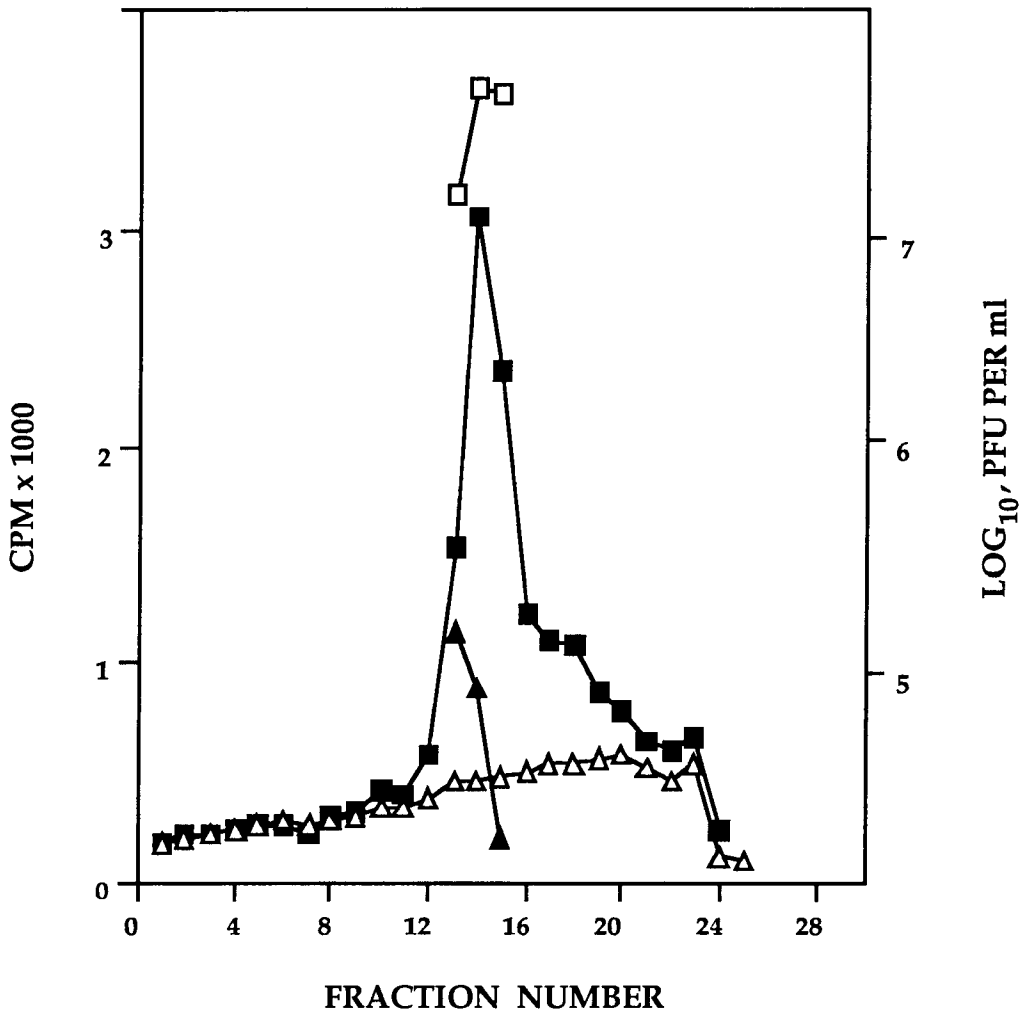

CPM x 1000
0
1
2
3
0
4
8
12
16
20
24
28
FRACTION NUMBER
5
6
7
LOG10, PFU PER ml

Figure II. 3

Figure II. 4. *Proteolytic processing of VV proteins.* BSC_{40} cell monolayers were infected with VV at 25 PFU per cell plus (+) or minus (-) 60 mM NIC, and viral and cellular polypeptides pulse labeled for 30 min with [^{35}S]methionine at 8 hpi. After a 4 h chase period with cold methionine, lysates were harvested, and subsequently analyzed by electrophoresis on a 12% SDS:PAGE gel. The two major VV core polypeptides 4A and 4B and their precursors P4A and P4B are indicated on the right. The molecular weights (x 10^3) of marker proteins are indicated on the left.

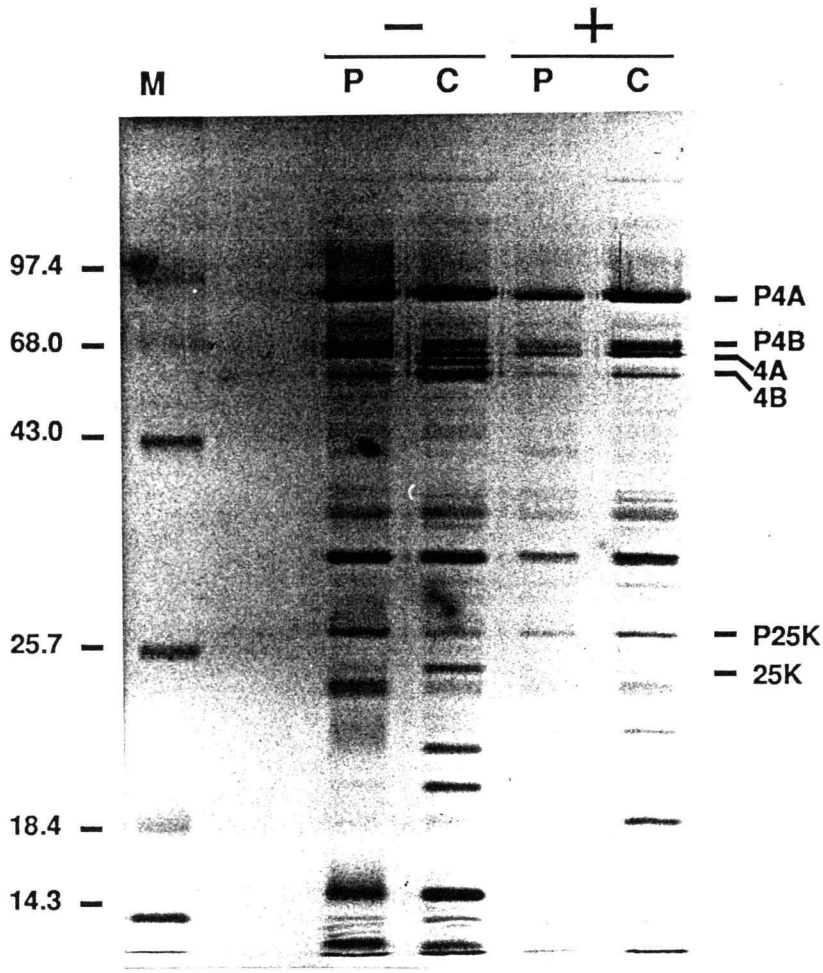


Figure II. 4

manner analogous to that of α-amanitin and rifampicin in preventing the proper proteolytic processing of the major VV core polypeptides.

ADP-ribosylation of viral proteins

Since NIC is known to inhibit ADP-ribosylation reactions (Déry *et al.*, 1986), we wanted to determine whether the NIC-mediated inhibition of VV replication might indicate that one or more VV proteins were subject to this modification reaction. To examine the possibility that VV proteins are ADP-ribosylated, infected cells were incubated in the presence of [^{3}H]adenosine, which, unlike nicotinamide adenine dinucleotide (NAD^+), the substrate for ADP-ribosylation reactions, is taken up by intact cells. It was postulated that some portion of the labeled adenosine would be converted to NAD^+, and would thus be made available as substrate for the ADP-ribosylation of cellular and VV proteins. A peak of [^{3}H]adenosine-labeled virus was purified from infected cells [However, the majority of this label was shown to be incorporated into viral DNA, as would be expected (data not shown)]. In order to establish whether any viral polypeptides were [^{3}H]adenosine-labeled, the purified virus was analyzed by SDS: polyacrylamide gel elecrophoresis (Figure II. 5). This data indicated that several labeled polypeptides, either cellular proteins which had been packaged by the virus or proteins of viral origin, are present in purified virus particles (thymidine-labeled purified virus was used as a negative control). It also appears that two of these proteins comigrate with the mature VV core polypeptides 4A and 4B. In order to determine the origin of the adenosine-labeled polypeptides, [^{3}H]adenosine-labeled mock infected (MI) and VV-infected (WT) cell lysates were also analyzed. Labeled proteins are present in both MI and VV-infected cells, with a protein cluster at 18-20 kDa being the most heavily labeled. It is also clear that the presence of NIC greatly reduces the amount of label seen in

Figure II. 5. A. *Continuous labeling of BSC_{40} cell and viral polypeptides with [^{3}H]adenosine.* BSC_{40} cells were mock infected (MI) or VV-infected (WT) at 25 PFU per cell in the presence (+) or absence (-) of 80 mM NIC, and labeled for 18 h with [^{3}H]adenosine. Samples were analyzed on a 12% SDS:PAGE gel. B. *Gel electrophoresis of [^{3}H]adenosine and [^{3}H]thymidine-labeled virus particles.* Labeled viral particles which had been collected by velocity sedimentation were analyzed by polyacrylamide gel electrophoresis as in panel A. The [^{3}H]adenosine-labeled virus-containing lane is designated A, the [^{3}H]tritium-labeled virus lane is marked T. The molecular weights (x 10^3) of marker proteins (M) are indicated on the right.

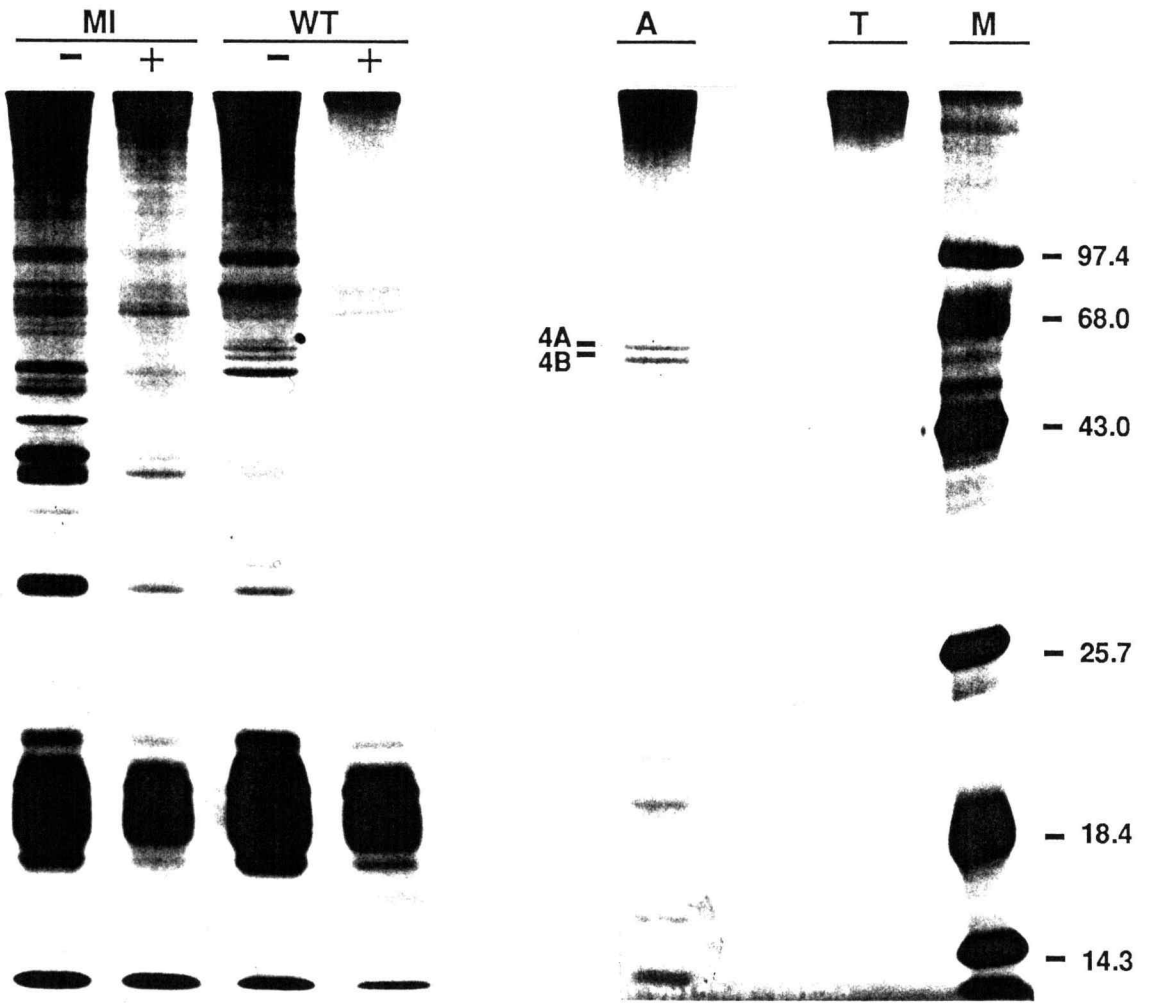


Figure II. 5

both MI and VV-infected cells. The presence of several new labeled bands in the VV-infected lane minus drug which comigrate with virion proteins suggests that some of the labeled proteins are virally-encoded, or that host cell proteins might be modified and thus migrate anomolously during infection by VV. It also appears that VV-infection induced the [^{3}H]adenosine-labeling of the proteins at 18-20 kDa. It is interesting to note that the proteins most heavily labeled with [^{3}H]adenosine migrate at the same position on SDS:polyacrylamide gels as the histone proteins are known to migrate, and that the histones are known to be modified by ADP-ribosylation (Burzio *et al.*, 1979; Ueda *et al.*, 1975). In order to better study the apparent induction of the bands at about 20 kDa, the MI and VV-infected lysates were electrophoresed on a 15% SDS polyacrylamide gel to better resolve the clustered proteins, and an autoradiogram of the induced bands scanned on a densitometer (Figure II. 6). The results of this scanning, shown below the gel in a bar graph, indicate that the intensity of the bands is induced by 4-fold in the presence of viral infection. This suggests that infection by VV induces either synthesis of these proteins or modification of pre-existing host cell proteins.

Figure II. 6. *Densitometric analysis of [^{3}H]adenosine-labeled polypeptides.* The [^{3}H]adenosine-labeled mock infected (MI) and VV-infected (WT) cell lysates used in Fig. 5. A. were subjected to electrophoresis on a 15% SDS:PAGE gel. Densitometric analysis of the superinduced polypeptide bands (18-20,000 MW) was performed using an autoradiograph of the gel on a Biomed densitometric scanner, and the results plotted as a bar graph. The "+" and "– " designations indicate samples incubated in the presence or absence of 80 mM NIC, and the molecular weights of marker proteins (M) are shown at the left.

