IN VIVO AND IN VITRO MODELS OF DEMYELINATING DISEASE: A PHOSPHOPROTEIN PHOSPHATASE IN HOST CELL ENDOSOMES DEPHOSPHORYLATING THE NUCLEOCAPSID PROTEIN OF CORONAVIRUS JHM

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Coronavirus JHM (JHMV) shows specific tropism for oligodendrocytes of the CNS.[1] Primary oligodendrocytes induced to differentiate using agents like dibutyryl cyclic AMP (dbcAMP) develop resistance to JHMV infection.[1,2] Several studies suggest that the virus enters the host cell through receptor mediated endocytosis.[3,4] Earlier studies also found that the nucleocapsid protein (NC) of JHMV is reduced in molecular weight (MW) from a 56K to a 50K component during the early stages of infection.[5] A change in the molecular weight of this magnitude with phosphorylated proteins can be accounted for by dephosphorylation.[6] The reversible phosphorylation-dephosphorylation of a capsid polypeptide-nucleic acid binding protein has been shown to influence the binding of nucleic acid in a retrovirus.[7] This information suggests that a phosphoprotein phosphatase (PPPase) dephosphorylating the NC of JHMV effects the uncoating of the RNA genome during the early stages of infection. Here we present evidence for such a dephosphorylating activity in neural and other cells which are hosts for JHMV.

[^{32}P]-labelled NC protein was prepared from JHMV infected L-2 mouse fibroblasts labelled with [^{32}P] orthophosphate using conventional biochemical methods. The final product, isolated from preparative SDS-PAGE, was examined and confirmed to be pure by autoradiography and Western blotting. The assay mixture for PPPase consisted of 25 mM Tris-maleate buffer (pH 7.0), 1 mM $MnCl_2$, 0.1% Triton X-100 and [^{32}P]-labelled NC protein in a total volume of 100 ul. The reaction was terminated after 90 min. at 30°C by precipitating on ice with 25% TCA. The [^{32}P] released from the substrate was determined by scintillation counter according to Maene and Greengard.[8] To separate the NC as a band the reaction mixture was boiled in Laemmli's dissociation buffer and subjected to SDS-PAGE[9] and autoradiography.

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Modulation of the 56K Nucleocapsid Protein

Addition of phosphate was made to infected L-2 cell monolayers, when about 10% of cells had formed syncytia. Upon completion of syncytia formation the cells were harvested, disrupted, centrifuged at 7,000 x g for 15' and the supernatant treated with 0.5% NP-40. Separation by 10% SDS-PAGE gel and Western blotting with anti-NC monoclonal antibody revealed the homogeneity of the NC component. Following exposure of L-2 cell monolayers infected with JHMV to varying concentrations of phosphate two MW species of NC of 56 and 50K were evident in the cell extracts. The proportion of each MW type present depended on the phosphate concentration applied (Figure 1). The results, shown as relative percentage of NC antigen, are calculated from densitometer scanning of the 56K and 50K bands in the Western blots. As shown in Figure 1, the amount of 50K protein decreased in relation to increasing phosphate concentrations, being completely absent at 5 mM phosphate. These data imply that phosphate concentration in the growth medium determines the amount of 56K NC protein which is produced presumably in relation to the phosphorylation of the 50K protein.

