

Regulation of the Initiation of Coronavirus JHM Infection in Primary Oligodendrocytes and L-2 Fibroblasts

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Upon maturation, primary rat oligodendrocytes become resistant to coronavirus JHM (JHMV) infection at an early stage. Involvement of cAMP-dependent protein kinase (PK) in the regulation of oligodendrocyte differentiation has been established (S. Beushausen *et al.* (1987). *J. Virol.* 61, 3795–3803). An inducer which accelerates maturation, dibutyl cyclic AMP (dbcAMP) also upregulates the expression of the regulatory subunit, R1 of PK1. Since (i) early block preventing infection of mature oligodendrocytes can be bypassed when transfection with genomic RNA is used and (ii) inhibitors of PKs counteract the dbcAMP effect, so as to alleviate the inhibition of JHMV, enhanced expression of R1 appeared to be connected with virus restriction. This idea was confirmed following upregulation of the R1 gene in fully permissive L-2 cells. There was a connection between an effect due to R1 and dephosphorylation of the nucleocapsid protein N by an endosomal phosphoprotein phosphatase (PPPase) having the properties of types 1 or 2A enzyme which occurs during penetration of inoculum virions. An inhibition *in vitro* (cell free) of N dephosphorylation by R1 together with evidence that *in vivo* (cell culture) overexpression of R1 inhibited the endosomal PPPase as well as replication of JHMV supports the hypothesis that uncoating of the JHMV inoculum occurs after dephosphorylation, a step obligatory for dissociation of the N protein from the genome. Thus inhibition by R prevents uncoating and thereby interferes with the commencement of replication. These observations intimate the existence of a novel mechanism controlling a virus infection of specific cell target(s) undergoing a process of differentiation and maturation in the central nervous system. © 1996 Academic Press, Inc.

INTRODUCTION

The appearance and type of neurological disease apparent in the central nervous system of rats inoculated with the neurotropic coronavirus JHM (JHMV) is related to the postpartum age at challenge. Onset of resistance to a demyelinating form of white matter disease occurs around the time that CNS myelination is at the stage of completion and may be due to the paucity of undifferentiated progenitor cells of the oligodendrocytic lineage (Sorensen *et al.*, 1980). This presumption is in line with data from *in vitro* studies revealing that JHMV replication is arrested at an early stage when explanted oligodendrocytes are allowed to mature slowly or are driven into rapid differentiation by modulation of the adenylate cyclase metabolic pathway (Beushausen and Dales, 1985; Beushausen *et al.*, 1987; Wilson *et al.*, 1986). It is known that infection of neural cells by other agents can also be influenced, either positively or negatively, by elevation of intracellular cAMP. In the case of measles, infection of neuroblastoma cells is suppressed (Miller and Carrigan,

1982; Robbins and Rapp, 1980), but neural cell infections with rubella virus (Van Alstyne and Paty, 1983) and polyoma virus (Baru and Manor, 1988) are enhanced. In the above systems the cAMP effect has not been explained, although previous evidence (Beushausen *et al.*, 1987) has suggested that the regulatory (R) subunit of cAMP-dependent protein kinase (PK) may somehow be involved.

With respect to gene expression as it may be connected to differentiation, elevation of intracellular cAMP is believed to increase amounts of the cAMP-dependent PKs of type 1 (PK1) and 2 (PK2) (Beushausen *et al.*, 1985; Flockhart and Corbin, 1982; Scott, 1991; Rubin and Rosen, 1975). However, when Wistar Furth (WF) primary oligodendrocytes, from which the PK1 isoform is absent, are induced to differentiate by application of dbcAMP, R1 is massively induced without increasing PK2 activity (Beushausen *et al.*, 1987). While the assumed function of R as a component of the holoenzyme is to form a reversible complex with the catalytic (C) subunit, thereby controlling phosphorylating activity, the R2 of PK2 was shown to exert a second property, as an inhibitor of some phosphoprotein phosphatase(s) (PPPase) (Jurgensen *et al.*, 1985; Khatra *et al.*, 1985; Shenolikar, 1994; Srivastava *et al.*, 1988).

Primary oligodendrocytes are susceptible to JHMV at a distinct stage during their differentiation, between the

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period they are in a highly proliferative O-2A progenitor phase and the time they become fully mature oligodendrocytes (Pasick and Dales, 1991). The block in virus replication found with oligodendrocytes at an early stage in the viral cycle, before onset of transcription but after adsorption, does not occur in fully permissive L-2 fibroblasts, which served as the controls throughout the present investigation. Previous studies revealed that during virus assembly the nascent N protein of JHMV becomes phosphorylated on serine residues, which is the only posttranslational modification to occur (Cheley and Anderson, 1981; Stohlman *et al.*, 1983; Wilbur *et al.*, 1986). Conversely, during virus disassembly and uncoating, N undergoes a molecular weight reduction from 56 to 50 kDa, perhaps due to specific dephosphorylation of N by an endosome-associated PPPase (Mohandas and Dales, 1991), evidently an essential step for initiation of the JHMV replication cycle. This study was concerned with regulation over virus infections by host cells from the central nervous system (CNS), exemplified by interactions between JHMV and differentiating oligodendrocytes.

MATERIALS AND METHODS

Culture of L cells, preparations of virus, and assays

Details concerning L-2 cell cultures, preparation of JHMV stocks, methods of virus infection, and plaque assays were as previously described (Beushausen and Dales, 1985).

Treatment of cells with protein kinase and phosphatase inhibitors

Primary WF cultures enriched over 90% with respect to oligodendrocytes were established from cerebral cortices of neonatal rats as in Beushausen and Dales (1985). Multiwell dishes containing 5×10^5 cells per cm^2 were maintained for 3 days at 37° in modified basal Eagle's medium plus 10% fetal calf serum (BME10) or in BME10 supplemented with dibutyl cAMP (dbcAMP) (Sigma) and/or with the protein kinase inhibitors H-8 and HA1004 (Seikagaku Kogyo Co.) (Hidaka *et al.*, 1984). For the subsequent inoculation, JHMV was used at a multiplicity of infection (m.o.i.) of 1 plaque forming unit (PFU) per cell. Virus was adsorbed at 37° for 1 hr, cells washed $3 \times$ with 1 ml phosphate-buffered saline (PBS), pH 7.2, and incubated at 37° in BME10, with supplements where appropriate. Extracellular JHMV was quantitated in the medium, which was changed daily, as previously described (Lucas *et al.*, 1977).