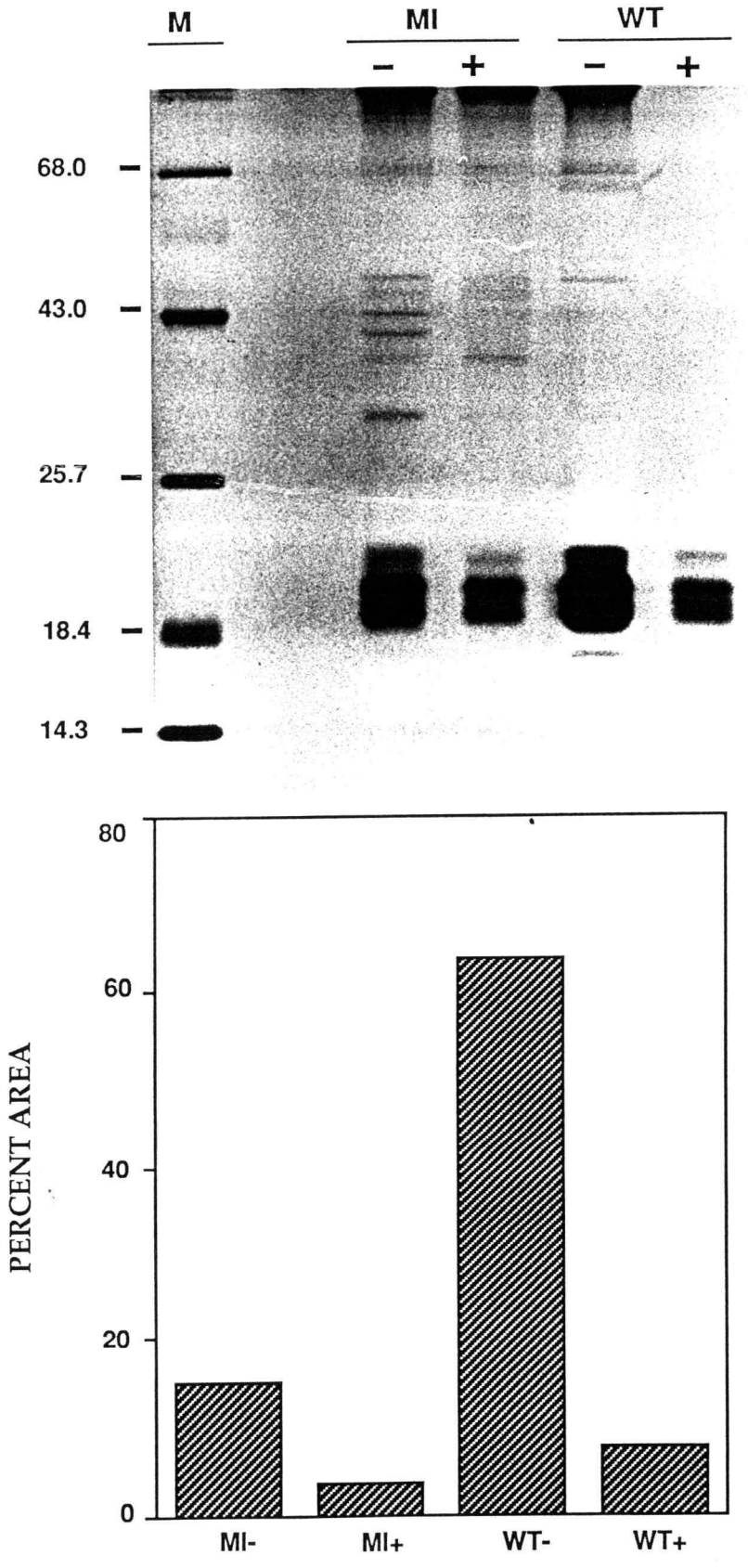


Figure II. 6

DISCUSSION

The results presented here suggest that one or more VV proteins are subject to an ADP-ribosylation post-translational modification reaction in order to function properly during the viral replicative cycle. This conclusion is based on two lines of evidence. First, the replication of VV is blocked by the drug nicotinamide, a competitive inhibitor of ADP-ribosylation. Although most of the viral macromolecular syntheses occur in the presence of this drug, one or more of the essential virion components are apparently absent or nonfunctional, as progeny particles are not assembled. Second, a number of virion-associated proteins are metabolically labeled when VV infections are carried out in the presence of [^{3}H]adenosine. These proteins comigrate with adenosine-labeled proteins found in extracts of infected, but not uninfected cells, indicating that they are either virally-encoded or virally-induced proteins. An alternative explanation for the NIC-induced inhibition of VV replication is that it is due to a generalized inhibition of late viral protein synthesis which blocks viral assembly. This seems less likely in view of previous work (Villareal *et al.*, 1984) which demonstrated that VV drug resistant mutants were able to produce normal yields of progeny virions in the presence of the drug α-amanitin, which also substantially affects late viral protein synthesis.

We have interpreted the incorporation of [^{3}H]adenosine into VV proteins as evidence that these proteins are being post-translationally ADP-ribosylated. Another explanation of this data is that the labeled adenosine is being degraded and the tritium label recycled within the cell into the amino acid pool. This latter possibility seems unlikely in view of the fact that of the more than 200 VV encoded proteins (Moss, 1985), only a small subset of eight polypeptides become labeled. If amino acids were radioactive one would

anticipate that all of the VV proteins should become labeled. Furthermore, if the adenosine label was being mostly channeled into the amino acid pool, one would expect to see the major cytoplasmic protein actin (56 kDa) labeled, but this is not the case (Figure II. 5). It would, of course, be more satisfactory to use radioactive NAD^+, the direct precursor in the ADP-ribosylation reaction, as the substrate for labeling VV proteins. Unfortunately, this compound is not taken into intact cells without resorting to harsh treatments which render them unlikely to support a subsequent VV infection. It is likely that future experiments will be directed towards developing an appropriate cell-free ADP-ribosylation system, as well as a direct molecular characterization and identification of the [^{3}H]adenosine-labeled VV proteins to circumvent this problem.

Nicotinamide has previously been shown to be an effective inhibitor of adenovirus replication. In that system, it was found that some of the virion capsid proteins were ADP-ribosylated and it was postulated that this modification was important for maintaining capsid integrity and catalyzing subsequent decapsidation reactions after infection (Déry *et al.*, 1986). Thus it is of interest to note that the VV proteins which were thought to be ADP-ribosylated are apparently virion proteins, including two polypeptides which comigrate with with the major virion structural proteins 4A and 4B, although the identity of these latter two proteins remains to be firmly established using immunological reagents. Therefore, by analogy to the adenovirus system, one could suggest that the function of ADP-ribosylation of VV proteins is to allow virion proteins to assume an active configuration necessary for assembly and maintenance of the VV virion.

It was of interest to note the four-fold VV-induced increase in adenosine labeling of proteins which migrate at 18-20 kDa. While the evidence is far

from conclusive, the size and quantity of these proteins, previous reports of ADP-ribosylation, and the fact that these proteins are present in both infected and uninfected cells is consistent with the hypothesis that these proteins are cellular histones (Poirer *et al.*, 1982; Ueda *et al.*, 1975). If this hypothesis is correct, it is totally unknown how or why a cytoplasmically-replicating virus such as VV would induce the hyper-modification (or perhaps overexpression) of these proteins and what role, if any, they might have within the viral life cycle.

The identity of the enzyme which is responsible for adding ADP-ribose residues to VV proteins, and perhaps cellular histones, is unknown. Possibilities include the pre-existing cellular enzyme, a cellular enzyme which is modified by viral infection, or a virally encoded ADP-ribose polymerase activity. Since VV is a large, complex animal virus that has already been shown to encode a considerable number of enzyme activities (Dales and Pogo, 1981; Moss, 1985), the latter possibilty must be considered. However, in an effort to address this question, we have attempted to generate a nicotinamide-resistant VV mutant using a strategy similar to that successfully employed in the past to generate α-amanitin-resistant and phosphonoacetic acid-resistant mutants (Villarreal *et al.*, 1984; Traktman *et al.*, 1984). This effort has been unsuccessful (data not shown). This result might be anticipated if a cellular enzyme is being employed to modify eight different viral proteins as opposed to the virus encoding its own enzyme. Experiments are currently in progress to test this conclusion.

CHAPTER III

BIOCHEMICAL EVIDENCE FOR THE MODIFICATION OF VACCINIA VIRUS CORE PROTEINS BY ADP-RIBOSYLATION

Stephanie J. Child and Dennis E. Hruby

SUMMARY

Growth of vaccinia virus (VV) in the presence of radiolabeled adenosine ([2, 8, 5'-^{3}H]- or [8-^{14}C]adenosine) or [^{32}P]orthophosphate results in the metabolic labeling of at least eight proteins which become packaged into mature virus particles. Three of these proteins were identified as the major viral core proteins 4A, 4B, and 25K based on comigration of adenosine- and [^{32}P]orthophosphate-labeled proteins from purified VV with [^{35}S]methionine-labeled viral proteins and by immunoprecipitation with monospecific polyclonal antisera. Boronate affinity chromatography and HPLC analyses suggested that this set of viral proteins is modified by a *cis*-diol-containing adenylate compound. When the VV virion proteins were treated with pH and temperature conditions known to disrupt protein-ADP-ribose bonds, the electrophoretic mobility of the major core proteins on phosphate gels was altered in comparison to proteins in untreated control samples. Together these results provide several lines of independent biochemical evidence that the major VV core proteins are subject to ADP-ribosylation or some related form of modification.

INTRODUCTION

Vaccinia virus (VV), the prototype member of the poxvirus family, has a 192 kilo base-pair double-stranded DNA genome which encodes approximately 200 open reading frames (Wittek 1982; Goebel *et al.*, 1991). Expression of these genes is tightly regulated and appears to occur in a cascade fashion, with specific transcription factors activating the expression of different sets of genes at early (prior to DNA replication), intermediate, and late times during the infection cycle (Moss, 1990). Since VV replicates within the cytoplasmic compartment of infected cells, it follows that the virus must contain or encode many, if not all, of the enzymes and regulatory factors essential for the replication and expression of its genetic information (Moss, 1985). This, along with the essentially haploid genome of the virus (Baroudy *et al.*, 1982), makes VV an excellent system for the genetic and biochemical study of viral replication and gene expression, and provides an accessible model for examining cognate regulatory mechanisms employed by the host cell. Regulation of gene expression can be exerted at a variety of levels, including transcriptional, translational, and post-translational points of control (Creighton, 1983). In addition to transcriptional regulatory controls, a variety of post-translational modifications, including proteolytic cleavage, glycosylation, phosphorylation, acylation, and possibly ADP-ribosylation, have been identified on VV proteins (VanSlyke and Hruby, 1990).

With regard to the latter modification, ADP-ribosylation, previous studies have shown that although 60 mM nicotinamide (NIC), a competitive inhibitor of ADP-ribosylation reactions, inhibits VV replication by greater than 99%, the drug has minimal effects on viral DNA and protein synthesis (Child *et al.*, 1988). In addition, mature virus particles are not formed in the presence of NIC, nor does proteolytic cleavage of the major core proteins

occur, an event which is obligatory during virion morphogenesis (Child *et al.*, 1988). Although it is unclear what potential roles ADP-ribosylation may play during the VV replication cycle, this modification has been implicated in a diverse array of cellular functions including responses to DNA damage, alteration of enzyme activity, involvement in cell cycle progression, alteration of chromatin conformation during various physiological processes, and in the case of mono-ADP-ribosylation, regulation of signal transduction, enzyme activity, and perhaps of the release of Ca^{+2} from mitochondria (Althaus and Richter, 1987). It has also been suggested that ADP-ribosylation of adenovirus core proteins might play a role in virus decapsidation, and in the relaxation of the core chromatin structure during viral replication (Déry *et al.*, 1986). We have sought to determine whether, like adenovirus, VV core proteins might also be modified by ADP-ribosylation, and whether such a modification could serve a comparable function during the VV replication cycle. The work described here provides biochemical and immunological evidence that the major VV core proteins 4A, 4B, and 25K, as well as several other virion associated proteins, are subject to modification by an adenosine-containing moiety, and suggests that this modification is likely to be ADP-ribosylation.

MATERIALS AND METHODS

Cells and virus

BSC_{40} cells were maintained in monolayer culture in Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, and 50 μg/ml gentamycin sulfate. VV (WR strain) was maintained and purified as previously described (Hruby *et al.*, 1979). Infected cell extracts were prepared by washing the cell monolayers 2-3 times with phosphate buffered saline containing 1 mM Mg^{+2} (PBS-M) followed by solubilization in 2% SDS containing 20 μg/ml PMSF at 65°C . Cellular and viral DNA was sheared by repeated passage of the extracts through a 25 gauge needle and the extracts stored at -20°C until subsequent gel analyses.

Preparation of radiolabeled VV proteins

Confluent BSC_{40} cell monolayers were infected with VV at a multiplicity of infection (MOI) of 20 plaque forming units (PFUs) per cell, and then labeled continuously in the presence of [2,8,5'-^{3}H]adenosine, [8-^{14}C]adenosine, [^{32}P]orthophosphate (all purchased from Amersham), or [^{35}S]methionine (New England Nuclear) for 18 to 24 hrs., or pulse-labeled for 30 min at 6 to 8 hours post infection (hpi) with [^{35}S]methionine prior to harvesting. The radiolabeled infected cells were then used for the preparation of purified virus particles (Hruby *et al.*, 1979) or infected cell lysates as described above. Discontinuous polyacrylamide gel electrophoresis (SDS:PAGE) was carried out on the labeled viral proteins as previously described by Studier (1973). Following electrophoresis, gels were fixed, fluorographed with EN^{3}HANCE (New England Nuclear), dried, and exposed to Kodak XAR-5 X-ray film at -70°C.

Immunoprecipitation

Samples were prepared by solubilizing 5 to 10 µl of purified radiolabeled virus in an equal volume of 2% SDS at 100°C for 3 min, and then adding 1 ml of RIPA buffer [1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 50 mM Tris-Cl (pH 7.4)]. Five µl of polyclonal rabbit antiserum with specificity for the 4A, 4B, or 25K VV core proteins was then added to each reaction tube, and the samples incubated for 2 hr on ice. Two hundred µl of a 10% (v/v) suspension of Protein A-Sepharose beads (prepared as previously described by Miner and Hruby, 1989) was then added to each tube, and the samples were incubated at 4°C on a rocking shaker overnight. The beads were pelleted and washed three times in RIPA buffer, with the samples being transferred to new tubes during the final wash. The beads were then resuspended in 50 µl of SDS:PAGE loading buffer, heated to 100°C for 3 min, centrifuged briefly and the supernatant analyzed by SDS:PAGE. Following electrophoresis of the samples, the gels were fixed, fluorographed, dried, and exposed to film as described above.

