Journal of General Virology (1994), 75, 819–827. *Printed in Great Britain*

Reversal of the measles virus-mediated increase of phosphorylating activity in persistently infected mouse neuroblastoma cells by anti-measles virus antibodies

Y. Segev,[1] B. Rager-Zisman,[1] N. Isakov,[1] S. Schneider-Schaulies,[2] V. ter Meulen,[2] S. Udem,[3] S. Segal[1] and M. Wolfson[1]*†

[1] *Ben Gurion University of the Negev, Department of Microbiology and Immunology, P.O. Box 653, 84105 Beer Sheva, Israel,* [2] *Institut für Virologie und Immunbiologie, Universität Würzburg, Versbacher Strasse 7, 97078 Würzburg, Germany and* [3] *New Jersey Medical School-UMDNJ, Department of Medicine, Newark, New Jersey 07163, U.S.A.*

To investigate the effect of persistent measles virus infection on signal transduction in cells of neuronal origin, the mouse neuroblastoma cell line NS20Y/MS, which is persistently infected with measles virus, was used. The results demonstrate an approximate 50% increase in total phosphorylation and a similar increase in protein kinase C (PKC) activity. Western blot analysis with anti-total PKC or anti-PKC-α antibodies revealed a significant increase in the level of an 80K immunoreactive PKC in NS20Y/MS cells. Following incubation of NS20Y/MS cells with polyclonal anti-measles virus antibodies, which down-regulate the level of measles virus proteins, total and PKC-mediated phosphorylation returned to the basal level of uninfected cells. This effect was reversible and removal of the antibodies resulted in restoration of the high level of total and PKC-mediated phosphorylation. The release of infectious measles virus was strongly inhibited by incubation of NS20Y/MS cells with the PKC inhibitor, 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7). These results demonstrate that measles virus induces elevation in cellular phosphorylation which is essential for measles virus production.

Introduction

Measles virus (MV), a paramyxovirus, is highly contagious and is one of the major causes of infant mortality in underdeveloped countries (Bloom, 1989). Sporadic outbreaks of acute measles still occur in industrialized countries despite vaccination (Centers for Disease Control, 1989) and a recent epidemic has caused more than 100 deaths in North America. MV can cause four distinct syndromes in humans: the classical epidemic acute illness of children, an autoimmune-like condition known as post-infectious encephalomyelitis (Norrby & Oxman, 1990; Kipps *et al.*, 1983), late and usually fatal complications of acute measles infection known as subacute sclerosing panencephalitis (SSPE) and measles inclusion body encephalitis (MIBE). These complications develop as a result of the persistence of measles virus in brain cells (Baczko *et al.*, 1984; Basle *et al.*, 1987; Carter *et al.*, 1983; Cattaneo *et al.*, 1987, 1988; Kipps *et al.*, 1983; Payne *et al.*, 1969). MV replication in the brains of SSPE and MIBE patients is characterized by defective expression of the viral envelope proteins (Baczko *et al.*, 1984, 1988; Cattaneo *et al.*, 1988). In these cases the virus persists despite the presence of high titres of antiviral antibodies and competent immune cells.

Protein phosphorylation is a common mechanism for the regulation of physiological functions of cells. Many key regulatory proteins exist in cells in either a phosphorylated or a dephosphorylated form, their steady-state levels of phosphorylation reflecting the relative activities of the protein kinases and protein phosphatases that catalyse the interconversion processes. These kinases and phosphatases are often targets themselves for regulation by protein phosphorylation as part of a cascade in signal transduction pathways. The basic mechanism for signal transduction is that the extracellular signal affects the activity of a protein kinase cascade that modulates transcription factor activity by phosphorylation and results in alterations in cellular gene expression (Karin, 1991, 1992; Nigg, 1990; Schwartz & Greenberg, 1987).

One signal transduction pathway that is of special importance in the regulation of brain cell functions uses

† Present address: Department of Molecular Pharmacology, Albert Einstein School of Medicine of Yeshiva University, 1300 Morris Park Avenue, Bronx, New York, New York 10461, U.S.A.

phosphatidylinositol 4,5-bisphosphate (PIP2) as a precursor molecule for two second messengers. Ligand binding by receptors that operate via this pathway results in activation of a phospholipase C that hydrolyses PIP2 to yield diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). DAG activates protein kinase C (PKC) which leads to phosphorylation of numerous endogenous substrates, and IP3 releases Ca^{2+} from intracellular stores and activates various Ca^{2+}-dependent enzymes (Berridge, 1987). PKC is a ubiquitous calcium/lipid-dependent enzyme which phosphorylates a variety of membrane, cytosolic and nuclear proteins on serine/threonine residues (for reviews see Nishizuka, 1986*a*, *b*, 1988, 1992). PKC exists as a family of at least eight structurally and functionally related isoenzymes with minor differences in enzymatic properties, substrate specificity and tissue distribution (Kikkawa *et al.*, 1987; Kuo *et al.*, 1986; Huang *et al.*, 1986, 1988; Huang, 1989; Ono *et al.*, 1988; Akita *et al.*, 1990; Sekiguchi *et al.*, 1987; Coussens *et al.*, 1986). Physiologically, PKC is activated by endogenous DAG in response to occupation of membrane receptors by the appropriate ligand (Nishizuka, 1986*a*, 1988; Huang, 1989). An enzymatically active PKC binds tightly to membranes (Kikkawa *et al.*, 1982). This phenomenon, known as translocation, designates activation of the enzyme.

Taking into account the principal role of phosphorylation processes and PKC activity in neuronal functions, one may suggest that any alteration in this enzyme's activity will result in impairment of the normal function of these cells. Indeed, changes in PKC activity have been observed in various patho-physiological conditions. For instance, in short- or long-term forebrain ischaemia, translocation of PKC from cytosol to membranes and its subsequent downregulation have been observed (Louis *et al.*, 1988, 1991; Wieloch *et al.*, 1991). Enhanced PKC activity and alterations in its distribution, as well as phosphorylation of a neuron-specific PKC substrate, were found in the neocortex of patients at early stages of Alzheimer's disease (Clark *et al.*, 1991; Florez *et al.*, 1991; Masliah *et al.*, 1991). Several studies have indicated that viral infection may indeed affect phosphoinositide (PI) turnover and the phosphorylating activity of PKC. Mouse fibroblasts infected with herpes simplex virus type 2 exhibited elevated PKC activity (Roddick *et al.*, 1988), whereas mouse fibroblasts infected with human T cell leukaemia virus or adenovirus exhibited an increased turnover of PI (Nevins, 1989; Tan *et al.*, 1989).