Confluent L-2 cells in 35-mm dishes were either treated for 30 min before JHMV inoculation at 4° for 60 min or 2 hr after inoculation with various concentrations of calyculin A (Research Biochemical Inc., MA). After in-

cubation for 10 hr at 37° , the supernatant containing progeny virus was titrated on L-2 cells.

The effect of calyculin A on the processing of N within the virus inoculum was determined using confluent L-2 cultures pretreated 30 min prior to JHMV absorption at 4° for 60 min using various concentrations of the drug. After 1 hr incubation at 37° , following absorption, the cytoplasmic fraction was processed for immunoblotting with anti-N antibodies.

Preparation of purified N protein

Isolation of unlabeled and ^{32}P -labeled N protein from infected L-2 mouse fibroblasts conformed to procedures previously described by Mohandas and Dales (1991).

Photoaffinity labeling of R subunit in cell extracts

After treatment with H-8 and/or dbcAMP, individual cultures of 2×10^6 oligodendrocytes per 35-mm plate were rinsed twice with ice-cold PBS, the cells were released by scraping and collected into pellets, then resuspended at 0° in 500 μl buffer containing 10 mM Tris-HCl at pH 7.4, 1 mM ethylene glycol-bis-(*a*-aminoethyl ether)*N,N,N',N'*-tetra acetic acid (EGTA), and 50 μg PMSF/ml. Thorough cell disruption was effected by squeezing the cell suspension repeatedly ($\times 10$) through a No. 30 gauge needle of a 1-ml hypodermic syringe. The homogenates obtained were frozen and thawed twice, sedimented at 15,000g for 10 min using an Eppendorf microfuge at 4° . The cytosol fraction was affinity-labeled with 8-azido- ^{32}P cAMP (61.4 Ci/nmol; ICN Pharmaceutical Inc., Irvine, CA) as described (Budzilowicz *et al.*, 1985). Autoradiograms were prepared after separating the proteins in 10% SDS-PAGE gels (Laemmli *et al.*, 1970).

Preparation of catalytic and regulatory subunits from the holoenzyme and PK1 assay

Affinity columns were employed to separate the R and C subunits, as previously outlined (Beavo *et al.*, 1974). Briefly, dehydrated preparations of rabbit PK1 (Sigma) were reconstituted in distilled water to give a final concentration of 5 mg/ml. The material was introduced into columns of 5 ml packed volume containing N6-2C-cAMP sepharose preequilibrated with buffer containing 5 mM 2-[*N*-Morpholino] ethane sulfonic acid (MES), 9 mM NaCl, 15 mM B-ME, pH 6.5, termed MES buffer. The columns were then washed at 20° with 2 M NaCl in MES buffer, followed by washes with MES buffer alone. The C subunit was eluted in the void volume and concentrated on Amicon YM-10 membranes as outlined below. To elute R1 from the columns, 3 vol of 30 mM cAMP in MES buffer were added during incubation for 1 hr at 30° . The eluate, containing R1, was concentrated by Amicon YM-10 membrane and dialyzed at 4° for 2 days with 4×1 liter

changes of MES buffer. Protein determinations were performed using a Bio-Rad kit with BSA as standard. Another preparation of R1, employed as a control, was obtained from skeletal muscle of Wistar rats (a gift from B. D. Sanwal, Department of Biochemistry, UWO).

Samples of isolated C and R subunits were analyzed for presence of residual PK activity, using histone IIA (Sigma) as substrate by the procedure of Corbin *et al.* (1975); briefly, 84 mM KH_2PO_4 , pH 6.8, 28 mM Mg acetate, 1.75 mM ATP, histone IIA at 50 mg/ml, 10 μM cAMP, and H_2O was mixed with an equal volume of 50 μCi [^{32}P]ATP (Amersham; sp act 75,000 Ci/mmol). Then, 50- μl aliquots of the above mixture were added to approximately 3 μg PK protein in glass tubes, incubated for 15 min at 30°, then "spotted" onto GF/C glass fiber filters (Millipore). The reaction was terminated by immersing the filters in 10% ice-cold trichloroacetic acid (TCA) and extraction achieved by agitation for 1 hr on a shaker apparatus. Following three washes with TCA the filters were rinsed twice with 100% ethanol and dried at -20°. The [^{32}P]dpm were determined in a scintillation counter.

Isolation of the endosomal fraction and PPPase assay

The endosomal/prelysosomal fractions were isolated from disrupted L-2 cells by use of a dual percoll gradient procedure and characterized with respect to the enzyme activities as previously described (Mohandas and Dales, 1991). Enzyme assays included those for acid phosphatase and a "neutral" PPPase with specificity for N as a substrate. Where appropriate the additions were 1 mM 3'5' cAMP and 3 μg of either PK holoenzyme or regulatory (R) or catalytic (C) subunits. The mixtures were preincubated for 30 min at 30°. The ^{32}P -labeled or unlabeled N was then added and the reaction continued at 30° for a further 90 min. The reactions were terminated by addition of 1% BSA and 25% TCA. The released [^{32}P]dpm were determined as described previously (Mohandas and Dales, 1991).