Boronate affinity chromatography

An *m*-aminophenyl boronic acid glutaryl hydrazide polyacrylamide affinity resin was produced as previously described (Romaschin *et al.*, 1981; Inman, 1974). Briefly, Bio-Gel P-300 beads (Bio-Rad), whose porosity excludes macromolecules with molecular weight exceeding 400,000 daltons, were reacted with 6 N hydrazine, followed by the addition of glutaric anhydride to provide a 7 carbon atom spacer arm. The derivitized beads were then coupled with *m*-aminophenyl boronic acid under conditions which should yield 2.5 mmol of affinity label per gram of resin. The boronate resin, when synthesized in this manner, displays a high degree of selectivity at pH 8.2 for proteins that contain covalently bound ADP-ribose moieties (Romaschin *et*

al., 1981). For affinity chromatography, the equivalent of 1 gram dry weight of boronate resin was equilibrated in 6 N guanidine HCl-200 mM morpholine buffer (pH 8.2) for 5 to 6 hr, and the resin was then poured into a Bio-Rad disposable column. [^{35}S]methionine-labeled purified VV was solubilized in 1.5 ml of equilibration buffer for 15 min at 65°C, and the insoluble material was removed by centrifugation for 10 min at 15,000 rpm. The supernatant was passed over the boronate affinity column 4 times. The column was then washed with 15 column volumes of equilibration buffer, followed by elution of bound proteins with 6 N guanidine HCl-200 mM acetate buffer (pH 4.0). The eluted samples were then desalted by dialysis against 10% glycerol-0.1% SDS, concentrated by acetone precipitation, and analyzed by SDS:PAGE and fluorography as described above.

HPLC analysis

[2,8,5'-^{3}H]adenosine-labeled purified VV was incubated in 10 mM Tris-Cl (pH 12.0)-1 mM EDTA for 3 hr at 60°C to hydrolyze putative protein-ADP-ribose bonds. The soluble fraction of this mixture was extracted twice with an equal volume of phenol:chloroform, ether extracted several times, and the residual ether removed under a stream of nitrogen. The sample was brought up to a total volume of 1 ml with 1 M ammonium carbonate and passed over an Affigel 601 boronate affinity column (Bio-Rad) which had been prepared in a Bio-Rad disposable column after swelling 0.2 g of the resin in 1 M ammonium carbonate (equilibration buffer) for 2 hr. The column was washed with 4 column volumes of equilibration buffer, and the sample passed over the column 4 times. At this point the column was washed with 15 mls of equilibration buffer, and the bound material was then eluted with 1 M triethylammonium acetate (pH 6.3) and 0.5 ml fractions collected. Fractions

which contained radioactive counts were pooled and lyophilized in a Savant Speed-Vac concentrator prior to HPLC analysis.

HPLC analyses were performed using a Waters model 600 multisolvent delivery system, a Waters WISP model 712 automatic sample injector, and a Waters Lambda-Max LC spectrophotometer model 481. Samples were collected with an ISCO Foxy fraction collector, and analyzed for radioactive counts on a Beckman LS 3801 scintillation counter. The column utilized was a VYDAK C18 analytical reverse phase column (5 μM, 0.46 x 25 cm i.d.). Chromatography was carried out at room temperature with the following buffer system: buffer A, 0.1 M KPO_4 (pH 4.25); buffer B, 0.1 M KPO_4 (pH 4.25)-20% MeOH, and buffer C, 0.1 M KPO_4 (pH 7.0)-1 M urea-50% acetonitrile (Hakam *et al.*, 1986). The flow rate was 1 ml/min, with the gradient progressing from 100% buffer A to 100% buffer B in 20 min with a concave gradient. The gradient remained at 100% buffer B for 2 min, and then went from 100% buffer B to 100% buffer C with a linear gradient over 6 min, with elution continuing at 100% buffer C for 10 min (Hakam *et al.*, 1986).

Phosphate gel analysis

[^{35}S]methionine-labeled pulse and pulse-chase extracts were prepared by infecting confluent BSC_{40} cell monolayers at an MOI of 20 PFU per cell, and pulse-labeling at 6 hpi as described. At the end of the 30 min pulse, one sample was harvested by washing with PBS-M, followed by the addition of 0.5 ml of 2% SDS containing 20 μg/ml PMSF (65°C), and the extract processed as described above. The radiolabeled proteins in the second sample were chased for 4 hr in the presence of a 100-fold excess of unlabeled methionine prior to extract preparation. Each sample was then split into two aliquots, one of which was placed directly at -20°C to serve as the untreated control. The other aliquots were adjusted to pH 9.0 by the addition of Tris-Cl (20 mM final

concentration), and the samples were incubated for 2 hr at 60°C prior to storage at -20°C and subsequent gel analysis.

Gels consisted of 10% acrylamide, 0.2% bis acrylamide, 100 mM sodium phosphate (pH 7.0), 1 M urea, and were poured using 20 x 32 cm plates (Weber and Osborn, 1969). Before loading, gels were pre-run at 80 V for 1 hr in buffer made up of 100 mM sodium phosphate (pH 7.0), 0.1% SDS, 8.4 mM 3-mercaptoproprionic acid, and 8.9 mM NaOH. Twenty µl of each sample was mixed with 30 µl of sample buffer [5% Ficoll, 100 mM sodium phosphate (pH 7.0), 2% SDS] and the samples heated for 2 min at 100°C. The samples were subjected to electrophoresis at 80 V for 20 to 24 hr, and the gels fixed, dried, and exposed to film at room temperature.

RESULTS

Comigration of [^{35}S]methionine-, [2,8,5'-^{3}H]adenosine-, [^{32}P]orthophosphate- and [8-^{3}H]adenosine-labeled proteins from purified VV

Nicotinamide adenine dinucleotide (NAD^+) is the direct donor molecule for both mono- and poly- ADP-ribosyltransferases (Ferro and Oppenheimer, 1978; Moss *et al.*, 1979; Alvarez-Gonzalez *et al.*, 1988). However, due to the inability of NAD^+ to pass through intact cell membranes, various labeled precursor molecules or techniques to disrupt cell membranes are generally used for *in vivo* labeling of ADP-ribose moieties (Pope, *et al.*, 1985; Adolph, 1987; Fendrick and Iglewski, 1989; Elia *et al.*, 1991). Studies of ADP-ribosylated proteins have proven to be difficult due to the low steady-state levels of ADP-ribose residues relative to other adenine-containing compounds within cells such as nucleic acids (Aboul-Ela *et al.*, 1988; Elia *et al.*, 1991). Likewise, the possibility for the interconversion of [2,8,5'-^{3}H]adenosine into amino acids such as histidine can lead to the incorporation of radiolabel into proteins.

In order to determine that the labeling of the eight VV core proteins which was previously detected when VV was grown in the presence of [2,8,5'-^{3}H]adenosine (Figure III. 1A; Child *et al.*, 1988) was due to modification of the proteins and not to interconversion of label, VV-infected cells were also labeled with [8-^{14}C]adenosine, which, because of the position of the radiolabel, should not be subject to similar interconversion reactions. Due to the low specific activity of the [8-^{14}C]adenosine label, the amount of radioactivity associated with the virion proteins was reduced when compared to [2,8,5'-^{3}H]adenosine-labeling, yet it is evident in Figure III. 1B that proteins which comigrate with the major virion core proteins 4A, 4B, and possibly 25K and 23K were labeled during viral replication in the presence of [8-^{14}C]adenosine, indicating the presence of an adenylate modifying group on these proteins.

Figure III. 1. *Electrophoretic analysis of radiolabeled VV virion proteins.* Radiolabeled VV-infected cell extracts or purified virus particles were prepared and subjected to electrophoresis on 12% polyacrylamide gels. (A) SP, [^{35}S]methionine pulse labeled infected cell lysate; SC, pulse-chase labeled infected cell lysate; and HV, [2,8,5'-^{3}H]adenosine-labeled purified virus particles. (B) SV, [^{35}S]methionine- labeled virions; HV, [2,8,5'-^{3}H]adenosine-labeled virions; and CV, [8-^{14}C]adenosine-labeled virions. (C) PV, [^{32}P]orthophosphate-labeled virions; and SV, [^{35}S]methionine-labeled virions. The molecular weights of marker proteins are indicated in kDa at the left of each panel, and the positions of the major core proteins are indicated at the right.

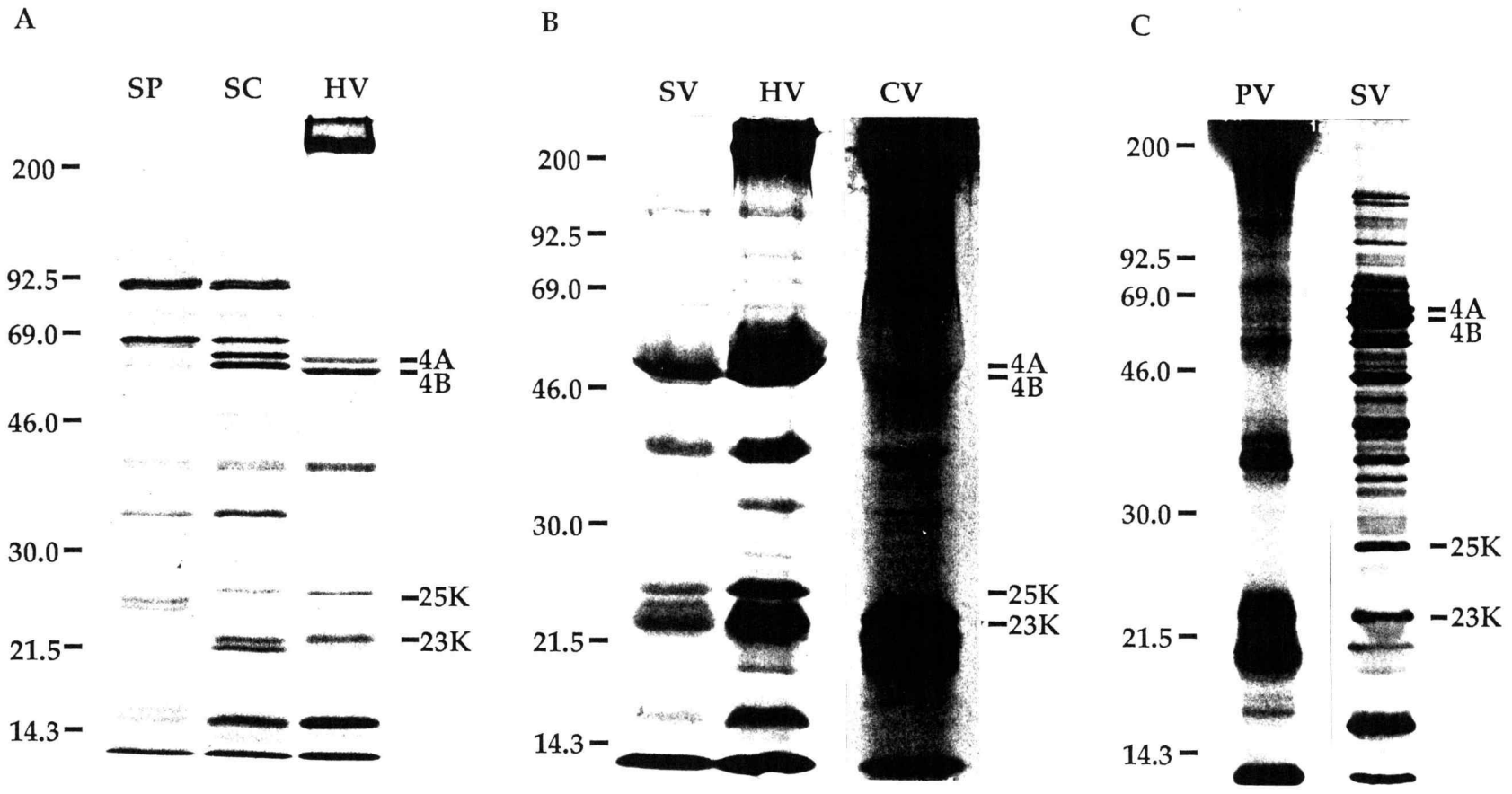

A
SP
SC
HV
200
92.5
69.0
46.0
30.0
21.5
14.3
4A
4B
25K
23K
B
SV
HV
CV
200
92.5
69.0
46.0
30.0
21.5
14.3
4A
4B
25K
23K
C
PV
SV
200
92.5
69.0
46.0
30.0
21.5
14.3
4A
4B
25K
23K

Figure III. 1

Figure III. 2. *Immunoprecipitation of [^{35}S]methionine- and [2,8,5'-^{3}H]adenosine-labeled VV core proteins.* Purified virus particles which had been grown in the presence of either [^{35}S]methionine (SV lanes) or [2,8,5'-^{3}H]adenosine (HV lanes) were disrupted by heating in 2% SDS, diluted into RIPA buffer, and immunoprecipitated with monospecific polyclonal antisera directed against the 4A, 4B, or 25K core proteins. The immunoreactive proteins were then analyzed by gel electrophoresis and fluorography. The molecular weights of the marker proteins (M) are indicated in kDa at the left, and the positions of the major core proteins are shown at the right.

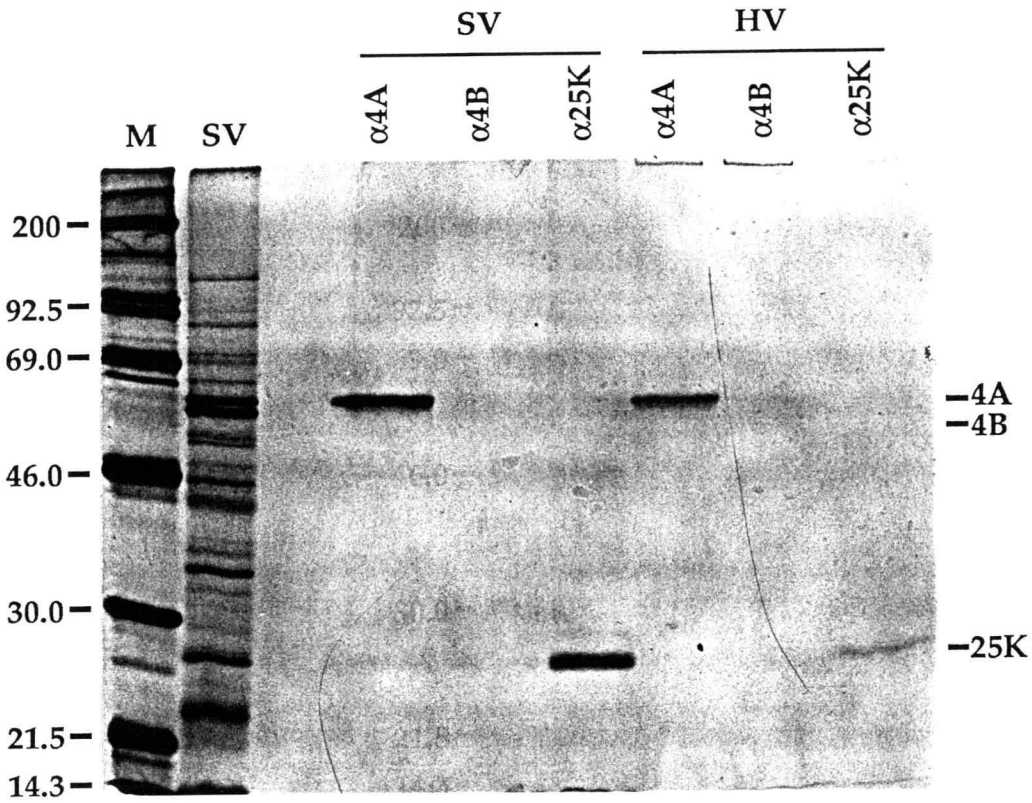

SV
HV
α4A
α4B
α25K
α4A
α4B
α25K
M
SV
200
92.5
69.0
46.0
30.0
21.5
14.3
4A
4B
25K

Figure III. 2

This same set of proteins appears to be labeled by [^{32}P]orthophosphate as well (Figure III. 1C), although the labeling is relatively weak by comparison to the labeling of the viral phosphoproteins (Rosemond and Moss, 1973). It is of interest to note that there are several proteins of approximately 20 kDa molecular weight which become heavily labeled in the presence of both [8-^{14}C]adenosine and [^{32}P]orthophosphate, although the identities of these proteins are not known at this time. These results suggest that the modifying groups present on the eight virion proteins contain both adenosine and phosphate, lending support to the hypothesis that these proteins are modified by ADP-ribosylation.