To investigate the effects of persistent MV infection on cells of the central nervous system we have used a mouse neuroblastoma cell line persistently infected with MV, NS20Y/MS (Rager-Zisman *et al.*, 1984). This cell line was established from clone NS20Y of the C1300 murine neuroblastoma which is capable of differentiating *in vitro* and retains neuron-specific enzymes and receptor markers (Augusti-Tocco & Sato, 1969; Oldstone *et al.*, 1977). Recent studies by Schneider-Schaulies *et al.* (1992) have also demonstrated that the restriction of MV gene expression in these cells is similar to that found in brain cells of SSPE patients and experimentally infected rats. Our previous studies demonstrated that NS20Y/MS cells exhibit elevated PKC activity and augmented expression of the c-*fos* proto-oncogene (Wolfson *et al.*, 1991).

The present study was undertaken to investigate the regulation of protein phosphorylation and PKC activity in NS20Y/MS cells. We demonstrate that the enhanced total cellular phosphorylation and PKC activity could be downregulated by treatment of the persistently infected cells with polyclonal anti-MV antibodies. Following treatment of infected cells with these antibodies, but not with monoclonal antibodies, total and PKC-mediated protein phosphorylation returned to the basal levels of uninfected neuroblastoma cells (NS20Y). In addition we have shown that inhibition of PKC activity by 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7) decreased the release of infectious virus from these cells. These results suggest that the enhanced phosphorylating activity of persistently infected cells is induced by the virus and that PKC-mediated phosphorylation plays an important role in the maturation of infectious MV particles.

Methods

Cells and viruses. The C1300 neuroblastoma, clone NS20Y, of the A/J mouse strain was used. The establishment of MV persistently infected NS20Y cells termed NS20Y/MS is described in detail elsewhere (Rager-Zisman *et al.*, 1984). Cells were routinely grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), glutamine and antibiotics.

Antibodies. Polyclonal anti-MV hyperimmune serum was raised in rabbits by immunization with antigen in complete Freund's adjuvant, followed by repeated intramuscular injections with solubilized virus alone (Ju *et al.*, 1978). The preparation and characterization of monoclonal antibodies against measles virus N protein were described by Birrer *et al.* (1981) and against H protein (NC32) by Carter *et al.* (1983). Polyclonal rabbit anti-PKC antibodies were prepared as described previously (Isakov *et al.*, 1990). Monoclonal anti-bovine spleen PKCα antibodies were purchased from Seikagaku.

Measurement of total phosphorylation and PKC activity. Total phosphorylation and PKC activity were determined in cytosolic and particulate fractions. Cytosolic fractions were obtained by the following procedure. Cells were resuspended in an extraction buffer containing 20 mM-Tris–HCl pH 7·5, 1 mM-PMSF, 2 mM-EGTA and 10 mM-DTT, disrupted by sonication on ice and centrifuged for 30 min at 20000 *g*. The supernatant was designated the cytosolic fraction. To obtain membrane-associated proteins, corresponding pellets were extracted for 1 h on ice in the same buffer containing 1% Triton X-100. After centrifugation at 20000 *g* for 30 min the resulting supernatant was designated the particulate fraction. Both fractions were further purified

by chromatography on DE52 cellulose columns (Whatman) (Thomas *et al.*, 1987). Histone H1 was used as an exogenous substrate, [γ-^{32}P]ATP (NEN, sp. act. 10 to 50 Ci/mmol) as the phosphate group donor and DAG, phosphatidylserine (PS) and $CaCl_2$ as PKC activators (Kikkawa *et al.*, 1982; Wolfson *et al.*, 1985). The standard assay mixture (0·2 ml) contained 20 mM-Tris–HCl pH 7·5, 10 mM-DTT, 0·4 mM-EGTA, 10 mM-$MgCl_2$, 1 mM-$CaCl_2$, 2 μg/ml DAG and 80 μg/ml PS, histone H1 (30 μg/ml) and [γ-^{32}P]ATP (2×10^6 c.p.m./assay, in a final concentration of 50 μM). Where indicated, 50 nM-phorbol 12-myristate 13-acetate was used instead of DAG. Optimal concentrations of the activators were determined in preliminary experiments. After 3 min incubation at 30 °C, the reaction was stopped by the addition of 25% TCA and the reaction mixture was filtered through nitrocellulose filters (0·45 μm) in a dot blot apparatus. The nitrocellulose filter was washed three times with cold 10% TCA, dried, cut and ^{32}P incorporation was measured by liquid scintillation counting. Assays were performed in duplicate in the presence or absence of calcium and lipids. Enzymatic activity was measured as the incorporation of ^{32}P from [γ-^{32}P]ATP into histone H1. Activity is expressed as picomoles of ^{32}P incorporated per min per mg of sample protein. Total phosphorylating activity was calculated in the same units in the absence of PKC cofactors (calcium or lipids). Net PKC activity was calculated by subtracting the activity measured in the absence of the cofactors from that measured in the presence of cofactors. Calculation of the cytosol:particulate ratio is used to demonstrate enzyme translocation.

Endogenous protein phosphorylation in vitro. For analysis of total protein phosphorylation, cell suspensions were washed twice with PBS, resuspended in extraction buffer, disrupted by sonication on ice and centrifuged for 30 min. Reactions were initiated by the addition of [γ-^{32}P]ATP as the phosphate group donor (2×10^6 c.p.m./assay in a final concentration of 50 mM), for 3 min at 30 °C. The reaction was terminated by boiling for 5 min in 2 × SDS sample buffer as described by Laemmli (1970). Phosphorylated samples containing 150 to 200 μg protein were then separated by 10% SDS–PAGE for 18 h. Gels were dried and the phosphorylated proteins were detected by autoradiography.

For analysis of PKC-mediated phosphorylation, the same reaction mixture was used with the addition of 2 μg/ml DAG, 80 μg/ml PS and 1 mM-$CaCl_2$ as PKC cofactors.

