IN VIVO AND IN VITRO MODELS OF DEMYELINATING DISEASE. POSSIBLE RELATIONSHIP BETWEEN INDUCTION OF REGULATORY SUBUNIT FROM cAMP DEPENDENT PROTEIN KINASES AND INHIBITION OF JHMV REPLICATION IN CULTURED OLIGODENDROCYTES

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Among the parameters controlling coronavirus (CV) replication within the nervous system of rodents is the state of glial-cell maturation.[1,14] Thus differentiation of primary cultures of oligodendrocytes with dibutyryl cAMP (dbcAMP) effectively results in a restriction of CV-JHMV replication. Treatment of cells with dbcAMP, which directly raises the intracellular concentration of cAMP, stimulates the adenylate cyclase system. In primary rat oligodendrocytes stimulation of the adenylate cyclase system results in an induction of the regulatory subunit (R) of cAMP-dependent protein kinase type 1 (PK_1).[2] It is conceivable that the function of increase in free R_1 within host-cells may be related to inhibition of phosphoprotein phosphatases (PPPase), as previously demonstrated in rabbit skeletal muscle with R_2 from PK_2.[6,7]. Evidence from our studies indicates that a PPPase may participate in the early stages of CV infection, perhaps during traverse through endosomes of the host.[12] The present study was undertaken to ascertain whether the R protein plays any role in restricting JHMV replication in mature glial cells by inhibiting an endosomal PPPase.

Inhibition of Endosome PPPase Activity with the R_1 Protein

Purified endosomes, obtained through percoll gradient fractionation of L-2 mouse fibroblasts[12] were used in an in-vitro PPPase assay to detect phosphoserine phosphatase activity against purified preparations of [^{32}P]-labeled JHMV nucleocapsid (NC) protein. This assay has previously been shown to result in the specific dephosphorylation of labeled NC preparations.[12]

Briefly, reactions were initiated containing 25 mM Tris-maleate pH 7.0, 10 mM 3':5' cAMP, 25 mM $MgCl_2$, 1% Triton X-100, 25 ug of endosomes and varying quantities of affinity-

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purified R and catalytic (C) subunits or the PK holoenzyme. After incubation at 30°C for 30 min the [^{32}P]-NC protein substrate was added to the reaction mixture and incubation continued for 90 min. at 30°C. Reactions were terminated by addition of 1% BSA and 25% TCA and the contents analysed either for [^{32}P] released or, following dissociation in 3x Laemmli's buffer for NC polypeptide by means of SDS-PAGE. The amount of [^{32}P] released into the supernatant was determined,[9] permitting us to calculate the amount of NC dephosphorylation.

The data in Figure 1 indicate that as little as 1 ug of free R_1 protein is capable of inhibiting NC protein dephosphorylation due to the endosome PPPase by 32%. With increasing concentration of R_1 there was a corresponding increase in PPPase inhibition up to a maximum of 50% when 12 ug of R_1 were added. Since the reaction was not affected appreciably at concentrations of R_1 above 3 ug, this concentration was employed in all subsequent experiments. Inhibition of PPPase by R_1 could be visualized after separating the [^{32}P]-NC protein by 10% SDS-PAGE and employing autoradiography (Figure 2). The results demonstrate a substantial reduction of the [^{32}P]-signal following the PPPase reaction (lane 2) as compared to the control sample (lane 1). Upon addition of 3 ug R_1 to the PPPase reaction less dephosphorylation occurred (lane 3) corresponding to approximately a 40% inhibition of PPPase activity, as determined by densitometric scanning of the autoradiogram. From these findings it is concluded that (1) the endosomal PPPase activity possesses specificity for the NC protein of JHMV and (2) R_1 subunit of PK can inhibit the endosomal PPPase, albeit incompletely.

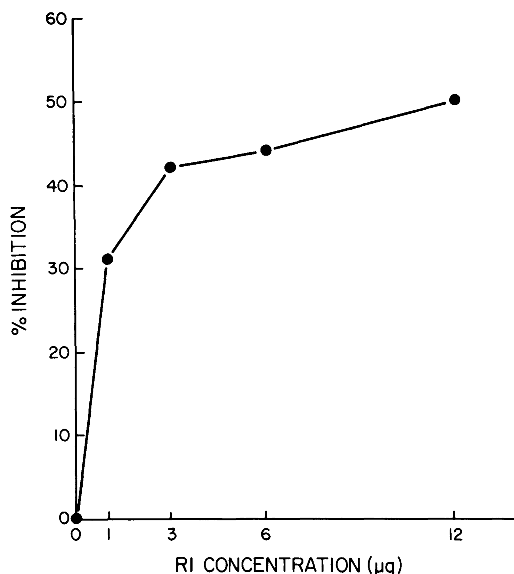

Figure 1. Effects of increasing R_1 concentration on PPPase activity: % inhibition of NC protein dephosphorylation as a function of increasing R_1 concentration.