Identification of three of the adenosine-labeled core proteins as 4A, 4B, and 25K by immunoprecipitation

As an approach to identifying one or more of the labeled virion proteins, immunoprecipitation reactions were carried out using monospecific polyclonal antisera directed against the individual major core proteins 4A, 4B and 25K (unpublished data; VanSlyke *et al.*, 1991). As shown in Figure III. 2, using [2,8,5'-^{3}H]adenosine-labeled VV each of these sera was able to specifically recognize the cognate radiolabeled protein species (although reactive proteins are not apparent in the 4B lanes, they are detectable with longer film exposures). Unfortunately, the low specific activity of [8-^{14}C]adenosine or [^{32}P]orthophosphate labeled VV coupled to the inefficient recovery of protein label by immunoprecipitation procedures did not allow these preparations to be tested in this manner. However, based on the comigration of the labeled species using all three labeling protocols, the identity of the radiolabeled proteins are likely to be the same. This data confirms the identities of three of the [^{3}H]adenosine-labeled core proteins as

Figure III. 3. *Boronate affinity chromatography of [^{35}S]methionine-labeled VV core proteins.* Proteins were solubilized and bound to the resin in 6 N guanidine HCl-morpholine buffer (pH 8.2), and the column washed with 15 column volumes of this buffer prior to elution of bound proteins with 6 N guanidine HCl-acetate buffer (pH 4.0). The sample was then desalted, concentrated, and subjected to polyacrylamide gel electrophoresis and fluorography. Shown are the starting material (total) and the eluted proteins (eluate). The molecular weights of the marker proteins (M) are indicated in kDa at the left, and the positions of the major core proteins are marked at the right.

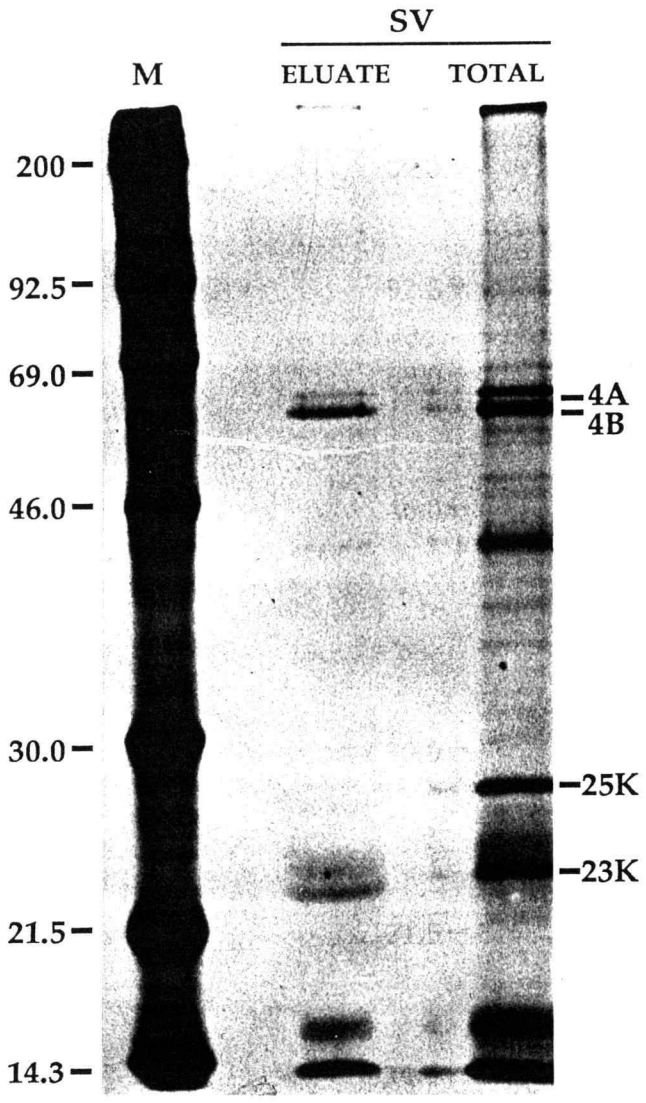


Figure III. 3

4A, 4B and 25K, and further suggests that these major core proteins are subject to modification by an adenosine-containing moiety.

Boronate affinity chromatography of [^{35}S]methionine-labeled proteins from purified VV

Since adenosine-labeling of VV core proteins provided some indirect evidence that these proteins were modified by ADP-ribosylation, boronate affinity chromatography of solubilized [^{35}S]methionine-labeled proteins from purified virus particles was undertaken to confirm or disprove this hypothesis. The affinity resin used in these studies had a pore size such that protein complexes of up to 400,000 daltons were not excluded from the resin, and was synthesized in such a manner that the resin contained no unsubstituted carbohydrate resin base, assuring specificity in the pH-dependent binding of *cis*-diol-containing substances to the resin (Romaschin *et al.*, 1981). After binding of the protein-containing sample to the resin in 6N guanidine HCl-200 mM morpholine (pH 8.2), the column was washed with approximately 15 column volumes of the same buffer prior to elution of bound proteins with 6N guanidine HCl-200 mM acetate pH 4.0. SDS:PAGE analysis of the eluted fractions revealed proteins which comigrate with the major viral core proteins 4A, 4B, 25K, and 23K, as well as several lower molecular weight protein species (Figure III. 3). This result confirms that the major VV core proteins are subject to modification by a moiety which interacts with boronate affinity resin in a manner similar to that of ADP-ribose.

Reverse phase HPLC analysis of protein-attached adenylate moieties isolated from purified VV proteins

HPLC analyses of the modifying groups isolated from [2,8,5'-^{3}H]adenosine-labeled purified VV were carried out to identify the modifying group present

Figure III. 4. *Reverse phase HPLC analysis of adenylate moieties isolated from purified VV proteins.* Protein-bound moieties containing adenosine were removed from purified [2,8,5'-^{3}H]adenosine-labeled VV particles and prepared for HPLC analysis by boronate affinity chromatography. The samples were analyzed by HPLC on a VYDAK C18 reverse phase column as described in the *Materials and Methods* section. The elution profiles of the labeled samples (solid lines) and of unlabeled adenylate standards (patterned lines) are indicated.

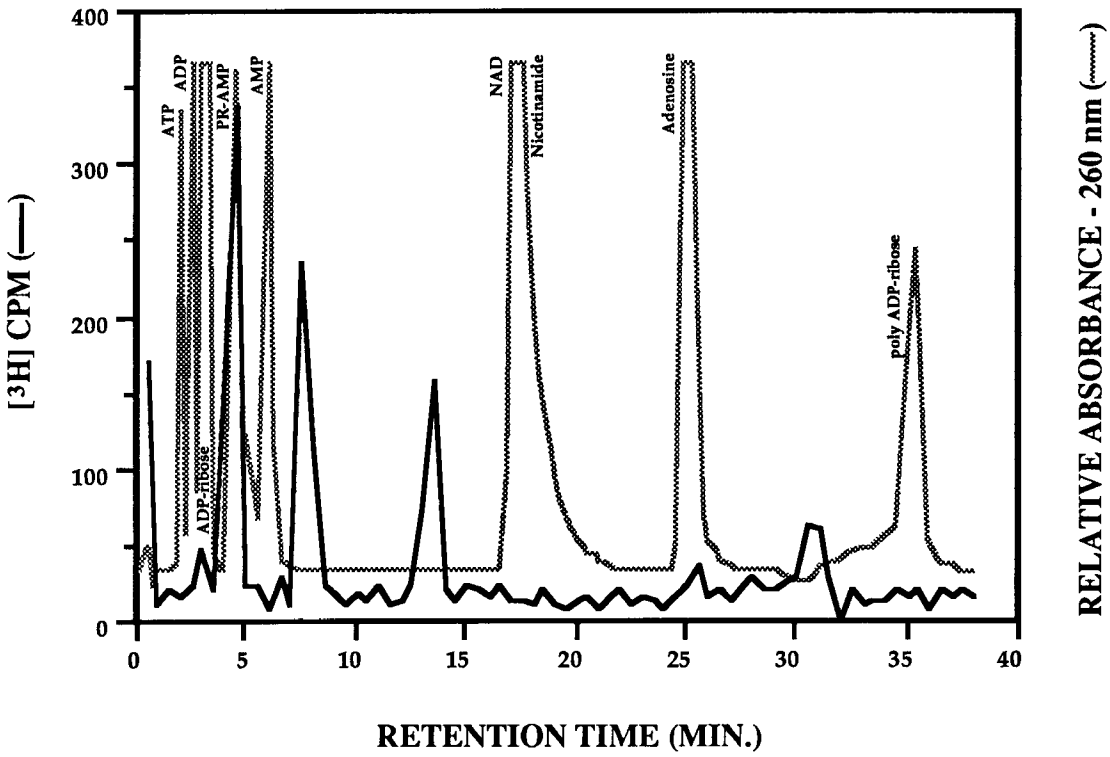

[3H] CPM (—)
RELATIVE ABSORBANCE - 260 nm (.....)
RETENTION TIME (MIN.)
ATP
ADP
ADP-ribose
PR-AMP
AMP
NAD
Nicotinamide
Adenosine
poly ADP-ribose
400
300
200
100
0
0
5
10
15
20
25
30
35
40

Figure III. 4

on the VV core proteins. Adenosine-labeled purified VV was disrupted and incubated under conditions designed to cleave potential protein-ADP-ribose bonds. The extract was then subjected to boronate affinity chromatography on an Affigel 601 column to eliminate extraneous protein, DNA, and RNA products prior to HPLC analysis (Wielckens *et al.*, 1984). The eluate from the boronate affinity column was then analyzed together with unlabeled adenylate standards by reverse phase HPLC, with separate trials yielding reproducible results. The results of a typical analysis are shown in Figure III. 4. The major radiolabeled peak comigrates with the unlabeled phosphoribosyl-AMP (PR-AMP) standard. Two other major unidentified peaks of radioactivity are also detected during HPLC analysis. Based on the fact that the sample represents only compounds with affinity for a boronate resin, it is unlikely that any of these peaks represent amino acid derivatives. Furthermore, when partially purified VV preparations, which are likely to contain the modifying groups from a variety of cytoplasmic cellular proteins as well as from the VV proteins were analyzed, a peak which comigrates with ADP-ribose is obtained in addition to the PR-AMP and unidentified peaks (data not shown). Thus, these results appear to indicate that some adenylate compound is associated with purified VV proteins. However, the modifying group displays some novel characteristics and its precise biochemical identity remains to be established.

Gel analysis of untreated proteins vs. proteins treated to remove potential ADP-ribose moieties

Following previously reported methods (Lowery *et al.*, 1986), we attempted to demonstrate a shift in electrophoretic mobility during SDS:PAGE analysis when VV proteins were treated to remove putative ADP-ribose residues. These initial experiments failed to demonstrate a shift in migration of the

Figure III. 5. *Effect of high pH treatment on the electrophoretic mobility of VV core proteins.* BSC_{40} cell monolayers were infected with VV and pulse- or pulse-chase labeled with [^{35}S]methionine. Cell extracts were prepared and treated at pH 9.0 to remove ADP-ribose moieties prior to phosphate gel analysis. The indicated lanes contain pulse-labeled extracts (SP) or pulse-chase labeled extracts (SC) which have been treated at high pH (9) or left untreated (7). The molecular weights of the marker proteins (M) are indicated in kDa at the left, and the major core proteins are denoted at the right.