Western blotting. For protein analysis cell extracts were prepared in cold RIPA buffer [(50 mM-Tris–HCl pH 7·5, 0·25% w/v sodium deoxycholate, 0·1% w/v SDS and 0·5% w/v NP40). Extracts were mixed with 2 × sample buffer and boiled for 5 min. Then 150 to 200 μg portions of sample protein were loaded in each gel lane and subjected to 10% SDS–PAGE. The resolved proteins were transferred electrophoretically to nitrocellulose filters which were then blocked for 1 h in PBS containing 10% low fat dry milk, followed by 1 h incubation with the first antibody diluted in PBS containing 1% dry milk. The filters were washed three times in PBS and the immunoblots were incubated with peroxidase-conjugated second antibody for 1 h, washed three times with PBS and developed with 4-chloronaphthol (0·3 mg/ml) and H_2O_2.

Treatment of cells with anti-MV antibodies. For antibody treatment, the medium of semiconfluent cultures was replaced with fresh medium containing anti-MV polyclonal or monoclonal antibodies sufficient to neutralize 10^4 p.f.u. of MV. Every third day, the cells were split and fresh medium containing antibodies was added. Antibody-treated or untreated cells were harvested at different times after antibody treatment. For reactivation, the cells were cultured in medium without antibodies and harvested at different times after antibody removal.

Treatment of cells with H-7. To study its effect on virus release, H-7 (dissolved in DMSO) was added to confluent cell cultures, 24 h after subculture, at a final concentration of 100 μM. Twenty-four hours later, supernatants were collected and used for titration of the virus. The solvent (DMSO) had no effect on the virus release at the concentration used in the experiments.

Virus titration. The titres of virus in culture supernatants were determined by a semiquantitative plaque assay (Rager-Zisman *et al.*, 1984). Vero cell monolayers were trypsinized and plated in 24-well plastic trays. Cells (3×10^5) were seeded in each well and incubated at 37 °C overnight. Tenfold dilutions of virus samples were prepared in Dulbecco's MEM supplemented with 2% FCS. The growth medium from each well was aspirated, and duplicate wells were inoculated with 0·2 ml of viral dilutions. After an adsorption period of 2 h at 33 °C, 1 ml of growth medium containing 0·75% carboxymethyl cellulose was added. The monolayers were then incubated at 37 °C for 5 days, fixed with 10% formol–saline and stained with crystal violet.

Results

In vitro *phosphorylation of endogenous proteins in NS20Y/MS cells*

The effect of MV persistent infection on total protein phosphorylation was investigated by measuring *in vitro* incorporation of ^{32}P into proteins as described in Methods. The results demonstrate a 50% increase in total phosphorylating activity in the cytosolic fraction of infected cells NS20Y/MS, as compared with uninfected NS20Y (Fig. 1*a*). Separation of the phosphorylated proteins by SDS–PAGE showed an overall increase in phosphorylation of endogenous protein substrates in NS20Y/MS cells (Fig. 1*b*).

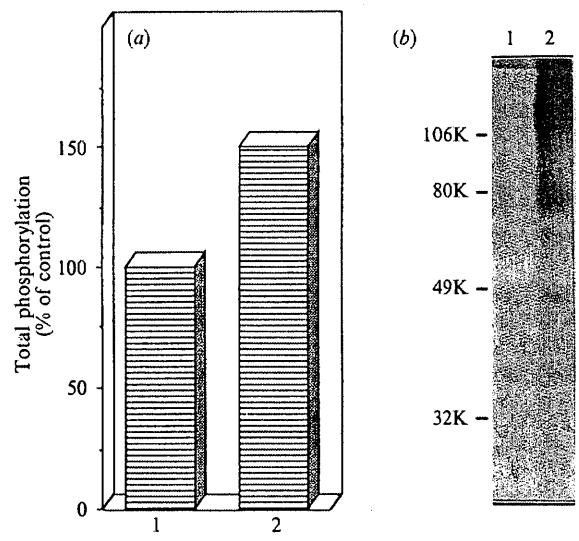

Fig. 1. Total protein phosphorylation activity in NS20Y and NS20Y/MS cells. (*a*) Phosphorylation of histone H1 by cytosolic extracts of NS20Y or NS20Y/MS cells. (*b*) *In vitro* endogenous protein phosphorylation of crude cytosolic extracts incubated with [^{32}P]ATP for 3 min, followed by SDS–PAGE. Gels were dried and phosphoproteins were visualized by autoradiography. Lane 1, NS20Y; lane 2, NS20Y/MS. Results were representative of at least six experiments.

Table 1. *PKC activity in NS20Y/MS and NS20Y cells**

Cell	Fraction	TPA	DAG	Increase (%)	Cytosolic: particulate
NS20Y	Cytosol	364±11	329±34		
NS20Y/MS	Cytosol	564±27	523±31	37%	1·28
NS20Y	Particulate	278±18	256±43		
NS20Y/MS	Particulate	426±17	396±25	36%	1·32

* Endogenous activity of PKC (pmol/min/mg protein) was measured in the cytosolic and particulate extracts of NS20Y and NS20Y/MS cells as described in Methods. TPA (50 nM) or DAG (2 μg/ml) were added to the reaction mixture and PKC activity was determined on 50 mg protein samples. Results are the mean of three experiments±S.D.

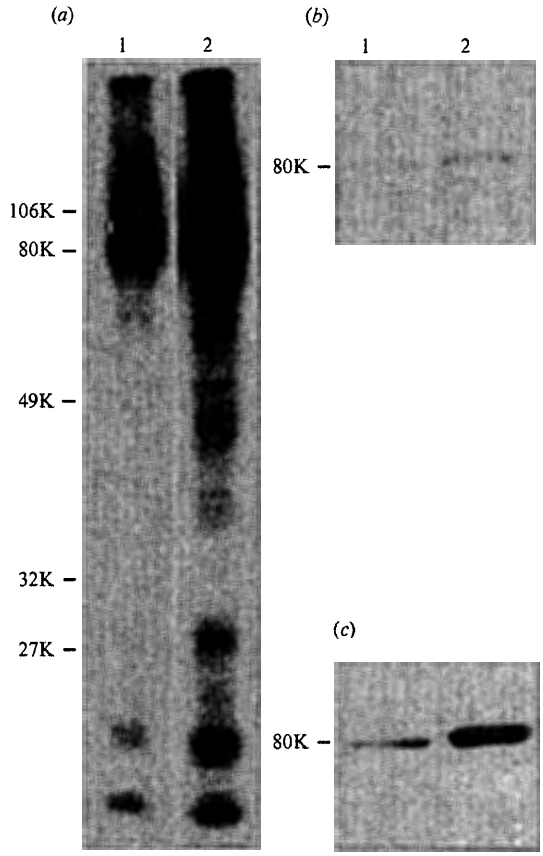

Fig. 2. PKC-mediated phosphorylation in NS20Y or NS20Y/MS cells. Lanes 1, uninfected cells; lanes 2, MV-infected cells. (*a*) PKC activity was measured in a reaction mixture specific for PKC with histone H1 (30 μg/ml), DAG (2 μg/ml) and PS (80 μg/ml), as described in Methods. The proteins were then separated by SDS–PAGE and subjected to autoradiography. PKC protein was detected by Western blotting as described in Methods using polyclonal rabbit anti-PKC antibodies (*b*) or monoclonal anti-PKC-α antibodies (*c*).