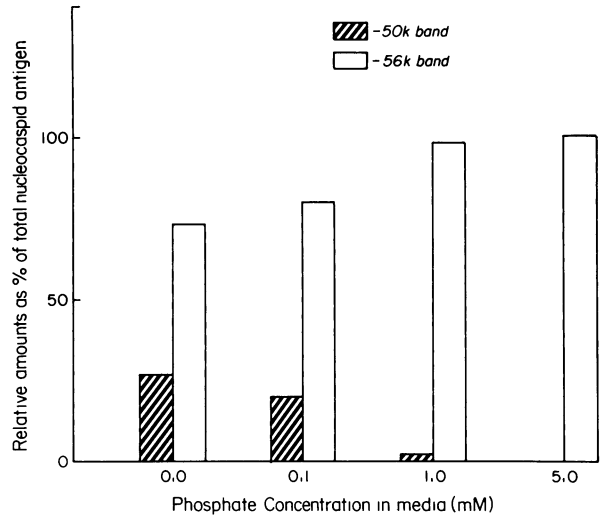

Figure 1. Relationship between phosphate concentration (mM) added to the NM and relative percentage of 56K and 50K nucleocapsid antigen. Following inoculation and incubation to allow virus replication virus-cell complexes were disrupted and the larger cell organelles were removed by centrifugation. The remaining supernatant material was treated with detergent NP-40, prior to separation of the components by SDS-PAGE. The 50K and 56K NC antigens were detected by immunoblotting. Relative antigen concentrations were determined by scanning densitometer.

Subcellular Localization of PPPase Acting on 56K NC Protein

The presumed association of inoculum JHMV with endosomes during early cell-virus interactions led us to isolate an endosomal fraction from host L-2 cells. For this purpose the method of Merion and Poretz[10] was followed, using a two step centrifugation through percoll gradients. The individual fractions collected from the gradients were identified by contents of marker enzymes (data not shown). Some of these results on one enzyme activity within various cellular fractions are summarized in Table 1. After addition of Triton X-100 to the reaction mixture, specific activity of the PPPase with a neutral pH optimum was enhanced more than 3-fold, indicating that this PPPase is membrane associated. The neutral PPPase was found to be concentrated in the endosomal fraction of L-2 cells, since in this cell fraction a 25-fold increase in specific activity was evident, compared to that in the homogenate (Table 1).

Table 1. Subcellular distribution of a 'neutral' phosphoprotein phosphatase from L-2 cells, active on JHMV nucleocapsids

Cell fraction	Specific activity units/mg protein	Amplification in Sp. activity relative to homogenate
Total homogenate with 0.1% Triton X-100	65	1.0
Total homogenate without detergent	19	0.3
Nuclear pellet	162	2.5
Post-nuclear supernatant	96	1.5
Lysosomal	333	5.1
Cytosolic	288	4.5
Endosomal/prelysosomal	1,660	25.5

For comparative purposes specific PPPase activity is a measure of the percentage of [^{32}P] released from NC, one unit being equivalent to release of 1% [^{32}P] dpm from the NC substrate. The representative data shown are from one of several such experiments.

Table 2. Characteristics of PPPase

1. pH optimum	7.0
2. Cation requirement	Mn^{++}, 1 mM
3. Effect of 30 mM NaF inhibition	30%
4. Effect of commercial acid and alkaline phosphatase on the NC substrate	No activity
5. Effect of PPPase on [^{32}P]-labelled Casein and Histone	Active

Partial Characterization of the Neutral PPPase

In order to define the endosomal PPPase further, partial characterization was undertaken. A summary of the findings is presented in Table 2. The pH optimum for the PPPase activity was found at about neutrality, in line with characteristics of other phosphoprotein phosphatases.[11] This finding suggests that the PPPase under study is more likely to function in the prelysosomal/endosomal subpopulations termed 'early endosomes'[12] which have been shown to contain a milieu at pH of about 6.5. Divalent cation requirement for Mn^{++} is also in line with characteristics of other protein phosphatases.[13] Exposure to 30 mM NaF inhibited the endosomal PPPase by only 30%, distinguishing it from the acid phosphatase activity present in the same cell material, which was inhibited by over 95% with NaF, when acting on the non-specific substrate p-nitrophenyl phosphate (pNPP). Commercial acid phosphatase from potato and alkaline phosphatase from E. coli were inert against the NC protein as substrate when used at concentrations which caused comparable dephosphorylation of pNPP to that obtained with phosphatases in the endosomal extract, indicating that the endosomal PPPase activity is distinguishable from that of the acid and alkaline phosphatases. PPPase in the endosomal extracts was able to dephosphorylate [^{32}P]-labelled -casein and histone when used as substrates, implying that the neutral endosomal PPPase activity acts on phosphorylated serine or threonine residues in the NC protein.