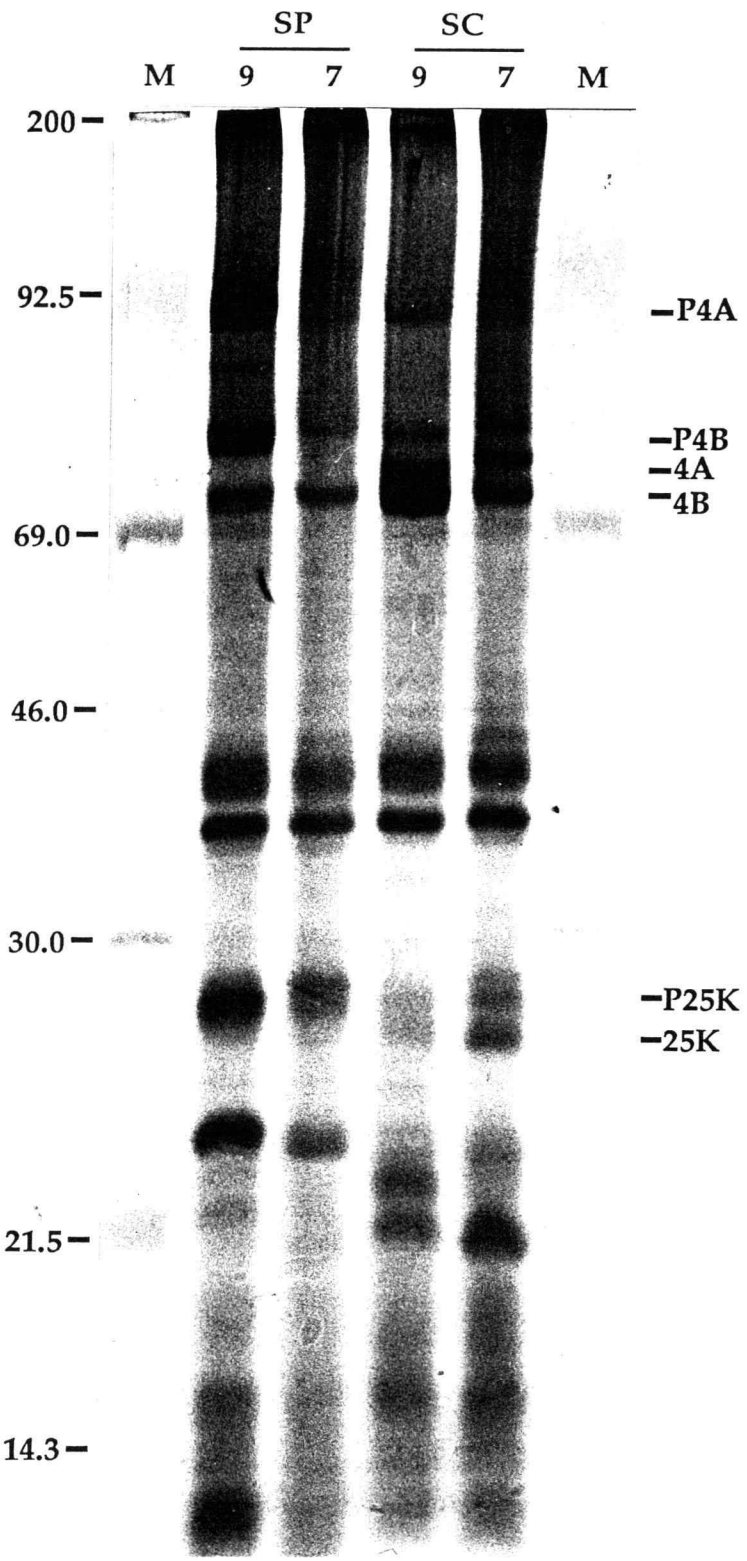


Figure III. 5

core proteins following incubation of infected cell extracts for 2 h at pH 9.0 and 60°C (data not shown). This result could have several potential explanations, including loss of modifying groups from the control samples during harvesting, loss of ADP-ribose residues due to the pH of the buffers used in SDS:PAGE, the presence of reducing agents, the percentage of acrylamide used, or it could be due to the high MW of the more prominent modified proteins (62 kDa and 60 kDa, respectively, for 4A and 4B), which are considerably larger than the 30 kDa protein studied by Lowery and colleagues (1986). As an alternative approach, continuous phosphate gels (pH 7.0, 10% acrylamide, minus reducing agents) were used to analyze the treated and untreated infected cell extracts. As can be seen in Figure III. 5, the mobility of the 4A and 4B core proteins, and possibly the 25K and 23K proteins as well, appears to be affected by incubation at pH 9.0 (SC lanes), while the migration of other proteins in these lanes is not affected by this incubation. It is also interesting to note that there seems to be no change in the migration of the major core protein precursors P4A, P4B, and P25K (SP lanes) following incubation at high pH, a result which is in agreement with the inability to detect any modifying groups on these precursor proteins (Lowery *et al.*, 1986). These results give additional support to the hypothesis that the major core proteins are subject to some form of post-translational modification which shares similarity to ADP-ribosylation.

DISCUSSION

Monitoring ADP-ribosylation *in vivo* is difficult due to the inability to use direct precursors for radiolabeling, the low percentage of ADP-ribose residues with respect to contaminants, the lability of the protein-ADP-ribose bond, and the difficulty in maintaining the integrity of this bond during such manipulations as viral purification and subsequent protein analyses. Although studies of ADP-ribosylation *in vivo* have proven challenging, improved methods have made these studies feasible in a variety of systems (Aboul-Ela *et al.*, 1988; Adolph, 1987; Déry *et al.*, 1986). Evidence for the modification of adenovirus core proteins by ADP-ribosylation (Déry *et al.*, 1986) led us to question of whether any of the VV core proteins might be ADP-ribosylated, and initial experiments showing nicotinamide inhibition of viral replication supported this notion (Child *et al.*, 1988). The results of the experiments reported here support our original observation and provide biochemical evidence for the post-translational modification of VV core proteins by an adenylate compound.

A subset of approximately eight VV core proteins was shown to be metabolically labeled by growth of the virus in the presence of [2,8,5'-^{3}H]adenosine, [8-^{14}C]adenosine, or [^{32}P]orthophosphate. The identities of four of these labeled proteins, 4A, 4B, 25K, and 23K were suggested by co-migration with [^{35}S]methionine-labeled VV proteins. In the case of 4A, 4B, and 25K these identities were confirmed by immunoreactivity of the proteins with monospecific polyclonal antisera. Chromatography of proteins from purified VV particles on a boronate affinity resin, synthesized in such a manner as to render the affinity matrix specific for the binding of *cis*-diol-containing compounds (Romaschin *et al.*, 1981), provided strong evidence that these major core proteins are modified by ADP-ribosylation. Reverse phase HPLC

analyses of the isolated modifying groups provided results which suggest that the viral core proteins are modified by some adenylate compound, although the elution pattern of the prosthetic group was somewhat unusual. A peak of radioactivity which co-elutes with PR-AMP as well as two thus far unidentified radioactive peaks were present under our assay conditions, indicating the possibility of multiple types of modifications. It should be noted that these analyses could have been affected by the type of protein-adduct linkage present on the VV proteins, for while linkages such as glutamate-ADP-ribose bonds are quite sensitive to conditions of high pH, linkages to other amino acid residues might not be disrupted under these conditions (Aktories *et al.*, 1988). The alteration in electrophoretic mobility of the major core proteins following incubation to disrupt potential protein-ADP-ribose bonds gives an indication that the linkage is at least somewhat labile under these reaction conditions, and provides further evidence that the VV core proteins are subject to post-translational modification. Additional evidence in this regard comes from the chromatographic behavior of two of the VV core proteins. The predicted pIs of the VV 4B and 25K proteins are 7.06 and 10.22, respectively. However, on a chromatofocusing column these two proteins elute at approximately pH 5.0 (4B) and pH 7.5 (25K) (data not shown). One potential explanation for this alteration in the apparent net charge of these proteins would be the charge contribution from the phosphate groups present within an ADP-ribose prosthetic group. While this data does not preclude the possibility that the modifying group we have analyzed represents some compound other than ADP-ribose, taken together the results of these experiments strongly indicate that the major VV core proteins are modified by some *cis*-diol-containing adenylate compound which shares characteristics in common with ADP-ribose. Confirmation of the biochemical

identity of this moiety must await improvements in our methods for isolating large quantities of purified, modified proteins so that the modifying group can be subjected to more rigorous forms of experimentation such as mass spectroscopy or nuclear magnetic resonance analysis.

Experiments are currently in progress to obtain information about the potential role played by ADP-ribosylation during the VV replication cycle. Previous experiments using the ADP-ribosylation inhibitor nicotinamide demonstrated that while overall levels of DNA and protein synthesis were relatively unaffected by the drug, the proteolytic cleavage of the major core protein precursors P4A, P4B, and P25K, an event necessary for the formation of mature virus particles, was inhibited (Child *et al.*, 1988). Whether this was due to prevention of the post-translational modification of these proteins or interference at some other level is not yet known. Further inhibitor studies are currently in progress in an effort to gain more information regarding the effect of this modification on the proteolytic processing of the major VV core proteins, and thus on viral replication.

CHAPTER IV

THE EFFECT OF ADP-RIBOSYLATION INHIBITORS ON VARIOUS STAGES OF VACCINIA VIRUS REPLICATION

Stephanie J. Child and Dennis E. Hruby

SUMMARY

Replication of vaccinia virus (VV) was prevented by the ADP-ribosylation inhibitors nicotinamide (NIC), 3-aminobenzamide (3-AB), and *meta*-iodobenzylguanidine (MIBG). None of these compounds significantly affected viral DNA synthesis at lower concentrations (10-20 mM NIC and 3-AB; 0.1-0.2 mM MIBG), although at higher concentrations of the three drugs a reduction in viral DNA synthesis was observed. This result was not unexpected since larger doses of the inhibitors are known to affect nucleotide synthesis. Total VV protein synthesis was decreased at higher inhibitor levels ($\geq$ 40 mM NIC and 3-AB; $\geq$ 0.4 mM MIBG) as well, and the proteolytic processing of the major virion core proteins was also greatly diminished within this range of drug concentrations. The effects of different inhibitor concentrations on the labeling of the viral core proteins and cellular histone proteins with [8-^{14}C]adenosine, which is incorporated into ADP-ribosylated proteins, were also noted; as inhibitor levels increased, the amount of label associated with the VV core proteins and the histones decreased. In addition, mature, infectious virus particles were not formed in the presence of either 60 mM NIC or 3-AB, or 0.6 mM MIBG. These results provide evidence which supports our hypothesis that the VV core proteins are subject to ADP-ribosylation.

INTRODUCTION

Vaccinia virus (VV) replicates within the cytoplasmic compartment of infected cells, and expression of its approximately 200 open reading frames occurs via a tightly regulated cascade of events (Goebel *et al.*, 1991; Moss, 1990). While at least some of the regulatory controls on VV gene expression take place at the transcriptional level (Moss, 1990), a variety of post-translational modifications, including proteolytic cleavage, glycosylation, phosphorylation, acylation, and possibly ADP-ribosylation have been identified on VV proteins (VanSlyke and Hruby, 1990).

ADP-ribosylation refers to the modification of proteins by the covalent attachment of ADP-ribose in either monomeric or homo-polymeric form. The biological significance of this modification is not yet well understood, although ADP-ribosylation appears to play a role in regulating a variety of cellular functions, including DNA repair and chromatin conformation (Gaal *et al.*, 1987; Shall, 1989), cell cycle progression, modulation of enzyme activity, signal transduction, and the efflux of Ca^{2+} from mitochondria (Althaus and Richter, 1987). While the ADP-ribosylation inhibitors available to date lack absolute specificity, these compounds have been important in many studies aimed at elucidating the biological role of ADP-ribosylation (Althaus and Richter, 1987). When chemically diverse compounds which would be unlikely to share common metabolic side effects are utilized, these types of studies are capable of yielding meaningful results, especially when these results can be corroborated with experiments which do not rely on the use of inhibitors.

Our previous experiments demonstrated that the competitive ADP-ribosylation inhibitor nicotinamide (NIC) inhibits VV replication by greater than 99% at 60 mM concentrations, and that proteolytic cleavage of the major

core proteins and the formation of mature virus particles do not occur in the presence of the drug (Child *et al.*, 1988). Due to the difficulty of obtaining enough modified viral proteins for direct biochemical analysis of the potentially ADP-ribosylated VV proteins, we were interested in performing further inhibitor studies with a variety of drugs in order to see whether more information could be gained concerning the potential role of ADP-ribosylation during the VV replication cycle. The experiments described here demonstrate the effects of three inhibitors of ADP-ribosylation reactions on viral DNA synthesis, protein synthesis, proteolytic cleavage and [8-^{14}C]adenosine-labeling of the major core proteins, and on the formation of infectious progeny virions. NIC and 3-aminobenzamide (3-AB) both act as competitive poly-ADP-ribosylation inhibitors (Purnell and Whish, 1980; Milam and Cleaver, 1984; Smets *et al.*, 1990), while *meta*-iodobenzylguanidine (MIBG), a functional analog of norepinephrine appears to selectively and potently inhibit mono-ADP-ribosylation reactions (Loesberg *et al.*, 1990 a,b). The results of these experiments provide strong, albeit indirect, evidence that a subset of the VV core proteins are subject to modification by ADP-ribosylation, and suggest that this is an obligatory event during VV replication.

MATERIALS AND METHODS

Cells and virus

BSC_{40} cells were maintained in monolayer culture in Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, and 50 μg/ml gentamycin sulfate. VV (WR strain) was maintained and purified as previously described (Hruby *et al.*, 1979). Crude virus stocks were prepared by harvesting infected cells into 1 ml of phosphate buffered saline containing 1 mM Mg^{+2} (PBS-M), then lysing the cells by three cycles of freeze-thawing to release intracellular virus. The crude stocks were then titrated on BSC_{40} cell monolayers as described earlier (Hruby *et al.*, 1979). Infected cell extracts for polyacrylamide gel analysis were prepared by washing the cell monolayers 2-3 times with PBS-M followed by solubilization in 2% SDS containing 20 μg per ml PMSF at 65°C. Cellular and viral DNA was sheared by repeated passage of the extracts through a 25 gauge needle and the extracts stored at -20°C prior to gel analysis. Nicotinamide (NIC) and 3-aminobenzamide (3-AB) (Sigma) were added at the indicated concentrations immediately following infection, and the cell monolayers were treated with the designated concentrations of *meta*-iodobenzylguanidine (MIBG) (a generous gift from the laboratory of Dr. Lou Smets) starting four hours prior to infection.

Dot blot analysis of VV DNA

A 24-well plate containing confluent BSC_{40} cells was infected with VV at a multiplicity of infection (MOI) of 1 plaque forming unit (PFU) per cell, and varying concentrations of NIC and 3-AB (from 0-80 mM), and MIBG (0-0.8 mM) added as indicated above. The infected monolayers were incubated at 37°C in 5% CO_2 until 8 hours post infection (hpi), when lysates were prepared by washing the monolayers twice with PBS-M, adding 0.25 ml of PBS-M to

each well of the 24-well plate, subjecting the plate to three cycles of freeze-thawing, then treating the lysates with a 0.25% trypsin solution at 37°C for 30 min. The lysates containing viral and cellular DNA were collected on a NYTRAN membrane by filtration through a microsample manifold (Schleicher and Schuell, Inc.). The NYTRAN-bound DNA was then prepared for hybridization, hybridized with a cloned [^{32}P]-labeled VV *Hind*III DNA fragment, and exposed to Kodak XAR-5 X-ray film as has been described (Child *et al.*, 1988). Quantitation of the counts associated with each "dot" was performed both by scanning the blot with the AMBIS Radioanalytic Imaging System (provided by the Center for Gene Research and Biotechnology Central Service Laboratory) and by detection of radioactive counts utilizing a Beckman LS 3801 scintillation counter.

SDS:PAGE analysis of [^{35}S]-labeled VV proteins

Confluent monolayers of BSC_{40} cells (60 mm dishes) were infected with VV at an MOI of 5 PFU per cell, treated with 0-80 mM concentrations of NIC and 3-AB, and 0-0.8 mM MIBG as described above, then incubated at 37°C in 5% CO_2 for 7 hr. At this time the infected cells were pulse-labeled with medium containing 10 μCi per ml [^{35}S]methionine + cysteine protein labeling mix (EXPRE^{35}S^{35}S; New England Nuclear, 1128 Ci/mmol) plus the indicated concentrations of inhibitor, followed by a three hour chase period with medium containing a 100-fold excess of unlabeled methionine and the proper inhibitor concentration. Infected cell lysates were harvested as described above, and analyzed by discontinuous polyacrylamide gel electrophoresis (SDS:PAGE) on 12% gels by the methods of Studier (1973). Following electrophoresis, the gels were fixed, dried, and exposed to Kodak XAR-5 X-ray film at room temperature.