Protein kinase C-mediated activity and phosphorylation in NS20Y/MS cells

To examine the extent of phosphorylation mediated by PKC, we assayed the activity of these enzymes using either DAG with PS or TPA with PS as specific PKC cofactors. The results summarized in Table 1 demonstrate an increased PKC activity in both fractions of NS20Y/MS cells being 37% and 36% in cytosolic and particulate fractions, respectively. The ratio of cytosolic:particulate PKC activity remained practically unchanged, 1·32 in NS20Y/MS cells and 1·28 in uninfected NS20Y cells. These results indicate that the accumulation of viral proteins in NS20Y/MS cells did not have an effect on the subcellular distribution of PKC.

PKC-mediated phosphorylation of endogenous protein substrates *in vitro* was studied next. Cellular extracts were incubated with the PKC reaction mixture and [γ-^{32}P]ATP as described in Methods and the phosphorylated proteins were separated by SDS–PAGE. In these experiments PKC-mediated phosphorylation was detected in both cell types; nonetheless, it was substantially higher in NS20Y/MS cells. In extracts of MV-infected cells intensely phosphorylated proteins ranging in M_r from 27K to 60K were observed (Fig. 2*a*, compare lane 1 with lane 2). To test whether phosphorylation was associated with viral proteins, MV proteins were immunoprecipitated from NS20Y/MS cell extracts using polyclonal rabbit anti-MV antibodies. No phosphorylated viral proteins were detected (data not shown).

To test whether the augmented activity of PKC is reflected by an increase in PKC synthesis, immunoblotting was performed with polyclonal anti-PKC or monoclonal anti-PKCα antibodies. The results, shown in Fig. 2(*b*) and (*c*) respectively, revealed a significant increase in the detected level of the 80K PKC proteins expressed in NS20Y/MS cells with both antibodies.

Downregulation of total protein phosphorylation activity in NS20Y/MS cells by anti-MV antibodies

In the following experiments the effect of anti-MV antibody treatment on total phosphorylating activity in cytosolic fractions of NS20Y/MS cells was investigated. Cells were grown in the presence of polyclonal anti-MV antibodies under conditions shown to downregulate MV protein synthesis (Schneider-Schaulies *et al.*, 1992). A decrease in total phosphorylating activity was first

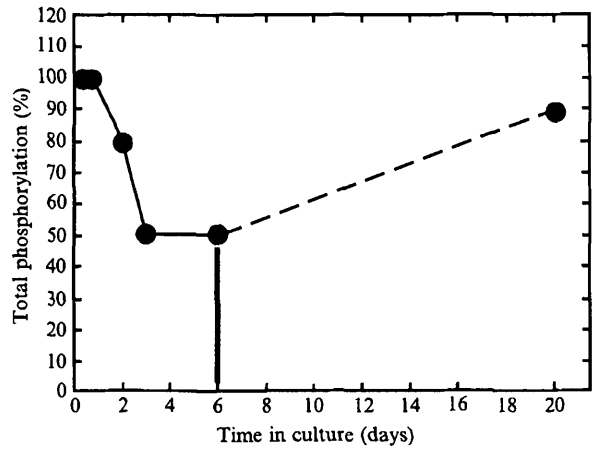

Fig. 3. Downregulation of total protein phosphorylation by anti-MV antibodies. Cells were grown in the presence of polyclonal anti-MV antibodies (solid line). Total phosphorylation activity was determined at different times during antibody treatment in the cytosolic fraction by using a standard kinase assay. Recovery of the level of total protein phosphorylation following growth in the absence of antibody is indicated by a broken line. Data are representative of three independent assays.

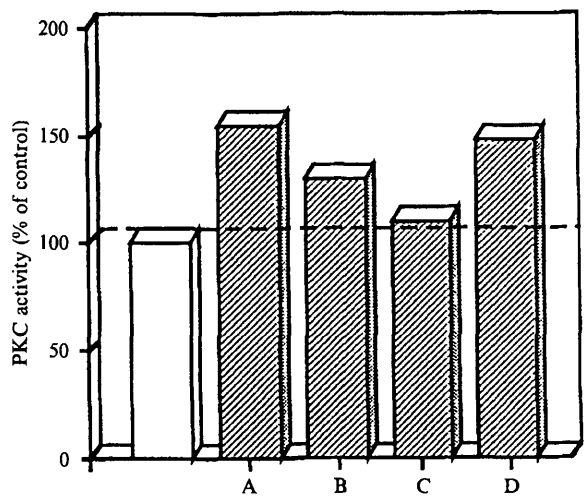

Fig. 4. Downregulation of PKC activity by anti-MV antibodies. Cells were grown in the presence of polyclonal anti-MV antibodies. PKC activity was determined, at various times during antibody treatment, in the cytosolic fraction as described in Methods. PKC activity in NS20Y cells was taken to be 100%. (A) untreated NS20Y/MS cells; (B) 2 days and (C) 6 days of growth in the presence of antibodies; (D) 6 days with antibodies followed by 14 days growth in the absence of antibodies. The results are the mean of four independent experiments. In all cases these determinations did not differ by more than 10%. (□), NS20Y cells, (▨), NS20Y/MS cells.

detected after 2 days of treatment with anti-MV antibodies and reached the baseline levels of uninfected cells after 4 to 6 days (Fig. 3). This effect was reversible since the original level of phosphorylating activity of NS20Y/MS cells was gradually restored when the cultures were grown in fresh medium without antibodies. Treatment of the cells with either anti-H or anti-N protein monoclonal antibodies or polyclonal antiserum non-specific for MV had no such effect.

Table 2. *Effect of H-7 on MV release by NS20Y/MS cells*

Cell	Treatment	Virus release*
NS20Y	None	–
NS20Y	H-7	–
NS20Y/MS	None	$1{\cdot}5 \times 10^3$
NS20Y/MS	H-7	$7{\cdot}0 \times 10^0$

* Cells were incubated with 100 μM-H-7 for 24 h. Virus release, expressed in p.f.u./ml, was determined by plaque formation on Vero cells, as described in Methods.