Characterization of the endosomal PPPase was made on the abundantly available L-2 murine fibroblasts because of the difficulty in obtaining sufficient quantities of primary oligodendrocytes. Presence of a neutral PPPase in neural tissue was determined with brain homogenates from Wistar Furth rat neonates and homogenates from cultured primary oligodendrocytes. The neural material possessed higher specific activities than homogenates of L-2 cells (Figure 2). the closest comparison between L-2 cells and oligodendrocytes which could be undertaken by us utilized endosomes isolated from Roc-1 cells, a hybrid C_6 astrocytoma x primary oligodendrocyte cell line (a kind gift from Dr. F.A. McMorris, The Wistar Institute, PA). It is evident from Figure 2 that Roc-1 cell endosomes possess a neutral PPPase activity comparable to that of L-2 cell endosomes.

SUMMARY

We have identified a phosphoprotein phosphatase which dephosphorylates efficiently the NC protein of coronavirus JHM. The activity was found in L-2 murine fibroblasts, Wistar Furth rat neonatal brain extracts, Wistar Furth rat oligodendrocyte primary cells and in Roc-1 cells, an oligodendrocytic hybrid cell line. In both L-2 cells and Roc-1 cells the enzyme was found to be localized predominantly in the endosomal fraction. The enzyme is optimally active at pH 7.0 and has a requirement for Mn^{++} ions. This PPPase activity is distinguishable from acidic and alkaline phosphatases. In view of the specificity

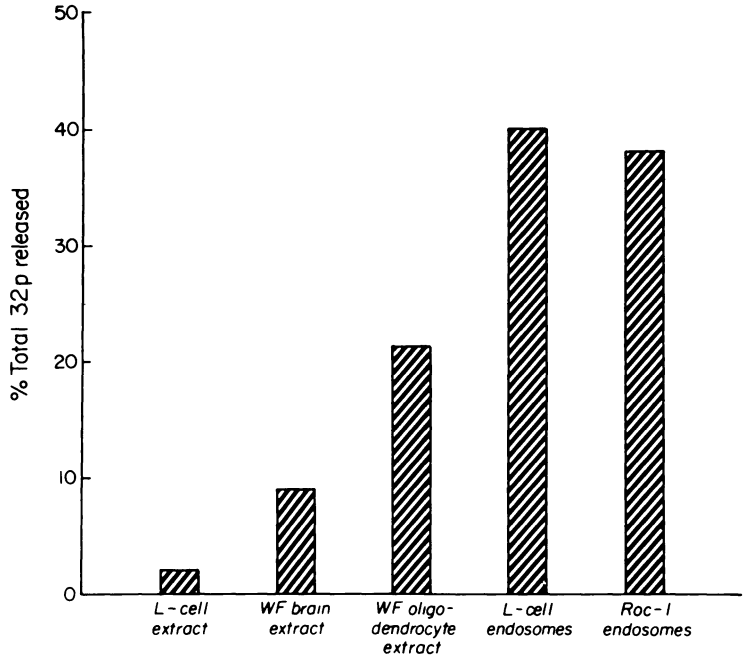

Figure 2. Comparison of specific activity of 'neutral' PPPase in brain tissue and neural cells with that of L-2 cells. The representative data are expressed from one of several such experiments as percent dephosphorylation of [^{32}P]-NC by enzyme in samples containing 50 ug protein during 90 min reaction at 30°C.
WF = Wistar Furth rats.

of the endosomal PPPase for the phosphory-lated NC protein it is hypothesized that this enzyme may have a function during early stages of coronavirus infection.

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