SDS:PAGE analysis of [8-^{14}C]adenosine-labeled VV and cellular proteins

Confluent monolayers of BSC_{40} cells (30 mm dishes) were infected with VV at an MOI of 5 PFU per cell, and treated with 10, 20, or 40 mM NIC and 3-AB or 0.1, 0.2, or 0.4 mM MIBG as described above. The infected cells were then incubated at 37°C, 5% CO_2 with medium containing 0.5 μCi per ml [8-^{14}C]adenosine (Amersham, 50 mCi/mmol). At 18 hpi infected cell lysates were prepared as indicated above, and the lysates were subsequently analyzed by electrophoresis on a 12% SDS:PAGE gel. Following electrophoresis the gel was fixed, fluorographed with EN^{3}HANCE (New England Nuclear), dried, and exposed to Kodak XAR-5 X-ray film at -70°C. The histone-containing portion of the autoradiograph was subsequently scanned using a Biomed Instruments densitometric scanner (kindly provided by the laboratory of Dr. JoAnne Leong).

Velocity sedimentation of VV virions

Confluent 100 mm dishes of BSC_{40} cells were infected with VV at an MOI of 5 PFU per cell, and the infected cells treated as described with either 60 mM NIC or 3-AB, 0.6 mM MIBG, or incubated in the absence of drug. The medium added to the cells following infection also contained 1 μCi per ml [^{3}H]thymidine (Amersham, 83 Ci/mmol). The infected cells were incubated at 37°C in 5% CO_2 for 24 hrs, at which time standard purification procedures were utilized to prepare the labeled virus particles for velocity sedimentation through 25-40% sucrose gradients (Hruby *et al.*, 1979). After centrifugation at 13,500 rpm, 40 min, 4°C in a SW41 rotor, 0.5 ml fractions were collected from the gradients, and 50 μl aliquots of each fraction analyzed for radioactive counts using a Beckman LS 3801 scintillation counter. Fractions corresponding to the peak fractions from the minus-drug samples were

titered as has been described (Hruby *et al.*, 1979) to assay for the presence of infectious virus particles.

RESULTS

Effect of the ADP-ribosylation inhibitors on VV replication

The effect of various concentrations of NIC, 3-AB, and MIBG on VV replication was measured by infecting confluent monolayers of BSC_{40} cells with VV and treating the cells as described in the Materials and Methods section with the concentrations of the three compounds indicated in Figure IV. 1 (0, 10, 20, 40, 60, 80, and 100 mM NIC or 3-AB; 0, 0.1, 0.2, 0.4, 0.6, and 0.8 mM MIBG). The bar graphs in Figure IV. 1 represent the averaged results from three separate trials with standard deviations indicated by cross-bars. For both NIC and 3-AB, which are known to be potent inhibitors of poly-ADP-ribosylation reactions (Purnell and Whish, 1980; Milam and Cleaver, 1984; Rankin *et al.*, 1989), fairly high inhibitor concentrations in comparison to those usually used in studies on intact cells (Smets *et al.*, 1990) were needed in order to elicit a significant reduction in viral titer. However, the two compounds demonstrated very similar inhibition profiles; in both cases 20 mM drug reduced VV titers by about 2 to 3-fold, while increasing the drug concentration to 40 mM caused an abrupt 200-fold reduction in titer. This could be an indication that the VV core proteins are subject to mono- rather than poly-ADP-ribosylation, because NIC is known to inhibit mono-ADP-ribosyltransferase activity by only 32-39% at even a 40 mM concentration (Smets *et al.*, 1990).

In contrast to NIC and 3-AB, much lower concentrations of the mono-ADP-ribosylation inhibitor MIBG (Loesberg *et al.*, 1990 a,b) are necessary to cause a reduction in VV titer, and increasing concentrations of MIBG produce a complex and apparently biphasic profile of inhibition, which is in agreement with the results reported by Smets *et al.* (1990). As can be seen in Figure IV. 1, 0.1 or 0.2 mM concentrations of MIBG cause an approximate 11-

Figure IV. 1. *Inhibition of VV replication by the ADP-ribosylation inhibitors NIC, 3-AB, and MIBG.* Monolayers of BSC_{40} cells were infected with VV at an MOI of 5 PFU per cell and incubated in the presence of the indicated mM concentrations of NIC (A), 3-AB (B), or MIBG (C) for 24 hr. The infected cell lysates were then harvested and subjected to plaque titration to determine the yield of progeny virus.

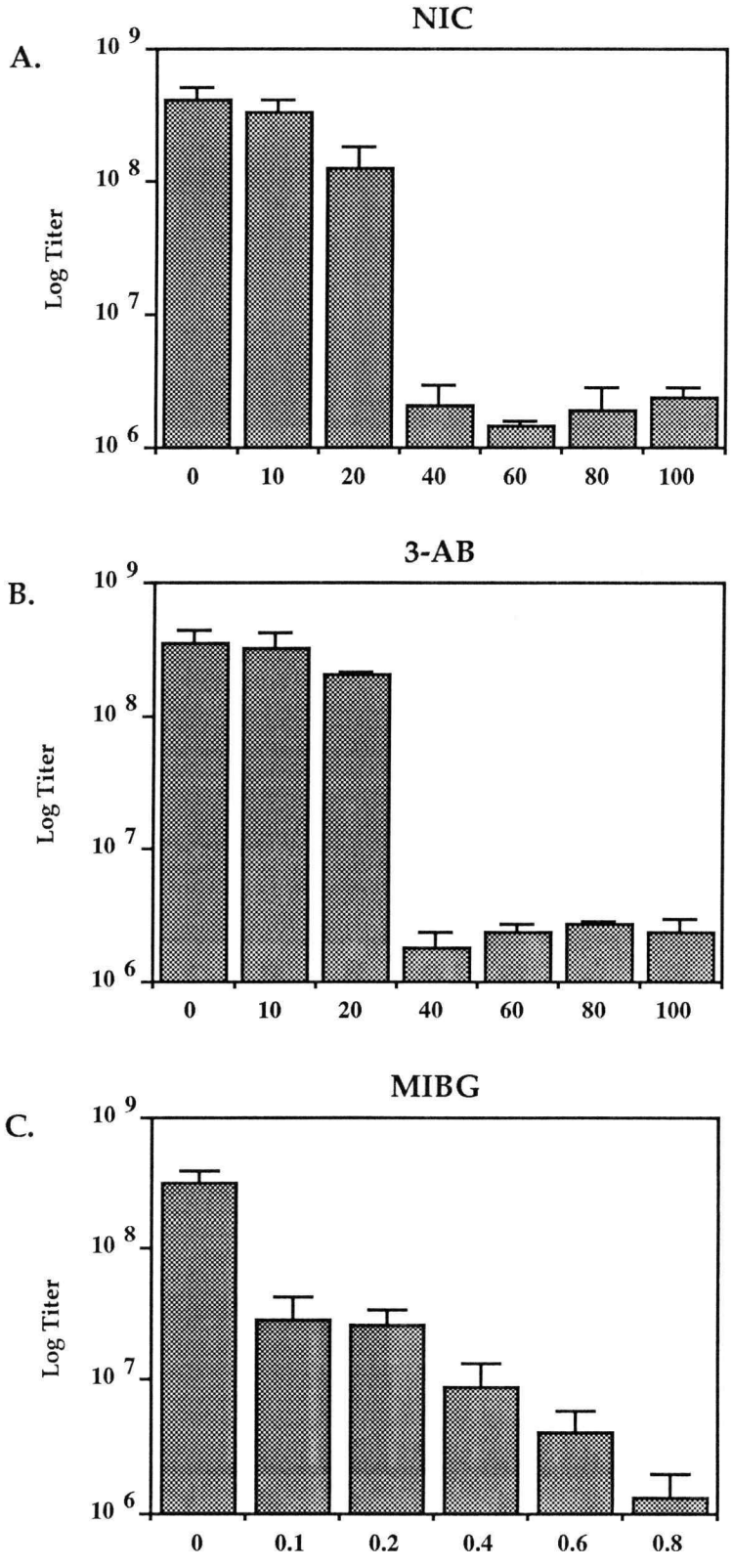

A.
NIC
Log Titer
10 9
10 8
10 7
10 6
0
10
20
40
60
80
100
B.
3-AB
Log Titer
10 9
10 8
10 7
10 6
0
10
20
40
60
80
100
C.
MIBG
Log Titer
10 9
10 8
10 7
10 6
0
0.1
0.2
0.4
0.6
0.8

Figure IV.1

fold reduction in viral replication levels, while concentrations of 0.4 mM and above lead to reductions in VV titer which follow a fairly linear curve (0.4 mM = 35-fold reduction; 0.6 mM = 74-fold; and 0.8 mM = 230-fold).

Effect of the ADP-ribosylation inhibitors on VV DNA synthesis

To determine what stage(s) of VV replication the inhibitors were influencing, we first measured the effect of NIC, 3-AB, and MIBG on VV DNA synthesis (Figure IV. 2). For this experiment, BSC_{40} monolayers were infected with VV, treated as previously indicated with 0, 10, 20, 40, 60, and 80 mM NIC or 3-AB and 0, 0.1, 0.2, 0.4, 0.6, and 0.8 mM MIBG, and infected cell lysates prepared at 8 hpi and analyzed as described in the Materials and Methods section. As is apparent both from inspection of the dot blot and analysis of the actual radioactive counts associated with each "dot", increasing inhibitor concentrations yielded decreasing levels of VV DNA synthesis. All three drugs exerted similar effects on DNA synthesis; little abrogation of DNA production was apparent at up to 20 mM concentrations of NIC and 3-AB and 0.2 mM MIBG, but 40 mM or greater concentrations of NIC or 3-AB and 0.4 mM and higher levels of MIBG reduced DNA synthesis by at least 50%. Interestingly, 3-AB seems to have had a greater effect on DNA synthesis than either NIC or MIBG in these studies. That the inhibitors affect the synthesis of VV DNA is not surprising since reduction of nucleotide synthesis is a known metabolic side effect for all three compounds (Purnell and Whish, 1980; Milam and Cleaver, 1984; Loesberg *et al.*, 1990 a). This reduction of nucleotide synthesis appears to be due to depletion of phosphoribosyl diphosphate (Purnell and Whish, 1980) and perhaps other nucleotide precursor pools rather than to a reduction in the production of nucleotide-synthesizing enzymes, suggesting an effect on enzyme activity rather than

Figure IV. 2. *Effect of the ADP-ribosylation inhibitors on VV DNA synthesis.* Monolayers of BSC_{40} cells were infected with VV at an MOI of 1 PFU per cell in the presence of the indicated concentrations of NIC, 3-AB, or MIBG [the numbers 0, 1, 2, 4, 6, 8 correspond to 0, 10, 20, 40, 60, 80 mM NIC or 3-AB; 0, 0.1, 0.2, 0.4, 0.6, 0.8 mM MIBG], and the infected cells were harvested at 8 hpi. The cell lysates were then subjected to dot-blot filtration, and the filter-bound DNA was hybridized with a VV DNA-specific radiolabeled probe. Panel A shows a film exposure of the dot blot, while panel B indicates the radioactive counts associated with the corresponding "dots" in panel A.

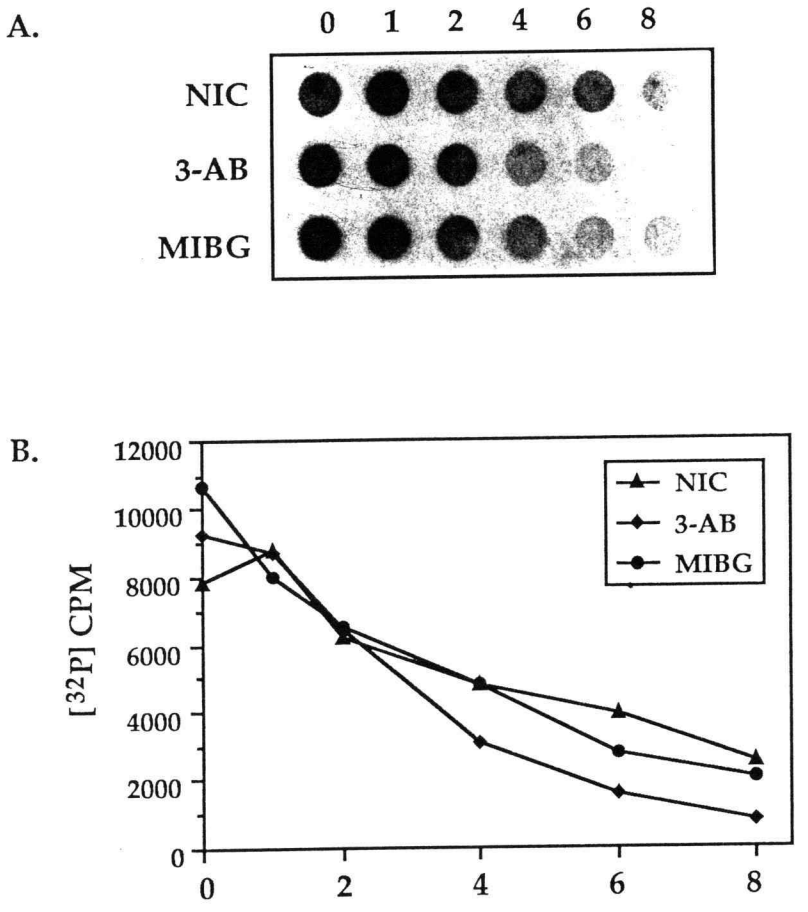

A.
0 1 2 4 6 8
NIC
3-AB
MIBG
B.
12000
10000
8000
6000
4000
2000
0
[32P] CPM
0 2 4 6 8
NIC
3-AB
MIBG

Figure IV.2

protein synthesis, although the specific effect of the three inhibitors on VV early protein synthesis was not monitored in these studies.

Effect of the ADP-ribosylation inhibitors on VV protein synthesis and proteolytic processing.

To determine how NIC, 3-AB, and MIBG affect viral protein synthesis and proteolytic processing, BSC_{40} cell monolayers were infected with VV and treated with various concentrations of the inhibitors as indicated in Figure IV. 3. At 7 hpi the infected monolayers were pulse-chase labeled as described in the Materials and Methods section, and infected cell lysates were prepared and analyzed by SDS:PAGE. As is evident in Figure IV. 3, concentrations greater than or equal to 40 mM NIC or 3-AB or 0.4mM MIBG begin to inhibit overall levels of viral protein synthesis. When replicates of the gels shown in Figure IV. 3 were subjected to immunoblot analysis utilizing a mixture of antisera with specificity for the major core proteins 4A, 4B, and 25K (unpublished data; VanSlyke et al., 1991), it was evident that the proteolytic processing of the major core protein precursors P4A (94 kDa), P4B (66 kDa), and P25K (28 kDa) to their mature forms 4A (62 kDa), 4B (60 kDa), and 25K (25 kDa) was inhibited beginning at concentrations of about 20 mM NIC or 3-AB and 0.4 mM MIBG (data not shown). Thus, all three compounds interfere with proteolytic processing, and at slightly higher inhibitor levels VV protein synthesis is affected as well, although whether this is due to prevention of ADP-ribosylation of the core proteins or to metabolic side effects of the drugs is not known.