Immunoblotting to demonstrate modulation of the N antigen

L-2 monolayers were inoculated with JHMV at an m.o.i. of about 50 PFU/cell. After adsorption at 4° for 60 min unattached virus was removed by thorough washing with cold BME10. Penetration was initiated by incubation in fresh BME10 at 37°. Cultures were sampled at intervals for 120 min, commencing with a sample taken at the end of adsorption, designated t0. At the time of sampling, monolayers were washed twice with cold PBS, the cells were scraped off and placed into 500 μl of ice cold buffer, containing 10 mM Tris-HCl at pH 7.4, 1 mM EGTA, 50 $\mu\text{g}/\text{ml}$ PMSF, and 2 $\mu\text{g}/\text{ml}$ leupeptin (TPEL). A cytosol fraction was derived from infected cells as above under "Photoaffinity labeling" except that cells were squeezed

through a syringe needle only 5 rather than 10 cycles and sedimented for only 5 min to remove the larger particulate. Membranes in the cytosol fraction were disrupted by means of NP-40 added to a final concentration of 0.5%, and brief agitation on a vortex mixer followed by 15 min at 4° on a rotary mixer. Proteins in the fractions were separated by SDS-PAGE then transferred to nitrocellulose filters for Western blotting (Towbin *et al.*, 1979); N antigen was identified with monoclonal (MAb) as in Beushausen *et al.* (1987).

Specificity of binding between N protein and cDNA

Specific binding of N with a cDNA copy of the gene was tested using the overlay method of Robbins *et al.* (1986). After immobilizing N in nitrocellulose, the blots were rinsed for 30 min with standard binding buffer (SBB) consisting of 0.05 M NaCl, 1 mM disodium EDTA, 10 mM Tris-HCl at pH 7, 0.02% BSA, 0.02% Ficoll, and 0.02% polyvinyl pyrrolidone (PVP-360), incubated for 90 min at room temperature with ^{32}P -labeled plasmid cDNA probe plus calf thymus DNA, washed 6 \times or more with SSB over a 60-min period, then air-dried. Autoradiograms were obtained by exposing X-ray film in the presence of an intensifying screen. The cDNA was constructed from plasmid G344 recombined with 1800-bp cDNA from the N gene of CV A59 (Budzilowicz *et al.*, 1985), kindly provided by S. Weiss (University of Pennsylvania, Philadelphia, PA). Labeling of plasmid DNA with [^{32}P] as described previously (Feinberg and Vogelstein, 1983) and purification by means of a spin column according to Maniatis *et al.*, (1982).

Transfections with RNA of JHMV

The genomic RNA was derived from JHMV propagated on confluent L-2 monolayers in 150-cm² flasks. After inoculation using an m.o.i. of 1 for 60 min at ~20°, growth medium was added and incubation carried out at 37° until the monolayer became about 95% virus-induced syncytium. Virus released into the supernatant was collected by centrifugation and used for preparing genomic RNA as described previously (Banner *et al.*, 1990). For transfection, 5 μg of RNA was combined with 15 μl of Lipofectin (Life Technologies) and 200 ml Opti MEM (Life Technologies). After gently mixing the reagents, the RNA was added to purified primary oligodendrocytes, prepared as described by Pasick and Dales (1991) and seeded at from 2.5 to 5.0 $\times 10^5$ cells per cm². Experimental cultures were treated with 3 mM dbcAMP for 24 hr. As an additional control, L-2 cultures were transfected in the same manner. The cells were exposed for 12 hr to the RNA transfection mixture and then growth medium was added and supernatants fluid monitored at 48 and 72 hr for the virus progeny released.

TABLE 1
Transfection of Mature, Primary Oligodendrocytes with RNA of JHMV

Treatment of cells	Titer (PFU/ml) ^a			
	48 ^b		72	
	Infection		Transfection	
Untreated	1,300 ± 125	4,680 ± 250	30 ± 5	50 ± 7
1 mM dbcAMP postinfection	3,340 ± 208	10,000 ± 270	ND	ND
1 mM dbcAMP before infection	0	0	10 ± 5	30 ± 14
L-2 cells ^c			38,000 ± 900	

^a Mean of three experiments with standard deviation. ND, not done.

^b Hours postinfection.

^c Titer with L-2 cells > 10⁷ PFU/ml within 24 hr. Oligodendrocyte cultures were treated with 1 mM dbcAMP either 24 hr before addition of 2 PFU/cell or viral RNA or 48 hr after inoculation. Supernatant virus was titrated on L-2 cells.

Transfection of L-2 cells with the R1 gene

Multiple copies of the DNA of the rat R1 gene, which had been cloned into the vector HL-REV (Corell *et al.*, 1989), were used for transfecting semiconfluent cultures of L-2 cells, as described above. After incubation with the plasmid for 72 hr, the cells were released and subcultured with growth medium containing 500 µg G418 (Life Technologies). Stable individual transfectants (clones) were selected, grown into cultures for assaying the quantity of R1 expressed by each clone, employing anti-R1 antibodies for immunoblotting. As a control the wild-type cells were transfected with the plasmid from which the R1 gene was absent. The high producer clones 1.2 and 1.1 were amplified and challenged with JHMV to determine their infectability. Efficiency of virus replication was determined by the PFU present in the supernatant at 10 hr PI. Changes in MW of N during the early stages of virus–cell interactions were monitored by precooling (4°) of host cells and then inoculating them for 60 min at 4° at an m.o.i. of 10 PFU/cell. The unattached inoculum was washed away, warm nutrient medium added, then incubation carried out for 1 hr. Samples were taken and processed for immunoblotting.

RESULTS

Do glial cells exert control over the virus infection at an early stage?