Downregulation of PKC activity in NS20Y/MS cells by anti-MV antibodies

Next we investigated whether the enhanced PKC activity in NS20Y/MS cells could also be modulated by treatment with anti-MV antibodies. As was mentioned above, PKC activity was higher in NS20Y/MS than in NS20Y cells. Reduction in PKC activity was observed on the second day of antibody treatment (Fig. 4, compare B with A) reaching the level of PKC activity in NS20Y cells by day 6 (Fig. 4, C). Removal of the antibodies and growth of the cells for an additional 14 days resulted in the restoration of PKC activity almost to the original level of NS20Y/MS cells (Fig. 4, D). Treatment of the cells with monoclonal anti-H or anti-N protein antibodies had no such effect (data not shown).

Inhibition of MV release by H-7

To study the involvement of PKC in the life cycle of MV, the release of infectious virus from NS20Y/MS cells was measured after treatment with H-7. NS20Y/MS cells were cultured in the presence or absence of 100 μM-H-7 for 24 h. As can be seen from Table 2, the yield of infectious virus was strongly inhibited, decreasing from $1{\cdot}5 \times 10^3$ to 7×10^0 p.f.u. in the presence of H-7. The compound had no effect on the level of MV proteins as measured by Western blot analysis of cell extracts (data not shown). These results may indicate that there is an intimate relationship between PKC-mediated phosphorylation and the assembly and budding of infectious MV particles.

Discussion

Clone NS20Y/MS of the murine neuroblastoma C1300 was infected with the wild-type Edmonston strain of MV and, after transition to a carrier culture, became

persistently infected (Rager-Zisman *et al.*, 1984). The cultures maintained a morphology indistinguishable from that of the parental uninfected NS20Y cells; nonetheless, immunofluorescence studies revealed accumulation of viral proteins in the majority of cells. We have previously investigated the effect of persistent MV infection of NS20Y cells and have shown that NS20Y/MS cells are less tumorigenic and express enhanced amounts of major histocompatibility complex class I glycoproteins (Gopas *et al.*, 1992). In addition increased expression of the c-*fos* proto-oncogene and PKC genes was also detected (Wolfson *et al.*, 1989, 1991).

In the present report we demonstrate that an intimate relationship exists between the synthesis of MV proteins and an increase in the phosphorylating activity as detected in the persistently infected cells. The endogenous phosphorylating activity in NS20Y/MS cells was 50% higher than that in the uninfected cells. The increase in total protein phosphorylation suggests that several kinases may be activated by MV. In this context we have recently shown that the activity of a dsRNA-dependent kinase was significantly elevated in NS20Y/MS cells (Gopas *et al.*, 1992). We have shown previously that the increase in PKC activity paralleled the elevation of PKC gene expression (Wolfson *et al.*, 1989, 1991). Here we confirm this observation at the protein level, demonstrating an increase in the 80K protein detectable by using anti-total PKC or anti-PKCα antibodies. It appears that MV requires a PKC-mediated phosphorylation step as shown for other viruses including human immunodeficiency virus (Chowdhury, *et al.*, 1990; Kinter *et al.*, 1990) and bovine leukaemia virus (Jensen *et al.*, 1992).

In the present study we show that although the extent of phosphorylation of endogenous proteins was consistently higher in NS20Y/MS cells, differences between the protein substrates could not be detected, indicating that these were primarily cellular proteins. In contrast, when PKC-mediated phosphorylation was examined, additional species of phosphorylated proteins could be observed in NS20Y/MS cells. The M_rs of these proteins were between 30K and 80K, similar to those of MV proteins. The results of immunoprecipitation with anti-MV antibodies were negative suggesting that the phosphorylated proteins are of cellular origin and that their phosphorylation is induced by the virus. Although it is well established that MV proteins P, N and V are phosphorylated, from this study it appears that PKC is not involved in this process. Another serine kinase, such as casein kinase II, shown by Barik & Banerjee (1992) to phosphorylate the P protein of vesicular stomatitis virus, is a probable candidate. The strong correlation found between the high MV protein content and both Ca^{2+}/lipid-dependent or -independent phosphorylating activity may indicate that MV infection can directly or indirectly mediate the activity of these kinases. The mechanism underlying this effect is as yet unknown although it has been described for a variety of DNA and RNA viruses, including polyoma virus, hepatitis B virus, adenovirus, herpes simplex virus, and human T cell leukaemia virus types I and II (De Wind *et al.*, 1992; Giordano *et al.*, 1991; Nevins, 1989, 1991; Shiroki *et al.*, 1992; Suzuki *et al.*, 1992; Tan *et al.*, 1989; Yoakim *et al.*, 1992; Maguire *et al.*, 1991; Wu *et al.*, 1990; Can & Chen, 1990).

We have shown recently that binding of polyclonal antibodies against MV or a mixture of monoclonal anti-H protein antibodies to NS20Y/MS cells resulted in downregulation of MV mRNA and protein synthesis (Schneider-Schaulies *et al.*, 1992). When the effect of this treatment on total phosphorylation or PKC activity was examined, downregulation occurred only when polyclonal antibodies were used. Treatment of the infected cells with antibodies against the intracellular N protein also had no such effect and in this case it appears that binding to a membrane entity is required. Furthermore it also appears that, in order to downregulate total phosphorylation or PKC activity, binding to more than one viral epitope is required since treatment with a single anti-H monoclonal antibody that binds and neutralizes MV had no effect. In this context, it is worth noting that monoclonal antibodies that bind to the same antigen may recognize epitopes with different structures since their binding sites are distinct in shape (Simpson *et al.*, 1993). Therefore, it seems possible that binding to more than one epitope is essential for inducing the conformational changes that will trigger the intracellular signalling.

The nature of the transduction pathway involved in the delivery of the signal triggered by the binding of anti-MV antibody is still unknown. Weinemann-Dorsch & Koschel (1989) reported that treatment of rat glioma cells, persistently infected with the SSPE strain of MV, with anti-MV antibodies stimulated phosphatidylinositol breakdown and inositol phosphate formation (Weinemann-Dorsch & Koschel, 1989; Tas & Koschel, 1991). Alterations in PIP2 metabolism do not necessarily result in the activation of PKC, and because in these experiments PKC activity was not measured it is difficult to compare the results of both systems.

