Cross-reactivity of the anti-PML antibody PG-M3 with the herpes simplex virus type 1 immediate early protein ICP4

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The PML protein is one of the components of ND10, nuclear matrix-associated structures which undergo rapid disintegration at the onset of herpes simplex virus type 1 (HSV-1) infection. This disruption event has been frequently visualized in immunofluorescence assays using the anti-PML mouse monoclonal antibody PG-M3. This antibody was surprisingly found to also stain nuclear virus replication compartments when employed at higher concentrations. This was shown to be due to an unexpected cross-reactivity of the PG-M3 antibody with the HSV-1 immediate early protein ICP4, a known component of replication compartments. The sequences of ICP4 recognized by PG-M3 were found to map to the extreme amino-terminal end of the protein, which includes a 21 amino acid segment that is partially homologous to the peptide of PML that was used to make PG-M3. These results suggest that PG-M3 may no longer represent an appropriate antibody for use in visualizing the fate of PML and ND10 during HSV-1 infection.

ND10 are complex nuclear matrix-associated structures which contain a number of associated proteins, including NDP55, Sp100 and PML. The actual function of ND10 within the cell remains unclear, but their structure, distribution and size can be affected by external stimuli such as interferon treatment, stress and viral infection (reviewed in Sterndsorf et al., 1997). One alteration in ND10 structure which has been particularly well characterized over the past several years is the disruption event which occurs at the onset of herpes simplex virus type 1 (HSV-1) infection (reviewed in Everett, 1999). After entry of the HSV-1 virus particle into the cell, the viral DNA genome is released and migrates to sites adjacent to ND10 (Ishov & Maul, 1996; Maul et al., 1996). As a consequence of the initial phase of viral gene transcription, the immediate early protein ICP0 is produced, which then associates with ND10 structures and mediates their disruption (Everett & Maul, 1994; Maul & Everett, 1994; Maul et al., 1993). The latter event appears to be dependent upon the specific proteasome-dependent degradation of SUMO-1-modified forms of PML (Everett et al., 1998). Continued expression of later classes of viral proteins ultimately results in the appearance of large globular structures called replication compartments near the former sites of ND10 within the nucleus. These contain viral-encoded proteins such as the immediate early polypeptide ICP4 and various enzymes involved in genome replication, and represent active sites of viral gene expression and DNA synthesis within the cell (de Bruyn Kops & Knipe, 1988; DeLuca & Schaffer, 1988; Quinlan et al., 1984). Thus, disruption of ND10 by the ICP0 protein appears to represent a crucial prerequisite for efficient virus replication during low multiplicity HSV-1 infection of many cell lines.

ND10 disruption events during HSV-1 infection have been readily monitored through immunofluorescence staining of ND10 components such as PML or Sp100. One antibody that has been frequently used in these studies is the anti-PML mouse monoclonal antibody PG-M3 (Flenghi et al., 1995). During the course of immunofluorescence analyses of HSV-1-infected HeLa cells, it was discovered that elevated concentrations of PG-M3 could stain nuclear structures that were identical in appearance to virus replication compartments. In these experiments, cells were plated in two-well chamber slides at a density of 6 x 10^4 cells per well and allowed to adhere overnight. The cells were mock- or HSV-1 (strain 17+) infected for 6 h, fixed and permeabilized in methanol at −20 °C for 20 min, washed in PBS, and then blocked in 3% BSA in 0.5% Triton X-100 for 30 min. The cells were then incubated with primary antibody [PG-M3 (Santa Cruz Biotechnology) at a 1:30 dilution or anti-ICP4 antibody 1101 (Goodwin Institute for Cancer Research) at a 1:1000 dilution] in 0.5% BSA–0.5% Triton X-100–PBS for 1 h, followed by three 5 min washes in 0.5% Triton X-100–PBS. The cells were then incubated with secondary FITC-conjugated goat anti-

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mouse antibody (Kirkegaard & Perry Laboratories) at a 1:250 dilution for 1 h, followed by three 5 min washes in 0.5% Triton X-100–PBS and one PBS wash. The cells were then mounted in Vectashield mounting medium (Vector Laboratories Inc.) and viewed with a Nikon Optiphot inverted microscope with 40× magnification.

Fig. 1. Staining of virus replication compartments by the anti-PML antibody PG-M3 in HSV-1-infected cells. HeLa or Vero cells were infected with wild-type HSV-1 and subjected to immunofluorescence analysis. Staining was performed with PG-M3 or an anti-ICP4 antibody as indicated.
Mock-infected HeLa cells that were stained with PG-M3 antibody typically exhibited punctate nuclear structures that were indicative of the presence of intact ND10 (Fig. 1). However, in PG-M3-stained HSV-1-infected HeLa cells, most of these bodies had been disrupted and replaced by much larger globular structures that were identical in appearance to replication compartments; the latter structures were readily visualized by staining infected cells with an antibody specific for the viral immediate early protein ICP4 (Fig. 1). One possible explanation for this observation is that the PML protein that is recognized by the PG-M3 antibody migrates to replication compartments after the disruption of ND10 early in infection. To address this possibility, the experiment was repeated using Vero cells, whose PML protein is not recognized by PG-M3 (Burkham et al., 1998). As expected, no ND10 structures were detected in PG-M3-stained mock-infected Vero cells (Fig. 1). However, replication compartments were readily stained in HSV-1-infected Vero cells in immunofluorescence analyses which employed either the PG-M3 or anti-ICP4 antibodies (Fig. 1). These results indicated that PG-M3 was able to recognize a component of virus replication compartments that was distinct from PML.