JHMV has a tropism for explanted rat oligodendrocytes, although infectability is confined to a discrete stage in the differentiation process, sometime between the mitotically active progenitors (O-2A cells) and terminally differentiated oligodendrocytes (Pasick and Dales, 1991). Permissiveness is apparently determined by intracellular factor(s) acting after adsorption, which itself is not affected when the nonpermissive cells are challenged (Beushausen *et al.*, 1987). To elaborate on our

previous results demonstrating that the restriction occurs at an early step, prior to onset of transcription, isolated JHMV genomic RNA was transfected into primary telencephalic cultures established as previously described (Pasick and Dales, 1991) from neonatal Wistar Furth pups. After 8–10 days in culture, the more loosely adherent O-2A lineage cells, which can be released by sharply tapping the culture flask, were plated into fresh growth medium and then treated with 1 mM dbcAMP either 24 hr before inoculation or 2 days following inoculation with JHMV. As shown in Table 1, pretreatment of cultures to induce oligodendrocyte maturation completely blocked replication, whereas posttreatment had no effect as compared with untreated cells, confirming our previous data (Beushausen *et al.*, 1987). To circumvent events related to penetration and uncoating connected with inoculum virions, virus genomes were introduced by transfection. The genomic RNA was isolated, combined with Lipofectin (Gibco), and added to mature oligodendrocyte cultures made nonpermissive by pretreatment with dbcAMP. Such transfected cells were infectable and were able to replicate JHMV as compared with controls inoculated with virions (Table 1), demonstrating as previously suggested (Beushausen *et al.*, 1987) that restriction does, indeed, pertain to an early event. Inevitably, the efficiency of infecting with isolated RNA was much lower than with virions, less than 1%, as evident with both L-2 cells and primary oligodendrocytes (Table 1). The relatively small but consistent disparity between yields from untreated oligodendrocytes and those treated postinoculation with dbcAMP, reported previously (Pasick and Dales, 1991), remains unexplained.

Effect of PK inhibitors on JHMV replication in oligodendrocytes treated with inducers of differentiation

The previously made connection relating modulation of the cAMP dependent-PK(s) caused by dbcAMP with

oligodendrocyte differentiation and inhibition of coronavirus expression (Beushausen and Dales, 1985; Beushausen *et al.*, 1987; Wilson *et al.*, 1986) led us to question whether PK inhibitors can counteract the dbcAMP effect and alleviate the restriction on virus replication. The answer was sought by pretreating WF oligodendrocytes with two isoquinoline sulfonamide derivatives, H-8 and HA1004 (Hidaka *et al.*, 1984), which are able to inactivate cAMP-dependent PKs by binding to the C subunits.

As a confirmation of our previous findings and a control for the inhibition of infection due to differentiation, cultures of oligodendrocytes were pretreated with 1 mM dbcAMP before inoculation. It is evident in Fig. 1A that due to pretreatment, JHMV titer was reduced by 80% within 24 hr and over 90% in 48 hr. Application of 3 mM dbcAMP caused almost complete virus suppression in 48 hr (Fig. 1B). By contrast, after simultaneous exposure of oligodendrocytes to 1 mM dbcAMP and 25 μ M H-8 the virus was replicated by 48 hr at the same rate as in

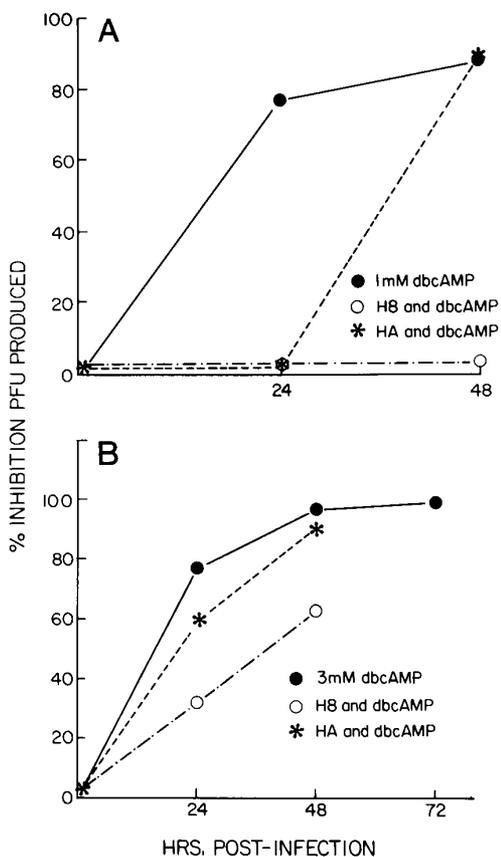


FIG. 1. Effect of protein kinase inhibitors on JHMV replication in cultures of glial cells induced to differentiate with dbcAMP. (A) cultures of glial cells at 2.5×10^5 cells per cm^2 were pretreated for 3 days with 1 mM dbcAMP, 1 mM dbcAMP + 25 μ M H-8, or +50 μ M HA1004 prior to inoculation with JHMV, m.o.i. 1 PFU/cell. The culture fluid was assayed for infectivity at the times indicated. Data are expressed as percentage of virus produced in the controls, usually 5×10^2 – 10^3 PFU/ml. (B) Experimental conditions as in (A) except that dbcAMP was added at 3 mM.

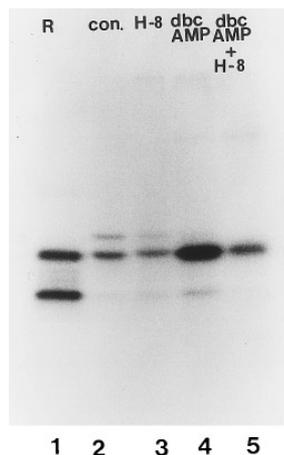


FIG. 2. Suppression by the PK inhibitor H-8 of R1 induction in oligodendrocytes treated with dbcAMP. About 2×10^6 oligodendrocytes per 35-mm plate were cultured in BME10 and for 3 days in the presence of either 25 mM H-8 or 1.5 mM dbcAMP or in the presence of both compounds. For dual treatment H-8 was added 1 day prior to dbcAMP. The cells were harvested and prepared for photoaffinity labeling with 8-azido- $[^{32}\text{P}]\text{cAMP}$ as described under Materials and Methods. Lane 1, commercially obtained (Sigma) 4 μ g cAMP-dependent PK1; lane 2, untreated cells; lane 3, grown in presence of H-8; lane 4, exposed to presence of dbcAMP; lane 5, treated with H-8 + dbcAMP. The lower MW R1 band evident with the purchased PK1, in lane 1, is presumably a degradation product.

the untreated controls (Fig. 1A). After similar treatment with 3 mM dbcAMP the addition of 25 μ M H-8 was able to relieve the inhibition only partially, as shown in Fig. 1B. The other inhibitor of PK, HA1004, when added at 50 μ M together with 1 mM dbcAMP reversed the dbcAMP effect during the initial 24 hr of the experiment (Fig. 1A). However, simultaneous treatment with 3 mM dbcAMP and HA1004 affected the inhibition of JHMV replication very little (Fig. 1B).