Modulation of viral protein synthesis and cellular phosphorylation by anti-MV antibodies in NS20Y/MS demonstrates that there is a dynamic equilibrium between the persistent virus state and cellular metabolism. Viruses often use cellular phosphoprotein kinases to phosphorylate their own proteins in order to activate their functions. Examples of this include potentiation of the v-*fms* transforming potential of feline sarcoma virus

by phosphorylation on threonine-939, phosphorylation of the v-*erb* A on serine 16/17 which is essential for its oncogenic potential, and reactivation of herpes simplex virus type 1 from latency by PKC activators (Glineur *et al.*, 1990; Leib *et al.*, 1991; Tamura *et al.*, 1991). Although MV neither transforms the cells nor causes immortalization, phosphorylation may be a necessary step in its replication. It was indeed reported that TPA, which strongly activates PKC, was a very strong inducer of productive MV infection in peripheral blood monocytes (Vanionpää *et al.*, 1991). These results are in agreement with our observation that treatment with H-7 decreased the release of infectious virions. Treatment of NS20Y/MS with H-7 for 24 h led to a profound inhibition of the release of infectious MV. This suggests that phosphorylation mediated by PKC and/or other kinases such as protein kinase A, is an essential step in assembly and maturation of the virions.

In conclusion, the results presented here suggest that the augmented PKC activity is a necessary step in the maintenance of the persistent infection as well as in viral maturation. It is proposed that viral antigens expressed on the host cell membrane behave as receptors transducing signals to maintain viral replication without affecting cell viability.

We thank Dr Rivki Ofir for the helpful discussion of this manuscript. This work was supported in part by grants from the Chief Scientist Office, Ministry of Health, the Institute of Psychobiology, Israel and the Deutsche Forschungsgemeinschaft, Germany. Y.S. was supported by a fellowship from Robert and Rena Lewin, London, U.K.

References

AKITA, Y., OHNO, S., KONNO, Y., YANO, A. & SUZUKI, K. (1990). Expression and properties of two distinct classes of the phorbol ester receptor family, four conventional protein kinase C types and a novel protein kinase C. *Journal of Biological Chemistry* **265**, 354–362.

AUGUSTI-TOCCO, G. & SATO, G. (1969). Establishment of functional clonal lines of neurons from mouse neuroblastoma. *Proceedings of the National Academy of Sciences, U.S.A.* **64**, 311–316.

BACZKO, K., CARTER, M. J., BILLETER, M. & TER MEULEN, V. (1984). Measles virus gene expression in subacute sclerosing panencephalitis. *Virus Research* **1**, 585–595.

BACZKO, K., LIEBERT, U. G., CATTANEO, R., BILLETER, M. A., ROOS, R. P. & TER MEULEN, V. (1988). Restriction of measles virus gene expression in measles virus inclusion body encephalitis. *Journal of Infectious Diseases* **158**, 144–150.

BARIK, S. & BANERJEE, A. K. (1992). Phosphorylation by cellular casein kinase II is essential for transcriptional activity of vesicular stomatitis virus phosphoprotein P. *Proceedings of the National Academy of Sciences, U.S.A.* **89**, 6570–6574.

BASLE, M. F., REBEL, A., FOURNIER, J. G., RUSSELL, W. C. & MALKANI, K. (1987). On the trail of paramyxoviruses in Paget's disease of bone. *Clinical Orthopedics* **21**, 9–15.

BERRIDGE, B. (1987). Inositol triphosphate and diacylglycerol: two interacting second messengers. *Annual Review of Biochemistry* **56**, 159–178.

BIRRER, M. J., BLOOM, B. R. & UDEM, S. (1981). Characterization of measles polypeptides by monoclonal antibodies. *Virology* **108**, 381–390.

BLOOM, B. R. (1989). Vaccines for the Third World. *Nature, London* **342**, 115–120.

CANN, A. J. & CHEN, I. S. Y. (1990). Human T-cell leukemia virus types I and II. In *Virology*, 2nd edn., pp. 1501–1527. Edited by B. N. Fields & D. M. Knipe. New York: Raven Press.

CARTER, M. J., WILLCOCKS, M. M. & TER MEULEN, V. (1983). Defective translation of measles virus matrix proteins in subacute sclerosing panencephalitis. *Nature, London* **305**, 153–155.

CATTANEO, R., REBMANN, G., SCHMID, A., BACZKO, K., TER MEULEN, V. & BILLETER, M. A. (1987). Altered transcription of a defective measles virus genome derived from a diseased human brain. *EMBO Journal* **6**, 681–687.

CATTANEO, R., SCHMID, A., ESCHLE, D., BACZKO, K., TER MEULEN, V. & BILLETER, M. A. (1988). Biased hypermutation and other genetic changes in defective measles virus in human brain infections. *Cell* **55**, 255–265.

CENTERS FOR DISEASE CONTROL (1989). Measles: United States, first 26 weeks. *Morbidity and Mortality Weekly Reports* **38**, 863–873.

CHOWDHURY, I. H., KOYANAGI, Y., KOBAYASHI, S., HAMAMOTO, Y., YOSHIYAMA, H., YOSHIDA, T. & YAMAMOTO, N. (1990). The phorbol ester TPA strongly inhibits HIV-1-induced syncytia formation but enhances virus production: possible involvement of protein kinase C pathway. *Virology* **176**, 126–132.

CLARK, E. A., LEACH, K. L., TROJANOWSKI, J. Q. & LEE, V. M. (1991). Characterization and differential distribution of the three major protein kinase C isozymes (PKC alpha, PKC beta, PKC gamma) of the central nervous system in normal and Alzheimer diseased brain. *Laboratory Investigation* **64**, 35–44.

COUSSENS, L., PARKER, D. J., RHEE, L., YANG-FENG, T., CHEN, E., WATERFIELD, M. D., FRANCKE, U. & ULLRICH, A. (1986). Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signalling pathways. *Science* **233**, 859–866.

DE WIND, N., DOMEN, J. & BERNS, A. (1992). Herpesviruses encode an unusual protein-serine/threonine kinase which is nonessential for growth in cultured cells. *Journal of Virology* **66**, 5200–5209.

FLOREZ, J. C., NELSON, R. B. & ROUTTENBERG, A. (1991). Contrasting pattern of protein phosphorylation in human normal and Alzheimer brain: focus on protein kinase C and protein F1/GAP-13. *Experimental Neurology* **112**, 264–272.