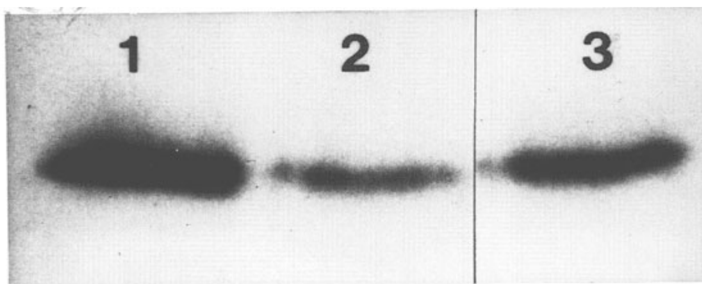

Figure 2. Autoradiogram of PPPase assay. Lane 1, undephosphorylated NC protein. Lane 2, NC protein dephosphorylated using L cell endosomes. Lane 3, NC protein dephosphorylation using L cell endosomes and 3 ug R_1 protein.

Specificity of PPPase Inhibition with R_1 Protein

Additional assays were undertaken using PK_1 and PK_2 holoenzymes as well as isolated catalytic subunits to determine if the PPPase inhibition was specific for the regulatory subunit. The results (Table 1) show the inability of catalytic subunits to inhibit PPPase activity as compared with R_1 subunit. Holoenzyme preparations of PK_1 and PK_2 were inert. However, upon addition of 10 mM 3':5' cAMP to promote R and C dissociation, small but significant PPPase inhibition was observed. This result further draws attention to the specificity of R in suppressing the endosomal PPPase. The present findings are supported by previous studies demonstrating inhibition by R_2 of a PPPase from rabbit skeletal muscle.[6,7] Our data and those from the cited articles draw attention to other functions of R_1 and R_2, such as inhibition of PPPases, apart from that of controlling the phosphorylation by the catalytic subunits.

Effects of NC dephosphorylation on nucleic acid binding potential

Previous evidence with the P56 protein of CV A59 and JHMV has indicated the affinity for binding nucleic acid by this protein.[2,13] The question of an altered nucleic acid binding capacity following dephosphorylation has significance for trying to understand the sequence of events which occur during CV uncoating process which may involve the endosome.

The procedure was essentially as previously described.[2] Following incubation of NC protein with endosomal PPPase the material was separated by 10% SDS-PAGE, then transferred to nitrocellulose paper.[3] Blots were probed with [^{32}P]-labeled CV-A59 g344 plasmid DNA,[3] washed and subjected to autoradiography. The dephosphorylated NC protein had a greatly reduced ability

Table 1. Dephosphorylation of NC protein by an endosomal PPPase in the presence of PK and R or C enzyme subunits

Addition	Activity in Units/mg endosomal[1] protein	% Inhibition[2]
none	1,440 (9)	
PK_1 holoenzyme	1,480 (4)	0
PK_2 holoenzyme+cAMP	1,240 (5)	14 $\pm$ 3.2
PK_2 holoenzyme	1,480 (2)	0
PK_2 holoenzyme+cAMP	1,120 (4)	22 $\pm$ 2.6
R_1 3 ug	960 (3)	33 $\pm$ 2.7
R_1 3 ug+cAMP	760 (9)	47 $\pm$ 7.0
C_1 5 ug+cAMP	1,680 (4)	0

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

[2] Average values with standard error of the mean.

to bind the nucleic acid probe (data not shown). This, as yet preliminary, evidence indicates that NC protein dephosphorylation reduces its binding capacity for nucleic acids. This observation implies that normal uncoating may require NC dephosphorylation. Therefore, differentiation of oligodendrocytes associated with elevation of R_1 creates an intracellular environment in which dephosphorylation of the NC by an endosomal PPPase is inhibited, thereby affecting the uncoating process.

SUMMARY

The results in this study suggest a unique possible association between inhibition of JHMV replication and the induction of R_1 proteins in differentiating oligodendrocytes, involving inhibition of a PPPase found in the endosome fractions of cells. Free R_1 protein appears to prevent the dephosphorylation of purified JHMV-NC substrate thereby possibly blocking the normal sequence of events necessary for NC release of viral genomes. Inhibition of host-cell endosome PPPase's may have relevance to controlling JHMV infection since CV particles adsorbing to cell surface receptors are believed to penetrate via clathrin coated pits into endosomes.[4,5,8,10,11] Thus, the early stages of CV infection, including dephosphorylation of NC protein within endosomes, provide a possible site for controlling the initial stages of viral infection.

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