To ascertain independently whether reversal of the inhibitory effect due to dbcAMP on JHMV replication by H-8 involves the PK's, oligodendrocytes were treated with either the inducer or the inhibitor or with both compounds. It is evident from lanes 2 and 4 of the autoradiogram in Fig. 2 that, as predicted from Beushausen *et al.* (1987), dbcAMP induced the synthesis of large amounts of R1. Treatment with H-8 alone did not appreciably decrease the quantity of R1 present in controls (lane 2 vs 3). When both H-8 and dbcAMP were added R1 induction was abrogated (lane 5), demonstrating that in oligodendrocytes presence of low amounts of R1 appears to be correlated with permissiveness of JHMV and vice versa.

Taken together, the above findings offer evidence supporting the hypothesis that in differentiating oligodendrocytes modulation of PK(s) is connected with inhibition of JHMV expression at an early stage in the replication cycle, which can be bypassed upon transfection with genomic RNA.

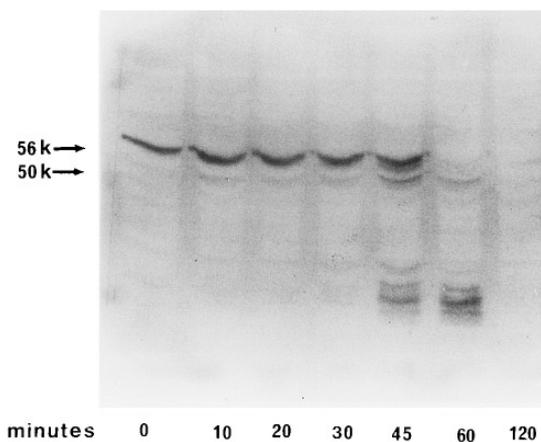


FIG. 3. Molecular Weight modulation of JHMV N protein during initial 120 min of JHMV penetration into L-2 cells. Infection of L-2 cells, preparation of samples, and immunoblotting are described under Materials and Methods.

Evidence that MW modulation of N is related to the state of phosphorylation

A relationship between the 56-kDa N present within virions and a related 50-kDa protein was previously established using 2-D tryptic peptide mapping (Cheley and Anderson, 1981) and anti-N monoclonal antibodies (MAb) (Coulter-Mackie *et al.*, 1985). Upon its synthesis, N becomes phosphorylated at serine residues (Stohlman *et al.*, 1983; Wilbur *et al.*, 1986). To determine whether MW modulation of N following its synthesis is due to phosphorylation, the nutrient medium of infected cultures was varied in the phosphate concentration. The 50-kDa species of N was reduced in proportion to increasing phosphate concentration, being absent when phosphate was at 5 mM (data not shown), demonstrating a regulation of the relative amounts of 50- and 56-kDa N protein in infected cells by phosphate concentration in the growth medium. The 56-kDa MW of N is the predominant form within mature JHMV particles.

From previous evidence suggesting that N is modified during JHMV penetration, MW of the inoculum N protein was ascertained by immunoblotting at intervals for the first 120 min. The results shown in Fig. 3 indicated that between t₀ and 30 min after warming to 37°, N existed predominantly as the 56-kDa species. At 45 min, there was some processing of the 56-kDa antigen evident as several lower MW bands, including one at 50 kDa and some below 40 kDa. At 60 min, the 56-kDa material was completely absent and only a faint 50-kDa band remained while the remainder of N was at lower MW. By 120 min, the N antigen had disappeared altogether. These observations established that during the time-course of JHMV penetration, N was reduced from 56 to 50 kDa and subsequently hydrolyzed to lower MW components.

The above results led us to extend our enquiry into changes in phosphorylation of N during virus penetration, specifically whether dephosphorylation is required for initiating JHMV replication. For this purpose, we employed calyculin A, a reversible inhibitor of PPPases (Ishihara *et al.*, 1989). L-2 cells were treated with the drug for 30 min either before inoculation or 2 hr after initiating penetration. As a measure of JHMV replication, titers of virus released by 10 hr postinfection into the medium were determined. As evident from Fig. 4, when 100 nM calyculin A was applied in advance of inoculation, the JHMV titer was reduced about 1.5 log₁₀ PFU/ml as compared with virus generated by cells treated with calyculin A commencing 2 hr postinfection. These data are consistent with the idea that dephosphorylation by a PPPase activity may be required to promote virus replication. To relate this evidence with any *in vivo* effect of calyculin A on phosphorylation of N, L-2 cells were treated with various concentrations of the inhibitor prior to inoculation as described for Fig. 4. Samples taken 1 hr postinoculation were subjected to immunoblotting employing anti-N antibodies. As evident from Fig. 5, conversion of N from the 56- to 50-kDa MW form was reduced by calyculin A in a concentration-dependent manner, as was to be anticipated if dephosphorylation of N was being affected. From these combined results, it was concluded that dephosphorylation of N is an early event during virus-cell interactions which may be essential for commencement of replication.