GIORDANO, A., LEE, J. H., SCHEPPLER, J. A., HERRMANN, C., HARLOW, E., DEUSCHLE, E., BEACH, D. & FRANZA, B. R., JR (1991). Cell cycle regulation of histone H1 kinase activity associated with the adenoviral protein E1A. *Science* **253**, 1271–1275.

GLINEUR, C., ZENKE, M., BEUG, H. & GHYSDAEL, J. (1990). Phosphorylation of the c-erb A protein is required for its function as an oncogene. *Genes and Development* **4**, 1663–1676.

GOPAS, J., ITZHAKI, D., SEGEV, Y., SALZBERG, S., TRINK, B., ISAKOV, N. & RAGER-ZISMAN, B. (1992). Persistent measles infection enhances major histocompatibility complex class I expression and immunogenicity of murine neuroblastoma cells. *Cancer Immunology and Immunotherapy* **34**, 313–320.

HUANG, K. P. (1989). The mechanism of protein kinase C activation. *Trends in Neuroscience* **12**, 425–432.

HUANG, K. P., NAKABAYASHI, H. & HUANG, F. L. (1986). Isozymic forms of rat brain Ca^{2+}-activated and phospholipid-dependent protein kinase. *Proceedings of the National Academy of Sciences, U.S.A.* **83**, 8535–8539.

HUANG, K. P., HUANG, F. L., NAKABAYASHI, H. & YOSHIDA, Y. (1988). Biochemical characterization of rat brain protein kinase C isozymes. *Journal of Biological Chemistry* **263**, 1439–1445.

ISAKOV, N., MCMAHON, P. & ALTMAN, A. (1990). Selective post transcriptional down-regulation of protein kinase C isoenzymes in leukemic T cells chronically treated with phorbol esters. *Journal of Biological Chemistry* **256**, 2091–2097.

JENSEN, W. A., WICKS-BEARD, B. J. & COCKERELL, G. L. (1992). Inhibition of protein kinase C results in decreased expression of bovine leukemia virus. *Journal of Virology* **66**, 4427–4433.

JU, G., UDEM, S., RAGER-ZISMAN, B. & BLOOM, B. R. (1978). Isolation of a heterogeneous population of temperature sensitive mutants of measles virus from persistently infected human lymphoblastoid cell lines. *Journal of Experimental Medicine* **147**, 1637–1652.

KARIN, M. (1991). Signal transduction and gene control. *Current Opinion in Cell Biology* **3**, 467–473.

KARIN, M. (1992). Signal transduction from cell surface to nucleus in development and disease. *FASEB Journal* **6**, 2582–2590.

KIKKAWA, U., TAKAI, Y., MINAKUCHI, R., INOHARA, S. & NISHIZUKA, Y. (1982). Calcium-activated, phospholipid-dependent protein kinase from rat brain. *Journal of Biological Chemistry* **257**, 13341–13348.

KIKKAWA, U., ONO, Y., OGITA, K., FUJI, T., ASAOKA, Y., SEKIGUCHI, K., KOSAKA, Y., IGARASHI, K. & NISHIZUKA, Y. (1987). Identification of the structures of multiple subspecies of protein kinase C expressed in rat brain. *FEBS Letters* **217**, 227–231.

KINTER, A. L., POLL, G., MAURY, W., FOLKS, T. M. & FAUCI, A. S. (1990). Direct and cytokine-mediated activation of protein kinase C induces human immunodeficiency virus expression in chronically infected promonocytic cells. *Journal of Virology* **64**, 4306–4312.

KIPPS, A., DICK, G. & MOODIE, J. W. (1983). Measles and the central nervous system. *Lancet* ii, 1406–1410.

KUO, J. F., SHOJI, M., GIRARD, P. R., MAZZEI, G. J., TURNER, R. S. & SU, H. D. (1986). Phospholipid/calcium-dependent protein kinase (protein kinase C) system: a major site of bioregulation. *Advances in Enzyme Regulation* **25**, 387–400.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.

LEIB, D. A., MADEAU, K. C., RUNDLE, S. A. & SCHAFFER, P. A. (1991). The promoter of the latency-associated transcripts of herpes simplex virus type 1 contains a functional cAMP-response element: role of the latency-associated transcripts and cAMP in reactivation of viral latency. *Proceedings of the National Academy of Sciences, U.S.A.* **88**, 48–52.

LOUIS, J. C., MAGAL, E. & YAVIN, E. (1988). Protein kinase C alterations in the fetal rat brain after global ischemia. *Journal of Biological Chemistry* **263**, 19282–19286.

LOUIS, J. C., MAGAL, E., BRIXI, A., STEIBERG, R., YAVIN, E. & VINCEDON, G. (1991) Down-regulation of protein kinase C in the adult rat brain following transient forebrain ischemia. *Brain Research* **541**, 171–174.

MAGUIRE, H. F., HOEFFER, J. P. & SIDDIQUI, A. (1991). HBV X protein alters the DNA binding specificity of CREB and ATF-2 by protein–protein interaction. *Science* **252**, 842–844.

MASLIAH, E., COLE, G. M., HANSEN, C. A., MALLERY, M., ALBRIGHT, T., TERRY, R. D. & SAITOH, T. (1991). PKC alterations is an early biochemical marker in Alzheimer disease. *Journal of Neuroscience* **11**, 2759–2767.

NEVINS, J. R. (1989). Mechanisms of viral-mediated trans-activation of transcription. *Advances in Virus Research* **37**, 35–83.

NEVINS, J. R. (1991). Transcriptional activation by viral regulatory proteins. *Trends in Biochemical Sciences* **16**, 435–439.

NIGG, E. A. (1990). Mechanisms of signal transduction to the cell nucleus. *Advances in Cancer Research* **55**, 271–310.

NISHIZUKA, Y. (1986*a*). Studies and perspectives of protein kinase C. *Science* **233**, 305–312.

NISHIZUKA, Y. (1986*b*). Perspectives on the role of protein kinase C in stimulus–response coupling. *Journal of National Cancer Institute* **76**, 363–370.

NISHIZUKA, Y. (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature, London* **334**, 661–665.

NISHIZUKA, Y. (1992). Intercellular signalling by hydrolysis of phospholipids and activation of PKC. *Science* **258**, 607–614.

NORRBY, E. & OXMAN, M N. (1990). Measles virus. In *Virology*, 2nd edn., pp. 1013–1044. Edited by B. N. Fields & D. M Knipe. New York: Raven Press.