In order to identify the replication compartment component that was recognized by PG-M3, lysates of HSV-1-infected Vero cells were analysed on Western blots using this antibody. Preparation of 24 h post-infection lysates and Western blot analysis were carried out as described previously (Spatz et al., 1996), and antibodies were used at the following concentrations: primary antibody PG-M3 at a 1:40 dilution, primary anti-ICP4 antibody 1101 at a 1:2000 dilution, and secondary alkaline phosphatase-conjugated goat anti-mouse antibody (Kirkegaard & Perry Laboratories) at a 1:2000 dilution. PG-M3 was found to react strongly with a 175 kDa protein species in these experiments (Fig. 2). The molecular mass of this large polypeptide suggested that it might be the 175 kDa HSV-1 immediate early protein ICP4, a known component of replication compartments (DeLuca & Schaffer, 1988). To investigate this possibility, lysates were prepared from cells infected with d120, an HSV-1 mutant from which most of the sequences encoding the ICP4 gene have been deleted (DeLuca & Schaffer, 1988). As expected, the 175 kDa ICP4 protein could not be detected in lysates from d120-infected cells in Western blots using an anti-ICP4 antibody. Instead, the antibody recognized a 38 kDa truncated version of ICP4 that was previously identified by DeLuca & Schaffer (1988) and contains the first 150 amino acids of ICP4 (Fig. 2). Identical results were obtained with the PG-M3 antibody: the 175 kDa protein detected by PG-M3 in wild-type HSV-1-infected cells was absent in d120-infected cells, and the 38 kDa truncated ICP4 protein was detected instead (Fig. 2). These results identified ICP4 as the component of replication compartments that is stained by PG-M3 in HSV-1-infected cells. This conclusion was supported by the absence of any distinctive staining pattern in cells that had been infected with d120 and stained with either PG-M3 or an anti-ICP4 antibody, and by the presence of a diffuse nuclear staining pattern in cells that had been transfected with an ICP4-expressing plasmid and stained with either antibody (S. L. Boulware & P. C. Weber, unpublished observations).

It was of interest to determine the molecular basis for the unexpected cross-reactivity of the PG-M3 antibody with the HSV-1 ICP4 protein. The observation that PG-M3 could recognize the severely truncated ICP4 protein encoded by d120 on Western blots (Fig. 2) indicated that the epitope...
recognized by PG-M3 mapped to the first 150 amino acids of ICP4. When homology searches were carried out on the amino acid sequence of the ICP4 protein remaining in d120, a 21 amino acid segment (amino acids 20–40) within this amino-terminal region of ICP4 was found to be partially homologous to the 15 amino acid peptide of PML (amino acids 37–51) that was used to make PG-M3 (Flenghi et al., 1995) (Fig. 3). It is this homology that most likely accounts for the cross-reactivity of this antibody with ICP4.

It should be noted that recognition of the ICP4 protein in either immunofluorescence assays or Western blots does require elevated concentrations of PG-M3 antibody, as this cross-reactivity is understandably weaker than the interactions that are normally observed between ICP4 and an ICP4-specific antibody. The affinity of the PG-M3 antibody for ICP4 was compared to that of other antibodies which were specifically raised against this protein by Western blot analysis. Using identical dilutions of blotted ICP4 protein and identical protein concentrations of primary antibody, the affinity of PG-M3 for ICP4 was found to be 25-fold lower than the HSV-1 anti-ICP4 antibody 1101 used throughout this study, and up to 125-fold lower than that of other commercially available antibodies that were specifically raised against this polypeptide (S. L. Boulware & P. C. Weber, unpublished observations). Nevertheless, this interaction was still strong enough to allow weak ICP4 signals to be detected at the lower antibody concentrations that are normally employed in immunofluorescence assays and Western blots (S. L. Boulware & P. C. Weber, unpublished observations).

In summary, the anti-PML antibody PG-M3 has unexpectedly been found to be cross-reactive with the HSV-1 immediate early protein ICP4. This conclusion was supported by the following observations: (a) ICP4-containing replication compartments were readily stained by PG-M3 in HSV-1-infected Vero cells, whose PML protein is not reactive with this antibody; (b) this staining by PG-M3 was lost when cells were infected with the ICP4 deletion mutant virus d120; (c) the 175 kDa ICP4 protein could be readily detected by PG-M3 in Western blot analyses of lysates of cells infected with wild-type HSV-1 but not d120; and (d) the first 150 amino acids of ICP4 contain a region of obvious homology to the PML peptide used to make PG-M3, and this portion of ICP4 was found to be reactive with PG-M3. Since this antibody has been frequently used to characterize the fate of PML and ND10 during HSV-1 infection (Burkham et al., 1998; Lukonis et al., 1997), the results in this study suggest that caution should be exercised when PG-M3 is used for this purpose. For example, Burkham et al. (1998) reported that the PML protein is recruited to virus replication compartments in HSV-1-infected cells several hours after ND10 disruption takes place. Since PG-M3 was the antibody used in these immunofluorescence analyses, an alternate explanation for this observation could be that ICP4 rather than PML was being detected in these replication compartments. However, Burkham et al. did employ a PG-M3 antibody concentration that was 7-fold lower than that used in the immunofluorescence assays in this study, and did perform controls to confirm that antibody cross-reactivity was not apparent under these experimental conditions. It is likely that similar precautions will necessarily become a prerequisite for the future use of PG-M3 in studies of ND10 structure in HSV-1 infected cells.

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References


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