To connect more directly dephosphorylation of the 56-kDa form of N with an endosomal PPPase activity, *in vitro* assays were conducted. As shown in Fig. 6, after

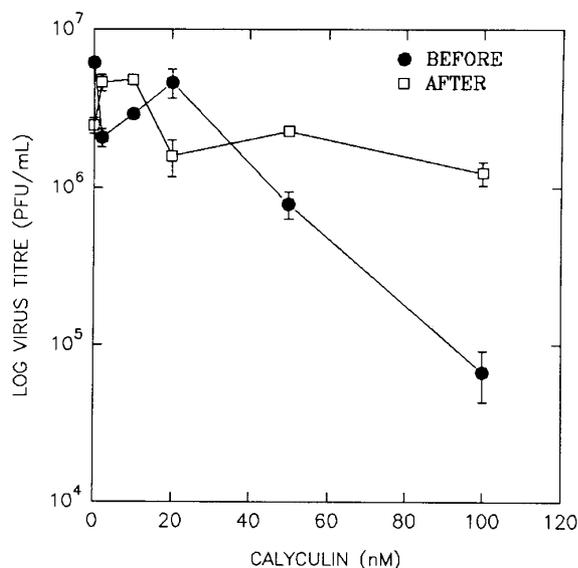


FIG. 4. Effect of calyculin A on JHMV replication. A range of calyculin A concentrations were added to the BME10 either 30 min prior to or 2 hr after virus inoculation. The PFU/ml in supernatant fluid were determined in samples taken 10 hr after inoculation. Pretreated, closed circles; treated after inoculation, open squares.

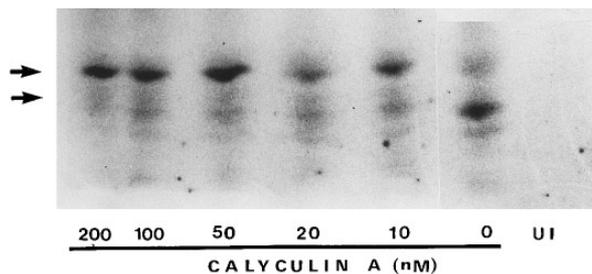


FIG. 5. Inhibition of N processing by calyculin A. L-2 cells were pretreated for 30 min with calyculin A prior to inoculation. Two hours after addition of virus, cell samples were taken for immunoblotting employing anti-N antibodies.

adding 56-kDa N to the endosomal fraction, the amount of this material converted into a lower MW form was increased according to the quantity of endosomes used. When ^{32}P -labeled N was reacted with endosomes in the same manner, about one-half of the protein bound [^{32}P] was solubilized (Mohandas and Dales, 1991). Evidently, conditions used for the *in vitro* assays were appropriate for demonstrating that dephosphorylation of N brings about a shift down of the MW.

As a confirmatory approach for detecting the MW modulation of N due to the endosomal PPPase activity, we correlated the binding of a radioactive cDNA probe with the N antigen, bound to nitrocellulose as detected by immunoblotting. Samples were monitored following incubation with the endosomal fraction, as described above. The results presented in Fig. 7A indicated that the cDNA copied from N RNA could recognize both the 56- and 50-kDa forms of N in a nondiscriminating manner. However, a specific binding of the 56-kDa form of N to viral RNA has been noted (Baric *et al.*, 1988). The specificity of the endosomal PPPase activity for N as a substrate was checked by means of okadaic acid which, like caly-

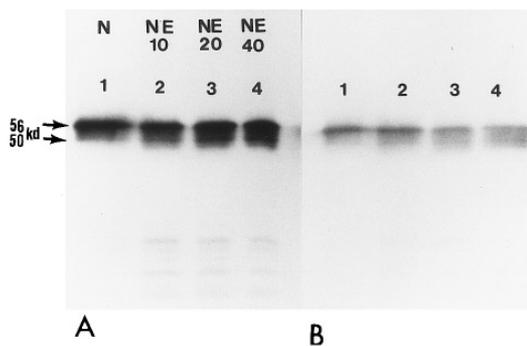


FIG. 6. Effect of endosomal fraction on the *in vitro* processing of N monitored by immunoblotting. Purified N was treated with varying amounts of the endosomal preparation and then monitored by immunoblotting. (A) 0.5 μg of purified N; (B) 1.0 μg of purified N. Lane 1, untreated N; lanes 2, 3, and 4, N incubated with, respectively, 10, 20, and 40 μl of the endosomal fraction. Duration of exposure to X-ray film was 48 hr in A and 24 hr in B.

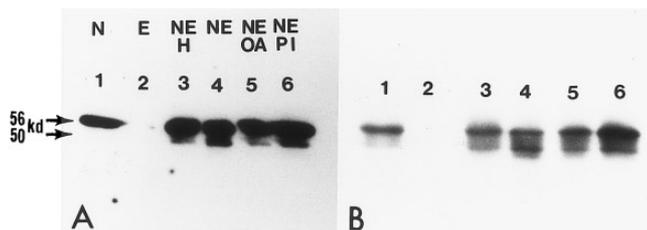


FIG. 7. Effect of a PPPase inhibitor on the processing of N in the presence of endosomes as revealed in (A) nucleic acid binding assays and in (B) by immunoblotting. Purified N protein was exposed to endosomes. [^{32}P] nucleic acid binding assay and Western blotting were performed as described under Materials and Methods. Lane 1, N enzyme absent; lane 2, E, endosomal fraction only; lane 3, NE/H in the presence of heat-inactivated endosomes; lane 4, NE, normal reaction with active endosomes; lane 5, NE/OA with endosomes and okadaic acid, following pretreatment for 30 min with okadaic acid; lane 6, NE/PI with endosomes and protease inhibitor, after pretreatment for 30 min with TPEL.

culin A, acting as a specific inhibitor of neutral PPPases, depressed the conversion of 56 to 50 kDa (lane 5 in Figs. 7A and 7B). Since the MW shift was unaffected in the presence of protease inhibitors in the reaction mixture, the observed change in MW of N must have been due to dephosphorylation, not proteolysis. From the above observations, it was concluded that, during the interaction of JHMV with host cells, the dephosphorylation of N by an endosomal PPPase most probably causes the 56- to 50-kDa MW conversion, as a step in the uncoating of inoculum genomes.