OLDSTONE, M. B. A., HOLMSTOEN, J. & WELSH, R. M., JR (1977). Alterations of acetylcholine enzymes in neuroblastoma cells persistently infected with lymphocytic choriomeningitis virus. *Journal of Cellular Physiology* **91**, 459–472.

ONO, Y., FUJI, T., OGITA, K., KIKKAWA, U., IGARASHI, K. & NISHIZUKA, Y. (1988). The structure, expression and properties of additional members of the protein kinase C family. *Journal of Biological Chemistry* **263**, 6927–6932.

PAYNE, F. E., BAUBLIS, J. V. & ITABASHI, H. H. (1969). Isolation of measles virus from cell cultures of brain from a patient with subacute sclerosing panencephalitis. *New England Journal of Medicine* **281**, 585–590.

RAGER-ZISMAN, B., EGAN, J. E., KRESS, Y. & BLOOM, B. R. (1984). Isolation of cold-sensitive mutants of measles virus from persistently infected murine neuroblastoma cells. *Journal of Virology* **51**, 845–855.

RODDICK, V. L., KREBS, C. R., KUCERA, L. S., DANIEL, L. W. & WAITE, M. (1988). Phospholipid-sensitive, Ca^{2+}-dependent protein kinase activity in rat embryo fibroblasts transformed by herpes simplex virus type 2. *Oncology* **45**, 197–201.

SCHNEIDER-SCHAULIES, S., LIEBERT, U. G., RAGER-ZISMAN, B., SEGEV, Y., WOLFSON, M. & TER MEULEN, V. (1992). Antibody dependent transcriptional regulation of measles virus in persistently infected neuronal cells. *Journal of Virology* **66**, 5534–5541.

SCHWARTZ, J. H. & GREENBERG, S. M. (1987). Molecular mechanisms for memory: second-messenger induced modifications of protein kinases in nerve cells. *Annual Review of Neurosciences* **10**, 459–476.

SEKIGUCHI, K., TSUKUDA, M., OGITA, K., KIKKAWA, U. & NISHIZUKA, Y. (1987) Three distinct forms of rat brain protein kinase C: differential response to unsaturated fatty acids. *Biochemical and Biophysical Research Communications* **145**, 797–802.

SHIROKI, K., YAMAKAWA, A., SHIBATA, M., TAKENAWA, T., SUGANO, S. & NOMOTO, A. (1992). Adenovirus E1A proteins stimulate inositol phospholipid metabolism in PC12 cells. *Journal of Virology* **66**, 6093–6098.

SIMPSON, J. A., CHOW, J. C., BAKER, J., AVADALOVIC, N., YUAN, S., AU, D., CO, M. S., VASQUEZ, M., BRITT, W. J. & COELINGH, K. L. (1993). Neutralizing monoclonal antibodies that distinguish three antigenic sites on human cytomegalovirus glycoprotein H have conformationally distinct binding sites. *Journal of Virology* **67**, 489–496.

SUZUKI, Y., OHSUGI, K. & ONO, Y. (1992). EBV increases phosphoinositide kinase activities in human B cells. *Journal of Immunology* **149**, 207–213.

TAMURA, T., HADWIGER-FANGMEIER, A., SIMON, E., SMOLA, U., GESCHWILL, H., SCHUTZ, B., TROULIARIS, S., BOSCHEK, B., NIEMANN, H. & BAUER, H. (1991). Transforming mechanism of the feline sarcoma virus encoded v-fms oncogene product. *Behring Institut Mitteilungen* **89**, 93–99.

TAN, T. H., JIA, R. & ROEDER, R. G. (1989). Utilization of signal transduction pathway by the human T-cell leukemia virus type I transcriptional activator tax. *Journal of Virology* **63**, 3761–3768.

TAS, P. W. & KOSCHEL, K. (1991). Loss of the endothelin signal pathway in C6 rat glioma cells persistently infected with measles virus. *Proceedings of the National Academy of Sciences, U.S.A.* **88**, 6736–6739.

THOMAS, T. P., GOPALAKRISHNA, R. & ANDERSON, W. B. (1987). Hormone and tumor promoter-induced activation of membrane association of protein kinase C in intact cells. *Methods in Enzymology* **141**, 399–411.

VANIONPÄÄ, R. T., HYYPIÄ, T. & ACKERMAN, K. E. (1991). Early signal transduction in measles virus-infected lymphocytes is unaltered, but second messengers activate virus replication. *Journal of Virology* **65**, 6743–6748.

WEINEMANN-DORSCH, C. & KOSCHEL, K. (1989). Coupling of viral membrane proteins to phosphatidylinositide signalling system. *FEBS Letters* **247**, 185–188.

WIELOCH, T., CARDELL, M., BINGREN, H., ZIVIN, J. & SAITOH, T. (1991). Changes in the activity of protein kinase C and the differential subcellular redistribution of its isozymes in the rat striatum during and following transient forebrain ischemia. *Journal of Neurochemistry* **56**, 1227–1235.

WOLFSON, M., MCPHAIL, L. & SNYDERMAN, R. (1985). Phorbol myristate acetate mediates redistribution of protein kinase C in human neutrophils: potential role in the activation of respiratory burst enzyme. *Journal of Immunology* **135**, 2057–2062.

WOLFSON, M., KATORZA, A., SEGAL, S. & RAGER-ZISMAN, B. (1989). Effect of persistent measles virus infection on protein kinase C activity and c-*fos* protooncogene expression in neuroblastoma cells. *FEBS Letters* **250**, 367–370.

WOLFSON, M., GOPAS, J., KATORZA, A., UDEM, S. A., SEGAL, S. & RAGER-ZISMAN, B. (1991). Regulatory effects of persistent measles

virus infection on tumorigenicity and protooncogene expression in neuroblastoma cells. *Cancer Detection and Prevention* **15**, 171–176.

WU, J. Y., ZHOU, Z.-Y., JUDD, A., CARTRIDGE, C. A. & ROBINSON, W. S. (1990). The hepatitis B virus-encoded transcriptional transactivator hbx appears to be a novel protein serine/threonine kinase. *Cell* **63**, 687–695.

YOAKIM, M., HAU, W., LIU, Y., CARPENTER, C. L., KAPELLER, R. & SCHAFFHAUSEN, B. S. (1992). Interactions of polyomavirus middle T with the SH2 domains of the pp85 subunit of phosphatidylinositol-3-kinase. *Journal of Virology* **66**, 5485–5491.

(Received 1 September 1993; Accepted 12 November 1993)