Effect of the ADP-ribosylation inhibitors on [8-^{14}C]adenosine-labeling of the VV core proteins and cellular histone proteins

We were also interested in determining the effect of NIC, 3-AB, and MIBG on the labeling of viral and cellular proteins with [8-^{14}C]adenosine, which

Figure IV. 3. *Effect of the inhibitors on VV protein synthesis and proteolytic processing.* BSC_{40} monolayers were infected with VV at an MOI of 5 PFU per cell, and infections carried out in the presence of the indicated mM concentrations of NIC (A), 3-AB (B), or MIBG (C). At 7 hpi the infected cells were pulse-labeled for 30 min with 10 μCi per ml [^{35}S]protein labeling mix, followed by a 3 hr chase period prior to harvesting of the extracts. Extracts were subjected to electrophoresis on 12% polyacrylamide gels followed by autoradiography. The molecular weights of marker proteins (M) are indicated in kDa at the left of each panel, and the positions of the major core proteins are indicated at the right.

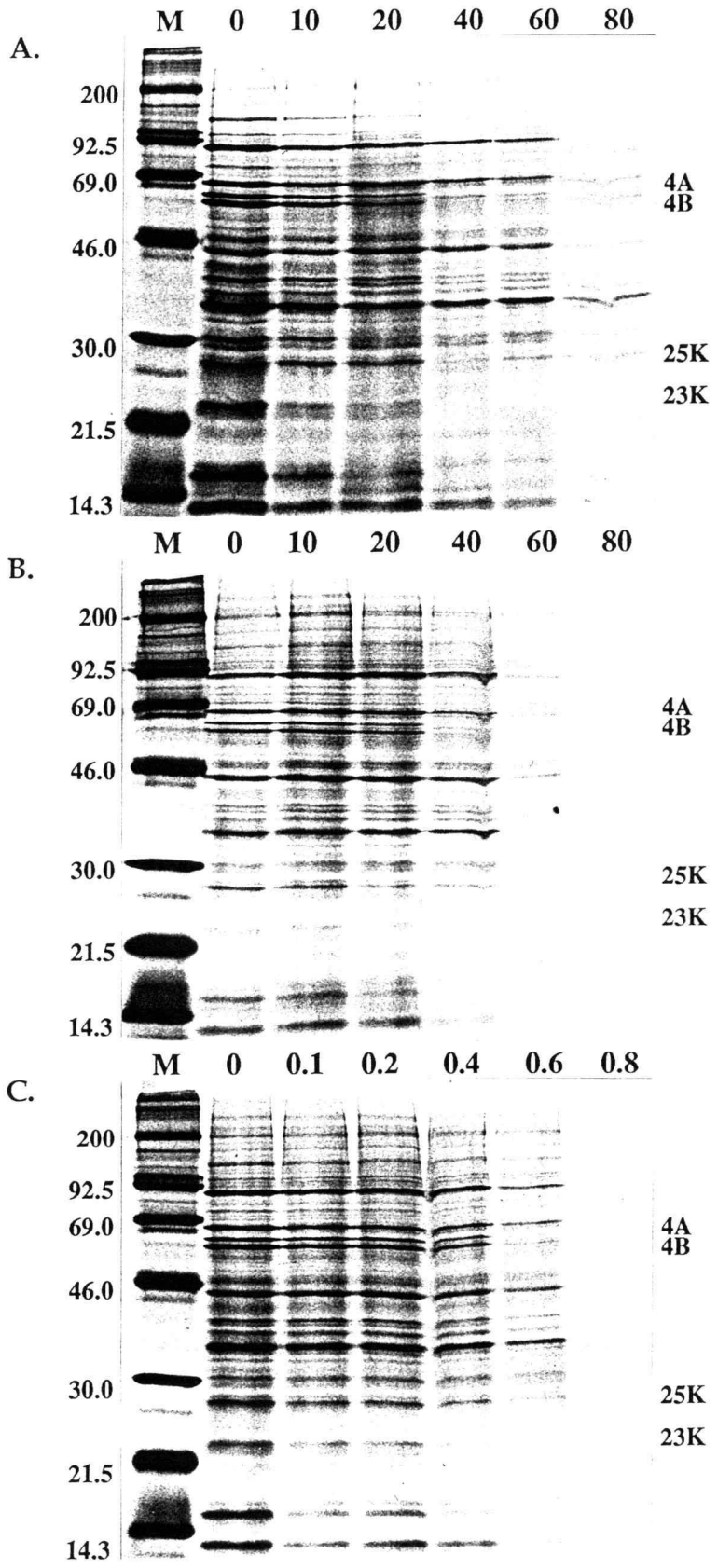
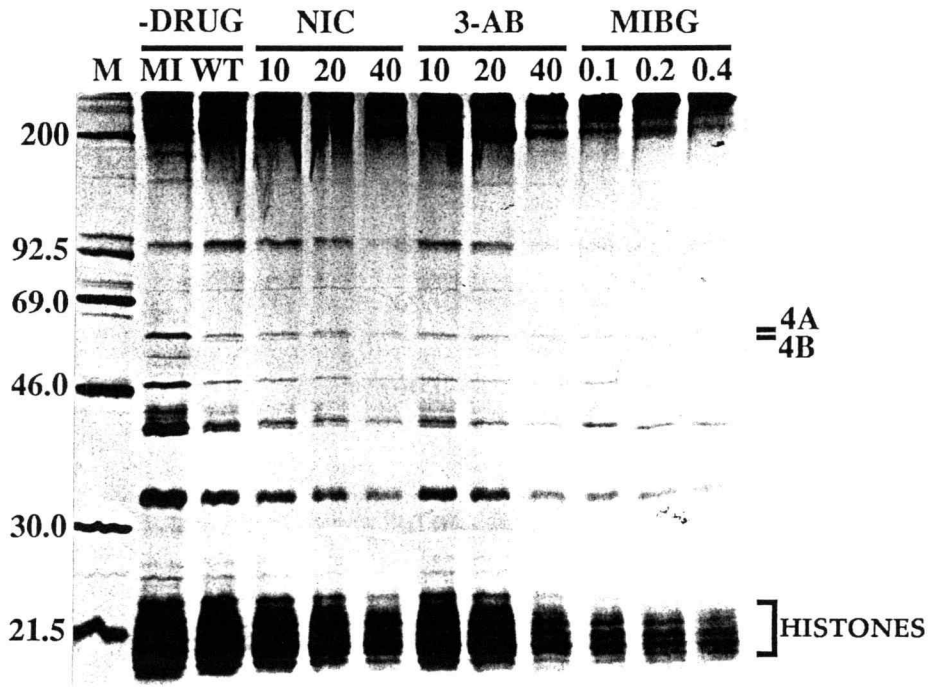

A.
M 0 10 20 40 60 80
200
92.5
69.0
46.0
30.0
21.5
14.3
4A
4B
25K
23K
B.
M 0 10 20 40 60 80
200
92.5
69.0
46.0
30.0
21.5
14.3
4A
4B
25K
23K
C.
M 0 0.1 0.2 0.4 0.6 0.8
200
92.5
69.0
46.0
30.0
21.5
14.3
4A
4B
25K
23K

Figure IV.3

Figure IV. 4. *Effect of the ADP-ribosylation inhibitors on [8-^{14}C]adenosine labeling of the VV core proteins and cellular histone proteins.* VV-infected cell extracts which were grown in the presence of 0.5 μCi per ml [8-^{14}C]adenosine plus the indicated mM concentrations of each of the three inhibitors were analyzed by gel electrophoresis and fluorography as shown in Panel A. The molecular weights of the marker proteins (M) are indicated in kDa at the left, and the positions of the major VV core proteins and the histone proteins are shown at the right. MI designates a mock-infected control. Panel B demonstrates the results of densitometric scanning of a shorter film exposure of the histone-containing portion of the autoradiograph. Total area refers to the area under the peaks generated during densitometric scanning.

A.



B.

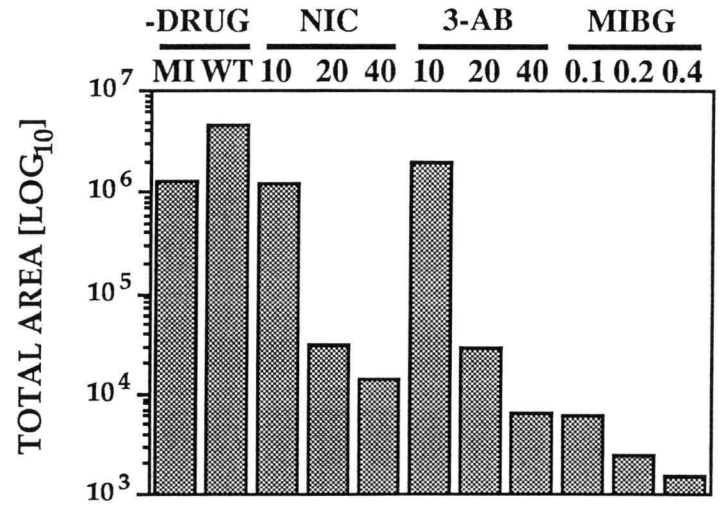


Figure IV.4

should be specifically incorporated into ADP-ribosylated proteins due to the position at which the compound is labeled. For this experiment, BSC_{40} cell monolayers were infected with VV, treated with 10, 20, and 40 mM NIC or 3-AB and 0.1, 0.2, and 0.4 mM MIBG, and continuously labeled with [8-^{14}C]adenosine as described in the Materials and Methods section. Infected cell lysates were analyzed on a 12% SDS:PAGE gel, exposed to X-ray film (Figure IV. 4, panel A), and the histone-containing portion of the autoradiograph (~ 18-22 kDa region) was subjected to densitometric scanning. Because the histone proteins are subject to modification by both mono- and poly-ADP-ribosylation, they were used here as a means of monitoring the effects of the three ADP-ribosylation inhibitors.

When the mock-infected (MI) and VV-infected (WT) samples minus drug were analyzed, bands which comigrated with the major core proteins 4A and 4B were identifiable in the VV-infected samples, although these proteins were labeled less intensly than proteins such as the histones. The core proteins have recurringly proven to be difficult to distinguish in infected cell lysates. It is clear here, however, that as concentrations of the three inhibitor compounds were increased, a concomitant decrease in the labeling of both the VV core proteins and the cellular histone proteins occurred. In addition, MIBG seemed to have a much greater effect on diminishing the [8-^{14}C]adenosine labeling of theses proteins than did either NIC or 3-AB.

The results of densitometric scanning of a lighter film exposure of the histone proteins (Figure IV. 4, Panel B) paralleled those which can be obtained by direct visualization of the autoradiograph shown in Panel A, and provided a more quantitative measure of the effects of the inhibitors on [8-^{14}C]adenosine labeling of the viral and cellular proteins. As we have noted before (Child *et al.*, 1988), VV infection appears to stimulate labeling of the

histone proteins by approximately 4-fold in comparison to the histones in mock-infected cells, although the explanation for this phenomenon is still unknown. Also, while 10 mM NIC or 3-AB had little effect on histone labeling, a 20 mM concentration of either drug reduced labeling by 150-fold, while 40 mM NIC reduced it by 300-fold, and 40 mM 3-AB by 700-fold. MIBG showed a much greater effect; 0.1 mM and greater drug concentrations diminished labeling by about 1000-fold. This study provides some indication that the metabolic effects of NIC, 3-AB, and MIBG might be due at least in part to prevention of the modification of the viral and cellular proteins by ADP-ribosylation.

Effect of the inhibitors on infectious VV particle formation

In order to see whether mature, infectious virus particles were formed in the presence of the three inhibitors, cells were infected, treated with 60 mM NIC or 3-AB and 0.6 mM MIBG, viral DNA labeled with [^{3}H]thymidine, and virus particles purified and analyzed by velocity sedimentation as described in the Materials and Methods section. Figure IV. 5 shows the pooled results from 3 or 4 replicates for each sample. In the absence of inhibitor, a radiolabeled peak which reflects the presence of VV virions was detectable, while upon growth of the virus in the presence of 60 mM NIC or 3-AB, no peak representing virus particles could be seen. Interestingly, a very small quantity of virus particles appeared to be formed when VV was grown in 0.6 mM MIBG, and this observation was readily reproducible. In order to assess the presence of mature, infectious virus particles, fractions from each radiolabeled sample corresponding to the peak virus fractions in the minus drug control were subjected to plaque titration (Figure IV. 5). As is indicated in Figure IV. 5, treatment with either NIC or 3-AB reduced VV titers to approximately 5% of those measured for the untreated samples (a 3 log

Figure IV. 5. *Infectious VV particle formation in the presence or absence of the ADP-ribosylation inhibitors.* Monolayers of BSC_{40} cells were infected with VV at an MOI of 5 PFU per cell in the presence of 60 mM NIC (▲), 60 mM 3-AB (◆), 0.6 mM MIBG (●), or in the absence of drug (■), and the infections allowed to proceed for 24 hr in the presence of 1 μCi per ml [^{3}H] thymidine to label viral DNA. The labeled virus particles were purified on sucrose gradients by velocity sedimentation, the gradients fractionated, and the radioactive counts associated with each fraction determined (open symbols). The peak radioactive fractions were then subjected to plaque titration in order to determine the yield of infectious particles (patterned symbols).