Inhibition of endosomal PPPase activity by the R subunit of cAMP-dependent PKs

We obtained evidence in this study that N is dephosphorylated during JHMV penetration into L-2 cells. Both previously and in the present study, it was demonstrated that an endosome-associated PPPase of L-2 and glial cells can specifically dephosphorylate N *in vitro* (Mohandas and Dales, 1991), indicating that this enzyme activity has a function during the interaction between JHMV and explanted oligodendrocytes. Since inducers of differentiation, among them cAMP, can regulate PK expression and suppress JHMV replication in oligodendrocytes at an early stage (Beushausen and Dales, 1985; Beushausen *et al.*, 1987), it was hypothesized that PK, or one of the holoenzyme components, can influence the endosomal PPPase. This idea is consistent with the well-recognized interplay between PKs and PPPases in metabolic pathways involving these enzymes (for reviews, see Cohen, 1989; Ingebritsen and Cohen, 1983; Shenolikar, 1994). When the dose-response effect of R1 was examined, it became evident that R1 inhibits the endosomal PPPase to almost the maximum extent at 3 μg . Increasing R1 up to 12 μg caused only very little additional inhibition of N dephosphorylation (data not shown).

TABLE 2

Dephosphorylation of N Protein by an Endosomal PPPase in the Presence of PK and Enzyme Subunits

Addition	Activity as % dephosphorylation/25 μ g endosomal ^a protein	% Inhibition ^b
None	36 (9)	0
PK ₁ holoenzyme	37 (4)	0
PK ₁ holoenzyme + cAMP	31 (5)	14 \pm 3.2
PK ₂ holoenzyme	37 (2)	0
PK ₂ holoenzyme + cAMP	28 (4)	22 \pm 2.6
R ₁ 3 μ g	24 (3)	33 \pm 2.7
R ₁ 3 μ g + cAMP	19 (9)	47 \pm 7.0
C ₁ 5 μ g + cAMP	42 (4)	0

^a Data from a representative experiment repeated the number of times shown in brackets. One unit of activity is defined as 1% of [³²P] released in 90 min at 30°.

^b Average values with standard error of the mean.

Effects of adding PK1, PK2, R1, or C subunits to the reaction mixture containing endosomal PPPase and ³²P-labeled N protein are summarized in Table 2. For making valid comparisons between various experiments as to the amount of [³²P] hydrolyzed, the data were calculated and expressed as percentage dephosphorylation per standard 25 μ g quantity of endosomal protein. The maximum [³²P] released from N was not above 50%, but usually 30–40% of the [³²P] residues were solubilized. It is evident that presence of either holoenzyme PK1 or PK2 had no inhibitory effect on the dephosphorylation reaction. Inclusion of cAMP along with the holoenzymes, which brings about PK dissociation into R and C subunits, resulted in a minor inhibition of [³²P] release. However, addition of the purified R1 subunit, whether in the absence or presence of cAMP, reduced PPPase more profoundly, but addition of the C subunit had no effect. These results imply that the R subunit can inhibit directly the endosomal PPPase. A graphic confirmation of the R1 effect was obtained by subjecting to autoradiography isotopically labeled N following SDS–PAGE in 10% gels. It is evident from Fig. 8 that N dephosphorylation, demonstrated in lane 2 was inhibited by the presence of R1, as evident from lane 3.

The species specificity of R1 was tested by comparing R1 from rabbit skeletal muscle with R1 isolated from skeletal muscle of Wistar Furth rats. At the standard concentration of 3 μ g per reaction mixture, the rabbit and rat subunits inhibited endosomal PPPase to the same extent (data not shown).

The consequence of overexpressing R1 on the MW of N and JHMV replication

The above evidence that R1 can inhibit endosomal-PPPase activity *in vitro* and may affect virus replication

in mature oligodendrocytes led us to test whether overexpression of R1 in a nondifferentiating, fully permissive continuous line of host cells can also influence the JHMV infectious process. For this purpose, plasmids encoding the rat R1 gene were transfected into L-2 cells. Stable transfectant clones were established and selected in the presence of G418 for comparison with normal L-2 cells for levels of R1 expression by means of immunoblotting employing anti-R1 antibodies, as illustrated in Fig. 9C. Influence of the amount of R1 expressed on virus replication was determined by titration of JHMV present in the supernatant at 8 hr PI. As shown in Fig. 9B, PFU/ml of JHMV produced by cells overexpressing R1 was about 1.5 log₁₀ lower than in untransfected L-2 cells. To examine the *in vivo* effect of R1 on modulation of N within inoculum virions, cells taken at 2 hr postinoculation were processed for immunoblotting with anti-N antibodies. As evident from Fig. 9A, N processing from 56 to 50 kDa was inhibited at an early stage of replication in the transfectant clones, with a greater restriction evident in the higher R1 expressing clone 1.2 (Fig. 9C). These findings are entirely consistent with above data from cell-free assays, which demonstrated that R1 can inhibit the dephosphorylation of N.

DISCUSSION

The multifactorial control over replication of coronaviruses in the CNS has been of continuing interest in this laboratory. One of the most challenging aspects in virus–cell interactions concerns the influence of differentiation on the infectability of oligodendrocytes by JHMV. When this cell type is driven toward differentiation by modulating the adenylate cyclase pathway, resistance becomes manifested at a stage subsequent to adsorption and internalization but antecedent to expression of the viral genome. Since the restriction in mature oligodendrocytes of JHMV could be circumvented by transfecting into them isolated genomic RNA, the arrest involves an early step of the replication cycle. Such resistance to infection appears to be connected with regulation of the cAMP-dependent PK, as evident following dbcAMP treatment which induces abundant synthesis of R1, despite absence of PK1 from these cells (Beushausen *et al.*, 1987). Involvement of PKs was extended by our current data

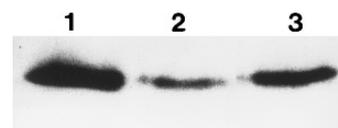


FIG. 8. Inhibition of N dephosphorylation by the endosomal PPPase in the presence of R1. In the autoradiogram the signal is due to ³²P-labeled N protein associated with N protein of JHMV. Lane 1, N in the absence of PPPase; lane 2, following reaction for 90 min at 30°; lane 3 as in lane 2 but in the presence of 3 μ g R1.