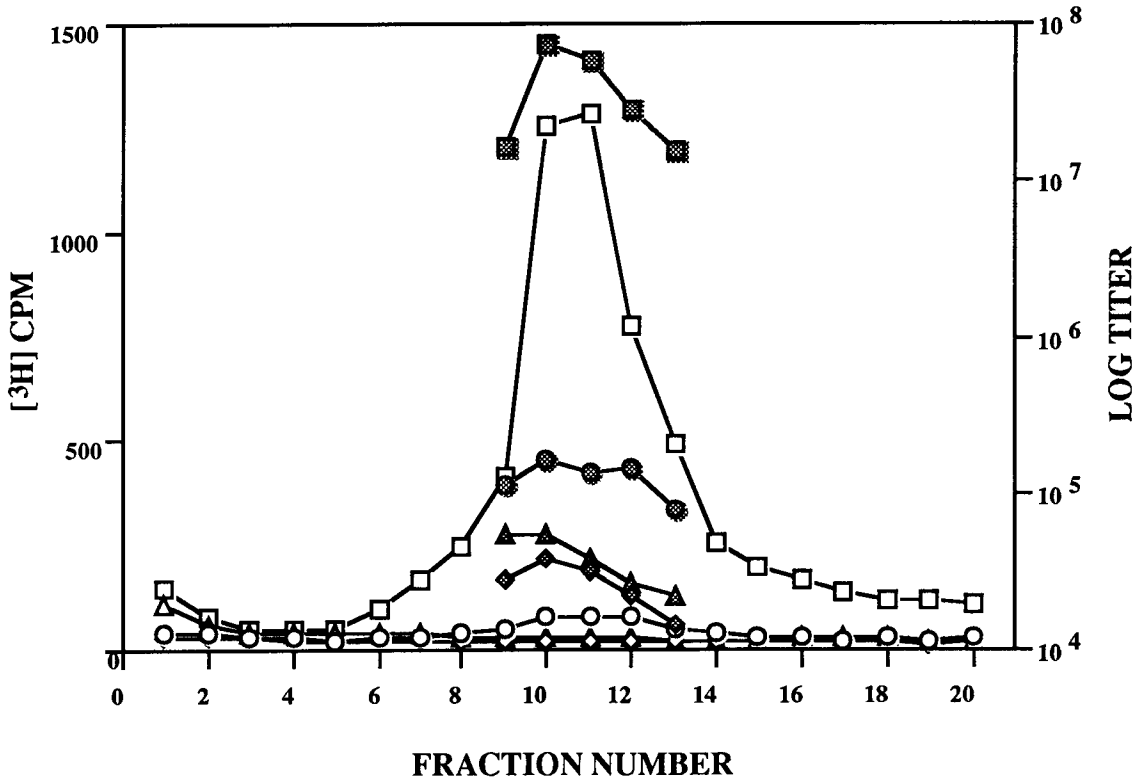

1500
1000
500
0
[3H] CPM
10^8
10^7
10^6
10^5
10^4
LOG TITER
0
2
4
6
8
10
12
14
16
18
20
FRACTION NUMBER

Figure IV.5

reduction in titer), while MIBG lowered the titers to about 20% of control levels (a 2 to 3 log decrease in titer).

The results of these experiments indicate that when VV was grown in the presence of the three ADP-ribosylation inhibitors, few, if any, mature progeny virions were produced.

DISCUSSION

The experimental results presented here demonstrate the effects of three inhibitors of ADP-ribosylation reactions, NIC, 3-AB, and MIBG, on various stages of the VV replication cycle. Fairly high concentrations (≥ 20 mM) of the competitive poly-ADP-ribosylation inhibitors NIC and 3-AB relative to those used in the majority of *in vivo* studies (Smets *et al.*, 1990) were necessary in order to demonstrate an effect on viral titer (Figure IV. 1), proteolytic processing (Figure IV. 3), [8-^{14}C]adenosine-labeling of the major VV core proteins (Figure IV. 4), and the formation of mature, infectious virions (Figure IV. 5), which may indicate that the viral core proteins are subject to mono- rather than poly-ADP-ribosylation events (Smets *et al.*, 1990). In contrast, low levels of MIBG (≥ 0.1 mM), which appears to be a highly specific inhibitor of mono-ADP-ribosylation reactions (Loesberg *et al.*, 1990 a,b), reduced VV titers (Figure IV. 1) and [8-^{14}C]adenosine-labeling of the core proteins (Figure IV. 4), although slightly higher MIBG concentrations (≥ 0.4 mM) were necessary in order to see an effect on either proteolytic processing of the major core proteins (Figure IV. 3) or infectious virion formation (Figure IV. 5).

It is not possible at this point to quantitate how much of the observed attenuation of viral replication is due to specific inhibition of ADP-ribosyltransferase activity and how much is due to side effects of the drugs. The cellular histone proteins, which are modified by both mono- and poly-ADP-ribosylation (Ueda *et al.*, 1975; Poirer *et al.*, 1982; Althaus and Richter, 1987), served as an effective control for monitoring the influence of varying concentrations of the three inhibitors on ADP-ribosylation reactions (Figure IV. 4B). It seems likely that the abrogation of VV DNA synthesis (Figure IV. 2) and protein synthesis (Figure IV. 3) evident at higher concentrations of the

three inhibitors was due, at least in part, to toxic metabolic side effects of the compounds (Purnell and Whish, 1980; Milam and Cleaver, 1984; Loesberg *et al.*, 1990 a), although these studies provided some indication (as demonstrated by the characteristic profiles of inhibition of VV titers (Figure IV. 1) and the effect of the three compounds on the [8-^{14}C]adenosine-labeling of the major core proteins (note the 4A and 4B core proteins in Figure IV. 4B)) that the inhibitors were also acting to prevent the modification of a subset of the VV core proteins by ADP-ribosylation.

In a previous study, Déry and colleagues found that several adenovirus core proteins are subject to ADP-ribosylation, and that this modification appears to function in virus decapsidation and the relaxation of the virion core chromatin structure during replication (Déry *et al.*, 1986). It is possible that ADP-ribosylation could play a similar role during VV replication.

Another attractive hypothesis for the functional role of ADP-ribosylation during the VV replication cycle is that the major core protein precursors must be ADP-ribosylated in order for their maturation *via* proteolytic processing to occur (although our inability to detect labeled precursor proteins indicates that if this is the case, the modification and cleavage events must be quite tightly temporally linked), or that modification of the mature cleaved proteins serves a role in the proper incorporation or positioning of the core proteins into mature virions. If one of these scenarios was accurate, the action of the ADP-ribosylation inhibitors could be attributed to the specific prevention of ADP-ribosylation of the VV core proteins, but it must be noted that a variety of different drugs (such as α-amanitin and rifampicin) and conditional-lethal mutants in the VV genome also prevent the proteolytic processing of the major core proteins by unknown mechanisms (Moss and Rosenblum, 1973; Silver and Dales, 1982; Villarreal *et al.*, 1984).

While the results of these studies in and of themselves are not conclusive regarding whether or not ADP-ribosylation is a necessary event during the VV replication cycle, when taken in concert with the similar findings of Smets *et al.* (1990) with respect to the inhibition profiles and differential effects of NIC, 3-AB, and MIBG on cellular mono- and poly-ADP-ribosylation events, and the results of our previous experiments which indicate the presence of an ADP-ribose-like, adenylate-containing moiety on the major VV core proteins (i.e. boronate affinity chromatography and reverse phase HPLC analysis), these results support our hypothesis that the viral core proteins are modified by ADP-ribosylation, and indicate the likelihood that the modifying moieties are mono-ADP-ribose residues.

CHAPTER V

ADDENDUM AND CONCLUSIONS

One of the recurring themes throughout this thesis has been the technical difficulties involved in the study of ADP-ribosylation reactions. The purpose of this chapter is to provide some further information about our attempts to study this modification in VV, including brief descriptions of some of the methods utilized which did not yield fruitful results, and some discussion as to potential reasons for our lack of success in applying these techniques. It is hoped that this information will be of some value in that it describes further methods which might be of use in studying the ADP-ribosylation of the major VV core proteins (or ADP-ribosylation reactions in general), as well as providing some insight into purification procedures which can be utilized for these proteins.

Endeavors to Increase the Radiolabeling of the Modified VV Proteins

Because certain protein-ADP-ribose linkages are known to be labile to conditions of neutral or alkaline pH (Dam *et al.*, 1981; Althaus and Richter, 1987; Aktories *et al.*, 1988), a gel system designed to prevent the hydrolysis of protein-ADP-ribose bonds (Jackowski and Kun, 1983) was used in an attempt to increase the intensity of the radiolabeled viral core proteins upon visualization by autoradiography. The gel system, which contained 5 M urea and sodium acetate (pH 5.0), caused the VV proteins to migrate more as

smears than distinct bands, making identification of the various core proteins difficult. In addition, the system did not seem to produce any substantial increase in the stability of the label associated with the VV core proteins, and so we discontinued the use of this method. Whether the lack of enhancement of the labeled proteins was due to the presence of a non-labile protein-adduct linkage or some other factor is not known.

Other efforts were directed at increasing the efficiency of radiolabeling of the core proteins, and these included transient expression of plasmids containing the P4A, P4B, and P25K open reading frames in VV-infected cells in the presence of [^{3}H]adenosine. This did not yield an increase in the production of [^{3}H]adenosine-labeled core proteins, which could indicate a low efficiency of transient expression, a need for the proper temporal expression, or for the correct contextual localization of the core proteins in order for modification to occur. Because repeated attempts proved to be unsuccessful, transient expression was discarded as a technique for producing increased levels of modified core proteins. With the newly available lipofection methods of transfection, which result in very high frequencies of transient expression (with up to 96% of transfected cells expressing), this line of research might now prove to be more successful (Rose et al., 1991). Permeabilization of cells has been utilized as a method for introducing nicotinamide adenine dinucleotide (NAD^+), the direct substrate for ADP-ribosylation reactions, into cells (Althaus and Richter, 1987). We made attempts at permeabilizing cells for the introduction of [^{3}H]NAD^+ both with the detergent digitonin (Peppers and Holz, 1986; Sarafian *et al.*, 1987) and by "bead loading", which involves the introduction of macromolecules into cells by transient mechanical disruption of the cell membrane with glass beads (McNeil, 1989). Both of these methods gave unsatisfactory results,

which may have been overcome had more time been available for working out experimental conditions. Finally, we also attempted to radiolabel ADP-ribose residues subsequent to their isolation from partially purified preparations of the 4A and 4B core proteins by *in vitro* sodium [^{3}H]borohydride labeling (Hakam, *et al.*, 1984; Hakam *et al.*, 1986). While high levels of radiolabel were incorporated into several species of molecules (indicating the sensitivity of this method), as was evident upon reverse phase HPLC analysis on a VYDAK C18 column, the number of labeled compounds and their lack of comigration with sodium [^{3}H]borohydride-labeled standards made interpretation of the results of these experiments difficult at best, and so this method was abandoned in favor of the HPLC analysis techniques described in Chapter III. It is probable, however, that this labeling method could prove useful in quantitating ADP-ribose residues or similar modifying groups which are present in very low levels within cells.

Efforts to Develop a Cell-free VV ADP-ribosylation System

In vitro ADP-ribosylation assays have been developed for several cellular systems (Hakam *et al.*, 1984; Scovassi, *et al.*, 1984; Cherney *et al.*, 1985; Alvarez-Gonzalez, 1988). These assays utilize cell lysates (or VV-infected cell lysates in our case) to which radiolabeled NAD^+ is added as the substrate along with Tris.Cl (~pH 8.0), nicked DNA, Mg^{+2}, and in some cases other factors which have been shown to stimulate ADP-ribosylation activity (spermidine, histone proteins, *etc.*). With various reaction conditions we were able to optimize levels of production of poly-ADPR in VV-infected BSC_{40} cells, but were unable to obtain any apparent specific labeling of VV or cellular proteins. The poly-ADP-ribose synthesized by this method was an effective control for our HPLC analyses, however, and cleavage of the polymers with snake venom phosphodiesterase (Oka *et al.*, 1978) yielded mono-ADP-ribose controls

(5'AMP and PR-AMP) for these analyses. There are also gel systems available for analysis of poly ADPR chain length following its synthesis (Hacham and Ben-Ishai, 1990; Naegeli *et al.*, 1989; Panzeter & Althaus, 1990), and we were able to effectively visualize polymers of up to ~ 60 residues using these procedures.

Attempts to Determine Whether the VV Core Protein Precursors are Modified by ADP-ribosylation

Because we were interested in determining whether the major core protein precursors are modified, rather than just the mature, cleaved products, we utilized a cleavage-deficient VV temperature sensitive mutant (ts 101) as well as rifampicin and NIC drug blocks in an attempt to visualize labeling of the precursors upon growth of VV in the presence of [^{3}H]adenosine. These attempts were unsuccessful, which could be due to several factors; the specific activity of the label incorporated into the core protein precursors might not have been high enough for detection, the modification and cleavage events may be closely temporally associated, or alternatively, the proteins might not be modified until after proteolytic processing occurs.

Methods Utilized for the Attempted Purification of Modified VV Core Proteins

Much of our effort during the later stages of this work was concentrated on attempts to purify one of the major core proteins so that HPLC and Mass Spectral analyses could be performed on the modifying moiety. A method for the purification of the 25K core protein has been developed (Yang and Bauer, 1988). This purification scheme involves the use of DEAE-cellulose, carboxymethyl-cellulose, and hydroxylapatite chromatography, and we were able to successfully purify reasonable amounts of 25K by these methods. While 25K is quite a bit more soluble and apparently more loosely associated

with VV virions than the 4A and 4B core proteins, unfortunately, 25K is also less strongly radiolabeled by [^{3}H]adenosine than the other two proteins. Because of this, we were more interested in purifying either the 4A or 4B core proteins, although the methods developed for 25K purification could not be utilized successfully for 4A and 4B due to their insolubility. If fact, these two proteins were insoluble without the presence of at least 0.1% SDS in the chromatography buffers. Various other attempts at solubilization of these proteins, including 60% formic acid (Heukeshoven and Dernick, 1985), non-ionic detergents such as Triton X-100, heating, and numerous salt conditions were not successful. Most of the techniques we tested were affected by either lack of solubility of the core proteins or the presence of SDS, including immunoaffinity chromatography with an α4B column, hydroxylapatite chromatography, chromatofocusing with Pharmacia PBE 94 polybuffer exchanger, isoelectric focusing utilizing the Bio-Rad Rotofor cell, hydrophobic interaction chromatography with Pharmacia Phenyl Sepharose CL-4B resin and a linear gradient of 1 M ammonium sulfate-50 mM KPO_4 (pH 7.0) to 50 mM KPO_4 (pH 7.0), and ammonium sulfate fractionation. One technique which proved to be effective was size fractionation over Pharmacia Sephacryl S-100 or S-300 resins, because this type of chromatography is compatible with buffers containing high concentrations of SDS, although this step alone was not sufficient for purification of 4A and 4B to any significant extent.

Conclusions

Although determining whether the major VV core proteins are modified by ADP-ribosylation and what potential role this modification might play during the VV replication cycle has proven to be difficult at best, the studies described in this thesis do provide some insights into experimental methods for studying post-translational modification events in an *in vivo* system, as

well as additional, albeit not definitive, information about the modification of VV structural proteins and various aspects of viral replication. As has been mentioned, the use of ADP-ribosylation inhibitors has many limitations, but the lack of genetic approaches for studying this modification event makes their use of importance in understanding the physiological functions of ADP-ribosylation (Rankin et al., 1989). Although ADP-ribosylation is an event which has proven to date to be technically difficult to study in intact viral and cellular systems, as more sensitive means of detection and more effective methods of protein purification and analysis are developed, and especially in the event that systems can be developed which allow ADP-ribosylation to be studied through genetic approaches, analysis of this and other post-translational modifications should prove to be far less difficult in the future.

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