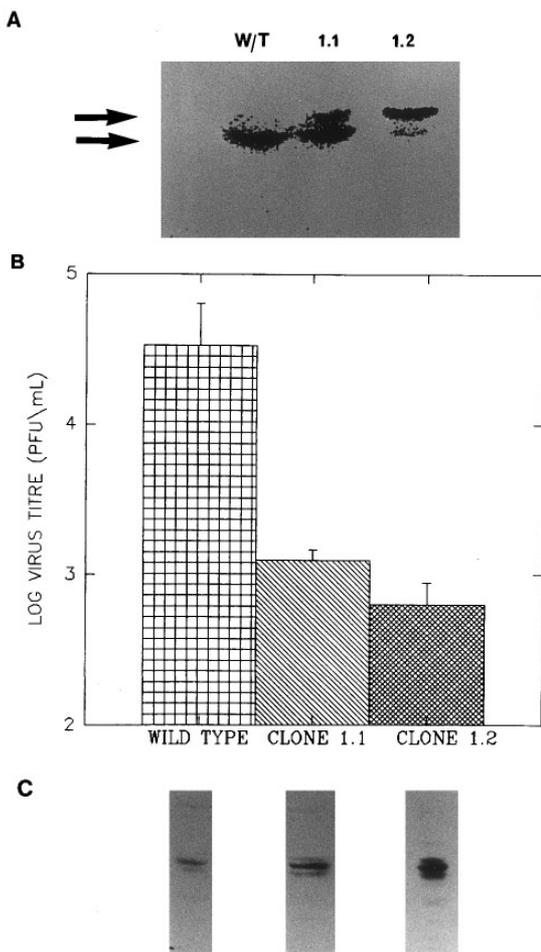


FIG. 9. Influence of overexpression of R1 on JHMV replication. Stable transfected clones of L-2 cells overexpressing the R1 subunit were identified. (A) Clones 1.1, 1.2 and normal L-2 cells were infected with JHMV. Two hours postinfection, samples were processed for immunoblotting with anti-N antibodies: lane 1, normal L cells; lane 2, clone 1.1; lane 3, clone 1.2. The location of 56- and 50-kDa N is indicated by the arrows. (B) Supernatant was collected 8 hr postinfection and the cells were assayed for PFU of virus produced. (C) Equivalent amount of cell material was processed for immunoblotting with anti-R1 antibodies, as above.

showing that application of specific PK inhibitors can counteract the dbcAMP effect so as to maintain the oligodendrocytes in a permissive state, depending on both the concentration of dbcAMP inducer used and inhibitor affinity for the PKs. While data from studies employing inhibitors do not provide unambiguous identification as to which among the various cellular PKs is pertinent to JHMV, our previous investigation (Beushausen *et al.*, 1987) indicated that neither the type C or cGMP-dependent PK's are involved in controlling the infection.

Data obtained from other studies, among them cell fusion analyses, demonstrated that other cellular factors apart from the presence of specific viral receptors are required for JHMV penetration (Asanaka and Lai, 1993; Flintoff and Van Dinter, 1989; Kooi *et al.*, 1988; van Dinter

and Flintoff, 1987; Yokomori *et al.*, 1993) and facilitation of the uncoating process (Asanaka and Lai, 1993; Beushausen *et al.*, 1987; Flintoff and van Dinter, 1989; Kooi *et al.*, 1988). Other evidence from several laboratories supports the view that MHVs, including JHMV, can penetrate by receptor-mediated endocytosis into vesicles possessing the structure of "coated pits" (David-Ferreira and Manakar, 1965; Flintoff and van Dinter, 1989; Krzystyniak and Dupuy, 1984; Mizzen *et al.*, 1985). The current study reveals that dephosphorylation of N from inoculum virions is most probably catalyzed by a PPPase associated with endosomes. Combined evidence from this study and a previous investigation (Mohandas and Dales, 1991) characterized the relevant PPPase as one which functions optimally at neutral pH and is sensitive to okadaic acid and calyculin A, two inhibitors of phosphatases type 1 and 2A. These observations led us to the hypothesis that JHMV uncoating may be initiated within early endosomes. The uncoating process whereby the RNA genome dissociates from the capsid N protein, follows dephosphorylation and subsequent breakdown of N. Presumably, the relevant endosomal PPPase is maximally active at neutrality, the pH milieu likely to prevail on the cytosolic face of the membrane, regardless of the pH optimum for virus-cell membrane fusion (Gallagher *et al.*, 1991; Kooi *et al.*, 1988). Latency of this PPPase, i.e., it becomes more active after solubilization of membranes, as demonstrated in Mohandas and Dales (1991), implies that in host cells the enzyme occurs in membranes at a site where it can function to dephosphorylate N as the N-RNA complex is released from inoculum virions. It has been suggested by Stohlman *et al.* (1983) that the conformation of N is drastically altered according to its state of phosphorylation. By catalyzing the removal of phosphorus residues, the endosomal PPPase may effect the requisite conformation change to bring about separation of N capsid from the genome, antecedent to hydrolysis of this protein. Thus an inhibition of the endosomal PPPase becomes directly relevant to interference with the uncoating process of JHMV.

Based on the assumption that dephosphorylation of N and release of the viral genome are connected, it is quite possible that JHMV uncoating is depressed in mature oligodendrocytes because R1 synthesis is upregulated, as demonstrated by Beushausen *et al.* (1987). This concept has support from our *in vitro* experiments and those of Jurgensen *et al.* (1985), Khatra *et al.* (1985), and Srivastava *et al.* (1988), who demonstrated that R can inhibit PPPase, including the endosomal enzyme. One should not, however, overlook other metabolic derangements effected by R, including a reduction in the basal level of cAMP-responsive transcription (Mellon *et al.*, 1989; Shenolikar, 1994); however, in the present context, an effect by R on transcription does not explain the resistance manifested by mature oligodendrocytes because

early block to infection can be circumvented by transfection with free JHMV RNA.

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